Endocytic Regulation of Chemokine Receptor Expression

Silène Tuija Wavre

Medical Research Council
Laboratory for Molecular Cell Biology and Cell Biology Unit
University College London

Thesis submitted for the degree of Doctor of Philosophy

December 2006

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

The activity of stimulated cell surface signalling receptors is frequently regulated by endocytosis, which provides a mechanism to internalise and either degrade or reprogram the protein. Clathrin-mediated endocytosis (CME) is one of the principal mechanisms responsible for these events. Although clathrin and many of its associated proteins are relatively well characterised, many aspects of how CME operates are yet to be established. In particular, the early steps of internalisation, the formation of clathrin-coated pits (CCPs) and the mechanism by which receptors are recruited into CCPs remain controversial. This thesis investigates the early events of CME occurring at the plasma membrane using as a model various cell lines expressing CC Chemokine receptor 5 (CCR5), a G-protein coupled receptor (GPCR).

Upon agonist binding, CCR5 undergoes rapid phosphorylation by a GPCR kinase (GRK) and protein kinase C (PKC) on four C-terminal serines. β-arrestin is subsequently recruited to the receptor, abrogates CCR5 interaction with the G-protein and links the receptor to the endocytic machinery by binding to AP-2 and clathrin.

Using confocal immunofluorescence microscopy and electron microscopy, I show that, on agonist binding, CCR5 relocates in the plasma membrane and clusters into flat clathrin lattices. CCPs were observed to invaginate at the edge of these lattices and proteins from the endocytic machinery were identified by immunolabelling within these domains. As C-terminal serine phosphorylation has been found to be important for efficient agonist-induced internalisation of CCR5, the requirement for specific serines for plasma membrane relocation, association with clathrin lattices and endocytosis were analysed. A mutant lacking all serines failed to be recruited into flat clathrin lattices. Further analysis showed that specific GRK phosphorylation of CCR5 was sufficient for this recruitment, suggesting a distinct role for different kinases in receptor desensitisation and internalisation.

This study brings new insights into the mechanism of recruitment of activated GPCRs into CCPs and reveals the importance of flat clathrin lattices in the formation of endocytic clathrin-coated vesicles and CME. Part of the results presented in this thesis have been published (Signoret et al., 2005) (see appendix).

Table of Contents

Abbreviations

7TM	seven trans-membrane
aa	amino acids
AAK1	adaptin-associated kinase-1
ANTH	AP180 N-terminal homology
AOP	aminooxypentane
AP	adaptor protein (originally assembly polypeptide)
ARF6	ADP-ribosylation factor 6
ARFGAP	ARF GTPase-activating proteins
ARH	autosomal excessive hypercholesterolemia
AT1aR	angiotensin II type 1a receptor
β_2AR	beta 2 adrenergic receptor
BAR	Bin/Amphiphysin/Rvs-homology
BM	binding medium
BSA	bovine serum albumin
CALM	clathrin assembly lymphoid myeloid leukaemia protein
CCL5	CC chemokine 5
CCP	clathrin-coated pit
CCR5	CC chemokine receptor 5
CCV	clathrin-coated vesicle
CD	cluster of differentiation
Dab-2	disabled-2
DAG	diacylglycerol
DNA	desoxyribonucleic acid
CHO	Chinese hamster ovary
CME	clathrin-mediated endocytosis
COP	coatomer protein
Cv	caveolae
CVAK104	104 kD coated vesicle-associated protein
EDTA	ethylene-diamine-tetraacetic acid
EE	early endosome
EEA-1	early endosomal antigen-1

EF	evanescent field
EGF	epidermal growth factor
EH	esp15 homology
EM	electron microscopy
ENTH	epsin N-terminal
Eps15	EGF receptor pathway substrate 15
Epsin	Eps15 interactor
FACS	fluorescence-activated cell sorting
FcεRI	high affinity receptor for IgE
FCS	foetal calf serum
FPR	*N*-formyl peptide receptor
GA	gluteraldehyde
GAG	glycosaminoglycan
GAM	goat-anti-mouse
GAR	goat-anti-rabbit
GDP	guanosine diphosphate
GEF	guanine nucleotide-exchange factor
GFP	green fluorescent protein
GGA	Golgi-localised, γ-ear-containing, ADP ribosylation factor-binding proteins
GPCR	G-protein coupled receptor
GRK	GPCR kinase
GTP	guanosine triphosphate
HB	hepes buffer
HEK	human embryonic kidney
HIV	human immunodeficiency virus
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate
Ig	Immunoglobulin
kD	kiloDalton
L	lattice
LDL	low-density lipoprotein
LE/L	late endosome/lysosome
MβCD	methyl-β-cyclodextrin

MCP	monocyte chemotactic peptide
MEF	mouse embryonic fibroblast
mM	milli molar
μm	micrometre
MIP	macrophage inflammatory protein
ml	millilitre
mv	microvilli
Mv-1-lu	mink epithelial lung cells
n/a	not applicable
n/d	not determined
nM	nanomolar
nm	nanometre
N-WASP	neural Wiskott Aldrich syndrome protein
PAG	protein A gold
PB	phosphate buffer
PBS	phosphate buffer saline
PDZ	postsynaptic density 95/disc-large/zona occludens
PFA	paraformaldehyde
PIPKIγ	phosphatidylinositol 4-phosphate 5-kinase type Iγ
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PP	protein phosphatase
PtdIns(4,5)P_2	phosphatidylinositol (4,5)-bisphosphate
RANTES	regulated on activation, normal T cell expressed and secreted
RBL	rat basophilic leukaemia
RE	recycling endosome
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RTK	receptor tyrosine kinases
SDF-1	stromal cell-derived factor-1
SDS	sodium dodecyl sulphate
SMAP-1	stromal membrane-associated protein 1

SV40	Simian virus 40
TEM	transmission electron microscopy
TGFβR	transforming growth factor β receptor
TGN	*trans*-Golgi network
TIRF	total internal reflection fluorescence
UIM	ubiquitin interacting motifs
WT	wild-type

List of Figures

List of Tables

1 Introduction

Endocytosis is an essential property of eukaryotic cells and consists of several mechanisms by which macromolecules and fluids, which cannot diffuse through the membrane, are transported into the cell via vesicles budding from the plasma membrane. Endocytosis is also used to regulate responses to extracellular cues. Indeed, extracellular signals are often transmitted through plasma membrane receptors, whose expression at the cell surface and resensitisation are regulated through endocytosis. For example, upon agonist binding, seven transmembrane domains (7TM), G-protein coupled receptors (GPCRs) are activated and signal through interaction with a G-protein. In order to stop their signalling activity, receptors are desensitised and uncoupled from G-proteins. Finally, they are removed from the plasma membrane via internalisation and enter cellular compartments where they dissociate from their ligand and can be resensitised, or are degraded.

The best-characterised endocytic pathway is clathrin-mediated endocytosis (CME). Since its first description by Porter and Roth in 1964, CME has been extensively studied and the complexity of the process has become apparent (Roth and Porter, 1964). A growing number of proteins that play a role in this process has been identified, and recent advances in live cell imaging have allowed insights into the dynamic nature of the process. Clathrin structures differing in size and mobility have been described (Perrais and Merrifield, 2005). The endocytic activity of these different structures has been at the centre of debates in the last few years. This thesis examines the early steps in the CME of chemokine receptors, part of the superfamily of GPCRs, and presents a detailed morphological study of the clathrin structures in which the receptors cluster prior to endocytosis. Furthermore, the importance of agonist-induced, post-translational modifications of CCR5 in receptor recruitment into clathrin structures and subsequent internalisation has been investigated. The first half of the introduction gives an overview of CME and the network of proteins involved in the process. The second half describes the chemokine receptor system and CC chemokine receptor 5 (CCR5) regulation through endocytosis.

1.1 Endocytosis

The plasma membrane regulates the exchange of molecules and the communication between the cell and its extracellular environment. The uptake of macromolecules and fluids via vesicles derived from the plasma membrane is termed endocytosis. This process plays a major role in a plethora of functions including development, immune responses and neurotransmission. It is highly regulated and involves a complex network of specific proteins and lipids dependent on the cargo and the cell type. There are two main processes of endocytosis. In each case, the process used is determined by the size of the cargo. Engulfment of large pathogens of >0.5μm, apoptotic cells and cell debris, which occurs primarily in specialised cells such as macrophages, monocytes and neutrophils, is termed phagocytosis (Stuart and Ezekowitz, 2005). The uptake of fluids, macromolecules and smaller particles is effected through pinocytosis. Several mechanisms of pinocytosis exist depending on the cargo. The two best-characterised pathways depend on the structural proteins clathrin and caveolin for the formation of endocytic vesicles (Edeling et al., 2006b; Okamoto et al., 1998). Other pathways, involving vesicles devoid of both proteins have been observed in some cells but remain poorly understood (Damm et al., 2005; Glebov et al., 2006; Kirkham et al., 2005; Lamaze et al., 2001). Finally, macropinocytosis resulting from membrane ruffling is responsible for the uptake of large volumes of extracellular fluid. Figure 1.1 illustrates different endocytic pathways observed in mammalian cells.

1.1.1 Clathrin-mediated endocytosis

CME was first described by Roth and Porter in their study on the uptake of the yolk protein in mosquito oocytes (Roth and Porter, 1964). Using electron microscopy, they observed a bristle-like coat on vesicles and invaginations at the plasma membrane, and proposed a role for these structures in the transport of cargo from the plasma membrane to intracellular structures. CME occurs constitutively in almost all mammalian cells, and is responsible for a variety of important processes. Firstly, nutrient uptake such as cholesterol via the low-density lipoprotein (LDL) and iron via transferrin relies on the internalisation of LDL and transferrin receptors, respectively. In addition, regulation of receptor signalling is in many cases tightly

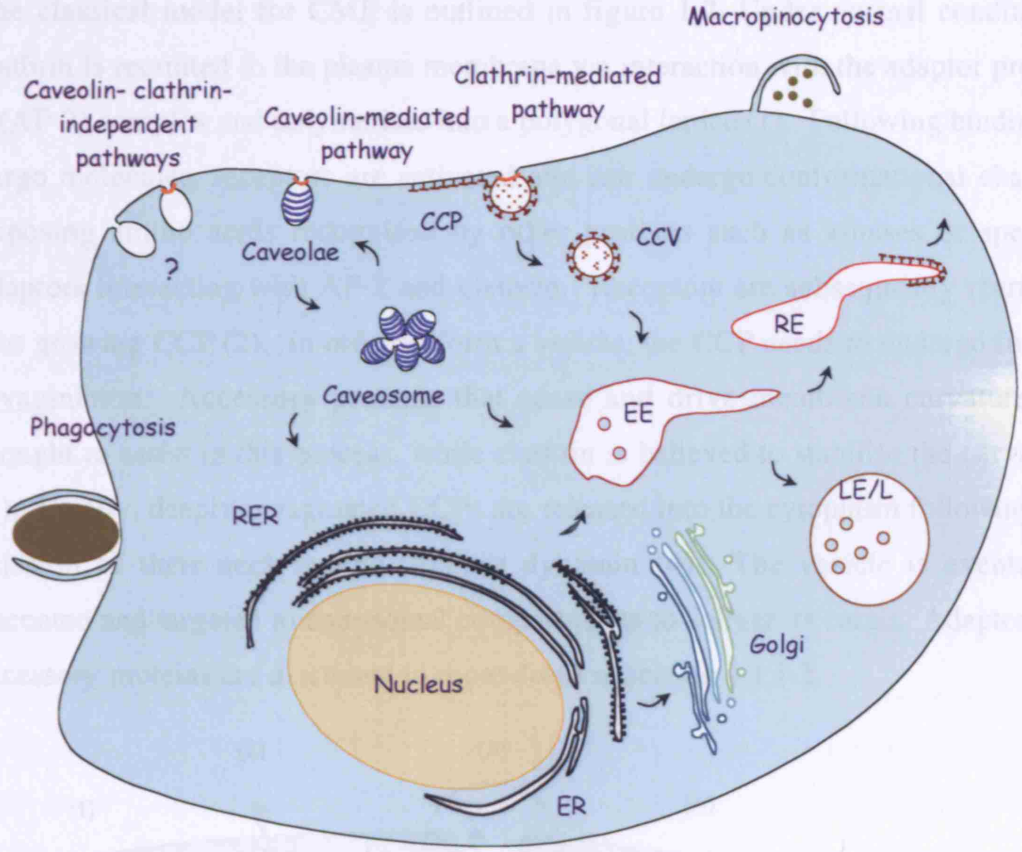


Figure 1.1: Endocytic pathways in mammalian cells

Internalisation pathways in mammalian cells depend on the size and the nature of the cargo. Phagocytosis mediates engulfment of large pathogens or cell debris and pinocytosis is responsible for the uptake of fluids and smaller particles. From left to right, poorly understood caveolin- and clathrin-independent pathways of internalisation, caveolin-mediated and clathrin-mediated endocytosis dependent on dynamin (orange rings at the neck of caveolae and CCP) and macropinocytosis. (CCP) clathrin-coated pit, (CCV) clathrin-coated vesicle, (EE) early endosome, (RE) recycling endosome, (LE/L) late endosome/lysosome, (RER) rough endoplasmic reticulum.

linked to receptor endocytosis via clathrin-coated vesicles (Le Roy and Wrana, 2005). Receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs) are two large groups of signalling receptors. Upon ligand binding receptors are activated and interact with a variety of proteins. This results in signal transduction and internalisation of the receptors. Endocytosis of the receptors correlates with the attenuation of their signalling activity (Sorkin and Von Zastrow, 2002). CME has also been proposed as the main mechanism for recycling of synaptic vesicles in neurons (Granseth et al., 2006). Finally, pathogens also take advantage of endocytic machinery to enter cells. For example, some adenoviruses use clathrin-coated pits (CCPs) and vesicles to gain access to the cytoplasm (Meier and Greber, 2004).

The classical model for CME is outlined in figure 1.2. Under normal conditions, clathrin is recruited to the plasma membrane via interaction with the adaptor protein 2 (AP-2) complex and polymerises into a polygonal lattice (1). Following binding of cargo molecules, receptors are activated and can undergo conformational changes exposing amino acids recognised by other proteins such as kinases or specific adaptors interacting with AP-2 and clathrin. Receptors are subsequently recruited into growing CCP (2). In order to form a vesicle, the CCP needs to undergo further invagination. Accessory proteins that sense and drive membrane curvature are thought to assist in this process, while clathrin is believed to stabilise the curvature (3). Finally, deeply invaginated CCPs are released into the cytoplasm following the scission of their neck by the GTPase dynamin (4). The vesicle is eventually uncoated and targeted to endosomal compartments to deliver its cargo. Adaptor and accessory proteins are discussed in more detail in section 1.1.1.2.

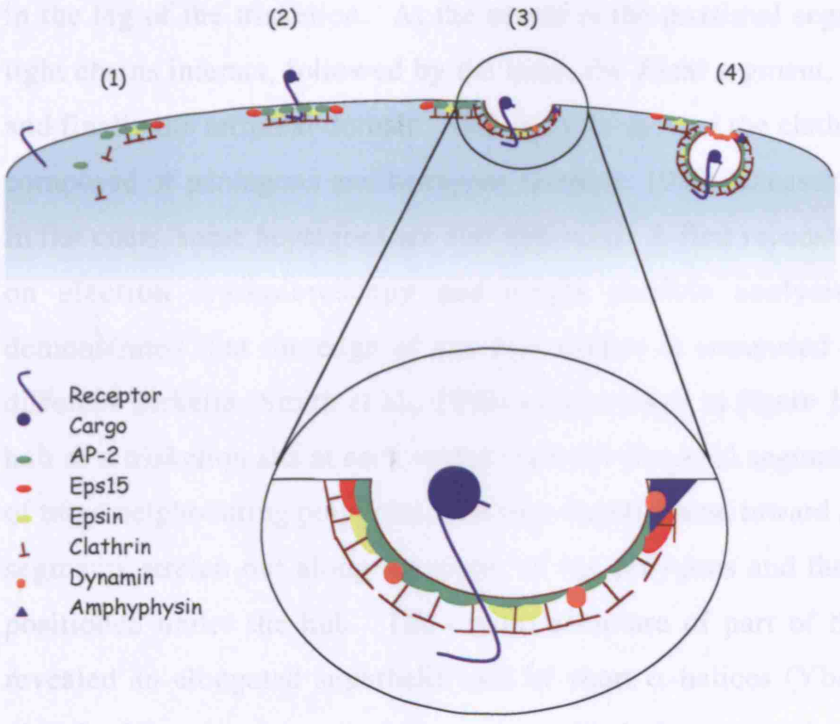


Figure 1.2: Clathrin-mediated endocytosis

Clathrin triskelia assemble at the plasma membrane into a polygonal lattice via interaction with AP-2 (1). Upon agonist binding, receptors interact with proteins from the clathrin endocytic machinery (2). Accessory protein, Eps15 (red), Epsin (yellow), dynamin (orange) and amphyphysin (blue) help the formation of CCP (3). Finally, dynamin moves to the neck of deeply invaginated CCP where it forms a constricting ring, allowing the CCP to pinch off and become a CCV (4).

1.1.2 Clathrin structure

More than 30 years ago, Pearse purified coated vesicles from pig brain and showed that their coat consisted essentially of a 180 kD protein (Pearse, 1975). Extensive studies of the coat of CCVs revealed that clathrin triskelia were the assembly unit of the polygonal coat (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981). The triskelia obtained from purified clathrin reconstituted *in vitro* could be visualised by electron microscopy using rotary shadowing and negative staining with uranyl acetate (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981) (fig. 1.3 A). In addition, the quick-freeze, deep-etch, rotary-replication method allowed the visualisation of extended polygonal clathrin coats in fibroblasts (Heuser, 1980).

Clathrin triskelia consist of three 180kD heavy chains (Hc) each associated with a 25kD light chain (Lc), as shown in figure 1.3 (B). Six domains can be distinguished in the leg of the triskelion. At the centre is the proximal segment with which the light chains interact, followed by the knee, the distal segment, the ankle, the linker, and finally the terminal domain. EM analysis showed the clathrin coat to be mainly composed of pentagons and hexagons (Heuser, 1980; Kanaseki and Kadota, 1969). In flat coats, some heptagons are also observed. A first reconstruction at 21Å, based on electron cryomicroscopy and single particle analysis image averaging, demonstrated that the edge of any one vertice is composed of segments of four different triskelia (Smith et al., 1998) as illustrated in figure 1.3 (C) and (D). The hub of a triskelion sits at each vertex with the proximal segments forming the edges of three neighbouring polygons. The legs slightly bend inward at the knee, the distal segments stretch out along the edges of the polygons and the terminal domain is positioned under the hub. The crystal structure of part of the proximal domain revealed an elongated superhelix coil of short α-helices (Ybe et al., 1999). The globular N-terminal domain forms a seven-blade β propeller in contrast to the linker, which is entirely α-helical and composed of a zigzag of ten short α-helices (ter Haar et al., 1998). A recent study has combined the known crystal structures of the proximal leg, the terminal domain and linker with the electron microscopy density map to obtain a higher resolution (8 Å) model of the triskelia assembly (Fotin et al., 2004), as shown in figure 1.3 (E). This study also revealed the importance of the "hub assembly" in holding the lattice together and in interacting with adaptors and

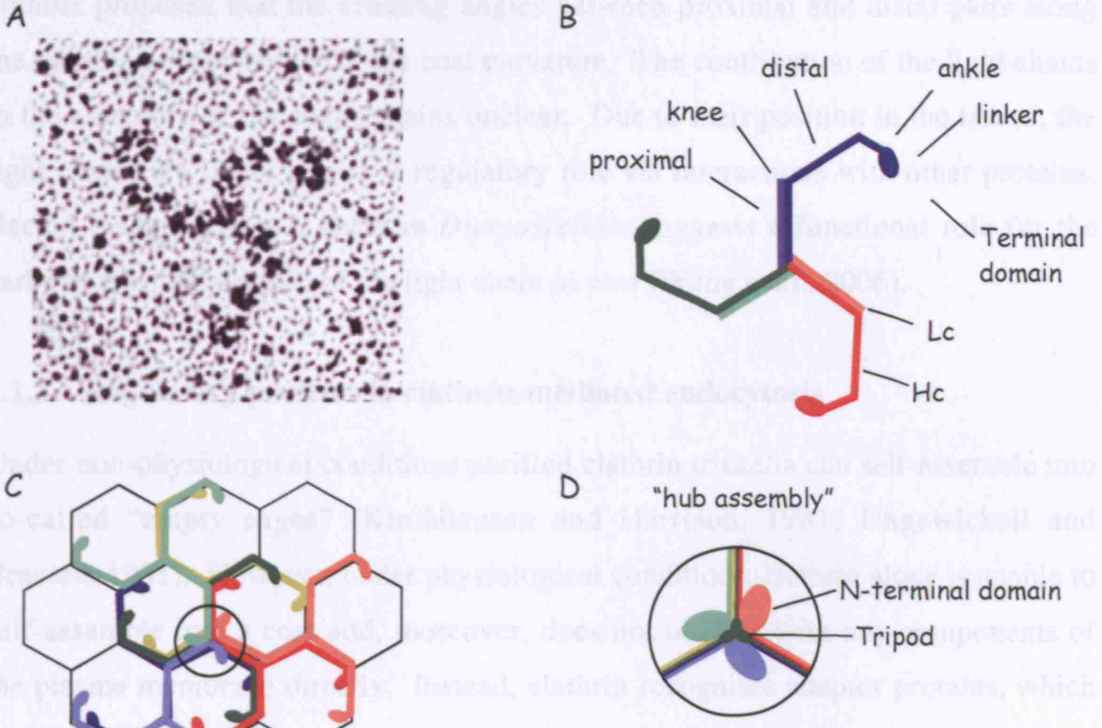


Figure 1.3: Structure of clathrin triskelia
(A) Electron micrograph of 8.4s clathrin triskelion after rotary shadowing (picture kindly given by Prof. E. Ungewickell). (B) A clathrin triskelion is composed of three heavy chains (dark blue, green and red) and three light chains (light blue, green and orange). The leg of the trsikelia is divided in six domains, proximal, knee, distal, ankle, linker, and terminal. (C) Triskelia assemble to form a polygonal lattice. Each side of a polygon is composed of four triskelia and the terminal domain of each triskelion is bent inward. (D) The center of the triskelia sits at each vertex. The tripod (dark green) buried under the triskelion interacts with the terminal domain of three second neighbouring triskelia to form the "hub assembly". (E) 8 Å resolution model of a clathrin hexagonal barrel (from Fotin et al, 2004).

accessory proteins. The "hub assembly" consists of a clathrin heavy-chain repeat (CHCR7), a proximal hairpin and a tripod (α-helix of three heavy-chains) projecting inwards from the top of the hub, and interacting with the ankle of the triskelia centred at the second nearest-neighbour vertices (fig. 1.3 (D)). In addition, the

authors proposed that the crossing angles between proximal and distal pairs along the edges are responsible for the coat curvature. The contribution of the light chains in the assembly of the coat remains unclear. Due to their position in the lattice, the light chains are likely to play a regulatory role via interactions with other proteins. Recent evidence from a study in *Dictyostelium* suggests a functional role for the carboxy terminal domain of the light chain *in vivo* (Wang et al., 2006).

1.1.3 Regulatory proteins in clathrin-mediated endocytosis

Under non-physiological conditions purified clathrin triskelia can self-assemble into so-called "empty cages" (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981). However, under physiological conditions, clathrin alone is unable to self-assemble into a coat and, moreover, does not interact with any components of the plasma membrane directly. Instead, clathrin recognises adaptor proteins, which not only bind membrane phospholipids and cargo proteins, but also facilitate the assembly of clathrin lattices. In addition to adaptor proteins, other accessory and regulatory proteins of the clathrin endocytic pathway that assist the formation of CCP and CCV have been identified. Figure 1.2 illustrates the involvement of adaptor and accessory proteins in the different steps of CME. So far more than 20 proteins have been shown to be involved in CME and these form a dynamic network (Schmid et al., 2006), the complexity of which is illustrated in figure 1.4.

Here, the endocytic machinery will be reviewed in detail following the order of events leading to CCV formation as depicted in figure 1.2. First, the membrane requirement for nucleation of clathrin lattices will be discussed. Secondly, the main clathrin adaptor, AP-2, will be described, followed by accessory proteins shown to assist CCV formation. Finally, the GTPase dynamin and its interactors will be examined. Only a subset of adaptors and accessory proteins of the clathrin endocytic machinery that pertain to the work in this thesis will be discussed here.

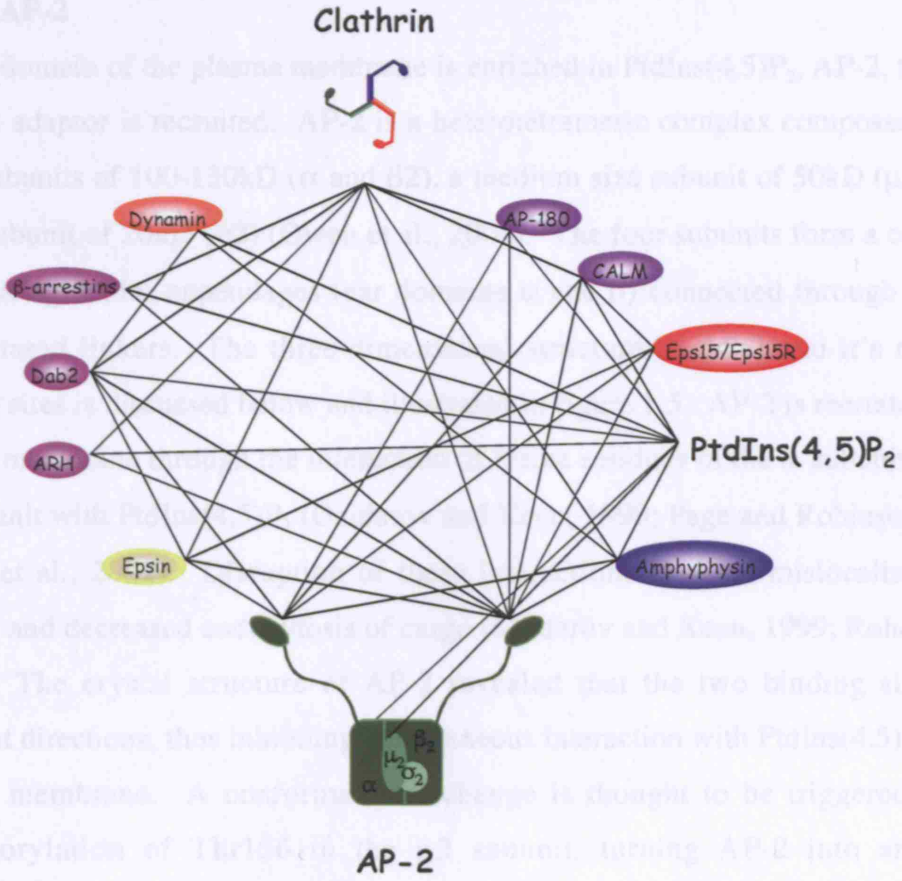


Figure 1.4: Interactions between adaptor and accessory proteins with clathrin and plasma membrane PtdIns(4,5)P_2

Adaptor and accessory proteins involved in the clathrin endocytic machinery are recruited to the plasma membrane by interacting with phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2), α and β2 appendages from AP-2 and/or clathrin. The complexity of these interactions is illustrated here with only 10 proteins involved in CME out of more than 20 identified to date.

1.1.3.1 Recruitment of adaptor proteins to the plasma membrane

The assembly of the clathrin endocytic machinery is proposed to start with the accumulation of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) on the inner leaflet of the plasma membrane, which is necessary for the recruitment of adaptor proteins. The small GTPase ADP-ribosylation factor 6 (ARF6) was shown to activate phosphatidylinositol 4-phosphate 5-kinase type Iγ (PIPKIγ) and may therefore stimulate clathrin assembly (Krauss et al., 2003). In addition, specific ARF GTPase-activating proteins (ARFGAP) were shown to bind proteins in separate pathways. For example, the stromal membrane-associated protein 1 (SMAP-1), an ARFGAP involved in transferrin receptor endocytosis, was found to interact directly with the clathrin heavy chain (Tanabe et al., 2005).

1.1.3.2 AP-2

Once a domain of the plasma membrane is enriched in PtdIns(4,5)P_2, AP-2, the main clathrin adaptor is recruited. AP-2 is a heterotetrameric complex composed of two large subunits of 100-130kD (α and β2), a medium size subunit of 50kD (μ2) and a small subunit of 20kD (σ2) (Owen et al., 2004). The four subunits form a core with two small globular appendages (ear domains α and β) connected through flexible unstructured linkers. The three-dimensional structure of AP-2 and it's different binding sites is discussed below and illustrated in figure 1.5. AP-2 is recruited to the plasma membrane through the interaction of lysine residues in the α-subunit and the μ2-subunit with PtdIns(4,5)P_2 (Gaidarov and Keen, 1999; Page and Robinson, 1995; Rohde et al., 2002). Disruption of these interactions leads to mislocalisation of clathrin and decreased endocytosis of cargo (Gaidarov and Keen, 1999; Rohde et al., 2002). The crystal structure of AP-2 revealed that the two binding sites face different directions, thus inhibiting simultaneous interaction with PtdIns(4,5)P_2 at the plasma membrane. A conformational change is thought to be triggered by the phosphorylation of Thr156 in the μ2 subunit, turning AP-2 into an active conformation, where the two binding sites are exposed on the same side of the protein (Collins et al., 2002; Olusanya et al., 2001). AP-2 interacts with the clathrin N-terminal domain via a clathrin-box motif in the unstructured linker of the β subunit (Lundmark and Carlsson, 2002; Owen et al., 2000; Shih et al., 1995). The adaptor was shown to be required for clathrin assembly under physiological conditions. In addition, AP-2 depleted cells show defects in CME (Hinrichsen et al., 2003; Motley et al., 2003). However, internalisation is not completely abrogated in these cells, suggesting that clathrin recruitment to the plasma membrane is not exclusively dependent on AP-2 (Motley et al., 2003).

AP-2 binds cargo proteins via specific sequences; the acidic dileucine motifs, [D/E]XXXL[L/I] (where X corresponds to any residue), found in clathrin endocytic cargos is thought to interact with the β2 and μ2 subunits, and YXXΦ (where Φ corresponds to a hydrophobic residue), another motif found in cargoes binds to the μ2 subunit of AP-2 and involves a hydrophobic pocket for each of the bulky residues (Bonifacino and Traub, 2003; Owen and Evans, 1998; Honing et al., 2005). However, the crystal structure of the core domain of AP-2 revealed that the binding

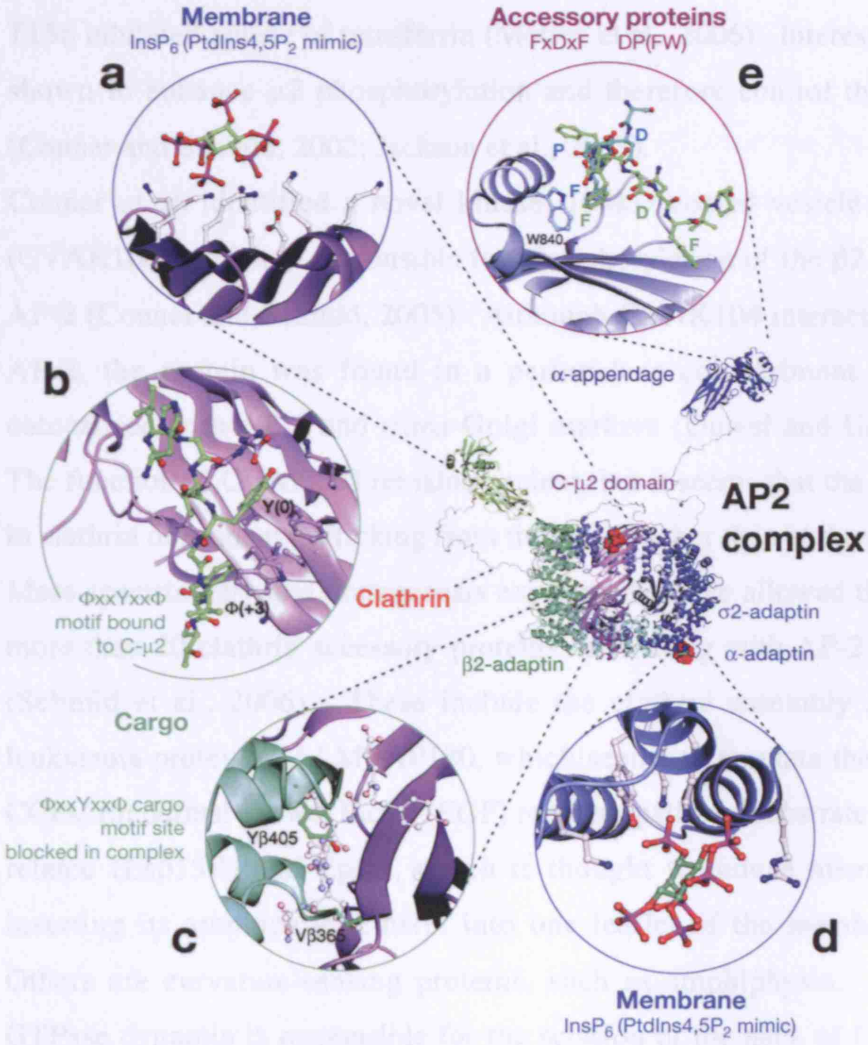


Figure 1.5: Three-dimensional structure of AP-2 and its binding sites

The AP-2 complex is composed of four subunits, α- (blue), σ2- (grey), β2- (green) and μ2-adaptin (purple). μ2-subunit contains a binding site for PtdIns(4,5)P_2 (a) and for ΦXXYXXΦ motif in cargo proteins (b). Unless μ2-subunit is phosphorylated on T156, the pocket for ΦXXYXXΦ motif is buried and not accessible (c). Another binding site for PtdIns(4,5)P_2 is found in the α trunk domain (d). In addition, the α-appendage binds to FXDXF and DP(FW) motifs in accessory proteins (e). (Figure from Owen et al., 2004)

site for YXXΦ is buried and requires a change in the conformation of the adaptor in order to be accessible (Collins et al., 2002). Interactions with cargo proteins are regulated through phosphorylation of the μ2 subunit on T156 by α-adaptin-associated kinase-1 (AAK1) (Conner and Schmid, 2002; Olusanya et al., 2001) suggesting that phosphorylation could induce this conformational change. Indeed, a recent functional analysis showed that mutations in the YXXΦ binding motif or

T156 inhibited uptake of transferrin (Motley et al., 2006). Interestingly, clathrin was shown to enhance μ2 phosphorylation and therefore control the activity of AP-2 (Conner and Schmid, 2002; Jackson et al., 2003).

Conner *et al.* identified a novel kinase, 104kD coated vesicle-associated protein (CVAK104), which is responsible for phosphorylation of the β2-adaptin subunit of AP-2 (Conner and Schmid, 2005). Although CVAK104 interacts with clathrin and AP-2, the protein was found in a perinuclear compartment in cells, where it colocalised with AP-1 and *trans*-Golgi markers (Duwel and Ungewickell, 2006). The function of CVAK104 remains unclear, but it seems that the protein is involved in clathrin dependent trafficking from the Golgi rather than at the plasma membrane.

Mass spectrometry and mutagenesis experiments have allowed the identification of more than 20 clathrin accessory proteins interacting with AP-2 appendages so far (Schmid et al., 2006). These include the clathrin assembly lymphoid myeloid leukaemia protein (CALM)/AP180, which seems to regulate the size and shape of CCPs, Epidermal growth factor (EGF) receptor pathway substrate 15 (Eps15), Eps15 related (Esp15R) and Epsin, which is thought to induce membrane bending by inserting its amphipatic α-helix into one leaflet of the membrane lipid bilayer. Others are curvature-sensing proteins, such as amphiphysin. Finally, the small GTPase dynamin is responsible for the scission of the neck of CCP, leading to the release of CCV into the cytoplasm. In addition, cargo-specific adaptors such as β-arrestin, disabled-2 (Dab2) and autosomal recessive hypercholesterolemia (ARH) have been reported. The role of these specific accessory proteins is discussed below.

1.1.3.3 Cargo-specific and alternative adaptors

Some receptors require other adaptor proteins in addition to, or instead of, AP-2 to access the endocytic pathway. These adaptors are usually cargo-specific or recognise a family of cargo proteins. For example, the arrestin family, that comprises visual arrestins and β-arrestin 1 and 2 (arrestin 2 and 3, respectively), is involved in GPCR endocytosis (Claing et al., 2002) and is discussed in more detail in section 1.2.5. Two other adaptors, Dab2 and ARH, recognise the short peptide sequence FXNPXY within the cytoplasmic tail of the members of the low-density lipoprotein receptor (LDLR) family (Stolt and Bock, 2006).

Work from Motley *et al.* and Conner and Schmid, in which AP2 was knocked down to undetectable level or inactivated, showed that although CME was impaired, it was not completely abrogated (Motley et al., 2003; Conner and Schmid, 2003). This suggested that AP-2 is not essential to CME and that alternative adapters can, at least partially or for some cargos, fulfill its role. In the case of LDLR and EGFR, endocytosis was shown to be clathrin-dependent, but AP-2-independent (Motley et al., 2003; Conner and Schmid, 2003). The interaction of the two cargo-specific adaptors, Dab2 and ARH, with LDLR might be sufficient for the receptor endocytosis. On the other hand, EGFR ubiquitination may promote interaction with Epsin, which was recently proposed to be an adaptor for sorting of ubiquitinated cargo via CME (Hawryluk et al., 2006). Future work monitoring cargos internalisation in the absence of AP-2 and potential adaptors, will allow the identification of cargo-specific and alternative adaptors for CME.

1.1.3.4 CALM and AP180

Morphological studies indicate that endocytic CCV usually have a diameter of 100-200 nm. Two proteins, the ubiquitously expressed CALM, and its neuron-specific homologue AP180, were shown to regulate the shape and the size of CCV (Ahle and Ungewickell, 1986; Dreyling et al., 1996; Morris et al., 1993). Work both *in vitro* and *in vivo* has provided evidence of a role for AP180 in regulating the size and shape of synaptic vesicles (Ahle and Ungewickell, 1986; Nonet et al., 1999; Zhang et al., 1998). Similarly, CALM depleted cells exhibit irregular elongated clathrin-coated buds instead of round CCPs. In addition, unusual small cages were observed budding from flat clathrin lattices (Meyerholz et al., 2005). CALM/AP180 interacts with phosphoinositides in the plasma membrane through their AP180 N-terminal homology (ANTH) domain. ANTH domains are similar to the Epsin N-terminal homology (ENTH) domain, also binding to $PI(4,5)P_2$. However, ANTH domains are not inserted into the lipid bilayer as are ENTH domains (Stahelin et al., 2003).

In addition, CALM/AP180 interact with the clathrin N-terminal domain through a series of DLL motifs (Morgan et al., 2000). However, CALM only associates weakly with clathrin. CALM also binds the α-appendage domain of AP-2 (Mishra et al., 2004). AP180 was shown to bind simultaneously to $PI(4,5)P_2$ and clathrin and

induce the formation of clathrin lattices *in vitro*. In the presence of AP-2, invaginated CCPs were observed (Ford et al., 2001).

Overexpression of AP180 or CALM was shown to inhibit the uptake of EGF and transferrin (Ford et al., 2001; Tebar et al., 1999). However, in CALM-depleted cells, no defect in transferrin uptake was observed, although the intracellular trafficking of the protein appeared to be affected as it did not accumulate in the perinuclear region (Huang et al., 2004; Meyerholz et al., 2005). Under the same conditions, CALM depleted cells showed impaired uptake of EGF.

1.1.3.5 Eps15, Eps15R and Epsin

Eps15 was originally identified as a substrate of EGFR tyrosine kinase (Fazioli et al., 1993). Eps15R was identified shortly after because of the N-terminal domain similarity with Eps15 (Eps15 homology (EH) domain) (Wong et al., 1995). It soon became apparent that Eps15 and Eps15R were linked to the clathrin endocytic machinery as they constitutively bind AP-2 at the plasma membrane through their C-terminal domain (Benmerah et al., 1996; Benmerah et al., 1995; Coda et al., 1998). Further studies demonstrated that Eps15 dimers associate with AP-2 complexes (Tebar et al., 1997) and the protein was observed at the rim of CCP and on CCV (Edeling et al., 2006a; Tebar et al., 1996). Finally, a role for Eps15 in CME was confirmed when the expression of a dominant negative mutant of Eps15 (unable to bind AP-2) blocked transferrin and EGF uptake (Benmerah et al., 1998). Recent structural studies of the β2 appendage of AP-2 and its interactors showed that clathrin competes with Eps15 for binding to AP-2, and therefore displaces it from the complex (Edeling et al., 2006a; Schmid et al., 2006). This explains the localisation of the protein at the edge of CCPs (Tebar et al., 1996).

A number of proteins interact with the EH domain of Eps15 and Eps15R. Among them is Eps15 interacting protein (Epsin) (Chen et al., 1998). Epsin interacts with various components of the clathrin machinery. Its ENTH domain drives its recruitment to the plasma membrane through binding to PtdIns(4,5)P_2. The DPW and NPF motifs in Espin bind to the ear domains of AP-2 and to Eps15 respectively, and a clathrin-binding motif is present in the C-terminus of the protein (Chen et al., 1998; Drake et al., 2000; Itoh et al., 2001). Like Eps15, Epsin contains ubiquitin interacting motifs (UIMs) (Polo et al., 2002). In contrast to the Eps15 UIM, which

has not yet been shown to be involved in any endocytic events, the Epsin UIM is thought to be involved in polyubiquitinated cargo endocytosis (Hawryluk et al., 2006; Klapisz et al., 2002). The Epsin ENTH domain was proposed to induce membrane curvature by penetrating the membrane with an amphipathic helix (Ford et al., 2002; Stahelin et al., 2003). Indeed, Epsin was shown to induce tubulation of liposomes *in vitro*. Furthermore, Epsin was able to recruit and promote polymerisation of clathrin and invagination of CCP onto a monolayer of PtdIns(4,5)P_2 in the absence of AP-2 (Ford et al., 2002; Ford et al., 2001). However, a role for Epsin in CCP invagination remains unclear as flat clathrin lattices are extensively labelled for epsin, and epsin-depleted cells exhibit no obvious defect in transferrin and EGF uptake (Huang et al., 2004; Mishra et al., 2004).

The recruitment of adaptors and accessory proteins to the plasma membrane and their interactions with clathrin have been well described. However, the nature of the forces driving invagination of the membrane to form a CCP, which will eventually pinch off to give rise to a CCV, remains unclear.

1.1.3.6 Dynamin and N-BAR domain containing proteins

The last step of CCP formation at the plasma membrane is the fission reaction that releases a CCV into the cytoplasm. The GTPase dynamin, found in flat clathrin lattices and at the neck of CCPs is thought to play a major role in this process (Baba et al., 1999; Damke et al., 1994). The first evidence came from the ultra-structural EM analysis of a temperature-sensitive mutant of *Drosophila melanogaster*, *shibire* (*shi*) (Kosaka and Ikeda, 1983). At the permissive temperature these flies are normal, but when shifted to the non-permissive temperature, they become rapidly paralysed. EM analysis of the flies' brains under these conditions indicated numerous clathrin-coated profiles in nerve terminals, many of which were still connected to the plasma membrane. *Shi* was subsequently found to encode the fly homologue of the mammalian protein dynamin. When the *Shi* mutation was introduced into mammalian cells, accumulation of similar clathrin-coated invaginations at the plasma membrane was seen (Damke et al., 1994; Damke et al., 2001). In these cells, although transferrin and EGF receptors recruitment in CCP was not impaired, their uptake was inhibited (Damke et al., 1994).

While the precise mechanism of the scission remains unclear, several models in which dynamin is proposed to work as a mechanochemical enzyme or a regulator protein have been suggested and are reviewed by Sever *et al.* (Sever et al., 2000). In addition, recent studies suggest a role for dynamin earlier in the formation of CCPs besides the fission step (Macia et al., 2006; Merrifield et al., 2002).

Dynamin is recruited to the plasma membrane through its interactions with PtdIns(4,5)P_2 (Zheng et al., 1996) and the amphiphysin SH3 domain (Takei et al., 1999). In addition, dynamin binds other SH3 domain-containing proteins including endophilin 1, intersectin and syndapin 1, which are thought to regulate its activity (Simpson et al., 1999).

Amphiphysin also comprises a so-called BAR (Bin/Amphiphysin/Rvs-homology) domain and an N-terminal amphipatic helix; together they form a N-BAR domain (Peter et al., 2004). BAR domains have been shown to promote membrane invagination and *in vitro* and *in vivo* (McMahon and Gallop, 2005; Masuda et al., 2006). Hence, amphiphysin might help to maintain the negative curvature at the neck of the CCP while interacting with dynamin.

While the number of accessory proteins shown to be involved in CME keeps increasing, their precise roles remain unclear. Huge efforts in the last few years have led to the discovery of a web of interactions between adaptors and accessory proteins, clathrin, and the components of the plasma membrane. However, how exactly these proteins interact and combine to form CCVs in cells remains poorly understood.

1.1.4 Dynamics of clathrin endocytosis

Recent advances in live microscopy, combined with the use of fluorescent proteins, has shed light on the dynamic of CCP formation (Gaidarov et al., 1999; Merrifield et al., 2002). In a first study by Gaidarov *et al.*, using green fluorescent protein (GFP) fused to the clathrin light chain a (LCa), clathrin assembly was observed at defined sites of the plasma membrane, described as "hot spots" of endocytosis (Gaidarov et al., 1999). Various intensities of clathrin structures were observed, probably corresponding to different sizes of CCPs, with different lifetimes ranging from 20 to 80 seconds. In addition, clathrin structures exhibited restricted movement at the plasma membrane, suggesting association with the actin cytoskeleton. However, this

study could not distinguish between CCPs attached to the plasma membrane or cytoplasmic CCVs. Subsequent work, using so-called evanescent field (EF) or total internal reflection fluorescence (TIRF) microscopy (Steyer and Almers, 1999), allowed this distinction to be made by imaging <100 nm into the cell. Furthermore, the final events leading to vesicle fission were shown to involve not only dynamin but a series of dynamin interacting proteins, including sorting nexin-9, arp3, neural Wiskott Aldrich syndrome protein (N-WASP), WASP interacting protein, cortactin and actin (reviewed in Perrais and Merrifield, 2005). Although a role for actin polymerisation in vesicle fission has emerged from many studies (reviewed in Merrifield, 2004), work from Fujimoto *et al.* suggested that the degree of actin involvement is cell line-dependent (Fujimoto et al., 2000).

While the chronology of the final events of CCP formation have been the focused of a number of studies in the past few years, the assembly process and the role of the accessory proteins remain vague.

1.1.5 Endocytic activity of clathrin structures

Several populations of clathrin structures that differ mainly in size, lifetime and dynamics are observed at the plasma membrane and have distinct endocytic activity (Ehrlich et al., 2004; Gaidarov et al., 1999; Merrifield et al., 2005). The first of these encompasses small, short-lived, endocytically inactive CCPs, resulting from structures collapsing catastrophically due to their instability (Ehrlich et al., 2004). Bigger structures corresponding to single CCPs of 100-200 nm in diameter have a longer lifetime of 30-60s (Ehrlich et al., 2004; Merrifield et al., 2002). These structures are thought to be stabilised by cargo interacting with endocytic adapters, until dynamin and actin initiate the fission events, leading to the formation of a CCVs.

Larger structures with a lifetime as long as 200s are also observed in all cell lines analysed (Bellve et al., 2006; Ehrlich et al., 2004; Gaidarov et al., 1999; Merrifield et al., 2002; Rappoport and Simon, 2003). These probably correspond to the flat clathrin lattices found both, at the top and the bottom of cells, and extensively described in EM analysis (Heuser, 1980; Signoret et al., 2005). Successive vesicle budding events were seen to occur from these structures, resulting in the term "hot spots" of endocytosis (Gaidarov et al., 1999; Merrifield et al., 2002; Merrifield et al.,

2005; Rappoport et al., 2005). In neuronal dendrites, Blanpied *et al.* also observed "hot spots" for clathrin assembly (Blanpied et al., 2002). In addition, flat clathrin lattices were suggested to be endocytically active in synaptic boutons (Mueller et al., 2004). This is consistent with electron microscopy studies of clathrin structures showing multiple CCPs forming at and budding off the edge of flat clathrin lattices (Heuser, 1980). However, Ehrlich *et al.* have not observed any endocytic events from these stable clathrin structures (Ehrlich et al., 2004). Figure 1.6 outlines three different models proposed for the formation of CCV. In the first model, a growing CCP collapses if no cargo stabilises it. The second suggests that one CCP stabilised by an endocytic cargo will give rise to one CCV only. Finally, the third model shows a flat clathrin lattice where CCVs can form at the edge.

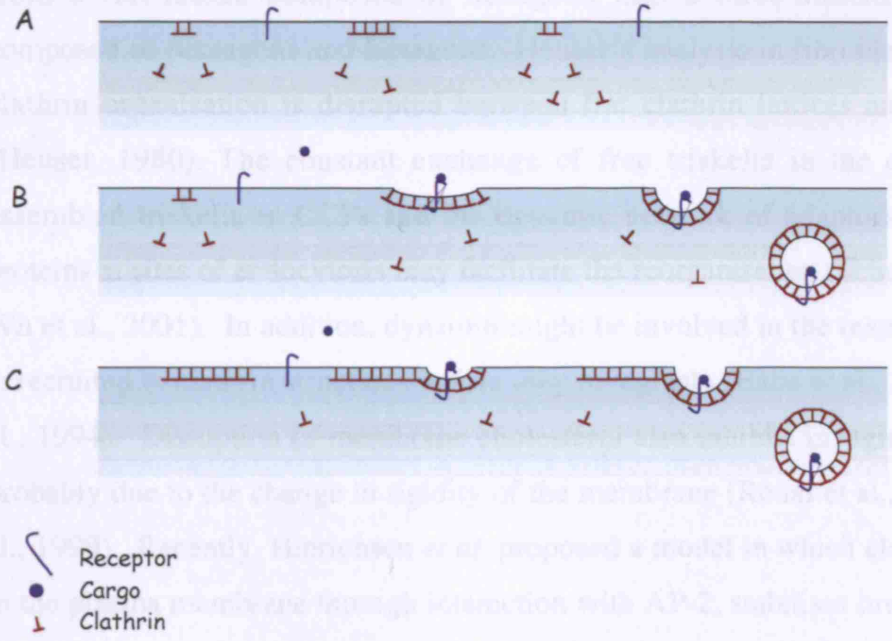


Figure 1.6: Formation of clathrin-coated pits

(A) Clathrin triskelia at the plasma membrane are stabilised by receptors. In the absence of cargo, growing CCPs eventually collapse. (B) In the presence of cargo, assembling CCPs are stabilised and generate single CCVs. (C) Activated cargos are internalised via CCPs forming at the edge of flat clathrin lattices, where multiple budding events may occur.

A study using transferrin as a cargo to further investigate the activity of clathrin structures support the third model illustrated in figure 1.4 (Bellve et al., 2006). Indeed, Bellve *et al.* reported that the transferrin receptor was internalised through vesicles budding from large stable patches of clathrin (Bellve et al., 2006). In

addition, ultra-structural studies of isolated plasma membrane showed clustering of the receptor into flat clathrin lattices (Damke et al., 1994; Lamaze et al., 2001; Miller et al., 1991). Some GPCRs have been shown to be recruited into pre-existing CCPs, together with β-arrestin upon activation (Santini et al., 2002; Scott et al., 2002). In some cases, receptor clustering into CCPs was also shown to affect the size of the pre-existing clathrin structures (Santini et al., 2002). In our studies on agonist-induced endocytosis of CCR5 and CXCR4, receptors are recruited into flat clathrin lattices, where CCPs form at the edge (Signoret et al., 2005 and unpublished results). Altogether, these findings suggest that flat clathrin lattices are endocytically active. However, the mechanism by which curvature is initiated at the edge of the flat lattices is not clear. In order to form a vesicle, the clathrin coat must be rearranged from a flat lattice composed of hexagons into a three-dimensional structure composed of pentagons and hexagons. Heuser's analysis in fibroblasts showed that clathrin organisation is disrupted between flat clathrin lattices and budding pits (Heuser, 1980). The constant exchange of free triskelia in the cytoplasm with assembled triskelia in CCPs and the dynamic network of adaptors and accessory proteins at sites of endocytosis may facilitate the reorganisation (Schmid et al., 2006; Wu et al., 2001). In addition, dynamin might be involved in the rearrangement, as it is recruited to clathrin structures before they invaginate (Baba et al., 1999; Damke et al., 1994). Disruption of membrane cholesterol also inhibits invagination of CCPs, probably due to the change in rigidity of the membrane (Rodal et al., 1999; Subtil et al., 1999). Recently, Hinrichsen *et al.* proposed a model in which clathrin, recruited to the plasma membrane through interaction with AP-2, stabilises inward bending of the membrane to form endocytic vesicles (Hinrichsen et al., 2006).

1.1.6 Different cargos targeted to different CCPs

In addition to their differences in size, lifetime and dynamics, CCPs were suggested to form distinct sub-populations. Indeed, Cao *et al.* showed that although the β_2-adrenergic (β_2AR) and transferrin receptors were internalised through a clathrin mediated pathway, the two receptors used distinct sub-populations of CCPs in human embryonic kidney (HEK) 293 and Hela cells (Cao et al., 1998). β_2AR endocytosis was blocked at 16°C, whereas constitutive internalisation of the transferrin receptor was only partially inhibited under the same conditions.

Furthermore, CCPs differed in their protein coat composition; only a subpopulation of CCPs contained β-arrestins. Interestingly, β_2AR was selectively recruited in these pits. Recent work on two purinergic GPCRs, $P2Y_1$ and $P2Y_{12}$, also suggested the existence of two distinct populations of CCPs in astrocytoma cells (Mundell et al., 2006). Both receptors were internalised via a clathrin-mediated pathway. However, upon activation, $P2Y_1$ and $P2Y_{12}$ were sorted into distinct CCPs on the plasma membrane. In addition, while $P2Y_{12}$ endocytosis was GRK and β-arrestin dependent, $P2Y_1$ endocytosis required PKC and was GRK- and β-arrestin-independent. Although sorting occurred at the plasma membrane, the receptors rapidly converged to the same intracellular endosomal compartment, as also reported for the transferrin receptor and the β_2AR (Cao et al., 1998).

Although these two studies indicate the existence of distinct populations of CCPs at the plasma membrane, it remains to be determined how the protein cargoes are specifically targeted to one or the other. Moreover, if sorting occurs at the cell surface, it is not clear why cargoes are then targeted to the same intracellular compartment. Additional work combining live microscopy and ultrastructural analysis will further elucidate these mechanisms.

1.1.7 Endocytic pathways independent of clathrin

1.1.7.1 Caveolar pathway

The key proteins of the caveolar pathway are caveolins; caveolin-1 and-2 are not ubiquitous but are mainly expressed in epithelial cells and fibroblastic cell types, whereas caveolin-3 is only found in muscles (Okamoto et al., 1998). The expression of caveolin-1 was shown to be sufficient to induce the formation of 70 nm, smooth flask-shape invaginations of the plasma membrane, caveolae (Fra et al., 1995). Caveolae were originally thought to mediate transcytosis of serum proteins from the bloodstream into tissues across endothelial cells. To date, a handful of cargoes have been shown to be internalised via the caveolar pathway, including EGFR and the transforming growth factor β receptor (TGFβR), which also both use the clathrin mediated pathway (Di Guglielmo et al., 2003; Sigismund et al., 2005). In addition to caveolae, intracellular structures of neutral pH containing caveolins were identified as caveosomes (Pelkmans et al., 2001). Caveolae and caveosomes are illustrated in figure 1.1. The lipid composition of caveolae is similar to that of lipid rafts, rich in

cholesterol and sphingolipids, and regulates the rate of caveolar endocytosis (Sharma et al., 2004). Moreover, caveolae are rich in GPI-anchored proteins (Sargiacomo et al., 1993).

Simian virus 40 (SV40) enters cells via caveolae and has proved to be an excellent tool to study the caveolar pathway (Pelkmans and Helenius, 2002). Actin and dynamin were shown to be involved in the initial stages of SV40 internalisation via caveolae (Pelkmans et al., 2002). Once inside the cells, caveolae can fuse with early endosomes and caveosomes but retain their identity; no exchange of caveolin occurs in contrast to other membrane coat proteins, which assemble and disassemble continuously (Pelkmans et al., 2004; Tagawa et al., 2005).

Beside SV40, a series of ligands, membrane constituents, toxins and viruses are also internalised through the caveolar pathway (Pelkmans and Helenius, 2002). In addition, some GPCRs have been observed in caveolae, such as the adenosine A1 and the somatostatin SST2 receptors (Escriche et al., 2003; Gines et al., 2001; Krisch et al., 1998; Lasley et al., 2000; Mentlein et al., 2001). Other reports suggested association of GPCRs with caveolin, however, significant inconsistency exist across these reports (Chini and Parenti, 2004). For example, the chemokine receptor CCR5 was reported to associate with lipid raft domains (Manes et al., 1999; Venkatesan et al., 2003), and suggested to be internalised through both the caveolae and the clathrin-mediated pathway (Mueller et al., 2002; Venkatesan et al., 2003). Yet, CCR5 association with rafts was questioned (Percherancier et al., 2003). Moreover, evidence for internalisation through caveolae comes from experiments using drugs disturbing membrane cholesterol, thought to block specifically the caveolar pathway. However, these drugs not only abolish agonist-binding to the receptor (Nguyen and Taub, 2002; Nguyen and Taub, 2003a), but also affect CME (Rodal et al., 1999; Subtil et al., 1999). In addition, no evidence for CCR5 internalisation through caveolae by electron microscopy has been observed (Signoret et al., 2005 and our unpublished results).

1.1.7.2 Caveolae-independent pathways

In addition to clathrin- and caveolin-dependent pathways, endocytic pathways independent of both coat proteins have been reported. For example, SV40 was observed entering cells devoid of caveolin-1 in a clathrin-, dynamin 2- and Arf6-

independent manner (Damm et al., 2005). A similar pathway was described for cholera toxin uptake in caveolin-1 *null* mouse embryonic fibroblasts (Kirkham et al., 2005). In addition, receptor endocytosis was also suggested to occur independently of clathrin and caveolin in some cases. For example, the IL-2 receptor internalisation pathway in lymphocytes was Eps15 and AP-2 independent, but dynamin-dependent (Lamaze et al., 2001). Recently, flotillin-1 was reported to define an endocytic pathway independent of clathrin, caveolin, and dynamin (Glebov et al., 2006). Although evidence is accumulating for these new endocytic pathways, little is known on their mechanisms and significance.

1.1.8 Clathrin association with intracellular membranes

Clathrin coats are not only found at the plasma membrane but also at the *trans*-Golgi network (TGN), on some endosomes and vesicles or tubules derived from these compartments (Bonifacino and Lippincott-Schwartz, 2003). Clathrin is targeted to intracellular membranes via interaction with adaptor proteins similar to AP-2, which are recruited to specific intracellular membranes due to the composition in their phosphoinositide (Robinson, 2004). In addition, a family of monomeric adaptors has recently been identified, the Golgi-localised, γ-ear-containing, ADP ribosylation factor-binding proteins (GGAs). As their name suggests, GGAs are components of clathrin coats at the TGN. Finally, clathrin coats were also observed on early endosome but found to differ from both, plasma membrane and TGN clathrin coats, in composition and organisation (Sachse et al., 2002). By EM the coat seemed composed of two electron-dense layers in which no AP complexes were detected. Clathrin was the main component of the outer layer, whereas the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) was present in the inner layer (Raiborg et al., 2002).

1.1.9 Other types of coated vesicles

Clathrin- and caveolin-coated vesicles are not the only types of coated vesicles in cells. Coatomer protein I and II (COPI and COPII) coated vesicles mediate trafficking between the Golgi and the endoplasmic reticulum (ER) (McMahon and Mills, 2004). Conversely, they are not present at the plasma membrane.

1.2 CC chemokine receptor 5 regulation

The plasma membrane is a barrier where receptors translate extracellular cues into intracellular signals. For example, the family of 7TM GPCRs, comprising more than 600 members in the human genome, responds to a huge range of signals including light, odours, nucleotides, ions, lipids and peptides (Gether, 2000). GPCRs transmit extracellular signals to the intracellular milieu via interaction with heterotrimeric G-proteins. Upon recruitment to the receptor, the GDP associated with the α-subunit of the G-protein is exchanged for GTP. The α-subunit separates from the βγ-dimer and both can then bind to effectors and mediate distinct cellular signalling pathways. In order to generate an appropriate cellular response, receptor activation, de-activation and expression at the plasma membrane require tight regulation. This occurs through interaction with cellular proteins and endocytosis. The best-characterised and most common GPCR internalisation pathway is the one mediated by clathrin. However, clathrin-independent mechanisms such as the caveolar pathway have been proposed to regulate internalisation of activated receptors (Chini and Parenti, 2004).

Figure 1.7 outlines agonist-induced internalisation of GPCRs via the clathrin pathway based on the β_2AR. First, agonist binding activates the receptor and promotes guanine nucleotide exchange in a heterotrimeric G-protein, which mediates downstream signalling (1). GPCR kinases (GRKs) subsequently phosphorylate serines or threonines in the C-terminal tail of the receptor (2). Phosphorylation of these residues induces recruitment of β-arrestin, which uncouples the G-protein from the receptor and links the receptor to the endocytic machinery (3). Once internalised, receptors are either degraded (4) or recycled back to the plasma membrane in a resensitised form (5). The fate of the receptor depends on various factors such as the agonist, the cellular environment and endocytic signal sequences in the cytoplasmic tail of the receptor.

Chemokine receptors form a sub-family of GPCRs, that mediate leukocyte activation and recruitment to sites of inflammation by binding to chemokines in the extracellular environment. CC chemokine receptor 5 (CCR5), which is expressed mainly on memory T cells, macrophages and dendritic cells, has been extensively studied in the past decade (Berger et al., 1999). In addition to its role in leukocyte trafficking, CCR5 has been identified as a co-receptor for human immunodeficiency

virus-1 (HIV-1), as has the CXC chemokine receptor 4 (CXCR4). Interestingly, individuals homozygous for a 32bp deletion within the coding region of the CCR5 gene, encoding a truncated receptor that fails to reach the plasma membrane, are highly resistant to HIV-1 infection (Benkirane et al., 1997; Liu et al., 1996). In addition, the natural ligands of the co-receptors have been shown to be powerful inhibitors of virus entry in tissue culture models by inducing receptor endocytosis (Bleul et al., 1996; Cocchi et al., 1995; Oberlin et al., 1996). In this section, the regulation of CCR5 will be reviewed.

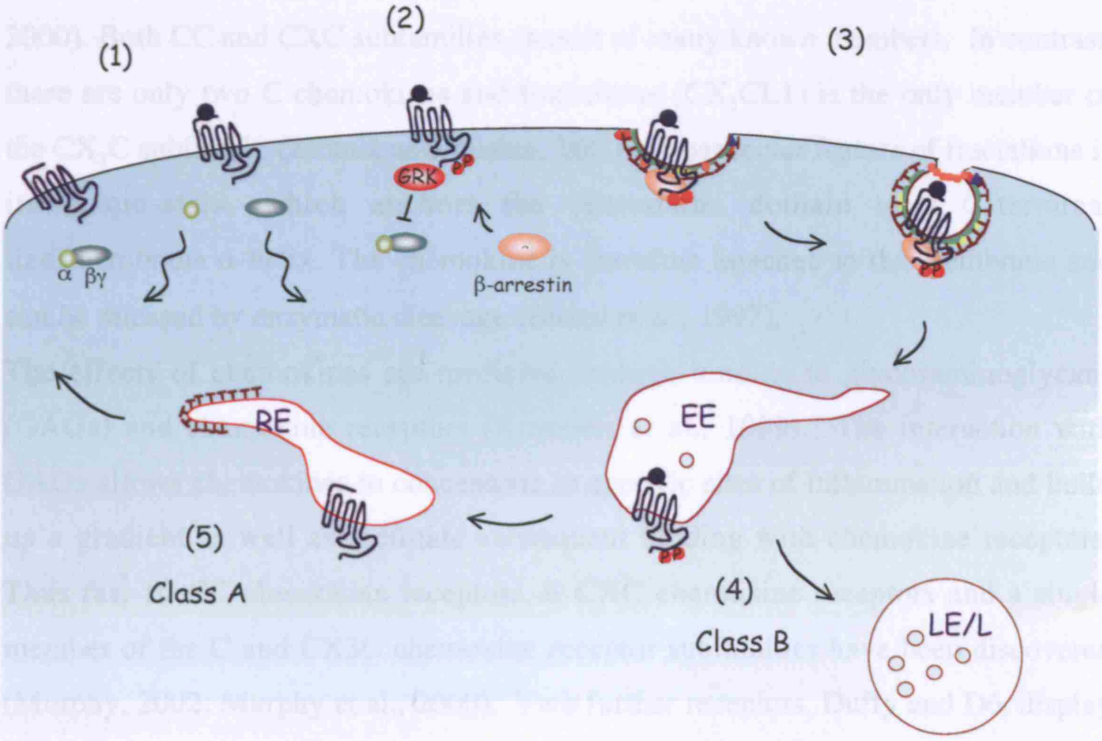


Figure 1.7: Model for agonist-induced internalisation of GPCRs based on the β_2AR internalisation via clathrin-coated pits

Agonist binding to GPCRs triggers activation of a G-protein, which then mediates downstream signalling (1). Desensitisation occurs through phosphorylation of C-terminal residues in the C-terminal cytoplasmic domain of the receptor, and recruitment of β-arrestins (2). β-arrestins serve both, to uncouple the G-protein and attenuate signalling, and to act as an adaptor, linking GPCRs to the endocytic machinery by interacting with clathrin and AP-2 (3). Once in early endosomes (EE), the receptors can take two different routes; they can be directed to late endosomes and lysosomes (LE/L) where they will eventually be degraded (4), or the receptor-ligand complexes are dissociated (due to the acidic pH in EE in the case of β_2AR) and receptors are sent back to the plasma membrane in a resensitised form through the recycling endosomes (RE) (5).

1.2.1 Chemokine receptors: definition and nomenclature

Chemokines are a family of chemotactic cytokines that influence cell activity through interaction with specific cell surface receptors. More than 50 chemokines have now been identified, as shown in table 1.1. They are divided into four subgroups according to the position of conserved cysteine residues in their amino termini. In the CC chemokines, the two cysteine residues are adjacent; in the CXC chemokines, the two cysteine residues are separated by an amino acid; in the C chemokines, the first cysteine residue is missing; and finally in the CX_3C chemokines, three amino acids separate the two cysteine residues (Rossi and Zlotnik, 2000). Both CC and CXC subfamilies consist of many known members. In contrast, there are only two C chemokines and fractalkine (CX_3CL1) is the only member of the CX_3C subfamily (Zlotnik and Yoshie, 2000). A particular feature of fractalkine is its mucin-stalk, which anchors the chemokine domain to a C-terminal transmembrane α-helix. The chemokine is therefore attached to the membrane and can be released by enzymatic cleavage (Bazan et al., 1997).

The effects of chemokines are mediated through binding to glycosaminoglycans (GAGs) and chemokine receptors (Kuschert et al., 1999). The interaction with GAGs allows chemokines to concentrate in specific sites of inflammation and build up a gradient as well as facilitate subsequent binding with chemokine receptors. Thus far, 10 CC chemokine receptors, 6 CXC chemokine receptors and a single member of the C and CX3C chemokine receptor subfamilies have been discovered (Murphy, 2002; Murphy et al., 2000). Two further receptors, Duffy and D6, display structural similarity with chemokine receptors and are able to bind chemokines, but to date have failed to show any signalling activity (Bonini et al., 1997; Neote et al., 1994; Nibbs et al., 1997). Receptors of the same subfamily share high amino acid sequence similarity, but are poorly related to the other subfamilies. In addition to sequence similarity, chemokine receptors can also be divided into two groups according to their role in homeostasis and inflammation; CXCR4, CXCR5, CCR4, CCR7 and CCR9 are homeostatic receptors, involved in immune surveillance, while CXCR1, CXCR2, CXCR3, CCR1, CCR2, CCR3, CCR5, CCR6 are inflammatory receptors. Chemokine receptors and their ligands are presented in table 1.1.

1.2.2 Various roles of chemokines and their receptors

Originally chemokines were found to play a key role in inflammation by regulating the trafficking and activation of leukocytes. It is now clear that chemokines act on a wide variety of cells and are not only involved in the immune system but also in other biological processes including homeostasis, haematopoiesis, angiogenesis, and the development of the immune, circulatory and central nervous systems. Some chemokines and their receptors have also been shown to be up-regulated in tumour cells and hijacked or mimicked by pathogens and viruses (Rosenkilde, 2005; Zlotnik, 2006). For example, HIV-1 envelope interaction with CCR5 and/or CXCR4

Table 1.1 Chemokine receptors and their ligands

Receptor	Ligands
CC chemokine receptors	
CCR1	CCL3, 5, 7, 8, 13, 14, 15, 16, 23
CCR2	CCL2, 7, 8, (12)*, 13
CCR3	CCL5, 7, 8, 11, 13, 15, 24, 26
CCR4	CCL17, 22
CCR5	CCL3, 4, 5, 8
CCR6	CCL20
CCR7	CCL19, 21
CCR8	CCL1
CCR9	CCL25
CCR10	CCL27
CXC chemokine receptors	
CXCR1	CXCL1, 6, 8
CXCR2	CXCL1, 2, 3, 5, 6, 7, 8
CXCR3	CXCL9, 10, 11
CXCR4	CXCL12
CXCR5	CXCL13
CXCR6	CXCL16
C chemokine receptor	
XCR1	XCL1, 2
CX_3C chemokine receptor	
CX_3CR1	CX_3CL1

Table modified from (Murphy, 2002)

* unknown in human, but known in mouse

together with CD4 enables the viral membrane to fuse with the plasma membrane of target cells and the subsequent release of the viral capsid into the host cytoplasm (Lusso, 2006). There is some functional redundancy within some members of the chemokine receptor family; mouse knock-outs for CCR5, or CCR5-deficient humans, appear to be healthy and only have partial defects in their immune system (Benkirane et al., 1997; Liu et al., 1996; Zhou et al., 1998). In contrast, the majority

of CXCR4 knock-out mice die perinatally and exhibit defects in B cell lymphopoeisis (Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998).

1.2.3 CCR5 structure

CCR5 is a 40.6 kD protein composed of 352 amino acids. As all GPCRs, CCR5 has an extracellular N-terminus, seven trans-membrane domains and a cytoplasmic C-terminus. Although the three-dimensional structure of CCR5 has not been determined, models based on the rhodopsin x-ray structure have been proposed and have been useful in understanding the specificity of chemokine binding and the interaction with HIV envelope proteins (Dragic et al., 2000; Govaerts et al., 2003; Paterlini, 2002). Further information on the conformation of the receptor has been obtained using monoclonal antibodies (Blanpain et al., 2002; Lee et al., 1999; Olson et al., 1999). A schematic representation of CCR5 is presented in figure 1.8. Highlighted residues are described below.

Four cysteines (orange in fig. 1.8) form disulfide bonds between the N-terminus segment and the third extracellular domain (C20 and C269) and between the first and second extracellular segments (C101 and C178). These two disulfide bridges maintain the receptor in a conformation that is able to bind agonists and signal properly (Blanpain et al., 1999a). Activation of CCR5 by chemokines has also been shown to rely on a conserved aromatic motif TVP (yellow in fig. 1.8) in the second transmembrane domain (Govaerts et al., 2001; Govaerts et al., 2003). Another conserved feature of the chemokine receptor involved in G-protein and β-arrestins interaction is the DRYLAVVHA (blue in fig. 1.8) motif found in the second cytoplasmic loop (Farzan et al., 1997; Lagane et al., 2005). Finally, the cytoplasmic C-terminal domain of the receptor contains three cysteines (C321, C323 and C324 green in fig. 1.8) and four serines (S336, S337, S342 and S349 red in fig. 1.8) that play key roles in agonist-induced CCR5 signalling, desensitisation and internalisation (Kraft et al., 2001).

1.2.3.1 CCR5 post-translational modifications

CCR5 undergoes post-translational modifications that not only affect the structure of the receptor and the binding to chemokines but also the signalling, desensitisation and internalisation of the receptor.

Tyrosines (purple residues in fig. 1.8) in the amino-terminal domain of CCR5 are modified by sulphate, which modulates binding of chemokine and HIV-1 envelope glycoprotein gp120 (Farzan et al., 1999). The first tyrosine, Y3, is definitely sulphated, and at least one other tyrosine Y10 and/or Y14 is modified. Additionally, O-link glycosylation of serine in position 6 (pink in fig. 1.8) is important for agonist binding (Bannert et al., 2001; Farzan et al., 1999). The carboxy-terminal domain of CCR5 also contains several key residues that can be modified. Three cysteines were shown to be palmitoylated (Blanpain et al., 2001; Kraft et al., 2001). Asides from its potential role for receptor association with raft domains in the plasma membrane, acylation of CCR5 was shown to be important for agonist-induced phosphorylation of serines 336, 337, 342 and 349 by GRK and PKC and in receptor desensitisation (Kraft et al., 2001). Table 1.2 summarises CCR5 post-translational modifications and all residues subjected to modification are highlighted in figure 1.8.

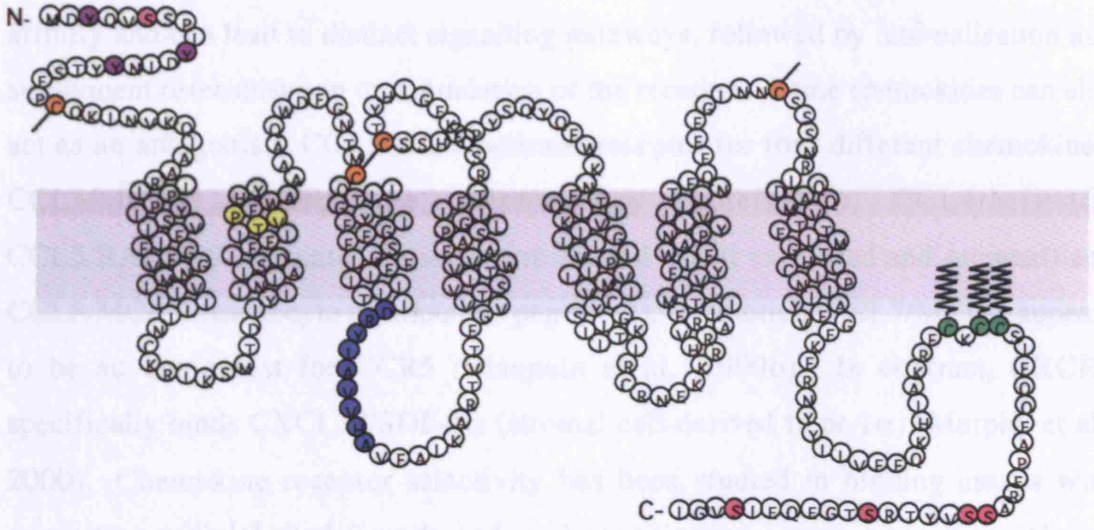


Figure 1.8: CCR5 sequence and model structure

A serpentine model illustrating CCR5 structure. Highlighted amino acids are important for maintaining the receptor structure or play important roles in receptor activation and desensitisation.

Table 1.2: Post-translational modifications of CCR5

Residues	Modification	Role	References
C20-C269 and C101-C178	Disulfide bridges	Conformation	(Blanpain et al., 1999a)
Y3 (and maybe Y10/14)	Sulphation	Chemokine and gp120 binding	(Farzan et al., 1999)
S6	O-linked glycosylation	Chemokine binding	(Bannert et al., 2001; Farzan et al., 1999)
C321/323/324	Palmitoylation	Association with rafts domains, 4th intracellular loop	(Blanpain et al., 2001; Kraft et al., 2001)
S336/337/342/349	GRK/PKC phosphorylation	Activation, desensitisation	(Kraft et al., 2001; Pollok-Kopp et al., 2003)

1.2.4 CCR5 activation and signalling

1.2.4.1 Chemokine binding

Chemokine receptors are activated following the binding of cognate chemokines (table 1.1). Interactions of different chemokines with the same receptor can differ in affinity and can lead to distinct signalling pathways, followed by internalisation and subsequent resensitisation or degradation of the receptor. Some chemokines can also act as an antagonist. CCR5 is a functional receptor for four different chemokines, CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL4/MIP-1β, CCL5/RANTES (regulated on activation normal T cell expressed and secreted) and CCL8/MCP-2 (monocyte chemotactic peptide-2). In addition, CCL7/MCP-3 appears to be an antagonist for CCR5 (Blanpain et al., 1999b). In contrast, CXCR4 specifically binds CXCL12/SDF-1α (stromal cell-derived fator-1α) (Murphy et al., 2000). Chemokine receptor selectivity has been studied in binding assays with competing radiolabelled ligands and in desensitisation assays measuring calcium mobilisation (Oppermann et al., 1999; Simmons et al., 1997). Blanpain *et al.* proposed the following model for chemokine interaction with CCR5; the core domain of the chemokine mediates high affinity binding to the receptor through interaction with residues in the N-terminus and the second extracellular loop, whereas the N-terminal domain of the chemokine regulates receptor activation through interaction with residues in the transmembrane domains (Blanpain et al., 2003).

1.2.4.2 Signalling through G-proteins

As with all GPCRs, chemokine receptors interact with G-proteins. Activation via chemokine binding induces dissociation of $G_{\alpha\beta\gamma}$ subunits and subsequent signalling through G_{α} and $G_{\beta\gamma}$. Different families of G_{α} subunits exist: $G_{\alpha s}$ stimulates adenylate cyclase, $G_{\alpha i/o}$ inhibits adenylate cyclase and mediates other signalling events, $G_{\alpha q/11}$ activates phospholipase C-β (PLCβ) and $G_{\alpha 12/13}$ regulates Rho proteins (Preininger and Hamm, 2004). In parallel, $G_{\beta\gamma}$ can activate various enzymes such as phospholipids kinases, lipases and guanine nucleotide-exchange factors (GEFs), which then lead to the production of second messengers such as Ins(1,4,5)P_3 (IP_3), Ca^{2+} and diacylglycerol (DAG). Among other cellular responses, chemotaxis is induced by chemokine receptor signalling through G-proteins (Lagane et al., 2005).

In the case of CCR5, the sensitivity of adenylyl cyclase regulation to pertussis toxin suggests a $G_{\alpha i}$-protein coupled mechanism (Aramori et al., 1997; Blanpain et al., 2001; Zhao et al., 1998). In contrast, the increase in intracellular Ca^{2+} induced by agonist binding is insensitive to pertussis toxin and therefore is likely to be coupled to a $G_{\alpha q}$-protein (Deng et al., 1996; Farzan et al., 1997; Lagane et al., 2005). CCR5 signalling also occurs through $G_{\beta\gamma}$, which activates protein kinase C (PKC) through PLC-β, and is important for receptor desensitisation, as discussed in the next section.

1.2.5 Desensitisation

GPCR signal transduction is tightly controlled and can be rapidly attenuated through desensitisation of the receptor, a process by which the G-protein is uncoupled from the receptor. A conserved mechanism of desensitisation involves phosphorylation of key residues in the C-terminus of the receptor and subsequent recruitment of β-arrestins, which leads to internalisation of the receptor and degradation or resensitisation.

1.2.5.1 GRK/PKC phosphorylation

GPCRs are phosphorylated by GRKs and second messenger-dependent kinases, such as cyclic AMP-dependent kinase and PKC. While GRKs only target agonist-activated receptors, second messenger-dependent kinases can phosphorylate non-

activated receptors. One of the first experiments suggesting a role for phosphorylation in GPCR signalling regulation came from a study by Sibley *et al.* showing that β2-adrenergic receptor (β2AR) desensitisation coincided with phosphorylation of the receptor at the plasma membrane (Sibley et al., 1986). The authors also showed that intracellular sequestration resulted in dephosphorylation of the receptor, and proposed a mechanism for desensitisation and recycling of the βAR relying on phosphorylation and dephosphorylation mediated by specific kinases (GRKs) and phosphatases. Since then, GRKs have been found to be involved in the regulation of numerous GPCRs. Seven GRKs have been described so far (Pierce et al., 2002). GRK1 and GRK7 are retinal enzymes, while GRK4 is specific to the brain, kidney and testes, and the remaining four GRKs are ubiquitously expressed.
In the case of CCR5, four C-terminal serines, S336, S337, S342 and S349, were shown to be phosphorylation sites for overexpressed GRKs in COS-7 or HEK 293 cells (Aramori et al., 1997; Oppermann et al., 1999). Further studies, in RBL cells, which have high levels of endogenous GRKs, showed that CCR5 was specifically phosphorylated by GRK2/3 on S349, whereas S337 phosphorylation was mediated by PKC (Pollok-Kopp et al., 2003). Moreover, the kinetics observed for these two phosphorylation events were different upon CCL5 binding; GRK-mediated phosphorylation was slower than that mediated by PKC with a $t_{1/2}$ of 1.5 to 2 minutes versus 10 seconds, respectively. The authors also proposed that dephosphorylation of S337 and S349 was mediated by different phosphatases, as $t_{1/2}$ of dephosphorylation of S337 after agonist removal was 90 seconds compared to 12 minutes for S349. In addition, an *in vitro* assay showed that okadaic acid, an inhibitor of protein phosphatases 1 (PP1) and 2a (PP2A), effectively blocked S337 dephosphorylation but had no effect on S349 dephosphorylation. These findings reveal how different these two serines are and suggest a distinct role for their phosphorylation in receptor desensitisation.

1.2.5.2 β-arrestins

Agonist-induced phosphorylation of the C-terminal serines of GPCRs induces rapid recruitment of arrestins to the plasma membrane. Four arrestin genes have been identified so far; visual arrestin (arrestin-1) and cone arrestin (arrestin-4) are found in the retina, whereas β-arrestin 1 and 2 (or non-visual arrestins 2 and 3) are

ubiquitously expressed (Pierce et al., 2002). Binding of β-arrestins to GPCRs uncouples the G-protein from the receptor and therefore inhibits signalling. Furthermore, β-arrestins have been linked to GPCR endocytosis through their interaction with AP-2 and the N-terminal domain of clathrin (Goodman et al., 1997; Goodman et al., 1996; Krupnick et al., 1997; Laporte et al., 2000; Laporte et al., 1999), and their ability to mediate the recruitment of receptors into CCPs (Goodman et al., 1996; Santini et al., 2002; Scott et al., 2002). It is notable that β-arrestin 2 has a higher affinity for clathrin than β-arrestin 1, and visual arrestins do not interact with clathrin (Goodman et al., 1996; Krupnick et al., 1997). Indeed, the clathrin binding domain in β-arrestin 2 is localised to a short C-terminal stretch of amino acids highly conserved in β-arrestin 1, but absent in visual arrestins (Krupnick et al., 1997). Moreover, binding of β-arrestins to AP-2 is independent of the association of β-arrestins with clathrin (Laporte et al., 2000).

Differential roles for β-arrestin 1 and 2 have emerged from various studies modifying the level of expression of both β-arrestins or investigating different GPCRs (Kohout et al., 2001; Santini et al., 2000). In RBL cells, where both β-arrestins are endogenously expressed at comparable level, only β-arrestin 2 was recruited to activated β_2AR and m1 muscarinic acetylcholinergic receptor (m1AChR) (Santini et al., 2000). In addition, a study in mouse embryonic fibroblasts from mouse knock-outs for only one or both isoforms of β-arrestins, suggested that both isoforms could support desensitisation of some GPCRs, while internalisation was regulated differently depending on the receptor. These authors also showed that β-arrestin 2 was much more efficient than β-arrestin 1 in β2AR internalisation (Kohout et al., 2001). Although most GPCRs require β-arrestins for efficient desensitisation and internalisation, β-arrestin-independent mechanisms have also been reported (Claing et al., 2002).

A few chemokine receptors have been shown to interact with β-arrestins upon ligand binding. Among them are CCR5 and CXCR4 (Aramori et al., 1997; Cheng et al., 2000; Orsini et al., 1999). β-arrestin binding to CCR5 was shown to be dependent on phosphorylation of C-terminal serines and on a conserved sequence motif, Asp-Arg-Tyr (DRY), in the second intracellular loop (Huttenrauch et al., 2002; Kraft et al., 2001). In particular, a translocation assay measuring the recruitment of β-

arrestins to the plasma membrane revealed the requirement of any two phosphorylation sites out of four (Huttenrauch et al., 2002).

Internalisation of CCR5 was shown to be β-arrestin-dependent (Fraile-Ramos et al., 2003). However, a phosphorylation deficient mutant of CCR5, CCR5 ΔS, which fails to recruit β-arrestin, was endocytosed upon agonist binding, but at a much slower rate than the wild-type receptor (Huttenrauch et al., 2002; Kraft et al., 2001). As the constitutive rate of internalisation of CCR5 has not yet been determined, it remains unknown as to whether CCR5 ΔS internalisation was activated by agonist binding or reflected the basal endocytosis of the receptor.

Beside their roles in receptor desensitisation and in CME, β-arrestins were recently shown to interact with several signalling proteins including Src family tyrosine kinases, and components of the ERK1/2, JNCK3 and p38 MAP kinases cascades (Shenoy and Lefkowitz, 2005). β-arrestins are thought to function as scaffolds for signalling complex, thus linking GPCRs to intracellular signalling. In the case of chemokine receptors, specifically CCR5 and CXCR4, β-arrestin signalling scaffolds have been implicated in chemotaxis (Sun et al., 2002). However, a study in macrophages suggested that chemotaxis is mediated by $G_{\alpha i}$-protein signalling via PI3Kγ (Weiss-Haljiti et al., 2004). Further investigations are required in order to fully understand the molecular mechanisms and the effects of β-arrestin signalling scaffolds. Nevertheless, these studies shed light on the complexity of signalling events elicited by agonist binding to these receptors.

1.2.6 Agonist-induced internalisation, recycling and resensitisation

1.2.6.1 Models for internalisation and recycling of GPCRs

Following recruitment of β-arrestins to the plasma membrane, most GPCRs undergo efficient internalisation, a process required for degradation or resensitisation of receptors. Although most receptors share a common mechanism of internalisation via the clathrin-mediated pathway, their intracellular trafficking can differ and was shown to depend on their association with β-arrestins (Oakley et al., 1999). In addition, the stability of the receptor/β-arrestin complex was shown to be determined by the ubiquitination status of β-arrestin (Shenoy and Lefkowitz, 2003). According to their interaction with β-arrestins and their fate following internalisation, receptors are divided into two classes, A and B (Oakley et al., 2000). Class A receptors such

as the β_2AR have a low affinity for β-arrestins, quickly dissociate from the adaptor once internalised and are recycled back to the plasma membrane in a resensitised form. Conversely, class B receptors, such as the angiotensin II type 1a receptor (AT1aR), have a much higher affinity for β-arrestins, exhibit a prolonged intracellular association with the adaptor proteins and can be directed to degradation rather than recycled to the plasma membrane (Anborgh et al., 2000). In addition, Shenoy and Lefkowitz showed that this stable association was dependent on ubiquitination of specific lysines in β-arrestin 2 (Shenoy and Lefkowitz, 2005). The differential trafficking of the two classes of receptors is illustrated in figure 1.7. The process of receptor resensitisation of class A GPCRs requires dissociation of the ligand and dephosphorylation of C-terminal residues. This occurs in early endosomes in the case of β_2AR, where receptor-ligand complexes are exposed to acidic pH and phosphatases (Krueger et al., 1997; Pitcher et al., 1995). A third class of receptors has been described following studies of the *N*-formyl peptide receptor (FPR) (Vines et al., 2003). Indeed, FPR internalisation was shown to be β-arrestin-independent, whereas recycling of the receptor required β-arrestin interaction.

1.2.6.2 CCR5 internalisation and recycling

Agonist-induced CCR5 endocytosis has been extensively studied in the past few years as it was shown to prevent HIV infection in tissue culture models (Berger et al., 1999). As mentioned in the previous section, CCR5 was shown to interact with β-arrestins upon agonist binding and down-modulation of the receptor was found to be β-arrestin-dependent (Aramori et al., 1997; Fraile-Ramos et al., 2003; Huttenrauch et al., 2002; Kraft et al., 2001; Vila-Coro et al., 1999). A recent study combining morphological and biochemical analyses demonstrated the involvement of clathrin and AP-2 in CCL5-stimulated internalisation of the receptor (Signoret et al., 2005). This is in contrast to other reports suggesting CCR5 endocytosis to be mediated via the caveolar pathway (Mueller et al., 2002; Venkatesan et al., 2003). However, the latter studies were based on the effect of membrane cholesterol extracting agents, which were subsequently shown to affect agonist binding to CCR5 (Nguyen and Taub, 2002; Nguyen and Taub, 2003a; Nguyen and Taub, 2003b; Signoret et al., 2005). Following endocytosis, the receptor accumulates in perinuclear recycling endosomes (Pollok-Kopp et al., 2003; Signoret et al., 2000;

Signoret et al., 1998), where it remains associated with β-arrestins (our unpublished data). Although CCR5 interacts with both β-arrestin 1 and 2 and shows prolonged association with them intracellularly, the receptor is not targeted to degradation but recycles back to the plasma membrane like the β_2AR (Mueller et al., 2002; Signoret et al., 2004; Signoret et al., 2000). However, unlike the β_2AR, which reaches the cell surface in a resensitised form ready for further stimulation after recycling, CCR5 can recycle back to the plasma membrane in an agonist-bound form (Signoret et al., 2000). Under these circumstances, the receptor can undergo multiple rounds of internalisation and recycling. This may be important for agonist dissociation, the mechanism of which remains unclear. Furthermore, an N-terminal modified form of CCL5, Aminooxypentane (AOP)-RANTES, which has a greater affinity for the receptor than CCL5, was shown to induce accumulation of the receptor in the recycling endosome, but the receptor did not reappear at the plasma membrane following agonist removal (Mack et al., 1998). It was later shown that AOP-RANTES binding to CCR5 does not inhibit recycling but induces continuous endocytosis through recycling endosomes. The agonist was suggested to modify the receptor in such a way that it is constantly recognised by the endocytic machinery and cannot re-accumulate at the plasma membrane (Signoret et al., 2000). Although these studies have provided insights into CCR5 internalisation pathway, they also demonstrated the complexity of the desensitisation process.

1.2.6.3 Resensitisation of CCR5

Intracellular trafficking of CCR5 might be required for dephosphorylation of the receptor and dissociation of the agonist. However, the mechanisms leading to the resensitisation of CCR5 remain unclear. Unlike the situation with the β_2AR, CCR5/agonist complex dissociation is not dependent on acidic endosomal pH (Signoret et al., 2004). Moreover, the association of CCR5 with β-arrestins in early/sorting endosomes (unpublished results) is likely to render dephosphorylation of the receptor more difficult, as phosphatases might not have access to phosphorylated residues. Further studies will be required to identify the molecular events leading to CCR5 resensitisation. Figure 1.9 illustrates CCR5 internalisation and the recycling pathway upon agonist binding.

1.3 Aim of this thesis

Although the clathrin endocytic machinery has been well studied, the early steps of endocytosis, including the recruitment of clathrin to activated GPCRs at the plasma membrane and the formation of CCV, remain poorly understood. The aim of my thesis was to shed light on the molecular mechanisms leading to GPCR endocytosis via the clathrin pathway following agonist binding. CCR5 expressed in rat basophilic leukaemia (RBL) cells was used as a model receptor and its internalisation was analysed in detail morphologically and biochemically. All methods used in this thesis are described in Chapter 2.

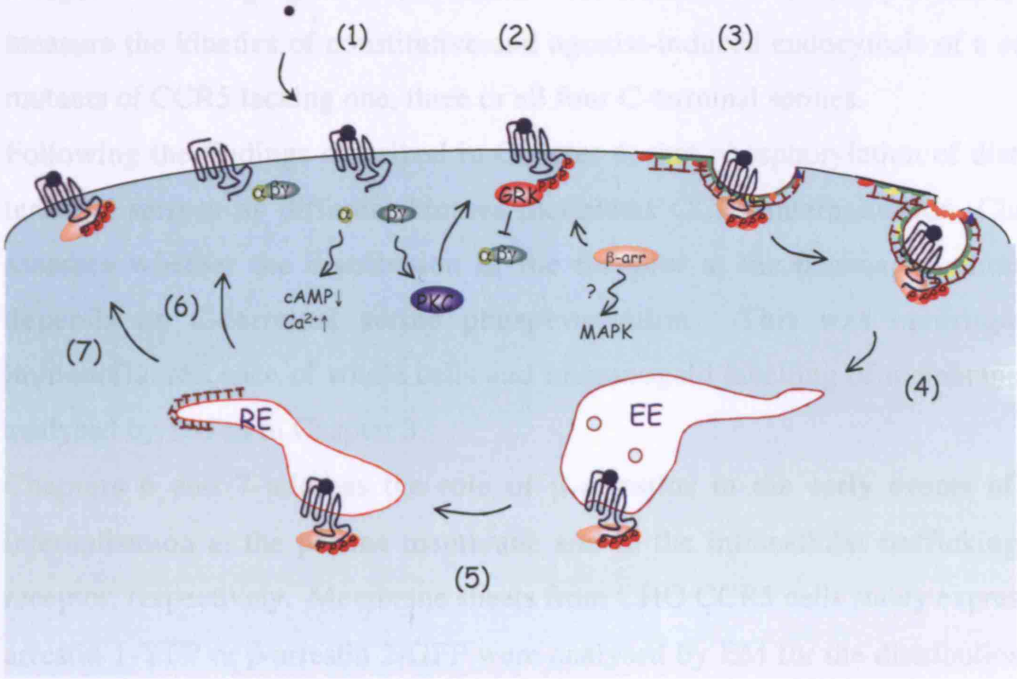


Figure 1.9: Agonist-induced CCR5 trafficking and signalling

Upon agonist binding, CCR5 interacts with heterotrimeric G-proteins (αβγ), which initiate downstream signalling (1). PKC (blue) and GRK (red) are subsequently recruited to the plasma membrane and phosphorylate the receptor, which then has a higher affinity for a β-arrestin (orange) (2); together, these events initiate desensitisation. β-arrestins also link the receptor to the endocytic machinery by binding to clathrin and AP-2 (3). CCR5 is subsequently recruited into CCP, internalised in CCV and delivered to early endosomes (EE) (4) and recycling endosomes (RE) (5). The receptor is thought to recycle back to the plasma membrane in a resensitised form, ready to bind the agonist again (6) or in a phosphorylated ligand-bound form (7). In the latter case, the receptor does not stay at the cell surface but undergoes endocytosis. Several cycles may be required for the receptor to be resensitised.

Chapter 3 presents a detailed analysis of the model system by immunofluorescence and EM and examines the role of clathrin in agonist-induced CCR5 endocytosis. Immunolabelling of the receptor in membrane sheets obtained from the upper surface of RBL CCR5 cells identified flat clathrin lattices where activated receptors cluster and CCPs can form. Moreover, analysis of membrane sheets from several cell lines revealed that these clathrin domains are a common feature of cells, contain various proteins from the endocytic machinery and also play a role in agonist-induced CXCR4 endocytosis.

Chapter 4 investigates the importance and the role of serine phosphorylation by different kinases in the C-terminal domain of CCR5 for the down-modulation of the receptor following agonist stimulation. An internalisation assay was set up to measure the kinetics of constitutive and agonist-induced endocytosis of a series of mutants of CCR5 lacking one, three or all four C-terminal serines.

Following the findings described in Chapter 4, that phosphorylation of distinct C-terminal serines by different kinases modulates CCR5 internalisation, Chapter 5 assesses whether the distribution of the receptor at the plasma membrane also depends on C-terminal serine phosphorylation. This was investigated by immunofluorescence of whole cells and immunogold labelling of membrane sheets analysed by EM as in Chapter 3.

Chapters 6 and 7 address the role of β-arrestins in the early events of CCR5 internalisation at the plasma membrane and in the intracellular trafficking of the receptor, respectively. Membrane sheets from CHO CCR5 cells stably expressing β-arrestin 1-YFP or β-arrestin 2-GFP were analysed by EM for the distribution of the receptor and β-arrestins. Immunogold labelling of ultra-thin cryosections from RBL CCR5 cells was used to investigate the nature of the compartment in which CCR5 accumulates upon sustained agonist binding.

2 Materials and Methods

2.1 Reagents

Tissue culture media and supplements, and Nunc tissue culture plastics were from Gibco/Invitrogen (Paisley, UK). All chemicals were from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise indicated. Recombinant CCL5 (RANTES) and stromal cell derived factor-1α (SDF-1α) was provided by A. E. I. Proudfoot (Serono Pharmaceuticals Research Institute, Geneva, Switzerland). Fixatives and reagents for electron microscopy were from TAAB Laboratories (Aldermaston, Berks, UK) and Agar Scientific (Stanstead, United Kingdom), unless otherwise indicated.
Protein A-colloid gold conjugates (10 nm and 15 nm) were provided by the Cell Microscopy Center, University Medical Centre Utrecht, The Netherlands.

2.2 Cell culture

2.2.1 Cell lines and maintenance

The different cell lines used in this thesis are listed in table 2.1. Further details on hybridoma cells and their maintenance are given in section 2.3.2.
Rat basophilic leukaemia (RBL) cells expressing CCR5 or mutants of CCR5 were cultured in a 80% RPMI 1640 Glutamax/ 20% Medium 199 containing 10% foetal calf serum (FCS), 100 units/ml Penicillin and 100 μg/ml Streptomycin and 600 μg/ml G418 (Kraft et al., 2001). DHFR-deficient Chinese hamster ovary (CHO) cells stably expressing human CCR5 were maintained in nucleoside-free MEM-α containing Glutamax and supplemented with 10% FCS, 100 units/ml Penicillin and 100 μg/ml Streptomycin (Mack et al., 1998). Mouse embryonic fibroblast (MEF) from wild-type or β-arrestin knockout mice were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 2 mM glutamine, 100 units/ml Penicillin and 100 μg/ml Streptomycin (Kohout et al., 2001). Mink lung epithelial (Mv-1-lu) cells stably expressing CXCR4 or CCR5 were grown in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 units/ml Penicillin, 100 μg/ml Streptomycin and 1 mg/ml G418 (Signoret et al., 1998).
Confluent monolayers of cells were passaged every two days (RBL cells), or three to four days (CHO, MEF and Mv-1-lu cells). All cells were detached with 0.05% Trypsin/0.02%EDTA. RBL cells were pelleted by centrifugation (800 rpm,

3 minutes) to remove any debris or secretion from the cells before being replated at a ratio of 1:5. Other cells were directly replated.

Table 2.1: Cell lines used in this thesis

Cell lines	Clones	Over-expressed proteins	Sources
Rat Basophilic Leukemia (RBL) cells[a]			
RBL	3.3	CCR5	M. Oppermann, Georg August University, Goettingen, Germany.
RBL	2.5 22	CCR5 S337A	
RBL	5-19 2.2	CCR5 S349A	
RBL	P23.8	CCR5 S336/337/342A	
RBL	29/2C-12	CCR5 S336/342/349A	
RBL	20	CCR5 S336/337/342/349A	
Chinese Hamster Ovary (CHO) cells[b]			
CHO		CCR5 DHFR	M. Mack, University of Munich, Germany
CHO		CCR5 DHFR YFP-β-arrestin-1	N. Signoret (MRC-LMCB, UCL)
CHO		CCR5 DHFR GFP-β-arrestin-2	
Mouse Embryonic Fibroblasts (MEF)[c]			
MEF MEF KO β-arrestins			R. Lefkowitz, Duke University Medical Centre, Durham, USA
Mink epithelial lung cells (Mv-1-lu)[d]			
Mv-1-lu		CCR5	P.J. Klasse (MRC-LMCB, UCL)
Mv-1-lu		CXCR4	
Hybridoma from BALB/c mice[e]			
Hybridomas producing monoclonal antibody MC-5			M. Mack, University of Munich, Germany

[a](Huttenrauch et al., 2002) [b] (Mack et al., 1998) [c] (Kohout et al., 2001) [d] (Signoret et al., 1998), [e](Segerer et al., 1999)

2.2.2 Cell thawing and freezing

Frozen cells were rapidly thawed at 37°C, and diluted in 10 ml of warm tissue culture medium. Cells were pelleted by centrifugation (800 rpm, 3 min) and resuspended in 10 ml of the appropriate warm tissue culture medium and plated in a 10 cm tissue culture plate. 24 hour after plating, the medium was exchanged to remove any debris and dead cells.

All cell lines, except RBL cells, were frozen in 50% tissue culture medium, 40% FCS and 10% dimethyl sulfoxide (DMSO). RBL cells are very fragile and were therefore frozen in 90% FCS, 10% DMSO. Pellets of cells were resuspended in ice-cold freezing medium, immediately frozen at -70°C and subsequently transferred to liquid nitrogen.

2.3 Antibodies

2.3.1 Antibodies used in this thesis

Details of all antibodies used in this thesis are presented below in table 2.2.

Table 2.2: Antibodies used in this thesis

Antigen	Antibody	Species/Isotype	Concentration	Source
Antibodies against CCR5 and CXCR4				
CCR5 Nter	MC5 (supernatant)[1]	Mouse IgG2a	n/d	Produced in house (see section 2.3.2)
CCR5 Nter	MC5 (Purified)	Mouse IgG2a	1-2 µg/ml	
CCR5-S349-P	M5/4	Mouse IgG1	5 µg/ml	M. Oppermann, Georg August University, Goettingen, Germany
CCR5-S349-P	E11/19	Mouse IgG1	5 µg/ml	
CCR5-S337-P	V14/2	Mouse IgG2b	5 µg/ml	
CXCR4	12G5	Mouse IgG2a	4 µg/ml	J. Hoxie, University of Pennsylvania, Philadelphia, USA
Antibodies against cellular proteins				
Clathrin	X22	Mouse IgG1	6 µg/ml	Affinity Bioreagents, Nottingham, UK
Clathrin	αLC	Rabbit polyclonal	n/d	F. Brodsky, University of California San Francisco, CA, USA
Transferrin Receptor	H68.4	Mouse IgG1	2.5 µg/ml	Invitrogen/Molecular Probes, Paisley, UK
Lgp100/lgp80	Sunshine	Rabbit antiserum	n/d	[2]
Caveolin-1	C37120	IgG1	10 µg/ml	Transduction laboratories, BD Bioscience, Oxford, UK
AP-1		Rabbit polyclonal	n/d	M. S. Robinson, University of Cambridge, UK
AP-2	C8	Rabbit polyclonal	n/d	
Eps15		Rabbit polyclonal	n/d	H. McMahon, MRC, Cambridge, UK
Epsin-1		Rabbit polyclonal	n/d	
β-arrestin 2	182-4	Mouse monoclonal	n/d	J. L. Benovic, Thomas Jefferson University, Philadelphia
GFP		Rabbit polyclonal		D. Shima, ICRF, London
Bridging antibodies				
Anti-mouse	ZO412	Rabbit polyclonal	1.8 µg/ml	Dako Ltd, Ely, UK
Antibodies conjugated to a fluorophore				
CCR5	MC5-488	Mouse IgG2a	0.7 µg/ml	Produced in house
Control Antibodies				
hIgG	hIgG	Human IgG	6 µg/ml	Sigma-Aldrich, Gillingham, UK
Quenching Antibodies				
Alexa Fluor® 488	Anti Alexa Fluor® 488	Rabbit IgG	5 µl/ml	Molecular Probes
Secondary antibodies				
Mouse IgG	Alexa Fluor® 488	Goat	n/d	Invitrogen/Molecular Probes, Paisley, UK
Mouse IgG	Alexa Fluor® 594			
Mouse IgG1	Alexa Fluor® 594			
Mouse IgG2a	Alexa Fluor® 488			
Mouse IgG2b	Alexa Fluor® 594			
Rabbit IgG	Alexa Fluor® 488			
Rabbit IgG	Alexa Fluor® 594			

[1] 0.05% (w/w) sodium azide was added to supernatant kept at 4°C, [2] (Lewis et al., 1985), n/d, not determined

2.3.2 MC5 production from hybridomas

Hybridoma MC-5 (anti human CCR5) from M. Mack (Segerer et al., 1999) were grown in RPMI Glutamax containing 10% FCS, 100 units/ml Penicillin and 100 µg/ml Streptomycin, and supplemented with Hypoxanthine and thymidine (HT supplement 50X, Gibco) and sodium pyruvate 1mM, using an INTEGRA CELLine CL350 apparatus, which allows high cell concentrations and high secreted product concentrations.

On day 1, $8x10^6$ viable cells were prepared in 5 ml of fresh complete medium and inoculated into the cell compartment. 350 ml of warm nutrient medium were then added to the nutrient compartment. On day 7 the cell compartment was harvested and the nutrient medium exchanged. Half of the mixed cell suspension was returned to cell compartment, the other half was centrifuged and the supernatant containing MC-5 was collected and kept at –80°C. Every three days until day 21, the cell compartment harvest and nutrient medium exchange was repeated.

2.3.2.1 Purification

To estimate the concentration of antibody in the supernatant, two different amounts of supernatant, 0.5, 1, and 2 µg of BSA as well as 3 µg of human IgG (Sigma-Aldrich, Gillingham, UK) were prepared in non-reducing sample buffer and were loaded on a non-reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE) composed of a 4% stacking gel and a 12% separating gel. The stacking gel was made of 33.4% (v/v) protogel, 26% (v/v) protogel buffer, 0.1% (v/v) Ammonium persulphate (APS) and 0.1% (v/v) TEMED in deionised water, and the separating gel consisted of 13% (v/v) protogel, 25% (v/v) protogel stacking buffer, 0.1% (v/v) APS and 0.1% (v/v) TEMED in deionised water. Gels were run in a Hoefer mini-gel caster system (San Francisco, CA, USA) in SDS-PAGE running buffer (see section 2.7) at 20 mA per gel. Gels were stained for 15 minutes with 0.05% (w/v) Coomassie blue in 40% (v/v) methanol, 10% (v/v) acetic acid and deionised water, and destained in 50% (v/v) methanol 10% (v/v) acetic acid and dionised water. Gels were finally dried for 45 minutes at 80°C on Watmann paper.

The antibody was purified using the ImmunoPure® (A/G) IgG purification kit from Pierce (Perbio Science UK Ltd, Northumberland) according to the manufacturer's instruction. Briefly, supernatant containing 14 mg of antibody was diluted 1:1 in

binding buffer (pH 8.0, containing EDTA as a preservative) and loaded on a 2 ml AffinityPak™ Immobilised Protein A/G Column. The column was washed with 10-15 ml of binding buffer. The antibody was then eluted using 6-10 ml of elution buffer (pH 2.8) and 0.5 ml fractions collected. To neutralise the low pH of the elution buffer, 50 µl of 1M Tris pH 8.8 was immediately added to the fractions; these were subsequently analysed by SDS-PAGE in non-reducing conditions as described above to identify the antibody containing fraction and determine antibody concentration.

The fractions containing antibody were pooled together and the eluted buffer subsequently exchanged for PBS with a D-salt™ Dextran Desalting Columns 5kD molecular weight cut-off (5K MWCO). The antibody sample was loaded on the column, and 0.5 ml fractions collected immediately. PBS was added in order to collect approximately 10 fractions. Finally, the antibody level was determined using SDS-PAGE and, the BCA protein assay described below.

2.3.2.2 Concentration of Antibody solution

MC5 solution was concentrated using MICROCON® centrifugal filter device, YM-3 3,000 nominal molecular weight limit (NMWL), provided by Millipore (Watford, UK).

The antibody solution (80%) was added to the microcon sample reservoir into the vial, the assembly placed in centrifuged at 4°C and spun down 10 minutes at 14,000xg. The remaining antibody (20%) was added in the sample reservoir, which was then placed upside down in a new vial and spun 5 minutes at 1000 g to transfer concentrate to vial.

The concentration of the new antibody solution was determined by protein assay (BCA).

2.3.2.3 Determination of antibody concentrations

Antibody concentrations were determined using the BCA protein assay adapted from the Pierce recommended method (Smith et al., 1985). The assay is based on the biuret reaction (after Folin/Lowry) but is more flexible than the Lowry assay. The reaction at 60°C uses the peptide backbone rather than specific amino acid side groups and is therefore more reliable.

Reagents:

Micro Reagent A (MA):	20g $Na_2CO_3 \cdot H_2O$
	4 g NaOH
	0.4 g Sodium tartrate
	in 250ml ddH_2O, pH adjusted to 11.25 with $NaHCO_3$
Micro Reagent B (MB):	4 g bicinchoninic acid, sodium salt
	in 100 ml ddH_2O
Micro Reagent C (MC):	2 g $CuSO_4 \cdot 5H_2O$
	in 50 ml ddH_2O

A set of standards were prepared using a 2 mg/ml stock solution of BSA to cover a range of protein concentrations expected in the samples (0-0.25 mg/ml).
The micro-BCA working reagent (M-WR) was prepared by mixing precisely in the following order, 100µl of MC, 2.4 ml of MB and 2.5 ml of MA. The samples and the M-WR were mixed to a 1:1 ratio in a 96 well plate, incubated for 30 minutes at 60°C and analyzed with a spectrophotometer at A_{562}.

2.3.3 Coupling of MC5 to Alexa Fluor® 488

MC-5 was coupled to Alexa Fluor® 488 using the protein labelling kit (A-10235) from Molecular Probes (Paisley, UK) following the manufacturer's protocol. Briefly, 50 µl of 1M sodium bicarbonate were added to the antibody before transferring it into the vial of reactive dye. The reaction mixture was left for 1 hour at room temperature and run through a purification column. The concentration of protein in the sample was determined by measuring the absorbance of the conjugate solution at 280 nm and 494 nm in a cuvette with a 1 cm path length.

$$Concentration = \frac{\left[A_{280} - (A_{494} \cdot 0.11)\right] \cdot dilutionfactor}{203{,}000}$$

where the concentration is expressed in M, 203,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of a typical IgG, and 0.11 is a correction factor to account for absorption of the dye at 280 nm.

The degree of labelling was then calculated using the concentration of protein in the sample and the absorbance at 494 nm.

$$molesdye/moleprotein = \frac{A_{494} \cdot dilutionfactor}{71{,}000 \cdot concentration(M)}$$

where 71,000 $cm^{-1}M^{-1}$ is the approximate molar extinction coefficient of the Alexa Fluor 488 dye at 494 nm.

2.4 Immunofluorescence microscopy

All coverslips were examined using a Zeiss Axioskop microscope (Zeiss, Welwyn Garden City, UK) and analysed with the Openlab software (Improvision, Coventry, UK) or a Nikon Optiphot-2 microscope (Kingston upon Thames, UK) equipped with an MRC Bio-Rad 1024 confocal laser scanner (BioRad, Hemel Hemstead, UK) and in this case analyses were performed using the BioRad Lasersharp 2000™ software.

2.4.1 Cellular distribution of CCR5

RBL cells grown on coverslips for one day were rinsed with PBS and fixed in 3% paraformaldehyde (PFA) in PBS. Cells were subsequently rinsed three times in PBS and free aldehyde groups were quenched with 50mM NH_4Cl in PBS. To analyse cell surface receptor distribution, intact cells were incubated with blocking buffer (0.2% gelatin in PBS) for 15 minutes, before labelling for 1 hour with MC-5 in blocking buffer. Free antibody was removed with three 5 minute washes with PBS. Cells were then stained with a secondary goat-anti-mouse antibody conjugated to Alexa Fluor® 488 (^{488}GAM) for an hour. Finally, cells were washed twice with PBS for 5 minutes and rinsed in ddH_2O, before being mounted in Mowiol on a microscope slide. To analyse cell surface and intracellular distribution of CCR5, cells were permeabilised with 0.05% saponin in blocking buffer for 15 minutes prior to labelling with MC-5 in blocking buffer containing 0.05% saponin. Cells were then washed 3 x 5 minutes in PBS/0.05% saponin, and stained with ^{488}GAM in PBS/0.05% saponin. Unbound antibody was removed by washing the cells 2 x 5 minutes with PBS/0.05% saponin. Finally, the cells were washed 2 x with PBS for

5 minutes each, rinsed with ddH_2O and mounted in Mowiol (Calbiochem, La Jolla, USA) on a microscope slide.

2.4.2 Co-staining of clathrin and CCR5 at the plasma membrane

RBL cells grown on coverslips for one day were treated with binding medium (BM, see section 2.7) or BM containing 125nM CCL5 for 2, 5 or 10 minutes in 37°C BM. The coverslips were then plunged in ice-cold fixative (3% PFA in PBS), kept on ice for 20 minutes and rinsed three times in PBS. Free aldehyde groups were quenched with 50mM NH_4Cl in PBS. After three washes in PBS, non-specific binding sites were saturated using blocking buffer for 15 minutes and cell surface CCR5 was subsequently labelled. Following three 5 minutes washes with PBS, cells were then permeabilised with 0.05% saponin in blocking buffer for 15 minutes and incubated for 1 hour with a rabbit anti-clathrin light chain antibody in blocking buffer containing 0.05% saponin. Cells were washed carefully 3 x 5 minutes in PBS containing 0.05% saponin, and stained with two secondary antibodies, Alexa Fluor® 594 conjugated goat-anti-rabbit (^{594}GAR) and ^{488}GAM. To remove free antibody, cells were washed twice for 10 minutes with PBS containing 0.05% saponin. Finally, the cells were washed 2 x with PBS for 5 minutes and rinsed with ddH_2O, before being mounted in Mowiol on microscope slide.

2.4.3 Detection of phosphorylated CCR5

Cells were treated and processed as described previously in section 2.4.2. However, phosphatase inhibitors were added (at 1/100 dilution) to the fixing, washing and permeabilisation solutions and during the primary antibody incubations to avoid possible dephosphorylation of the receptor.

2.5 Electron microscopy

2.5.1 Transmission electron microscopes and equipment

2.5.1.1 Transmission electron microscopes and photographic material

Samples were examined on Philips EM420 and CM10 transmission electron microscopes (FEI company Ltd., Cambridge Business park, Cambridge, UK) and images recorded onto Kodak SO-163 electron image film. Negatives were

developed according to the manufacturer's instructions and digitized by scanning with a FlexTight precision II rotating drum CCD scanner (Imacon). TIFF images were acquired with the colorFlex 1.9.5 FlexTight Interface Software. Alternatively, samples were examined with a Tecnai G2 Spirit transmission electron microscope (FEI company UK Ltd.) and digital images were recorded with a Morada 11 Megapixel TEM camera (Olympus Soft Imaging System) and analysed with the iTEM software package. All TIFF images were further analysed and assembled into montages with Adobe Photoshop 6.0 and Illustrator 10.

2.5.1.2 Critical point dryer and thermo-circulator

Samples were critical point dried using the E3100 critical point dryer equipped with a E3500 thermo-circulator to control the temperature in the chamber (Quorum Technologies, East Sussex, UK).

2.5.1.3 Rotary shadow and platinum/carbon coating

Grids or cells were rotary shadowed with carbon/platinum and/or carbon coated using the MED020 modular high vacuum coating unit (BALTEC). Carbon/platinum and carbon were evaporated at 1.6kV and 80mA and 1.8kV and 90mA, respectively.

2.5.2 Cell surface replicas of whole mount preparations

Figure 2.1 illustrates the preparation of samples for whole mount replicas. Cells were grown to 50–70% confluence on glass coverslips and were rinsed in BM and incubated at 37°C in BM or in BM containing 125 nM CCL5 for various times. The cells were rinsed briefly in ice-cold PBS and fixed in 2% PFA/0.1% gluteraldehyde (GA) in 0.1 M phosphate buffer, pH 7.4 at room temperature. After washing-out the fixative with PBS, the cells were quenched in 50 mM glycine/50mM NH_4Cl 2 x 10 minutes, washed and blocked in PBS containing 2% BSA (blocking buffer) 3 x 5 minutes. CCR5 at the plasma membrane was labelled at room temperature with MC-5 (7.7 nM) in blocking buffer containing 0.1% Aurion BSA-c™ (Aurion, Wageningen, The Netherlands) for 1 hour with gentle reversible shaking. Unbound antibody was washed off extensively with blocking buffer and cells were incubated with Protein A conjugated to 15 nm-gold particles (PAG_{15}). After washing extensively in blocking buffer and PBS, the cells were fixed in 4% glutaraldehyde in

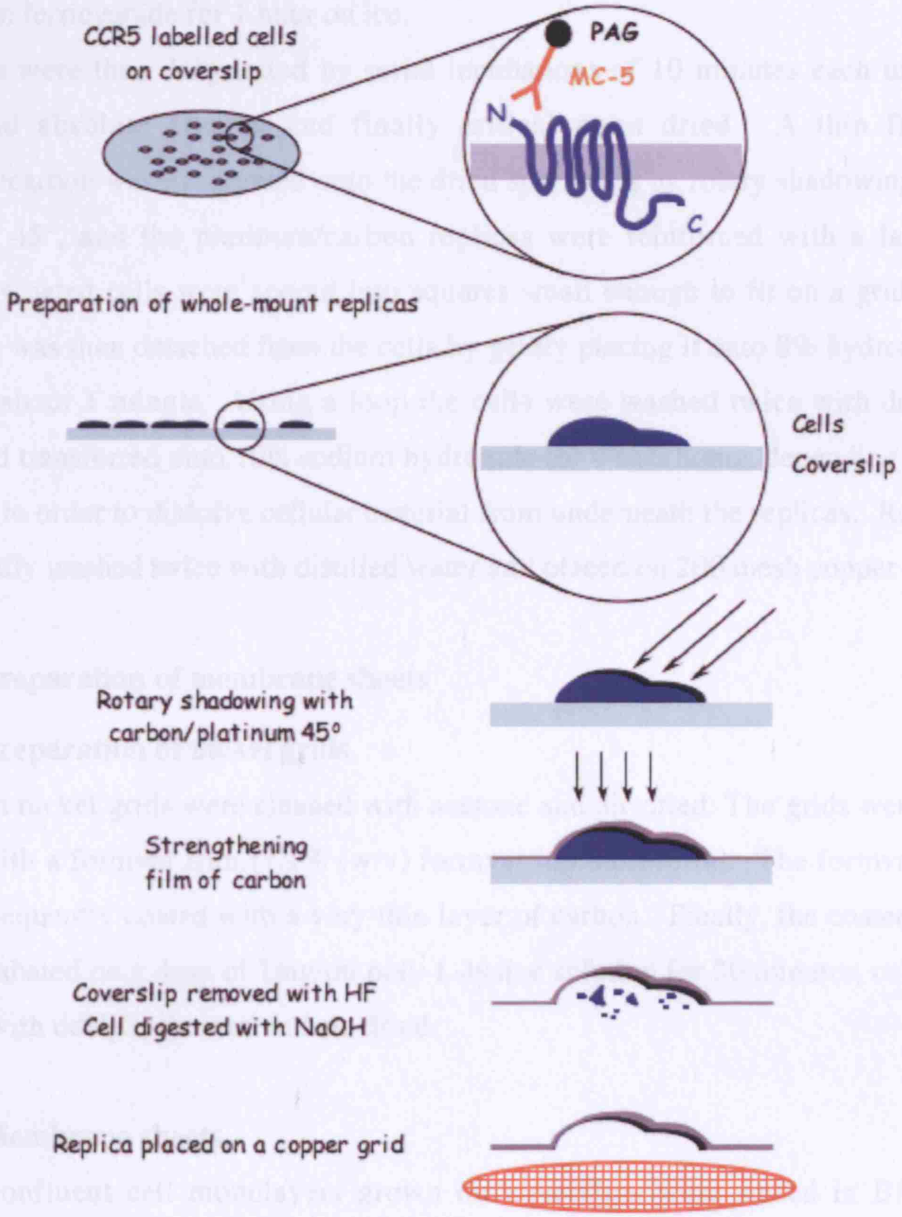


Figure 2.1: Preparation of cell surface replicas of whole mount

Cells grown on coverslips were fixed and labelled for plasma membrane receptor with antibodies and PAG. Cells were then critical point dried and shadowed with carbon and platinum. A film of carbon was subsequently evaporated on the samples to strengthen the replicas. Finally, the coverslip and the cells were digested away and the replicas placed on a nickel grid. (Adapted from Mol. Biol Of The Cell, 3rd Edition, Alberts *et al.*)

0.1 M sodium cacodylate buffer, pH 7.6 for 30 minutes at room temperature, washed with 0.1 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide/1.5% potassium ferricyanide for 1 hour on ice.

The cells were then dehydrated by serial incubations of 10 minutes each in 70%, 90%, and absolute ethanol and finally critical point dried. A thin film of platinum/carbon was evaporated onto the dried specimens by rotary shadowing at an angle of 45°, and the platinum/carbon replicas were reinforced with a layer of carbon. Coated cells were scored into squares small enough to fit on a grid. The coverslip was then detached from the cells by gently placing it onto 8% hydrofluoric acid for about 1 minute. Using a loop the cells were washed twice with distilled water and transferred onto 10N sodium hydroxide for 4 to 6 hours, depending on the cell type in order to dissolve cellular material from underneath the replicas. Replicas were finally washed twice with distilled water and placed on 200 mesh copper grids.

2.5.3 Preparation of membrane sheets

2.5.3.1 Preparation of nickel grids

300 mesh nickel grids were cleaned with acetone and air-dried. The grids were then coated with a formvar film (1.1% (w/v) formvar in chloroform). The formvar film was subsequently coated with a very thin layer of carbon. Finally, the coated grids were incubated on a drop of 1mg/ml poly-L-lysine solution for 30 minutes, carefully washed with ddH_2O, drained and air-dried.

2.5.3.2 Membrane sheets

Nearly confluent cell monolayers grown on coverslips were rinsed in BM and incubated at 37°C in BM or in BM containing 125 nM CCL5 for various times. The cells were then washed in ice-cold BM. CCR5 at the plasma membrane was labelled with 15.3 nM MC-5 in BM for 1 hour at 4°C. Unbound antibody was washed off carefully with ice-cold BM and the cells were incubated with PAG_{15} for 1 hour at 4°C. Samples were rinsed in ice-cold BM and HEPES buffer (HB).

Upper membranes were prepared using the "rip-off" technique described previously (Sanan and Anderson, 1991) and illustrated in figure 2.2. Prepared grids were positioned film side up on circular pieces of cellulose membrane, that had been permeated from underneath with HB, and placed on a sheet of clear ice-cold glass.

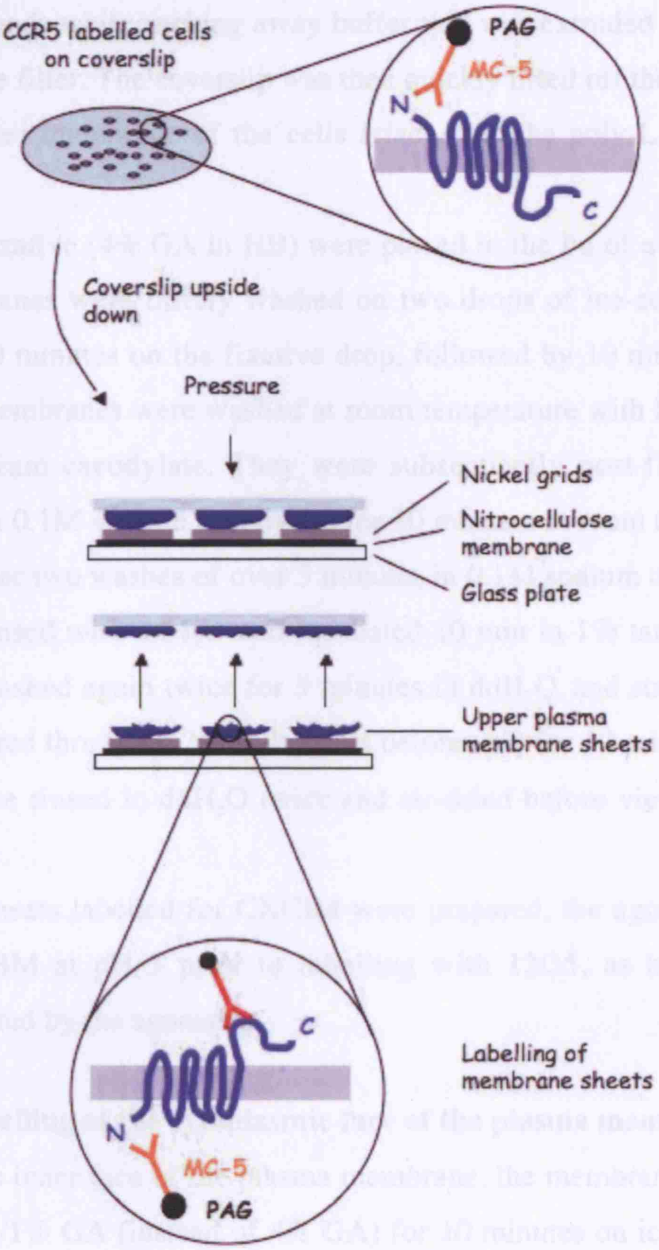


Figure 2.2: Preparation of membrane sheets

Cells on coverslips were labelled for plasma membrane receptors at 4°C with specific antibodies detected with PAG. Cells were subsequently washed with HB and membrane sheets obtained as described in the text. Upper membrane sheets left on the grids were then fixed, and could be labelled for the cytosolic domains of plasma membrane proteins and/or cytoplasmic protein associated with the plasma membrane.

Coverslips were drained slightly onto tissue and inverted carefully over grids. A 20 mm diameter rubber bung was then pressed on the coverslip with light finger pressure for 10 seconds while sucking away buffer that was extruded from between the coverslip and the filter. The coverslip was then quickly lifted off the filter leaving portions of the upper membrane of the cells attached to the poly-L-lysine-coated grids.

Drops of HB and fixative (4% GA in HB) were placed in the lid of a 96 well plate, on ice. The membranes were briefly washed on two drops of ice-cold HB before leaving them for 10 minutes on the fixative drop, followed by 10 minutes at room temperature. The membranes were washed at room temperature with HB and rinsed twice in 0.1M sodium cacodylate. They were subsequently post-fixed with 1% osmium tetroxide in 0.1M sodium cacodylate for 10 minutes at room temperature in the fume hood. After two washes of over 5 minutes in 0.1M sodium cacodylate, the membranes were rinsed with ddH_2O and incubated 10 min in 1% tannic acid. The membranes were washed again twice for 5 minutes in ddH_2O, and stained with 1% uranyl acetate (filtered through 0.2μm filter just before use) for 10 minutes. Finally, the membranes were rinsed in ddH_2O twice and air-dried before viewing with the electron microscope.

When membrane sheets labelled for CXCR4 were prepared, the agonist had to be stripped-off with BM at pH 3 prior to labelling with 12G5, as binding of the antibody was inhibited by the agonist.

2.5.3.3 Immunolabelling of the cytoplasmic face of the plasma membrane

To immunolabel the inner face of the plasma membrane, the membrane sheets were fixed with 2% PFA/1% GA (instead of 4% GA) for 10 minutes on ice followed by 10 minutes at room temperature. The membranes were then rinsed in HB, PBS, and quenched in 50 mM glycine/50mM NH_4Cl three times 5 minutes. After several washes in PBS, the membranes were treated with blocking buffer twice for 5 minutes and incubated with primary antibody in blocking buffer containing 0.1% Aurion BSA-c™ for 30-60 minutes (depending on the antibody) at room temperature. Unbound antibody was carefully washed away with blocking buffer and the membranes were incubated with PAG in blocking buffer for 30 minutes at room temperature.

After washing extensively in blocking buffer and PBS, immunolabelled membranes were fixed in 2% glutaraldehyde in PBS for 10 minutes at room temperature and subsequently post-fixed and stained as described in section 2.5.3.2.

2.5.4 Ultra-thin section of plastic embedded cells

2.5.4.1 Epoxy resin

Samples were embedded in epoxy resin (Epon 812) with the following composition:

TAAB 812 19.2 ml

DDSA 7.6 ml

MNA 13.2 ml

DMP-30 0.8 ml

2.5.4.2 Samples preparation

Nearly confluent monolayers of cells grown in 35mm dishes for 24 hours were fixed with 2% PFA/0.1% GA in PBS for 20 minutes on ice. Cells were subsequently washed three times with PBS at room temperature and free aldehyde groups quenched with 50 mM NH_4Cl/50 mM glycine in PBS 2 x 5 minutes. After further washes in PBS at room temperature, non-specific antibody binding sites were blocked with 2% BSA in PBS (blocking buffer). Cell surface CCR5 was labelled with MC-5 (1μg/ml) in blocking buffer containing Aurion BSA-c, 0.1% for an hour. Free antibody was removed by washing the cells 5 x 5 minutes with blocking buffer. The cells were then incubated with PAG_{10} in blocking buffer for an hour. Unbound PAG_{10} was washed away with PBS, and the cells were washed with sodium cacodylate and further fixed in 1.5% GA in cacodylate buffer for 30 minutes. Excess fixative was washed away with cacodylate buffer and cells prepared for osmication. The cells were incubated for an hour in 1.5% potassium ferricyanide in ddH_2O/1% osmium tetroxide solution in the fridge. After extensive washes with sodium cacodylate, cells were incubated at room temperature for 45 minutes in 1% tannic acid in 0.05% sodium cacodylate, and subsequently quenched with 1% sodium sulphate in 0.05% sodium cacodylate 5 minutes. Finally cells were rinsed three times with ddH_2O before dehydration.

Cell were dehydrated stepwise in ethanol by incubating them 2 x 5 minutes first in 70% ethanol, followed by 90% ethanol and finally absolute ethanol. While the cells

were in absolute ethanol, the monolayer was scored with a needle to break up the cell monolayer. In the fume hood, the absolute ethanol was replaced by propylene oxide, which dissolved the plastic surface and released the cells. Cells floating off the dish were collected in a thin 0.5 ml Eppendorf™ tube and spun down in 90° rotor for 10 minutes at 11,000 rpm. The supernatant was discarded and replaced with clean propylene oxide for 10 minutes, which was then replaced by a 1:1 mixture of propylene oxide and Epon for an hour at room temperature. The pellet was then cut out of the Eppendorf tube and further incubated in Epon in a glass jar on a rotating wheel for 4 hours (Epon was changed after 2 hours). Pellets were finally transferred to plastic moulds and baked overnight in a 60°C oven.
Ultra-thin sections were cut on an Ultracut (UCT) ultramicrotome (Leica Microsystems, Vienna, Austria) at room temperature and stained with lead citrate.

2.5.5 Ultra-thin cryosections (Tokuyasu technique)

2.5.5.1 Preparation of grids

Grids were cleaned with 25% NH_4OH in ddH_2O and dried on filter paper at 37°C. The grids were subsequently coated with a formvar film (1.1% (w/v) formvar in chloroform) and a very thin layer of carbon.

2.5.5.2 Sample embedding in gelatin

Confluent monolayers of cells were fixed by adding an equal volume of pre-warmed double-strength fixative (8% PFA in 0.1 PB) directly into the culture medium for 10 minutes. The fixative was then replaced with single-strength fixative (4% PFA) for 90 minutes. The cells were washed with PBS and free aldehyde groups quenched with 20 mM glycine/PBS for 10 minutes, before scraping them off in 1% gelatine (provided by the Cell Microscopy Centre, University Medical Centre Utrecht, The Netherlands) in PBS. The cells were spun down and 1% gelatin exchanged for 12% gelatin. After 10 minutes incubation at 37°C the cells were spun down into a pellet and transferred to ice for the gelatin to harden. The pellet was then cut into small blocs, infiltrated overnight in 2.3 M sucrose, mounted on pins (from Leica Microsystems, UK) and frozen in liquid nitrogen.

Cryosections were cut on an Ultracut UCT microtome equipped with an EM FCS cryochamber (Leica Microsystems, Vienna, Austria). Cryosections were picked up with 1% methyl cellulose/ 2.3M sucrose and transferred onto prepared grids.

2.5.5.3 Immuno-gold labelling of ultra-thin cryosections

For labelling of ultra-thin cryosections, grids were first incubated in 2% gelatin in order to remove the methyl cellulose/sucrose mixture from the pick up solution. Sections were incubated on drops 4 x 1 minute in 0.1% glycine in PBS to quench free aldehyde groups, and non-specific sites were blocked with 1% BSA in PBS for 3 minutes. The sections were labelled with primary antibody diluted in 1%BSA/PBS for 60 minutes, rinsed 4 x 2 minutes with PBS and incubated for 20 minutes with PAG in 1% BSA/PBS. Unbound PAG was removed by washing with PBS. Labelling was stabilised by fixing with 1% GA in PBS for 5 minutes. After 10 x 1 minute washes, sections were stained with 2% uranyl oxalate/acetate at pH 7 for 5 minutes. The sections were rinse in ice-cold ddH_2O and incubated for 5 minutes in 1% methyl cellulose/2% uranyl acetate pH 4 on ice. Finally, grids were picked up with loops and dried at RT to form a thin support film of methylcellulose and uranyl acetate.

For double labelling, sections were first labelled with a rabbit antibody or mouse IgG2a and PAG, fixed in 1% GA in PBS for 10 minutes (to neutralise any unoccupied binding site), quenched and stained with the second primary antibody and a different size of PAG. As protein A usually has a low affinity for mouse antibody, except IgG2a, a rabbit anti-mouse bridging antibody was used for mouse antibodies other than IgG2a.

2.5.6 Quantification

To assess receptor clustering by EM, to measure the sizes of clusters on membrane replicas, and the sizes of flat clathrin lattices on membrane sheets random fields were photographed. Pictures were then either printed at 44175x and gold particles counted, or digital pictures were analysed using the AnalySIS software package.

2.5.4.1 Whole mount analysis

Digital images were analysed using item Emarker software (Olympia SIS) and the total number of particles and their average density on the membrane were determined. To measure the density of gold particles in the CCR5 clusters on CCL5-treated cells, circles of 0.32 μm of diameter were placed on visible clusters and the number of gold particles was counted. The average diameter of clusters was determined by measuring the smaller and longer diameter in each cluster. As illustrated in figure 2.3.

2.5.4.2 Rip-off analysis

The area of membrane coated with flat clathrin lattice was determined using a test point grid (point spacing 4 mm) and following standard stereological procedures (Mayhew et al., 2002). The test point grid was placed over the photographs, and the proportion of test points over the lattices counted and converted to square micrometers. In addition to the total area of flat clathrin lattice, the average size of lattice was determined by dividing the total area covered of flat clathrin lattice on the membrane sheets by the number of flat lattices. To calculate the density of PAG per μm^2 of lattice, the number of gold particles in area of flat clathrin lattices was counted and divided by the area of flat clathrin lattice.

Membrane sheets containing two sizes of gold particles were first analysed with item Emarker software (Olympia SIS), which gave the total number of particles of each size. The distribution of the gold particles was subsequently determined by counting the number of particles in flat clathrin lattices and/or their association with each other. Doublets of gold particles probably due to the bridging antibody were counted as 2 particles rather than 1. However, 35% of the labeling with bridging antibody showed a single particle. PAG_{15} and PAG_{10} were considered to bind the same receptor molecule when less than 25 nm apart from each other.

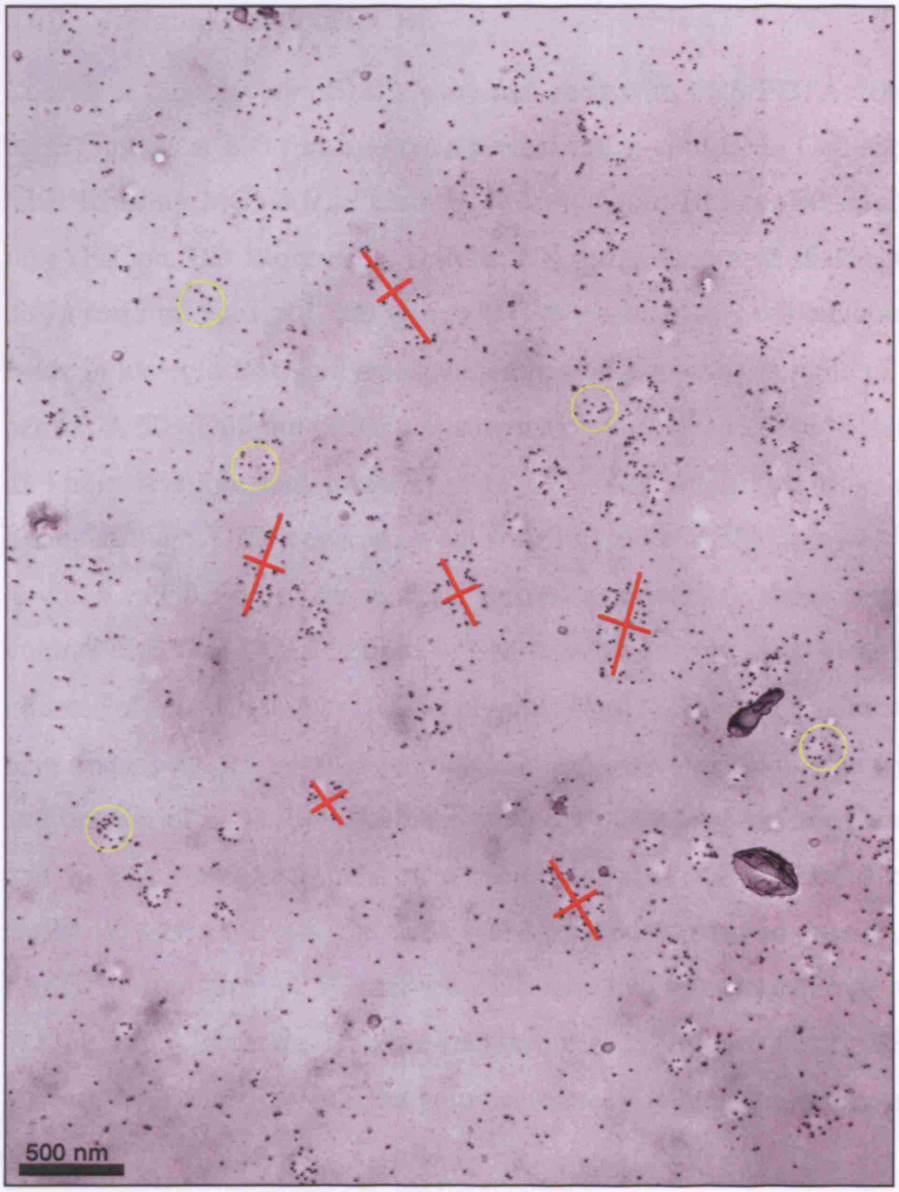


Figure 2.3: Analysis of CCR5 clusters on membrane replicas of RBL cells

To determine the size of the clusters the diameters (red) were measured in obvious clusters. The density was determined by placing a circle (yellow) over the centre of an obvious cluster, and counting the gold particles falling within this ring.

2.6 FACS® analysis

Samples were analysed using a FACSCalibur flow cytometer from Becton Dickinson (UK Ltd, Oxford, UK). The mean fluorescence intensity of each sample was determined by measuring 10,000 events.

2.6.1 Internalisation of CCR5

Confluent monolayers of cells were detached with PBS/EDTA 10mM, spun down for 3 minutes at 800 rpm and resuspended in ice-cold BM. Cell surface CCR5 was labelled using MC5-Alexa Fluor® 488 in ice-cold BM for 90 minutes in an assay tube (Falcon, BD Bioscience, Oxford, UK) on a reciprocal shaker. Cells were spun down for 5 minutes at 1,200 rpm at 4°C to wash out any unbound antibody, washed twice in ice-cold BM, and resuspended in an appropriate volume to have $2 \cdot 10^6$ cells per ml. A 50 µl aliquot of the cell suspension was taken as the '0 minute' time point of internalisation, and transferred to a 96-well plate U-bottom plate (Nalgene, Longhborough, UK) prepared on ice with 100 µl of BM in each well.

A volume of 0.5 ml of the cell suspension was added to tubes containing the same volume of BM or CCL5 250nM in BM in a 37°C water bath. After 5, 15, 30 and 60 minutes of incubation, two 100 µl aliquots were taken and transferred to the 96-well plate to stop CCR5 internalisation. Cells were then washed twice with ice-cold BM and one set of cells was incubated with the quenching antibody anti-Alexa Fluor® 488 in BM for 90 minutes on a reciprocal shaker in the cold room. Unbound antibody was removed, the cells were washed twice and finally resuspended in FACS® buffer for FACS® analysis. The samples were transferred to mini (RB738, Teklab Ltd., Durham, UK) or standard FACS® tubes (Falcon, BD Bioscience, Oxford, UK), depending on the volume, to measure their fluorescence.

2.7 Buffers and solutions

Phosphate Buffered Saline (PBS)

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

SDS-PAGE running buffer

25 mM Tris

192 mM Glycine

0.1% (w/v) SDS

Binding Medium (pH 7)

RPMI-1640 without bicarbonate

0.01 M HEPES pH 7

0.2% BSA in ddH_2O, pH adjusted to 7

Binding Medium (pH 3, to elute antibody or agonist)

RPMI-1640 without bicarbonate

0.01 M MES

0.2% BSA in ddH_2O, pH adjusted to 3

Phosphate Buffer 0.2M (PB)

0.16 M Na_2HPO_4

0.04 M NaH_2PO_4 pH adjusted at 7.4

HEPES Buffer (HB)

25 mM HEPES

25 mM KCl

2.5 mM Mg acetate pH adjusted to 7.0

FACS® wash buffer

1% FCS

0.01% NaN_3

in PBS

3 Morphological analysis of early events of agonist-induced CCR5 endocytosis

Previous work on CCR5 has shown that upon agonist binding, the receptor undergoes rapid internalisation and accumulates into a perinuclear region identified as the recycling endosome by the presence of the transferrin receptor (Mack et al., 1998; Signoret et al., 2000; Signoret et al., 1998). Although CCR5 intracellular localisation after agonist treatment is well described, it remains unclear how the receptor enters the endocytic pathway. Several studies have suggested a role for clathrin in CCR5 endocytosis. Mack *et al.* showed that agonist-induced internalisation was inhibited when cells were treated with hypertonic sucrose, which blocks receptor mediated endocytosis, including the clathrin-mediated pathway (Mack et al., 1998). Furthermore, in Chinese hamster ovary (CHO) cells treated with AOP-RANTES (a modified form of the CCR5 agonist CCL5/RANTES), Signoret *et al.* observed CCR5 in clathrin-coated vesicles (CCV) (Signoret et al., 2000). Finally, in the absence of β-arrestins, which interact with clathrin and other proteins from the clathrin-mediated pathway, agonist-induced CCR5 internalisation was inhibited (Fraile-Ramos et al., 2003). Altogether these findings point towards a role for clathrin in the internalisation of CCR5.

In contrast, Mueller *et al.* and Venkatesan *et al.* reported evidence for CCR5 internalisation through a clathrin-independent pathway (Mueller et al., 2002; Venkatesan et al., 2003). Among the few alternative endocytic pathways independent of clathrin, the caveolar pathway is the best-characterised (Marsh and Helenius, 2006). It involves caveolins, which are integral membrane proteins essential for the formation of caveolae, small 50 nm flask shaped invaginations of the plasma membrane (Okamoto et al., 1998). Enriched in cholesterol, sphingolipids and glycolipids, caveolae are part of microdomains in the plasma membrane resistant to non-ionic detergents. Internalisation via caveolae directs cargo to caveosomes, which are distinct from early, recycling and late endosomes, and can be disrupted with reagents that bind or extract membrane cholesterol.

The studies reporting a clathrin-independent pathway for agonist-induced CCR5 endocytosis were based on experiments using drugs, which disrupt cholesterol and

are known to block the caveolar pathway (Mueller et al., 2002; Venkatesan et al., 2003). However, Nguyen *et al.* have shown that membrane cholesterol is important for maintaining CCR5 in a conformation able to bind agonists (Nguyen and Taub, 2002; Nguyen and Taub, 2003a). Additionally, clathrin-mediated endocytosis (CME) has also been shown to be cholesterol-dependent (Moskowitz et al., 2003; Rodal et al., 1999; Subtil et al., 1999). Together these observations demonstrate that perturbing membrane cholesterol has multiple effects on the plasma membrane and is not a specific inhibitor of caveolin-mediated endocytosis. Moreover they question the proposed role for caveolae in CCR5 endocytosis.

The aim of the work described in this chapter was to study the early events of CCR5 endocytosis occurring at the plasma membrane after agonist binding. Immunofluorescence and electron microscopy were used to gain information on the cell surface distribution of the receptor and changes in this distribution induced by the agonist. The role of clathrin in CCR5 endocytosis was investigated and clathrin-coated domains of the plasma membrane, where CCR5 was seen following agonist treatment, were characterised in various cell lines. Finally, to see whether these events were specific to CCR5 internalisation, the early steps of endocytosis of CXCR4, another chemokine receptor co-receptor for human immunodeficiency virus (HIV-1) were investigated.

3.1 Rat basophilic leukaemia cells

CCR5 is usually expressed on subsets of leukocytes. However, the low level of expression of the receptor in primary cells renders morphological studies difficult. For this reason, CCR5 has been stably expressed in various cell lines, including CHO cells, mink epithelial lung cells (Mv-1-lu), and rat basophilic leukaemia (RBL) cells. Here, RBL cells were chosen for the study of CCR5 as they are of haematopoietic lineage and may therefore provide a more appropriate background for this receptor than non-haematopoietic cells. Furthermore, RBL cells have been used to study the endocytosis of other GPCRs as they express high endogenous levels of β-arrestins (Santini et al., 2000).

Typically, RBL cells are used to study the endogenous high affinity IgE receptor FcεRI. Cross-linking of FcεRI initiates signalling cascades leading to morphological

changes and degranulation (Pfeiffer and Oliver, 1994; Pfeiffer et al., 1985; Wilson et al., 2000). Pfeiffer and Oliver described resting cells as "compact cell bodies with arborised extensions" with very little F-actin at the cell-substrate interface (Pfeiffer and Oliver, 1994). Upon activation, the cells spread extensively, ruffle and F-actin redistributes into plaques at the adherent surface. In addition, the microvillous topography of the cell surface becomes lamellar in activated cells and fluid pinocytosis is increased (Pfeiffer et al., 1985; Wilson et al., 1995). Binding of IgE to FcεRI induces endocytosis of the receptor and its degradation (Xu et al., 1998). However, the receptor can also reach the secretory granules through the endocytic pathway and be released upon further activation.

3.1.1 RBL CCR5 cell line

RBL cells stably expressing human CCR5 (RBL CCR5 cells) were provided by M. Oppermann, Department of Immunology, Georg August University, Goettingen, Germany. The human CCR5 cDNA was tagged with an influenza virus haemaglutinin signal sequence and a FLAG epitope tag before the start codon (Oppermann et al., 1999).

3.1.2.1 CCR5 in secretory granules

CCR5 distribution in RBL cells was analysed by immunofluorescence (as described in Materials and Methods in section 2.4.1). The receptor was detected with MC-5, a monoclonal antibody against the extreme N-terminal extracellular domain of the protein (Blanpain et al., 2002; Segerer et al., 1999) and a secondary antibody goat-anti-mouse conjugated to Alexa Fluor® 488 (^{488}GAM). MC-5 specificity had previously been tested on CCR5-negative cells where no staining was detected. CCR5 was highly expressed at the plasma membrane (figure 3.1 A, Intact cell) and the staining delineated the stellar shape of the cells. However, when cells were permeabilised with 0.05% saponin, a significant pool of the receptor was found in intracellular structures that resemble granules (figure 3.1 A, Permeabilised cell). To identify this compartment, cells were labelled with antibodies against cellular proteins. Rabbit antiserum (sunshine) recognising the rat lysosomal proteins lgp100/lgp80 (Lewis et al., 1985) co-localised with intracellular CCR5 (not shown here). Granules in secretory cells have been well studied and described to contain

both, secretory and lysosomal markers (Dragonetti et al., 2000). The intracellular structures, in which CCR5 was seen at steady state, are probably secretory/lysosomal granules.

In order to investigate whether the receptor was trafficking from the plasma membrane to the granules upon agonist binding, cell surface receptors were first labelled with MC-5 prior to CCL5 binding. MC-5 recognises an epitope containing the first three residues of CCR5 and does not interfere with agonist binding to the receptor or agonist-induced activation (Blanpain et al., 2002; Signoret et al, 2000). Therefore both MC-5 and CCL5 can bind the receptor simultaneously. In addition when used on live cells, MC-5 binding to CCR5 does not induce any clustering or internalisation of the receptor (Blanpain et al., 2002). After 60 minutes of agonist treatment the cells were fixed, permeabilised and stained with ^{488}GAM to detect MC-5-labelled CCR5. Secretory granules were labelled with the rabbit antiserum against lgp100/lgp80 (sunshine) and detected with a secondary antibody goat-anti-rabbit conjugated to Alexa Fluor® 594 (^{594}GAR). Figure 3.1 B shows a confocal section of an agonist-treated cell with CCR5 (green) and secretory granules (red). Lgp100/lgp80 was located in the peripheral membrane of the secretory granules, while CCR5 was found in small endocytic vesicles as well as in larger ring-like intracellular structures where it colocalised with lgp100/lgp80 (figure 3.1 B, inset). It is not yet clear why and how some of the receptors traffic to the secretory granules. Nevertheless, when investigating the trafficking of CCR5 in these cells, it is important to keep in mind that there is an intracellular pool of receptor. Therefore suitable approaches have been developed to follow receptor trafficking from the plasma membrane into the cell.

The secretory granules were examined at higher magnification by transmission electron microscopy (TEM) of ultra-thin sections of epon embedded RBL CCR5 cells. Figure 3.2 shows intracellular compartments with the morphological characteristics of secretory granules (asterisks and enlargement A-D). Initially, intact cells were labelled for plasma membrane CCR5. However, probably due to fusion of the secretory granules with the plasma membrane or because of mechanical membrane breakage prior to or during the fixation, the content of some of the granules was exposed to the extracellular medium containing anti-CCR5 antibodies. This revealed some of the intracellular pool of receptor previously imaged by

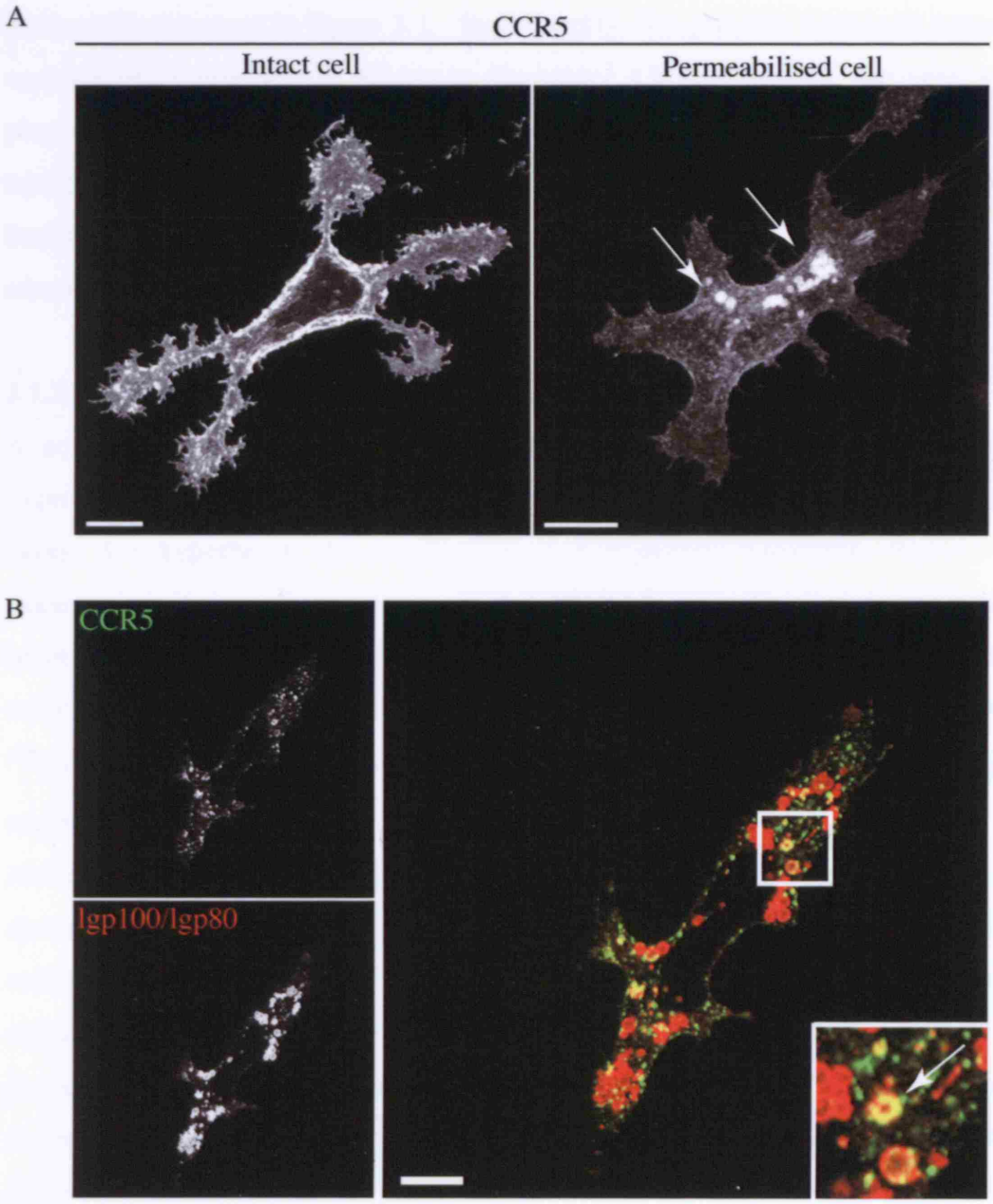


Figure 3.1: Immunofluorescence labelling of CCR5 in RBL CCR5 cells

(A) Untreated RBL CCR5 cells were fixed and stained with MC-5 and ^{488}GAM, either intact to label cell surface receptors or following saponin permeabilisation to detect both cell surface and intracellular CCR5 (permeabilised cell). A shorter exposure was used to distinguish the intracellular vesicles in permeabilised cells, hence the less bright staining at the plasma membrane. (B) Cell surface receptors were labelled with MC-5, and the cells were then incubated with 125 nM CCL5 for 60 minutes at 37°C. Cells were then fixed, permeabilised and labelled with a rabbit antiserum to lgp100/lgp80. ^{488}GAM and ^{594}GAR secondary antibodies were used to detect CCR5 and lgp100/lgp80, respectively. Scale bar = 10 μm.

immunofluorescence in figure 3.1. Significantly, the granules contained numerous vesicles of approximately 50 nm in diameter. CCR5 labelling was seen at the plasma membrane and on small vesicles within the granules. In addition, most of the labelling in granules appeared to be associated with the vesicles and not the granule limiting membrane. The labelled granules may be in the process of secretion stimulated by CCL5 treatment.

3.1.2.2 Endogenous transferrin receptor in secretory granules

A possible explanation for the presence of CCR5 in granules is that due to the expression of the receptor, some may be directed for degradation or secretion. To assess this hypothesis, the localisation of endogenous transferrin receptor was examined by immunofluorescence. RBL CCR5 cells were fixed, permeabilised and incubated with a monoclonal antibody against transferrin receptor (H68.4) and a rabbit antiserum against lgp100/lgp80 (sunshine). Two secondary antibodies, ^{488}GAM and ^{594}GAR, were used to detect the transferrin receptor and lgp100/lgp80, respectively.

Although these transfected cells had been cloned from a single transfected RBL cell, distinct morphologies were seen in the cultures. Figure 3.3 shows three different cells illustrating the variety of morphological phenotypes found within the same cell culture. In 90-95% of the cells, the transferrin receptor was predominantly localised in small vesicles dispersed through the cell and in the perinuclear region probably corresponding to recycling endosomes but did not colocalise with lgp100/lgp80 in secretory granules and lysosomes (Figure 3.3 cell 1 and cell 2. However, in a small proportion of cells (5-10%), which contained a larger pool of secretory granules, the transferrin receptor was observed inside the granules (figure 3.3 cell 3). No colocalisation of the two labelling was observed. This immunofluorescence analysis

Figure 3.2: Ultra-thin sections of epon embedded RBL CCR5 cells labelled for CCR5

RBL CCR5 cells were treated with 125 nM CCL5 for 5 min at 37°C, fixed with 2% PFA/0.1% GA, and labelled for cell surface CCR5 with MC-5 and PAG_{10}. Cells were scraped, pelleted and further processed for epon embedding and ultra-thin sectioning, as described in Materials and Methods 2.5.4. CCR5 is present at the plasma membrane and in secretory granules that had fused with the plasma membrane (enlargement A, B and C). D and E show vesicles labelled for CCR5 released from the granules. Asterisks mark secretory granules. Scale bars = 500 nm and 100 nm in enlargement A-C, 200 nm in D and 40 nm in E

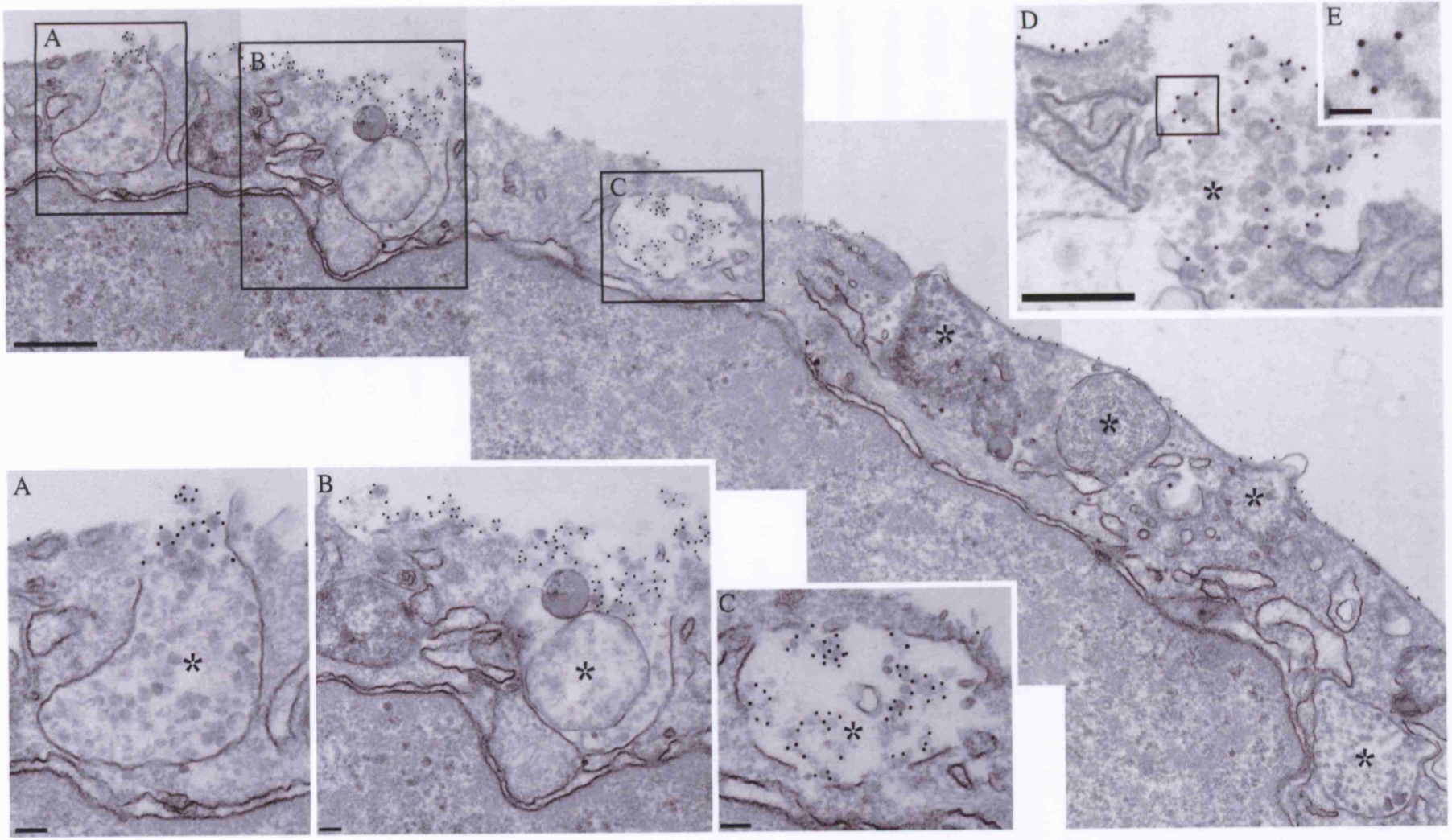


Figure 3.2: Ultra-thin sections of epon embedded RBL CCR5 cells labelled for CCR5

shows that the presence of CCR5 in secretory granules may indeed be due to the high expression of the receptor in these cells. Nevertheless, the endogenous transferrin receptor can also be found in secretory granules, although only in a small number of cells.

In summary, this morphological analysis shows that CCR5 is highly expressed at the surface of untreated RBL CCR5 cells. In addition, an internal pool is present in the secretory granules at steady state. Cell surface receptors can reach the granules through the endocytic pathway upon sustained agonist treatment. This agrees with earlier report showing that the secretory granules in RBL cells are linked to the endocytic pathway (Bonifacino et al., 1986; Bonifacino et al., 1989). Whether CCR5 targeting to the secretory granules has a functional role is unclear. Nevertheless, the high level of expression of plasma membrane CCR5 allows further characterisation of the early events leading to the receptor endocytosis upon agonist binding. The intracellular pool of receptor has to be carefully considered for further studies of CCR5 internalisation in RBL cells. In order to distinguish internalised receptor from the internal pool in the granules, cells have to be pre-labelled on ice prior to CCL5 treatment.

3.2 Cells surface localisation of CCR5

Studies in CHO cells have indicated some association of CCR5 with plasma membrane associated clathrin following agonist binding (Signoret et al., 2005). I investigated whether a similar association could be seen in RBL cells. The distribution of CCR5 at the plasma membrane prior to and following agonist binding was studied by immunofluorescence on intact cells using an antibody recognising residues at the N-terminus of the receptor. A more detailed analysis was subsequently carried out using TEM to image both the extra- and intracellular face of the plasma membrane.

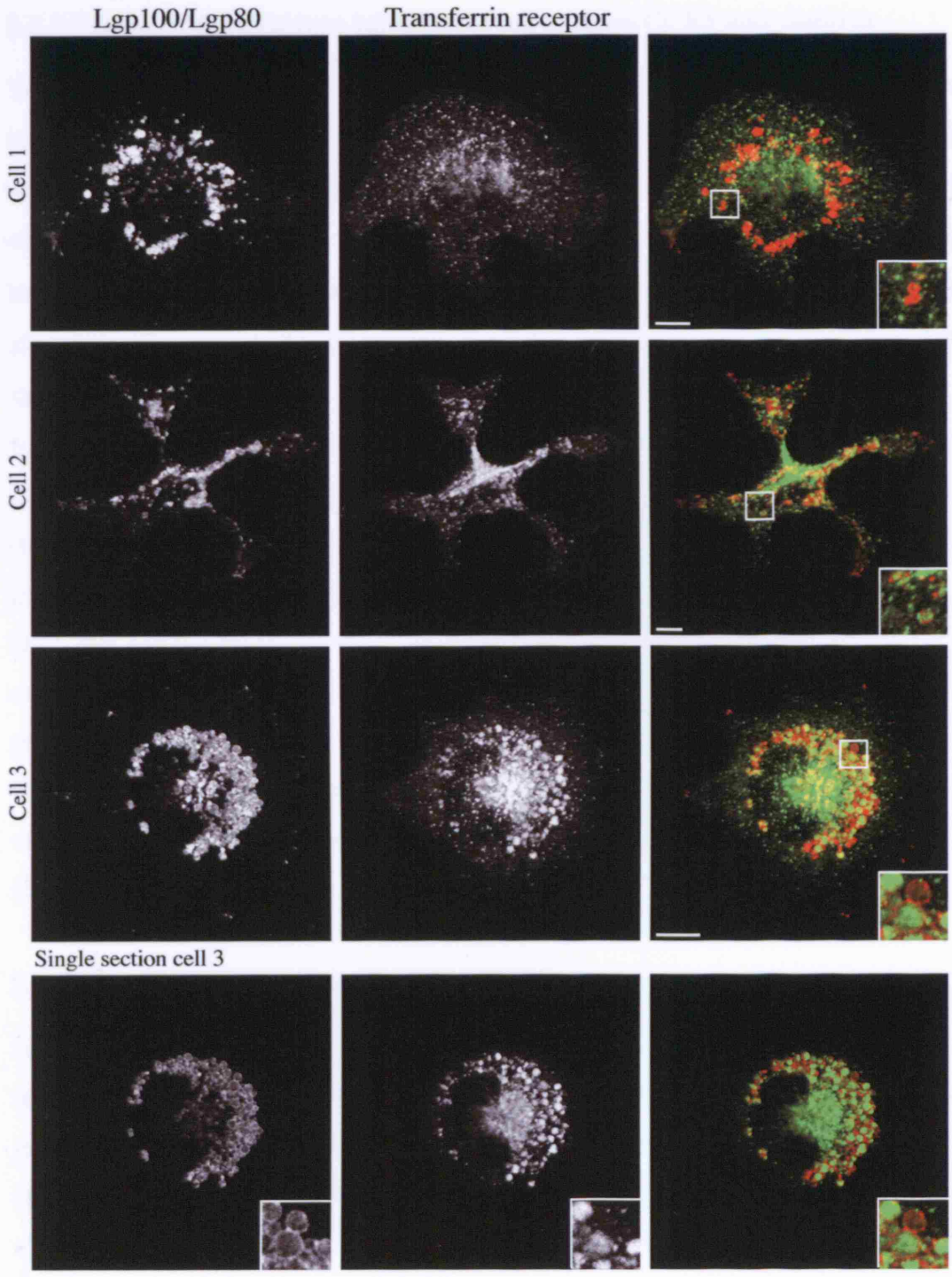


Figure 3.3: Immunofluorescence labelling of RBL CCR5 cells for the transferrin receptor and secretory granules

RBL CCR5 cells were fixed, permeabilised and labelled for lgp100/lgp80 (red) and transferrin receptor (green). Primary antibodies were detected with ^{594}GAR and ^{488}GAM secondary antibodies, respectively. The cells were analysed by confocal microscopy. Projections of z series of confocal sections of three representative cells are shown. The lower panel shows a single section from the stack of cell 3. Insets show an enlargement of the boxed areas in the merge images. Scale bar = 10 μm.

3.2.1 Immunofluorescence labelling of cell surface CCR5 and clathrin

To investigate whether clathrin was involved in agonist-induced CCR5 endocytosis, intact cells were incubated in BM or BM containing 125nM CCL5 (this concentration of agonist induces maximum down-modulation of the receptor) for 2 or 5 minutes at 37°C, then fixed and labelled for CCR5 with MC-5 and ^{488}GAM before permeabilisation. Subsequently, the cells were stained with a rabbit anti-clathrin antibody and ^{594}GAR, as described in Material and Methods section 2.4.2. Single confocal sections are shown in figure 3.4.

In untreated cells (A), plasma membrane CCR5 did not co-localise with clathrin. Upon agonist treatment (B and C), CCR5 partially colocalised with clathrin. Note the difference in cell morphology following CCL5 treatment; the cells spread, flattened and lost their star shape.

Only cell surface receptors were labelled in this experiment. Hence, the regions of overlap between CCR5 and clathrin following agonist binding correspond to clathrin positive domains of the plasma membrane rather than endocytic vesicles. Therefore, the immunofluorescence analysis suggests a role for clathrin in the very early events of agonist-induced activation of CCR5, as previously observed in CHO cells (Signoret et al., 2005).

3.2.2 Whole mount replicas of RBL CCR5 cells

Although useful, confocal microscopy is limited in the information it gives on receptor distribution. To study the distribution of CCR5 at the plasma membrane in greater detail, whole mount replicas of immunolabelled RBL CCR5 cells were prepared, as described in the Material and Methods section 2.5.2. These replicas allow visualisation of large areas of the extracellular face of the plasma membrane (Miller et al., 1991). Figure 2.1 in Materials and Methods illustrates the preparation of whole mount replicas.

Figure 3.5 shows replicas of RBL CCR5 cells incubated in BM only or BM containing 125nM CCL5 and subsequently labelled for CCR5 detected with PAG_{15}. Specific features of the plasma membrane such as microvilli (Mv) and invaginations (arrows) were observed in all samples. In untreated cells, the receptor was

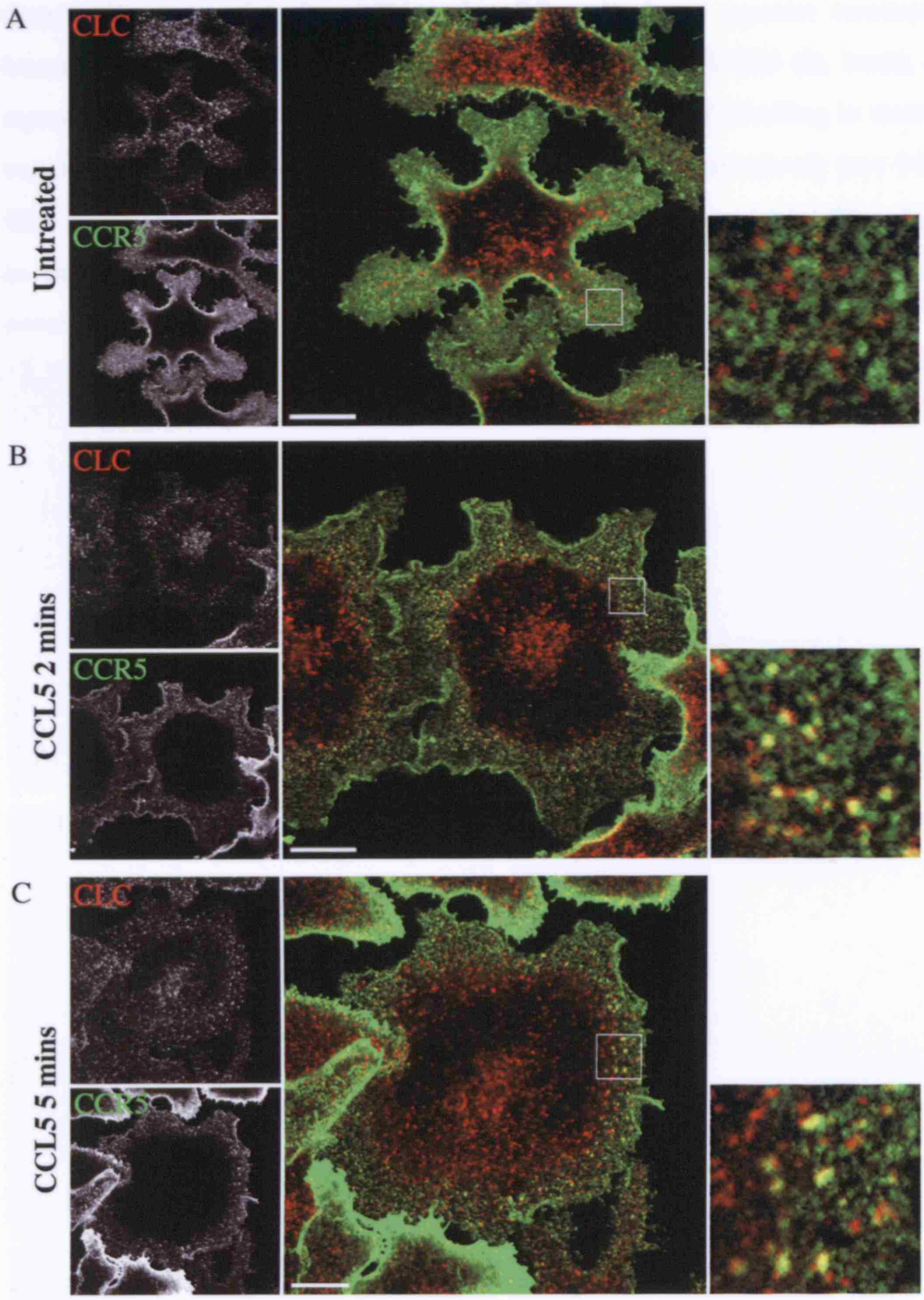


Figure 3.4: Immunofluorescence labelling of RBL CCR5 cells for surface CCR5 and clathrin

RBL CCR5 cells were incubated with BM alone (A) or with 125nM CCL5 for 2 min (B) or 5 min (C) at 37°C, fixed and labelled intact for CCR5 with MC-5, and subsequently permeabilised and labelled for clathrin with a rabbit antibody. Primary antibodies were detected with species-specific secondary antibodies ^{488}GAM and ^{594}GAR. The figure shows single confocal sections. Scale bar = 10 μm.

distributed evenly over the plasma membrane (A). In contrast, in CCL5-treated cells, CCR5 was seen in small clusters (B and C). The dramatic change in

distribution was already visible after 30 seconds of agonist treatment (B). Interestingly, some invaginations contained labelling for CCR5 (B, inset), perhaps representing endocytic uptake. In addition, the density of labelling in treated cells was lower than in untreated cells 24 and 47 PAG/μm^2, respectively (see table 3.1). This might reflect the internalisation of CCR5 following agonist binding. However, as the cells spread upon stimulation the decrease in density could also be a consequence of a larger surface of plasma membrane.

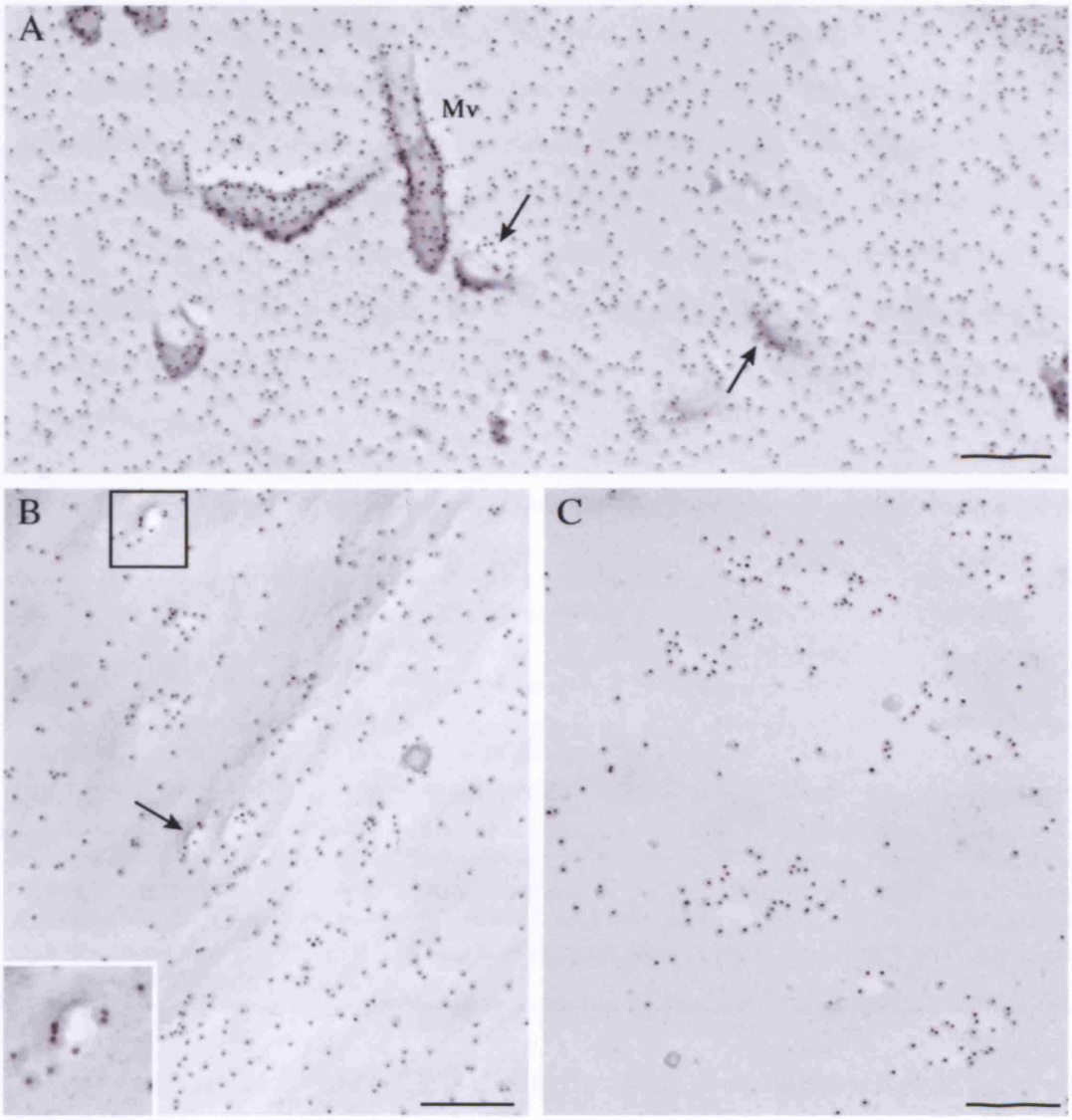


Figure 3.5: Whole mount replicas of immunolabelled RBL CCR5 cells

RBL CCR5 cells were incubated with BM alone (A) or with 125nM CCL5 for 30 sec (B) or 2 min (C) at 37°C before fixation at 4°C. CCR5 was detected by labelling intact cells with MC5 and PAG_{15}, and cell surface replicas were prepared as described in Materials and Methods section 2.5.2. Specimens were examined by EM. Invaginations of the membrane are shown with arrows. (Mv) microvilli. Scale bar = 300 nm.

The size of the clusters in cells treated for 2 min with CCL5 were measured as described in Materials and Methods section 2.5.6. Clusters had an average diameter of 0.44 ± 0.09 μm reaching to a maximum of 0.76 μm (see table 3.1). To establish whether the observed clustering of CCR5 was significant, the distribution of gold particles on untreated and CCL5-treated cells was analysed quantitatively. For each sample, a series of random images was examined and the density of gold particles per square micrometer of membrane was calculated as described in Material and Methods section 2.5.6. The results of the quantification are summarised in table 3.1. The density of gold particles in both samples varied somewhat, probably due to the heterogeneous expression of the receptors between cells. Indeed some untreated cells exhibited a density of labelling as low as 9 PAG/μm^2 while others had a density of 104 PAG/μm^2. In average the density of PAG on untreated cells was <50 PAG/μm^2. The gold particle density in clusters, on agonist-treated cells, averaged >170 PAG/μm^2, indicating an enrichment of 3.6-fold compared to untreated cells.

Table 3.1. Density of gold particles and diameter of clusters on membrane replicas of RBL CCR5 cells

	Average density (per μm^2± SD)		Cluster diameter (μm± SD)
	Random areas	Cluster (n=44)	
Untreated	47.11 ± 35.49	-	-
CCL5 2 min	24.46 ± 13.69	171.52 ± 58.87	0.44 ± 0.09

Random fields of membrane were photographed and the density of PAG was determined in the area of membrane or for CCL5 treated samples in apparent clusters. 10 different cells were analysed in untreated and CCL5-treated samples, 369.6 μm^2 and 406 μm^2 of membrane and 17086 and 9930 particles, respectively. On CCL5-treated samples, 44 clusters were analysed. Details are given in Materials and Methods section 2.5.6.

These experiments show that CCR5, initially diffusely distributed across the surface of RBL CCR5 cells, following agonist treatment rapidly redistributes into clusters. The fact that the density of receptor in clusters was higher than the density of receptor on untreated cells argues that CCR5 redistributed to clusters rather than these being patches of receptors left behind following endocytic clearance of adjacent areas.

3.2.3 Preparation of membrane sheets from RBL CCR5 cells

To determine whether the agonist-induced CCR5 clusters are associated with specific structures on the inner side of the plasma membrane, the so-called "rip-off" technique initially developed by Sanan and Anderson was used (Sanan and Anderson, 1991). By this method, large membrane sheets from the upper surface of cells can be isolated and visualised as described in figure 2.2 and in Materials and Methods section 2.5.3. RBL CCR5 cells treated with CCL5 were labelled on ice for cell surface receptors with MC-5 and detected with PAG_{15}. Subsequently, membrane sheets were generated, fixed immediately, stained and examined by TEM. Figure 3.6 shows membrane sheets from untreated (A) or agonist treated (B-H) RBL CCR5 cells labelled for CCR5 (PAG_{15}). Various features of the interior face of the plasma membrane were observed in these membrane sheets. The filamentous meshwork, which can be labelled with phalloidin (Wilson et al., 2000 and unpublished data from L. Hewlett), corresponds to the cortical actin cytoskeleton (arrow). Clathrin structures such as flat clathrin lattices (L) and invaginated clathrin-coated pits (CCP) were easily identified by their characteristic hexagonal pattern, and labelling for clathrin and AP-2 (Signoret et al., 2005).

On untreated cells, the gold particles labelling CCR5 were evenly distributed across the membrane sheets. However, their distribution did not appear to be random as particles were often located close to actin filaments (arrow), suggesting that in the absence of agonist CCR5 may be associated with the cortical actin cytoskeleton. Particles were rarely seen in areas coated with flat clathrin lattice. In cells treated with CCL5 (B), the gold particles showed the clustered distribution previously observed on the whole mount replicas. Interestingly, the clusters were located in flat clathrin lattices, and occasionally coated pits containing gold particles were seen budding from these areas (arrowhead in C). Budding profiles captured at different stages of their formation are shown in D-H. Fully formed pits had a tendency to

Figure 3.6: Membrane sheets of immunolabelled RBL CCR5 cells

RBL cells were incubated for 2 minutes at 37°C with BM (A) or BM containing 125nM CCL5 (B-H), were labelled intact with MC-5 followed by PAG_{15}. Membrane sheets were subsequently prepared as described in Materials and Methods section 2.5.3, fixed and examined by EM. (L) flat clathrin lattice, (CCP) clathrin-coated pit, (arrow) actin cytoskeleton, (arrowhead) CCP containing gold particles CCR5. Scale bars = 300 nm (A and B), 200 nm (C-F), 100 nm (G and H)

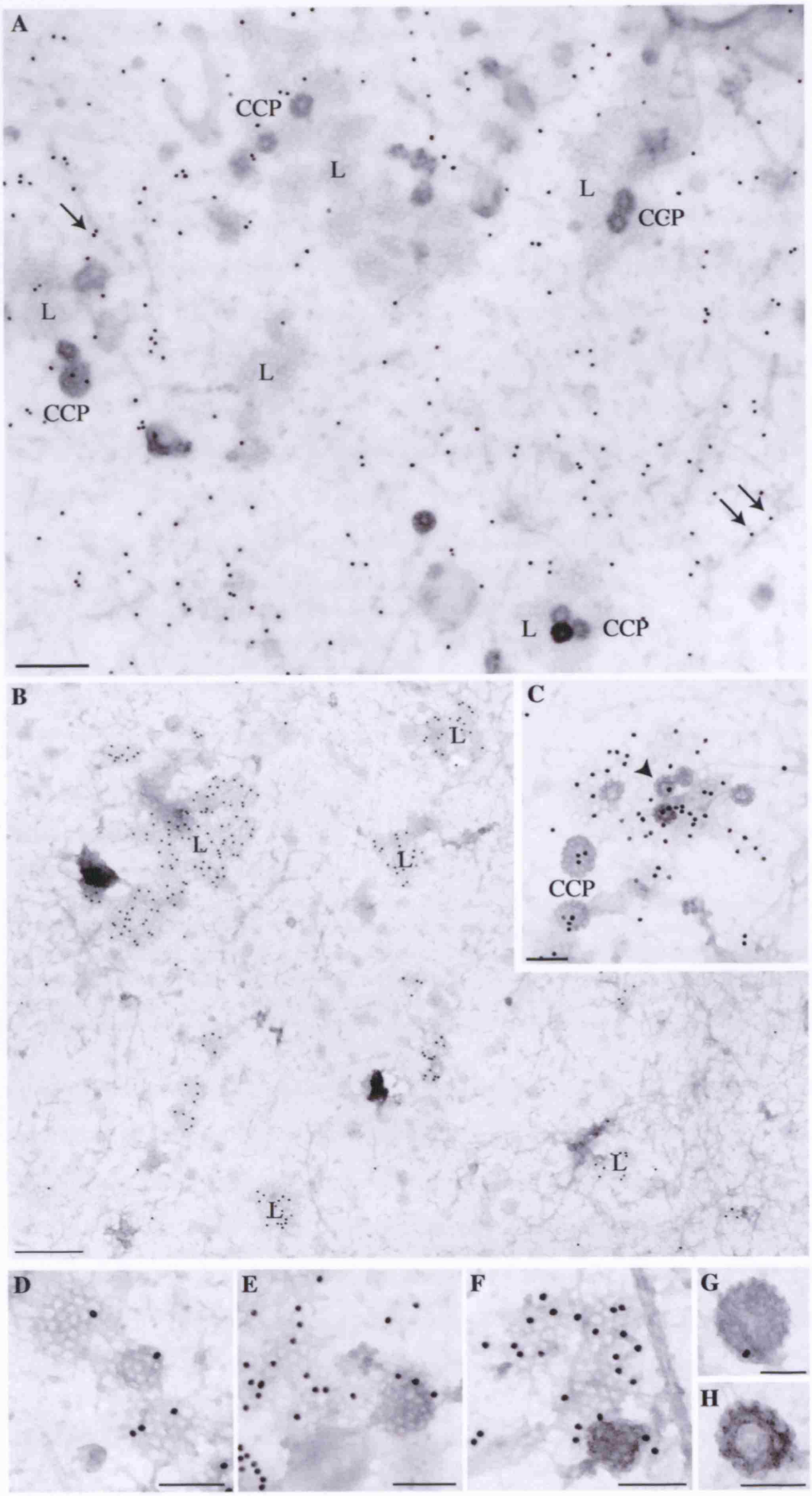


Figure 3.6: Membrane sheets of immunolabelled RBL CCR5 cells

collapse and therefore appear smaller than non-collapsed pits.

Quantitative analysis of membrane sheets revealed that before and after CCL5 treatment, equivalent areas of membrane were coated with clathrin lattice of similar average size (Table 3.2). This suggests that clathrin was not recruited to the plasma membrane following agonist treatment. In addition, the distribution of the gold particles on the membrane sheets was analysed and revealed a 4.9-fold enrichment of gold-labelled CCR5 molecules in lattices upon agonist stimulation. This is in contrast to the 16-fold enrichment observed in CHO cells, where receptors were rarely seen in clathrin lattices in untreated cells (Signoret et al., 2005). However, only 1% of the plasma membrane was coated with flat clathrin lattice in these cells compare to 4% in RBL CCR5 cells. Therefore, in untreated RBL cells, the receptor has a higher chance to be found associated with clathrin lattices than in CHO cells.

Table 3.2. Density of gold particles in flat clathrin lattices

	Average	
	Untreated	CCL5 2 min
Lattices analysed	124	63
Area of lattices (% of total membrane)	3.98±1.12	4.32±3.99
Area per lattice (μm^2)	0.09±0.03	0.09±0.06
Gold particles per μm^2 of lattice	37.65±14.49	183.27±28.47

Arbitrarily selected areas of membrane from untreated or CCL5 treated RBL CCR5 cells were photographed and analysed. As described in Materials and Methods section 2.5.6, the total area of clathrin lattice per area of membrane (expressed in %), the average size of a lattice and the number of gold particles per square micrometer of lattice were determined.

Together these results show that upon agonist binding, CCR5 is relocated into flat clathrin lattices on the plasma membrane and that clathrin-coated pits containing CCR5 bud from these domains. The membrane sheets analysed were obtained from the top of the cells as in (Signoret et al., 2005), in contrast to other studies that reported flat clathrin lattices at the bottom of the cells (Heuser, 1980; Hinrichsen et al., 2006). This analysis supports the immunofluorescence data (figure 3.4) and suggests a role for clathrin in CCR5 agonist-induced endocytosis.

3.3 Analysis of flat clathrin lattices

3.3.1 Visualisation of flat clathrin lattices by ultra-thin section

In 1964, Roth and Porter described protein endocytosis by coated vesicles (Roth and Porter, 1964). They observed coated areas on the inner side of the plasma membrane and predicted these to be the precursors of coated vesicles. Kanaseki and Kadota subsequently examined the coat around the vesicles and depicted a "basket work" composed of hexagons and pentagons (Kanaseki and Kadota, 1969). In 1980, Heuser gave a detailed description of flat clathrin lattices observed on the inside of the plasma membrane of chicken fibroblasts using the quick-freeze deep-etch technique (Heuser, 1980). The lattices were later shown to be present in various cells such as mouse peritoneal macrophages (Aggeler et al., 1983) human fibroblasts (Sanan and Anderson, 1991), Chinese hamster ovary cell (Sachse et al., 2001; Signoret et al., 2005) and lymphocytes (Lamaze et al., 2001). The main techniques used to visualise these structures were the quick-freeze, deep-etch, rotary replica developed by Heuser (Heuser, 1980), and the so-called "rip-off" developed by Sanan and Anderson (Sanan and Anderson, 1991). However, it is possible to visualise them with more traditional EM techniques such as ultra-thin section of epon embedded samples or ultra-thin cryosections (Maupin and Pollard, 1983; Sachse et al., 2001; Tokuyasu, 1997).

Here, ultra-thin sections of epon embedded RBL CCR5 cells were analysed by EM. Flat lattices are not usually seen using this technique, however careful examination of thin sections might provide supporting evidence for the presence of lattices in RBL cells on the top and the bottom of the cells.

RBL CCR5 cells were incubated in BM or BM containing 125nM CCL5, fixed with 2%PFA/0.1%GA and labelled for cell surface CCR5 with MC-5 and PAG_{15}. Following osmication, tannic acid treatment and dehydration the cells were detached from the plastic dish using propylene oxide, pelleted and embedded in epon (details in Materials and Methods section 2.5.4). Figure 3.7 shows a series of profiles from ultra-thin epon sections of RBL CCR5 cells treated with 125nM CCL5 for 5 minutes, and labelled intact for cell surface CCR5. In A and B, regions of the plasma membrane enriched with gold particles exhibit an extended electron dense coat characteristic of a clathrin coat. In addition, gold particles were observed in

budding profiles at the plasma membrane (C and D). These coats and budding profiles containing gold particles were not only present on the tops of cells (A, B, C, E) but also at the bottom (E, F and G), suggesting that CCR5 is internalised at the apical and basal plasma membrane. However, probably due to the limited accessibility of the antibody to the antigen between the cell and the coverslip after fixation, only coated structures at the edge of the cells were labelled for CCR5 (E and G). This analysis suggests the presence of the lattices at the plasma membrane and provides evidence for active endocytosis of CCR5 at the bottom and the top of the cells.

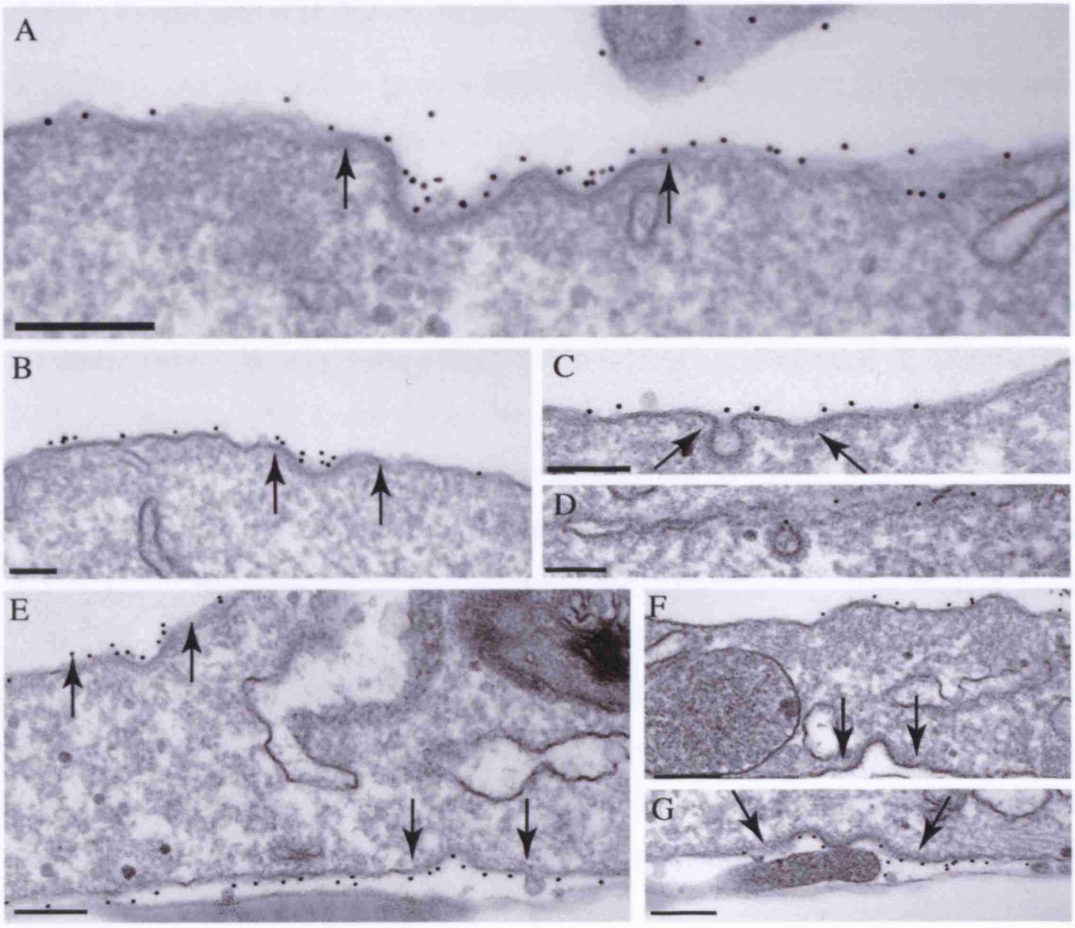


Figure 3.7: Ultra-thin section of RBL CCR5 cells

RBL CCR5 cells were treated with 125 nM CCL5 for 5 minutes, fixed in 2% PFA/0.1% GA, and intact cells were labelled for cell surface CCR5 with MC-5 and PAG_{10}. Cells were further processed for epon embedding and ultra-thin sectioning as described in Materials and Methods section 2.5.4. CCR5 is seen in extended coated area of the membrane and budding coated pits (A, B and C). Coated vesicles not opened to the plasma membrane do not contain gold particles (D). Coats and budding figures are not only seen on the upper plasma membrane but also at the bottom of the cells (E, F and G). Coats are shown between arrows. Scale bars = 200 nm (A and C-G) and 100 nm (B)

Together, the ultrastructural study shows that flat clathrin lattices can be seen in RBL CCR5 cells using an EM technique different to the rip-off. In addition, the analysis provided evidence for the presence of clathrin lattice domains, both at the bottom and the top of the cells, as previously imaged in membrane sheets. Nevertheless, preparation of membrane sheets remains the method of choice to visualise flat clathrin lattices, as it provides a large area of membrane for analysis and typical features of clathrin assembly such as the hexagonal pattern are easily identified.

3.3.2 Visualisation of flat clathrin lattices in different model cell lines

Regions of the flat clathrin lattices have been observed on the apical membrane of RBL (this thesis) and CHO cells (Signoret et al., 2005). To see whether this is a general feature of cells, several cell lines were analysed by EM for the presence of flat clathrin lattices at the plasma membrane. Membrane sheets were prepared from CHO cells expressing CCR5, mouse embryonic fibroblast (MEF) and mink lung epithelia (Mv-1-lu) expressing CXCR4 or CCR5 (as described in Materials and Methods section 2.5.3.2) and examined by EM. Figure 3.8 shows a series of profiles from different cells, where flat clathrin lattices can be seen. The presence of flat clathrin lattices was not dependent on the expression of CCR5 as wild-type MEF (A) and Mv-1-lu (B), which did not express CCR5, exhibited extended area of clathrin coat (L). In addition to clathrin structures, caveolae, identified by labelling for caveolin-1, were observed in these cells as shown in C. Flat clathrin lattices were also observed in CHO cells (D) where, similarly to RBL cells, CCR5 clustered upon agonist binding. This demonstrates that flat clathrin lattices are found in various cell lines and that the recruitment of CCR5 in these structures is not limited to RBL cells but also occurs in CHO cells.

3.3.3 Labelling of membrane sheets for endocytic proteins

In order to investigate the nature of the endocytic structures where CCR5 accumulate following agonist binding, membrane sheets from Mv-1-lu and CHO CCR5 cells were stained for proteins known to be involved in clathrin- and caveolae-mediated endocytosis. In these two cell lines flat clathrin lattices and caveolae were observed, in contrast to RBL cells where only clathrin structures were visible.

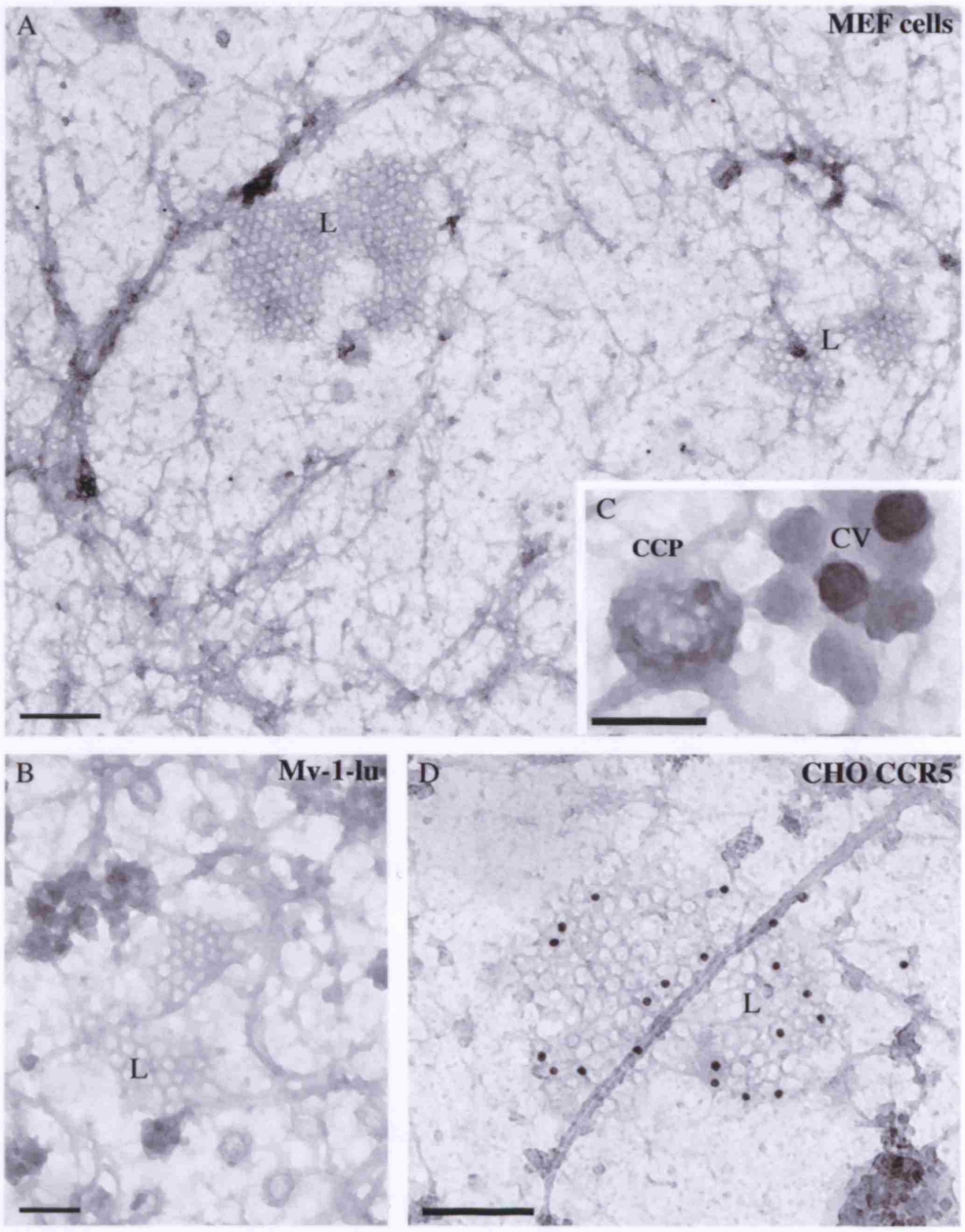


Figure 3.8: Membrane sheets obtained from different cell lines

Membrane sheets were prepared from various cell lines and analysed by EM. Untreated MEF WT cells (A), and Mv-1-lu cells (B and C). CHO CCR5 cells treated with 125 nM CCL5 for 5 minutes at 37°C, were labelled for CCR5 with MC-5 followed by PAG_{15} (C). (L) flat clathrin lattice, (Cv) caveolae, (CCP) clathrin-coated pit. Scale bars = 200 nm (A and D), 100 nm (B and C).

Figure 3.9 shows that CCR5 labelling was distinct from caveolae labelling in untreated (A) and CCL5 treated CHO cells (B). Similarly, in untreated (C) or CCL5-treated (D and E) Mv-1-lu cells, CCR5 was only observed in clathrin structures at the plasma membrane. Although the membrane sheets from Mv-1-lu cells have not been labelled for caveolin-1, caveolae can be identified by their characteristic morphology (Cv).

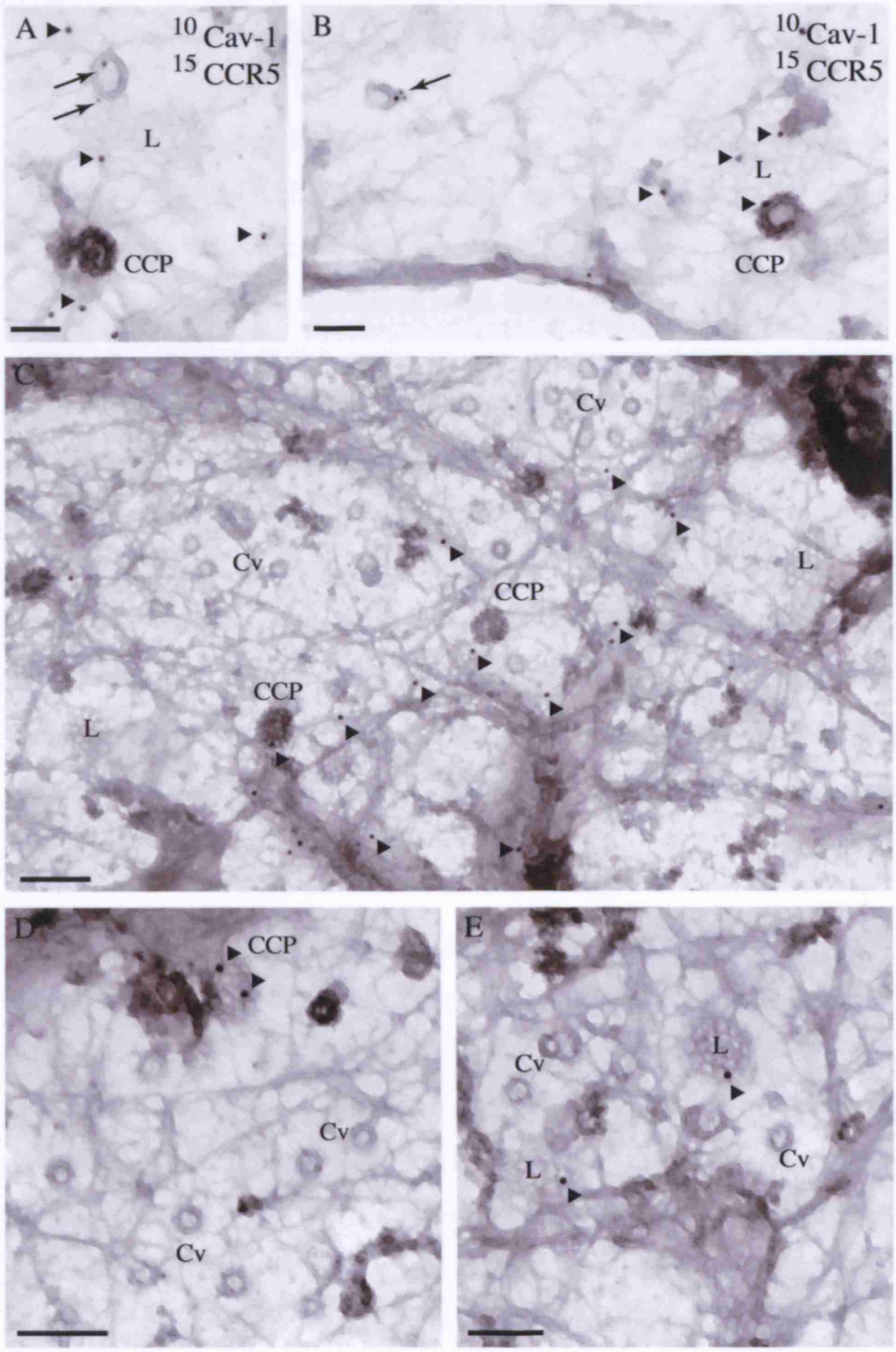


Figure 3.9: Membrane sheets from Mv-1-lu and CHO CCR5 cells labelled for CCR5 and caveolae

CHO CCR5 cells, incubated for 5 minutes at 37°C in BM (A) or BM containing 125 nM CCL5 (B), were labelled with MC-5 detected with PAG_{15} and membrane sheets prepared and labelled with antibodies against caveolin-1 and PAG_{10}. Membrane sheets from Mv-1-lu cells incubated for 5 minutes in BM (C) or BM containing 125 nM CCL5 (D and E) were labelled for CCR5 with MC-5 and PAG_{15}. Arrows indicate PAG_{10} detecting caveolin-1 and arrowheads point at PAG_{15} detecting CCR5. (L) lattices, (CCP) clathrin-coated pit, (Cv) caveolae. Scale bars = 100 nm (A and B), 200 nm (C-E).

Membrane sheets from MEF cells were also labelled for proteins of the clathrin endocytic machinery, Epsin-1, AP-2 and Eps15. The three proteins were present in flat clathrin lattices although with distinct distributions as shown in figure 3.10 A. AP-2 and Eps15 labelling were mainly associated with the edge of the lattices and CCP, whereas Epsin-1 did not show any particular distribution within the lattices, as recently described by Hawryluk *et al.* (Hawryluk et al., 2006). Whether AP-2 and Eps15 are present or not in the middle of lattices remains to be determined as the absence of labelling could just reflect a lack of antibody accessibility to the antigen. Indeed, recent reports showed labelling for AP-2 through out lattices (Hinrichsen et al., 2006; Signoret et al., 2005). On the other hand, Eps15 has only been observed at the edge of CCPs at the plasma membrane (Tebar et al., 1996).
Figure 3.10 B shows membrane sheets from CHO CCR5 cells labelled with MC-5 and PAG_{15} and with antibodies against Eps15 and Epsin-1 detected with PAG_{10}. Staining for Epsin-1 and Eps15 were similar to those seen in MEF cells in A. Upon agonist binding the receptor was recruited in clathrin structures labelled for Epsin-1 and Eps15.

In summary, the immunogold labelling of membrane sheets reveals the presence of proteins from the clathrin endocytic machinery in flat clathrin lattices independently of the presence of CCR5 or treatment with the agonist CCL5. This indicates that the lattice domains contain key accessory proteins for clathrin-mediated endocytosis. Although flat clathrin lattices have been reported in different studies, their role in endocytosis has been argued. Here, flat clathrin lattices were labelled for several proteins that have been implicated in the formation of clathrin coated vesicles. This strongly suggests flat clathrin lattices to be functionally active. Caveolae were also identified in MEF, Mv-1-lu and CHO cells but no association of CCR5 within these structures was observed. This is in direct contrast to studies suggesting CCR5 internalisation to occur via the caveolar pathway (Mueller et al., 2002; Venkatesan et al., 2003).

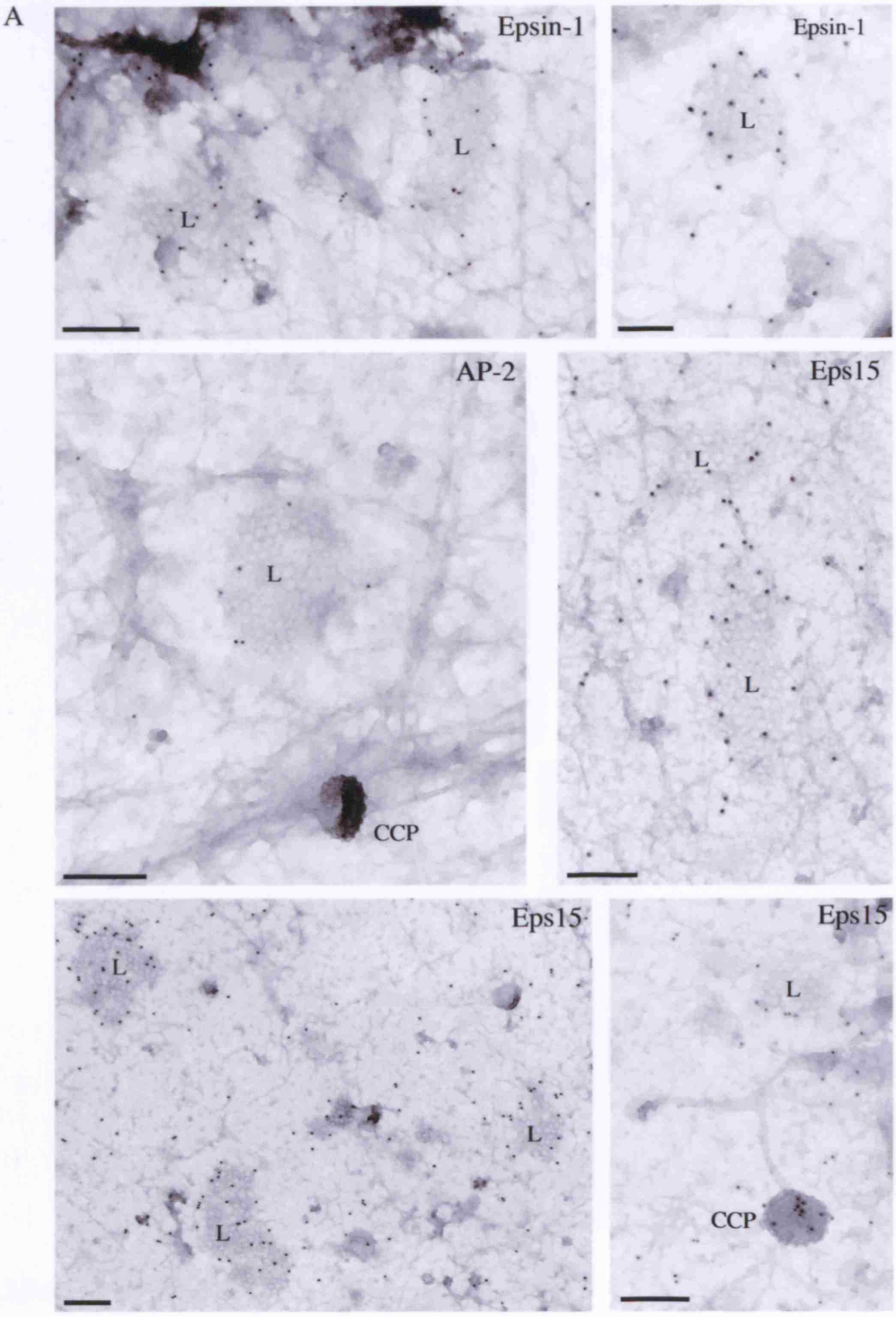
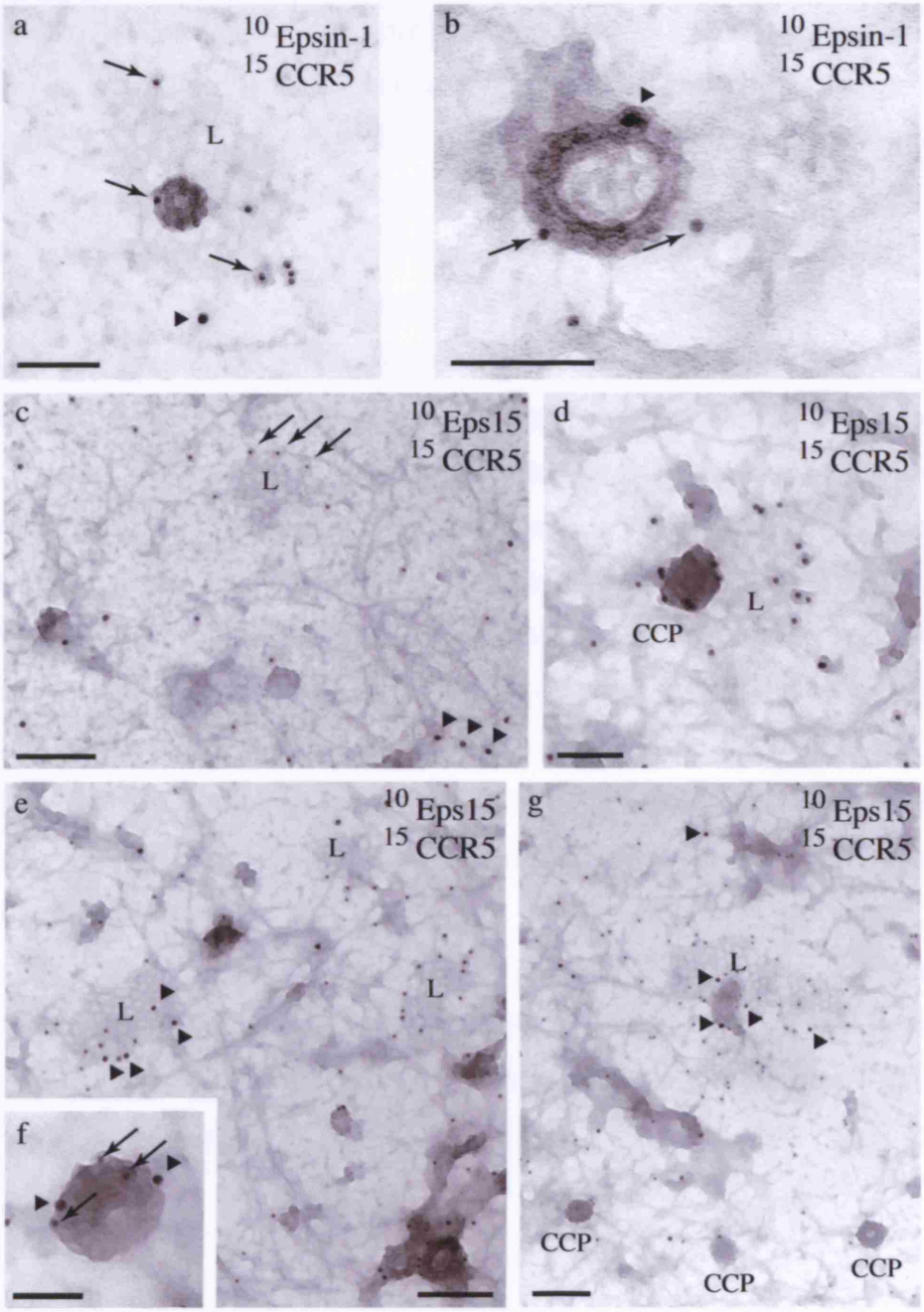


Figure 3.10: Labelling of flat clathrin lattices with antibody against endocytic proteins

(A) Wild type MEF cells were washed with Hepes buffer and membrane sheets prepared and labelled as described in Materials and Methods. The gold particles detect labelling for Epsin-1, AP-2 and Eps15 as indicated in each panel. Scale bars 200 nm. (B, see next page) CHO CCR5 cells incubated for 5 minutes in BM (a, c and d) or BM containing 125nM CCL5 (b, e-g) were labelled with MC-5 detected with PAG_{15} (Arrowhead) and membrane sheets prepared and labelled with antibodies against Epsin-1 and Eps15 detected with PAG_{10} (arrows). Scale bars = 100 nm (a, b, d and f) and 200 nm (c, e and g).

B



3.3.4 Early events in CXCR4 endocytosis

To examine whether flat clathrin lattice domains are important for the internalisation of other receptors, the early steps of CXCR4 endocytosis were investigated using the same approach as for CCR5. CXCR4 is down-modulated following agonist binding (stromal cell derived factor-1α (SDF-1α)) or activation of PKC by phorbol 12-myristate 13-acetate (PMA) treatment (Signoret et al., 1997). However, the receptor has different fates in the cell depending on the stimulus leading to its internalisation.

Following SDF-1α-induced internalisation, CXCR4 is rapidly endocytosed and directed to lysosomes for degradation (Marchese and Benovic, 2001), whereas following PMA treatment internalised receptor can recycle to the cell surface (Signoret et al., 1997). It is unknown whether the receptor uses the same endocytic machinery at the plasma membrane in each situation.

Mv-1-lu cells expressing CXCR4 were incubated in BM or BM containing the CXCR4 agonist SDF-1α or the phorbol ester PMA. The cells were labelled for the receptor and membrane sheets prepared as described in Materials and Methods section 2.5.3.3. As shown in figure 3.11, in untreated cells the receptor did not show any association with clathrin structures at the plasma membrane. However, upon agonist binding or PMA treatment CXCR4 was recruited into flat clathrin lattices and CCP, respectively. However, very few flat clathrin lattices were observed in the PMA treated samples. From this experiment it is hard to draw conclusions on possible differences between SDF-1α- and PMA-induced CXCR4 down-modulation. No association of the receptor with caveolae was observed. The antibody used here, 12G5, induced cross-linking upon binding to the receptor, even when incubated at 4°C on ice. This explains the quadruplets of gold particles usually observed on the plasma membrane.

Altogether these data show that CXCR4, another chemokine receptor, is recruited into flat clathrin lattices upon agonist binding. Whether the receptor is internalised through the same clathrin structures following agonist or phorbol ester treatment still remains to be determined.

3.4 Discussion

Immunofluorescence and electron microscopy analysis of RBL cells expressing human CCR5 indicate that the receptor is located both at the plasma membrane and on intracellular vesicles similar to secretory granules. These structures contained many small membrane vesicles, which appeared to be the principal site of the

Figure 3.11: Membrane sheets of immunolabelled Mv-1-lu CXCR4 cells

Mv-1-lu cells expressing CXCR4 were incubated with BM (A and B), BM containing SDF-1α (C) or phorbol ester PMA (D and E) for 5 minutes and labelled with 12G5 and PAG_{15}. Arrows indicate PAG_{15} in CCP. (L) flat clathrin lattice, (Cv) caveolae, (CCP) clathrin-coated pit. Scale bars = 200 nm (A and C), 100 nm (B, D and E)

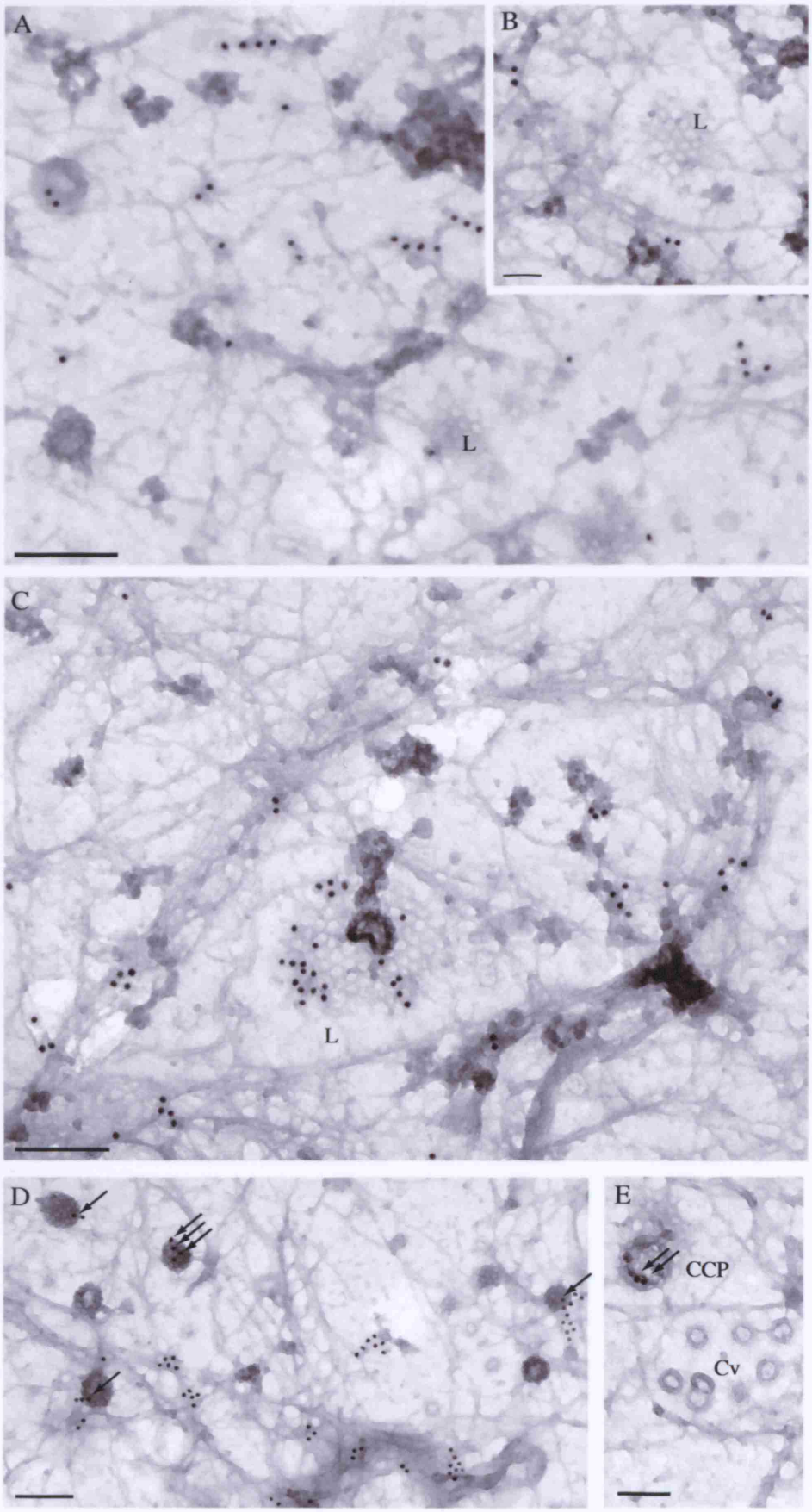


Figure 3.11: Membrane sheets of immunolabelled Mv-1-lu CXCR4 cells

intracellular CCR5. Moreover, these compartments could be induced to undergo fusion with the plasma and release their internal vesicles when cells were treated with the agonist CCL5. The cell surface pool of CCR5 was demonstrated to have at least some interaction with the secretory granule pool, as prolonged treatment with the agonist resulted in some relocation of antibody-labelled cell surface CCR5 into the vacuoles. However, for the analysis of cell-surface CCR5 distribution and the mechanism of CCR5 agonist-induced endocytosis, this internal pool of receptor was not an issue.

To analyse the cell surface distribution of CCR5 and possible agonist-induced changes in this distribution, two procedures to image plasma membrane CCR5 were set up. Firstly, immunofluorescence confocal microscopy was used to gain a view of the total population of receptors at the surface of the cells. The antibody MC-5, which recognises an epitope containing an N-terminal tyrosine residue in human CCR5 (Blanpain et al., 2002; Segerer et al., 1999), proved to be extremely effective, as this antibody bound with apparently high affinity without influence on receptor-activation, agonist-binding or agonist-induced activation. Secondly, two different ultra-structural electron microscopy techniques were used to visualise both the extracellular and cytosolic sides of the plasma membrane. In this case the receptor was detected with MC-5, followed by protein A conjugated to gold particles. These procedures revealed that CCR5 has a scattered distribution on the plasma membrane of untreated cells and suggested an association of the receptor with cortical actin under these conditions. Following stimulation with the agonist CCL5, the receptor clustered into large clathrin-coated domains of the plasma membrane identified as flat clathrin lattices. The presence of proteins from the endocytic machinery in these flat clathrin lattices and budding of CCPs containing CCR5 from these structures indicated that flat clathrin lattices are functionally active and involved in CCR5 internalisation. Together with the fact that in clathrin-depleted CHO cells agonist-induced CCR5 internalisation was inhibited (Signoret et al., 2005), these results confirm a role for clathrin in CCR5 endocytosis.

The molecular mechanism leading to CCR5 recruitment into flat clathrin lattices still remains to be established. Nevertheless, it is known that post-translational modifications are necessary for the receptor to be internalised (Kraft et al., 2001). Indeed, upon agonist binding CCR5 is rapidly phosphorylated and interacts with β-

arrestins, which play a key role in the endocytosis of many GPCRs by linking the receptors to the clathrin machinery. The importance of agonist-induced CCR5 post-translational modifications in the early events of internalisation will be investigated in the following chapters.

As caveolae had been proposed to play a role in CCR5 internalisation (Mueller et al., 2002; Venkatesan et al., 2003), ultra-structural analysis of cells labelled for caveolae and the receptor was carried out. However, in none of the samples analysed was CCR5 associated with caveolae. The evidence from Mueller *et al.* and Vankatesan *et al.* for a clathrin-independent pathway for CCR5 internalisation rely on the use of drugs that disturb membrane cholesterol (Mueller et al., 2002; Venkatesan et al., 2003). However, these drugs might have indirect effects on the endocytic pathway and the receptor itself. Indeed membrane cholesterol is required for maintaining CCR5 in a conformation able to bind its agonists (Nguyen and Taub, 2002; Nguyen and Taub, 2003a; Nguyen and Taub, 2003b; Signoret et al., 2005). Furthermore, Signoret *et al.* reported that transferrin receptor internalisation via clathrin-mediated pathway, was inhibited in the presence of fillipin (Signoret et al., 2005). The importance of membrane cholesterol in CME had previously been reported by two similar studies using methyl-β-cyclodextrin (MβCD) to selectively extract cholesterol from the plasma membrane (Rodal et al., 1999; Subtil et al., 1999). Transferrin and epidermal growth factor (EGF) uptake were inhibited by MβCD treatment. High resolution EM analysis of MβCD treated cells revealed that the invagination of clathrin pits was impaired; extended flat clathrin coats were found on the plasma membrane but no deeply invaginated pits were observed. In the present study, as well as in other reports, invaginated coated pits were observed at the edge of flat clathrin lattices. The fact that Eps15 interacts with dynamin and is only found at the periphery of the lattice might explain why budding occurs only there. In addition, invagination of regions of a flat clathrin lattice requires rearrangement of triskelia (Jin and Nossal, 1993). Indeed, flat lattices are mainly formed of hexagons. In order to obtain a vesicle-like structure, some of these hexagons have to be turned into pentagons or pentagons have to form independently. This may be possible at the edge of the lattice, where triskelia might be rapidly exchanged. In addition, a few CCPs were sometimes observed to form within the same lattice. This agrees with recent studies of live cell imaging by light microscopy, which reported 'hot

spots' of internalisation, where more than one budding events appeared to occur (reviewed in Perrais and Merrifield, 2005). The flat clathrin lattices could correspond to these 'hot spots' as they provide large amounts of readily assembled clathrin and exhibited multiple budding profiles.

To investigate whether flat clathrin lattices were part of a common mechanism of internalisation or whether they were particular to CCR5 endocytosis, another chemokine receptor, CXCR4, was studied. Preliminary data on CXCR4 internalisation suggested that the receptor was indeed recruited into flat clathrin lattices upon agonist binding. Following treatment with phorbol ester PMA, CXCR4 was also observed in clathrin structures. Whether these are similar to the ones used by CCR5 or CXCR4 upon agonist stimulation remains to be determined. The existence of distinct populations of pits has been suggested (Cao et al., 1998; Mundell et al., 2006). The two groups presented evidence for internalisation through different CCPs depending on phosphorylation of the cargo and interaction with β-arrestins. The involvement of β-arrestins in CCR5 and CXCR4 agonist-induced internalisation has previously been reported (Aramori et al., 1997; Fraile-Ramos et al., 2003) and will be further discussed in Chapter 6.

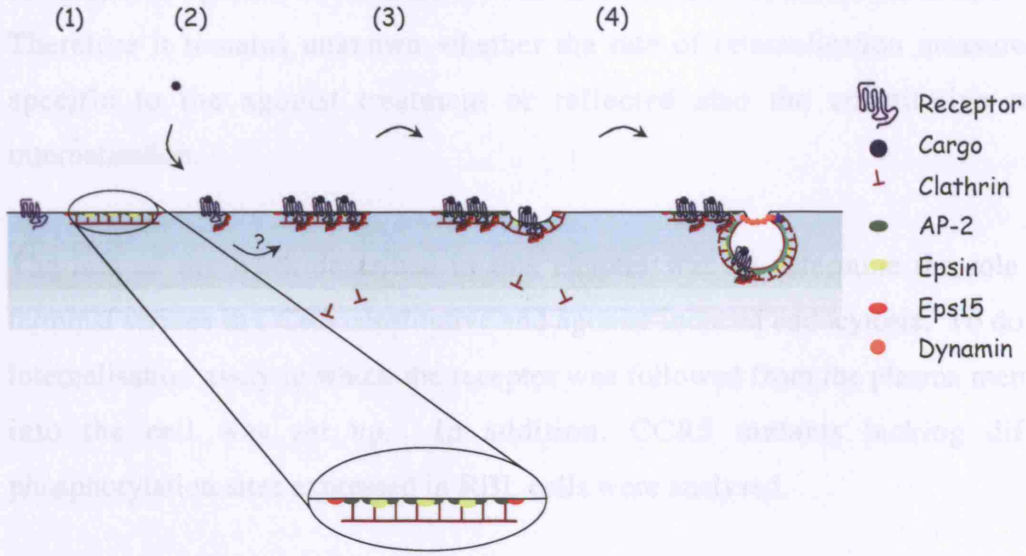


Figure 3.12: Model for early events of agonist-induced CCR5 endocytosis at the cell surface
Flat clathrin lattices (1) are extended clathrin-coated domains of the plasma membrane present at the top and the bottom surfaces of cells and contain proteins known to be involved in clathrin-mediated endocytosis, such as Epsin, Eps15, AP-2 and dynamin (Baba et al., 1999; Damke et al., 1994). Upon stimulation with agonist (2-4), CCR5 is recruited into flat clathrin lattices, where budding clathrin-coated pits are observed. The molecular mechanism behind the recruitment of the receptor into flat clathrin lattices remains unclear.

4 Importance of C-terminal phosphorylation for agonist-induced CCR5 internalisation

Agonist binding to chemokine receptors triggers conformational changes and post-translational modifications of the receptors prior to endocytosis. In particular, a cluster of serine residues in the C-terminal domain of some receptors are the targets of two kinases, protein kinase C (PKC) and G-protein coupled receptor kinase (GRK), and have been shown to be involved in the internalisation and desensitisation events. Human CCR5 contains four C-terminal serines in position 336, 337, 342 and 349, which become phosphorylated following agonist binding (Oppermann et al., 1999). Moreover, it is known that PKC and GRK mediate serine 337 and 349 phosphorylation, respectively (Pollok-Kopp et al., 2003). Huttenrauch *et al.* observed that mutants of CCR5 lacking one or two serines were internalised similarly to the wild-type receptor upon agonist binding (Huttenrauch et al., 2002). In contrast mutants lacking three or all four serines exhibited slower but efficient agonist-induced endocytosis. However, as the internalisation was measured using a radiolabelled agonist, the constitutive rate of CCR5 endocytosis was not determined. Therefore it remains unknown whether the rate of internalisation measured was specific to the agonist treatment or reflected also the constitutive rate of internalisation.

The aim of the work described in this chapter was to determine the role of C-terminal serines in CCR5 constitutive and agonist-induced endocytosis. To do so, an internalisation assay in which the receptor was followed from the plasma membrane into the cell was set up. In addition, CCR5 mutants lacking different phosphorylation sites expressed in RBL cells were analysed.

4.1 Analysis of agonist-induced CCR5 internalisation by FACS

An internalisation assay that tracks CCR5 directly has proven to be technically difficult. For example, CCR5 could not be successfully biotinylated and attempts to use antibodies were not successful either as, for example, MC-5 is not easily eluted from the receptor. Therefore, all assays set up so far to measure CCR5

internalisation were based on the uptake of labelled agonist, assuming that it would stay bound to the internalised receptor. If the agonist uptake is strictly receptor-dependent it does reflect the receptor internalisation. However, if the agonist binds other structures at the plasma membrane the internalisation rate can be biased. This is the case with chemokines because of their propensity to bind glycosaminoglycans (GAGs) such as heparan sulphate (Kuschert et al., 1999; Trkola et al., 1999). Moreover, an assay following the agonist does not allow the constitutive rate of internalisation to be determined.

An internalisation assay had been developed to analyse CD4 endocytosis (Pelchen-Matthews et al., 1989). A radiolabelled antibody against the extracellular domain of the receptor was pre-bound before inducing internalisation by a temperature shift. Endocytosis was stopped by cooling the cells to 4°C on ice and, in order to measure the pool of internalised CD4, the antibody associated with plasma membrane CD4 was acid stripped. The rate of internalisation was then determined according to the amount of radioactivity resistant to the acid strip. Unfortunately this approach is not possible to study CCR5 endocytosis, since most antibodies against the N-terminus or extracellular loops of the receptor induce CCR5 endocytosis, block the agonist-binding site or inhibit agonist binding by affecting the receptor conformation. The only antibody binding CCR5 without any effect on the receptor internalisation cannot be stripped off the receptor (Signoret et al., 2000).

Therefore a slightly different approach was taken using the high affinity antibody, MC-5, directly coupled to a fluorophore, Alexa Fluor® 488 (method modified from Peden et al., 2004). Labelled MC-5 still binds efficiently to CCR5 (figure 4.2) and can be used to follow the receptor from the plasma membrane into intracellular compartments. In addition, the fluorescence associated with cell surface receptor can be quenched using an antibody against Alexa Fluor® 488, and the amount of internalised CCR5 determined. The internalisation assay is described in figure 4.1.

Figure 4.1: Internalisation assay

An antibody against the N-terminus of CCR5, MC-5, was directly coupled to Alexa Fluor 488. CCR5 positive cells in suspension were labelled at 4°C and internalisation was then induced by bringing the cells to 37°C in the presence or absence of agonist. Two aliquots were taken at different time points and transferred on ice in order to stop endocytosis. The total cell-associated fluorescence was determined on one sample, and the internal fluorescence was measured in the other after quenching the cell surface fluorescence with an antibody against Alexa Fluor® 488. Fluorescence was measured by flow cytometry, counting 10,000 events per samples.

Live cells in suspension
CCR5 labelling with 488MC-5
+ CCL5
Quenching of cell surface 488MC-5
No quenching
= internal fluorescence
= total cell-associated fluorescence
Flow cytometry analysis: quench vs total fluorescence
CCL5
CCR5 labelling with 488MC-5
Temperature (°C)
37
4
Time (min)

4.1.1 Analysis of the data

The internalisation of CCR5 was followed after the temperature shift, and aliquots were taken before starting the internalisation (time 0) and 5, 15 30 and 60 minutes after inducing endocytosis. At each time point, two aliquots were taken to measure the total fluorescence associated with the cells, and the internal fluorescence after quenching. At time zero, the total fluorescence corresponded to the maximum cell surface fluorescence as no internalisation had occurred. The fluorescence associated with the quenched sample at time zero is a background value and appears as the limit of the assay.

To quantify the internalisation of the receptor at anytime point, the background value at time 0 (Q_0), the fluorescence associated with the quenched sample (Q) and the total fluorescence (T) have to be considered. The percentage of internalised receptor at any time can be calculated according to the formula:

$$\% \text{ internalised CCR5} = \frac{Q - Q_0}{T - Q_0} \cdot 100$$

The percentage of internalised CCR5 can then be plotted versus time in a graphic as shown in figures 4.3, 4.5 and 4.6. The average rate of internalisation in the first five minutes can also be calculated by dividing the percentage of internalised receptor at 5 minutes by the number of minutes, and is then expressed as %/min as shown in table 4.1. By calculating the internalisation rate over five minutes, a value that is not affected by significant recycling is obtained.

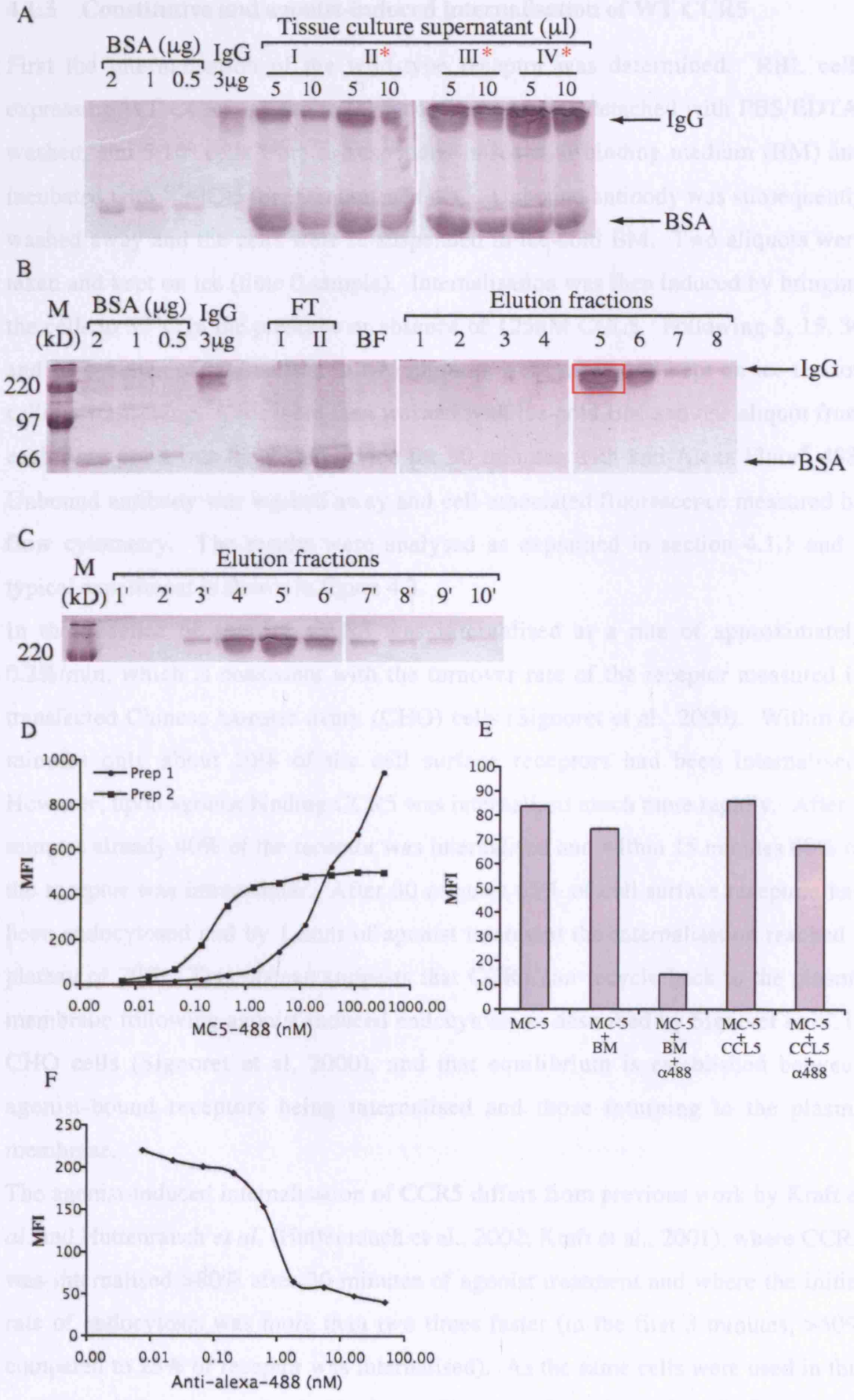
4.1.2 MC-5 coupling to Alexa Fluor® 488

MC-5 was prepared from hybridoma cells as described in Materials and Methods section 2.3.2. Supernatant was analysed for the presence of MC-5, and the antibody was purified and run through a desalting column (Figure 4.2 A-C). The purified antibody was subsequently coupled to the Alexa Fluor® 488 dye and the degree of labelling determined following the manufacturer instructions. Two different preparations of antibody were coupled to Alexa Fluor® 488. While the first one had a ratio dye:antibody of 9:1, the second had a ratio of 3:1. Both antibody preparations showed good signal when titrated by FACS, however the second had a greater

binding efficiency for CCR5 than the first (figure 4.2 D). The first failed to show saturable binding, whereas the second preparation reached saturation between 1 and 10 nM. This is probably due the lower degree of dye coupled to the antibody. The second preparation was therefore used for further work at 4.9 nM, where maximum labelling is obtained. Panel E illutrates the principle of the assay with the quenching antibody anti-Alexa Fluor® 488. All cells were labelled for plasma membrane CCR5 with ^{488}MC-5 on ice, and subsequently washed to remove unbound antibody. Samples, which were fixed immediately after labelling, had a maximum mean fluorescence intensity (MFI) of >80. When cells were incubated for 30 minutes in BM after labelling, the MFI decreased slightly (>70), probably due to some dissociation of the antibody. If these cells were then incubated with the quenching antibody anti-Alexa Fluor® 488, the MFI decreased to 15, indicating that most of the labelled-receptors were accessible to the quenching antibody at the plasma membrane, and therefore not internalised. Cells incubated for 30 minutes in BM containing 125mM CCL5 showed a maximum MFI (>80), indicating that the fluorescence was not lost upon agonist treatment (no degradation or dissociation of ^{488}MC-5 from CCR5). Finally, when the CCL5-stimulated cells were incubated with the quenching antibody, the MFI only decreased to 65, indicating that most of the labelled receptor was not accessible to the antibody, as a result of agonist-induced internalisation. In addition, the quenching antibody was titrated and showed a maximum of 80% quenching at 5 nM (figure 4.2 E and F). Thus, this concentration was chosen for the endocytosis assay.

Figure 4.2: MC-5 coupling to Alexa-488 and quenching with anti-Alexa Fluor® 488

(A) Supernatants from hybridoma cells producing MC-5 were analysed by SDS-PAGE under non-denaturing conditions. Supernatants II, III and IV were pooled, purified on a protein A/G column and analysed by SDS-PAGE (B). Fraction 5 of purified antibody was then run on a desalting column prior to coupling the Ig to Alexa Fluor® 488 (C). (D) Two preparations of ^{488}MC-5 were titrated. Preparation 1 (prep1), which was labelled three times more than preparation 2 (prep 2), did not show saturable bindind on RBL CCR5 cells. (E) Anti-Alexa Fluor® 488 antibody quenches ^{488}MC-5 at the plasma membrane. (F) Titration of anti-Alexa Fluor® 488. (IgG) purified human immunoglobulin G, (BSA) bovine serum albumin, (M) MW marker, (FT) flow through, (BF) binding buffer.


A
BSA (µg)
2 1 0.5
IgG 3µg
Tissue culture supernatant (µl)
I
II*
III*
IV*
5 10 5 10 5 10 5 10
IgG
BSA
B
M (kD)
BSA (µg)
2 1 0.5
IgG 3µg
FT
I II
BF
Elution fractions
1 2 3 4 5 6 7 8
220
97
66
IgG
BSA
C
M (kD)
Elution fractions
1' 2' 3' 4' 5' 6' 7' 8' 9' 10'
220
D
Prep 1
Prep 2
MFI
MC5-488 (nM)
E
MFI
MC-5
MC-5 + BM
MC-5 + BM + α488
MC-5 + CCL5
MC-5 + CCL5 + α488
F
MFI
Anti-alexa-488 (nM)

4.1.3 Constitutive and agonist-induced internalisation of WT CCR5

First the internalisation of the wild-type receptor was determined. RBL cells expressing WT CCR5, grown to 90% confluence, were detached with PBS/EDTA, washed, and $5 \cdot 10^6$ cells were re-suspended in ice-cold binding medium (BM) and incubated with ^{488}MC-5 for 90 minutes at 4°C. Unbound antibody was subsequently washed away and the cells were re-suspended in ice-cold BM. Two aliquots were taken and kept on ice (time 0 sample). Internalisation was then induced by bringing the cells to 37°C in the presence or absence of 125nM CCL5. Following 5, 15, 30 and 60 minutes of internalisation two aliquots were taken and kept on ice to stop cellular trafficking. Cells were then washed with ice-cold BM and one aliquot from each time point was incubated on ice for 90 minutes with anti-Alexa Fluor® 488. Unbound antibody was washed away and cell-associated fluorescence measured by flow cytometry. The results were analysed as explained in section 4.1.1 and a typical experiment is shown in figure 4.3.

In the absence of agonist, CCR5 was internalised at a rate of approximately 0.2%/min, which is consistent with the turnover rate of the receptor measured in transfected Chinese hamster ovary (CHO) cells (Signoret et al., 2000). Within 60 minutes only about 10% of the cell surface receptors had been internalised. However, upon agonist binding CCR5 was internalised much more rapidly. After 5 minutes already 40% of the receptor was internalised and within 15 minutes 60% of the receptor was intracellular. After 30 minutes 65% of cell surface receptors had been endocytosed and by 1 hour of agonist treatment the internalisation reached a plateau of 70%. This plateau suggests that CCR5 can recycle back to the plasma membrane following agonist-induced endocytosis as described by Signoret *et al.* in CHO cells (Signoret et al, 2000), and that equilibrium is established between agonist-bound receptors being internalised and those returning to the plasma membrane.

The agonist-induced internalisation of CCR5 differs from previous work by Kraft *et al.* and Huttenrauch *et al.* (Huttenrauch et al., 2002; Kraft et al., 2001), where CCR5 was internalised >80% after 30 minutes of agonist treatment and where the initial rate of endocytosis was more than two times faster (in the first 3 minutes, >50% compared to 25% of receptor was internalised). As the same cells were used in this study, the differences are probably due to the assay. In their studies, Kraft *et al.* and

Huttenrauch *et al.* measured the uptake of the agonist CCL5, which may well reflect the receptor endocytosis, but does not necessarily measure receptor recycling as the labelled agonist might dissociate from the receptor intracellularly before CCR5 returns to the plasma membrane. In contrast in the present assay, recycling receptor are still labelled and therefore might contribute to the lower internalised pool of CCR5. Nevertheless, this does not explain the higher initial rate of internalisation.

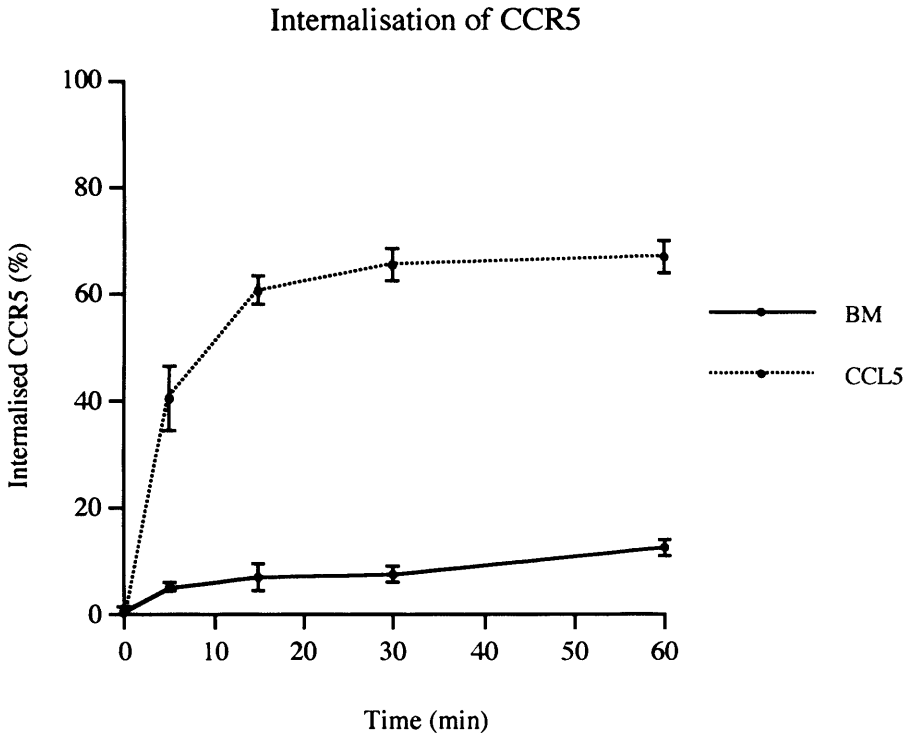


Figure 4.3: Constitutive and agonist-induced internalisation of WT CCR5

RBL cells expressing CCR5 WT were labelled in suspension with ^{488}MC-5 for cell surface receptors. Internalisation was induced by a temperature shift in the presence (dashed line) or absence (full line) of 125nM CCL5. Aliquots were taken at 0, 5, 15, 30 and 60 minutes and the fluorescence associated with each time point (total and internal) analysed by flow cytometry. The amount of internalised receptor was determined as described in section 4.1.1. Each time point indicates the internal fluorescence as a proportion of the total cell associated fluorescence. All data points show mean and standard deviations (S.D.) for duplicate samples from 6 independent experiments.

This is the first assay to be described that measures the basal (constitutive) and agonist-induced internalisation of CCR5. The data show that the constitutive rate of CCR5 endocytosis is very low, but following agonist binding the receptor is rapidly internalised. Probably due to the recycling ability of CCR5, the internalisation curve

reaches a plateau within 60 minutes of stimulation where 70% of the receptor is intracellular.

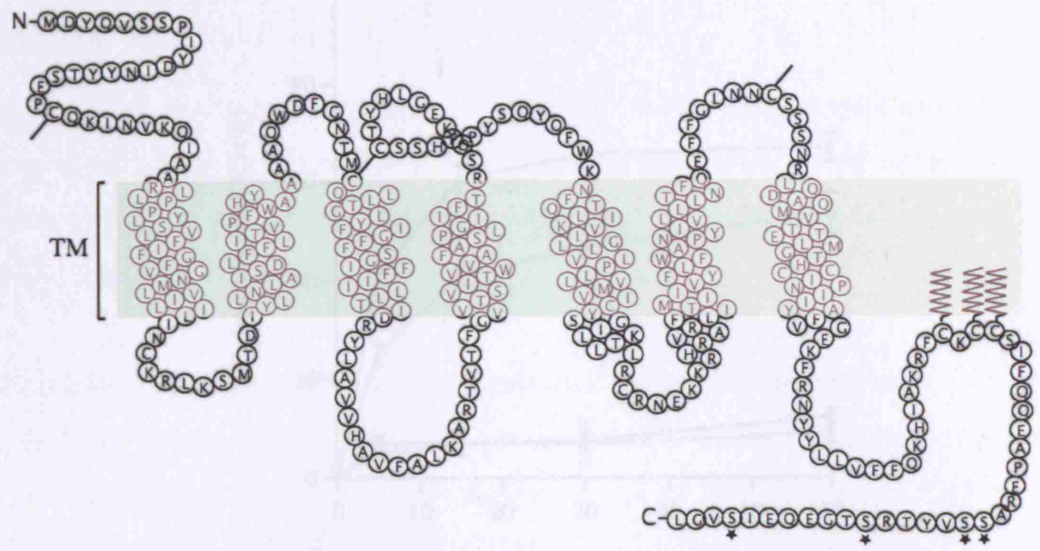
4.1.4 Constitutive and agonist-induced internalisation of phosphorylation deficient CCR5

To determine the importance of C-terminal serines in both agonist-induced and constitutive CCR5 endocytosis, the internalisation of different CCR5 mutants lacking some or all serines was analysed and compared with the wild-type receptor endocytosis. The different mutants detailed in figure 4.4 were all stably expressed in RBL cells and were provided by M. Oppermann. In CCR5 ΔS all serines were mutated to alanines, and the receptor cannot be phosphorylated. In order to study specifically the role of PKC phosphorylation, two mutants were used; in one S337 was mutated to alanine (CCR5 S337A), in the other all serines but S337 were substituted with alanine (CCR5 S337only). Similarly we used two mutants of CCR5 lacking only S349 (CCR5 S349A) or lacking all serines but S349 (CCR5 S349only) to study the role of GRK phosphorylation. All mutants were shown to be efficiently phosphorylated on their remaining serines upon agonist binding (see Chapter 5).
The receptor expression level varied between the cell lines. RBL CCR5 S337A cells had a higher level of expression compared to RBL CCR5 cells, and RBL CCR5 S349A cells had a lower expression level. The other cell lines had a comparable receptor expression levels to the RBL CCR5 cells.

4.1.4.1 Internalisation of CCR5 ΔS

A low level of constitutive endocytosis was observed for CCR5 ΔS (lacking all serines) as shown in figure 4.5. This kinetic was similar to the wild-type receptor, with only 10% of cell surface receptor internalised after 60 minutes in BM at 37°C. Surprisingly, upon CCL5 binding, CCR5 ΔS was rapidly endocytosed. In comparison to the WT receptor, the initial rate of internalisation was lower (5%/min versus 8%). The amount of internalised receptor kept increasing and by 60 minutes of agonist treatment over 50% of the receptor was internal. This is in contrast to the internalisation of WT CCR5, which plateaued after 30 minutes at around 70%.

A



B

	336 337 342 349
CCR5 WT:	-KHIAKRFCKCCSIFQQEAPERASSVYTRSTGEQEISVGL
CCR5 ΔS:	-KHIAKRFCKCCSIFQQEAPERAAAVYTRATGEQEIAVGL
CCR5 S337A:	-KHIAKRFCKCCSIFQQEAPERASAVYTRSTGEQEISVGL
CCR5 S336/342/349A:	-KHIAKRFCKCCSIFQQEAPERAASVYTRATGEQEIAVGL
CCR5 S349A:	-KHIAKRFCKCCSIFQQEAPERASSVYTRSTGEQEIAVGL
CCR5 S336/337/342A:	-KHIAKRFCKCCSIFQQEAPERAAAVYTRATGEQEISVGL

Figure 4.4: CCR5 serine mutants

(A) Serpentine model of CCR5. Asterisks indicate C-terminal serines modified upon agonist binding. (B) C-terminal residues of CCR5 and mutants of CCR5 used in this thesis. The four serines of interest are highlighted in red.

As described in Chapter 1, agonist-induced phosphorylation of C-terminal serines is believed to play a key role in CCR5 internalisation by providing a platform for recruitment of β-arrestins to the activated receptor that link the receptor to the clathrin endocytic machinery (Aramori et al., 1997; Kraft et al., 2001). Therefore, mutation of all C-terminal serines in CCR5 should impair the interaction with β-arrestins and thus inhibit CCR5 endocytosis. The observation that CCR5 ΔS internalisation was not abolished suggests that the receptor is still able to access some endocytic machinery. Whether it is internalised through the same pathway as the wild-type receptor remains to be determined.

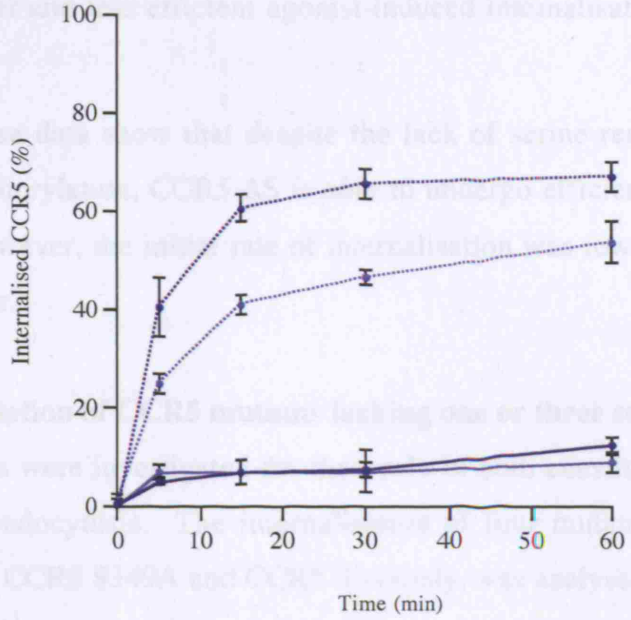


Figure 4.5: Constitutive and agonist-induced internalisation of WT CCR5 and CCR5 ΔS

RBL cells expressing CCR5 WT (dark blue) or ΔS (light blue) were labelled in suspension with ^{488}MC-5 for cell surface receptors. Internalisation was induced by a temperature shift in the presence (dashed line) or absence (full line) of 125nM CCL5. Aliquots were taken at the indicated times and the total and internal fluorescence analysed by flow cytometry. The amount of internalised receptor was determined as described in section 4.1.1. Each time point indicates the internal fluorescence as a proportion of the total cell-associated fluorescence. All data points show the mean ± S.D. for duplicate samples from 4 independent experiments for CCR5 ΔS and 6 for CCR5 WT.

Agonist-induced internalisation of CCR5 ΔS have previously been measured by Huttenrauch *et al.* and Kraft *et al.* where respectively 6% and 14% of CCR5 ΔS were internalised after 3 minutes (Huttenrauch et al., 2002; Kraft et al., 2001). The authors used the same assay and the same cells, and did not comment on this variation. Moreover, the standard deviation in Kraft *et al.* studies suggests minimal errors on their data. Huttenrauch *et al.*, however, did not show any standard deviation. As shown in figure 4.5, using this new assay 15% of receptor was internalised 3 minutes after agonist stimulation consistent with the data of Kraft *et al.* In addition to the differences in the initial rate of endocytosis, the receptor was internalised >65% after 30 minutes in Huttenrauch *et al.* and Kraft *et al.* studies, compared to 50% in figure 4.5. As mentioned in section 4.1.2, this difference probably reflected the different approach used to study the internalisation of the receptor. Nevertheless, in both assays the phosphorylation deficient receptor

exhibited a slower and less efficient agonist-induced internalisation than the wild-type receptor.

In summary, these data show that despite the lack of serine residues that can be targets for phosphorylation, CCR5 ΔS is able to undergo efficient agonist-induced endocytosis. However, the initial rate of internalisation was lower than that of the wild-type receptor.

4.1.4.2 Internalisation of CCR5 mutants lacking one or three serines

Individual serines were investigated for their role in both constitutive and agonist-induced CCR5 endocytosis. The internalisation of four mutants, CCR5 S337A, CCR5 S337only, CCR5 S349A and CCR5 S349only, was analysed and compared to that of the wild-type receptor. All mutants exhibited comparable level of constitutive internalisation to CCR5 WT with 5 to 10% of the receptor internalised after 60 minutes in BM at 37°C (although CCR5 S337only was not internalised as well as the other receptors, see section 4.1.5). However, differences were observed in the agonist-induced internalisation. CCR5 S337A underwent slightly slower endocytosis than the wild-type receptor reaching 55% of internalised receptors after 60 minutes. In the case of CCR5 S337only, the receptor internalisation resembled very much that of CCR5 ΔS: 5 minutes of agonist treatment induced 20% of CCR5 S337only internalisation and after 60 minutes 50% of the receptor was internal as in the case of CCR5 ΔS. These results suggest a minimal role for agonist-induced PKC phosphorylation of S337 in CCR5 internalisation.

CCR5 S349A showed a faster initial and much more efficient internalisation compare to CCR5 WT. In the first 5 minutes, already more than 60% of the mutant receptor was internalised compare to 40% of the wild-type receptor. The pool of internal receptor increased further during the 60 minutes of agonist treatment and reached 85%, in contrast to the wild type receptor internalisation, which plateaued after 30 minutes of treatment with >65% internal receptor. When all serines were mutated to alanines but S349 (S349only), internalisation of the mutant receptor showed very similar kinetics to the wild-type receptor. After 5 minutes of agonist treatment 35% of CCR5 S349only was internalised, compare to 40% of CCR5 WT. A plateau was reached after 30 minutes with >60% of mutant receptor internal.

Together, these results strongly suggest an important role for agonist-induced GRK phosphorylation of S349 in CCR5 endocytosis.

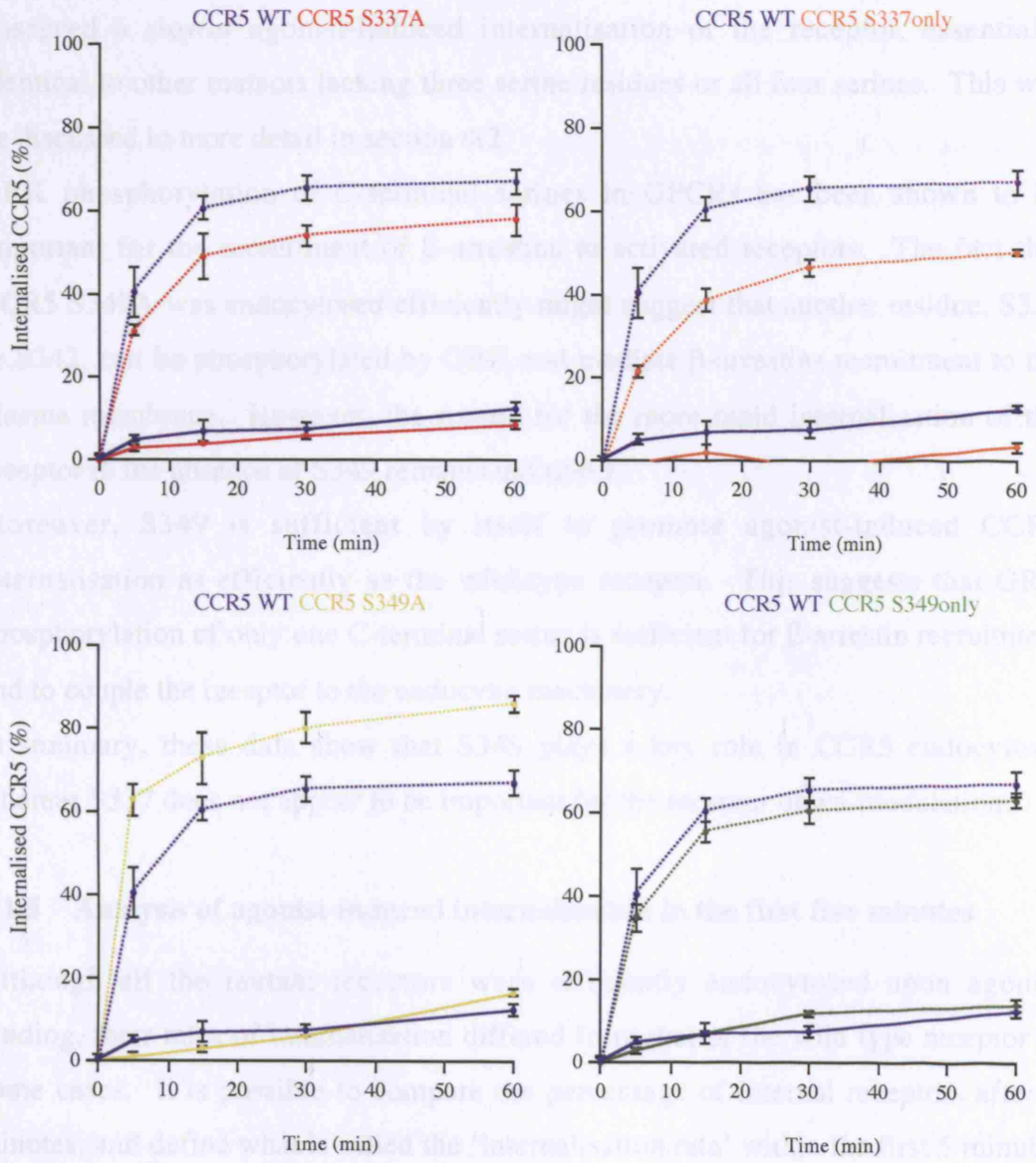


Figure 4.6: Constitutive and agonist-induced internalisation of CCR5 WT and CCR5 phosphorylation mutants

RBL cells expressing CCR5 WT (dark blue), CCR5 S337A (red), CCR5S337only (orange), CCR5 S349A (yellow) or CCR5 S349only (green) were labelled in suspension with ^{488}MC-5 for cell surface receptors. Internalisation was induced by shifting the cells to 37°C in the presence (dashed line) or absence (full line) of CCL5 125nM. Aliquots were taken at the indicated times and the fluorescence associated (total and internal) analysed by flow cytometry. The amount of internalised receptor was determined as described in section 4.1.1. Each time point indicates the cell-associated internal fluorescence as a proportion of the total fluorescence. All data points show the mean ± S.D. for duplicate samples from 4 independent experiments except for CCR5 S349A, where they represent 3 independent experiments.

Although Huttenrauch *at al.* reported similar internalisation of CCR5 S349A and CCR5 S337only relatively to the wild-type receptor, their data on the down-modulation of CCR5 S349only differ from that shown in figure 4.6. The authors observed a slower agonist-induced internalisation of the receptor, essentially identical to other mutants lacking three serine residues or all four serines. This will be discussed in more detail in section 4.2.

GRK phosphorylation of C-terminal serines in GPCRs has been shown to be important for the recruitment of β-arrestins to activated receptors. The fact that CCR5 S349A was endocytosed efficiently might suggest that another residue, S336 or S342, can be phosphorylated by GRK and mediate β-arrestins recruitment to the plasma membrane. However, the reason for the more rapid internalisation of the receptor in the absence of S349 remains unknown.

Moreover, S349 is sufficient by itself to promote agonist-induced CCR5 internalisation as efficiently as the wild-type receptor. This suggests that GRK phosphorylation of only one C-terminal serine is sufficient for β-arrestin recruitment and to couple the receptor to the endocytic machinery.

In summary, these data show that S349 plays a key role in CCR5 endocytosis whereas S337 does not appear to be important for the receptor down-modulation.

4.1.5 Analysis of agonist-induced internalisation in the first five minutes

Although all the mutant receptors were efficiently endocytosed upon agonist binding, their rates of internalisation differed from that of the wild type receptor in some cases. It is possible to compare the percentage of internal receptors after 5 minutes, and define what is called the 'internalisation rate' within the first 5 minutes of endocytosis expressed in % of total receptors per minute. The rates of internalisation of the wild type and the different mutant receptors are shown in table 4.1.

As expected, the constitutive rate of internalisation in the first 5 minutes did not differ significantly and varied between 0.5 and 1.3 %/min for the wild-type receptor, CCR5 ΔS, CCR5 S337A and CCR5 S349only. CCR5 S349A and CCR5 S337only exhibited a slower rate of 0-0.2 %/min. The rate of CCR5 S349A internalisation was constant and in 60 minutes about 10 % of receptors were internalised, whereas CCR5 S337only did not show much internalisation over the same time course. This

suggests a more stable association of the receptor with the plasma membrane. This low rate of constitutive internalisation is similar to that observed for CD4 in lymphocytes, where the receptor is retained at the cell surface through a mechanism preventing its association with coated pits for endocytosis (Pelchen-Matthews et al., 1991). However, further evidence is required to confirm this hypothesis.

In contrast, the rate of internalisation following agonist treatment fluctuated much more. The wild type receptor was internalised at a rate of 8.02 ± 1.19 %/min, while the two mutants, CCR5 S337A and CCR5 S349only, were slightly slower, 6.19 ± 0.25 %/min and 6.91 ± 0.66 %/min, respectively. The phosphorylation deficient mutant CCR5 ΔS, and CCR5 S337only were significantly slower with respective rates of 4.93 ± 0.4 %/min and 4.25 ± 0.28 %/min. Finally, CCR5 S349A was internalised 1.5 times faster than the wild type receptor with a rate of 12.54 ± 0.74 %/min.

Table 4.1. CCR5 internalisation rate in the first 5 minutes of endocytosis

		Internalisation rate (%/min)	
	n	Constitutive	Agonist-induced
WT CCR5	7	0.98 ± 0.18	8.02 ± 1.19
CCR5 ΔS	4	1.34 ± 0.33	4.93 ± 0.4
CCR5 S337A	4	0.55 ± 0.15	6.19 ± 0.25
CCR5 S349A	3	0.17 ± 0.20	12.54 ± 0.74
CCR5 S337only	4	0.0 ± 0.18	4.25 ± 0.28
CCR5 S349only	5	0.51 ± 0.14	6.91 ± 0.66

The rate of internalisation was determined by dividing the percentage of receptor internalised after 5 minutes of agonist stimulation by 5. The data shown are derived from n independent experiments done in duplicate.

Altogether these results suggest that C-terminal serines might not be important for constitutive endocytosis of CCR5, although CCR5 S337only did not show much internalisation. However, agonist-induced phosphorylation of C-terminal serines is required for rapid internalisation of the receptor in the first five minutes. In particular, GRK phosphorylation of S349 is sufficient to promote rapid internalisation of CCR5. On the contrary, PKC phosphorylation of S337 only leads to a slow internalisation. However, rapid internalisation does not only rely on GRK phosphorylation of S349 as CCR5 S349A showed similar kinetics to the wild-type receptor. The C-terminal serine in position 342 might be a substrate for GRK and compensate for the absence of S349.

4.2 Discussion

4.2.1 Internalisation of WT CCR5

To study CCR5 constitutive and agonist-induced endocytosis, I set up an internalisation assay to follow specifically the receptor from the plasma membrane into the cell. This assay is the first capable of monitoring CCR5 internalisation both in the presence and absence of agonist. The antibody MC-5, previously used for immunofluorescence studies, binds efficiently to the receptor when coupled to Alexa Fluor® 488 dye at low dye:protein ratio and allowed direct visualisation of CCR5 by FACS. Internalised ^{488}MC-5/CCR5 complexes were distinguished from cell surface complexes using a quenching antibody for the fluorescent dye. Moreover, as cell surface receptors were labelled prior to internalisation, the secretory vesicle pool of CCR5 was invisible in this analysis.

The assay revealed a low level of constitutive endocytosis of the wild-type receptor (10-15% over 60 minutes), which is consistent with the turnover rate of the protein and similar to the agonist-independent internalisation observed previously for CXCR4 (Signoret et al., 1997). In contrast, upon agonist binding CCR5 was rapidly endocytosed reaching a maximum of 70% of internalised receptors within 60 minutes. The ability of CCR5 to recycle following internalisation, previously studied in CHO cells (Signoret et al, 2000), was also suggested here as the amount of internalised receptor reached a steady state after 30 minutes.

4.2.2 Internalisation of CCR5 mutants lacking C-terminal serine residues

To investigate the molecular mechanisms responsible for CCR5 endocytosis, mutant receptors lacking C-terminal serine residues and therefore impaired in agonist-induced phosphorylation were analysed using the internalisation assay. Whether some or all C-terminal serines where mutated to alanines, the level of constitutive endocytosis remained low and similar to the wild-type receptor. An exception was observed for CCR5 S337only, where almost no internalisation was observed. In contrast, the lack of all phosphorylation sites resulted in slower agonist-induced endocytosis compared to the wild-type.

Further studies of CCR5 mutants indicated a minimal role for PKC phosphorylation site, S337, in CCR5 agonist-induced endocytosis. In contrast, they revealed a major

role for the GRK phosphorylation site, S349, in agonist-induced internalisation of CCR5, as a receptor lacking all serines but S349 exhibited similar endocytic properties to the wild-type receptor. This suggests that GRK phosphorylation of this residue alone is sufficient for internalisation of CCR5. Interestingly, the mutation of S349 resulted in a more rapid internalisation (figure 4.6). This suggests that although S349 is sufficient to mediate CCR5 internalisation another residue must have a similar role. A likely candidate is S342. It would be interesting to analyse the agonist-induced endocytosis of CCR5 lacking all serine residues but S342. However, due to low expression of this mutant in RBL cells, this analysis has not been possible.

The more rapid endocytosis observed for CCR5 S349A relative to the wild-type receptor was unexpected. A recent study proposed that C-terminal postsynaptic density 95/disc-large/zona occludens (PDZ) ligand might delay down-modulation of some GPCRs upon agonist binding by linking receptors to the actin cytoskeleton (Puthenveedu and von Zastrow, 2006). The authors used two chimeric δ-opioid receptors containing either the β1-adrenergic receptor (β1AR) PDZ ligand (db1) or the β1AR PDZ ligand followed by an alanine residue (ala) abolishing PDZ binding. The internalisation of db1 was slower than ala, as the receptor stayed associated longer with clathrin structures at the plasma membrane and the recruitment of dynamin was delayed. Although CCR5 does not have a classical C-terminus PDZ ligand, the last residues form a PDZ-like ligand. The mutation of S349 may affect the receptor association with the cortical actin cytoskeleton and therefore increase the rate of internalisation. However, further characterisation of the C-terminus of CCR5 is required to confirm this hypothesis.

The rates of internalisation measured in this thesis differ from those observed by Huttenrauch *et al.* and Kraft *et al.* where CCR5 internalisation was measured by following the uptake of radiolabelled CCL5 (Huttenrauch et al., 2002; Kraft et al., 2001). The cells used in this thesis were provided by M. Oppermann and are believed to be the same as those used by Huttenrauch *et al.* and Kraft *et al.* Therefore, the differences observed are likely to come from differences in the assays used. Table 4.2 summarises results obtained in these studies and this chapter. Although the initial rates of internalisation differ between the two assays, after 30 minutes the amount of internalised receptor relative to the wild-type receptor is

comparable. The main difference was found in the case of CCR5 S349only, for which a rapid initial internalisation was observed in this thesis, compare to a very slow initial rate similar to CCR5 ΔS in Huttenrauch *et al.* study.

Table 4.2: Percentage of agonist-induced internalised receptor – comparison between two different assays

	Huttenrauch *et al.* and Kraft *et al.* [1]		This thesis[2]	
	(% of receptor internalised)			
	3 min	30 min	3 min	30 min
CCR5 WT	55	90	25	65
CCR5 ΔS	6 or 14	65	18	45
CCR5 S337A	n/d	n/d	15	55
CCR5 S349A	40	100	37	80
CCR5 S337only	2	70	13	46
CCR5 S349only	1	75	24	60

[1]Values were obtained from (Huttenrauch et al., 2002; Kraft et al., 2001). [2]Values extrapolated from graphics in figures 4.4-4.6. n/d not determined.

From their studies, the authors concluded that receptor mutants lacking one or two C-terminal serines undergo agonist-induced internalisation with a similar kinetics to the wild-type receptor. When three C-terminal serines are mutated, agonist-induced receptor endocytosis is slowed down and resembles that of CCR5 ΔS. This did not suggest a particular role for any residue, but the requirement of any two serines for rapid down-modulation of the receptor. This is in contrast to the results presented in this chapter, where S349 alone clearly mediates CCR5 internalisation following agonist stimulation to the same extent as the wild-type receptor, whereas S337 alone does not.

Agonist-induced GRK phosphorylation of C-terminal serines is important for recruitment of β-arrestins to some activated GPCRs at the plasma membrane (Claing et al., 2002). β-arrestins play a major role in desensitisation of many GPCRs as they sterically inhibit interaction with G-proteins. In addition, β-arrestins link agonist-bound receptors to the clathrin endocytic machinery via their interaction with clathrin and the clathrin adaptor protein 2 (AP-2) complex. CCR5 has been shown to interact with β-arrestins following agonist-induced GRK phosphorylation (Aramori et al., 1997; Huttenrauch et al., 2002; Kraft et al., 2001). Furthermore, agonist-induced internalisation of the receptor is blocked in β-arrestin knock-out cells (Fraile-Ramos et al., 2003). Huttenrauch *et al.* showed that β-arrestin 1 recruitment to CCR5 not only depends on C-terminal phosphorylation but also

involves a conserved aspartic acid-arginine-tyrosine (DRY) motif in the second intracellular loop (Huttenrauch et al., 2002). Moreover, surface plasmon resonance (SPR) analysis showed that β-arrestin 1 has a low affinity for the non-phosphorylated C-terminal domain of CCR5. However, this interaction was not detected in their translocation assay.

The present study demonstrates that interfering with both GRK and PKC phosphorylation sites impairs but does not inhibit CCR5 internalisation. Hence, this suggests either that CCR5 ΔS is internalised in a β-arrestin-independent manner via a clathrin-dependent or -independent pathway or that β-arrestins are still recruited to the receptor but at a low level that cannot be detected with existing biochemical and morphological assays. In addition, the efficient internalisation of CCR5 S349only suggests that GRK phosphorylation of S349 might be sufficient for β-arrestin recruitment to the plasma membrane and subsequent coupling to the clathrin endocytic machinery. Further biochemical or morphological studies are needed to confirm the recruitment of β-arrestins to activated receptors deficient in C-terminal serine phosphorylation. Chapter 6 will address some of these issues.

5 Morphological analysis of CCR5 mutants distribution in RBL cells

The morphological analysis of CCR5 distribution described in Chapter 3, indicated a disperse but non-random distribution of the receptor at the plasma membrane. Interestingly, upon agonist binding CCR5 was observed to cluster into flat clathrin lattices, from where clathrin-coated pits (CCPs) containing the receptor were seen to bud. The rapid endocytosis of CCR5 observed in the first few minutes of agonist treatment might reflect the endocytic activity of the clathrin structures where CCR5 accumulates. As CCR5 S336/337/342/349A (CCR5 ΔS) lacks all phosphorylation sites and is internalised 60% more slowly than the wild-type receptor, it would be interesting to see whether it also associates with or accumulates in the same structures.

This chapter presents a detailed morphological analysis of the role of C-terminal serines in constitutive and agonist-induced endocytosis of CCR5. The phosphorylation state of the wild-type receptor and the C-terminal serine mutants of CCR5 before and after agonist binding was determined using phosphosite-specific antibodies against phosphorylated S337 (S337-P) and S349 (S349-P). Subsequently, using the same techniques previously described in Chapter 3, the distribution of CCR5 ΔS at the plasma membrane and its association with clathrin structures were investigated. Finally, to determine the role of protein kinase C (PKC) and G-protein coupled receptor kinase (GRK) phosphorylation of individual C-terminal serines in the early events of CCR5 endocytosis, the cell surface distribution of CCR5 mutants lacking either S337 or S349 or lacking all serines but S337 or S349 and their association with clathrin structures was analysed.

5.1 Detection of phosphorylated CCR5 using phosphosite-specific antibodies

Before investigating the role of agonist-induced phosphorylation of CCR5 in the early events of endocytosis, the phosphorylation state of the wild-type and mutant receptors was determined before and after agonist treatment. Phosphosite-specific

antibodies to S337-P and S349-P were used to visualise the CCR5 molecules phosphorylated on these particular residues (Pollok-Kopp et al., 2003). Figure 5.1 illustrates the specific epitope of each antibody.

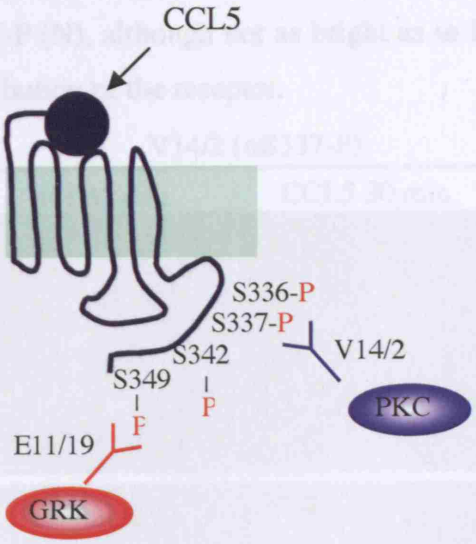


Figure 5.1: Phosphosite-specific antibodies
C-terminal serines 336, 337, 342 and 349 can be phosphorylated upon agonist stimulation. S337, at least, is phosphorylated by PKC and is recognised by the antibody V14/2 (blue), whereas S349 is a target of GRK and an epitope for the antibody E11/19 (red).

RBL cells expressing WT CCR5, CCR5 ΔS, CCR5 S336/342/349A (S337only) or CCR5 S336/337/342A (S349only) were incubated in BM or BM containing 125nM CCL5 for 30 minutes at 37°C. Cells were subsequently fixed, permeabilised, and labelled with an antibody against S337-P (V14/2) or S349-P (E11/19) detected with ^{488}GAM. Throughout the experiment, including the first primary antibody incubation, all solutions contained phosphatase inhibitors to avoid possible dephosphorylation. Projections of z series of confocal sections through selected cells are shown in figure 5.2.

The wild-type receptor did not show any staining for S337-P or S349-P when incubated in BM only (A and C). In contrast, after 30 minutes of CCL5 treatment, both serines, S337 (B) and S349 (D), were detected with the phosphosite-specific antibodies and revealed a punctate intracellular staining. No specific labelling was detected for CCR5 ΔS in any condition (E-H), although in G and H the antibody showed a higher background than in C. Staining of CCR5 ΔS provided a good control for the specificity of these antibodies as the mutant receptor does not have any C-terminal serines. It proved the specificity of V14/2 for S337-P while revealing more background for E11/19 staining of S349-P. Neverthless, as no other phosphosite-specific antibody is available, E11/19 was used in the rest of the study. No specific labelling was detected when CCR5 S349only and CCR5 S337only were

incubated in BM only (I, K, M and O). S337-P was not detected in CCR5 S349only after CCL5 treatment (J), and S349-P showed a similar intracellular staining as the wild-type receptor in D, although a bit weaker. Finally, S349-P was not detected after CCL5 stimulation of CCR5 S337only (P), but a staining was observed for S337-P (N), although not as bright as in B, and revealed a predominant cell surface distribution of the receptor.

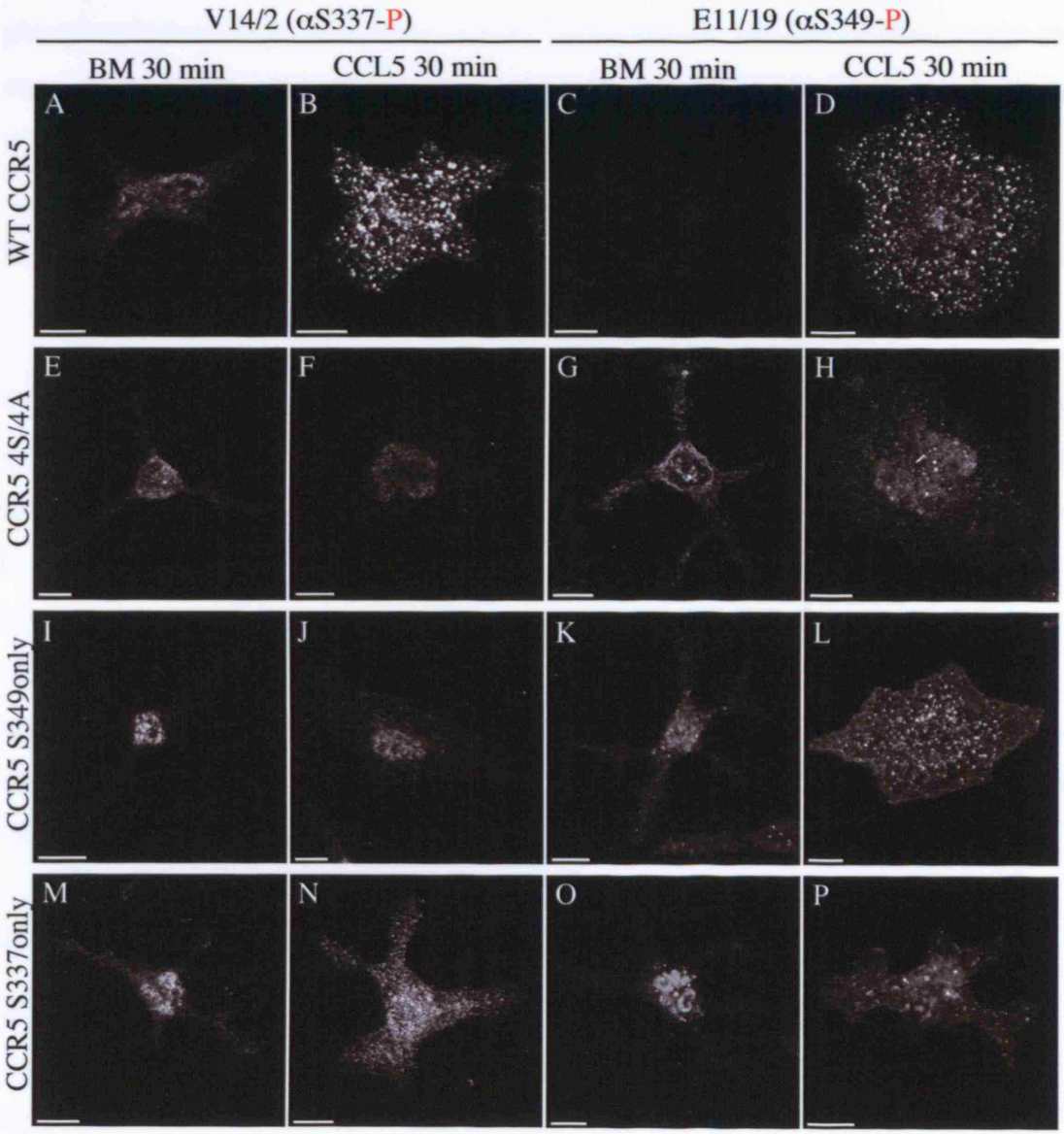


Figure 5.2: Agonist-induced phosphorylation of CCR5 and CCR5 mutants

RBL cells expressing WT CCR5 (A-D), CCR5 ΔS (E-H), CCR5 S349only (I-L) and CCR5 S337only (M-P) were incubated in BM or BM containing 125 nM CCL5 for 30 minutes at 37°C. Cells were subsequently fixed, permeabilised and labelled with antibodies against S337-P (V14/2) or S349-P (E11/19). Primary antibodies were detected with GAM^{488}. Projections of z series of confocal sections through selected cells are presented. Scale bar = 10μm

This immunofluorescence analysis shows that S337-P and S349-P were only detected after agonist treatment. Hence the residues were not phosphorylated in the absence of agonist, or at least not at detectable levels. When a serine was mutated to alanine, the antibody did not recognise the epitope anymore. Furthermore, the analysis demonstrated that phosphorylation could occur independently on each serine, as mutations of three C-terminal residues did not affect agonist-induced phosphorylation of the remaining serine. Nevertheless, phosphorylation might be more efficient with adjacent serines present, as staining in CCR5 C-terminal mutants was weaker.

5.2 Analysis of cell surface distribution of C-terminal serine mutants of CCR5

CCR5 ΔS is not phosphorylated upon agonist binding, and undergoes slower endocytosis compare to WT CCR5. In order to understand the molecular mechanism leading to differences in agonist-induced internalisation of the receptors, the early events of CCR5 ΔS endocytosis that occur at the plasma membrane were investigated. In addition, the internalisation study described in Chapter 4 suggested differential roles for agonist-induced GRK and PKC phosphorylation of CCR5 C-terminal serines S337 and S349 in the receptor endocytosis. Therefore, the contribution of each residue in determining the cell surface CCR5 distribution and association with clathrin was also investigated.

5.2.1 Agonist-activated CCR5 ΔS does not cluster into clathrin lattices

5.2.1.1 Immunofluorescence labelling of cell surface CCR5 ΔS and clathrin

As clathrin was previously shown to play an important role in agonist-induced CCR5 internalisation, the association of CCR5 ΔS with clathrin structures at the plasma membrane was investigated in the absence or presence of agonist. Intact RBL CCR5 ΔS cells were incubated in BM or BM containing 125nM CCL5, labelled for CCR5 with MC-5 and ^{488}GAM before permeabilisation, and subsequently stained for clathrin with a rabbit antibody and ^{594}GAR, as described in Material and Methods section 2.4.2. Single confocal sections are shown in figure 5.3.

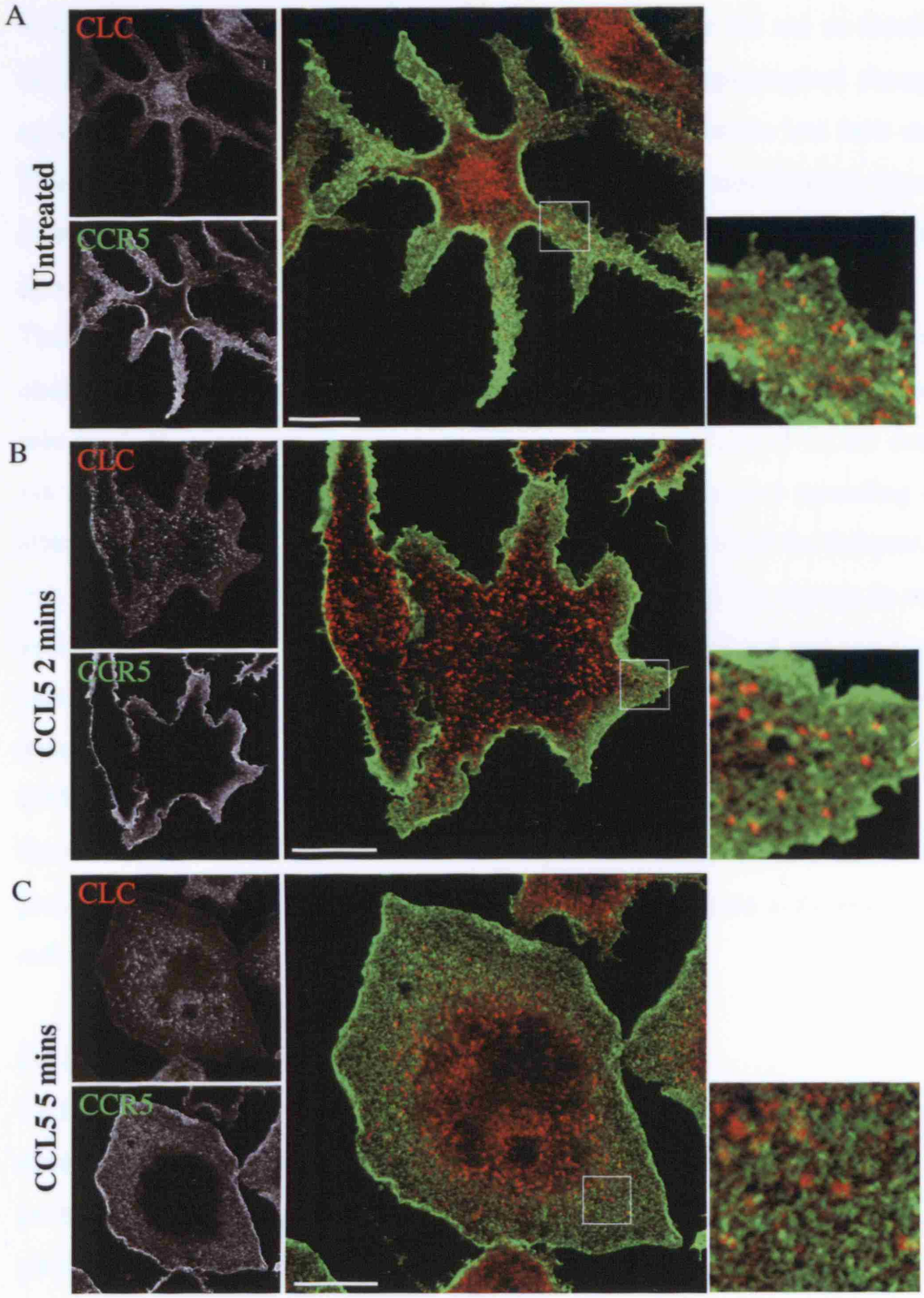


Figure 5.3: Immunolabelling of RBL CCR5 ΔS cells for surface receptors and clathrin

RBL CCR5 ΔS cells were incubated in BM alone (A) or with CCL5 for 2 min (B) or 5 min (C) at 37°C, fixed and labelled intact for CCR5 with MC-5 and subsequently permeabilised and labelled for clathrin with a rabbit antibody. Primary antibodies were detected with species-specific secondary antibodies ^{488}GAM and ^{594}GAR. The figure shows single confocal sections. Scale bar = 10 μm.

In untreated cells (A), the receptor showed a bright staining on the plasma membrane distinct from the clathrin labelling. Upon agonist binding (B and C), no difference

was observed in CCR5 ΔS distribution, and the receptor did not co-localised with clathrin. However, the cells underwent dramatic morphological changes upon agonist treatment; they flattened rapidly and within 5 minutes lost their star-shape. This morphological change and the sustained calcium mobilisation measured by Huttenrauch *et al.* for CCR5 ΔS upon CCL5 treatment confirm the activity of the agonist on the receptor (Huttenrauch et al., 2002).

This result indicates that, upon agonist binding, CCR5 ΔS fails to associate with clathrin-containing structures at the plasma membrane. Furthermore, it suggests a role for C-terminal serine phosphorylation in the early steps of CCR5 endocytosis via the clathrin-mediated pathway. In addition, the extensive spreading observed after CCL5 treatment suggests impairment in desensitisation of the receptor. Indeed, one of the characteristic features of RBL cell activation is the change in cell shape, as described in Chapter 3. Desensitisation is highly regulated and required for the cells to return to their resting state. For most G-protein coupled receptors (GPCRs) desensitisation is mediated through phosphorylation of the receptor by PKC and GRK and subsequent recruitment of β-arrestins, which uncouples the G-proteins from the receptor (Claing et al., 2002). As CCR5 ΔS lacks all C-terminal serines no phosphorylation can occur and the receptor is likely to remain activated longer at the cell surface, hence the dramatic morphological change.

5.2.1.2 Whole mount replicas of RBL CCR5 ΔS

To further investigate the distribution of CCR5 ΔS at the plasma membrane in the absence or presence of agonist, whole mount replicas of RBL CCR5 ΔS were produced as described in Materials and Methods section 2.5.2. Figure 5.4 shows plasma membrane replicas of RBL CCR5 ΔS cells incubated in BM (A) or BM containing 125nM CCL5 (B) for 2 minutes at 37°C and subsequently labelled for the receptor with MC-5 and protein A conjugated to 15nm-gold particles (PAG_{15}). In untreated cells, CCR5 ΔS showed a similar distribution to the wild-type receptor, disperse but not random, as PAG_{15} was often seen to be arranged in lines (arrowhead). Upon agonist binding, the receptor distribution did not change. However, the density of the PAG_{15} was lower, as shown in table 5.1. This could be due either to a rapid down-modulation of the receptor following CCL5 binding or to the change in cell morphology; as the cells spread upon CCL5 treatment, their

surface area was increase, if the receptor number remains the same, the labelling density will decrease.

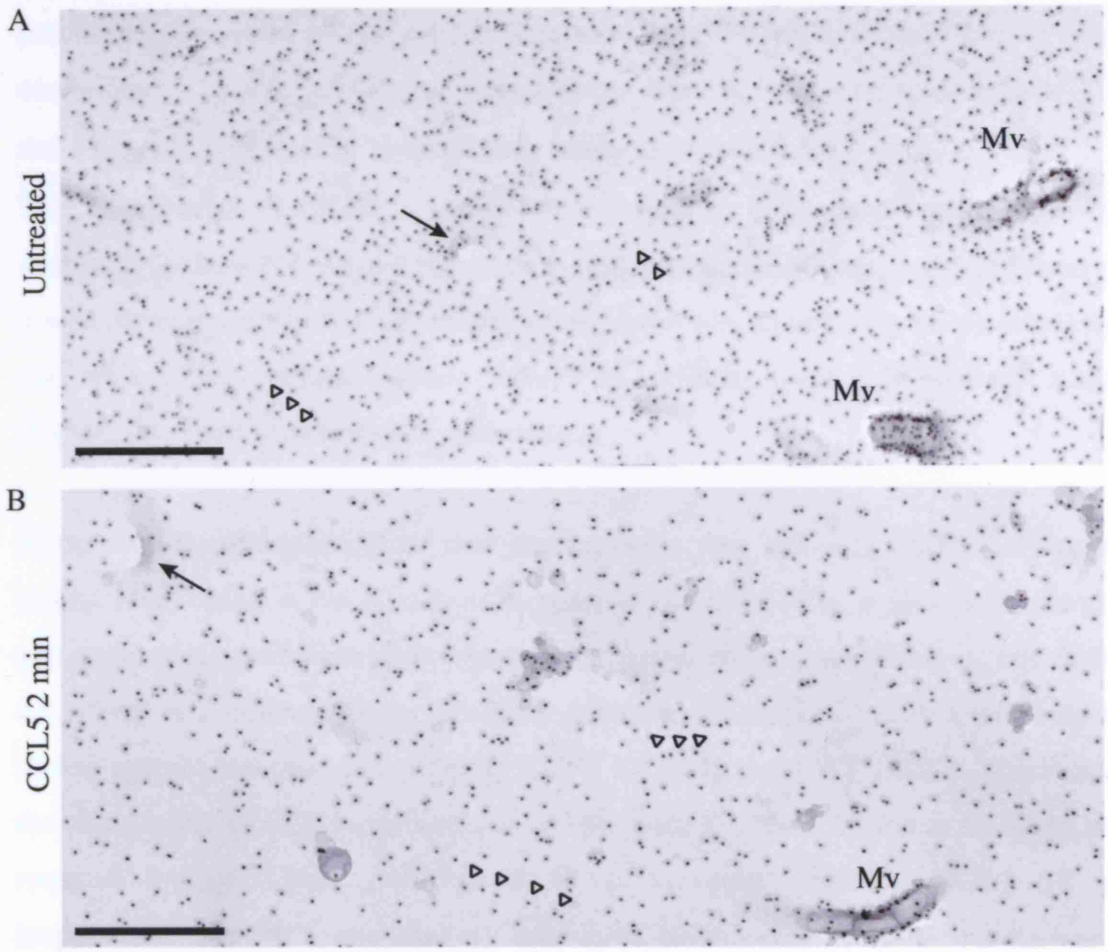


Figure 5.4: Whole mount replicas of immunolabelled RBL CCR5 ΔS cells

RBL CCR5 ΔS cells were incubated with BM alone (A) or with CCL5 for 2 minutes (B) at 37°C before fixation at 4°C. CCR5 ΔS was detected by labelling intact cells with MC5 and PAG_{15}, and cell surface replicas were prepared as described in Materials and Methods section 2.5.2. Specimens were examined by EM. Invaginations of the membrane are shown with arrows and arrowheads indicate lines of PAG_{15}. (Mv) microvilli. Scale bar = 300 nm.

In summary, this analysis indicates that upon agonist binding, CCR5 ΔS does not cluster on the plasma membrane as observed for the wild-type receptor. In addition, it reveals the importance of CCL5-induced phosphorylation in cell surface CCR5 redistribution.

5.2.1.3 Preparation of membrane sheets from RBL CCR5 ΔS cells

To further investigate the cell surface distribution of CCR5 ΔS, and the association of the receptor with membrane-bound cytoplasmic proteins, membrane sheets were prepared from cells incubated for 2 minutes at 37°C in BM only (A) or BM containing 125nM CCL5 (B) and subsequently labelled for the receptor with MC-5 and PAG_{15} as described in Materials and Methods chapter 2.5.3.

The distribution of CCR5 ΔS was very similar in both samples (figure 5.5). Although the receptor was not clustered in flat clathrin lattices upon agonist binding, it was not excluded from clathrin structures (arrows in A) and very rarely labelling was seen in flat clathrin lattices (inset). In addition, some gold particles were aligned with cortical actin (open arrowhead).

Altogether this morphological analysis indicates that although CCR5 ΔS has a similar distribution to the wild-type receptor in untreated cells, it does not undergo any obvious redistribution in the plasma membrane upon agonist binding, and does not cluster in clathrin lattices. The internalisation analysis in Chapter 4 indicated a slower agonist-induced endocytosis of CCR5 ΔS compare to WT CCR5. Therefore, the recruitment of CCR5 into clathrin lattices upon CCL5 treatment is likely to be required for more rapid endocytosis of the receptor. Whether CCR5 ΔS is internalised through a non-clathrin endocytic route remains to be determined. Nevertheless, these data suggest an important role for agonist-induced phosphorylation in the early events of CCR5 endocytosis and the receptor association with endocytic structures.

5.2.2 PKC phosphorylation of S337 is not necessary for agonist-induced redistribution

Analysis of the endocytosis of CCR5 mutants indicated a minimal role for agonist-induced PKC phosphorylation of S337 (Chapter 4). To further investigate the importance of S337 phosphorylation for CCR5 association with clathrin at the plasma membrane and in cell surface receptor distribution, morphological studies were carried out in RBL CCR5 S337A and RBL CCR5 S337only cells.

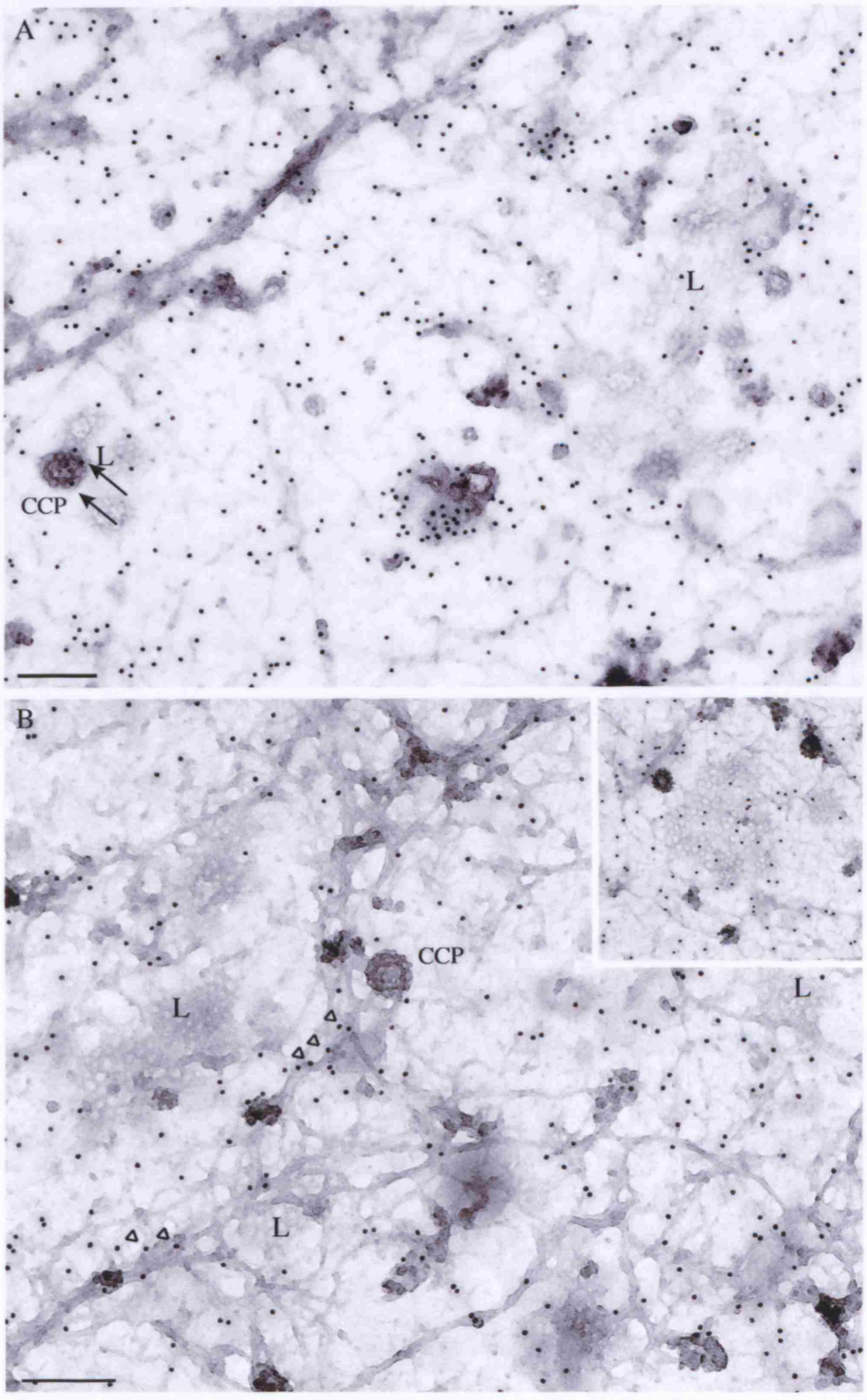


Figure 5.5: Membrane sheets of immunolabelled RBL CCR5 ΔS cells

RBL CCR5 ΔS cells were incubated for 2 minutes at 37°C with BM (A) or BM containing 125nM CCL5 (B) and labelled intact with MC-5 followed by PAG_{15}. Membrane sheets were prepared as described in Materials and Methods section 2.5.3, fixed and examined by EM. Scale bar = 200nm. (L) flat clathrin lattice, (CCP) clathrin-coated pit, (arrow) CCR5 in CCP, (arrowhead) CCR5 associated with cortical actin

5.2.2.1 Immunofluorescence labelling of RBL CCR5 S337A and RBL CCR5 S337only cells for surface receptors and clathrin

RBL cells expressing either CCR5 S337 or CCR5 S337only were stained for cell surface receptors and clathrin before and after agonist treatment. Figures 5.6 and 5.7 shows confocal sections of RBL CCR5 S337A and RBL CCR5 S337only cells, respectively, labelled for CCR5 with MC-5 and ^{488}GAM and for clathrin with a rabbit polyclonal antibody and ^{594}GAR, as described in Material and Methods section 2.4.2.

Both receptors showed distinct staining from clathrin when incubated in BM only (A). However, upon agonist binding, CCR5 S337A co-localised with clathrin structures at the plasma membrane (figure 5.6 B and C) whereas CCR5 S337only did not (figure 5.7 B and C). In addition, the two cell lines showed different morphological changes following agonist treatment. RBL CCR5 S337A cells spread rapidly and extensively upon CCL5 binding, similarly to RBL CCR5 ΔS cells. In contrast, RBL CCR5 S337only cells flattened moderately to the same extent as RBL CCR5 cells.

This analysis supports the biochemical data indicating that S337 does not play a role in CCR5 association with clathrin at the plasma membrane.

5.2.2.2 Whole mount replicas of RBL CCR5 S337A and RBL CCR5 S337only cells

The distribution of the two mutant receptors was further investigated using whole mount replicas of cells as described in Materials and Methods section 2.5.2. Figure 5.8 shows replicas of the plasma membrane of RBL cells expressing CCR5 S337A or CCR5 S337only incubated in BM only (A and B) or BM containing 125nM CCL5 (C-F) and labelled for the receptors with MC-5 and PAG_{15}. In untreated cells, both mutant receptors had a distribution similar to the wild-type receptor, disperse but not random as gold particles were observed in lines (open arrowheads in A and B). Upon agonist binding, CCR5 S337A was redistributed into clusters (arrowhead in C and D). Moreover, clusters appeared larger after 2 minutes than 30 seconds of CCL5 treatment. In contrast, CCR5 S337only distribution did not change upon agonist treatment; the receptor remained evenly distributed on the plasma membrane.

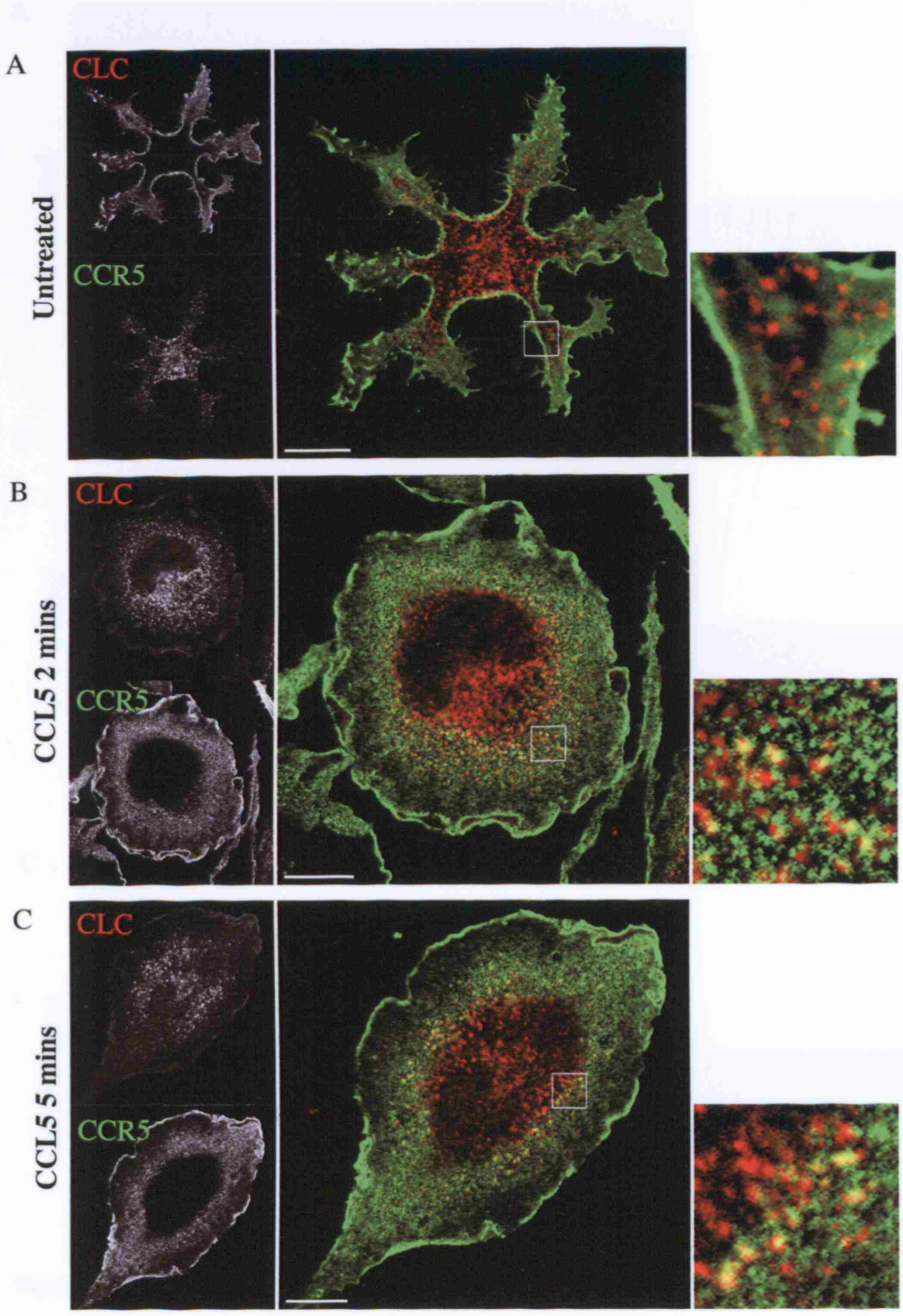


Figure 5.6: Immunofluorescence labelling of RBL CCR5 S337A cells for surface CCR5 and clathrin

RBL CCR5 S337A cells were incubated with BM alone (A) or with 125 mM CCL5 for 2 (B) or 5 min (C) at 37°C, fixed and labelled intact for CCR5 with MC-5 and subsequently permeabilised and labelled for clathrin with a rabbit antibody. Primary antibodies were detected with species-specific secondary antibodies ^{488}GAM and ^{594}GAR. The figure shows single confocal sections. Scale bar = 10 μm.

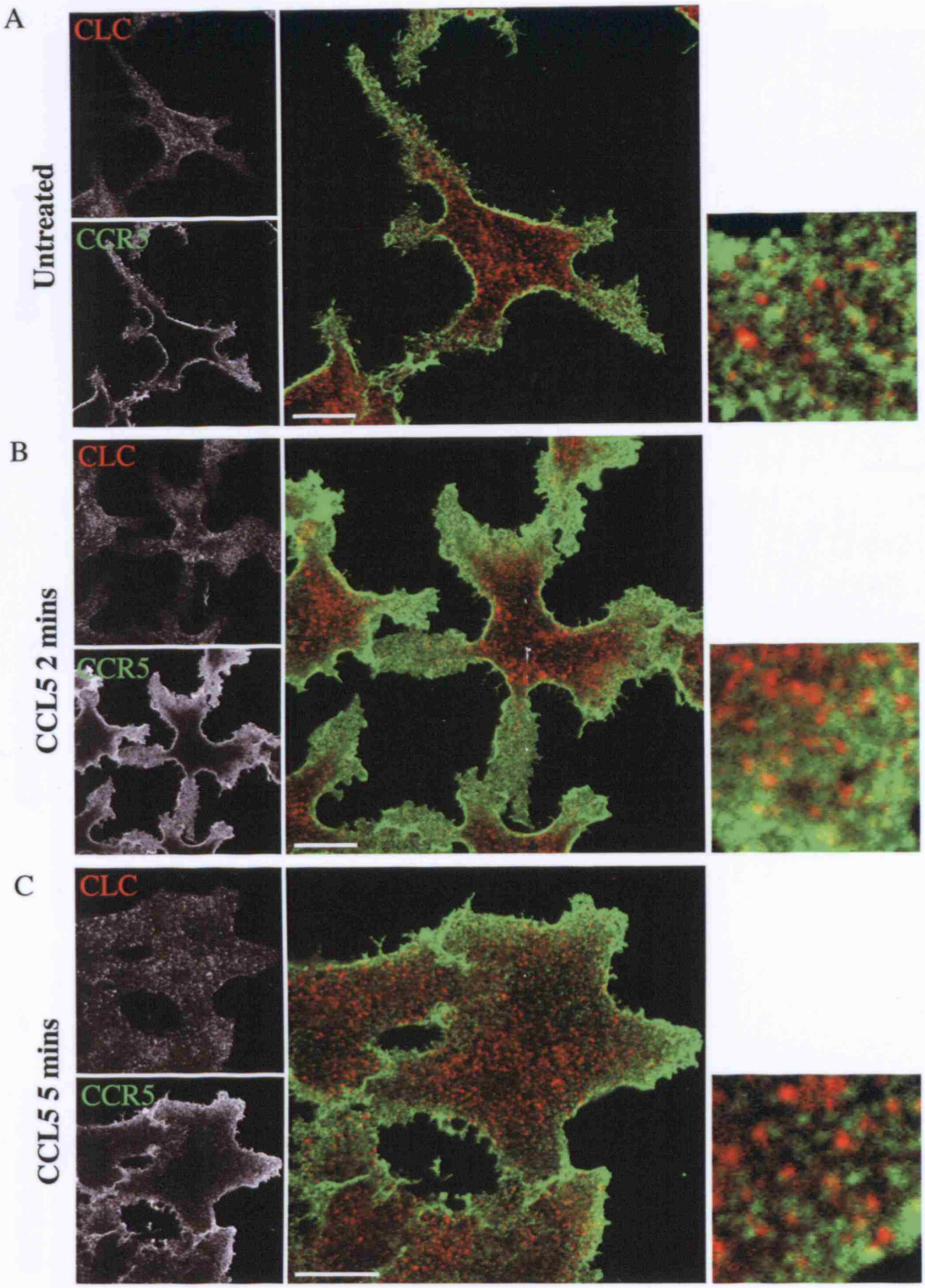


Figure 5.7: Immunofluorescence labelling of RBL CCR5 S337only cells for surface CCR5 and clathrin

RBL CCR5 S337only cells were incubated with BM alone (A) or with 125 mM CCL5 for 2 (B) or 5 min (C) at 37°C, fixed and labelled intact for CCR5 with MC-5 and subsequently permeabilised and labelled for clathrin with a rabbit antibody. Primary antibodies were detected with species specific secondary antibodies ^{488}GAM and ^{594}GAR. The figure shows single confocal sections. Scale bar = 10 μm.

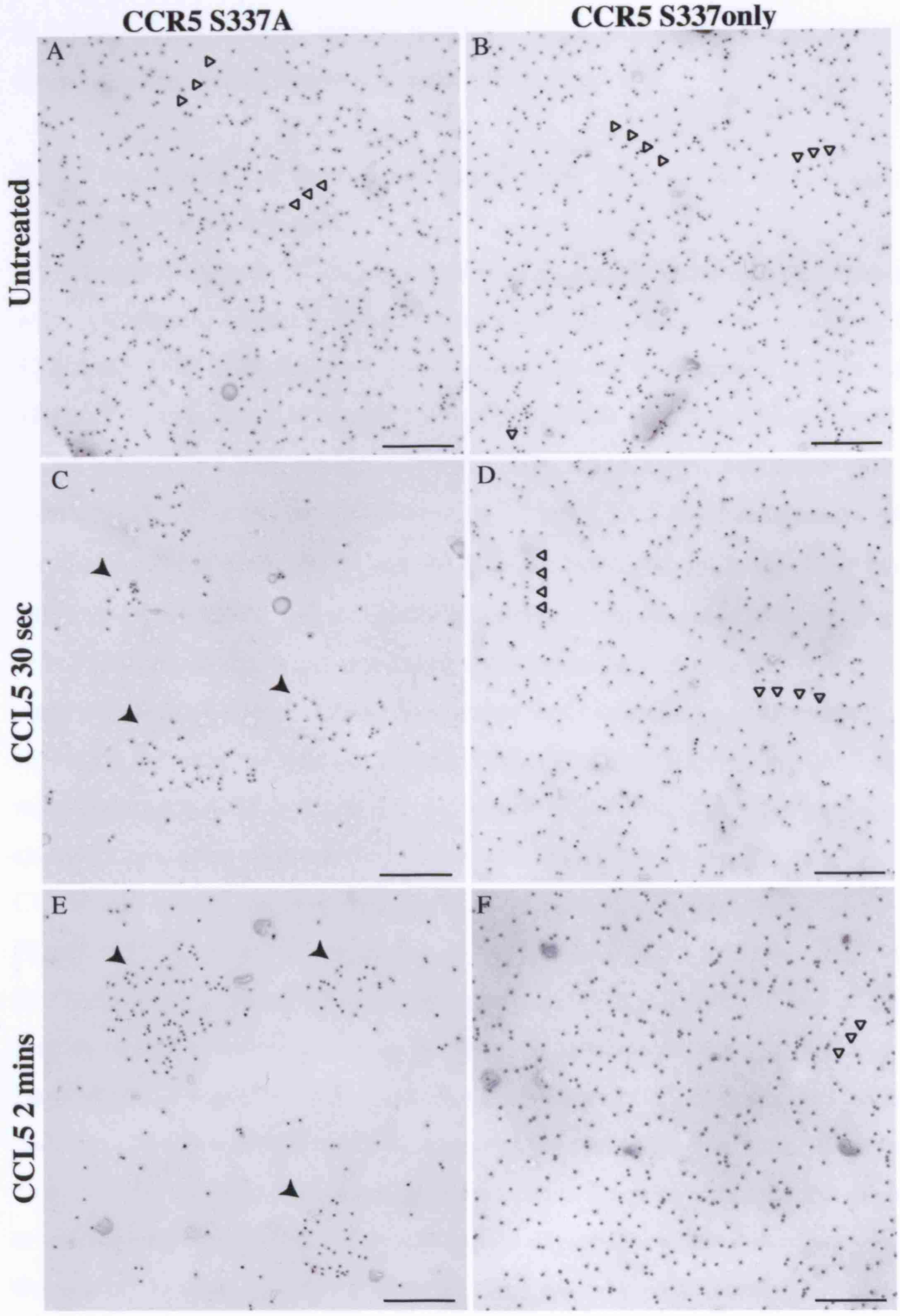


Figure 5.8: Whole mount replicas of immunolabelled RBL CCR5 S337A and S337only cells

Cells were incubated with BM alone (A and B) or with 125 nM CCL5 for 30 seconds (C an D) or 2 minutes (E an F) at 37°C before fixation at 4°C. CCR5 was detected by labelling intact cells with MC5 and PAG_{15}, and cell surface replicas were prepared as described in Materials and Methods section 2.5.2. Specimens were examined by EM. Filled arrowheads indicate clusters and open arrowheads point to lines of PAG_{15}. (Mv) microvilli. Scale bar = 300 nm.

In summary, this analysis implies that S337 is not required for CCR5 clustering at the plasma membrane following agonist stimulation.

5.2.2.3 Preparation of membrane sheets from RBL CCR5 S337A and RBL CCR5 S337only cells

To examine in more detail the distribution of mutant receptors and their association with cytoplasmic proteins at the plasma membrane, RBL cells expressing CCR5 S337A or CCR5 S337only were processed for 'rip-off' as described in Materials and Methods section 2.5.3. Figures 5.9 and 5.10 show membrane sheets from RBL CCR5 S337A and RBL CCR5 S337only cells, respectively, where the cells were incubated in BM alone or BM containing 125nM CCL5 and subsequently labelled for the receptors with MC-5 and PAG_{15}. In untreated cells, the gold particles labelling CCR5 S337A had a disperse distribution, and appeared to be aligned along actin filaments on the membrane sheets (open arrowheads in figure 5.9 A). The flat clathrin lattices observed in these membranes rarely contained any labelling (L in A). However, the receptor was not excluded from clathrin structures as gold particles were detected in CCP (inset in A). In agonist-treated cells, the gold particles were clustered over areas of flat clathrin lattice (B and C). CCPs containing labelling for CCR5 S337A were observed budding from these lattices (arrows in C).

Figure 5.10 A shows a membrane sheet obtained from untreated RBL CCR5 S337only cells. The gold particles labelling CCR5 S337only had a disperse distribution and as seen for other receptors in untreated conditions were in some cases aligned along actin filaments (open arrowheads). In addition, small clusters of particles (10-15) were occasionally observed (arrowhead). Flat clathrin lattices (L) were difficult to notice in these samples because of the poor quality of the membrane or the staining. However, under appropriate adjustment at the electron microscope the typical hexagonal pattern of assembled clathrin was discerned (B and B'). Unfortunately, with these settings, the gold particles were out of focus, hence the double rings in B'. Nevertheless, under these conditions, very few gold particles were observed in flat clathrin lattices. Upon agonist binding, gold particles did not show any major redistribution as observed previously for RBL CCR5 WT and RBL CCR5 S337A cells (C). However, labelling for CCR5 S337only was often found in isolated CCPs on the membrane sheets (inset in C) and, although some labelling was

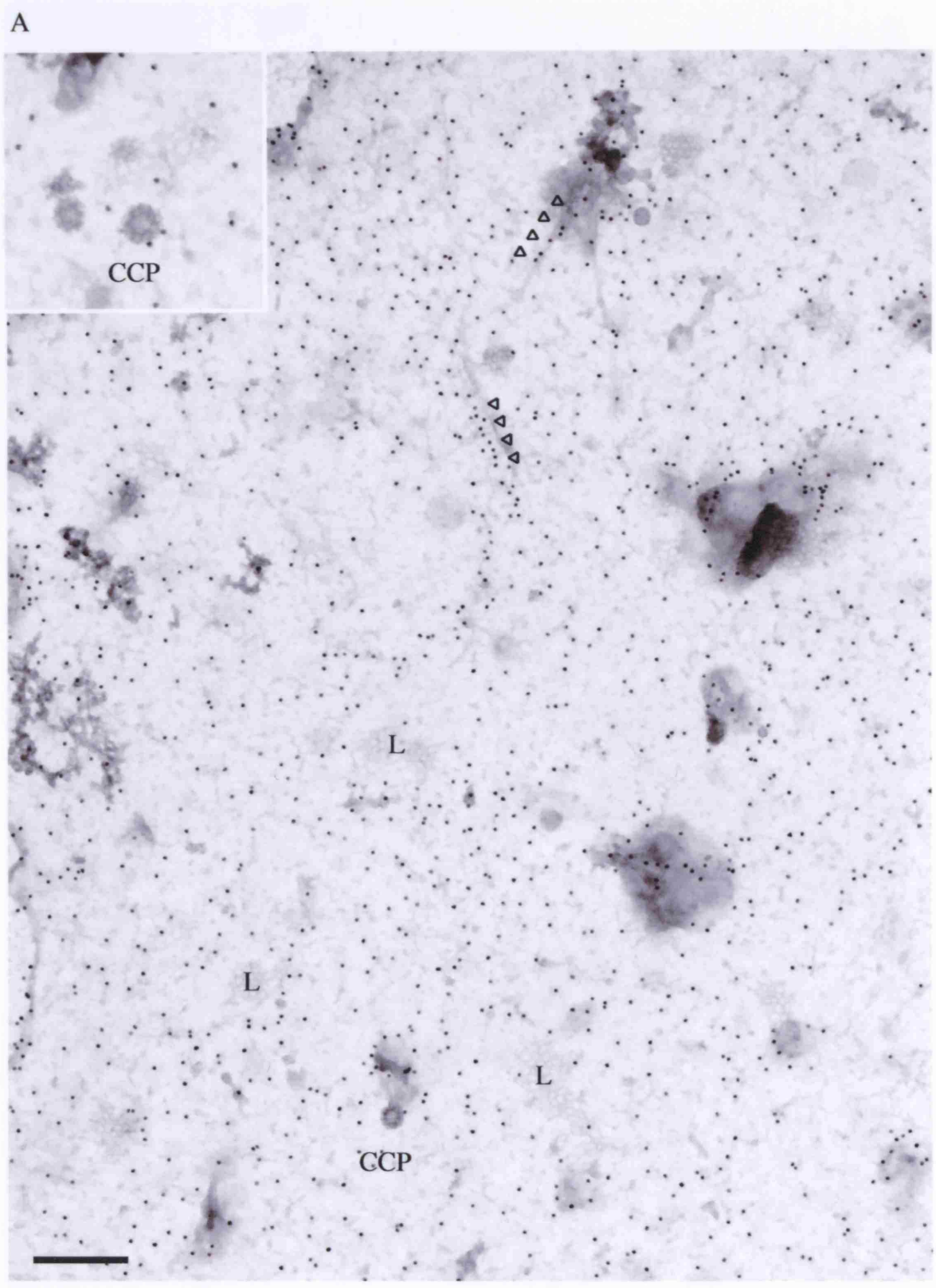
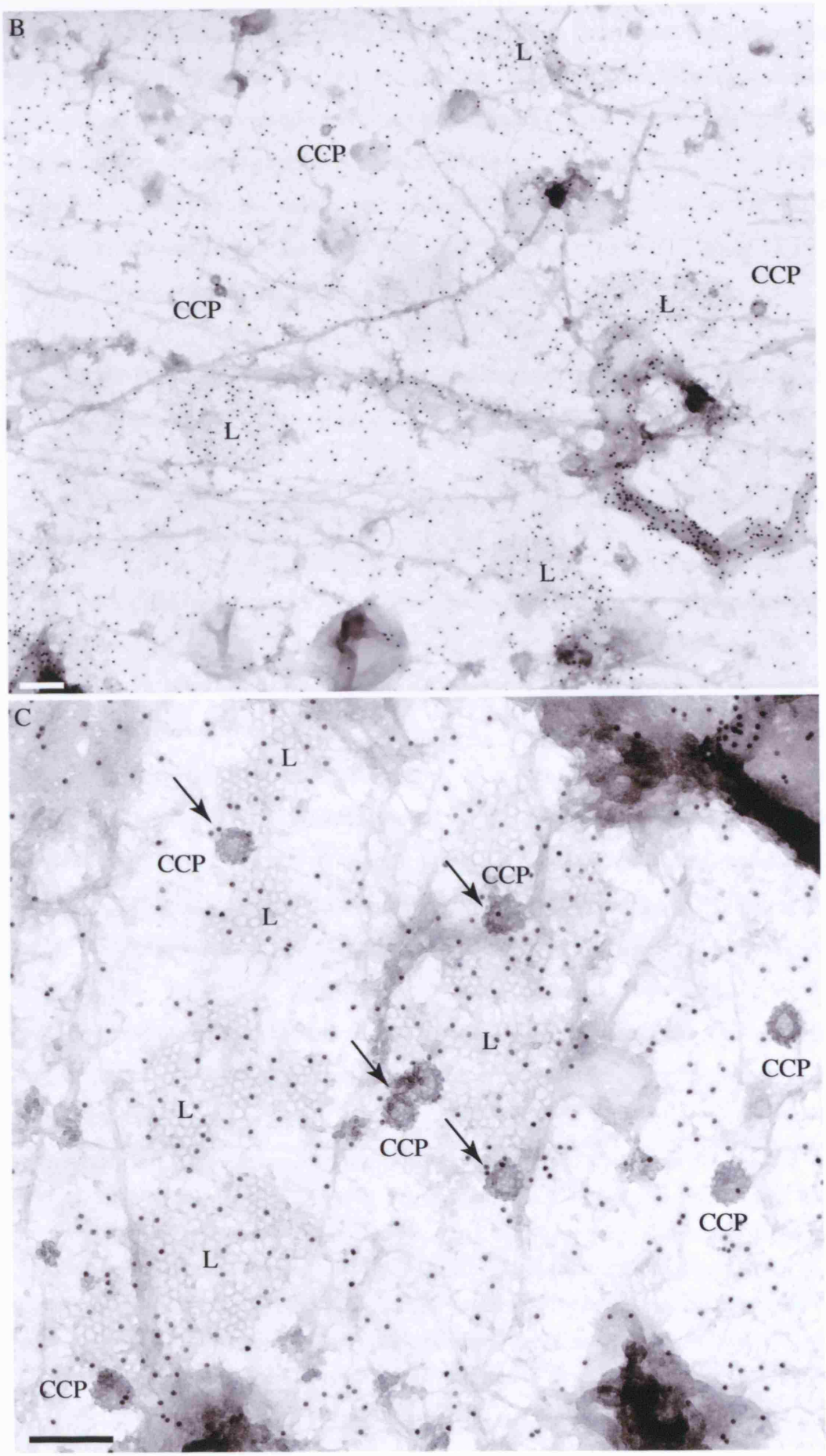


Figure 5.9: Membrane sheets of immunolabelled RBL CCR5 S337A cells

RBL CCR5 S337A cells were incubated with BM (A) or BM containing 125nM CCL5 (B and C) and labelled intact with MC-5 followed by PAG_{15}. Membrane sheets were subsequently prepared as described in Materials and Methods section 2.5.3, fixed and examined by EM. Scale bars = 300 nm (A, B), 200 nm (C). (L) flat clathrin lattice, (CCP) clathrin-coated pit, (open arrowheads) CCR5 S337A associated with cortical actin, (arrow) CCR5 S337A in CCP.


B
L
CCP
CCP
CCP
L
L
L
C
L
CCP
CCP
L
L
CCP
CCP
CCP
L
CCP

observed in flat clathrin lattices (C'), no CCP budding from these lattices contained gold particles. Whether the isolated pits have budded from lattices and moved laterally on the plasma membrane or are individual budding events independent from other clathrin structures remains to be determined. Nevertheless, quantification is required to confirm this result. In addition, gold particles accumulated in 'dark areas' on the membrane sheets, where CCP budding occurs (C'' and C'''). The nature of these areas of the plasma membrane remains obscure.

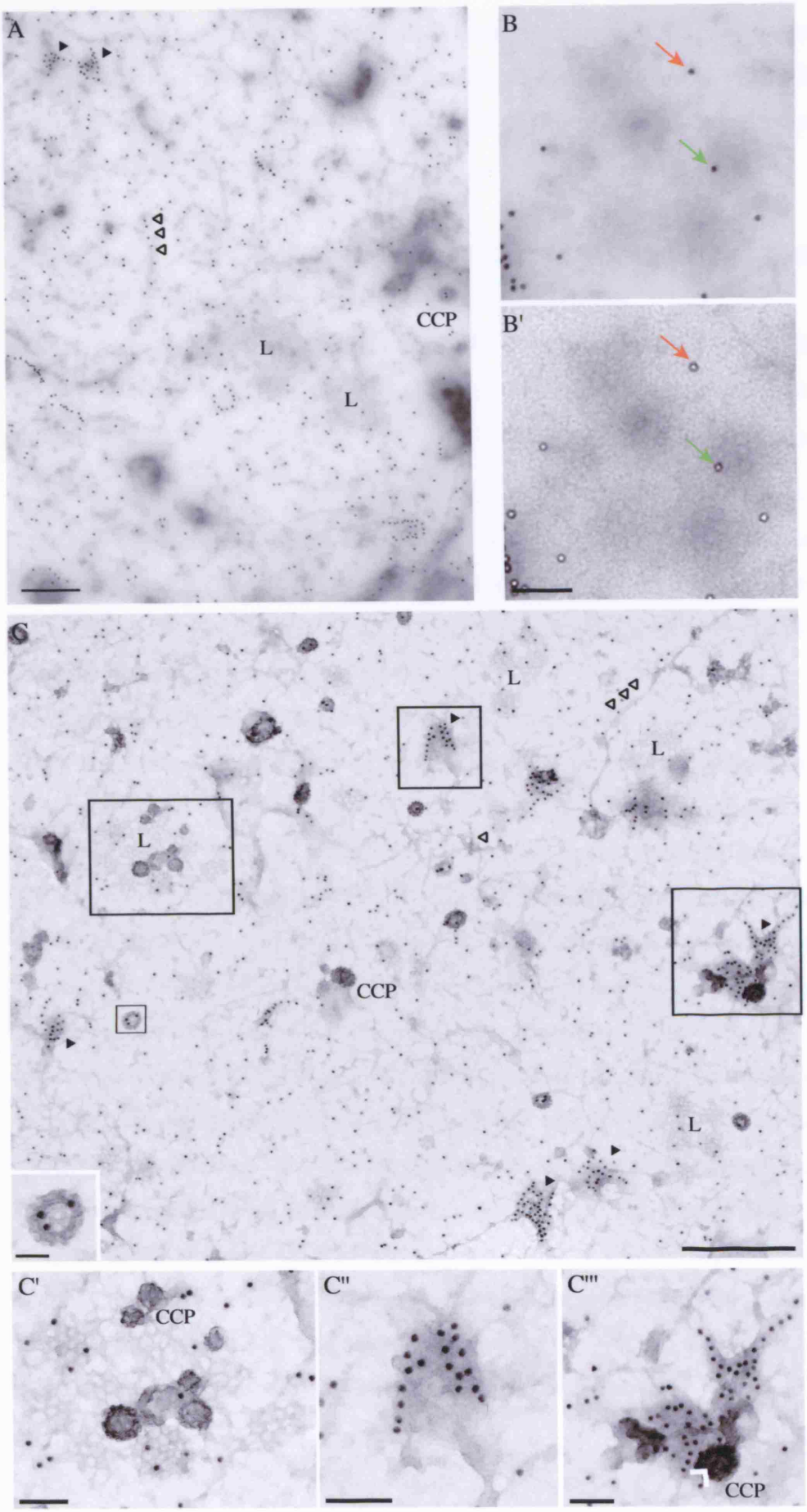
Altogether these data indicate that agonist-induced PKC phosphorylation of S337 does not contribute to CCR5 recruitment into flat clathrin lattices.

5.2.3 GRK phosphorylation of S349 is sufficient for agonist-induced redistribution

GRK phosphorylation plays a key role in GPCRs desensitisation and internalisation. Indeed, recruitment of β-arrestins to activated receptors is mediated by GRK phosphorylation of serine and threonine residues in the C-terminal domains of these receptors. Besides abrogating the interaction between activated receptors and G-proteins, β-arrestins bind to AP-2 and clathrin and therefore link the receptor to the endocytic machinery. Agonist-induced CCR5 endocytosis has been shown to involve β-arrestins. Moreover, analysis of CCL5-induced internalisation of a CCR5 mutant lacking all serines but S349, which is phosphorylated by GRK (Pollok-Kopp et al., 2003), revealed kinetics similar to the wild-type receptor. This suggested that CCR5 endocytosis upon agonist binding could be mediated by GRK phosphorylation of this specific residue alone. However, whether the early events of the receptors internalisation are similar to those of the wild-type receptor remains to be determined.

Figure 5.10: Membrane sheets of immunolabelled RBL CCR5 S337only cells

RBL CCR5 S337only cells were incubated with BM (A-B') or BM containing 125nM CCL5 (C-C''') and were labelled intact with MC-5 followed by PAG_{15}. Membrane sheets were subsequently prepared as described in Materials and Methods section 2.5.3, fixed and examined by EM. (L) Flat clathrin lattice, (CCP) clathrin-coated pit, (hx) hexagonal clathrin pattern, (open arrowheads) CCR5 S337only associated with cortical actin, (arrowheads) clusters of gold particles in 'dark area', (white arrowheads) gold particles in CCP budding from 'dark area'. Scale bars = 250 nm (A), 100 nm (B, B', C', C'' and C'''), 500 nm (C), 50 nm (inset in C).


A
CCP
L
L
B
B'
C
L
L
L
CCP
L
C'
CCP
C''
C'''
CCP

In this section, the association of two mutant receptors, CCR5 S349A and CCR5 S349only, with clathrin at the plasma membrane was investigated. In addition, the distribution of the two receptors at the cell surface was studied using whole-mount replicas and the 'rip-off' technique.

5.2.3.1 Immunofluorescence labelling of RBL CCR5 S349A and RBL CCR5 S349only cells for surface receptors and clathrin

RBL cells expressing CCR5 S349A or CCR5 S349only were incubated in BM alone or BM containing 125nM CCL5. The cells were subsequently labelled for cell surface CCR5 with MC-5 and ^{488}GAM and clathrin with a rabbit antibody and ^{594}GAR, as described in Materials and Methods. Figures 5.11 and 5.12 show confocal sections of RBL CCR5 S349A and RBL CCR5 S349only cells, respectively. In untreated cells, both receptors had a distinct staining from clathrin (A). However, on agonist treatment, both receptors partially co-localised with clathrin at the plasma membrane (B and C).

This immunofluorescence analysis demonstrates that in the absence of S349 or when S349 is the only C-terminal serine present in CCR5, the activated receptor associates with clathrin at the plasma membrane. This indicates a major role for GRK phosphorylation of S349 in the early events of agonist-induced endocytosis of CCR5. In addition, CCR5 S349only exhibited the same endocytic profile as WT CCR5 in Chapter 4. As the mutant receptor is also associated with clathrin at the cell surface, S349 seems to be the minimal requirement for sorting at the plasma membrane and efficient CCR5 endocytosis. However, in the absence of S349, agonist-induced CCR5 S349A internalisation is more efficient than for the wild-type receptor, and cell surface activated CCR5 S349A still associates with clathrin. This suggests that another C-terminal serine must be able to fulfil the same function as S349. Among the three other C-terminal serines, S337 has already been shown to play a minor role in CCR5 endocytosis. The kinases responsible for the phosphorylation S336 and S342 remain to be determined. However, because of its proximity to S337, S336 may also be a substrate for PKC, and S342 might be the other residue important for agonist-induced CCR5 endocytosis.

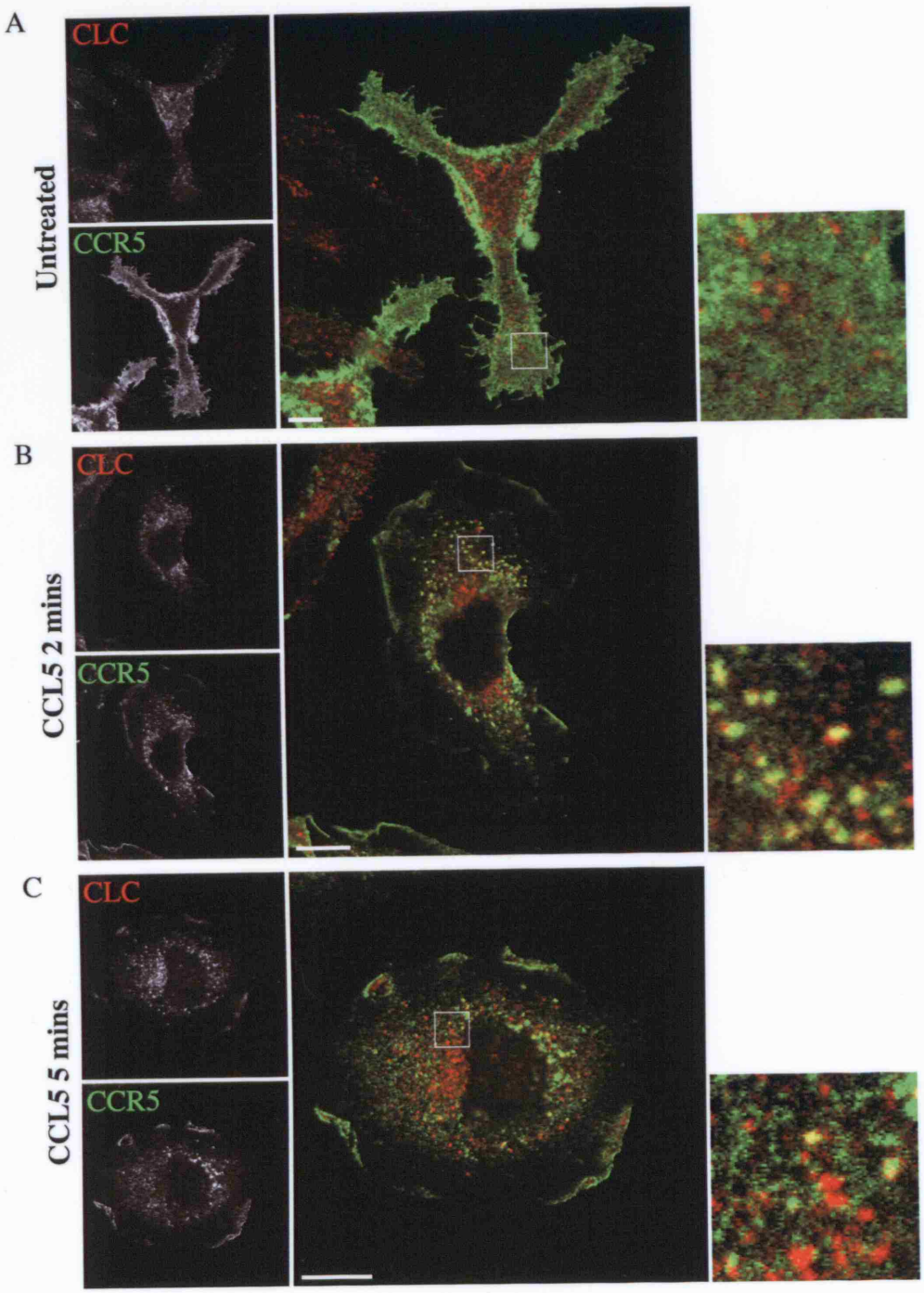


Figure 5.11: Immunofluorescence labelling of RBL CCR5 S349A cells for surface CCR5 and clathrin

RBL CCR5 S349A cells were incubated with BM alone (A) or with 125 mM CCL5 for 2 (B) or 5 min (C) at 37°C, fixed and labelled intact for CCR5 with MC-5 and subsequently permeabilised and labelled for clathrin with a rabbit antibody. Primary antibodies were detected with species-specific secondary antibodies ^{488}GAM and ^{594}GAR. The figure shows single confocal sections. Scale bar = 10 μm.

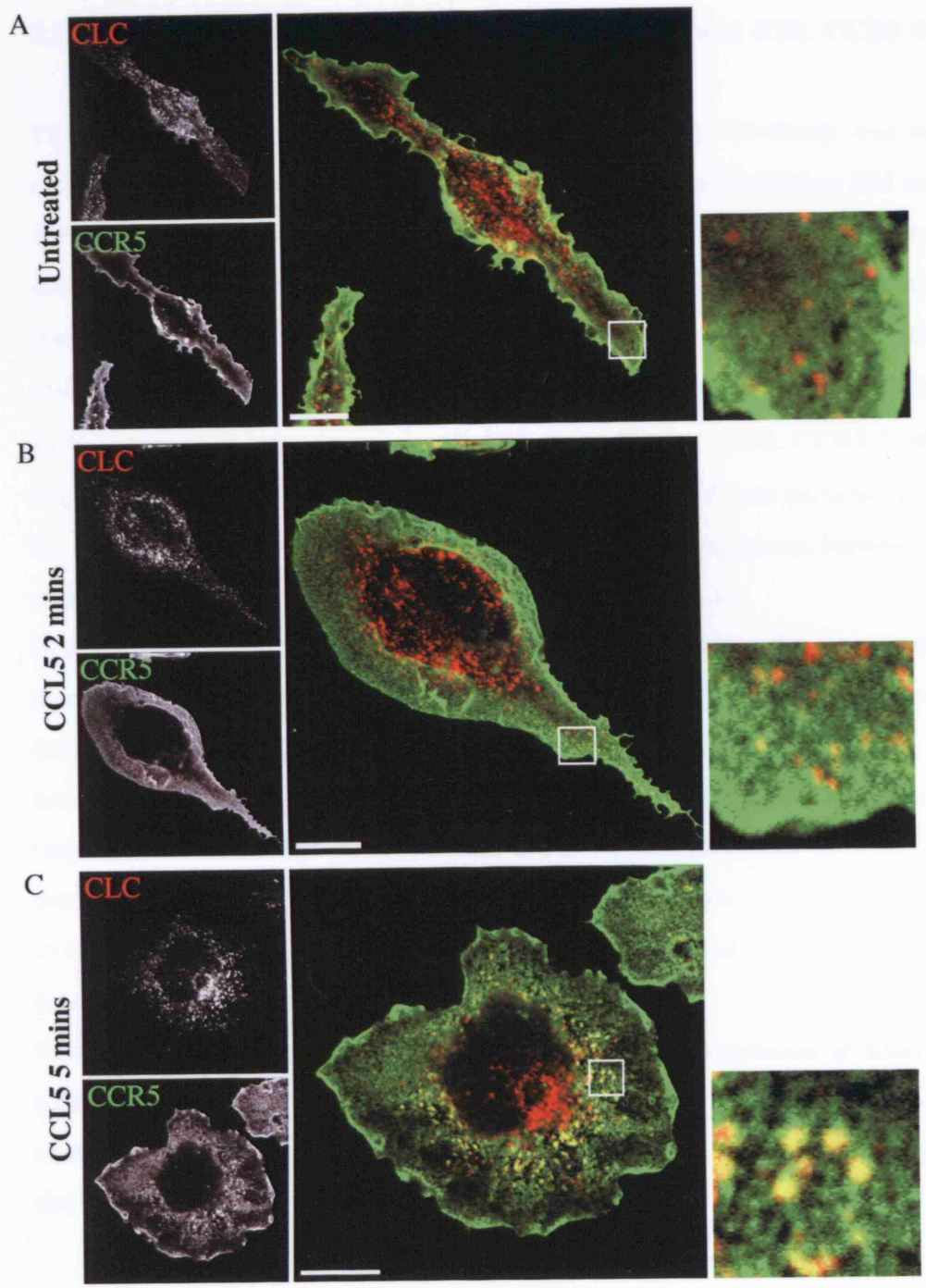


Figure 5.12: Immunofluorescence labelling of RBL CCR5 S349only cells for surface CCR5 and clathrin

RBL CCR5 S349only cells were incubated with BM alone (A) or with 125 mM CCL5 for 2 (B) or 5 min (C) at 37°C, fixed and labelled intact for CCR5 with MC-5 and subsequently permeabilised and labelled for clathrin with a rabbit antibody. Primary antibodies were detected with species-specific secondary antibodies ^{488}GAM and ^{594}GAR. The figure shows single confocal sections. Scale bar = 10 μm.

5.2.3.2 Whole mount replicas of RBL CCR5 S349A and RBL CCR5 S349only cells

The cell surface distribution of CCR5 S349A and CCR5 S349only was studied in details by whole mount replicas. RBL cells were incubated in BM or BM containing 125nM CCL5, labelled for the receptors at the plasma membrane and processed as described in Materials and Methods section 2.5.2. Figure 5.13 shows plasma membrane replicas of untreated (A and B), 30 seconds (C and D) or 2 minutes (E and F) activated RBL cells expressing CCR5 S349A or CCR5 S349only, where surface receptors were detected with MC-5 and PAG_{15}. RBL CCR5 S349A cells expressed a low level of receptor, hence the low density of gold particles in A, C and E. The expression level of CCR5 S349only was heterogeneous between cells, as shown in B, D and F and the standard deviation in table 5.1.

In untreated RBL CCR5 S349A cells, gold particles labelling for the receptor had a disperse distribution (A). Upon agonist binding, clusters of particles were observed (arrowhead in C and E). In addition, labelling for CCR5 S349A was often found in invaginations of the plasma membrane, probably corresponding to endocytic pits (arrows and inset in E). Gold particles in RBL CCR5 S349only cells had a similar distribution as in RBL CCR5 WT cells; some lines of particles could be distinguished among the disperse labelling (open arrowhead in B). Following CCL5 binding, the gold particles clustered.

This morphological analysis suggests that GRK phosphorylation of S349 alone is sufficient for plasma membrane redistribution of CCR5 into clusters. However, another residue must have a similar function to S349, as CCR5 S349only did not show any impairment in the receptor redistribution.

5.2.3.3 Preparation of membrane sheets from RBL CCR5 S349A and RBL CCR5 S349only cells

The recruitment of the two mutant receptors into flat clathrin lattices upon CCL5 treatment was subsequently investigated. Figure 5.14 shows membrane sheets from RBL cells expressing CCR5 S349A (A and B) or CCR5 S349only (C and D) labelled with MC-5 and PAG_{15}. In untreated cells, gold particles were mainly associated with cortical actin (arrowhead in C). In addition, some CCPs were found to contain labelling for the receptors (arrows in C). In contrast, following CCL5

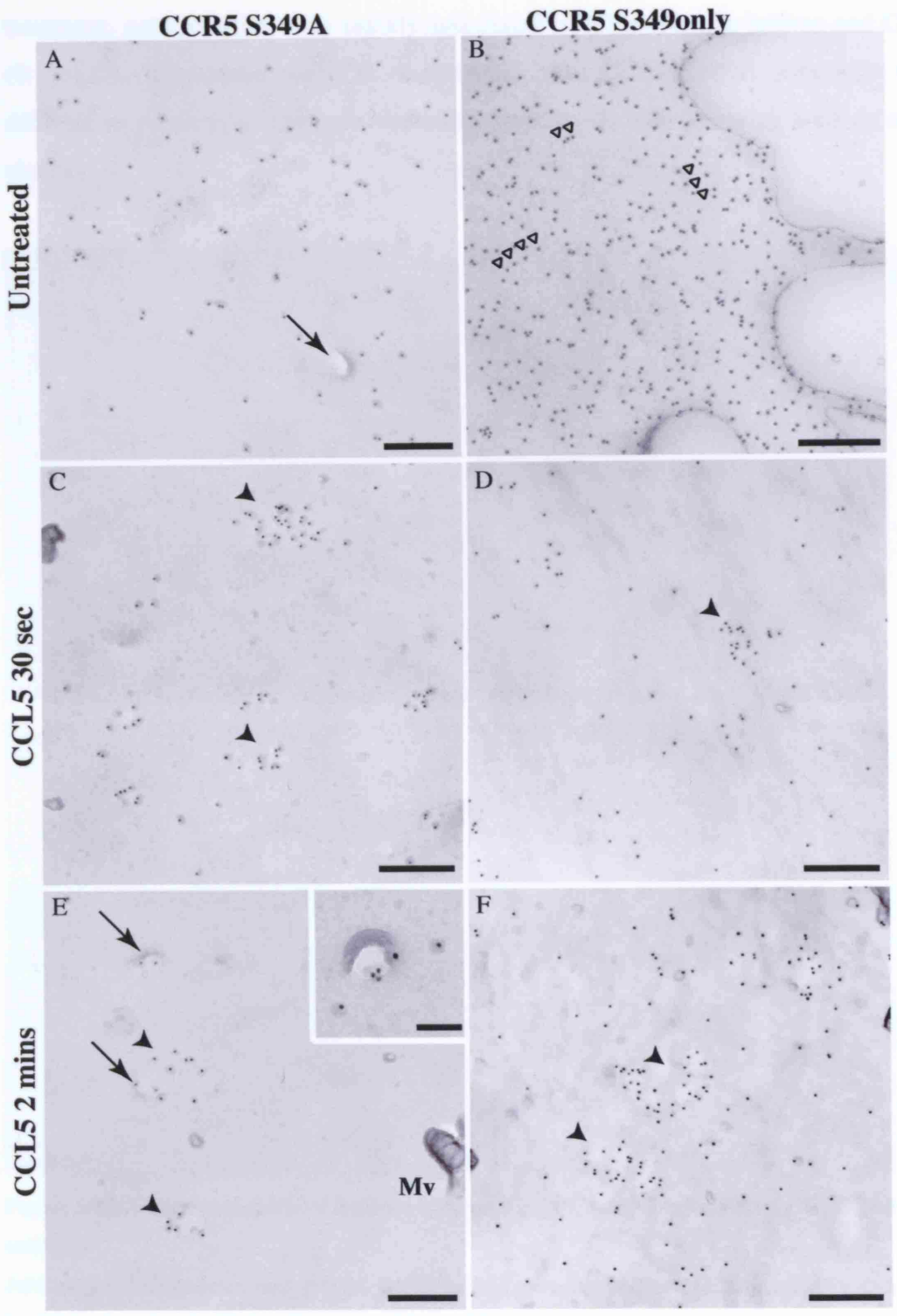


Figure 5.13: Whole mount replicas of immunolabelled RBL CCR5 S349A and S349only cells

RBL cells were incubated with BM alone (A and B) or with 125 mM CCL5 for 30 seconds (C an D) or 2 minutes (E and F) at 37°C, before fixation at 4°C. CCR5 was detected by labelling intact cells with MC-5 and PAG_{15}, and cell surface replicas were prepared as described in Materials and Methods section 2.5.2. Specimens were examined by EM. Invaginations of the membrane are shown with arrows, arrowheads indicate clusters and open arrowheads point to lines of PAG_{15}. (Mv) microvilli. Scale bar = 300 nm (A-F), 100 nm (inset in E).

treatment, gold particles were mainly associated with flat clathrin lattices and CCPs (B and D). Membrane sheets from untreated RBL CCR5 S349A cells were very difficult to prepare, as the cells did not adhere to the coverslips as well as other clones.

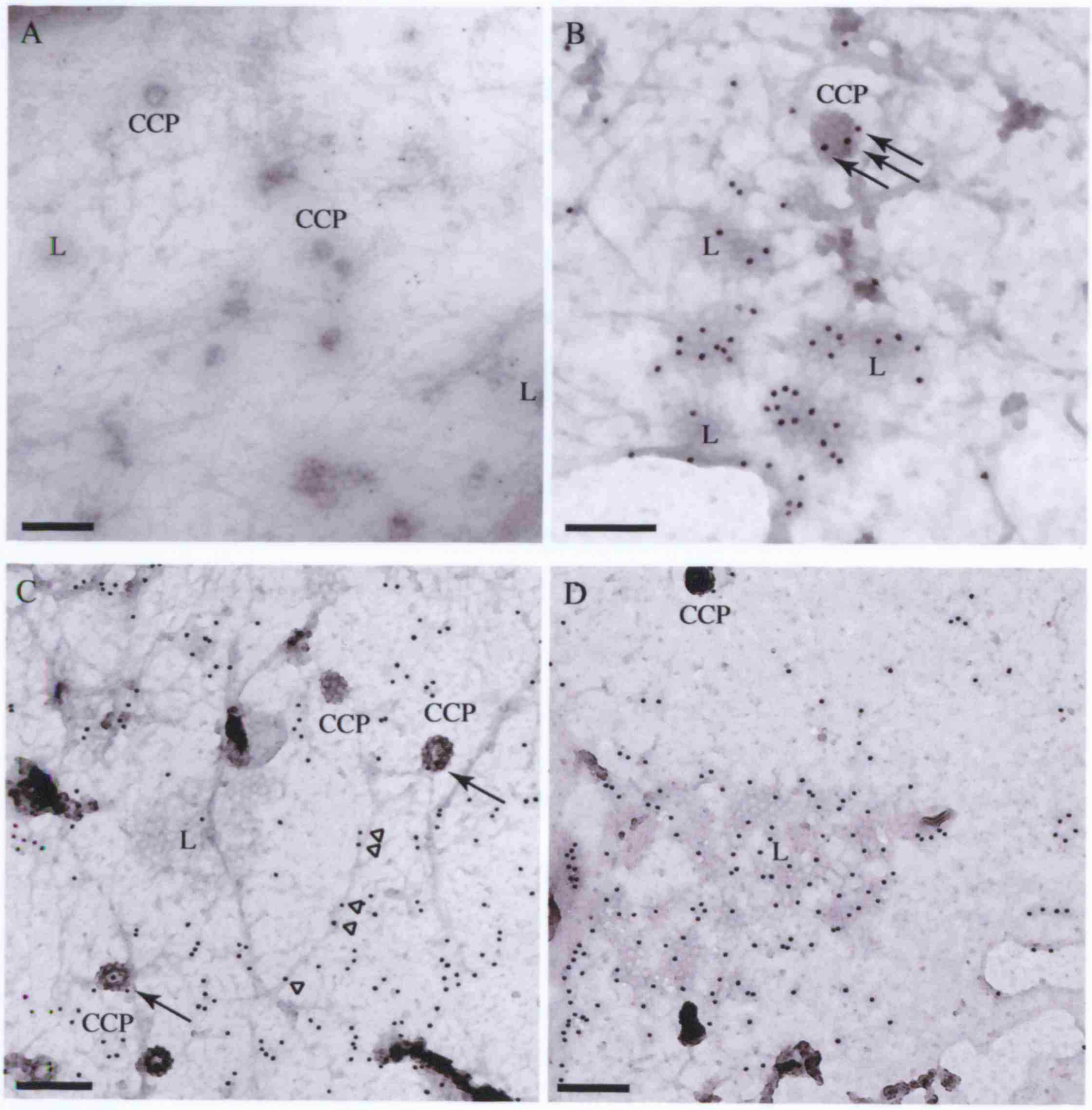


Figure 5.14: Membrane sheets of immunolabelled RBL CCR5 S349A and RBL CCR5 S349only cells

RBL cells were incubated with BM (A and C) or BM containing 125nM CCL5 (B and D) and were labelled intact with MC-5 followed by PAG_{15}. Membrane sheets were subsequently prepared as described in Materials and Methods section 2.5.3, fixed and examined by EM. Scale bar = 300nm (A) and 200nm (B-C), (L) flat clathrin lattice (CCP) clathrin-coated pit, (arrow) CCR5 in CCP, (arrowhead) CCR5 associated with cortical actin

In summary, this morphological analysis indicates that GRK phosphorylation of S349 alone is sufficient for agonist-induced recruitment of CCR5 into flat clathrin

lattices, but that another residue, likely to be S342, might have a similar activity. Additionally, as mentioned in the previous sections, the recruitment of receptors into flat clathrin lattices correlates with their ability to undergo rapid CCL5-induced endocytosis.

The density of the gold particles at the plasma membrane was analysed on whole-mount replicas of all the cell lines as described in Materials and Methods section 2.5.6. In all cases, the labelling density was higher in the untreated cells compare to agonist-stimulated cells. Whether this reflects the down-modulation of the receptor remains uncertain, as the cells also spread upon stimulation. Results of the quantification and the morphological analysis are summarised in table 5.1.
Agonist-induced clustering of the receptor into flat clathrin lattices correlated with the rapid internalisation of the receptor. This suggests an important role for these structures in clathrin-mediated endocytosis. Moreover, in Chapter 3, endocytic proteins involved in clathrin-coated vesicles formation were identified in flat clathrin lattices in the absence of CCR5 and in untreated cells. Altogether these data indicate that flat clathrin lattices represent a pool of clathrin, adaptors and accessory proteins rapidly available for efficient internalisation of receptors.

Table 5.1. Summary of cell surface distribution and density of gold particles in untreated and agonist-treated cells

	Agonist-induced internalisation	Clustering in flat clathrin lattices	Average PAG density (μm^2±SD)*	
			Untreated cells	CCL5 treated cells
WT CCR5	Rapid	+	47.1±35.5	24.5±13.7
CCR5 ΔS	Slower	-	97.7±31	71.5±48
CCR5 S337A	Rapid	+	94.3±58.9	43±31.6
CCR5 S337only	Slower	-	129.7±56.8	102±29.5
CCR5 S349A	Rapid	+	64.5±21.3	14.7±12.3
CCR5 S349only	Rapid	+	96.9±44	44.3±20.7

*Random fields of membranes were photographed and the density of PAG was determined in the area of membrane or for CCL5 treated samples in apparent clusters. 10 different cells were analysed for each cell line in untreated and CCL5-treated samples. Details of the analysis are given in Materials and Methods section 2.5.6.

In conclusion, these morphological analyses indicate that phosphorylation of C-terminal residues upon agonist binding is necessary for receptor recruitment into flat clathrin lattices. Furthermore, GRK phosphorylation of S349 is sufficient to

relocalise the receptor into these domains, in contrast to PKC phosphorylation of S337, which is probably involved in regulating receptor signalling. Finally, recruitment of the receptor into flat clathrin lattices correlates with its rapid endocytosis (as measured in Chapter 4). Altogether, this suggests that clathrin lattices play an important role in the rapid modulation of cell surface CCR5 level following agonist activation.

5.3 Discussion

In Chapter 4, endocytosis analysis of various mutants of CCR5 lacking C-terminal serines revealed differences in the kinetics of agonist-induced internalisation. In order to understand the origin of these differences, the early steps of internalisation of the receptor occurring at the plasma membrane were analysed using immunofluorescence microscopy and electron microscopy, similar to the morphological studies described in Chapter 3. When CCR5 ΔS distribution was analysed in the absence of agonist, no difference was observed compared to the wild-type receptor. However, upon stimulation, the receptor failed to co-localise with clathrin at the plasma membrane when analysed by immunofluorescence; no change in distribution was observed in whole mount replicas, and the receptor was not recruited into flat clathrin lattices. This indicates an important role for agonist-induced phosphorylation of C-terminal serines for CCR5 recruitment into clathrin-coated domains of the plasma membrane. Moreover, as CCR5 ΔS has been shown to be internalised upon agonist binding, although more slowly than the wild-type receptor, it suggests a role for flat clathrin lattices in the rapid endocytosis in response to agonist stimulation.

Analysis of CCR5 internalisation in Chapter 4 also indicated distinct roles for GRK and PKC phosphorylation in the down-modulation of the receptor. This was confirmed in the morphological analysis. Indeed, cell surface CCR5 S337only, phosphorylated by PKC, failed to co-localised with clathrin by immunofluorescence and did not cluster into flat clathrin lattices upon agonist binding, although gold particles were seen associated with CCP on membrane sheets. This is in contrast to CCR5 S349only, which did co-localise with clathrin by immunofluorescence and did cluster into flat clathrin lattices. In addition, as mentioned for CCR5 ΔS, the rapid

endocytosis of receptors measured in Chapter 4 correlated with recruitment into flat clathrin lattices upon agonist binding.

The morphological changes observed in RBL CCR5 S337A cells suggest a specific role for S337 phosphorylation in CCR5 desensitisation of receptor. When S337 was the only residue that could be phosphorylated following agonist binding, the signalling leading to cell spreading seemed to be regulated as in cells expressing wild-type CCR5. The cells did not exhibit sustained activation, as it appeared for CCR5 ΔS. This points to a role for S337 phosphorylation in regulating signalling rather than in the early events of agonist-induced internalisation. Interestingly, when CCR5 is only phosphorylated on S337 (in RBL CCR5 S337only cells) the receptor distribution after stimulation is similar to CCR5 ΔS. Moreover, the analysis in Chapter 4 showed that the two mutant receptors have the same endocytic profile following agonist binding. Altogether, these data suggest that agonist-induced phosphorylation of S337 has little if any role in CCR5 endocytosis.

The role of GRK phosphorylation in GPCRs endocytosis is important for the recruitment of β-arrestins to the activated receptor at the plasma membrane. β-arrestins play a major role in receptor desensitisation, by uncoupling the activated receptor from G-protein and linking them to the clathrin endocytic machinery (Claing et al., 2002). CCR5 internalisation has been shown to be β-arrestin-dependent (Fraile-Ramos et al., 2003). Therefore it may be possible that CCR5 phosphorylation by GRK on S349 induces recruitment of β-arrestins to the receptor at the plasma membrane. Subsequently, via interaction with AP-2 and clathrin, the receptor/β-arrestin complex could be recruited to flat clathrin lattices.

If GRK phosphorylation is required for recruitment of β-arrestins and the link to the clathrin endocytic machinery, how are CCR5 ΔS and CCR5 S337only internalised? Huttenrauch *et al.* showed that β-arrestins were not recruited to receptors lacking more than two C-terminal serines, and therefore proposed a β-arrestin-independent mode of internalisation for these receptors (Huttenrauch et al., 2002). In an earlier study from Kraft *et al.* a dileucine motif in the C-terminus of CCR5 was suggested to link the receptor directly to the endocytic machinery via AP-2 (Kraft et al., 2001). However, this still needs to be confirmed. Nevertheless, as labelling for both receptors was observed in clathrin structures on membrane sheets, endocytosis via the clathrin pathway is not excluded.

It would be interesting to know to what extent these mutated receptors co-localised with the wild-type receptors along the endocytic pathway and whether they use the same clathrin structures at the plasma membrane. A few years ago, work by Cao *et al.* indicated that GPCRs could be targeted to distinct CCPs depending on their interaction with β-arrestin (Cao et al., 1998). Similar evidence emerged from a more recent study from Mundell *et al.* (Mundell et al., 2006). The recruitment of β-arrestins to cell surface activated receptors and their role in the internalisation of CCR5 will be the subjects of the next two chapters.

6 Importance of β-arrestins in the early events of CCR5 internalisation

Originally, β-arrestins were only thought to play a role in GPCR desensitisation, by abrogating receptor interactions with G-proteins and mediating receptor internalisation through the clathrin endocytic machinery. Recently, β-arrestins have also been shown to function as signalling scaffolds and to interact with proteins from the MAP kinase signalling cascades.

Evidence for a role for β-arrestins in CCR5 internalisation has accumulated in the past decade and their association with this receptor was shown to rely on its phosphorylation by GPCR kinase 2 and 3 (GRK2/3) (Aramori et al., 1997; Fraile-Ramos et al., 2003; Huttenrauch et al., 2002; Kraft et al., 2001). Interestingly, in the previous chapters, GRK phosphorylation of CCR5 was shown to be important for recruitment of CCR5 into flat clathrin lattices and its subsequent rapid internalisation. These findings have raised the question whether β-arrestins are involved in CCR5 recruitment into these structures.

In this chapter, GRK phosphorylation of CCR5 on S349 was monitored before and after agonist stimulation, and the distribution of the phosphorylated receptor was analysed on membrane sheets. In addition, the localisation of β-arrestins has been investigated by immunofluorescence and electron microscopy in CCR5 expressing cells before and after agonist stimulation. Finally, the association of endogenous β-arrestin-2 with mutants of CCR5 was investigated.

6.1 Agonist-induced phosphorylation of CCR5 WT on serine 349

6.1.1 Immunofluorescence labelling of CCR5 S349-P

GRK phosphorylation of S349 was shown to be sufficient for agonist-induced recruitment of CCR5 into flat clathrin lattices and rapid endocytosis of the receptor. Thus, the phosphorylation of this residue in the wild-type receptor was examined before and after agonist treatment by immunofluorescence as described in Materials and Methods section 2.4.3. In order to determine whether the receptor was phosphorylated, I used M5/4, an antibody that specifically recognises S349 phosphorylated (S349-P) CCR5. Figure 6.1 shows single confocal sections of RBL

CCR5 cells incubated in BM (A an B) or BM containing 125nM CCL5 for 2 or 5 minutes at 37°C (C-F), stained for cell surface CCR5 (A, C and E), permeabilised and further labelled with M5/4 for CCR5 S349-P (B, D and F). The two primary antibodies were subsequently detected with isotype specific secondary antibodies ^{488}GAM-IgG2a and ^{594}GAM-IgG1. Very little staining was detected with M5/4 in untreated cells, indicating that the receptor, expression of which was confirmed by MC-5 labelling, was not phosphorylated on S349. In contrast, upon CCL5 treatment bright staining was observed first at the plasma membrane and at the lamellae. With time, a more punctate intracellular and perinuclear staining appeared.

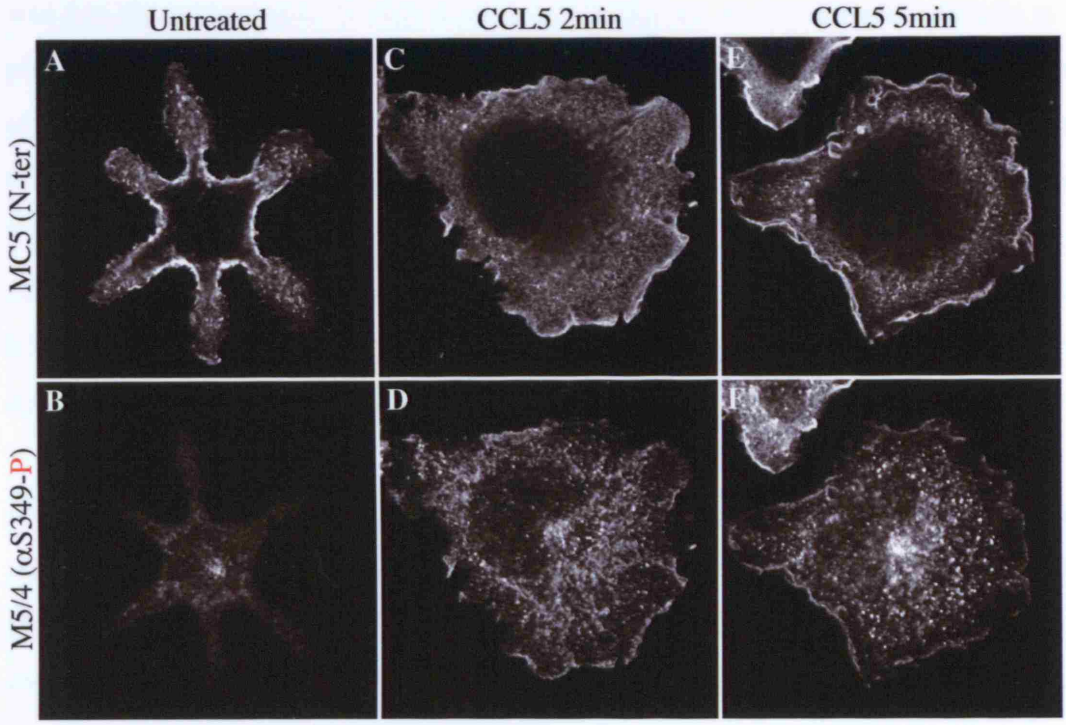


Figure 6.1: Immunofluorescence labelling of CCR5 S349-P in RBL CCR5 cells

RBL CCR5 cells were incubated in BM (A and B) or BM containing 125 nM CCL5 for 2 (C and D) or 5 minutes (E and F). To detect cell surface CCR5, intact cells were labelled with MC-5 and ^{488}GAM-IgG2a. Cells were then permeabilised to label S349-P with M5/4 followed by ^{594}GAM-IgG1. Scale bars = 10 μm.

In conclusion, this immunofluorescence analysis shows that CCR5 is not phosphorylated in untreated cells and that CCR5 S349-P can only be detected upon agonist treatment. Moreover, phosphorylation was shown to occur very rapidly within 2 minutes after agonist stimulation. Finally, the internalised phosphorylated receptors were observed in intracellular structures probably corresponding to endocytic vesicles and recycling endosomes (Signoret et al., 2000).

In Chapter 3, CCR5 was shown to co-localise with clathrin structures at the plasma membrane. In order to investigate whether the receptor was phosphorylated in these structures, RBL CCR5 were further analysed by immunofluorescence. Briefly, cells were incubated in BM or BM containing 125nM CCL5 for 30 seconds or 5 minutes, fixed and labelled intact for cell surface CCR5 with MC-5. The cells were then permeabilised and labelled for clathrin and CCR5 S349-P with a rabbit anti-clathrin and M5/4, respectively. The primary antibodies were detected using species and isotype specific secondary antibodies. The samples were analysed by confocal microscopy and single sections are shown in figure 6.2. In untreated cells, CCR5 was not phosphorylated (background in J) and did not co-localis with clathrin at the plasma membrane. In contrast, upon CCL5 treatment, the receptor was rapidly phosphorylated (K and L) and some cell surface receptors co-localised with clathrin (yellow puncta in enlargement). Although staining for S349-P was very bright, very little labelling was detected at the plasma membrane together with MC-5 labelling, suggesting that the receptor was not phosphorylated at the plasma membrane or that S349-P was not accessible to the antibody. Nevertheless, when the two labels were detected together, clathrin was also present (arrowheads in enlargement).

In summary, this immunofluorescence analysis shows that CCR5 S349 was phosphorylated within 30 seconds of agonist stimulation. In addition, the phosphorylated receptor was sometimes observed in clathrin structures at the plasma membrane. Although in agonist-treated cells clathrin structures at the plasma membrane often contained CCR5, only a few of them appeared to contain the phosphorylated receptor. This does not necessarily mean that the clathrin-associated CCR5 was not phosphorylated, but might indicate that the antibody does not have access to the antigen due to steric hindrance (see below).

Figure 6.2: Immunofluorescence labelling of CCR5 S349-P and clathrin in RBL cells

RBL cells were incubated in BM (Untreated) or BM containing 125nM CCL5 for 30 seconds or 5 minutes. Intact cells were labelled for cell surface CCR5 with MC-5 (G-I). The cells were subsequently permeabilised and stained for clathrin with a rabbit polyclonal antibody (D-F) and CCR5 S349-P with M5/4 (J-L). Primary antibodies were detected with ^{488}GAM-IgG2, GAR^{594} and GAM-IgG1-biotin followed by Cy-5 conjugated streptavidin, respectively. The area in the square in B, E, H and K is enlarged and arrows show the co-localisation of the three markers. Scale bar = 10 μm

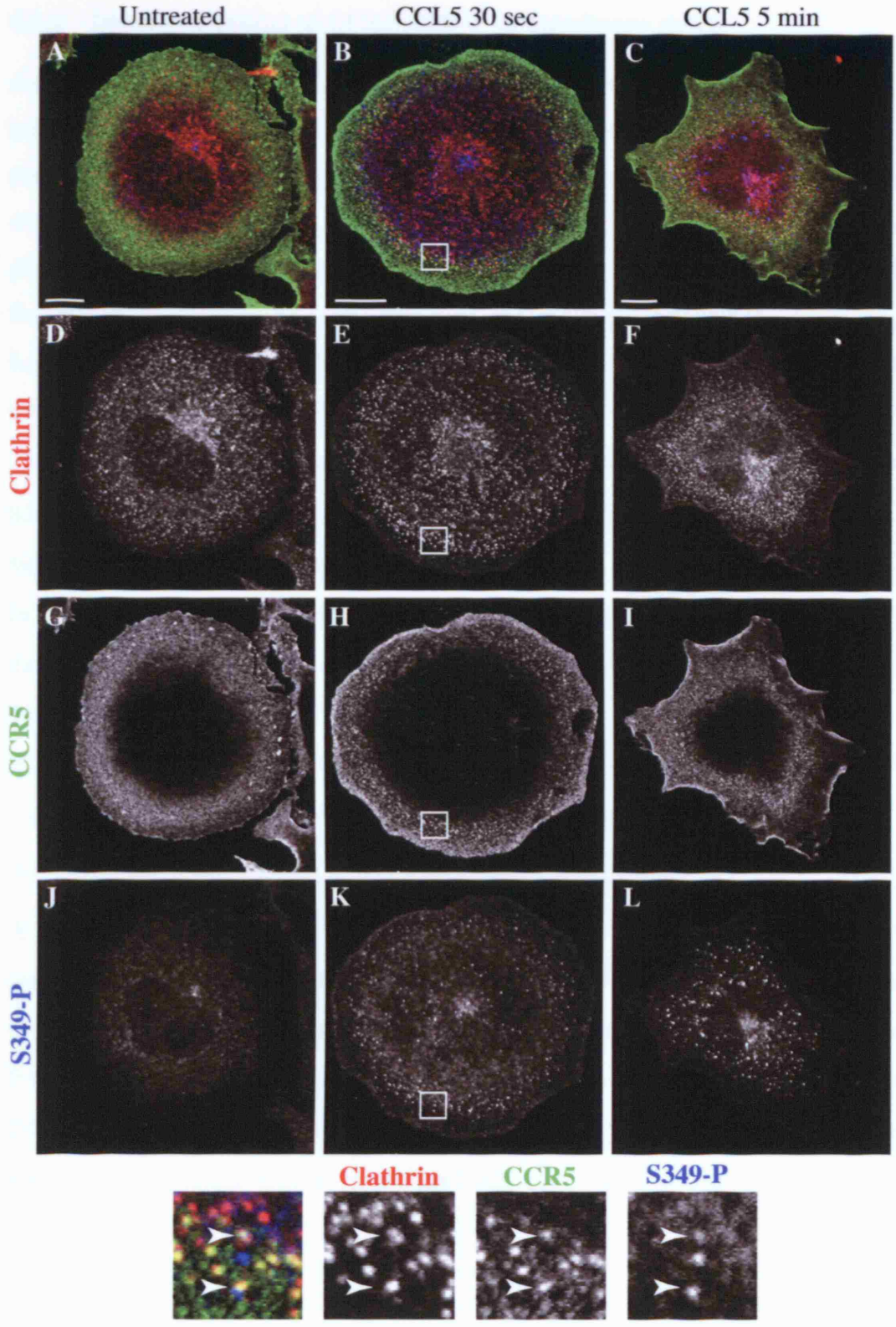


Figure 6.2: Immunofluorescence labelling of CCR5 S349-P and clathrin in RBL cells

6.1.2 Immunolabelling of CCR5 S349-P in membrane sheets

As the detection of S349-P at the plasma membrane seemed difficult by immunofluorescence, membrane sheets of the upper surface of the cells were prepared as described in Materials and Methods section 2.5.3. Membrane sheets are obtained from unfixed cells, consequently cytoplasmic proteins associated with the plasma membrane through low affinity interactions might be washed away before fixation, perhaps allowing better detection of the cytoplasmic tail of CCR5. Figure 6.3 shows the cytoplasmic side of the plasma membrane in RBL CCR5 cells, where cell surface receptors have been labelled with MC-5 and Protein A conjugated to 15 nm-gold particles (PAG_{15}) on one side of the membrane and with M5/4 against S349-P followed by a bridging antibody, rabbit anti-mouse, and PAG_{10}, on the other side of the lipid bilayer as shown in figure 6.3 A.

In untreated cells, PAG_{15} that detected MC-5 bound to the N-terminus of CCR5 was disperse throughout the membrane and, as described in Chapter 3, showed little association with flat clathrin lattices (B). In agreement with the immunofluorescence labelling in figures 6.1 and 6.2, little PAG_{10} detecting S349-P was observed (arrows and inset in B). In contrast, upon CCL5 treatment PAG_{15} was mainly found in flat clathrin lattices, and PAG_{10} was detected at a higher level than in untreated cells. Frequently, this PAG_{10} was seen close to PAG_{15} particles indicating phosphorylation of CCR5 molecules (arrows in C). Interestingly, PAG_{10} was only observed outside of or at the edges of lattices (arrowhead in C) and only a few particles were seen in the middle of the lattices or in CCPs (double arrowheads). Doublets of PAG_{10} were seen most of the time, as a result of using a bridging antibody as explained in panel A of figure 6.3.

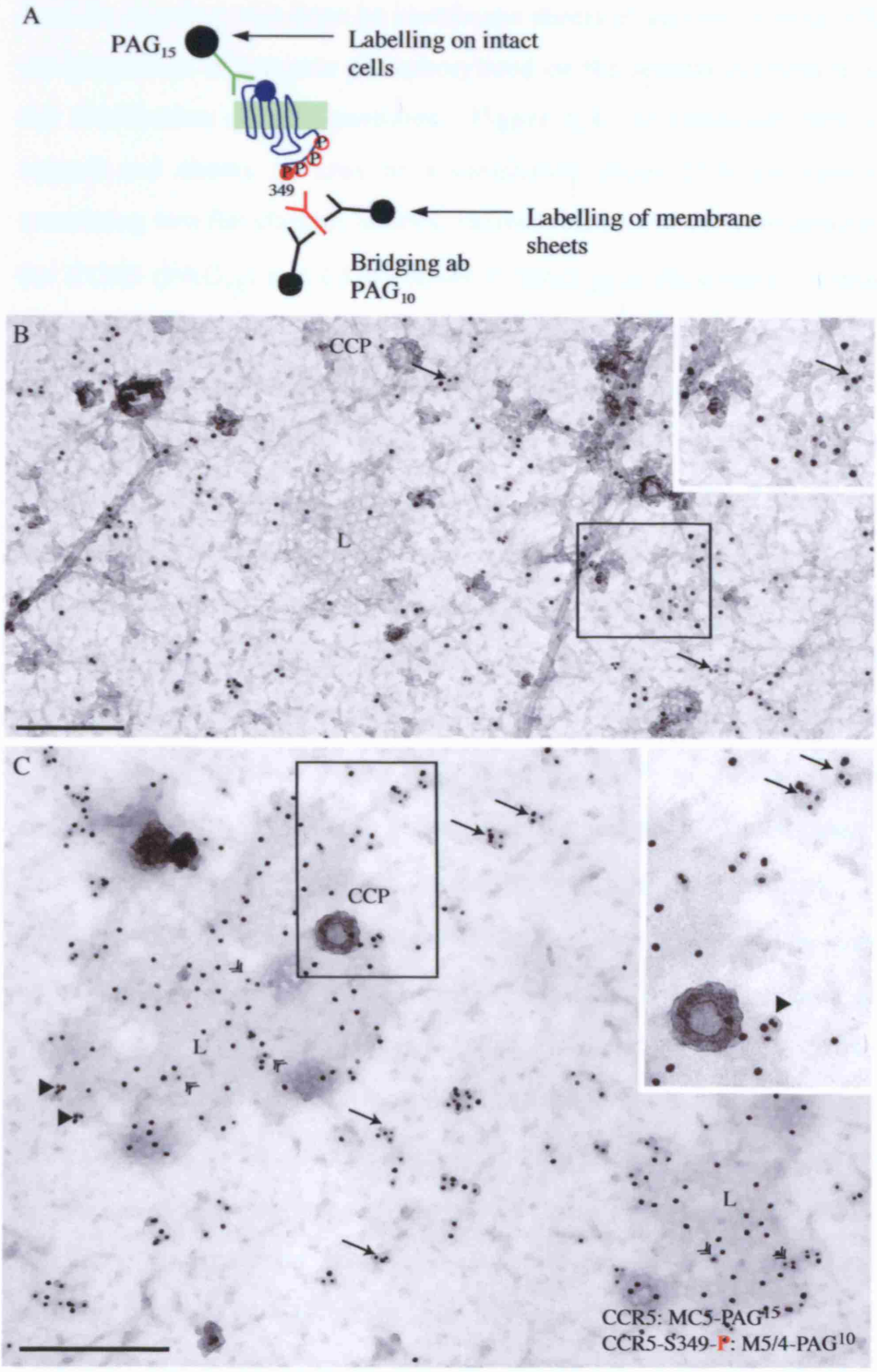


Figure 6.3: Visualisation of agonist-induced phosphorylation of CCR5 at the plasma membrane

RBL CCR5 cells were incubated in BM alone (B) or with CCL5 (C) for 2 minutes at 37°C, washed at 4°C, and labelled for CCR5 with MC-5 and PAG_{15} on ice and before being processed for rip-off as described in section 2.5.3. The membrane sheets were fixed and labelled with an antibody against CCR5 S349-P (M5/4) followed by a bridging antibody, RαMIgG1 and PAG_{10} (A). Arrows indicate large gold particles associated with small gold particles (phosphorylated CCR5) outside flat clathrin lattices. Arrowheads indicate association of small and large gold particles at the edges of flat clathrin lattices. Double arrowheads show PAG_{10} in the middle of the lattices. Scale bars = 300 nm

Particle counting was done on membrane sheets of agonist-treated cells to determine the proportion of receptor phosphorylated on the plasma membrane and to quantify the distribution of gold particles. Figure 6.4 (A) illustrates how particles were scored and shows an area of a membrane sheet from an agonist-treated cell containing two flat clathrin lattices, various features of the cytoskeleton and labelling for CCR5 (PAG_{15}) and CCR5-S349-P (PAG_{10}) is illustrated. Arrows indicate 10 nm-gold particles and circles illustrate double labelling of a receptor. The results of the analysis are summarised in figure 6.4 (B) and in table 6.1.

The distribution of PAG_{15} detecting MC-5 was not random; as seen previously, the receptor clustered into clathrin lattices, and just over 20% of the gold particles were localised in flat clathrin lattices (grey bar in B). In contrast, less than 4% of PAG_{10} was observed over lattices. Moreover, when labelling was detected it was mainly outside of or at the edge of lattices as shown in figures 6.3 and 6.4. Among the 15 nm-gold particles labelling for CCR5, 23% were associated with PAG_{10} detecting CCR5-S349-P (striped bar in B). Similarly, 27% of 10 nm-gold particles were associated with PAG_{15}. This indicates that a quarter of the receptors at the plasma membrane may be phosphorylated following agonist treatment. The remaining PAG_{15}, outside lattices and not associated with PAG_{10}, might be unphosphorylated or undetected phosphorylated receptors. Indeed, if a cytoplasmic protein such as GRK, protein kinase C (PKC) or β-arrestin is recruited to a cell surface receptor, the epitope recognised by M5/4 might be hidden and not accessible to the antibody. The isolated PAG_{10} is likely to be specific as very little if any labelling was observed on membrane sheets obtained from untreated cells. However, as the labelling for CCR5 was done on live cells prior to preparation of the membrane sheets, some gold particles might have dissociated from MC-5.

Together, this morphological analysis shows that phosphorylation occurs rapidly (within 2 minutes) on S349 upon agonist treatment, and can be detected on the membrane sheets outside and at the edge of flat clathrin lattices. Nevertheless, the receptor is likely to be phosphorylated in lattices as M5/4 intracellular staining co-localises with clathrin-coated vesicles and markers for early and recycling endosomes (see Chapter 7 and unpublished data from our laboratory).

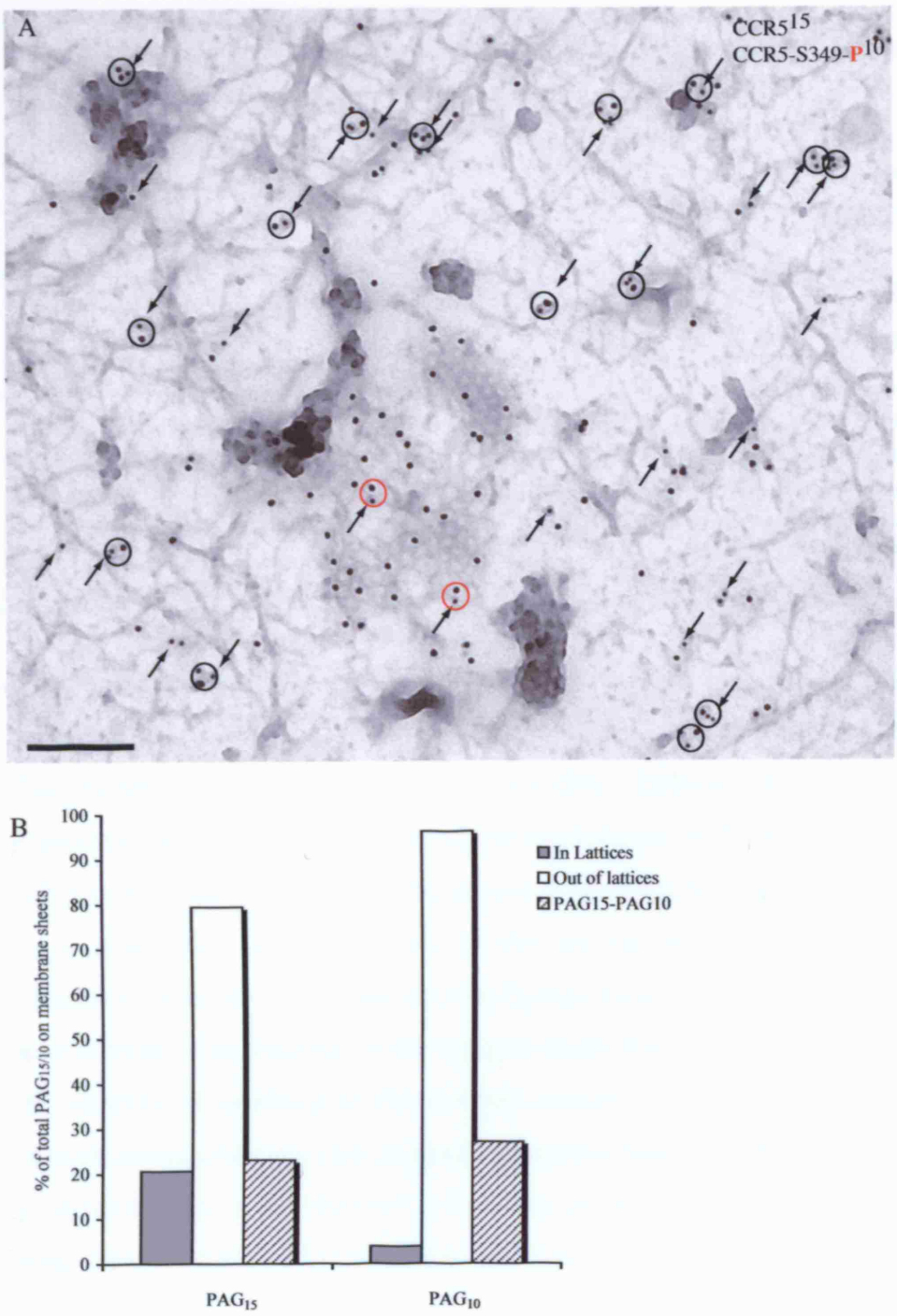


Figure 6.4: Quantification of CCR5 labelling on membrane sheets

(A) Membrane sheets obtained from RBL CCR5 cells treated with 125nM CCL5 for 2 minutes at 37°C and labelled for CCR5 with MC-5 and PAG_{15}, were incubated with M5/4, RαMIgG1 and PAG_{10} to detect CCR5-S349-P. Only part of a membrane sheet analysed is shown here. (Arrows) PAG_{10}, (circles) PAG_{10} and PAG_{15} likely to label the same receptor molecule as they are less than 25 nm apart. Scale bar = 200 nm. (B) Graphic illustrating the data in table 6.1 showing the percentage of gold particles in and out of lattices, as well as PAG_{10} associated with PAG_{15} and vice versa. Details of the quantification analysis are given in Materials and Methods section 2.5.6.

Table 6.1: Distribution of gold particles detecting CCR5 and CCR5-S349-P on membrane sheets from CCL5-stimulated RBL CCR5

	Total gold particles	Lattices[1]		PAG_{15} and PAG_{10} less than 25 nm apart
		In	Out	
PAG_{15} (CCR5)	1101	226	875	253
PAG_{10} (CCR5-S349-P)	1466	56	1410	397[2]

RBL CCR5 cells were incubated for 5 minutes in BM containing 125 nM CCL5 and labelled for cell surface receptor with MC-5 detected with PAG_{15}. Membrane sheets were subsequently prepared, fixed and CCR5-S349-P detected with M5/4, RαM-IgG and PAG_{10}. Gold particles were counted and their distribution analysed as described in Materials and Methods section 2.5.6. [1]Seven membrane sheets were analysed containing a total of 16 lattices. [2]Note that the ratio PAG_{10}/PAG_{15} was higher than 1 due to binding of the bridging antibody to different sites on M5/4 simultaneously.

The absence of labelling is probably due to the lack of access of the antibody to the S349 epitope. CCR5 might interact with proteins from the endocytic machinery in flat clathrin lattices in particular β-arrestins. Indeed, CCR5 internalisation is dependent on β-arrestins, and the two proteins interact with the receptor, clathrin and AP-2 at the plasma membrane. Moreover, β-arrestins binding to CCR5 is dependent on a conserved sequence motif in the second intracellular loop and on the phosphorylation of C-terminal serines (Huttenrauch et al., 2002; Kraft et al., 2001). Recruitment of β-arrestins to the receptor might hide the M5/4 epitope and explain the absence of labelling in flat clathrin lattices. Also, phosphorylated receptors outside lattices may indicate receptors that have been activated but have yet to bind β -arrestins or, as the network of protein-protein interaction is likely to be less established on the molecules outside pits, β-arrestins may dissociate more readily.

6.2 Localisation of β-arrestin in cells expressing CCR5

RBL cells provide an excellent background to investigate the role of β-arrestins in CCR5 internalisation as they express high endogenous level of both proteins (Santini et al., 2000). However, good antibodies against β-arrestins are not easily available for morphological studies. Therefore, Chinese hamster ovary cells (CHO) stably expressing CCR5 and β-arrestin 1-YFP or β-arrestin 2-GFP were used to study the recruitment of the β-arrestins to agonist-activated CCR5. In most respects the

internalisation of CCR5 in CHO cells is very similar to that reported here in RBL cells (Signoret et al., 2000).

6.2.1 Immunofluorescence labelling of cell surface CCR5 and β-arrestins

Immunofluorescence labelling was used to determine whether β-arrestins were recruited to cell surface CCR5 in CHO cells following agonist binding. Figures 6.5 and 6.6 show the distribution of CCR5 and β-arrestin 1-YFP or β-arrestin 2-GFP, respectively. Cells were incubated in BM (A) or BM containing 125 nM CCL5 for 2 or 5 min (B and C, respectively) and fixed. Intact cells were subsequently stained for plasma membrane CCR5 with MC-5 and ^{594}GAM. Single confocal sections are shown here.

In untreated cells, both β-arrestins were diffusely distributed in the cytoplasm (panels A in figures 6.5 and 6.6). β-arrestin 2 was excluded from the nucleus in most cells in contrast to β-arrestin 1, which always showed some nuclear localisation. This agrees with the previously reported distribution of the two proteins and can be explain by the presence of a nuclear export signal (NES) at the N-terminus of β-arrestin 2 that is absent in β-arrestin 1 (Wang et al., 2003a). CCR5 was highly expressed at the plasma membrane. Upon agonist binding, the distribution of the β-arrestins became more punctate and co-localised with CCR5 at the cell surface (panels B and C in both figures).

In summary, this immunofluorescence analysis shows that both β-arrestins are recruited to cell surface CCR5 upon stimulation with the agonist, and suggests that both proteins may be involved in the desensitisation and internalisation of the receptor at the plasma membrane.

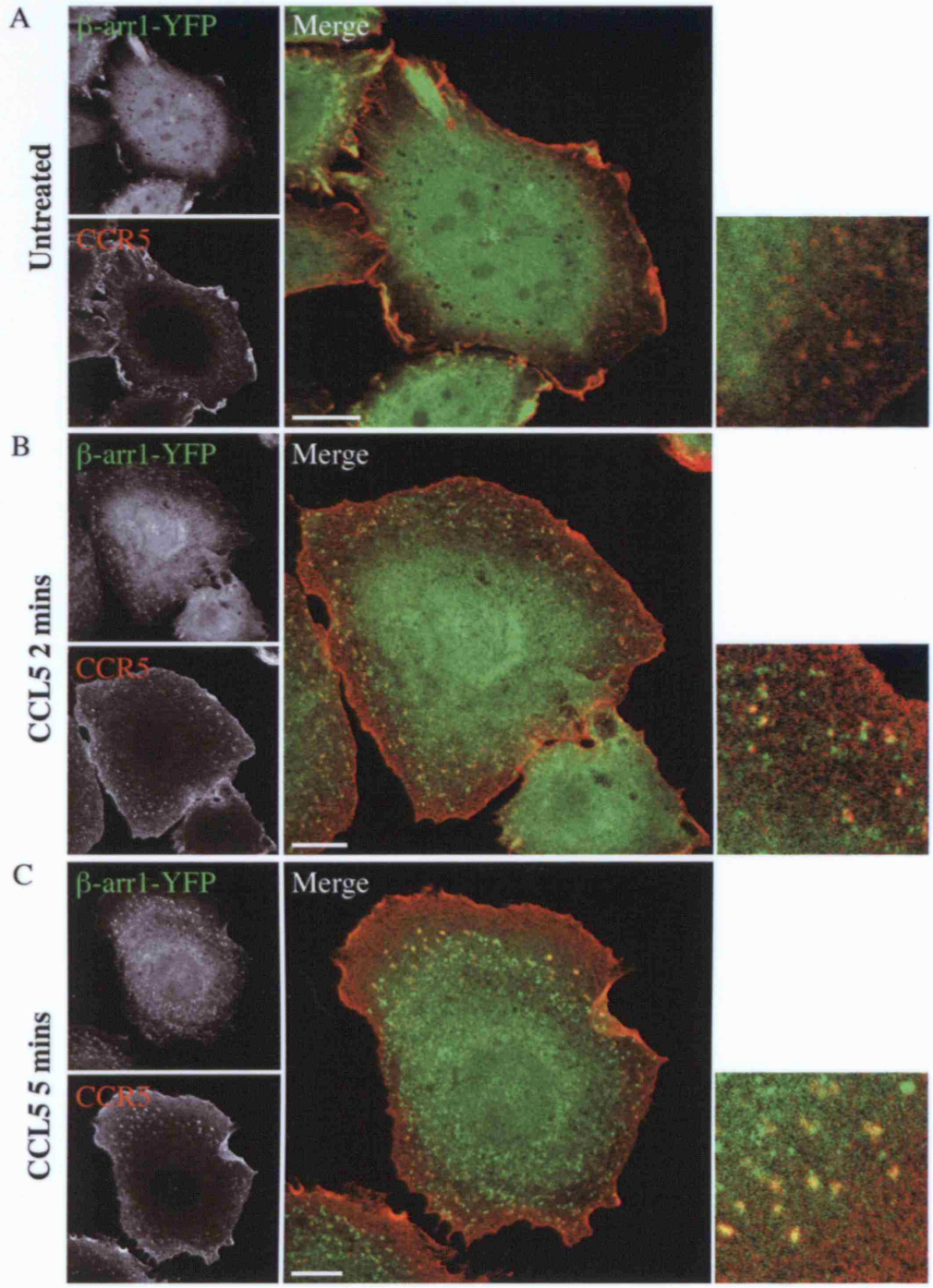


Figure 6.5: Immunofluorescence localisation of β-arrestin 1-YFP and immunofluorescence labelling of plasma membrane CCR5 in CHO cells

CHO CCR5 cells stably expressing β-arrestin 1-YFP were incubated in BM alone (A) or with CCL5 for 2 (B) or 5 min (C) at 37°C, fixed and labelled intact for CCR5 with MC-5 followed by ^{488}GAM. Single confocal sections are shown. Scale bar = 10 μm.

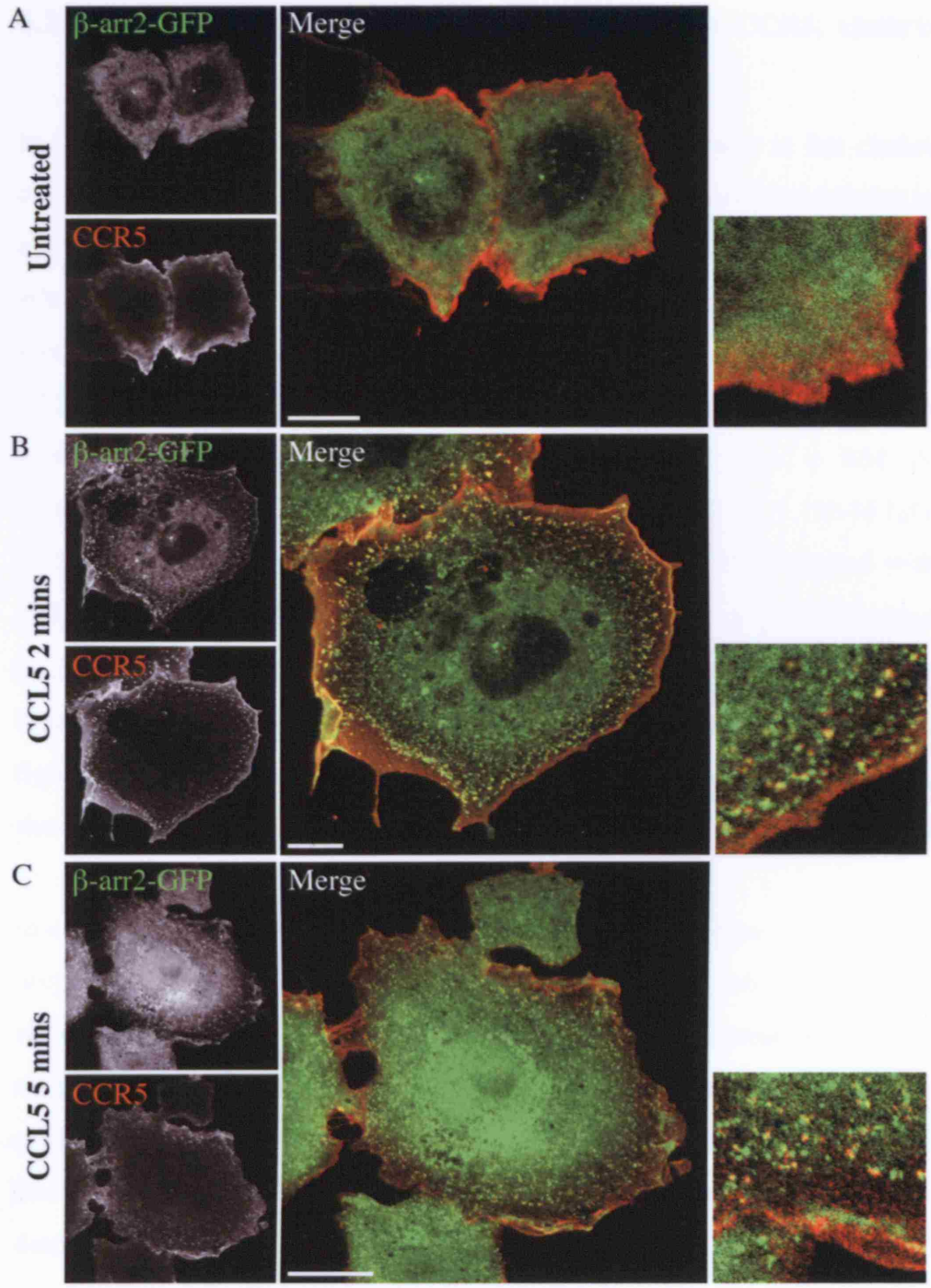


Figure 6.6: Immunofluorescence localisation of β-arrestin 2-GFP and immunofluorescence labelling of plasma membrane CCR5 in CHO cells

CHO CCR5 cells stably expressing β-arrestin 2-GFP were incubated in BM alone (A) or with CCL5 for 2 (B) or 5 min (C) at 37°C, fixed and labelled intact for CCR5 with MC-5 followed by ^{488}GAM. Single confocal sections are shown. Scale bar = 10 μm.

6.2.2 Immunofluorescence labelling of cell surface CCR5, clathrin and β-arrestins

In Chapter 3, agonist-activated CCR5 was shown to cluster in flat clathrin lattices containing the adaptor protein-2 (AP-2) complex. β-arrestins interact with both, clathrin and AP-2 (Goodman et al., 1997; Krupnick et al., 1997). To investigate whether β-arrestins and CCR5 co-localised in clathrin-coated domains of the plasma membrane I used immunofluorescence labelling. Figures 6.7 and 6.8 show CHO CCR5 β-arrestin 1-YFP and β-arrestins 2-GFP cells, respectively, stained for cell surface CCR5 and clathrin. Cells incubated for 5 minutes in BM (A) or BM containing 125 nM CCL5 (B) were labelled intact with MC-5, GAM-IgG2a-biotin and streptavidin Cy-5. After permeabilisation, clathrin was detected with a rabbit antibody and ^{594}GAR. As shown earlier, in untreated cells both β-arrestins were cytosolic, and did not co-localise with cell surface CCR5. In addition no co-localisation was observed between the three proteins and clathrin (panels A in both figures). In contrast, after 5 minutes of CCL5 treatment, β-arrestins and CCR5 showed some co-localisation with clathrin at the plasma membrane (panels B).

In conclusion, this analysis showed that upon CCL5 treatment cell surface CCR5 not only co-localise with clathrin, but also with β-arrestins, which are recruited to the activated receptor. Furthermore, as CCR5 and β-arrestins were only detected together with clathrin, this suggests that β-arrestins were recruited to the plasma membrane when CCR5 was already in flat clathrin lattices. However, the possibility that β-arrestins recruitment to the activated receptor induced its relocation to clathrin domains cannot be excluded. Indeed, the movement of the receptor/β-arrestin complexes into clathrin structures might occurs so rapidly that it may not be possible to detect the complex on its own on the membrane. Live microscopy studies with tagged proteins will give more details into the dynamics of CCR5 at the plasma membrane and how these dynamics change on agonist binding.

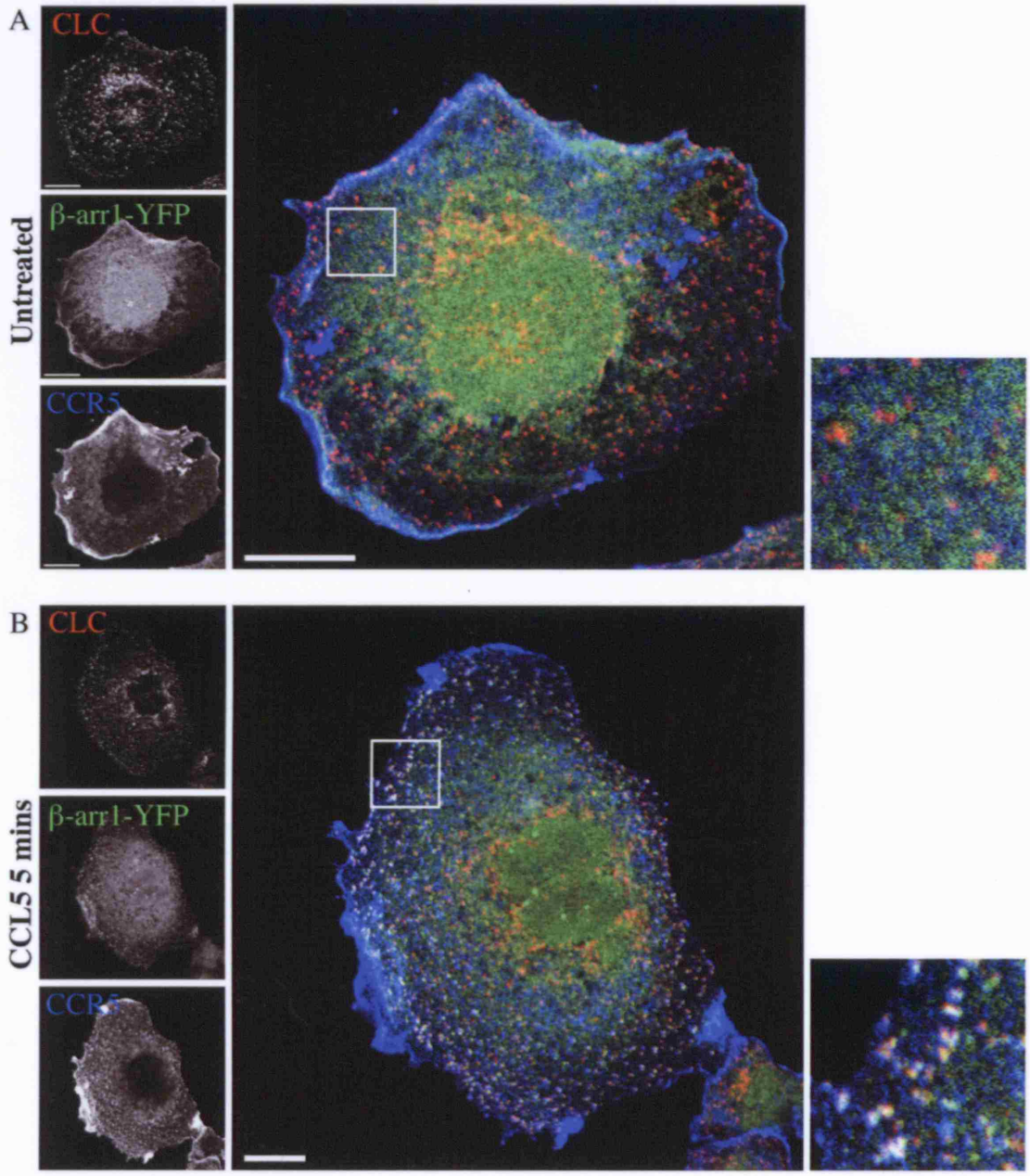


Figure 6.7: Immunofluorescence labelling of CCR5 and clathrin and localisation of β-arrestin 1-YFP in CHO cells

CHO CCR5 cells stably expressing β-arrestin 1-YFP were incubated in BM alone (A) or with CCL5 for 5 min (B) at 37°C before fixation. CCR5 was detected in intact cells with MC-5, GAM-IgG2a-biotin and streptavidin Cy-5. Cells were then permeabilised and labelled for clathrin with a rabbit anti-clathrin light chain antibody and ^{594}GAR. Single confocal sections are shown. Scale bar = 10 μm.

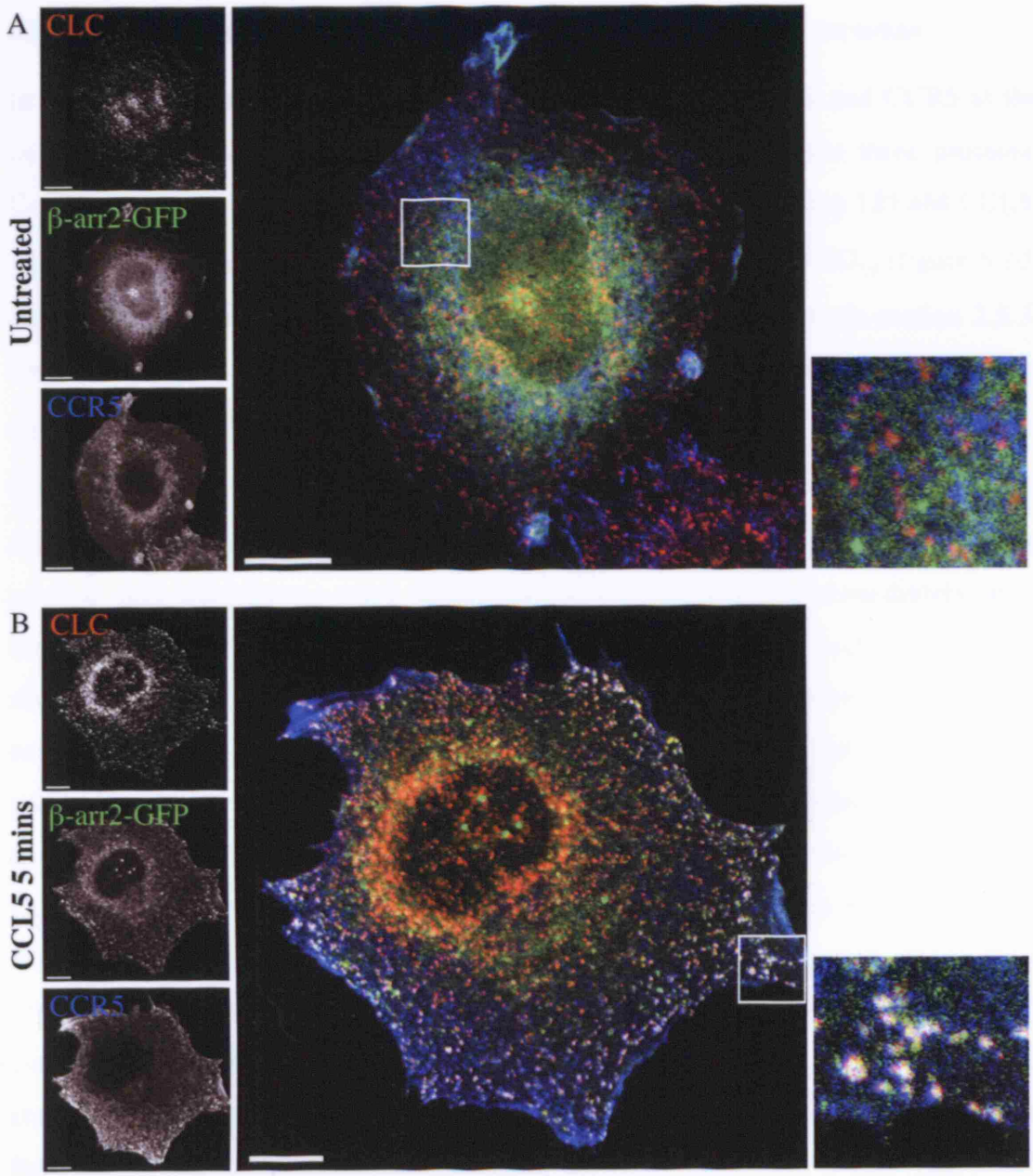


Figure 6.8: Immunofluorescence labelling of CCR5 and clathrin and localisation of β-arrestin 2-GFP in CHO cells

CHO CCR5 cells stably expressing β-arrestin 2-GFP were incubated in BM alone (A) or with CCL5 for 5 min (B) at 37°C before fixation. CCR5 was detected in intact cells with MC-5, GAM-IgG2a-biotin and streptavidin Cy-5. Cells were then permeabilised and labelled for clathrin with a rabbit anti-clathrin light chain antibody and ^{594}GAR. Single confocal sections are shown. Scale bar = 10 μm.

6.2.3 Immunolabelling of membrane sheets for CCR5 and β-arrestins

In order to analyse in more details the distribution of β-arrestins and CCR5 at the cell surface, membrane sheets were prepared and labelled for the three proteins. Cells were incubated for 5 minutes at 37°C in BM or BM containing 125 nM CCL5. The receptor was detected with MC-5 and PAG_{15} (figure 6.9) or PAG_{10} (figure 6.10) and cells processed for rip-off as described in Materials and Methods section 2.5.3. Membrane sheets were subsequently labelled for β-arrestins with a rabbit antibody against GFP and YFP and detected with a different size of PAG (PAG_{10} in figure 6.9, and PAG_{15} in figure 6.10). To verify that gold particles detecting the rabbit antibody did not cross-react with MC-5, the antibody against GFP was omitted in a control, and membranes were incubated with gold particles immediately after neutralisation with glutaraldehyde (see Materials and Methods section 2.5.3). As shown in figure 6.9 panel A, no small gold particles were detected. In untreated cells, very little labelling was found for either β-arrestin and, as described previously, CCR5 had a disperse distribution on the plasma membrane (figure 6.9 B and figure 6.10 A). Upon agonist binding, both β-arrestins were found with CCR5, clustered in flat clathrin lattices (figure 6.9 C and D and figure 6.10 B and D). Moreover, doublets of gold particles detecting receptor/arrestin complexes were often observed (arrowheads). Interestingly, doublets were rarely found outside clathrin lattices. Whether this reflects the recruitment of β-arrestins directly into flat clathrin lattices remains to be determined. Clathrin-coated pits (CCPs) budding from flat clathrin lattices were also labelled for β-arrestins (figure 6.9 D and figure 6.10 C). Some PAG detecting CCR5 in lattices was not associated with PAG detecting β-arrestins-GFP/YFP, maybe because the receptor was also associated with endogenous β-arrestins.

Figure 6.9: Membrane sheets of immunolabelled CHO CCR5 β-arrestin 1-YFP cells

CHO CCR5 β-arrestin 1-YFP cells were incubated in BM (B) or BM containing 125 nM CCL5 (A, C and D) for 2 minutes at 37°C. Internalisation was stopped on ice and cells labelled for CCR5 with MC-5 and PAG_{15}. Membrane sheets were subsequently prepared and after neutralisation of any remaining binding sites for PAG, the membranes were labelled for β-arrestin 1-YFP with anti-GFP antibody and PAG_{10} (B-D). In A, control membranes were only incubated with PAG_{10} after neutralisation. Arrows indicate some of the small gold particles (PAG_{10}) labelling β-arrestin 1, arrowhead indicate doublets of PAG_{10}/PAG_{15} labelling CCR5 and β-arrestin 1. Scale bars = 100 nm (A and D), 200 nm (B and C).

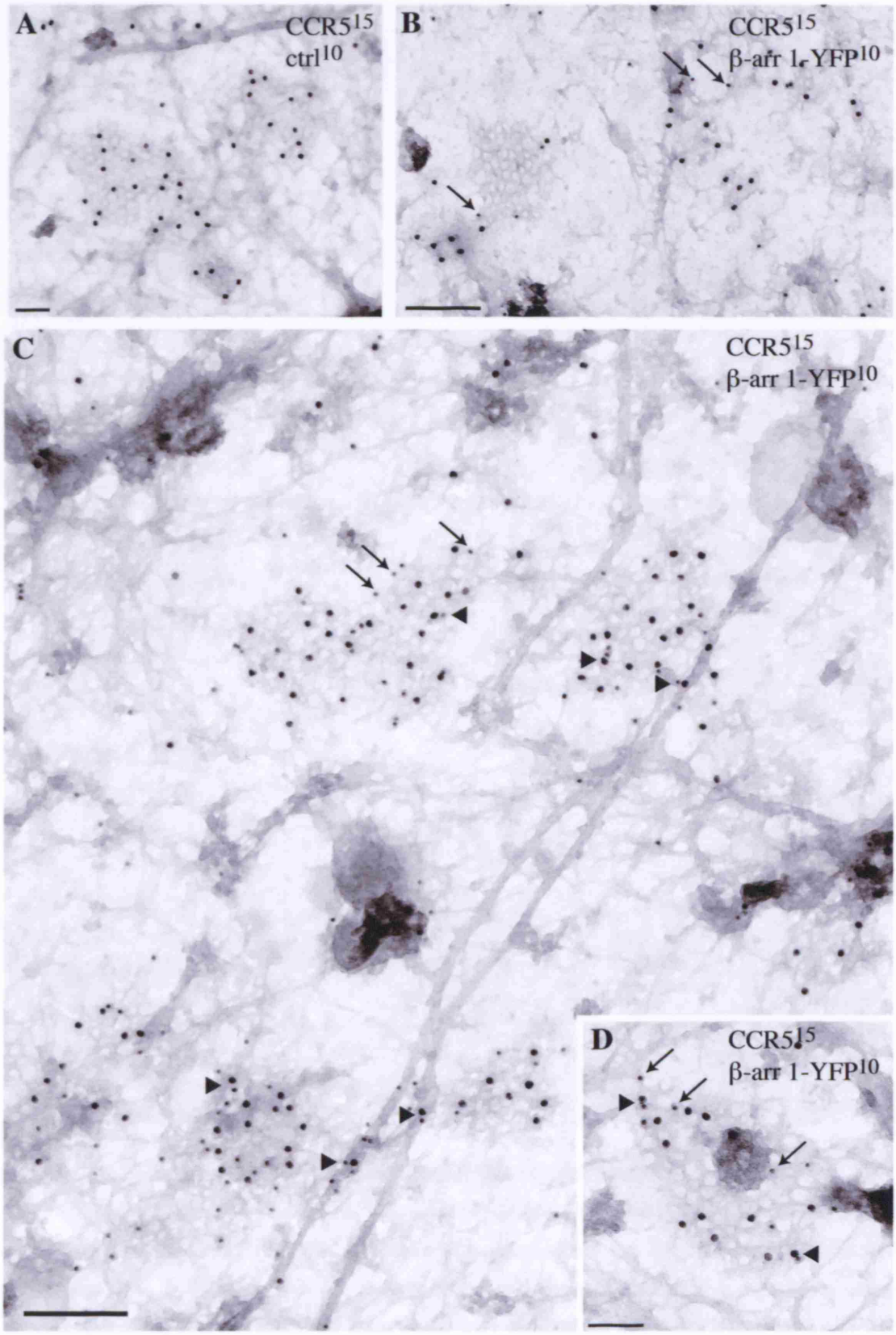


Figure 6.9: Membrane sheets of immunolabelled CHO CCR5 β-arrestin 1-YFP cells

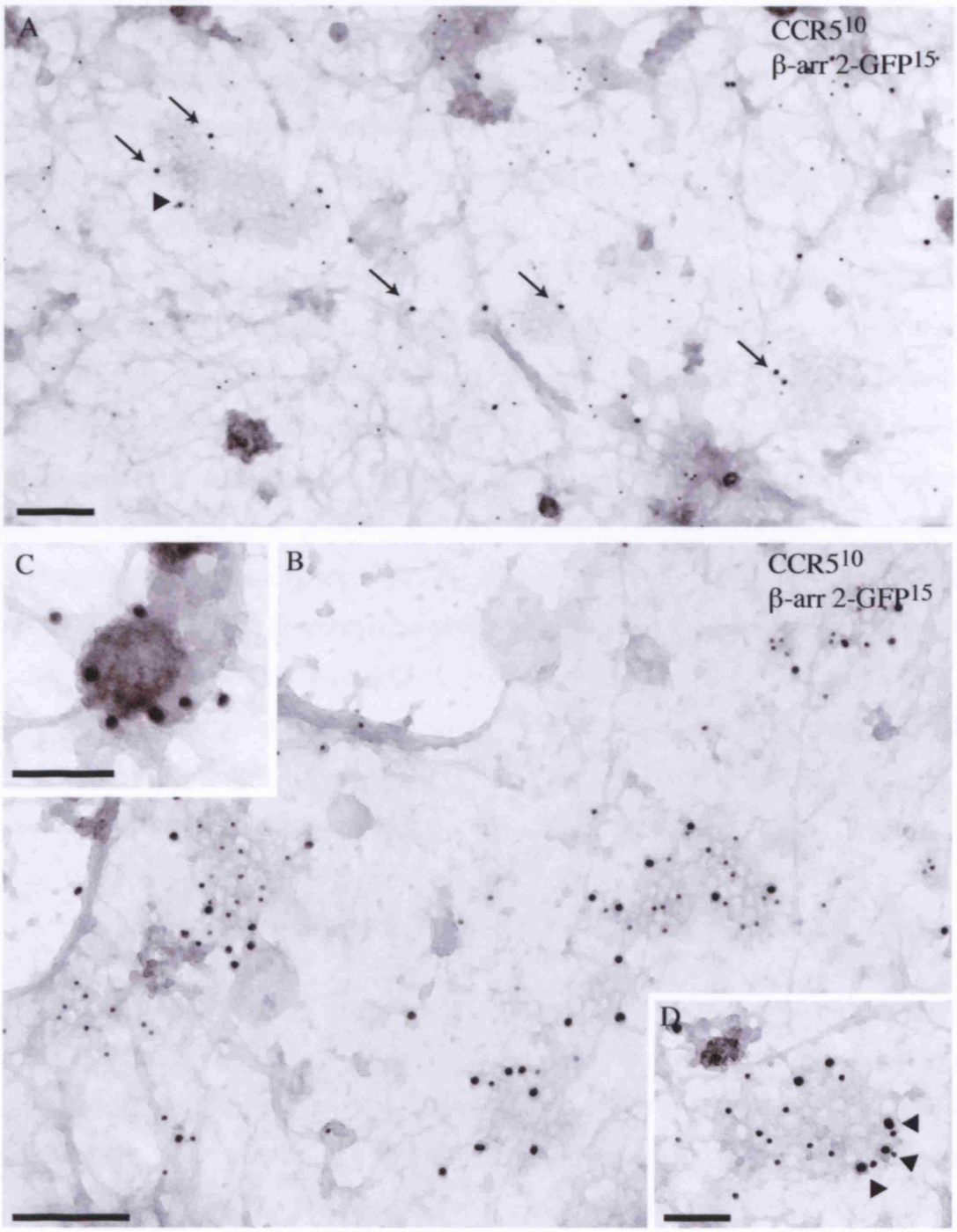


Figure 6.10: Membrane sheets of immunolabelled CHO CCR5 β-arrestin 2-GFP cells

CHO CCR5 β-arrestin 2-GFP cells were incubated in BM (A) or BM containing 125 nM CCL5 (B, C and D) for 2 minutes at 37°C. Internalisation was stopped on ice and cells labelled for CCR5 with MC-5 and PAG_{10}. Membrane sheets were subsequently prepared and after neutralisation of any remaining binding sites for PAG, the membranes were labelled for β-arrestin 2-GFP with anti-GFP antibody and PAG_{15}. In C, a budding clathrin-coated pit is labelled for β-arrestin 2. Arrows indicate large gold particles detecting β-arrestin 2 in A. Arrowheads indicate doublets of PAG_{10}/PAG_{15} labelling CCR5 and β-arrestin 2. Scale bars = 100 nm (C and D), 200 nm (A and B).

Altogether, this morphological analysis shows that both β-arrestins were specifically recruited to the plasma membrane upon agonist binding, and found with the activated receptor in flat clathrin lattices. In addition, no obvious difference in the distribution of the two β-arrestins was observed, suggesting a role for both proteins in CCR5 internalisation.

Whether β-arrestins induce recruitment of the receptor into flat clathrin lattices remains unknown. Further studies in cells knocked-out for both β-arrestins, where CCR5 endocytosis is impaired, would probably shed light on this mechanism (Fraile-Ramos et al., 2003). Indeed, if β-arrestins were required to link the receptor to the endocytic machinery, no clustering in flat clathrin lattices should be observed. On the other hand, if β-arrestins play a role once the receptor is relocated, endocytosis should be blocked in flat clathrin lattices. Due to low transfection efficiency of CCR5 in β-arrestin knock-out mouse embryonic fibroblasts, it was not possible to do ultra-structural analysis in these cells. Different approaches such as siRNA, or overexpression of dominant negative forms of β-arrestin, might be useful to test this hypothesis. In addition, live cell microscopy would also be useful to provide a spatial and temporal analysis of the process.

6.3 Localisation of β-arrestins in cells expressing CCR5 mutants

In the previous chapters, morphological and biochemical analysis showed that mutants of CCR5 lacking all phosphorylation sites failed to cluster into flat clathrin lattices and were internalised more slowly than the wild-type receptor. On the other hand, when all serines were mutated to alanines apart from S349, the receptor did relocate in flat clathrin lattices (Chapter 5) and exhibited a rapid internalisation similar to the wild-type receptor (Chapter 4). In order to investigate whether these differences in the localisation of the activated receptor and in the rate of internalisation corresponded to differential recruitment of β-arrestins, the distribution of endogenous β-arrestin 2 was monitored in RBL cells expressing WT CCR5, CCR5 ΔS or CCR5 S349only (figures 6.11-6.13).

In order to follow plasma membrane receptor, cells were pre-incubated with MC-5 on ice for one hour. They were subsequently incubated in BM (A) or BM containing 125 nM CCL5 for 5 and 15 minutes (B and C, respectively). After fixation, cells

were permeabilised, and labelled for β-arrestin-2 with a rabbit antibody, 182-4 (provided by Jeffrey L. Benovic, Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia). MC-5 and the 182-4 were detected with ^{488}GAM and ^{594}GAR. Projections of z series of single sections through selected cells are shown here. Note that CCL5-treated cells were much flatter than untreated cells and a whole cell could be scanned in less than 8 sections 0.5 μm apart. In contrast, untreated cells required sometimes twice as many sections. Images for only 13 sections were acquired. The tops of untreated cells are therefore missing in figures 6.11-6.13 A.

Figure 6.11 shows RBL cells expressing WT CCR5. In untreated cells, the β-arrestin 2 staining was mainly cytosolic, while CCR5 was localised at the plasma membrane. Upon agonist stimulation, β-arrestin 2 labelling became more punctate and showed some co-localisation with CCR5 in endocytic vesicles (arrows in B and C). Figure 6.12 shows RBL cells expressing CCR5 ΔS. Similarly to RBL WT CCR5, in untreated CCR5 ΔS cells β-arrestin 2 had a cytosolic distribution, while MC-5 labelling was associated with the plasma membrane. The large vesicle-like structures in the cell body are connected to the cell surface as they could be followed from the first sections close to the coverslip (A, right hand panel). Following agonist binding, the cells spread and lost their star-shape as previously shown in Chapter 5. β-arrestin 2 labelling did not change, and remained cytosolic. CCR5 was mainly localised at the plasma membrane, but some intracellular structures also appeared positive for the receptor (arrows in B and C). However, no co-localisation was observed between CCR5 ΔS and β-arrestin 2. This suggests the requirement for serine phosphorylation for β-arrestin 2 to interact with the receptor.

Figure 6.11: Immunofluorescence labelling of RBL cells for CCR5 and β-arrestin 2

RBL cells expressing CCR5 were pre-labelled on ice with MC-5, and subsequently incubated in BM (A) or BM with 125 nM CCL5 (B and C). Internalisation was stopped by cooling the cells to 4°C, and cells were fixed, permeabilised and labelled for β-arrestin 2 with the rabbit antibody 182-4. Primary antibodies were detected with ^{488}GAM and ^{594}GAR. Arrows indicate intracellular vesicles positive for the receptor and β-arrestin 2. Scale bars = 10 μm. Projections of z series of confocal sections through the cells are shown.

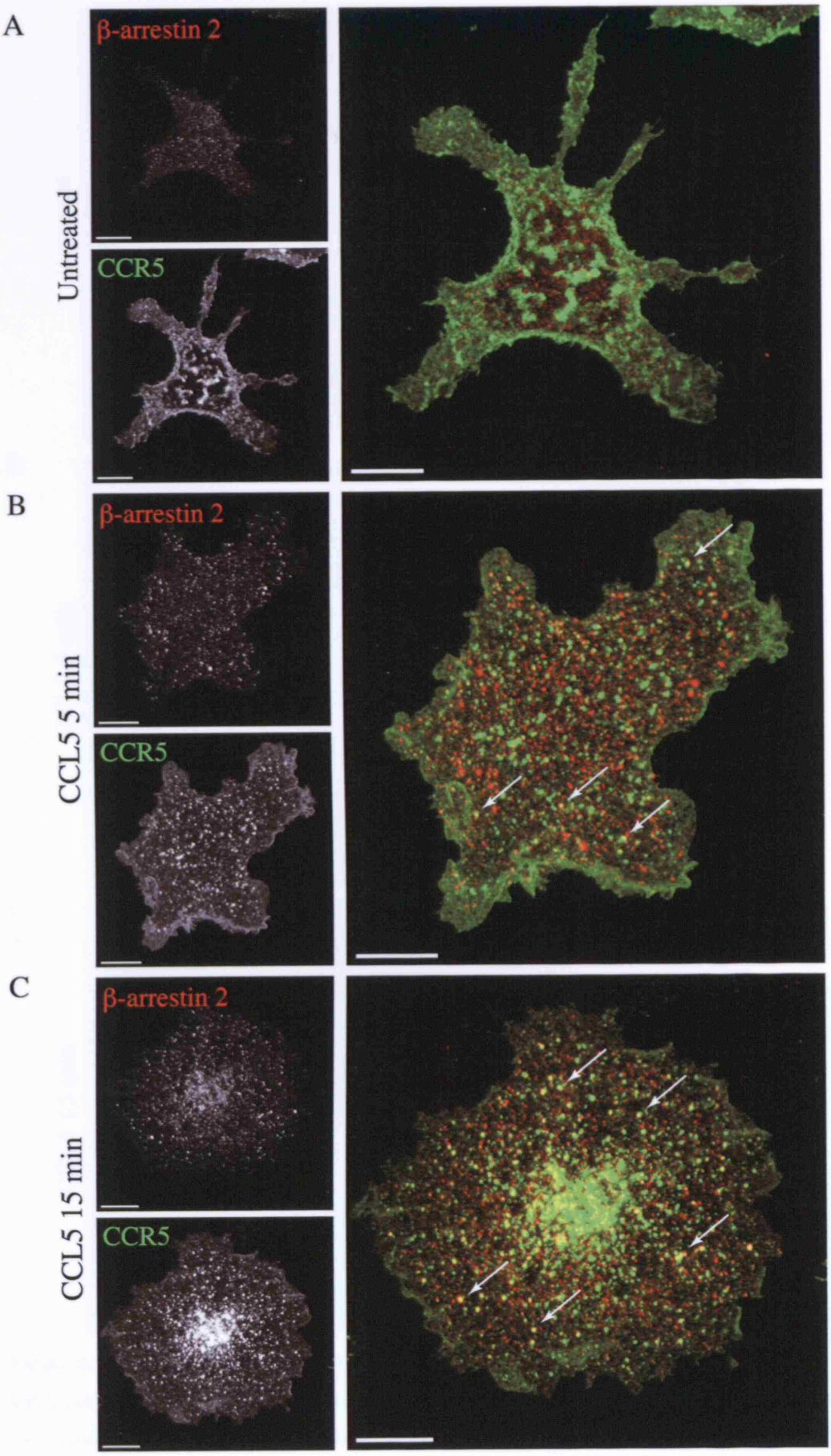


Figure 6.11: Immunofluorescence labelling of RBL cells for CCR5 and β-arrestin 2

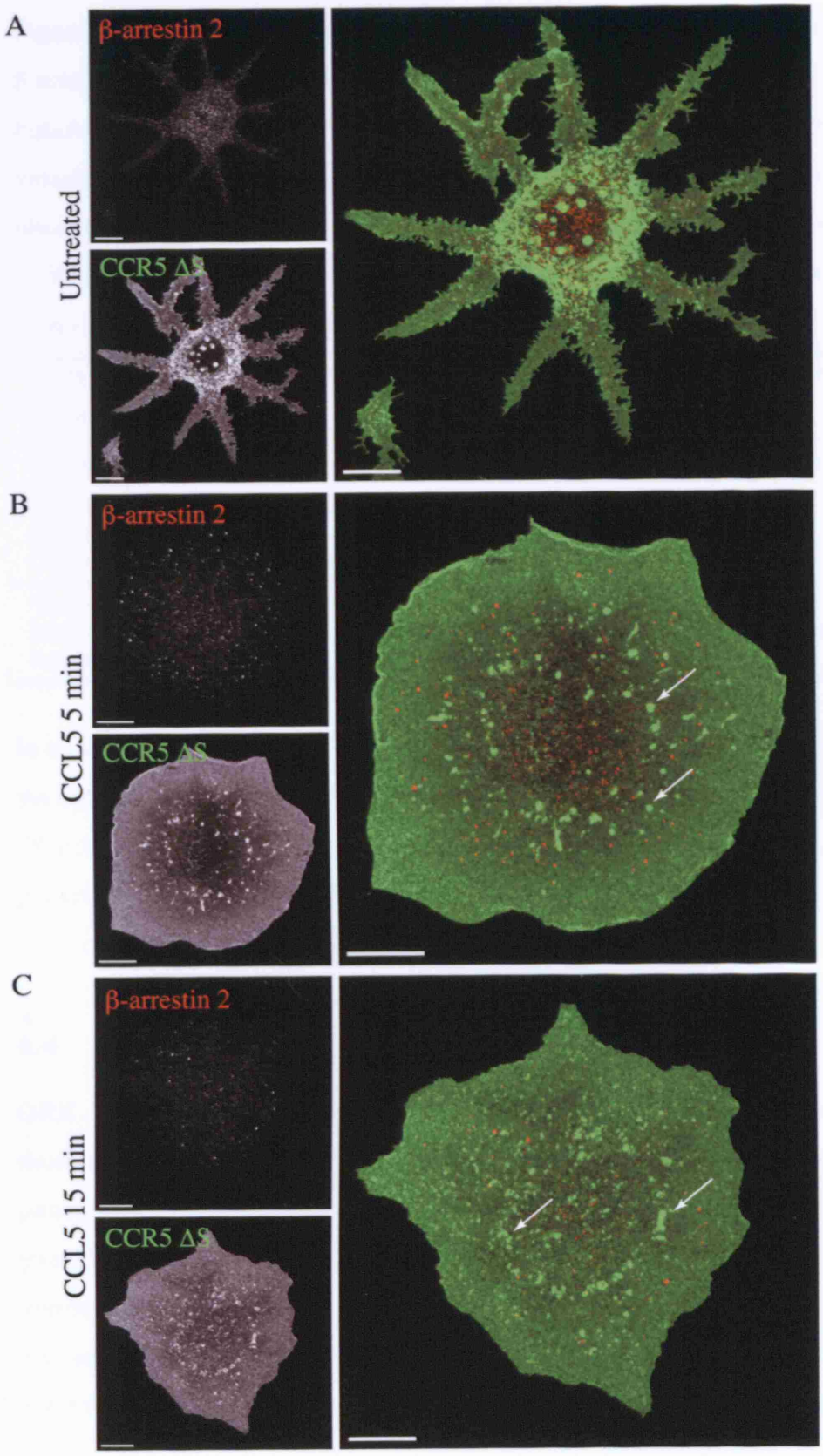


Figure 6.12: Immunofluorescence labelling of RBL cells for CCR5 ΔS and β-arrestin 2

RBL cells expressing CCR5 ΔS were treated as described in figure 6.11. Arrows indicates intracellular vesicles containing the receptor. Scale bars = 10 μm. Projections of z series of confocal sections through the cells are shown.

Figure 6.13 shows RBL cells expressing CCR5 S349only. Before agonist treatment, β-arrestin 2 was found in cytosol and CCR5 S349only on the plasma membrane (A). Following stimulation with CCL5, the receptor was internalised into small endocytic vesicles, where β-arrestin 2 was also recruited (arrows in B and C). Thus phosphorylation of serine 349 alone is sufficient for β-arrestin 2 recruitment to CCR5. Table 6.1 summarises the data from this immunofluorescence analysis and combines them with the results obtained in Chapters 3 to 5.

Table 6.2: Internalisation and clustering of receptors and their association with β-arrestin 2

Receptor	Internalisation[1]	Clusters[2]	β-arrestin 2 recruitment
CCR5 WT	Rapid	Yes	Yes
CCR5 ΔS	Slower	No	No
CCR5 S349only	Rapid	Yes	Yes

[1]Upon agonist binding CCR5 WT was internalised at 8.02 ± 1.19 %/min, CCR5 ΔS 4.93 ± 0.4 5/min and CCR5 S349only 6.91 ± 0.66 %/min. See Chapter 4 for more details. [2]See Chapters 3 and 5 for more details.

In conclusion, this immunofluorescence analysis shows that β-arrestin 2 is located in the cytosol of untreated cells, and upon agonist stimulation is recruited to activated CCR5. In addition, it indicates that the interaction relies mainly on the phosphorylation of C-terminal serines, in particular S349. Indeed β-arrestin 2 was not recruited to CCR5 ΔS, but did interact with CCR5 S349only.

6.4 Discussion

GRK phosphorylation of CCR5 C-terminal serine 349 has been investigated thoroughly in order to understand its importance for CCR5 internalisation. A phosphosite-specific antibody (M5/4) recognising specifically the receptor phosphorylated on S349 (CCR5 S349-P) proved to be extremely useful for immunofluorescence and electron microscopic analysis in RBL cells. Indeed, M5/4 was used in combination with MC-5, which binds to the N-terminus of CCR5, and allowed to determine whether the receptor was phosphorylated. Very little CCR5

Figure 6.13: Immunofluorescence labelling of RBL cells for CCR5 S349only and β-arrestin 2

RBL cells expressing CCR5 S349only were treated as described in figure 6.11. Arrows indicates intracellular vesicles positive for the receptor and β-arrestin 2. Scale bars = 10 μm. Projections of z series of confocal sections through the cells are shown.

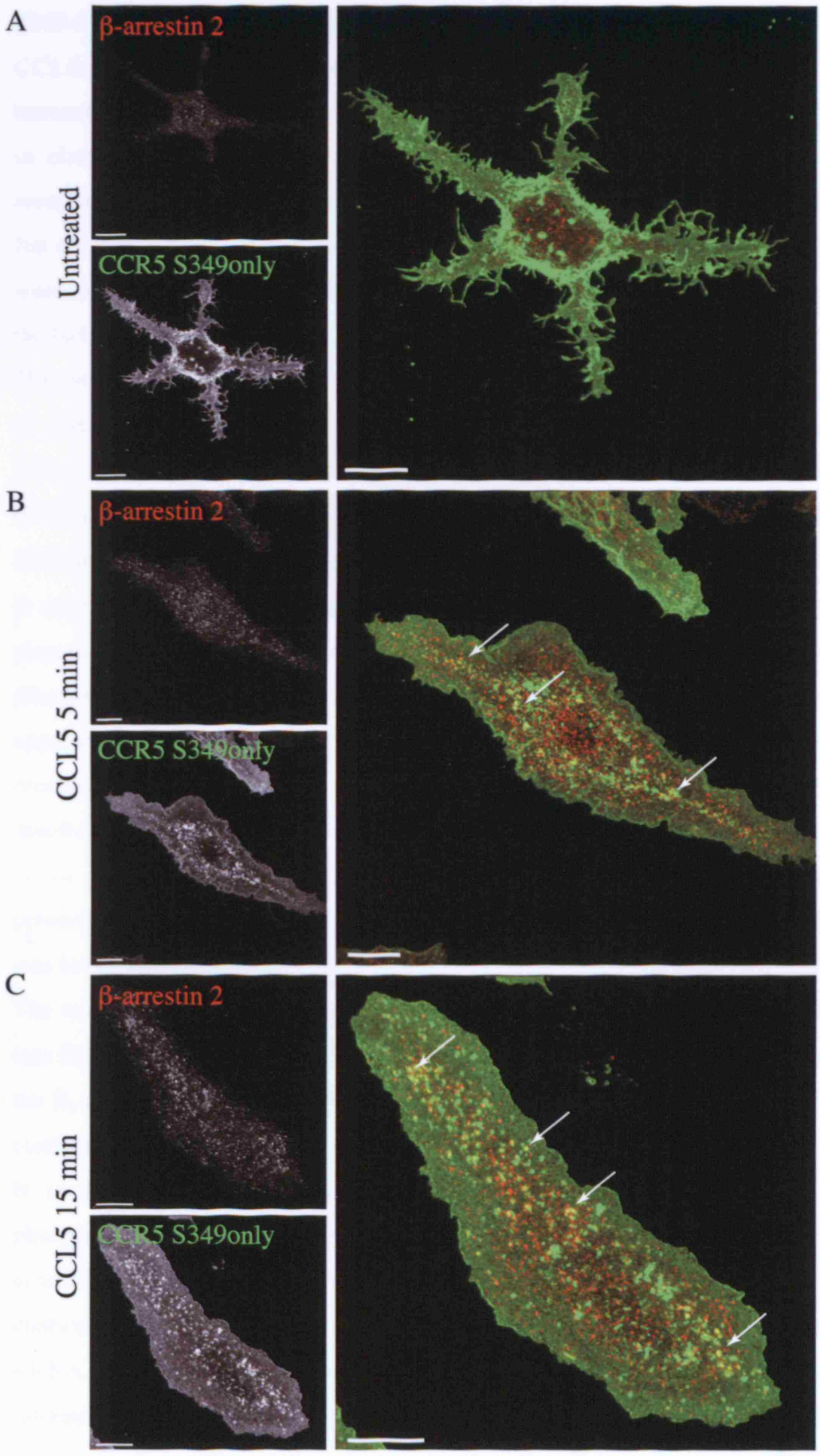


Figure 6.13: Immunofluorescence labelling of RBL cells for CCR5 S349only and β-arrestin 2

S349-P was detected in untreated cells. However, upon stimulation with the agonist CCL5, most receptors became phosphorylated and were detected in intracellularvesicles. Interestingly, very little phosphorylated receptor was detected in clathrin structures at the plasma membrane. Moreover, further analysis of membrane sheets revealed the absence of M5/4 labelling on receptors clustered in flat clathrin lattices. As β-arrestins interact with the cytoplasmic tail of CCR5 and were also detected in flat clathrin lattices, they might hide the M5/4 epitope, hence the lack of labelling.

The presence of β-arrestins with CCR5 in flat clathrin lattices led to further analysis of their recruitment to activated receptors deficient in phosphorylation. Interestingly, immunofluorescence analysis of endogenous β-arrestin 2 distribution in RBL cells expressing WT CCR5, CCR5 ΔS or CCR5 S349only revealed that S349 phosphorylation alone was sufficient for β-arrestin 2 binding to CCR5. This is in contrast to a study from Huttenrauch *et al.*, where β-arrestin translocation to the plasma membrane was shown to require at least two intact phosphorylation sites (Huttenrauch et al., 2002). This discrepancy is probably due the different approaches used. As CCR5 S349only has only one phosphorylation site, the receptor might still interact with β-arrestins but with a lower affinity. The membrane fractionation assay used by Huttenrauch *et al.* might lead to dissociation of the arrestins during fractionation. Indeed. in the immunofluorescence analysis presented in section 6.3 the co-localisation between β-arrestin 2 and CCR5 S349only was lower than with the wild-type receptor, suggesting an attenuated interaction.

The recruitment of β-arrestin 2 correlates with the capacity of receptors to relocate into flat clathrin lattices (as summarised in table 6.1). β-arrestins were shown to link the β_2-adrenergic receptor to the clathrin endocytic machinery by interacting with clathrin and AP-2 (Goodman et al., 1996; Laporte et al., 2000; Laporte et al., 1999). It is tempting to hypothesise that following agonist stimulation, GRK phosphorylation of S349 in the C-terminus of CCR5 triggers the recruitment of β-arrestins, which subsequently link the receptor to the endocytic machinery in flat clathrin lattices. To test this hypothesis approaches using β-arrestin knock-out cells, siRNA or dominant negative constructs of β-arrestins, are required.

Internalisation studies in Chapter 4 showed that CCR5 ΔS was endocytosed upon agonist binding, although at a slower rate than the wild-type receptor. The lack of

interaction between β-arrestins and the receptor suggests a β-arrestin-independent pathway of internalisation. Nevertheless, a C-terminal dileucine motif (L308 and L309), which can potentially interact with AP-2 and was shown to regulate CCR5 internalisation, might still link the receptor to the clathrin endocytic machinery in the absence of serine phosphorylation. Whether CCR5 ΔS uses the same clathrin pathway as the wild-type receptor remains to be determined. However, recent papers have suggested that receptors are targeted to different CCPs at the plasma membrane depending on their interaction with β-arrestins, and on the kinases that

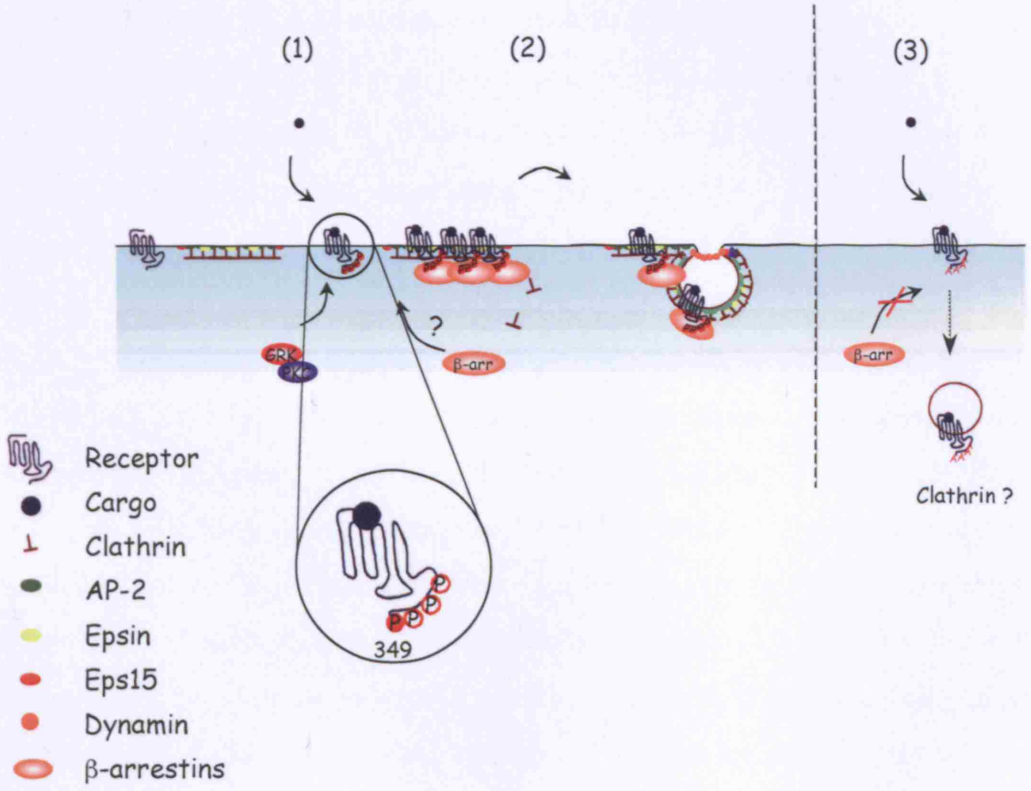


Figure 6.14: Summary

Upon agonist binding, CCR5 is phosphorylated by PKC and GRK on C-terminal serines (1). Following phosphorylation of S349, β-arrestin is recruited to the receptor. Whether β-arrestins induce clustering of CCR5 into flat clathrin lattices remains uncertain. Nevertheless, the two proteins are seen together in lattices and clathrin-coated pits are budding from these structures (2). CCR5 ΔS is not phosphorylated upon agonist binding, and does not cluster into flat clathrin lattices (3). Moreover, β-arrestins do not interact with the mutated receptor.

phosphorylate these receptors (Mundell et al., 2006; Puthenveedu and von Zastrow, 2006). Therefore if CCR5 ΔS is internalised via a clathrin-dependent pathway, the receptor may be targeted to CCPs different to those used by WT CCR5.

Figure 6.14 summarises the findings presented in Chapters 3 to 6, and illustrates the early events of CCR5 endocytosis at the plasma membrane following agonist binding.

7. Analysis of intracellular trafficking of CCR5 following agonist binding

As shown in the previous chapter, β-arrestins are recruited to plasma membrane CCR5 following agonist-induced phosphorylation of S349 by G-protein coupled receptor (GPCR) kinase (GRK). In addition, experiments in fibroblasts from β-arrestin knock-out mouse embryos have shown that the interaction of CCR5 with β-arrestins is essential for receptor internalisation following agonist binding (Fraile-Ramos et al., 2003). However, the phosphorylation-deficient receptor, CCR5 ΔS, which does not recruit β-arrestin at the plasma membrane is endocytosed upon agonist binding, although at a slower rate (see Chapter 4 section 4.1.3.1). Whether the intracellular trafficking of the wild-type receptor and CCR5 ΔS differs is unknown. Nevertheless, it is known that the intracellular trafficking of GPCRs differs depending on their association with β-arrestins. Indeed, receptors can be divided into two classes (Oakley et al., 2000). Class A receptors that have a lower affinity for β-arrestins, from which they dissociate rapidly once internalised, recycle back to the plasma membrane. By contrast, class B receptors that have a much higher affinity for β-arrestins, with which they show a sustained intracellular association, are usually degraded. However, this classification does not hold true in all cases, as CCR5 remains associated with β-arrestins following endocytosis, and recycles back to the plasma membrane (unpublished results from N. Signoret and T. Kershaw). Other studies have indicated an additional role for β-arrestins in recycling of the receptor N-formyl peptide receptor (FPR) (Vines et al., 2003). This suggests that β-arrestins regulate the intracellular trafficking of receptors in addition to their role in desensitisation and down-modulation through clathrin-mediated endocytosis (CME).

The aim of the work presented in this chapter was to investigate the intracellular trafficking of CCR5 following agonist binding and internalisation. Firstly, the distributions of the wild-type receptor and CCR5 ΔS upon stimulation with CCL5 were compared. Then, the phosphorylation state of CCR5 following sustained agonist treatment was analysed, and an ultra-structural electron microscopy study of the intracellular localisation of the receptor was carried out by labelling ultra-thin

cryosections of Chinese hamster ovary (CHO) cells. Finally, as β-arrestin 1 was shown to interact with the adaptor protein-1 (AP-1) complex (Nature Signalling Gateway yeast two-hybrid screen and personal communication from J. Pitcher) and was found with CCR5 in early and recycling endosomes (Nathalie Signoret and Tom Kershaw, unpublished results), the presence of AP-1 in CCR5-positive compartment was investigated.

7.1 β-arrestin 2-dependent intracellular trafficking of CCR5 in RBL cells

7.1.1 Intracellular localisation of WT CCR5 and CCR5 ΔS

To compare the intracellular localisation of wild-type CCR5 and CCR5 ΔS, RBL cells expressing either receptor were analysed by immunofluorescence after stimulation with 125 mM CCL5. In order to follow receptors originally at the plasma membrane, intact cells were pre-labelled with MC-5 for one hour on ice, prior to treatment with the agonist for 30 minutes at 37°C. Cells were subsequently fixed, permeabilised and labelled for β-arrestin 2 with the rabbit antibody 182-4 (provided by Jeffrey L. Benovic, Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia). Primary antibodies were detected with species-specific secondary antibodies, ^{488}GAM and ^{594}GAR. Figure 7.1 shows projections of a z series of 0.5 μm confocal sections through the cells.

In untreated cells, both receptors were localised at the plasma membrane and β-arrestin 2 staining was cytoplasmic (as shown in figure 6.11 and 6.12). Upon 30 minutes of agonist treatment the wild-type receptor accumulated intracellularly (A). β-arrestin 2 partially colocalised with the receptor in vesicular structures (B). Arrows indicate intracellular structures positive for both proteins. In contrast, CCR5 ΔS stimulation led to an intracellular localisation of the receptor in small structures scattered throughout the cells (D). In addition, as shown previously in Chapter 6, β-arrestin 2 was not recruited to activated CCR5 ΔS (E).

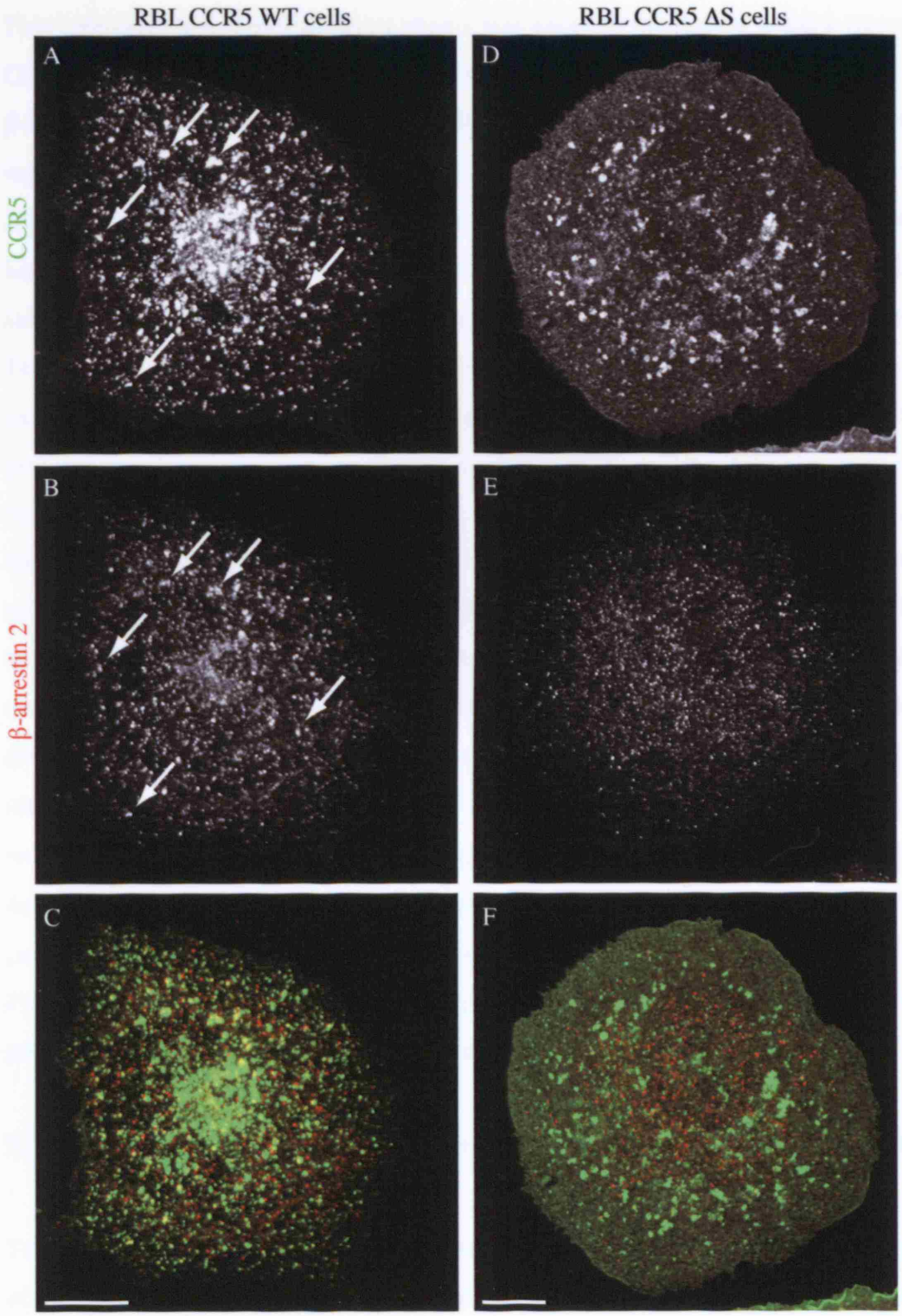


Figure 7.1: Immunofluorescence localisation of β-arrestin 2 and WT CCR5 or CCR5 ΔS

RBL cells expressing WT CCR5 (A-C) or CCR5 ΔS (D-F) were pre-labelled for cell surface receptors for an hour at 4°C with MC-5, and incubated in BM or BM containing 125nM CCL5. Internalisation was stopped on ice and cells fixed with 3% PFA. Following permeabilisation, cells were labelled with an antibody recognising β-arrestin 2. Primary antibodies against CCR5 (A and D) and β-arrestin (B and E) were detected with ^{488}GAM (green) and ^{594}GAR (red), respectively. Arrows indicate structures labelled for CCR5 and β-arrestin. Scale bar = 10 μm.

This immunofluorescence analysis shows that upon 30 minutes of agonist treatment, CCR5 accumulates in an intracellular compartment, where some co-localisation with β-arrestin 2 is observed. In CHO cells, the receptor was shown to be targeted to the recycling endosome (Mack et al., 1998; Signoret et al., 2000; Signoret et al., 1998). Although only β-arrestin 2 distribution is shown here, in CHO cells both β-arrestins have been reported in the recycling endosome together with the transferrin receptor and CCR5 by immunofluorescence (unpublished work from Nathalie Signoret and Tom Kershaw). The colocalisation between CCR5 and β-arrestins was more extensive in CHO than in RBL cells. This may be due to the over-expression of the proteins in CHO cells or may reflect the poor quality of the antibody staining for endogenous arrestins in RBL cells. Nevertheless, this suggests that CCR5 internalisation involves both, β-arrestin 1 and 2. However, evidence from work in mouse embryonic fibroblasts (MEF), obtained from mice knocked-out for either or both isoforms of β-arrestins, indicated that CCR5 endocytosis can occur normally in the absence of one β-arrestin, but is blocked when both β-arrestins are missing (Fraile-Ramos et al., 2003 and unpublished data). In addition, the prolonged intracellular association of CCR5 with β-arrestins suggests a potential role in the receptor trafficking. Indeed, the phosphorylation-deficient mutant CCR5 ΔS, which does not recruit β-arrestins upon agonist stimulation, does not accumulate in the perinuclear region, but is localised in vesicles scattered throughout the cytoplasm. Further immunofluorescence labelling with various markers of the endocytic pathway has to be completed in order to identify the nature of these structures.

7.1.2 Intracellular immunofluorescence labelling of agonist-induced phosphorylated CCR5

To determine whether intracellular CCR5 was still phosphorylated after 30 minutes of agonist stimulation, cells were labelled with a phosphosite-specific antibody against CCR5-S349-P, E11/19 (provided by M. Oppermann, Department of Immunology, Georg August University, Goettingen, Germany). In addition, cells were pre-labelled with MC-5 prior to CCL5 treatment to follow receptors from the plasma membrane into the cells. Figure 7.2 shows cells pre-labelled with MC-5 (A and C), incubated for 30 minutes in BM (A and B) or BM containing 125 nM CCL5 (C and D), fixed, permeabilised and labelled with E11/19 recognising CCR5-S349-P.

In untreated cells, the receptor was located at the plasma membrane and only background fluorescence was detected with E11/19. However, in agonist treated cells the internalised receptor was phosphorylated. The staining with E11/19 was almost indistinguishable from MC-5 staining, suggesting that all receptors initially on the cell surface had been activated by the agonist, subsequently phosphorylated by GRK, and had remained phosphorylated on S349 during trafficking to intracellular compartments.

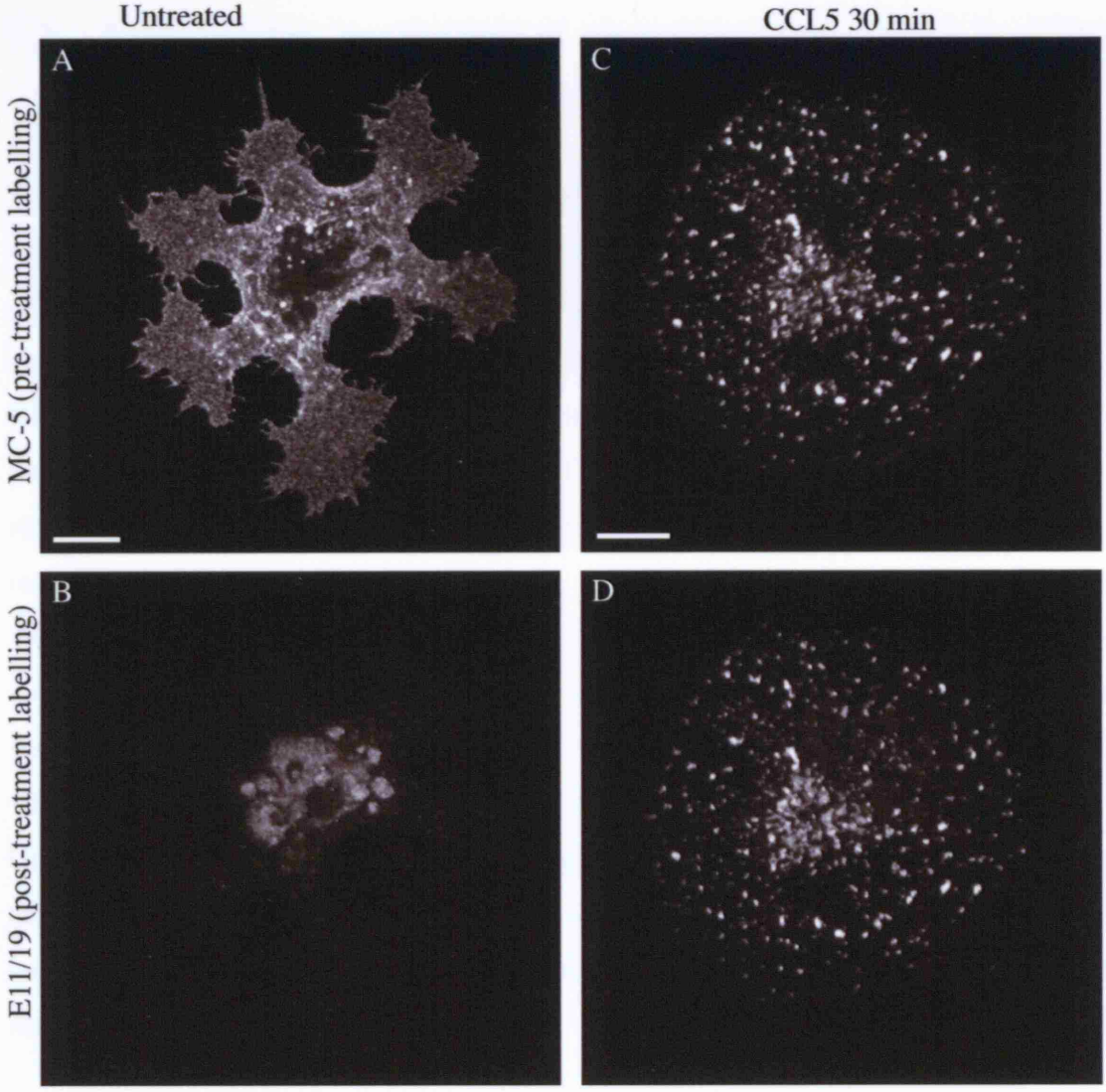


Figure 7.2: Immunofluorescence labelling of phosphorylated CCR5.

RBL CCR5 cells were incubated for 1 hour on ice with MC-5 prior to 30 minutes treatment with BM or BM containing 125 nM CCL5. Cells were subsequently fixed, permeabilised and labelled for phosphorylated CCR5, with E11/19, recognising CCR5-S349-P. MC-5 and E11/19 were detected with mouse isotype specific secondary antibody ^{488}GAM-IgG2a and ^{594}GAM-IgG1, respectively. Samples were analysed by confocal microscopy, projections of z series through the cells are shown. Scale bar = 10 μm.

In conclusion, this immunofluorescence analysis shows that prior to agonist treatment, cell surface CCR5 is not phosphorylated on S349. Moreover, agonist-induced internalised CCR5 is still phosphorylated on S349 30 minutes after initial stimulation. In the previous chapter, S349 phosphorylation was shown to be sufficient for interaction between CCR5 and β-arrestins, and this may explain the continuous association of β-arrestin 2 with CCR5 observed in figure 7.1.

7.2 Intracellular localisation of phosphorylated CCR5 in CHO cells – analysis of immunogold-labelled ultra-thin cryosections

To obtain more details on the compartment in which CCR5 accumulates upon agonist binding, immunolabelling of ultra-thin cryosections was carried out on CHO cells expressing CCR5. These cells were chosen over the RBL cells, as they do not have an intracellular pool of CCR5. Figures 7.3-7.5 show ultra-thin cryosections of CHO CCR5 cells stimulated for 60 minutes with 125nM CCL5 and labelled with phosphosite-specific antibodies against CCR5-S349-P or CCR5-S337-P, or for clathrin. Details on the preparation and labelling of ultra-thin cryosections are given in Materials and Methods section 2.5.5.

Figure 7.3 illustrates the intracellular compartments in which CCR5-S349-P was localised after one hour of CCL5 stimulation. In A and B, labelling for the receptor was observed in a perinuclear network of membrane tubules and vesicles, some of which were coated with clathrin (CCV) (easily identified by the 20-25 nm coat). CCR5-S349-P was also detected in larger intracellular structures, containing a thicker coat of 30-35 nm (arrowheads in C-E). In addition, in some cases, gold particles were observed in clathrin-coated pits (CCPs) budding from the plasma membrane (F).

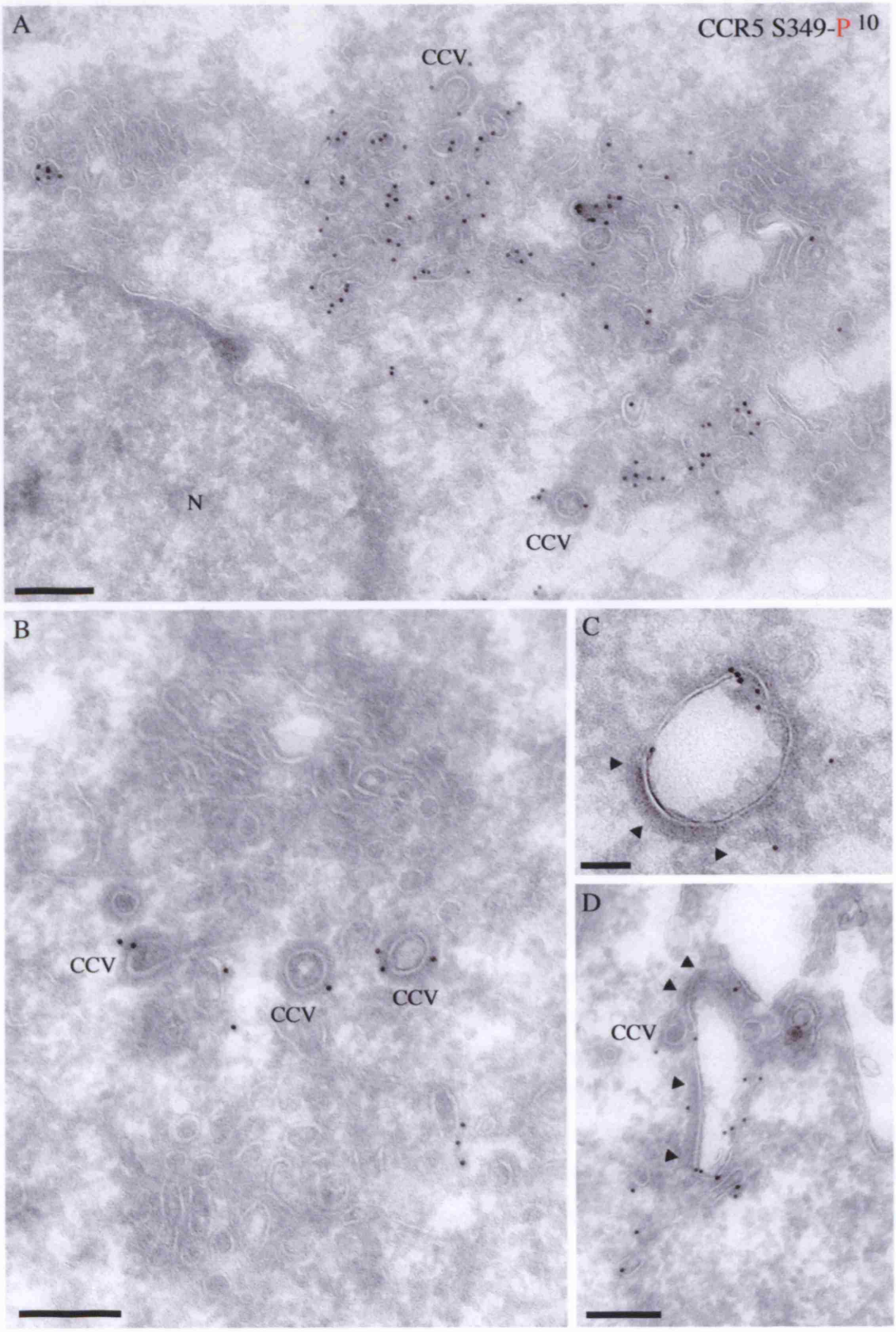


Figure 7.3: Immunogold-labelled ultra-thin cryosections for CCR5-S349-P

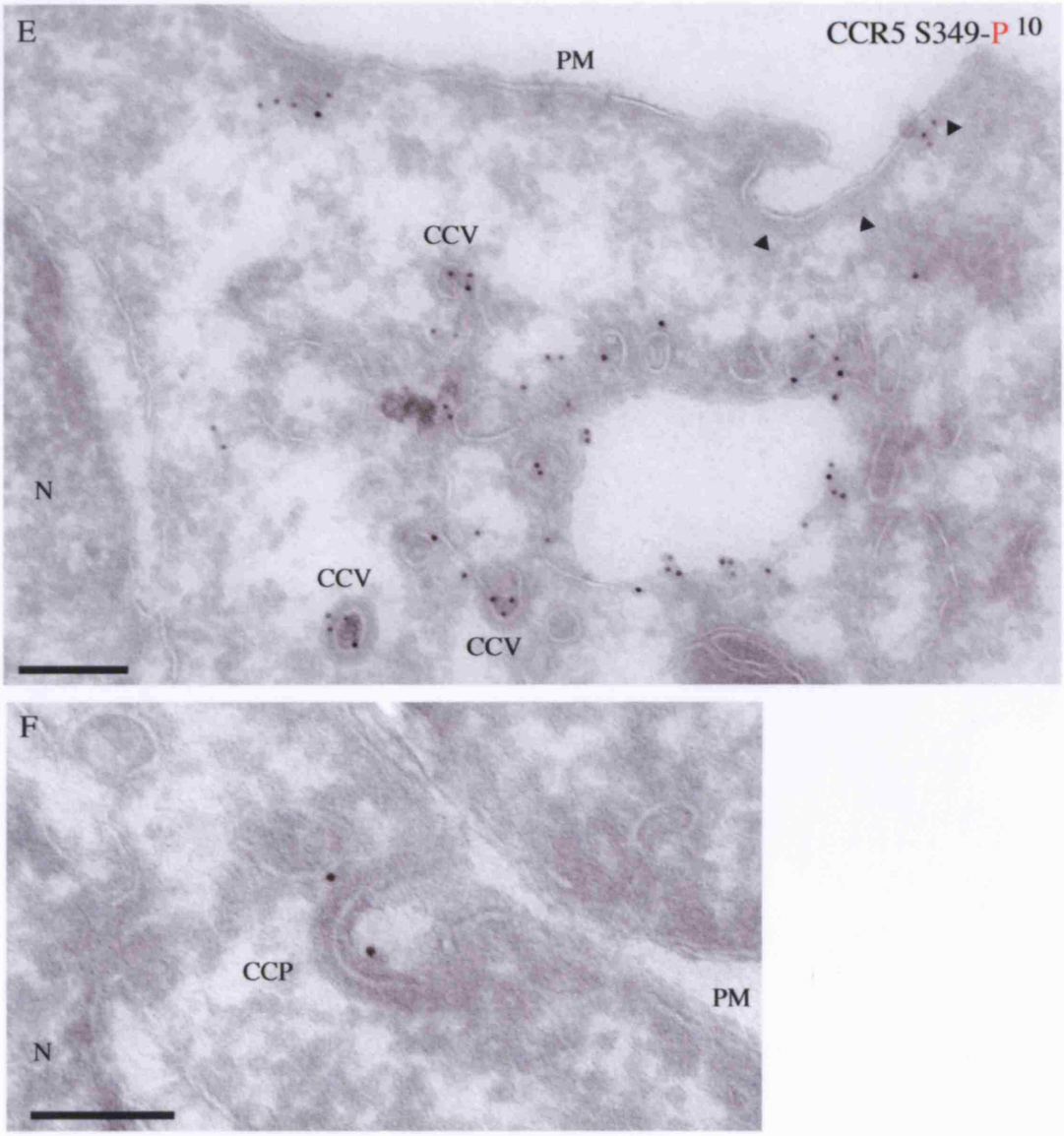


Figure 7.3: Immunogold-labelled ultra-thin cryosections for CCR5-S349-P

CHO CCR5 cells were incubated for one hour in BM containing 125 nM CCL5. Cells were subsequently fixed in 4% PFA and prepared for ultra-thin cryosectioning as described in Materials and Methods section 2.5.5. Cryosections were labelled with E11/19 followed by a bridging antibody rabbit-anti-mouse and detected with PAG_{10}. (Arrowhead) 30-35 nm thick coat on intracellular compartment and on the plasma membrane, (N) Nucleus, (CCP) clathrin-coated pit, (CCV) clathrin-coated vesicle (or bud), (PM) plasma membrane. Scale bars = 200 nm (A, B and D-F) and 100 nm (C).

To investigate whether agonist-internalised CCR5 was also phosphorylated on S337, cryosections were labelled with V14/2 a phosphosite-specific antibody against CCR5-S337-P. Figure 7.4 shows intracellular structures, similar to the ones in figure 7.3, labelled for CCR5-S337-P.

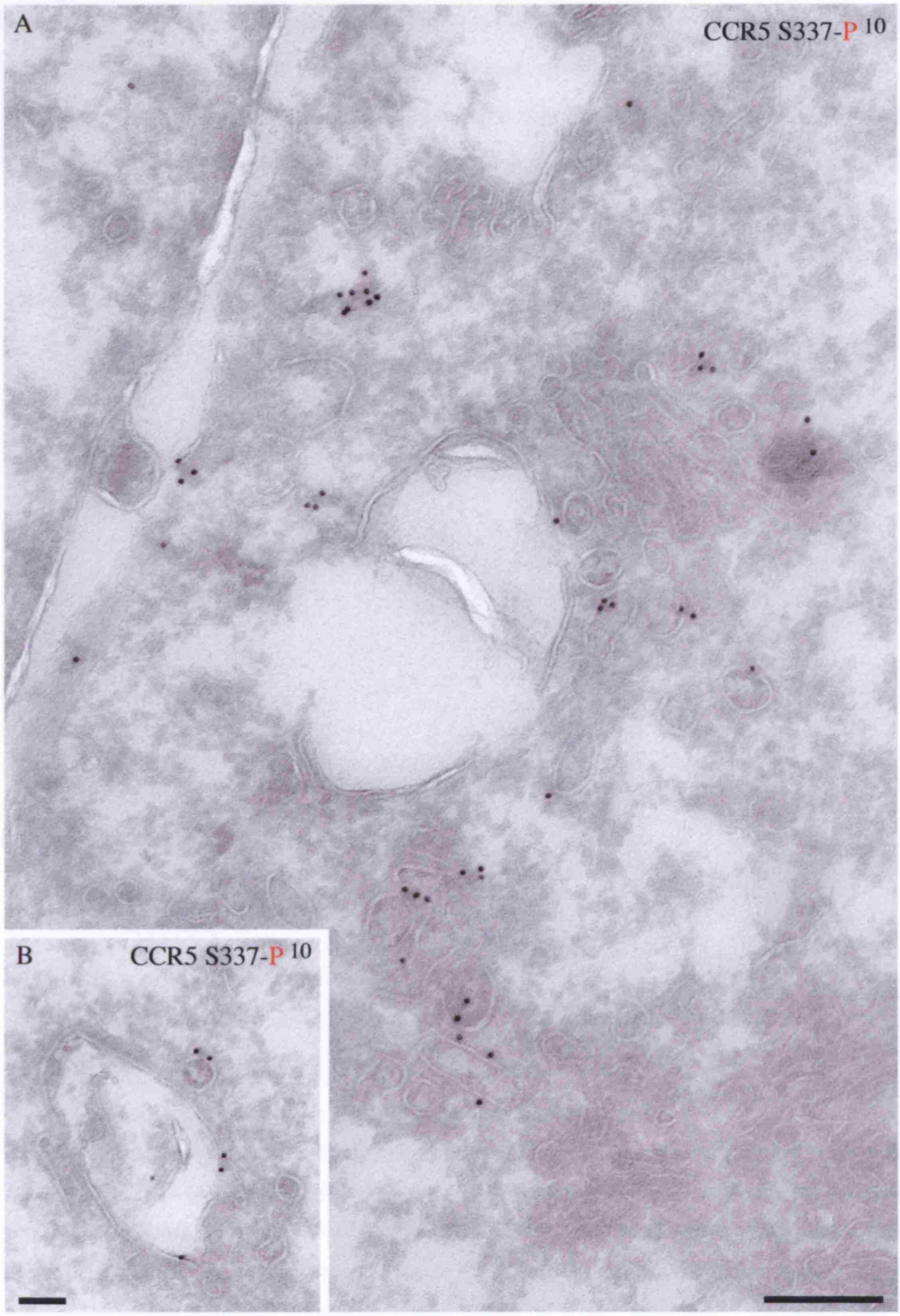


Figure 7.4: Immunogold-labelled ultra-thin cryosections for CCR5-S337-P

CHO CCR5 were incubated for one hour in BM containing 125 nM CCL5. Cells were subsequently fixed in 4% PFA and were prepared for ultra-thin cryosectioning as described in Materials and Methods section 2.5.5. Cryosections were labelled with V14/2 followed by a bridging antibody rabbit-anti-mouse and detected with PAG_{10}. Scale bars = 200 nm (A) and 100 nm (B).

Finally, to identify the nature of the coat on the intracellular structures where CCR5 accumulates upon sustained agonist-treatment, cryosections were labelled with an antibody against clathrin. Figure 7.5 presents three intracellular structures partially coated, similar to the ones shown in figures 7.3 and 7.4. Gold particles detecting clathrin were found on coated vesicles (A), and flat coats were observed on larger intracellular compartments (B and C). Interestingly, flat coats were slightly thicker (30-35 nm) than coats on pits and vesicles (20-24 nm).

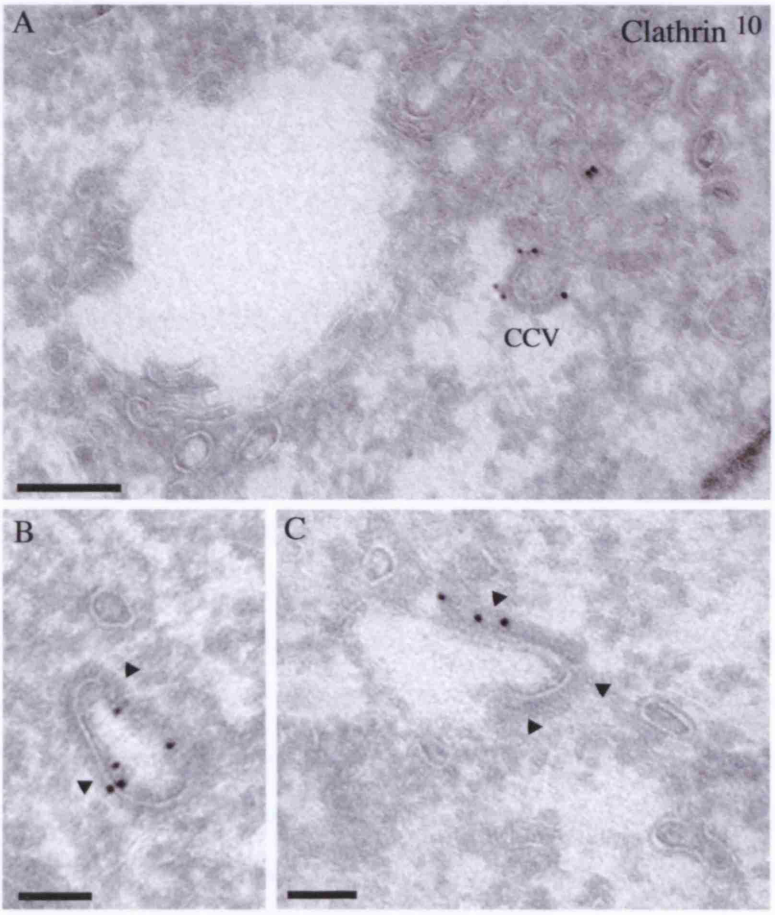


Figure 7.5: Immunogold-labelled ultra-thin cryosections for clathrin

CHO CCR5 cells were incubated for one hour in BM containing 125 nM CCL5. Cells were subsequently fixed in 4% PFA and prepared for ultra-thin cryosectioning as described in Materials and Methods section 2.5.5. Cryosections were labelled with an antibody against clathrin followed by a bridging antibody rabbit-anti-mouse detected with PAG_{10}. (Arrowhead) flat clathrin coat on intracellular compartment, (CCV) clathrin-coated vesicle (or buds). Scale bars = 200 nm (A) and 100 nm (B and C).

Altogether, this ultrastructural analysis shows that after one hour of CCL5 stimulation, CCR5 accumulates in a perinuclear compartment and can also be detected in clathrin-coated vesicles or buds. In addition, labelling with phosphosite-

specific antibodies demonstrated that internalised receptors are phosphorylated on S349 and S337. The intracellular structures in which CCR5 accumulates are partially coated. Although the receptor and the coat have not been labelled in the same sections yet, it is likely to be a clathrin coat. These structures also resemble the recycling endosomes in which the transferrin receptor accumulates and where CCR5 has previously been observed (Signoret et al., 2000). As intracellular CCR5 and β-arrestins co-localised in immunofluorescence analysis (figure 7.1 and unpublished data from N. Signoret and T. Kershaw), it would be interesting to confirm these findings by immunogold-labelling of cryosections for both proteins. In addition, it would be interesting to investigate the nature of the compartment in which CCR5 ΔS is found upon stimulation and compare it with the present results.

7.3 Immunogold-labelling of ultra-thin cryosections for CCR5 and AP-1

The Alliance for Cellular Signalling, which has undertaken a high-throughput yeast two-hybrid screen, recently reported an interaction between β-arrestin 1 and the β1 subunit of AP-1. This interaction has been confirmed in COS cells over-expressing β-arrestin 1 by immunoprecipitation (personal communication from Julie Pitcher). Interestingly, AP-1 only interacts with β-arrestin 1, whereas AP-2 was shown to interact with β-arrestins 1 and 2. AP-1 is recruited to intracellular membranes through binding to phosphatidylinositol 4 phosphate (PtdIns4P) (Wang et al., 2003b) and is therefore found associated with endosomal membranes and with the *trans*-Golgi network (TGN). Like AP-2, AP-1 interacts with clathrin and can be detected in CCV budding from endosomes and/or the TGN. The adaptor was proposed to mediate trafficking between endosomes and the TGN (Meyer et al., 2000).

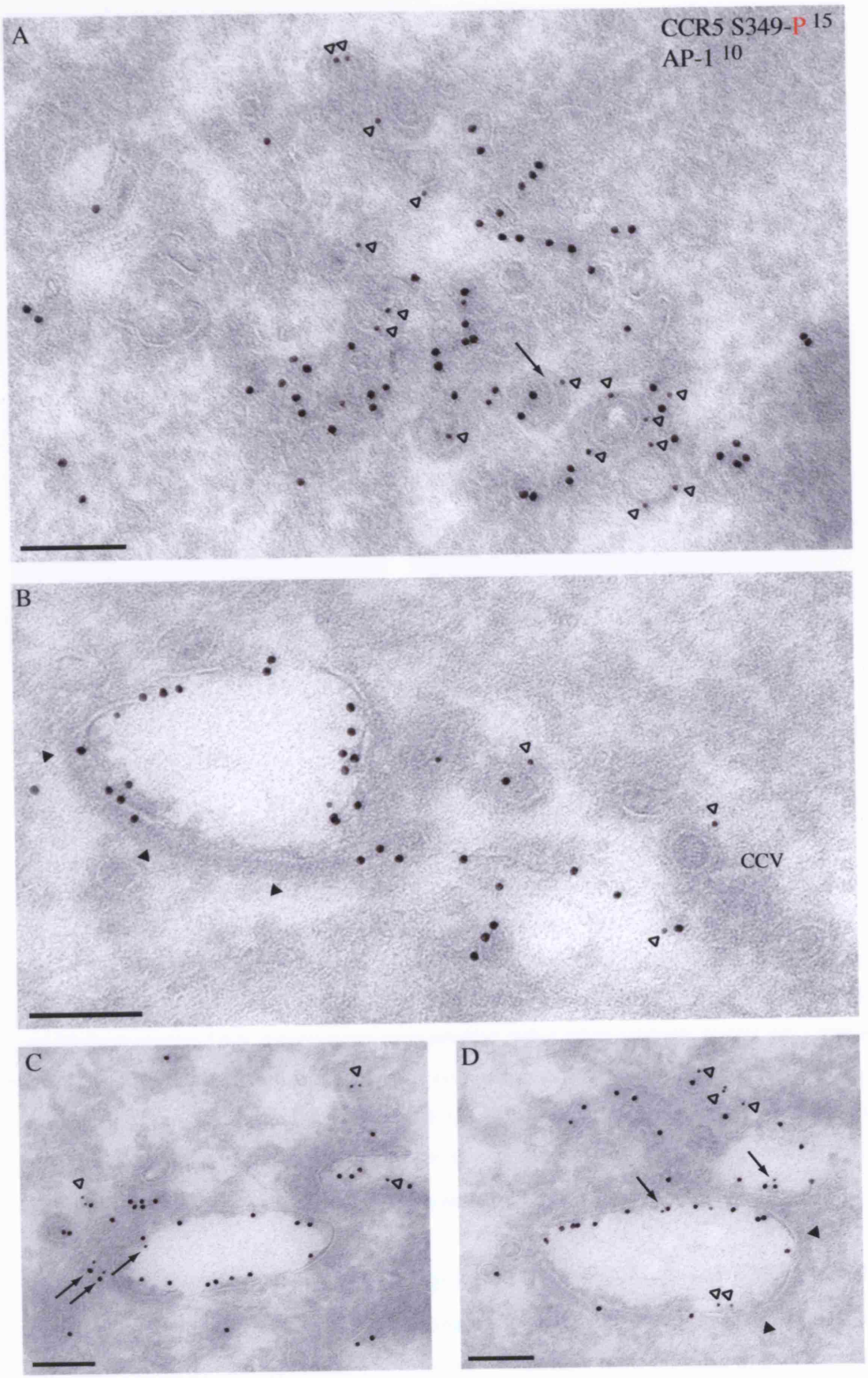
To investigate whether agonist-internalised CCR5, which is associated with both β-arrestins intracellularly, is found in structures containing AP-1, ultra-thin cryosections were labelled for CCR5 and AP-1. Figure 7.6 shows cryosections from CHO CCR5 cells stimulated for an hour with CCL5. Cells were labelled with E11/19 (anti-CCR5-S349-P) followed by PAG_{15} to detect desensitised receptors and with a rabbit antibody against AP-1 (provided by M. Robinson, Cambridge) detected with PAG_{10}. Both proteins were detected in CCV or clathrin-coated buds (A-C, F

and G) and larger structures partially coated with a 30 nm-coat (filled arrowhead in B, D, E and F). Gold particles detecting AP-1 were observed associated with these endosomal clathrin coats (white arrowheads in E and F) and with the coat of CCVs (white arrowheads in A, B, F and G). This indicated that the CCVs had budded off endosomal structures rather than the plasma membrane, where AP-2 rather than AP-1 would have been incorporated into the clathrin coat. Interestingly, gold particles labelling for CCR5-S349-P and AP-1 were often observed simultaneously in coated endosomes and vesicles (arrows). These AP-1 positive CCVs may be mediating recycling of the receptor from the recycling endosome back to the plasma membrane or further intracellular trafficking, to the TGN for example.

In summary, this ultra-structural analysis shows that the clathrin-coated vesicles or buds and endosomes, where CCR5 accumulates after one hour of agonist-stimulation, contain AP-1. As AP-1 is thought to be incorporated only in CCV budding from intracellular membranes and AP-2 in endocytic CCVs budding from the plasma membrane, the structures in which CCR5-S349-P was observed may control intracellular trafficking of CCR5. This suggests that the receptor can leave early or recycling endosomes still phosphorylated. This is in contrast to the β_2-adrenergic receptor (β_2AR), which is believed to be dephosphorylated in the early endosomes (Pitcher et al., 1995). Although labelling for β-arrestins has not been done yet, it will be interesting to see whether the receptor is associated with either or both isoforms of β-arrestins in these intracellular structures. It is very likely that β-arrestin 1 will be found, as it interacts with AP-1 and phosphorylated CCR5 (unpublished results from Tom Kershaw).

7.4 Discussion

Immunofluorescence analyses have shown that upon agonist treatment, wild-type CCR5 associates with both β-arrestins (this thesis and unpublished results from our laboratory) and that the receptor/β-arrestin complex is internalised and accumulates in a perinuclear compartment (Signoret et al., 2000). Previous reports have identified this compartment as the recycling endosomes by the presence of transferrin receptor (Mack et al., 1998; Signoret et al., 2000). Staining with


A
CCR5 S349-P 15
AP-1 10
B
CCV
C
D

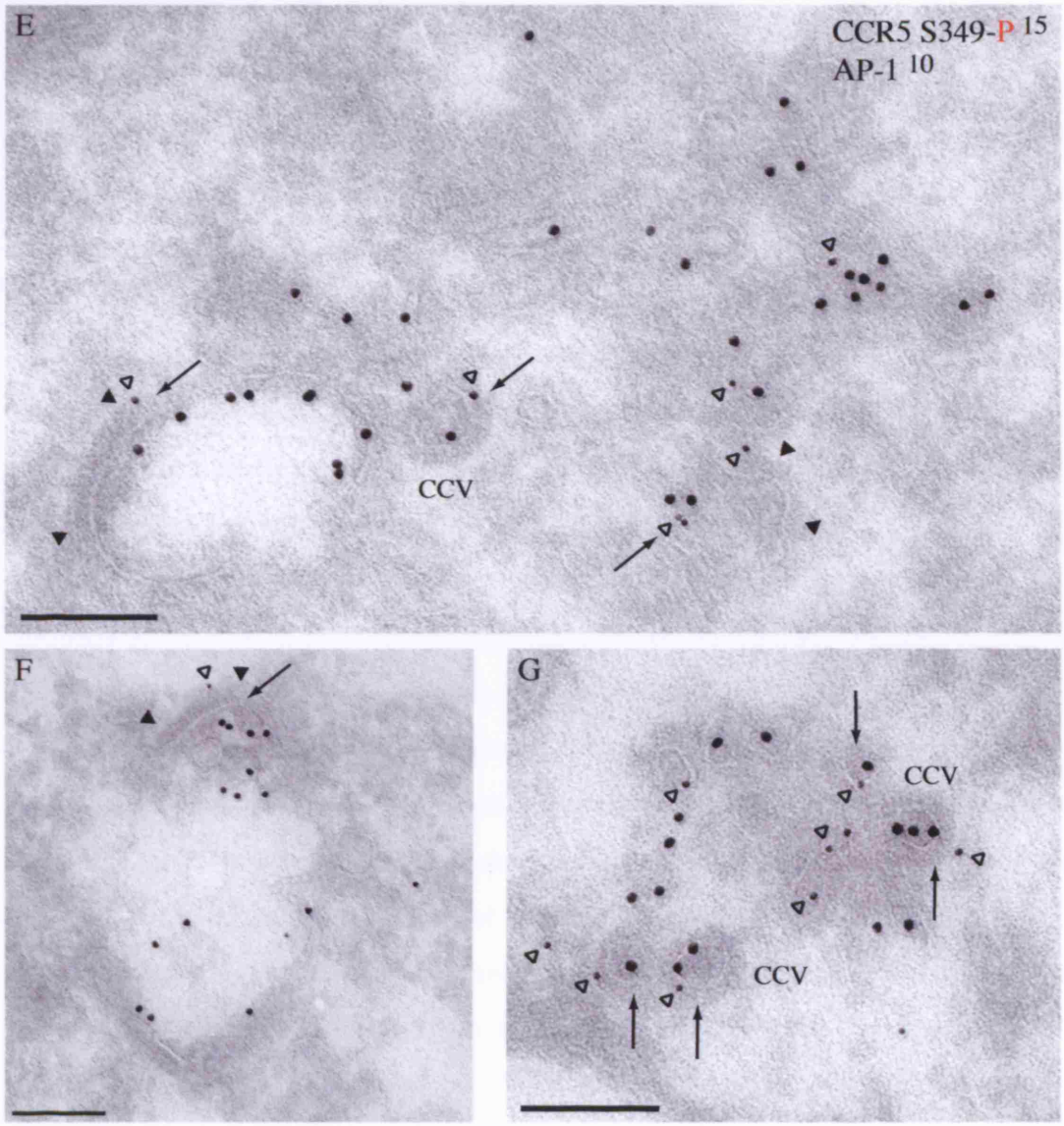


Figure 7.6: Immunogold-labelled ultra-thin cryosections for CCR5 and AP-1

CHO CCR5 cells were incubated for one hour in BM containing 125 nM CCL5. Cells were subsequently fixed in 4% PFA and prepared for ultra-thin cryosectioning as described in Materials and Methods section 2.5.5. Cryosections were labelled with a rabbit antibody against AP-1 detected with PAG_{10} and with E11/19 detected with a bringing antibody rabbit anti-mouse and PAG_{15}, as described in Materials and Methods section 2.5.5. (Filled arrowheads) endosomal clathrin coat, (CCV) clathrin-coated vesicles (or buds), (white arrowheads) AP-1 in clathrin coat, (arrows) PAG_{15} and PAG_{10} together in the same structure. Scale bars = 200 nm.

phosphosite-specific antibodies recognising CCR5-S337-P and CCR5-S349-P showed that following one hour of agonist treatment, internalised CCR5 was still phosphorylated in the recycling endosomes. Together with the fact that β-arrestins have a high affinity for phosphorylated CCR5 (Huttenrauch et al., 2002), this might explain the stable intracellular association of CCR5 with β-arrestins.

To investigate the nature of the perinuclear structures containing phosphorylated CCR5, immunogold labelled ultra-thin cryosections of CCL5-stimulated cells were analysed by EM. The receptor was found in large (400-700 nm of average diameter) intracellular compartments partially coated with clathrin probably corresponding to endosomes and in clathrin-coated buds. The presence of AP-1 in these clathrin-coated structures excluded the possibility of the CCVs being endocytic vesicles formed at the plasma membrane, but suggested they were vesicles budding from recycling endosomes. Therefore, the prolonged association of the receptor with β-arrestins, in particular β-arrestin 1, might link the receptor to AP-1 positive structures and promote recycling, or further intracellular trafficking of CCR5. This suggests a role for β-arrestin 1 in recycling of CCR5 to the plasma membrane, in addition to its role in their desensitisation and internalisation. Indeed, β-arrestins have been proposed to be involved in recycling of the FPR (Vines et al., 2003).

The study of agonist-induced internalisation of CCR5 ΔS, which cannot be phosphorylated and does not associate with β-arrestins, might help to understand the role of β-arrestins in the intracellular trafficking of CCR5. Indeed, the mutant receptor is internalised upon agonist binding, although at a slower rate (Chapter 4 and Huttenrauch et al., 2002). Preliminary immunofluorescence results suggest that CCR5 ΔS and CCR5 WT are targeted to different intracellular compartments. The nature of the organelle reached by CCR5 ΔS remains unknown. Co-labelling of internalised mutant receptor with cellular markers and ultrastructural analysis by EM would be of great interest and may shed light on a mechanism of internalisation independent of β-arrestins.

8 Discussion

Clathrin-mediated endocytosis (CME) is one of the main mechanisms regulating the biochemical composition and functional activities of the plasma membrane. The components of the clathrin endocytic machinery have been well characterised and described to form a complex dynamic network (Schmid et al., 2006). However, the molecular events leading to the recruitment of activated receptors into endocytic clathrin-coated structures and the formation of clathrin-coated vesicles (CCVs) remain somewhat controversial. This thesis presents a detailed morphological and biochemical analysis of the early events of CME leading to the internalisation of the CC chemokine receptor 5 (CCR5), a G-protein coupled receptor (GPCR) for several CC chemokines that also mediates entry of the human immunodeficiency virus 1 (HIV-1). Understanding the molecular events leading to CCR5 endocytosis will not only provide insights into the general mechanism by which GPCR cell surface expression is modulated, but may also help to find ways of preventing HIV-1 infection.

The work presented in this thesis shows that, following agonist stimulation, cell surface CCR5 is recruited into flat clathrin lattices, which contain accessory proteins from the clathrin endocytic machinery, and where clathrin-coated pits (CCP) containing CCR5 can bud (Chapter 3). These lattices are a common feature of the apical and basal membranes in many tissue culture cells. Clustering of activated CCR5 within these domains is not peculiar to this receptor as the CXC chemokine receptor 4 (CXCR4), another co-receptor for HIV-1, shows the same agonist-induced clustering. Interestingly, the association of CCR5 with these clathrin domains correlates with its rapid agonist-induced internalisation (Chapter 4). The recruitment of CCR5 into flat clathrin lattices relies on agonist-induced GPCR kinase (GRK) phosphorylation of C-terminal serines (Chapter 5), which promotes β-arrestin association with the activated receptor in flat clathrin lattices (Chapter 6). However, the role of β-arrestins might not be limited to desensitisation of the receptor or early endocytic events at the plasma membrane. Indeed, CCR5 shows a sustained association with β-arrestins intracellularly, and β-arrestin 1 may influence the recycling of the receptor by directing it into sorting endosomes via interaction with AP-1 (Chapter 7). The findings presented in this thesis and their importance in

understanding the mechanisms that modulate receptor expression at the plasma membrane via CME will be discussed in this chapter.

8.1 Agonist-induced CCR5 internalisation is clathrin-dependent

Regulation of GPCRs signal transduction relies on rapid and efficient desensitisation of the receptor and its subsequent internalisation leading to degradation or resensitisation before recycling (Tan et al., 2004). The series of events leading to the endocytosis of receptors varies between GPCRs and depends on their intrinsic properties. Desensitisation is mediated via phosphorylation of C-terminal serine and threonine residues by GRK, protein kinase C (PKC) and maybe other kinases such as PKA, and via recruitment of β-arrestins to activated receptors, which abrogates interaction with G-proteins (Moore et al., 2006). β-arrestins also interact with clathrin and the adaptor protein 2 (AP-2) complex (Goodman et al., 1997; Goodman et al., 1996; Krupnick et al., 1997; Laporte et al., 2000; Laporte et al., 1999) and were found to play a key role in GPCR endocytosis by linking desensitised receptors to the endocytic machinery (Goodman et al., 1996; Santini et al., 2002; Scott et al., 2002). However, not all GPCRs depend on β-arrestins for endocytosis (Claing et al., 2002). Indeed, the human cytomegalovirus encoded GPCR-like protein, US28, and the *N*-formyl peptide receptor (FPR) have been reported to be constitutively endocytosed via a β-arrestin-independent and clathrin-dependent mechanism (Fraile-Ramos et al., 2003; Vines et al., 2003).

Evidence has been accumulating in the past decade on the mechanism for agonist-induced CCR5 internalisation. Early studies indicated that agonist activated, internalised receptors accumulated in the perinuclear recycling compartment together with the transferrin receptor (Amara et al., 1997; Mack et al., 1998; Pollok-Kopp et al., 2003; Signoret and Marsh, 2000), β-arrestins were shown to be involved and necessary for CCR5 internalisation (Aramori et al., 1997; Fraile-Ramos et al., 2003) and finally several groups reported a direct association of CCR5 with β-arrestins at the plasma membrane (Huttenrauch et al., 2002; Kraft et al., 2001; Mueller et al., 2002; Vila-Coro et al., 1999). In addition, Mack *et al.* used high sucrose to block CCR5 down-modulation (Mack et al., 1998), and although the authors proposed this to indicate a role for clathrin, high sucrose does not block CME specifically. Signoret *et al.* showed CCR5 in CCVs in cell stimulated for 2

hours with CCL5 (Signoret et al, 2000). Nevertheless, the receptor was probably cycling under these conditions, and these CCVs may differ from the initial events in CCR5 endocytosis. Recent reports have suggested that CCR5 is internalised through a clathrin-independent, raft-mediated caveolar pathway (Mueller et al., 2002; Venkatesan et al., 2003). However, CME was shown to be the main mechanism responsible for the down-modulation of cell surface CCR5 upon agonist stimulation (Signoret et al., 2005). The evidence supporting a clathrin-independent down-modulation of CCR5 was based on studies using drugs that bind membrane cholesterol. Although thought to be specific inhibitors of raft-dependent internalisation, these drugs were shown to affect CCR5 conformation and its ability to bind agonists (Nguyen and Taub, 2002; Nguyen and Taub, 2003a; Nguyen and Taub, 2003b), as well as CME (Rodal et al., 1999; Signoret et al., 2005; Subtil et al., 1999).

The morphological and ultra-structural analysis in Chapter 3 presented evidence for the role of clathrin in the early steps of CCR5 internalisation: CCR5 was observed to cluster into flat clathrin lattices following agonist binding. As CCPs containing the receptor were observed to bud from the edges of these structures, it strongly suggests a clathrin-dependent mechanism leading to CCR5 down-modulation. Moreover, CCR5 was never seen in caveolae.

8.1.1 Molecular mechanism leading to recruitment into flat clathrin lattices

Oppermann *et al.* have previously shown that PKC and GRK mediate specifically S337 and S349 phosphorylation, respectively, upon agonist binding (Pollok-Kopp et al., 2003). The results presented in Chapters 4 and 5 suggest distinct roles for GRK and PKC mediated phosphorylation of the different C-terminal residues in agonist-induced CCR5 internalisation. PKC phosphorylation of S337 did not show any influence on the early events leading to CCR5 endocytosis following agonist binding. However, morphological changes observed in RBL cells following stimulation with CCL5 suggested a role for phosphorylation of S337 in abrogating signalling events. In contrast, ultra-structural analysis indicated that GRK phosphorylation of S349 plays a crucial role in CCR5 recruitment into flat clathrin lattices; this is likely to be mediated through recruitment of β-arrestins. Indeed, both, β-arrestin 1 and 2 were detected with the receptor in flat clathrin lattices (see

Chapter 6). Although β-arrestins have been shown to interact with clathrin and AP-2 (Goodman et al., 1997; Goodman et al., 1996; Krupnick et al., 1997; Laporte et al., 2000; Laporte et al., 1999), and were localised to cell surface receptors upon agonist binding (Goodman et al., 1996; Santini et al., 2002; Scott et al., 2002), the two proteins have never been shown in flat clathrin lattices. This thesis presents the first ultra-structural study showing β-arrestin/receptor complex recruited to CCPs and flat clathrin lattices.

Further experiments in β-arrestin knock-out cells or using siRNA or dominant negative mutants of β-arrestins will shed light on the role of the two proteins in the early events of CCR5 endocytosis. Indeed, identifying at which step CCR5 endocytosis is blocked in the absence of β-arrestins, or when β-arrestins ability to bind endocytic proteins is impaired, will determine precisely their key roles. In addition, live cell imaging of fluorescently labelled receptors, β-arrestins and endocytic proteins is essential to understand the sequence of events leading to CCR5 recruitment into flat clathrin lattices and the involvement of the various endocytic proteins.

Phosphorylation-deficient receptors, which do not interact with β-arrestins, fail to cluster into flat clathrin lattices (Chapter 5). Nevertheless, these receptors were efficiently internalised, although more slowly than the wild-type receptors (Chapter 4). As no morphological evidence for caveolae are seen in RBL cells, it is unlikely that this internalisation occurs through caveolae, however whether CCR5 ΔS endocytosis is clathrin-dependent remains to be determined.

Interestingly, β-arrestins have been reported to be associated with a subset of CCPs containing activated GPCRs at the plasma membrane, suggesting the existence two distinct classes of CCPs (Cao et al., 1998; Mundell et al., 2006; Puthenveedu and von Zastrow, 2006). In their studies of the early events of ADP-induced endocytosis of the two platelets GPCRs $P2Y_1$ and $P2Y_{12}$, Mundell *et al.* showed that these receptors were sorted to different CCPs on the plasma membrane (Mundell et al., 2006). $P2Y_{12}$ internalisation is GRK- and β-arrestin-dependent, like that of β_2AR and CCR5, whereas $P2Y_1$ internalisation is GRK- and β-arrestins-independent but PKC-dependent. In addition, the intracellular trafficking of the two receptors differs; $P2Y_{12}$ rapidly recycles to the plasma membrane like the β_2AR. In contrast, $P2Y_1$ is directed to the lysosomes for degradation. The authors suggested that the two

classes of CCPs were functionally distinct and would possibly influence the fate of the internalised receptors. As CCR5 internalisation is GRK- and β-arrestin-dependent, it is likely that the receptor uses the same CCPs as $P2Y_{12}$ and β_2AR. Whether CCR5 ΔS, which does not interact with β-arrestin, is directed to the second type of CCPs, similarly to $P2Y_1$, remains to be determined. In addition, it would be of interest to investigate the ultrastructure of the CCPs in which $P2Y_{12}$ and β_2AR are recruited in order to see whether they correspond to flat clathrin lattice structures reported here.

Puthenveedu *et al.* have recently suggested that GPCR cargoes themselves can increase the lifetime of CCPs (Puthenveedu and von Zastrow, 2006). Although others have reported a morphological effect on CCPs upon β_2AR activation (Santini et al., 2002), morphometric analysis of membrane sheets from untreated and CCL5-treated cells revealed no change in the size of CCPs and lattices of CHO and RBL CCR5 cells (Signoret et al., 2005 and this thesis). In addition, Puthenveedu *et al.* proposed that recruitment of the receptor/β-arrestin complex into a subset of CCP is a saturable phenomenon, limited by the amount of β-arrestin available in the cell (Puthenveedu and von Zastrow, 2006). This implies that in different cell lines, where the endocytic adaptors may be expressed at different levels, GPCR internalisation may be modulated differently when endocytosis depends on β-arrestins.

A recent study from Keyel *et al.*, looking at the uptake of transferrin and low-density lipoprotein (LDL), did not find any evidence for distinct subsets of CCPs (Keyel et al., 2006). These authors found that Disabled-2 (Dab2), autosomal excessive hypercholesterolemia protein (ARH) and AP-2 were present in most of the clathrin-structures at the plasma membrane, together with the transferrin and LDL receptors. However, the authors did not look at GPCR endocytosis or at the distribution of β-arrestin.

Although the work of Cao *et al.* and Mundell *et al.* proposed the existence of different subset of CCPs (Cao et al., 1998; Mundell et al., 2006), the reason why receptors might target a subset of CCPs, and the biochemical basis of this sorting remains unclear.

8.1.2 Role of CCR5 recruitment into flat clathrin lattices

The recruitment of receptors into flat clathrin lattices under specific conditions was shown to correlate with a faster rate of endocytosis. Indeed, all receptors clustering rapidly into flat clathrin lattices upon agonist binding exhibited similar kinetics of internalisation to the wild-type receptor. By contrast, phosphorylation-deficient receptors, or those exclusively phosphorylated on S337, were slower (Chapter 4).

Flat clathrin lattices have been shown to contain proteins from the endocytic machinery, including AP-2, Eps15, Epsin 1 and dynamin (this thesis and Edeling et al., 2006a; Hawryluk et al., 2006; Hinrichsen et al., 2006; Signoret et al., 2005; Tebar et al., 1996), suggesting their functional activity. They may provide a pool of clathrin and accessory proteins readily available for rapid down-modulation of activated receptors.

Beside their role in endocytosis, flat clathrin lattices might have additional functions. Sequestration of CCR5 in these clathrin domains with β-arrestins might help to abrogate G-protein signalling. Moreover, it is now clear that β-arrestins also function as scaffolds for signalling proteins; whether these signalling proteins are recruited to flat clathrin lattices via β-arrestins remains to be determined.

8.2 Flat clathrin lattices

8.2.1 Current views on clathrin-mediated endocytosis

The diversity of cargos, the multiple model systems, and the variety of techniques of investigation have shed light on the complexity of CME (Perrais and Merrifield, 2005). More than 40 years after the first description of coated structures and their potential role in endocytosis, the assembly of endocytic proteins leading to the formation of a CCP and subsequent internalisation of activated receptors is still only partially understood, and there is disagreement as to the nature of the clathrin-coated structures that mediate endocytosis. One idea is that clathrin forms stable assemblies at the plasma membrane (possibly flat clathrin lattices) from where multiple budding events can occur. They are the so-called 'hot spots' of endocytosis. Besides these 'hot spots' of endocytosis, other smaller structures, that appear and disappear within about a minute, were also proposed to be endocytically active. Evidence supporting this idea comes from live cell fluorescence imaging, total internal reflection

fluorescence (TIRF), and ultra-structural analysis by electron microscopy (EM) (Bellve et al., 2006; Gaidarov et al., 1999; Heuser, 1980; Hinrichsen et al., 2006; Merrifield et al., 2005; Rappoport et al., 2006; Signoret et al., 2005). The transferrin receptor was reported to be internalised through vesicles budding from large stable patches of clathrin and to cluster into flat clathrin lattices (Bellve et al., 2006; Damke et al., 1994; Lamaze et al., 2001; Miller et al., 1991). This suggests flat clathrin lattices are the "hot spots" of endocytosis.

On the other hand, although Ehrlich *et al.* saw large plasma membrane clathrin structures, they did not observe any endocytic events associated with these structures (Ehrlich et al., 2004). These authors suggested that nucleation of clathrin triskelia is random on the cell surface and most of the time leads to abortive events unless stabilised by a cargo. In this case, the stabilised CCP grows to form a single vesicle. The uptake of cargoes, such as LDL and transferrin, and reovirus was reported to be mediated by these short-lived clathrin structures (Ehrlich et al., 2004).

What is not clear from these studies is how a GPCR traffics. The recent work from Puthenveedu *et al.* suggests that some receptors might be internalised through single vesicles (Puthenveedu and von Zastrow, 2006). However, as the authors excluded larger and long-lived clathrin structure that possibly correspond to flat clathrin lattices from their data, the role that flat clathrin lattices play in GPCRs endocytosis remains unclear.

The data presented in this thesis clearly shows the recruitment of activated receptors/β-arrestin complexes into lattices containing necessary proteins for CME, where vesicles pinch off. Experiments with live cell imaging of CCR5 and CXCR4 will provide further evidence for this mechanism.

8.2.2 Flat clathrin lattices: Site of active endocytosis

The data presented in this thesis provides further evidence for the role of flat clathrin lattices in CME. Firstly, flat clathrin lattices were observed in all cell lines analysed, not only on the basal but also on the apical plasma membrane, suggesting a role other than simply adhesion. Moreover, although the access of antibodies was limited on the bottom of the cells, CCR5 could be detected in basal and apical CCPs and flat clathrin lattices in ultra-thin sections of epon-embedded RBL cells, indicating that CME can occur simultaneously at the top and the bottom of cells.

Flat clathrin lattices were also shown to contain proteins characteristic of the clathrin endocytic machinery, such as AP-2, Eps15, Epsin and, upon activation of CCR5, β-arrestins (Chapters 3 and 6). More than 20 clathrin accessory proteins have so far been identified (Schmid et al., 2006). To fully understand these structures, it will be essential to know which of these proteins are found in lattices, whether their distribution is uniform within the lattice as seen for Epsin, and whether some proteins are found in discrete regions (such as Eps15 seen at the periphery), or are specific to budding profiles. Moreover, combining ultra-structural studies with live cell imaging of the plasma membrane will provide a temporal analysis of the early events of CME, and may explain some of the differences observed between the clathrin structures described in the current models.

Often, a few CCPs were observed budding from flat clathrin lattices, reminiscent of the multiple endocytic events observed by live microscopy (Bellve et al., 2006; Ehrlich et al., 2004; Gaidarov et al., 1999; Merrifield et al., 2002; Rappoport and Simon, 2003). Interestingly, as reported earlier by others, budding profiles were only seen at the periphery of lattices. The presence of Eps15, interacting with dynamin, at the edge of lattices, could explain why budding only occurs there. In addition, as shown by Heuser in his structural analysis of flat clathrin lattices and CCP, the organisation of clathrin triskelia varies between flat and invaginated structures (Heuser, 1980). Lattices are essentially composed of hexagons and do not show any curvature, whereas budding profiles always contain both, hexagons and pentagons. In order to induce curvature and turn a flat lattice into a three-dimensional structure, reorganisation of the triskelia is required (Jin and Nossal, 1993). This is likely to happen at the periphery of the lattice, where constant exchange of free clathrin triskelia occurs, and where the dynamic network of accessory proteins might facilitate this process (Wu et al., 2001; Schmid et al., 2006).

Finally, agonist-induced internalisation via flat clathrin lattices is not specific to CCR5. Indeed, CXCR4 was also observed clustering in these structures upon stimulation (Chapter 3). Moreover, the transferrin receptor has also been detected in flat clathrin lattices, and has been shown to be internalised through vesicles budding from large stable areas of clathrin (Damke et al., 1994; Lamaze et al., 2001; Miller et al., 1991; Bellve et al., 2006). Whether the pre-existing clathrin structures in which

the thyrotropin-releasing hormone receptor-1, the β_2AR and the m1 muscarinic receptor are recruited upon activation are flat clathrin lattices remains to be determined (Santini et al., 2002; Scott et al., 2002).

8.2.3 Distinct classes of CCP

In Chapter 5, CCR5 S337only, a receptor that can only be phosphorylated on S337 upon agonist binding, was not observed clustered in flat clathrin lattices following CCL5 stimulation, but instead was seen associated with isolated CCPs (figure 5.10). Whether these CCPs have budded from and moved away from lattices, or are the result of the stabilisation of the random nucleation of clathrin triskelia, is unclear. In addition, these CCPs could correspond to the second class of CCPs described by Mundell *et al.* (Mundell et al., 2006) in which $P2Y_1$ is recruited following PKC phosphorylation. If indeed these pits do not arise from flat clathrin lattices, it would reconcile the two current models for the formation of CCPs. On one hand flat clathrin lattices might give rise to CCPs containing GPCRs undergoing GRK- and β-arrestin-dependent internalisation and, on the other hand, CCPs resulting from the stabilisation by activated receptors of random nucleation events might ensure PKC-dependent internalisation of GPCRs.

8.3 Additional roles for β-arrestins in GPCRs resensitisation

Until recently, β-arrestins were only thought to be involved in desensitisation and internalisation of GPCRs (Claing et al., 2002). In the past few years, β-arrestins have been identified as scaffolds for signalling complex and were shown to interact with Src family tyrosine kinases, and components of the ERK1/2, JNCK3 and p38 MAP kinase cascades (Shenoy and Lefkowitz, 2005). Thus, additional levels of signalling were revealed for GPCRs. β-arrestin 2 was also shown to mediate the internalisation of GRK2-phosphorylated seven transmembrane receptor not coupled to G-protein (Chen et al., 2004). Moreover, β-arrestin 1 could have a potential role in intracellular trafficking as it was recently found to interact with the β subunit of AP-1 (Alliance for Cellular Signalling and personal communication from Dr. Julie Pitcher). AP-1 is located in the TGN and endosomal membranes (Wang et al., 2003b). In addition, work on the FPR suggested β-arrestins might be required for

recycling of this receptor (Vines et al., 2003). Together, these data suggest that AP-1 and β-arrestin 1 may have a role in recycling or intracellular trafficking of GPCRs, therefore broadening the roles for these adaptors.

In the case of CCR5, β-arrestins are recruited to plasma membrane agonist-activated receptors and observed in flat clathrin lattices (Chapter 6). Moreover, they can be observed associated with internalised receptors in the perinuclear recycling endosomes (unpublished results from Nathalie Signoret and Tom Kershaw). The role of this prolonged interaction remains unclear. Interestingly, CCR5 is not dephosphorylated as it traffics through the early endosomes as has been described for the β_2AR (Signoret et al., 2004; Krueger et al., 1997; Pitcher et al., 1995). In addition ultrastructural analysis, showed that CCR5 was still phosphorylated in what were identified as recycling vesicles (Chapter 7 and Signoret et al., 2000). Whether the sustained phosphorylated state of CCR5 inhibits the dissociation of β-arrestins from the receptor, or whether the presence of β-arrestins at the C-terminus of the receptor inhibits phosphatases access or activity is not known. The long-lived complex might be responsible for the multiple rounds of internalisation and recycling observed by Signoret *et al.* (Signoret and Marsh, 2000).

The data presented in Chapter 7 may indicate a role of AP-1 in CCR5 intracellular trafficking. Although labelling for β-arrestin 1 was not done, it is likely that the protein will be found associated with phosphorylated CCR5 in the AP-1 positive CCVs. This would provide further evidence for the role of β-arrestin 1 in intracellular GPCR trafficking.

8.4 Alternative pathway of internalisation for phosphorylation-deficient receptors

CCR5 ΔS internalisation is likely to be β-arrestin-independent (this thesis and Huttenrauch et al., 2002). Moreover, as all C-terminal serines are mutated to alanines, endocytosis does not require PKC and GRK. Whether it is clathrin-mediated remains unclear. Although labelling for the receptor was sometimes observed in clathrin structures at the plasma membrane, the dependence on the clathrin pathway has to be assessed by efficiently blocking the pathway with, for

example, siRNA against clathrin, or using dominant negative mutants of Eps15 (Benmerah et al., 1998; Motley et al., 2003).

The intracellular distribution of CCR5 ΔS upon sustained agonist binding also appears to differ from the wild-type receptor. Whether the receptor reaches the sorting or the recycling endosome is unknown. Morphological studies using various markers of the endocytic pathway are required to determine the nature of the intracellular structures in which CCR5 ΔS is found following CCL5 stimulation. In addition, it would be interesting to investigate the recycling ability of the phosphorylation-deficient mutant. If, as suggested in Chapter 7, β-arrestins influence the intracellular trafficking of GPCRs, it is likely that a defect in CCR5 ΔS recycling to the plasma membrane will be observed.

Morphological studies of the agonist-induced intracellular distribution of the different mutants of CCR5 presented in this thesis will give further information on the role of each of the serines and their phosphorylation by GRK and PKC in CCR5 endocytosis intracellular trafficking.

8.5 Future directions

Although the work presented in this thesis has shed light on the molecular mechanisms leading to agonist-activated receptor coupling with the clathrin endocytic machinery, it also raised a number of questions. The findings that flat clathrin lattices are endocytically active, and that the recruitment of CCR5 into these domains leads to rapid down-modulation of the receptor, suggest a new mechanism for the modulation of cell surface expression of chemokine receptors and maybe other GPCRs. The nature of the driving force for the redistribution of plasma membrane receptors remains unknown. Furthermore, it remains to be determined whether these flat clathrin lattices have a role beyond their involvement in endocytosis, for example in G-protein-independent signalling.

The formation of buds from flat clathrin lattices requires reorganisation of clathrin triskelia from hexagons to pentagons and is still very poorly understood. This process may involve accessory proteins present at the edge of the lattice, such as Eps15, and its interaction with the GTPase dynamin. Ultrastructural analysis combined with live microscopy might provide information on this process.

The existence of distinct populations of CCPs has been suggested (Cao et al., 1998; Mundell et al., 2006; Puthenveedu and von Zastrow, 2006). Whether these are really different biochemically and delineate distinct clathrin-mediated pathways, or whether cargos dictate the differences between CCPs, as recently suggested, requires further investigation.

The findings that β-arrestin 1 interacts with AP-1 (Association for Cellular Signalling and Dr. Julie Pitcher personal communication) and that agonist-activated CCR5 co-localises with AP-1 in recycling vesicles also suggests a novel role for β-arrestins in intracellular trafficking of GPCRs beside their role in desensitisation and internalisation.

Finally, we have shown that the two C-terminal serine residues S337 and S349 have distinct roles in agonist-induced CCR5 internalisation, but haven't studied the involvement of S336 and S342 in this process. We hypothesised that S336 may have a similar role to S337 due to their proximity, and may also be phosphorylated by PKC. However, the role of S342 remains unclear. It would be interesting to analyse a mutant lacking all serines but S342, and see whether the residue is sufficient to induce rapid internalisation upon agonist treatment as is S349. Indeed, our results suggests that rapid endocytosis does not only depend on S349, as CCR5 S349A has a fast agonist-induced internalisation profile. Maybe S342 has a similar role to S349.

Acknowledgements

Firstly, my thanks go to my supervisor Mark Marsh, who offered me the chance to study a fascinating aspect of cell biology and to develop new skills, especially in electron microscopy. Mark also helped me to improve my approach to research by stimulating my intellect.

My project would not have been completed without the help of Nathalie Signoret, Lindsay Hewlett and Lucy Collinson. Natalie worked closely with me during the first two years of my PhD, taught me a great deal about the basic techniques of cell biology, shared with me her knowledge of CCR5 and read part of my thesis. Lindsay and Lucy were my "EM mentors". They allowed me to discover the amazing field of electron microscopy, in which I hope to develop more skills. Lucy also gave me useful advice while I was writing my thesis.

I would like to acknowledge the support that I received from my thesis committee, Dan Cutler, Stephen Nurrish and Julie Pitcher, throughout my PhD. In particular, I thank Julie for her help and her expertise on the β-arrestin project.

Thank you to all the past and present members of the lab: Alberto, Bessi, Natalie, Rahel, Annegret, Magda, Ezequiel, Claudia, Tom and Lars. Annegret shared with me her experience of EM techniques. Thanks to Lars for the useful discussions on clathrin lattices and for reading part of my thesis. Special thanks to Magda, "the wise Polish post-doc" and Bessi, for their support, advice and friendship which were essential during my PhD.

These few years would not have been fun without "the students": Emma, Helen, Andy, Doug, Tom and Sean, with whom I shared fantastic cakes, as well as Ale and Kevin with whom I shared serious EM discussions. Thanks to Doug for reading my introduction and give me useful advice.

Thank you also to Winnie and Marnie for their stimulating trafficking discussions, their advice and encouragement.

Importantly, I would like to thank my mother and my sisters for always being available on the other end of the phone. They, together with friends in Switzerland, cheered me up with a steady supply of chocolate and cheese.

Finally, a very special thanks to Dave for standing by me selflessly throughout my PhD, and especially during the finishing straight.

References

Aggeler, J., R. Takemura, and Z. Werb. 1983. High-resolution three-dimensional views of membrane-associated clathrin and cytoskeleton in critical-point-dried macrophages. *J Cell Biol.* 97:1452-8.

Ahle, S., and E. Ungewickell. 1986. Purification and properties of a new clathrin assembly protein. *Embo J.* 5:3143-9.

Amara, A., S.L. Gall, O. Schwartz, J. Salamero, M. Montes, P. Loetscher, M. Baggiolini, J.L. Virelizier, and F. Arenzana-Seisdedos. 1997. HIV coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J Exp Med.* 186:139-46.

Anborgh, P.H., J.L. Seachrist, L.B. Dale, and S.S. Ferguson. 2000. Receptor/beta-arrestin complex formation and the differential trafficking and resensitization of beta2-adrenergic and angiotensin II type 1A receptors. *Mol Endocrinol.* 14:2040-53.

Aramori, I., S.S. Ferguson, P.D. Bieniasz, J. Zhang, B. Cullen, and M.G. Cullen. 1997. Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. *Embo J.* 16:4606-16.

Baba, T., H. Ueda, N. Terada, Y. Fujii, and S. Ohno. 1999. Immunocytochemical study of endocytotic structures accumulated in HeLa cells transformed with a temperature-sensitive mutant of dynamin. *J Histochem Cytochem.* 47:637-48.

Bannert, N., S. Craig, M. Farzan, D. Sogah, N.V. Santo, H. Choe, and J. Sodroski. 2001. Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. *J Exp Med.* 194:1661-73.

Bazan, J.F., K.B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D.R. Greaves, A. Zlotnik, and T.J. Schall. 1997. A new class of membrane-bound chemokine with a CX3C motif. *Nature.* 385:640-4.

Bellve, K.D., D. Leonard, C. Standley, L.M. Lifshitz, R.A. Tuft, A. Hayakawa, S. Corvera, and K.E. Fogarty. 2006. Plasma membrane domains specialized for clathrin-mediated endocytosis in primary cells. *J Biol Chem.* 281:16139-46.

Benkirane, M., D.Y. Jin, R.F. Chun, R.A. Koup, and K.T. Jeang. 1997. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. *J Biol Chem.* 272:30603-6.

Benmerah, A., B. Begue, A. Dautry-Varsat, and N. Cerf-Bensussan. 1996. The ear of alpha-adaptin interacts with the COOH-terminal domain of the Eps 15 protein. *J Biol Chem.* 271:12111-6.

Benmerah, A., J. Gagnon, B. Begue, B. Megarbane, A. Dautry-Varsat, and N. Cerf-Bensussan. 1995. The tyrosine kinase substrate eps15 is constitutively associated with the plasma membrane adaptor AP-2. *J Cell Biol.* 131:1831-8.

Benmerah, A., C. Lamaze, B. Begue, S.L. Schmid, A. Dautry-Varsat, and N. Cerf-Bensussan. 1998. AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J Cell Biol.* 140:1055-62.

Berger, E.A., P.M. Murphy, and J.M. Farber. 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol.* 17:657-700.

Blanpain, C., B.J. Doranz, A. Bondue, C. Govaerts, A. De Leener, G. Vassart, R.W. Doms, A. Proudfoot, and M. Parmentier. 2003. The core domain of chemokines binds CCR5 extracellular domains while their amino terminus interacts with the transmembrane helix bundle. *J Biol Chem.* 278:5179-87.

Blanpain, C., B. Lee, J. Vakili, B.J. Doranz, C. Govaerts, I. Migeotte, M. Sharron, V. Dupriez, G. Vassart, R.W. Doms, and M. Parmentier. 1999a. Extracellular cysteines of CCR5 are required for chemokine binding, but dispensable for HIV-1 coreceptor activity. *J Biol Chem.* 274:18902-8.

Blanpain, C., I. Migeotte, B. Lee, J. Vakili, B.J. Doranz, C. Govaerts, G. Vassart, R.W. Doms, and M. Parmentier. 1999b. CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. *Blood.* 94:1899-905.

Blanpain, C., J.M. Vanderwinden, J. Cihak, V. Wittamer, E. Le Poul, H. Issafras, M. Stangassinger, G. Vassart, S. Marullo, D. Schlndorff, M. Parmentier, and M. Mack. 2002. Multiple active states and oligomerization of CCR5 revealed by functional properties of monoclonal antibodies. *Mol Biol Cell.* 13:723-37.

Blanpain, C., V. Wittamer, J.M. Vanderwinden, A. Boom, B. Renneboog, B. Lee, E. Le Poul, L. El Asmar, C. Govaerts, G. Vassart, R.W. Doms, and M. Parmentier. 2001. Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways. *J Biol Chem.* 276:23795-804.

Blanpied, T.A., D.B. Scott, and M.D. Ehlers. 2002. Dynamics and regulation of clathrin coats at specialized endocytic zones of dendrites and spines. *Neuron.* 36:435-49.

Bleul, C.C., M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, and T.A. Springer. 1996. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature.* 382:829-33.

Bonifacino, J.S., and J. Lippincott-Schwartz. 2003. Coat proteins: shaping membrane transport. *Nat Rev Mol Cell Biol.* 4:409-14.

Bonifacino, J.S., P. Perez, R.D. Klausner, and I.V. Sandoval. 1986. Study of the transit of an integral membrane protein from secretory granules through the plasma membrane of secreting rat basophilic leukemia cells using a specific monoclonal antibody. *J Cell Biol.* 102:516-22.

Bonifacino, J.S., and L.M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem.* 72:395-447.

Bonifacino, J.S., L. Yuan, and I.V. Sandoval. 1989. Internalization and recycling to serotonin-containing granules of the 80K integral membrane protein exposed on the surface of secreting rat basophilic leukaemia cells. *J Cell Sci.* 92 (Pt 4):701-12.

Bonini, J.A., S.K. Martin, F. Dralyuk, M.W. Roe, L.H. Philipson, and D.F. Steiner. 1997. Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3. *DNA Cell Biol.* 16:1249-56.

Cao, T.T., R.W. Mays, and M. von Zastrow. 1998. Regulated endocytosis of G-protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits. *J Biol Chem.* 273:24592-602.

Chen, H., S. Fre, V.I. Slepnev, M.R. Capua, K. Takei, M.H. Butler, P.P. Di Fiore, and P. De Camilli. 1998. Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature.* 394:793-7.

Chen, W., X.R. Ren, C.D. Nelson, L.S. Barak, J.K. Chen, P.A. Beachy, F. de Sauvage, and R.J. Lefkowitz. 2004. Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. *Science.* 306:2257-60.

Cheng, Z.J., J. Zhao, Y. Sun, W. Hu, Y.L. Wu, B. Cen, G.X. Wu, and G. Pei. 2000. beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. *J Biol Chem.* 275:2479-85.

Chini, B., and M. Parenti. 2004. G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? *J Mol Endocrinol.* 32:325-38.

Claing, A., S.A. Laporte, M.G. Caron, and R.J. Lefkowitz. 2002. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog Neurobiol.* 66:61-79.

Cocchi, F., A.L. DeVico, A. Garzino-Demo, S.K. Arya, R.C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science.* 270:1811-5.

Coda, L., A.E. Salcini, S. Confalonieri, G. Pelicci, T. Sorkina, A. Sorkin, P.G. Pelicci, and P.P. Di Fiore. 1998. Eps15R is a tyrosine kinase substrate with characteristics of a docking protein possibly involved in coated pits-mediated internalization. *J Biol Chem.* 273:3003-12.

Collins, B.M., A.J. McCoy, H.M. Kent, P.R. Evans, and D.J. Owen. 2002. Molecular architecture and functional model of the endocytic AP2 complex. *Cell.* 109:523-35.

Conner, S.D., and S.L. Schmid. 2002. Identification of an adaptor-associated kinase, AAK1, as a regulator of clathrin-mediated endocytosis. *J Cell Biol.* 156:921-9.

Conner, S.D., and S.L. Schmid. 2003. Differential requirements for AP-2 in clathrin-mediated endocytosis. *J Cell Biol.* 162:773-9

Conner, S.D., and S.L. Schmid. 2005. CVAK104 is a novel poly-L-lysine-stimulated kinase that targets the beta2-subunit of AP2. *J Biol Chem.* 280:21539-44.

Damke, H., T. Baba, D.E. Warnock, and S.L. Schmid. 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J Cell Biol.* 127:915-34.

Damke, H., D.D. Binns, H. Ueda, S.L. Schmid, and T. Baba. 2001. Dynamin GTPase domain mutants block endocytic vesicle formation at morphologically distinct stages. *Mol Biol Cell.* 12:2578-89.

Damm, E.M., L. Pelkmans, J. Kartenbeck, A. Mezzacasa, T. Kurzchalia, and A. Helenius. 2005. Clathrin- and caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. *J Cell Biol.* 168:477-88.

Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R.E. Sutton, C.M. Hill, C.B. Davis, S.C. Peiper, T.J. Schall, D.R. Littman, and N.R. Landau. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature.* 381:661-6.

Di Guglielmo, G.M., C. Le Roy, A.F. Goodfellow, and J.L. Wrana. 2003. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol.* 5:410-21.

Dragic, T., A. Trkola, D.A. Thompson, E.G. Cormier, F.A. Kajumo, E. Maxwell, S.W. Lin, W. Ying, S.O. Smith, T.P. Sakmar, and J.P. Moore. 2000. A

binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proc Natl Acad Sci U S A.* 97:5639-44.

Dragonetti, A., M. Baldassarre, R. Castino, M. Demoz, A. Luini, R. Buccione, and C. Isidoro. 2000. The lysosomal protease cathepsin D is efficiently sorted to and secreted from regulated secretory compartments in the rat basophilic/mast cell line RBL. *J Cell Sci.* 113 (Pt 18):3289-98.

Drake, M.T., M.A. Downs, and L.M. Traub. 2000. Epsin binds to clathrin by associating directly with the clathrin-terminal domain. Evidence for cooperative binding through two discrete sites. *J Biol Chem.* 275:6479-89.

Dreyling, M.H., J.A. Martinez-Climent, M. Zheng, J. Mao, J.D. Rowley, and S.K. Bohlander. 1996. The t(10;11)(p13;q14) in the U937 cell line results in the fusion of the AF10 gene and CALM, encoding a new member of the AP-3 clathrin assembly protein family. *Proc Natl Acad Sci U S A.* 93:4804-9.

Duwel, M., and E.J. Ungewickell. 2006. Clathrin-dependent Association of CVAK104 with Endosomes and the Trans-Golgi Network. *Mol Biol Cell.* 17:4513-25.

Edeling, M.A., S.K. Mishra, P.A. Keyel, A.L. Steinhauser, B.M. Collins, R. Roth, J.E. Heuser, D.J. Owen, and L.M. Traub. 2006a. Molecular switches involving the AP-2 beta2 appendage regulate endocytic cargo selection and clathrin coat assembly. *Dev Cell.* 10:329-42.

Edeling, M.A., C. Smith, and D. Owen. 2006b. Life of a clathrin coat: insights from clathrin and AP structures. *Nat Rev Mol Cell Biol.* 7:32-44.

Ehrlich, M., W. Boll, A. Van Oijen, R. Hariharan, K. Chandran, M.L. Nibert, and T. Kirchhausen. 2004. Endocytosis by random initiation and stabilization of clathrin-coated pits. *Cell.* 118:591-605.

Escriche, M., J. Burgueno, F. Ciruela, E.I. Canela, J. Mallol, C. Enrich, C. Lluis, and R. Franco. 2003. Ligand-induced caveolae-mediated internalization of A1 adenosine receptors: morphological evidence of endosomal sorting and receptor recycling. *Exp Cell Res.* 285:72-90.

Farzan, M., H. Choe, K.A. Martin, Y. Sun, M. Sidelko, C.R. Mackay, N.P. Gerard, J. Sodroski, and C. Gerard. 1997. HIV-1 entry and macrophage inflammatory protein-1beta-mediated signaling are independent functions of the chemokine receptor CCR5. *J Biol Chem.* 272:6854-7.

Farzan, M., T. Mirzabekov, P. Kolchinsky, R. Wyatt, M. Cayabyab, N.P. Gerard, C. Gerard, J. Sodroski, and H. Choe. 1999. Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry. *Cell.* 96:667-76.

Fazioli, F., L. Minichiello, B. Matoskova, W.T. Wong, and P.P. Di Fiore. 1993. eps15, a novel tyrosine kinase substrate, exhibits transforming activity. *Mol Cell Biol.* 13:5814-28.

Ford, M.G., I.G. Mills, B.J. Peter, Y. Vallis, G.J. Praefcke, P.R. Evans, and H.T. McMahon. 2002. Curvature of clathrin-coated pits driven by epsin. *Nature.* 419:361-6.

Ford, M.G., B.M. Pearse, M.K. Higgins, Y. Vallis, D.J. Owen, A. Gibson, C.R. Hopkins, P.R. Evans, and H.T. McMahon. 2001. Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science.* 291:1051-5.

Fotin, A., Y. Cheng, P. Sliz, N. Grigorieff, S.C. Harrison, T. Kirchhausen, and T. Walz. 2004. Molecular model for a complete clathrin lattice from electron cryomicroscopy. *Nature.* 432:573-9.

Fra, A.M., E. Williamson, K. Simons, and R.G. Parton. 1995. De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc Natl Acad Sci U S A.* 92:8655-9.

Fraile-Ramos, A., T.A. Kohout, M. Waldhoer, and M. Marsh. 2003. Endocytosis of the viral chemokine receptor US28 does not require beta-arrestins but is dependent on the clathrin-mediated pathway. *Traffic.* 4:243-53.

Fujimoto, L.M., R. Roth, J.E. Heuser, and S.L. Schmid. 2000. Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells. *Traffic.* 1:161-71.

Gaidarov, I., and J.H. Keen. 1999. Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. *J Cell Biol.* 146:755-64.

Gaidarov, I., F. Santini, R.A. Warren, and J.H. Keen. 1999. Spatial control of coated-pit dynamics in living cells. *Nat Cell Biol.* 1:1-7.

Gether, U. 2000. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr Rev.* 21:90-113.

Gines, S., F. Ciruela, J. Burgueno, V. Casado, E.I. Canela, J. Mallol, C. Lluis, and R. Franco. 2001. Involvement of caveolin in ligand-induced recruitment and internalization of A(1) adenosine receptor and adenosine deaminase in an epithelial cell line. *Mol Pharmacol.* 59:1314-23.

Glebov, O.O., N.A. Bright, and B.J. Nichols. 2006. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol.* 8:46-54.

Goodman, O.B., Jr., J.G. Krupnick, V.V. Gurevich, J.L. Benovic, and J.H. Keen. 1997. Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. *J Biol Chem.* 272:15017-22.

Goodman, O.B., Jr., J.G. Krupnick, F. Santini, V.V. Gurevich, R.B. Penn, A.W. Gagnon, J.H. Keen, and J.L. Benovic. 1996. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature.* 383:447-50.

Govaerts, C., C. Blanpain, X. Deupi, S. Ballet, J.A. Ballesteros, S.J. Wodak, G. Vassart, L. Pardo, and M. Parmentier. 2001. The TXP motif in the second transmembrane helix of CCR5. A structural determinant of chemokine-induced activation. *J Biol Chem.* 276:13217-25.

Govaerts, C., A. Bondue, J.Y. Springael, M. Olivella, X. Deupi, E. Le Poul, S.J. Wodak, M. Parmentier, L. Pardo, and C. Blanpain. 2003. Activation of CCR5 by chemokines involves an aromatic cluster between transmembrane helices 2 and 3. *J Biol Chem.* 278:1892-903.

Granseth, B., B. Odermatt, S.J. Royle, and L. Lagnado. 2006. Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. *Neuron.* 51:773-86.

Hawryluk, M.J., P.A. Keyel, S.K. Mishra, S.C. Watkins, J.E. Heuser, and L.M. Traub. 2006. Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. *Traffic.* 7:262-81.

Heuser, J. 1980. Three-dimensional visualization of coated vesicle formation in fibroblasts. *J Cell Biol.* 84:560-83.

Hinrichsen, L., J. Harborth, L. Andrees, K. Weber, and E.J. Ungewickell. 2003. Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. *J Biol Chem.* 278:45160-70.

Hinrichsen, L., A. Meyerholz, S. Groos, and E.J. Ungewickell. 2006. Bending a membrane: how clathrin affects budding. *Proc Natl Acad Sci U S A.* 103:8715-20.

Honing, S., D. Ricotta, M. Krauss, K. Spate, B. Spolaore, A. Motley, M. Robinson, C. Robinson, V. Haucke, D. J. Owen. 2005. Phosphatidylinositol-(4,5)-Bisphosphate regulates sorting signal recognition by the clathrin-associated adaptor complex AP2. *Mol. Cell.* 18:519-531

Huang, F., A. Khvorova, W. Marshall, and A. Sorkin. 2004. Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *J Biol Chem.* 279:16657-61.

Huttenrauch, F., A. Nitzki, F.T. Lin, S. Honing, and M. Oppermann. 2002. Beta-arrestin binding to CC chemokine receptor 5 requires multiple C-terminal receptor phosphorylation sites and involves a conserved Asp-Arg-Tyr sequence motif. *J Biol Chem.* 277:30769-77.

Itoh, T., S. Koshiba, T. Kigawa, A. Kikuchi, S. Yokoyama, and T. Takenawa. 2001. Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science.* 291:1047-51.

Jackson, A.P., A. Flett, C. Smythe, L. Hufton, F.R. Wettey, and E. Smythe. 2003. Clathrin promotes incorporation of cargo into coated pits by activation of the AP2 adaptor micro2 kinase. *J Cell Biol.* 163:231-6.

Jin, A.J., and R. Nossal. 1993. Topological mechanisms involved in the formation of clathrin-coated vesicles. *Biophys J.* 65:1523-37.

Kanaseki, T., and K. Kadota. 1969. The "vesicle in a basket". A morphological study of the coated vesicle isolated from the nerve endings of the guinea pig brain, with special reference to the mechanism of membrane movements. *J Cell Biol.* 42:202-20.

Keyel, P.A., S.K. Mishra, R. Roth, J.E. Heuser, S.C. Watkins, and L.M. Traub. 2006. A single common portal for clathrin-mediated endocytosis of distinct cargo governed by cargo-selective adaptors. *Mol Biol Cell.* 17:4300-17.

Kirchhausen, T., and S.C. Harrison. 1981. Protein organization in clathrin trimers. *Cell.* 23:755-61.

Kirkham, M., A. Fujita, R. Chadda, S.J. Nixon, T.V. Kurzchalia, D.K. Sharma, R.E. Pagano, J.F. Hancock, S. Mayor, and R.G. Parton. 2005. Ultrastructural identification of uncoated caveolin-independent early endocytic vehicles. *J Cell Biol.* 168:465-76.

Klapisz, E., I. Sorokina, S. Lemeer, M. Pijnenburg, A.J. Verkleij, and P.M. van Bergen en Henegouwen. 2002. A ubiquitin-interacting motif (UIM) is essential for Eps15 and Eps15R ubiquitination. *J Biol Chem.* 277:30746-53.

Kohout, T.A., F.S. Lin, S.J. Perry, D.A. Conner, and R.J. Lefkowitz. 2001. beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Natl Acad Sci U S A.* 98:1601-6.

Kosaka, T., and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of Drosophila melanogaster, shibirets1. *J Cell Biol.* 97:499-507.

Kraft, K., H. Olbrich, I. Majoul, M. Mack, A. Proudfoot, and M. Oppermann. 2001. Characterization of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization. *J Biol Chem.* 276:34408-18.

Krauss, M., M. Kinuta, M.R. Wenk, P. De Camilli, K. Takei, and V. Haucke. 2003. ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type Igamma. *J Cell Biol.* 162:113-24.

Krisch, B., J. Feindt, and R. Mentlein. 1998. Immunoelectronmicroscopic analysis of the ligand-induced internalization of the somatostatin receptor subtype 2 in cultured human glioma cells. *J Histochem Cytochem.* 46:1233-42.

Krueger, K.M., Y. Daaka, J.A. Pitcher, and R.J. Lefkowitz. 1997. The role of sequestration in G protein-coupled receptor resensitization. Regulation of beta2-adrenergic receptor dephosphorylation by vesicular acidification. *J Biol Chem.* 272:5-8.

Krupnick, J.G., O.B. Goodman, Jr., J.H. Keen, and J.L. Benovic. 1997. Arrestin/clathrin interaction. Localization of the clathrin binding domain of nonvisual arrestins to the carboxy terminus. *J Biol Chem.* 272:15011-6.

Kuschert, G.S., F. Coulin, C.A. Power, A.E. Proudfoot, R.E. Hubbard, A.J. Hoogewerf, and T.N. Wells. 1999. Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry.* 38:12959-68.

Lagane, B., S. Ballet, T. Planchenault, K. Balabanian, E. Le Poul, C. Blanpain, Y. Percherancier, I. Staropoli, G. Vassart, M. Oppermann, M. Parmentier, and F. Bachelerie. 2005. Mutation of the DRY motif reveals different structural requirements for the CC chemokine receptor 5-mediated signaling and receptor endocytosis. *Mol Pharmacol.* 67:1966-76.

Lakadamyali, M., M.J. Rust, and X. Zhuang. 2006. Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell.* 124:997-1009.

Lamaze, C., A. Dujeancourt, T. Baba, C.G. Lo, A. Benmerah, and A. Dautry-Varsat. 2001. Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Mol Cell.* 7:661-71.

Laporte, S.A., R.H. Oakley, J.A. Holt, L.S. Barak, and M.G. Caron. 2000. The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. *J Biol Chem.* 275:23120-6.

Laporte, S.A., R.H. Oakley, J. Zhang, J.A. Holt, S.S. Ferguson, M.G. Caron, and L.S. Barak. 1999. The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci U S A.* 96:3712-7.

Lasley, R.D., P. Narayan, A. Uittenbogaard, and E.J. Smart. 2000. Activated cardiac adenosine A(1) receptors translocate out of caveolae. *J Biol Chem.* 275:4417-21.

Le Roy, C., and J.L. Wrana. 2005. Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. *Nat Rev Mol Cell Biol.* 6:112-26.

Lee, B., M. Sharron, C. Blanpain, B.J. Doranz, J. Vakili, P. Setoh, E. Berg, G. Liu, H.R. Guy, S.R. Durell, M. Parmentier, C.N. Chang, K. Price, M. Tsang, and R.W. Doms. 1999. Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *J Biol Chem.* 274:9617-26.

Lewis, V., S.A. Green, M. Marsh, P. Vihko, A. Helenius, and I. Mellman. 1985. Glycoproteins of the lysosomal membrane. *J Cell Biol.* 100:1839-47.

Liu, R., W.A. Paxton, S. Choe, D. Ceradini, S.R. Martin, R. Horuk, M.E. MacDonald, H. Stuhlmann, R.A. Koup, and N.R. Landau. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell.* 86:367-77.

Lundmark, R., and S.R. Carlsson. 2002. The beta-appendages of the four adaptor-protein (AP) complexes: structure and binding properties, and identification of sorting nexin 9 as an accessory protein to AP-2. *Biochem J.* 362:597-607.

Lusso, P. 2006. HIV and the chemokine system: 10 years later. *Embo J.* 25:447-56.

Ma, Q., D. Jones, P.R. Borghesani, R.A. Segal, T. Nagasawa, T. Kishimoto, R.T. Bronson, and T.A. Springer. 1998. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A.* 95:9448-53.

Macia, E., M. Ehrlich, R. Massol, E. Boucrot, C. Brunner, and T. Kirchhausen. 2006. Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell.* 10:839-50.

Mack, M., B. Luckow, P.J. Nelson, J. Cihak, G. Simmons, P.R. Clapham, N. Signoret, M. Marsh, M. Stangassinger, F. Borlat, T.N. Wells, D. Schlondorff, and A.E. Proudfoot. 1998. Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J Exp Med.* 187:1215-24.

Manes, S., E. Mira, C. Gomez-Mouton, R.A. Lacalle, P. Keller, J.P. Labrador, and A.C. Martinez. 1999. Membrane raft microdomains mediate front-rear polarity in migrating cells. *Embo J.* 18:6211-20.

Marchese, A., and J.L. Benovic. 2001. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem.* 276:45509-12.

Marsh, M., and Helenius, A. 2006. Virus entry: open sesame. *Cell.* 124:729-740.

Masuda, M., S. Takeda, M. Sone, T. Ohki, H. Mori, Y. Kamioka, and N. Mochizuki. 2006. Endophilin BAR domain drives membrane curvature by two newly identified structure-based mechanisms. *Embo J.* 25:2889-97.

Maupin, P., and T.D. Pollard. 1983. Improved preservation and staining of HeLa cell actin filaments, clathrin-coated membranes, and other cytoplasmic structures by tannic acid-glutaraldehyde-saponin fixation. *J Cell Biol.* 96:51-62.

Mayhew, T.M., J.M. Lucocq, and G. Griffiths. 2002. Relative labelling index: a novel stereological approach to test for non-random immunogold labelling of organelles and membranes on transmission electron microscopy thin sections. *J Microsc.* 205:153-64.

McMahon, H.T., and J.L. Gallop. 2005. Membrane curvature and mechanisms of dynamic cell membrane remodelling. *Nature.* 438:590-6.

McMahon, H.T., and I.G. Mills. 2004. COP and clathrin-coated vesicle budding: different pathways, common approaches. *Curr Opin Cell Biol.* 16:379-91.

Meier, O., and U.F. Greber. 2004. Adenovirus endocytosis. *J Gene Med.* 6 Suppl 1:S152-63.

Mentlein, R., J. Held-Feindt, and B. Krisch. 2001. Topology of the signal transduction of the G protein-coupled somatostatin receptor sst2 in human glioma cells. *Cell Tissue Res.* 303:27-34.

Merrifield, C.J. 2004. Seeing is believing: imaging actin dynamics at single sites of endocytosis. *Trends Cell Biol.* 14:352-8.

Merrifield, C.J., M.E. Feldman, L. Wan, and W. Almers. 2002. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. *Nat Cell Biol.* 4:691-8.

Merrifield, C.J., D. Perrais, and D. Zenisek. 2005. Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. *Cell.* 121:593-606.

Meyer, C., D. Zizioli, S. Lausmann, E.L. Eskelinen, J. Hamann, P. Saftig, K. von Figura, and P. Schu. 2000. mu1A-adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors. *Embo J.* 19:2193-203.

Meyerholz, A., L. Hinrichsen, S. Groos, P.C. Esk, G. Brandes, and E.J. Ungewickell. 2005. Effect of clathrin assembly lymphoid myeloid leukemia protein depletion on clathrin coat formation. *Traffic.* 6:1225-34.

Miller, K., M. Shipman, I.S. Trowbridge, and C.R. Hopkins. 1991. Transferrin receptors promote the formation of clathrin lattices. *Cell.* 65:621-32.

Mishra, S.K., M.J. Hawryluk, T.J. Brett, P.A. Keyel, A.L. Dupin, A. Jha, J.E. Heuser, D.H. Fremont, and L.M. Traub. 2004. Dual engagement regulation of protein interactions with the AP-2 adaptor alpha appendage. *J Biol Chem.* 279:46191-203.

Moore, C.A., S.K. Milano, and J.L. Benovic. 2006. Regulation of Receptor Trafficking by GRKs and Arrestins. *Annu Rev Physiol.*

Morgan, J.R., K. Prasad, W. Hao, G.J. Augustine, and E.M. Lafer. 2000. A conserved clathrin assembly motif essential for synaptic vesicle endocytosis. *J Neurosci.* 20:8667-76.

Morris, S.A., S. Schroder, U. Plessmann, K. Weber, and E. Ungewickell. 1993. Clathrin assembly protein AP180: primary structure, domain organization and identification of a clathrin binding site. *Embo J.* 12:667-75.

Moskowitz, H.S., J. Heuser, T.E. McGraw, and T.A. Ryan. 2003. Targeted chemical disruption of clathrin function in living cells. *Mol Biol Cell.* 14:4437-47.

Motley, A., N.A. Bright, M.N. Seaman, and M.S. Robinson. 2003. Clathrin-mediated endocytosis in AP-2-depleted cells. *J Cell Biol.* 162:909-18.

Motley, A.M., N. Berg, M.J. Taylor, D.A. Sahlender, J. Hirst, D.J. Owen, and M.S. Robinson. 2006. Functional Analysis of AP-2 {alpha} and {micro}2 Subunits. *Mol Biol Cell.*

Mueller, A., E. Kelly, and P.G. Strange. 2002. Pathways for internalization and recycling of the chemokine receptor CCR5. *Blood.* 99:785-91.

Mueller, V.J., M. Wienisch, R.B. Nehring, and J. Klingauf. 2004. Monitoring clathrin-mediated endocytosis during synaptic activity. *J Neurosci.* 24:2004-12.

Mundell, S.J., J. Luo, J.L. Benovic, P.B. Conley, and A.W. Poole. 2006. Distinct Clathrin-Coated Pits Sort Different G Protein-Coupled Receptor Cargo. *Traffic.*

Murphy, P.M. 2002. International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacol Rev.* 54:227-9.

Murphy, P.M., M. Baggiolini, I.F. Charo, C.A. Hebert, R. Horuk, K. Matsushima, L.H. Miller, J.J. Oppenheim, and C.A. Power. 2000. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev.* 52:145-76.

Neote, K., J.Y. Mak, L.F. Kolakowski, Jr., and T.J. Schall. 1994. Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. *Blood.* 84:44-52.

Nguyen, D.H., and D. Taub. 2002. Cholesterol is essential for macrophage inflammatory protein 1 beta binding and conformational integrity of CC chemokine receptor 5. *Blood.* 99:4298-306.

Nguyen, D.H., and D.D. Taub. 2003a. Inhibition of chemokine receptor function by membrane cholesterol oxidation. *Exp Cell Res.* 291:36-45.

Nguyen, D.H., and D.D. Taub. 2003b. Membrane incorporation of 22-hydroxycholesterol inhibits chemokine receptor activity. *Exp Cell Res.* 285:268-77.

Nibbs, R.J., S.M. Wylie, J. Yang, N.R. Landau, and G.J. Graham. 1997. Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *J Biol Chem.* 272:32078-83.

Nonet, M.L., A.M. Holgado, F. Brewer, C.J. Serpe, B.A. Norbeck, J. Holleran, L. Wei, E. Hartwieg, E.M. Jorgensen, and A. Alfonso. 1999. UNC-11, a Caenorhabditis elegans AP180 homologue, regulates the size and protein composition of synaptic vesicles. *Mol Biol Cell.* 10:2343-60.

Oakley, R.H., S.A. Laporte, J.A. Holt, L.S. Barak, and M.G. Caron. 1999. Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization. *J Biol Chem.* 274:32248-57.

Oakley, R.H., S.A. Laporte, J.A. Holt, M.G. Caron, and L.S. Barak. 2000. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem.* 275:17201-10.

Oberlin, E., A. Amara, F. Bachelerie, C. Bessia, J.L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J.M. Heard, I. Clark-Lewis, D.F. Legler, M. Loetscher, M. Baggiolini, and B. Moser. 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature.* 382:833-5.

Okamoto, T., A. Schlegel, P.E. Scherer, and M.P. Lisanti. 1998. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem.* 273:5419-22.

Olson, W.C., G.E. Rabut, K.A. Nagashima, D.N. Tran, D.J. Anselma, S.P. Monard, J.P. Segal, D.A. Thompson, F. Kajumo, Y. Guo, J.P. Moore, P.J. Maddon, and T. Dragic. 1999. Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5. *J Virol.* 73:4145-55.

Olusanya, O., P.D. Andrews, J.R. Swedlow, and E. Smythe. 2001. Phosphorylation of threonine 156 of the mu2 subunit of the AP2 complex is essential for endocytosis in vitro and in vivo. *Curr Biol.* 11:896-900.

Oppermann, M., M. Mack, A.E. Proudfoot, and H. Olbrich. 1999. Differential effects of CC chemokines on CC chemokine receptor 5 (CCR5) phosphorylation and identification of phosphorylation sites on the CCR5 carboxyl terminus. *J Biol Chem.* 274:8875-85.

Orsini, M.J., J.L. Parent, S.J. Mundell, J.L. Benovic, and A. Marchese. 1999. Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the c-terminal tail that mediate receptor internalization. *J Biol Chem.* 274:31076-86.

Owen, D.J., B.M. Collins, and P.R. Evans. 2004. Adaptors for clathrin coats: structure and function. *Annu Rev Cell Dev Biol.* 20:153-91.

Owen, D.J., and P.R. Evans. 1998. A structural explanation for the recognition of tyrosine-based endocytotic signals. *Science.* 282:1327-32.

Owen, D.J., Y. Vallis, B.M. Pearse, H.T. McMahon, and P.R. Evans. 2000. The structure and function of the beta 2-adaptin appendage domain. *Embo J.* 19:4216-27.

Page, L.J., and M.S. Robinson. 1995. Targeting signals and subunit interactions in coated vesicle adaptor complexes. *J Cell Biol.* 131:619-30.

Paterlini, M.G. 2002. Structure modeling of the chemokine receptor CCR5: implications for ligand binding and selectivity. *Biophys J.* 83:3012-31.

Pearse, B.M. 1975. Coated vesicles from pig brain: purification and biochemical characterization. *J Mol Biol.* 97:93-8.

Peden, A.A., V. Oorschot, B.A. Hesser, C.D. Austin, R.H. Scheller, and J. Klumperman. 2004. Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. *J Cell Biol.* 164:1065-76.

Pelchen-Matthews, A., J.E. Armes, G. Griffiths, and M. Marsh. 1991. Differential endocytosis of CD4 in lymphocytic and nonlymphocytic cells. *J Exp Med.* 173:575-87.

Pelchen-Matthews, A., J.E. Armes, and M. Marsh. 1989. Internalization and recycling of CD4 transfected into HeLa and NIH3T3 cells. *Embo J.* 8:3641-9.

Pelkmans, L., T. Burli, M. Zerial, and A. Helenius. 2004. Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell.* 118:767-80.

Pelkmans, L., and A. Helenius. 2002. Endocytosis via caveolae. *Traffic.* 3:311-20.

Pelkmans, L., J. Kartenbeck, and A. Helenius. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol.* 3:473-83.

Pelkmans, L., D. Puntener, and A. Helenius. 2002. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science.* 296:535-9.

Percherancier, Y., B. Lagane, T. Planchenault, I. Staropoli, R. Altmeyer, J.L. Virelizier, F. Arenzana-Seisdedos, D.C. Hoessli, and F. Bachelerie. 2003. HIV-1 entry into T-cells is not dependent on CD4 and CCR5 localization to sphingolipid-enriched, detergent-resistant, raft membrane domains. *J Biol Chem.* 278:3153-61.

Perrais, D., and C.J. Merrifield. 2005. Dynamics of endocytic vesicle creation. *Dev Cell.* 9:581-92.

Peter, B.J., H.M. Kent, I.G. Mills, Y. Vallis, P.J. Butler, P.R. Evans, and H.T. McMahon. 2004. BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. *Science.* 303:495-9.

Pfeiffer, J.R., and J.M. Oliver. 1994. Tyrosine kinase-dependent assembly of actin plaques linking Fc epsilon R1 cross-linking to increased cell substrate adhesion in RBL-2H3 tumor mast cells. *J Immunol.* 152:270-9.

Pfeiffer, J.R., J.C. Seagrave, B.H. Davis, G.G. Deanin, and J.M. Oliver. 1985. Membrane and cytoskeletal changes associated with IgE-mediated serotonin release from rat basophilic leukemia cells. *J Cell Biol.* 101:2145-55.

Pierce, K.L., R.T. Premont, and R.J. Lefkowitz. 2002. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol.* 3:639-50.

Pitcher, J.A., E.S. Payne, C. Csortos, A.A. DePaoli-Roach, and R.J. Lefkowitz. 1995. The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proc Natl Acad Sci U S A.* 92:8343-7.

Pollok-Kopp, B., K. Schwarze, V.K. Baradari, and M. Oppermann. 2003. Analysis of ligand-stimulated CC chemokine receptor 5 (CCR5) phosphorylation in intact cells using phosphosite-specific antibodies. *J Biol Chem.* 278:2190-8.

Polo, S., S. Sigismund, M. Faretta, M. Guidi, M.R. Capua, G. Bossi, H. Chen, P. De Camilli, and P.P. Di Fiore. 2002. A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature.* 416:451-5.

Preininger, A.M., and H.E. Hamm. 2004. G protein signaling: insights from new structures. *Sci STKE.* 2004:re3.

Puthenveedu, M.A., and M. von Zastrow. 2006. Cargo regulates clathrin-coated pit dynamics. *Cell.* 127:113-24.

Raiborg, C., K.G. Bache, D.J. Gillooly, I.H. Madshus, E. Stang, and H. Stenmark. 2002. Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. *Nat Cell Biol.* 4:394-8.

Rappoport, J.Z., A. Benmerah, and S.M. Simon. 2005. Analysis of the AP-2 adaptor complex and cargo during clathrin-mediated endocytosis. *Traffic.* 6:539-47.

Rappoport, J.Z., S. Kemal, A. Benmerah, and S.M. Simon. 2006. Dynamics of clathrin and adaptor proteins during endocytosis. *Am J Physiol Cell Physiol.* 291:C1072-81.

Rappoport, J.Z., and S.M. Simon. 2003. Real-time analysis of clathrin-mediated endocytosis during cell migration. *J Cell Sci.* 116:847-55.

Robinson, M.S. 2004. Adaptable adaptors for coated vesicles. *Trends Cell Biol.* 14:167-74.

Rodal, S.K., G. Skretting, O. Garred, F. Vilhardt, B. van Deurs, and K. Sandvig. 1999. Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell.* 10:961-74.

Rohde, G., D. Wenzel, and V. Haucke. 2002. A phosphatidylinositol (4,5)-bisphosphate binding site within mu2-adaptin regulates clathrin-mediated endocytosis. *J Cell Biol.* 158:209-14.

Rosenkilde, M.M. 2005. Virus-encoded chemokine receptors--putative novel antiviral drug targets. *Neuropharmacology.* 48:1-13.

Rossi, D., and A. Zlotnik. 2000. The biology of chemokines and their receptors. *Annu Rev Immunol.* 18:217-42.

Roth, T.F., and K.R. Porter. 1964. Yolk Protein Uptake In The Oocyte Of The Mosquito Aedes Aegypti. L. *J Cell Biol.* 20:313-32.

Sachse, M., S. Urbe, V. Oorschot, G.J. Strous, and J. Klumperman. 2002. Bilayered clathrin coats on endosomal vacuoles are involved in protein sorting toward lysosomes. *Mol Biol Cell.* 13:1313-28.

Sachse, M., P. van Kerkhof, G.J. Strous, and J. Klumperman. 2001. The ubiquitin-dependent endocytosis motif is required for efficient incorporation of growth hormone receptor in clathrin-coated pits, but not clathrin-coated lattices. *J Cell Sci.* 114:3943-52.

Sanan, D.A., and R.G. Anderson. 1991. Simultaneous visualization of LDL receptor distribution and clathrin lattices on membranes torn from the upper surface of cultured cells. *J Histochem Cytochem.* 39:1017-24.

Santini, F., I. Gaidarov, and J.H. Keen. 2002. G protein-coupled receptor/arrestin3 modulation of the endocytic machinery. *J Cell Biol.* 156:665-76.

Santini, F., R.B. Penn, A.W. Gagnon, J.L. Benovic, and J.H. Keen. 2000. Selective recruitment of arrestin-3 to clathrin coated pits upon stimulation of G protein-coupled receptors. *J Cell Sci.* 113 (Pt 13):2463-70.

Sargiacomo, M., M. Sudol, Z. Tang, and M.P. Lisanti. 1993. Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J Cell Biol.* 122:789-807.

Shenoy, S. K., R. J. Lefkowitz. 2005. Receptor-specific ubiquitination of β-arrestin directs assembly and targeting of seven-transmembrane receptor signalosomes. *J. Biol. Chem.* (280) 15:15315-15324

Schmid, E.M., M.G. Ford, A. Burtey, G.J. Praefcke, S.Y. Peak-Chew, I.G. Mills, A. Benmerah, and H.T. McMahon. 2006. Role of the AP2 beta-Appendage Hub in Recruiting Partners for Clathrin-Coated Vesicle Assembly. *PLoS Biol.* 4.

Scott, M.G., A. Benmerah, O. Muntaner, and S. Marullo. 2002. Recruitment of activated G protein-coupled receptors to pre-existing clathrin-coated pits in living cells. *J Biol Chem.* 277:3552-9.

Segerer, S., K.M. Mac, H. Regele, D. Kerjaschki, and D. Schlondorff. 1999. Expression of the C-C chemokine receptor 5 in human kidney diseases. *Kidney Int.* 56:52-64.

Sever, S., H. Damke, and S.L. Schmid. 2000. Garrotes, springs, ratchets, and whips: putting dynamin models to the test. *Traffic.* 1:385-92.

Sharma, D.K., J.C. Brown, A. Choudhury, T.E. Peterson, E. Holicky, D.L. Marks, R. Simari, R.G. Parton, and R.E. Pagano. 2004. Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. *Mol Biol Cell.* 15:3114-22.

Shenoy, S.K., and R.J. Lefkowitz. 2003. Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. *J Biol Chem.* 278:14498-506.

Shenoy, S.K., and R.J. Lefkowitz. 2005. Seven-transmembrane receptor signaling through beta-arrestin. *Sci STKE.* 2005:cm10.

Shih, W., A. Gallusser, and T. Kirchhausen. 1995. A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes. *J Biol Chem.* 270:31083-90.

Sibley, D.R., R.H. Strasser, J.L. Benovic, K. Daniel, and R.J. Lefkowitz. 1986. Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc Natl Acad Sci U S A.* 83:9408-12.

Sigismund, S., T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P.P. Di Fiore, and S. Polo. 2005. Clathrin-independent endocytosis of ubiquitinated cargos. *Proc Natl Acad Sci U S A.* 102:2760-5.

Signoret, N., T. Christophe, M. Oppermann, and M. Marsh. 2004. pH-independent endocytic cycling of the chemokine receptor CCR5. *Traffic.* 5:529-43.

Signoret, N., L. Hewlett, S. Wavre, A. Pelchen-Matthews, M. Oppermann, and M. Marsh. 2005. Agonist-induced endocytosis of CC chemokine receptor 5 is clathrin dependent. *Mol Biol Cell.* 16:902-17.

Signoret, N., and M. Marsh. 2000. Analysis of chemokine receptor endocytosis and recycling. *Methods Mol Biol.* 138:197-207.

Signoret, N., J. Oldridge, A. Pelchen-Matthews, P.J. Klasse, T. Tran, L.F. Brass, M.M. Rosenkilde, T.W. Schwartz, W. Holmes, W. Dallas, M.A. Luther, T.N. Wells, J.A. Hoxie, and M. Marsh. 1997. Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. *J Cell Biol.* 139:651-64.

Signoret, N., A. Pelchen-Matthews, M. Mack, A.E. Proudfoot, and M. Marsh. 2000. Endocytosis and recycling of the HIV coreceptor CCR5. *J Cell Biol.* 151:1281-94.

Signoret, N., M.M. Rosenkilde, P.J. Klasse, T.W. Schwartz, M.H. Malim, J.A. Hoxie, and M. Marsh. 1998. Differential regulation of CXCR4 and CCR5 endocytosis. *J Cell Sci.* 111 (Pt 18):2819-30.

Simmons, G., P.R. Clapham, L. Picard, R.E. Offord, M.M. Rosenkilde, T.W. Schwartz, R. Buser, T.N. Wells, and A.E. Proudfoot. 1997. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science.* 276:276-9.

Simpson, F., N.K. Hussain, B. Qualmann, R.B. Kelly, B.K. Kay, P.S. McPherson, and S.L. Schmid. 1999. SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nat Cell Biol.* 1:119-24.

Smith, C.J., N. Grigorieff, and B.M. Pearse. 1998. Clathrin coats at 21 A resolution: a cellular assembly designed to recycle multiple membrane receptors. *Embo J.* 17:4943-53.

Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem.* 150:76-85.

Sorkin, A., and M. Von Zastrow. 2002. Signal transduction and endocytosis: close encounters of many kinds. *Nat Rev Mol Cell Biol.* 3:600-14.

Stahelin, R.V., F. Long, B.J. Peter, D. Murray, P. De Camilli, H.T. McMahon, and W. Cho. 2003. Contrasting membrane interaction mechanisms of AP180 N-terminal homology (ANTH) and epsin N-terminal homology (ENTH) domains. *J Biol Chem.* 278:28993-9.

Steyer, J.A., and W. Almers. 1999. Tracking single secretory granules in live chromaffin cells by evanescent-field fluorescence microscopy. *Biophys J.* 76:2262-71.

Stolt, P.C., and H.H. Bock. 2006. Modulation of lipoprotein receptor functions by intracellular adaptor proteins. *Cell Signal.* 18:1560-71.

Stuart, L.M., and R.A. Ezekowitz. 2005. Phagocytosis: elegant complexity. *Immunity.* 22:539-50.

Subtil, A., I. Gaidarov, K. Kobylarz, M.A. Lampson, J.H. Keen, and T.E. McGraw. 1999. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci U S A.* 96:6775-80.

Sun, Y., Z. Cheng, L. Ma, and G. Pei. 2002. Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem.* 277:49212-9.

Tachibana, K., S. Hirota, H. Iizasa, H. Yoshida, K. Kawabata, Y. Kataoka, Y. Kitamura, K. Matsushima, N. Yoshida, S. Nishikawa, T. Kishimoto, and T. Nagasawa. 1998. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature.* 393:591-4.

Tagawa, A., A. Mezzacasa, A. Hayer, A. Longatti, L. Pelkmans, and A. Helenius. 2005. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. *J Cell Biol.* 170:769-79.

Takei, K., V.I. Slepnev, V. Haucke, and P. De Camilli. 1999. Functional partnership between amphiphysin and dynamin in clathrin-mediated endocytosis. *Nat Cell Biol.* 1:33-9.

Tan, C.M., A.E. Brady, H.H. Nickols, Q. Wang, and L.E. Limbird. 2004. Membrane trafficking of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol.* 44:559-609.

Tanabe, K., T. Torii, W. Natsume, S. Braesch-Andersen, T. Watanabe, and M. Satake. 2005. A novel GTPase-activating protein for ARF6 directly interacts with clathrin and regulates clathrin-dependent endocytosis. *Mol Biol Cell.* 16:1617-28.

Tebar, F., S.K. Bohlander, and A. Sorkin. 1999. Clathrin assembly lymphoid myeloid leukemia (CALM) protein: localization in endocytic-coated pits, interactions with clathrin, and the impact of overexpression on clathrin-mediated traffic. *Mol Biol Cell.* 10:2687-702.

Tebar, F., S. Confalonieri, R.E. Carter, P.P. Di Fiore, and A. Sorkin. 1997. Eps15 is constitutively oligomerized due to homophilic interaction of its coiled-coil region. *J Biol Chem.* 272:15413-8.

Tebar, F., T. Sorkina, A. Sorkin, M. Ericsson, and T. Kirchhausen. 1996. Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits. *J Biol Chem.* 271:28727-30.

ter Haar, E., A. Musacchio, S.C. Harrison, and T. Kirchhausen. 1998. Atomic structure of clathrin: a beta propeller terminal domain joins an alpha zigzag linker. *Cell.* 95:563-73.

Tokuyasu, K. 1997. Cells, a laboratory manual. Cold Spring Harbor Laboratory Press. 131.1-131.27 pp.

Trkola, A., C. Gordon, J. Matthews, E. Maxwell, T. Ketas, L. Czaplewski, A.E. Proudfoot, and J.P. Moore. 1999. The CC-chemokine RANTES increases the attachment of human immunodeficiency virus type 1 to target cells via glycosaminoglycans and also activates a signal transduction pathway that enhances viral infectivity. *J Virol.* 73:6370-9.

Ungewickell, E., and D. Branton. 1981. Assembly units of clathrin coats. *Nature.* 289:420-2.

Venkatesan, S., J.J. Rose, R. Lodge, P.M. Murphy, and J.F. Foley. 2003. Distinct mechanisms of agonist-induced endocytosis for human chemokine receptors CCR5 and CXCR4. *Mol Biol Cell.* 14:3305-24.

Vila-Coro, A.J., M. Mellado, A. Martin de Ana, A.C. Martinez, and J.M. Rodriguez-Frade. 1999. Characterization of RANTES- and aminooxypentane-RANTES-triggered desensitization signals reveals differences in recruitment of the G protein-coupled receptor complex. *J Immunol.* 163:3037-44.

Vines, C.M., C.M. Revankar, D.C. Maestas, L.L. LaRusch, D.F. Cimino, T.A. Kohout, R.J. Lefkowitz, and E.R. Prossnitz. 2003. N-formyl peptide receptors internalize but do not recycle in the absence of arrestins. *J Biol Chem.* 278:41581-4.

Wang, J., Y. Wang, and T.J. O'Halloran. 2006. Clathrin light chain: importance of the conserved carboxy terminal domain to function in living cells. *Traffic.* 7:824-32.

Wang, P., Y. Wu, X. Ge, L. Ma, and G. Pei. 2003a. Subcellular localization of beta-arrestins is determined by their intact N domain and the nuclear export signal at the C terminus. *J Biol Chem.* 278:11648-53.

Wang, Y.J., J. Wang, H.Q. Sun, M. Martinez, Y.X. Sun, E. Macia, T. Kirchhausen, J.P. Albanesi, M.G. Roth, and H.L. Yin. 2003b. Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell.* 114:299-310.

Weiss-Haljiti, C., C. Pasquali, H. Ji, C. Gillieron, C. Chabert, M.L. Curchod, E. Hirsch, A.J. Ridley, R.H. van Huijsduijnen, M. Camps, and C. Rommel. 2004. Involvement of phosphoinositide 3-kinase gamma, Rac, and PAK signaling in chemokine-induced macrophage migration. *J Biol Chem.* 279:43273-84.

Wilson, B.S., N. Kapp, R.J. Lee, J.R. Pfeiffer, A.M. Martinez, Y. Platt, F. Letourneur, and J.M. Oliver. 1995. Distinct functions of the Fc epsilon R1 gamma and beta subunits in the control of Fc epsilon R1-mediated tyrosine kinase activation and signaling responses in RBL-2H3 mast cells. *J Biol Chem.* 270:4013-22.

Wilson, B.S., J.R. Pfeiffer, and J.M. Oliver. 2000. Observing FcepsilonRI signaling from the inside of the mast cell membrane. *J Cell Biol.* 149:1131-42.

Wong, W.T., C. Schumacher, A.E. Salcini, A. Romano, P. Castagnino, P.G. Pelicci, and P.P. Di Fiore. 1995. A protein-binding domain, EH, identified in the receptor tyrosine kinase substrate Eps15 and conserved in evolution. *Proc Natl Acad Sci U S A.* 92:9530-4.

Wu, X., X. Zhao, L. Baylor, S. Kaushal, E. Eisenberg, and L.E. Greene. 2001. Clathrin exchange during clathrin-mediated endocytosis. *J Cell Biol.* 155:291-300.

Xu, K., R.M. Williams, D. Holowka, and B. Baird. 1998. Stimulated release of fluorescently labeled IgE fragments that efficiently accumulate in secretory granules after endocytosis in RBL-2H3 mast cells. *J Cell Sci.* 111 (Pt 16):2385-96.

Ybe, J.A., F.M. Brodsky, K. Hofmann, K. Lin, S.H. Liu, L. Chen, T.N. Earnest, R.J. Fletterick, and P.K. Hwang. 1999. Clathrin self-assembly is mediated by a tandemly repeated superhelix. *Nature.* 399:371-5.

Zhang, B., Y.H. Koh, R.B. Beckstead, V. Budnik, B. Ganetzky, and H.J. Bellen. 1998. Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron.* 21:1465-75.

Zhao, J., L. Ma, Y.L. Wu, P. Wang, W. Hu, and G. Pei. 1998. Chemokine receptor CCR5 functionally couples to inhibitory G proteins and undergoes desensitization. *J Cell Biochem.* 71:36-45.

Zheng, J., S.M. Cahill, M.A. Lemmon, D. Fushman, J. Schlessinger, and D. Cowburn. 1996. Identification of the binding site for acidic phospholipids on the pH domain of dynamin: implications for stimulation of GTPase activity. *J Mol Biol.* 255:14-21.

Zhou, Y., T. Kurihara, R.P. Ryseck, Y. Yang, C. Ryan, J. Loy, G. Warr, and R. Bravo. 1998. Impaired macrophage function and enhanced T cell-dependent immune response in mice lacking CCR5, the mouse homologue of the major HIV-1 coreceptor. *J Immunol.* 160:4018-25.

Zlotnik, A. 2006. Chemokines and cancer. *Int J Cancer.* 119:2026-9.

Zlotnik, A., and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity.* 12:121-7.

Zou, Y.R., A.H. Kottmann, M. Kuroda, I. Taniuchi, and D.R. Littman. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature.* 393:595-9.

Appendix