The Human Cytomegalovirus US28 Protein Is Located in Endocytic Vesicles and Undergoes Constitutive Endocytosis and Recycling

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Genes encoding chemokine receptor-like proteins have been found in herpes and poxviruses and implicated in viral pathogenesis. Here we describe the cellular distribution and trafficking of a human cytomegalovirus (HCMV) chemokine receptor encoded by the *US28* gene, after transient and stable expression in transfected HeLa and Cos cells. Immunofluorescence staining indicated that this viral protein accumulated intracellularly in vesicular structures in the perinuclear region of the cell and showed overlap with markers for endocytic organelles. By immunogold electron microscopy US28 was seen mostly to localize to multivesicular endosomes. A minor portion of the protein (at most 20%) was also expressed at the cell surface. Antibody-feeding experiments indicated that cell surface US28 undergoes constitutive ligand-independent endocytosis. Biochemical analysis with the use of iodinated ligands showed that US28 was rapidly internalized. The high-affinity ligand of US28, the CX_3C-chemokine fractalkine, reduced the steady-state levels of US28 at the cell surface, apparently by inhibiting the recycling of internalized receptor. Endocytosis and cycling of HCMV US28 could play a role in the sequestration of host chemokines, thereby modulating antiviral immune responses. In addition, the distribution of US28 mainly on endosomal membranes may allow it to be incorporated into the viral envelope during HCMV assembly.

INTRODUCTION

Chemokine receptors are a subgroup of the superfamily of seven transmembrane domain (7TM) G-protein–coupled receptors (GPCRs) that bind inflammatory chemokines and regulate leukocyte migration (Murphy *et al.*, 2000). Open reading frames (ORFs) with the potential to encode 7TM

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Abbreviations used: BM, binding medium; CCR5, CC-chemokine receptor 5; CHO, Chinese hamster ovary; CXCR4, CXC chemokine receptor 4; DARC, Duffy antigen receptor for chemokines; EM, electron microscopy; FCS, fetal calf serum; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; HA, hemagglutinin; HCMV, human cytomegalovirus; HRP, horseradish peroxidase; Ig, immunoglobulin; ORF, open reading frame; PBS, phosphate-buffered saline; RANTES, regulated on activation normal T cell expressed and secreted; SDF-1, stromal cell-derived factor 1; 7TM, seven-transmembrane domain; YFP, yellow fluorescent protein.

proteins with features similar to chemokine receptors have been identified in the genomes of herpes and poxviruses. Some of these genes have been implicated in pathogenesis and the proteins demonstrated to bind chemokines (Ahuja and Murphy, 1993; Neote *et al.*, 1993; Cesarman *et al.*, 1996; Arvanitakis *et al.*, 1997; Isegawa *et al.*, 1998; Kledal *et al.*, 1998; Milne *et al.*, 2000). The genome of human cytomegalovirus (HCMV) contains four ORFs encoding putative 7TM chemokine receptor *UL33*, *UL78*, *US27*, and *US28* (Chee *et al.*, 1990; Gompels *et al.*, 1995). Although the proteins encoded by these genes are not required for HCMV replication in culture (Mocarski and Kemble, 1996), their importance for virus persistence in vivo has been suggested by gene deletion experiments in related viruses. Mouse and rat CMVs that lack the homologue of HMCV *UL33* (*M33* and *R33*, respectively) are unable to replicate in salivary glands and their dissemination is compromised in the host populations (Davis-Poynter *et al.*, 1997; Beisser *et al.*, 1998).

Although important, little is known about the properties of the HCMV 7TM proteins. As yet only US28 has been characterized pharmacologically. This protein binds with

high affinity to several CC-chemokines such as macrophage inflammatory polypeptide (MIP)-1α, MIP-1β, monocyte chemotactic protein (MCP)-1, and regulated on activation normal T cell expressed and secreted (RANTES) (Neote *et al.*, 1993; Gao and Murphy, 1994; Kuhn *et al.*, 1995) as well as the CX$_3$C-chemokine fractalkine (Kledal *et al.*, 1998). At the cellular level, HCMV infection induces cellular changes, some of which are associated with G-protein signaling (Albrecht *et al.*, 1990). Signal transduction through US28 has been observed as a transient increase in intracellular calcium in response to high CC-chemokine concentrations (Gao and Murphy, 1994; Vieira *et al.*, 1998) and as an activation of the mitogen-activated protein kinase pathway in response to RANTES (Billstrom *et al.*, 1998). Furthermore, expression of US28 induces migration of smooth muscle cells in response to RANTES and MCP-1, implicating HCMV in the pathogenesis of atherosclerosis and restenosis (Streblow *et al.*, 1999). Recently it was shown that US28 displays constitutive signaling activity that can be modulated in part by fractalkine (Casarosa *et al.*, 2001; T. Kledal and T. Schwartz, unpublished data).

In addition to signaling activities, the 7TM proteins of HCMV have also been implicated in immune system evasion. HCMV has developed a number of strategies to escape immune surveillance and to persist in the host after primary infection. These include down-regulation of major histocompatibility complex class I molecules from the cell surface (Grulher and Früh, 2000). Recently it was suggested that the ability of US28 to bind and sequester cellular chemokines from the environment around HCMV-infected cells may also be a strategy to evade the immune system (Bodaghi *et al.*, 1998; Vieira *et al.*, 1998).

In the present study we examined the cellular distribution and trafficking of the HCMV chemokine receptor US28. In contrast to most cellular chemokine receptors that are expressed at the cell surface, we found that US28 was mostly located intracellularly in perinuclear endosomes. Interestingly, previous studies have indicated that HCMV particles complete their assembly and obtain their final envelope by budding into endosomal compartments (Smith and De Harven, 1973; Tooze *et al.*, 1993). Thus, association of US28 with endosomal organelles may facilitate its incorporation into the viral membrane during HCMV assembly. We also found that US28 undergoes rapid constitutive agonist-independent endocytosis, similar to that of activated chemokine receptors, and that the internalized receptors can recycle to the cell surface. This constitutive endocytosis and cycling may play a role in the sequestration of chemokines from the milieu around HCMV-infected cells (Bodaghi *et al.*, 1998; Vieira *et al.*, 1998).

MATERIALS AND METHODS

Reagents

Tissue culture reagents and Nunc tissue culture plastic were from Life Technologies (Paisley, UK), and other chemicals were from Sigma Aldrich (Poole, UK), unless otherwise indicated. Radioactive reagents were from Amersham Pharmacia Biotech (Little Chalfont, UK). Recombinant RANTES (Proudfoot *et al.*, 1996) was provided by Dr. Amanda Proudfoot (Serono Pharmaceutical Research Institute SA, Geneva, Switzerland), and recombinant stromal cell-derived factor 1α (SDF-1α) was provided by Dr. Mike Luther (Glaxo Wellcome, Research Triangle Park, NC). The chemokine domain

corresponding to amino acid 1 through 69 of the CX$_3$C-chemokine fractalkine and the cDNA encoding the US28 chemokine receptor from HCMV Towne strain (GenBank accession number P09704) were provided by Dr. Timothy Wells (Serono Pharmaceutical Research Institute).

Receptor Constructs

The US28 cDNA was inserted into the pEGFP-N1 expression vector (Clontech Laboratories, Palo Alto, CA) with the use of cohesive end ligation. Fusion proteins of the receptor with the yellow fluorescent protein (YFP), the 2 N-terminal immunoglobulin (Ig) domains of CD4 or a hemagglutinin (HA)-tag (the epitope YPYDVPDYA from the influenza virus HA) were generated with the use of polymerase chain reaction and inserted into the pTEJ8 expression vector (Johansen *et al.*, 1990). All receptor constructs were confirmed by sequencing.

Antibodies

Antibodies used in this study were as follows: anti-CD4, Q4120 (Healey *et al.*, 1990) was obtained from the AIDS Reagent Project of the United Kingdom Medical Research Council (Potters Bar, UK); anti-CXC chemokine receptor 4 (CXCR4), 12G5 (Endres *et al.*, 1996) was provided by Dr. James Hoxie (University of Pennsylvania, Philadelphia, PA); anti-CC chemokine receptor 5 (CCR5), MC-5 (Signoret *et al.*, 2000) was provided by Dr. Matthias Mack (Medizinische Poliklinik, Ludwig-Maximilians-University of Munich, Munich, Germany); anti-HA, 12CA5 was provided by Dr. David Drechsel (University College London, London, UK); rabbit anti-green fluorescent protein (GFP) was provided by Dr. David Shima (Imperial Cancer Research Fund, London, UK); anti-CD63, 1B5 was prepared in house (see below); anti-Lgp120, rabbit antibody against human LAMP1 was provided by Dr. Sven Carlsson (University of Umeå, Umeå, Sweden); anti-transferrin receptor H68.4 was purchased from Zymed Laboratories (San Francisco, CA); rabbit antibody against the γ subunit of AP-1 was provided by Dr. Margaret Robinson (University of Cambridge, Cambridge, UK); anti-vesicular stomatitis virus G-protein, P5D4 was provided by Dr. Thomas. Kreis (University of Geneva, Geneva, Switzerland); anti-HCMV-IE1, MAB810 (Chemicon, Temecula, CA); rabbit anti-mouse labeled with Alexa Flour-594 (Molecular Probes, Eugene, OR). Horseradish peroxidase (HRP)-conjugated and other fluorescent second antibody reagents were from Pierce & Warriner (Chester, UK).

Q4120 and MC-5 were ^{125}I-labeled with the use of Bolton and Hunter reagent (Amersham Pharmacia Biotech) as described (Signoret *et al.*, 1997). Specific activities of 400–500 Ci/mmol were obtained for different iodinations. Radioiodinated proteins, diluted in phosphate-buffered saline (PBS) containing 0.25% gelatin and 0.02% NaN$_3$ and stored in small aliquots at $-20°$C, were stable for at least 4 months.

Generation of the Monoclonal Anti-CD63 Antibody Mice were immunized with a heavy membrane fraction from the human epidermoid cell line Hep2. Spleen cells were fused with NS-1 myeloma cells and the fusions were plated into 96-well plates. Wells containing viable hybridomas were screened by immunofluorescence on Hep2 cells. 1B5 was one of several wells that gave strong punctate fluorescence on permeabilized cells. A hybridoma line was cloned by limiting dilution and the cognate antigen identified by Western blotting and cDNA expression as follows: Western blotting of Hep2 cell lysates indicated a broad band of mean molecular mass of approximately 60 kDa, similar to bands recognized by commercial anti-CD63 antibodies (Bowers and Marsh, unpublished results). Expression of the cDNA for human CD63 (kindly provided by Dr. Paul Luzio, University of Cambridge, Cambridge, UK) in Chinese hamster ovary (CHO) cells confirmed that 1B5 recognizes human CD63 (Bowers and Marsh, unpublished results). Subsequent studies indicated that this monoclonal antibody is specific to simian and

human CD63 and recognizes an epitope in the ecto (luminal) domain of this tetraspanin.

Cells

HeLa cells were maintained in DMEM containing 4% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (PenStrep). Cos-7 cells were maintained in DMEM containing 10% FCS, glutamine, and PenStrep as above. Mink Mv-1-Lu cells stably expressing the CD4-chemokine receptor hybrid CD4(2D)CXCR4, consisting of the 2 N-terminal Ig domains of CD4 linked directly to the N terminus of human CXCR4 (Klasse *et al.*, 1999) and CHO cells stably expressing human CCR5 (Mack *et al.*, 1998), were maintained in DMEM containing 10% FCS, glutamine, PenStrep, and 1 mg/ml G418 as above. HeLa and Cos-7 cells were transfected by electroporation (Bio-Rad, Hemel Hempstead, UK). HeLa CD4-US28 stable transfectants were selected in medium containing 1 mg/ml G418, and colonies were screened for CD4-US28 expression by immunofluorescence with the use of Q4120. HeLa CD4-US28 cells were maintained in DMEM containing 4% FCS, glutamine, PenStrep, and G418 as above. Human foreskin fibroblasts were grown in DMEM containing 10% NU-serum I culture supplement (Becton Dickinson, Bedford, MA), 100 U/ml penicillin, and 100 mg/ml streptomycin.

Western Blotting

HeLa cells expressing US28 constructs were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.8, 150 mM NaCl supplemented with 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) containing the protease inhibitors phenylmethylsulfonyl fluoride (at 1 mM) and CLAP (5 μg/ml each of chymostatin, pepstatin A, antipain hydrochloride, and 10 μg/ml leupeptin hemisulfate). After removal of the nuclei and cell debris by centrifugation at 13,000 × g for 10 min at 4°C the lysates were loaded on 10% SDS-polyacrylamide gels under nonreducing conditions without heating. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The blots were incubated in blocking buffer (10% skimmed milk powder, 0.1% Tween-20 in PBS) for 1 h at room temperature. Incubations with primary and secondary antibodies were in blocking buffer for 1 h each at room temperature. To detect CD4-US28, Q4120 (2.5 μg/ml) and HRP-conjugated goat anti-mouse antibodies were used. The rabbit antibody against GFP and HRP-conjugated goat anti-rabbit antibody (both at 1:2000) were used to detect US28-GFP. Blots were developed with the use of enhanced chemiluminescence (Amersham Pharmacia Biotech) and visualized with autoradiography film (Fuji Photo Film, Tokyo, Japan)

Immunofluorescence Microscopy

HeLa cells stably expressing CD4-US28, and HeLa or Cos cells transiently transfected with US28 constructs, were grown on glass coverslips and used 2 d after electroporation. Unless indicated otherwise, cells were first fixed with 3% paraformaldehyde in PBS for 10 min at room temperature, quenched with NH_4Cl, and then stained with appropriate antibodies with or without permeabilization with 0.05% saponin, essentially as described (Signoret *et al.*, 1997). After staining, cells were mounted in Moviol and analyzed with the use of an Optiphot-2 microscope (Nikon, Tokyo, Japan) equipped with an MRC Bio-Rad 1024 confocal laser scanning system. Digital images were transferred to Adobe Photoshop (Adobe Systems, Mountain View, CA) and adjusted so that all intensity values were in the measurable range (0–255 gray levels). Single channel and overlay images were printed directly from Adobe Photoshop.

Immunolabeling of Cryosections for Electron Microscopy

HeLa cells transiently transfected with US28-GFP were grown in 90-mm tissue culture dishes. After 2 d 30–40% of the cells expressed the GFP-tagged proteins, although <10% of the cells showed high expression levels. The cells were fixed by adding an equal volume of prewarmed double-strength fixative (8% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4) directly into the culture medium. After 10 min, the medium was replaced with single-strength fixative (4% paraformaldehyde, 0.05% glutaraldehyde). Fixed cells were washed in PBS containing 20 mM glycine and embedded in 10% gelatin (ICN Pharmaceuticals, Costa Mesa, CA), infiltrated with 2.3 M sucrose, and frozen in liquid nitrogen as described (Raposo *et al.*, 1997). Cryosections (~60 nm thick) were labeled with a rabbit antibody against GFP, and bound antibodies were detected with protein A-gold (EM Lab, Utrecht University, The Netherlands). Sections were examined with an EM420 transmission electron microscope (Phillips, Eindhoven, The Netherlands).

HCMV Infections

Human foreskin fibroblasts grown in chamber slides were transfected with US28-YFP with the use of Superfect (Qiagen, Valencia, CA) and immediately after transfection were infected with the Towne strain of HCMV at a multiplicity of infection of infection unit/cell. At the chosen time points after infection, cells were fixed in 3.7% paraformaldehyde in PBS at room temperature and stained with anti-IE1 antibody (1:1000) after permeabilization in 0.2% Triton X-100 for 20 min on ice. Secondary rabbit anti-mouse antibody labeled with Alexa Flour-594 as used at 1:1000. Cell nuclei were stained with 1 μg/ml Hoechst (Molecular Probes) in PBS. After staining, cells were mounted in FluoroGuard antifade reagent (Bio-Rad, Hercules, CA) and analyzed with the use of an Applied Precision DeltaVision Deconvolution system (Applied Precision, Issaquah, WA) connected to an IX-70 inverted microscope (Olympus, New Hyde Park, NY) with a mercury arc bulb as the illumination source. After image acquisition, the raw data were deconvoluted using the modifications made to the inverse matrix algorithm by Agard and Sedat (1983).

Antibody-feeding and Antibody-recycling Experiments

For antibody-feeding experiments HeLa CD4-US28-expressing cells were grown on coverslips for 48 h. The cells were washed in binding medium (BM: RPMI-1640 without bicarbonate, containing 0.2% bovine serum albumin, 10 mM HEPES, and adjusted to pH 7.4) at room temperature. Subsequently, the cells were incubated in BM containing anti-CD4 Q4120 (~60 nM) at 37°C. After 1 h the coverslips were placed on ice and washed with cold BM. To remove cell surface-bound antibody, the cells were washed twice in BM adjusted to pH 3.0, followed by two 3-min incubations in the same medium, and returned to BM, pH 7.4. The cells were then fixed in PBS containing 3% paraformaldehyde for 10 min, stained with rhodamine-conjugated goat anti-mouse antibody (Pierce & Warriner), with or without permeabilization with 0.05% saponin, and examined by confocal microscopy.

For antibody-recycling experiments HeLa CD4-US28 cells on glass coverslips were incubated in BM with anti-CD4 Q4120 antibody for 1 h, acid washed as above, and returned to pH 7.4. The cells were then reincubated in BM with a biotin-conjugated anti-mouse antibody (Amersham Pharmacia Biotech) at 37°C. After 1 h the coverslips were placed on ice, washed with cold BM, acid washed, and fixed in PBS containing 3% paraformaldehyde. Cells, either intact or permeabilized with 0.05% saponin, were stained with fluorescein-conjugated streptavidin (Amersham Pharmacia

Biotech) for 45 min. Subsequently the coverslips were mounted in Moviol and examined as above.

Endocytosis Assays

Endocytosis assays on adherent cells were performed essentially as described (Pelchen-Matthews *et al.*, 1991). Briefly, cells were seeded in 16-mm wells in 24-well plates and grown for 2 d to a final density of $\sim2.5 \times 10^5$ cells per well. The cells were cooled on ice, washed with DMEM containing 4% FCS, and incubated for 2 h at 4°C with 250 μl of either 0.5 nM ^{125}I-Q4120 antibody or 125 pM ^{125}I-RANTES in DMEM. Subsequently, the cells were washed in DMEM to remove free ligand and then warmed by addition of 1 ml DMEM at 37°C. At the indicated times the cells were returned to 4°C and washed with cold DMEM. For each time point at least four wells were used. For half of the wells, the cells were collected directly in 400 μl of 0.2 M NaOH and transferred to tubes for γ counting (total cell-associated activity). To determine the intracellular activity, the remaining wells were acid washed to remove cell surface ligand (acid-resistant activity). The cells were harvested in NaOH as above. The proportion of internalized activity for each time point was determined by dividing the acid-resistant activity by the total cell-associated activity and endocytic rates were calculated by analysis of the data from the first 5 min of warming.

Down-Modulation Assays

Cells plated in 16-mm wells of a 4-well plate were incubated in DMEM or in DMEM containing Q4120, RANTES, or fractalkine at 37°C as indicated in the text. After treatment, the cells were placed on ice and cooled by addition of 1 ml of ice-cold DMEM and four washes with the same medium. The cells treated with Q4120 were acid washed to remove the cell surface-bound antibody. The cells were returned to pH 7.4 by washes in cold DMEM and then labeled with 250 μl of 0.5 nM ^{125}I-Q4120 for 2 h at 4°C under agitation. Subsequently, the cells were washed again in cold DMEM, and harvested in 400 μl of 0.2 M NaOH, and the bound radioactivity was measured as above.

RESULTS

Subcellular Localization of US28

The chemokine receptor-like putative 7TM proteins encoded by HCMV and related viruses have important, yet poorly understood, roles in viral tropism and pathogenesis. Little is known of where these proteins are located in infected cells or how this distribution is achieved and related to the function of the proteins because few antibodies are available to HCMV 7TM proteins. We used tagged constructs to examine the distribution of the HCMV chemokine receptor US28. GFP was fused to the C-terminal cytoplasmic domain of US28, or epitope tags containing the 2 N-terminal Ig-domains of human CD4 (Klasse *et al.*, 1999) or a nine-amino acid epitope of influenza HA were fused to the extracellular N-terminal domain (Figure 1A). We analyzed the expression of US28-GFP and CD4-US28 in HeLa and Cos cells by Western blot and immunoprecipitation (Figure 1B). Both constructs showed a single band. Under the nonreducing conditions required for CD4 blotting, the electrophoretic mobilities may not precisely reflect the molecular masses. Nevertheless, CD4-US28 and US28-GFP showed a relative mobility corresponding to ~60 kDa. US28-GFP occasionally yielded a higher band at ~120 kDa that may be due to receptor dimerization. Immunoprecipitation of CD4-US28 from ^{35}S-Met–labeled cells produced a band of similar rela-
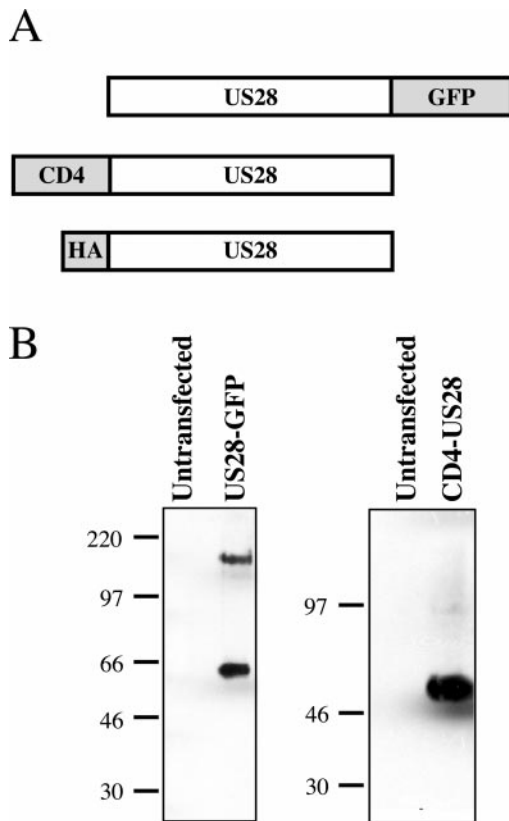

Figure 1. Schematic representation and Western blot of US28 constructs. (A) GFP was linked to the C terminus of US28 (US28-GFP). The two N-terminal Ig domains of CD4 or a nine-amino acid epitope from the influenza virus HA were fused to the N-terminal ectodomain of US28 (CD4-US28 and HA-US28). (B) Western blots of lysates of HeLa cells transiently transfected with US28-GFP or CD4-US28. The CD4 moiety was detected with Q4120 and GFP with a rabbit antibody against GFP. Molecular weight standards (the masses are given in kDa) migrated as indicated on the left. The mass of the US28 moiety predicted from the amino acid sequence is ~41 kDa; two domains of CD4 or the GFP moiety have predicted masses of 22 and 26 kDa, respectively.

tive mobility (Fraile-Ramos and Marsh, unpublished results).

Initial analysis of constructs expressed in Hela or COS cells indicated that the majority of US28-GFP was located intracellularly in punctate structures frequently concentrated on one side of the nucleus (Figure 2A). Little fluorescence was seen at the cell surface. To exclude the possibility that GFP tagging caused mislocalization of US28, the chemokine receptor CXCR4 was similarly tagged (CXCR4-GFP) and expressed in Hela cells and found to be distributed primarily at the cell surface (Fraile-Ramos, Kledal, Schwartz, and Marsh, unpublished results), as previously described (Signoret *et al.*, 1997). In addition, we expressed CD4-US28 and HA-US28. After staining with anti-CD4 or anti-HA antibodies, prominent intracellular staining was observed with both constructs (Figure 2C). Coexpression of CD4-US28 with US28-GFP indicated that both proteins were mostly located within the same cytoplasmic structures. Staining of CD4-
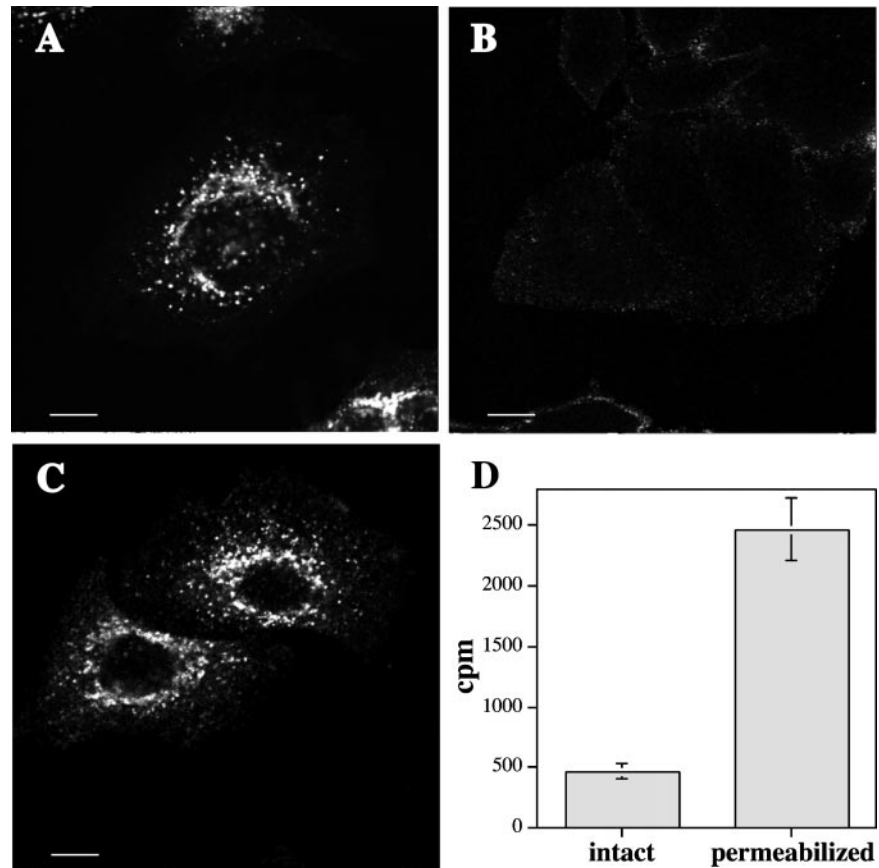
Figure 2. Subcellular localization and quantification of US28. Localization of US28-GFP in transiently transfected HeLa cells (A). HeLa cells expressing CD4-US28 were stained with an antibody against CD4 either without (B) or after permeabilization with saponin (C). Scale bars = 10 μm. (D) ^{125}I-Q4120 was used to quantify CD4-US28 distribution. Intact HeLa CD4-US28 cells were incubated with ^{125}I-Q4120 to determine the protein at the cell surface, and cells permeabilized with saponin were used to determine the total protein present in the cell. HeLa cells, intact or permeabilized with saponin, were incubated with ^{125}I-Q4120 to determine the unspecific binding. The plots show the bound radioactivity as counts per minute per well after subtraction of the nonspecific binding.

US28–expressing cells without permeabilization indicated a low level of the transfected construct on the cell surface (Figure 2B). CD4-US28 showed a similar staining pattern, both after transient transfection and in stably transfected cell lines. Biochemical analysis of Hela CD4-US28 cells with ^{125}I-labeled anti-CD4 antibodies, with or without permeabilization with saponin, indicated that at most 20% of the CD4-US28 construct was present at the cell surface (Figure 2D).

We next investigated where US28 was located by double-label immunofluorescence. US28-GFP–expressing cells were stained with antibodies against the transferrin receptor, or against Lgp120 and CD63, markers for recycling endosomes and late endosomes/lysosomes, respectively. In addition, an antibody against the γ subunit of the AP-1 adaptor complex was used as a marker for the *trans*-Golgi network, although this antibody may also label components of early endosomes (Futter *et al.*, 1998). Significant overlap of US28 with markers for recycling endosomes (Fraile-Ramos and Marsh, unpublished results) and late endosomes/lysosomes was seen, although in all cases US28-containing vesicles that lacked these markers were also observed (Figure 3). Little overlap of US28 with γ-adaptin was seen (Fraile-Ramos and Marsh, unpublished results).

Very similar distributions were seen with US28 proteins carrying markedly different tags, arguing that the distribution is due to intrinsic properties of US28 and is not influenced by tag addition. Together, these results indicate that

the HCMV chemokine receptor US28 was mostly distributed in intracellular organelles, although a minor proportion was seen at the cell surface. Moreover, intracellular US28 showed overlap with markers for the endocytic pathway including early and late endosomes.

Electron Microscopy (EM) Immunolocalization of US28

To identify the distribution of US28 at higher resolution, we studied the GFP chimera by EM of immunolabeled cryosections. HeLa cells transiently transfected with US28-GFP were grown for 2 d before fixation and embedding for immuno-EM. Cryosections were cut and labeled with a rabbit anti-GFP antibody and protein-A gold. Specific labeling for US28-GFP was found to be associated with multivesicular endosomal structures located in the juxtanuclear region of the cell (Figure 4, A and B). Gold particles were found over both the limiting membrane of the multivesicular endosomes and over the internal vesicles. This labeling is very similar to the distribution of the late endosome/lysosome marker CD63, which also labels multivesicular bodies (Figure 4C). Lower levels of US28-GFP were also found at other sites. Gold particles could be observed at the cytoplasmic face of the plasma membrane of US28-GFP–expressing cells. Occasionally, cell surface gold particles could be seen associated with coated pits (Figure 4A), suggesting that the protein may be internalized by the clathrin-mediated path-
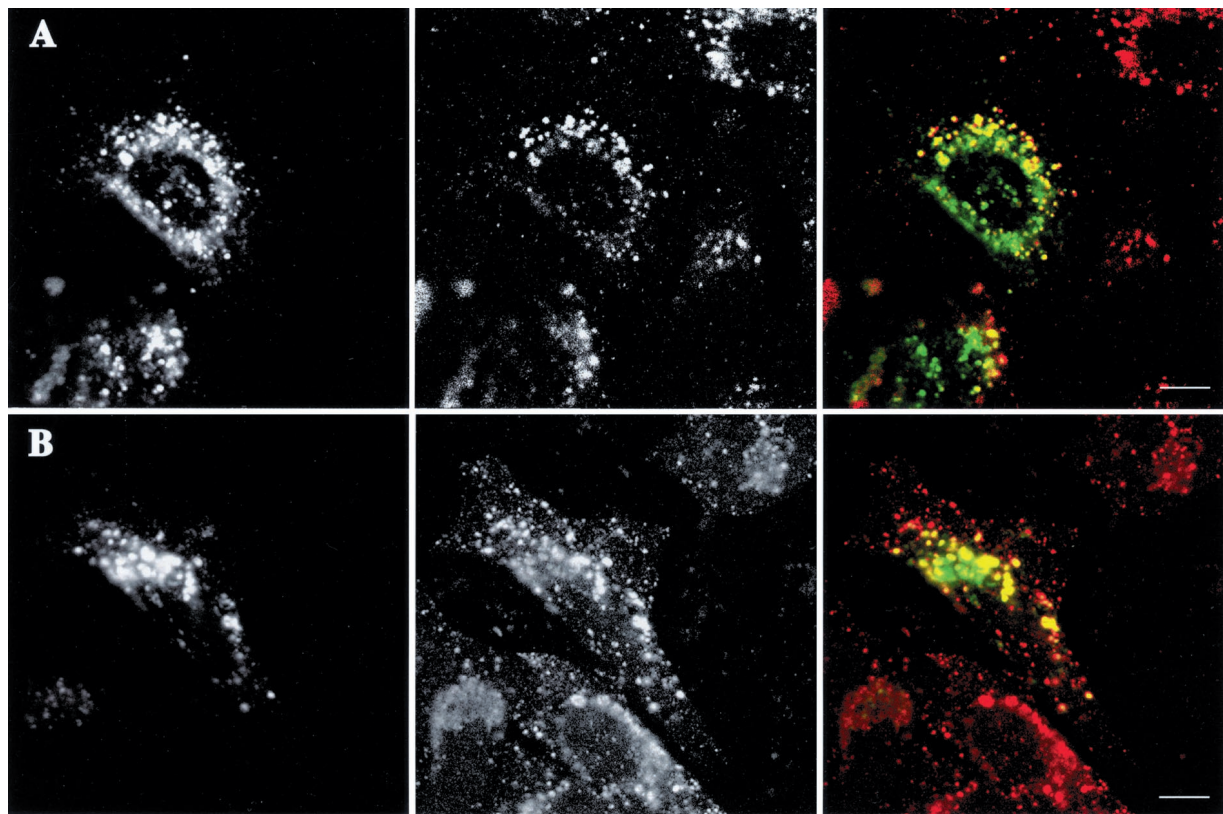
Figure 3. Colocalization of US28 with markers for late endosomes and lysosomes. HeLa cells transfected with US28-GFP (left-hand panels and in green) were stained with an antibody against CD63 and LAMP-1 (A and B, respectively, middle panels and in red), markers for late endosomes and lysosomes. Vesicles positive for both markers are seen in yellow on the right, merged panels. Scale bars, 10 μm.

way. In addition, there was some labeling of small tubules and vesicles, which may correspond to early endosomes.

US28 Undergoes Endocytosis

Although most US28 is found intracellularly, some of the protein is seen at the cell surface as indicated above and by previous pharmacological studies (Neote *et al.*, 1993; Gao and Murphy, 1994; Kledal *et al.*, 1998). To investigate whether cell surface US28 undergoes endocytosis we carried out antibody-feeding experiments. HeLa cells stably expressing CD4-US28 were incubated in medium containing an antibody against CD4, Q4120, at 37°C. Cell surface antibody was removed by acid washing, and antibody uptake was assessed by immunofluorescence staining of permeabilized cells with a fluorescent anti-mouse antibody. After 60 min at 37°C a clear pattern of intracellular vesicular staining was seen (Figure 5A). Staining of cells without permeabilization showed no fluorescence, indicating that little or no antibody remained at the cell surface after the acid wash (Figure 5B). To ensure that the labeling was dependent on CD4-US28–mediated uptake of the antibody, internalization of an irrelevant antibody (anti–VSV-G) was assessed on CD4-US28–expressing cells. In addition, the anti-CD4 and anti–VSV-G antibodies were added to untransfected HeLa cells. No staining of these controls was seen (Fraile-Ramos

and Marsh, unpublished results). These results support the hypothesis that cell surface US28 can undergo endocytosis.

To measure CD4-US28 endocytosis directly we used ^{125}I-Q4120. HeLa CD4-US28 cells were labeled with ^{125}I-Q4120 for 2 h at 4°C, washed, and then warmed to 37°C for various times to allow endocytosis. Antibody remaining at the cell surface was removed by acid wash, and the remaining acid-resistant activity was determined. Control experiments indicated that >95% of the antibody bound to the cell surface at 4°C was eluted under these conditions.

As indicated in Figure 6A bound ^{125}I-Q4120 was internalized rapidly (~7% of the cell surface pool/min, measured over the first 5 min of warm up). After 60 min at 37°C ~90% of the initial cell-associated radioactivity was intracellular. To compare this uptake to the endocytosis of a related 7TM GPCR, internalization of CXCR4 on Mink Mv-1-Lu cells expressing an equivalent CD4-CXCR4 hybrid (CD4(2D)CXCR4) was measured (Klasse *et al.*, 1999). The rapid constitutive US28 endocytosis was faster than the phorbol ester-induced endocytosis of CD4-CXCR4 and was similar to ligand-induced endocytosis of CD4-CXCR4 (Figure 6B), suggesting that the endocytic properties of US28 are similar to those of a ligand-activated receptor.

It has been established that agonist binding can initiate signaling responses and rapid internalization of cell surface chemokine receptors (Pelchen-Matthews *et al.*, 1999). US28
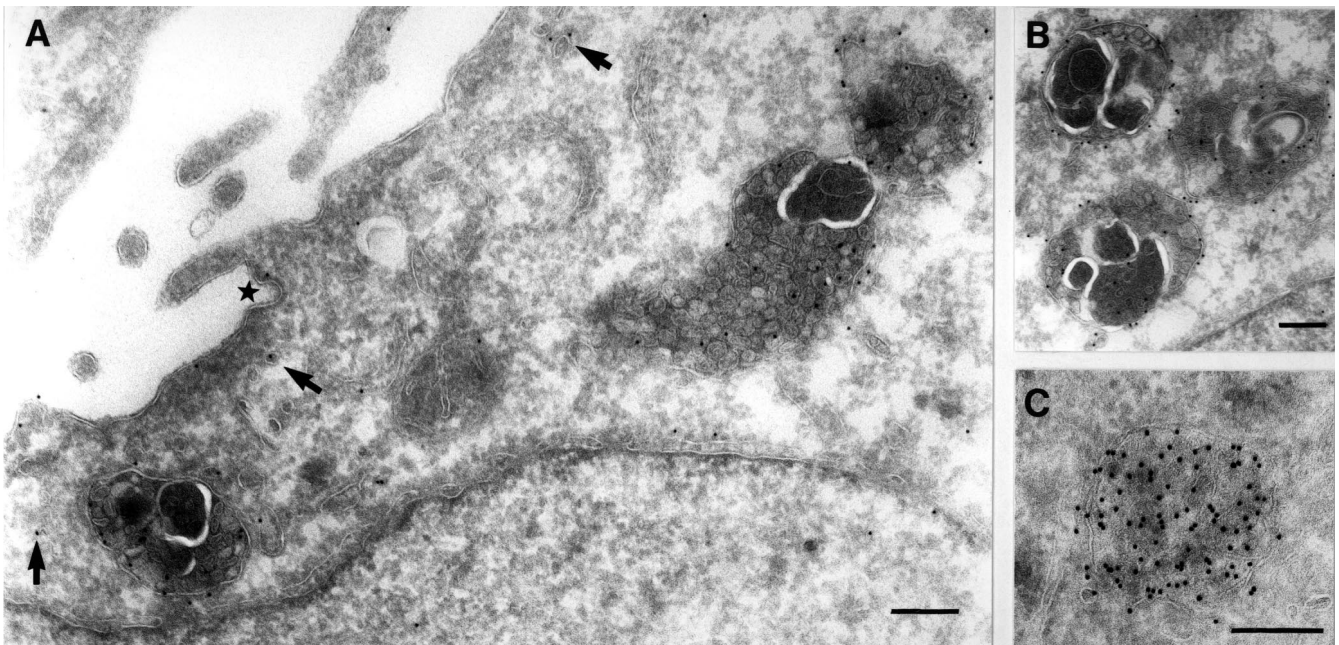
Figure 4. EM immunolocalization of US28. (A and B) Cryosections of HeLa cells transiently expressing US28-GFP were stained with an anti-GFP antibody and 10-nm protein A-gold particles (PAG$_{10}$). Most of the gold particles were found associated with multivesicular endosomes, both over the limiting membrane and over internal vesicles. Some gold particles were also found at the plasma membrane (including coated pits, asterisk in A) and in small tubular structures and vesicles that could correspond to endosomes (arrows in A). (C) Cryosections of HeLa cells stained with the anti-CD63 antibody 1B5 and PAG$_{10}$ also showed enriched labeling of multivesicular bodies. Scale bars, 200 nm.

binds several CC-chemokines and fractalkine with high affinity (Neote *et al.*, 1993; Gao and Murphy, 1994; Kledal *et al.*, 1998). To determine whether the CC-chemokine RANTES induced endocytosis of US28, we used ^{125}I-RANTES (Signoret *et al.*, 2000). HeLa CD4-US28 cells were labeled with 125

pM ^{125}I-RANTES (a concentration at which RANTES shows little binding to cell surface glycosaminoglycans) for 2 h at 4°C, washed, and then warmed to 37°C. The amount of internalized ligand was determined by acid washing the cells with media adjusted to pH 2.0, which eluted >90% of
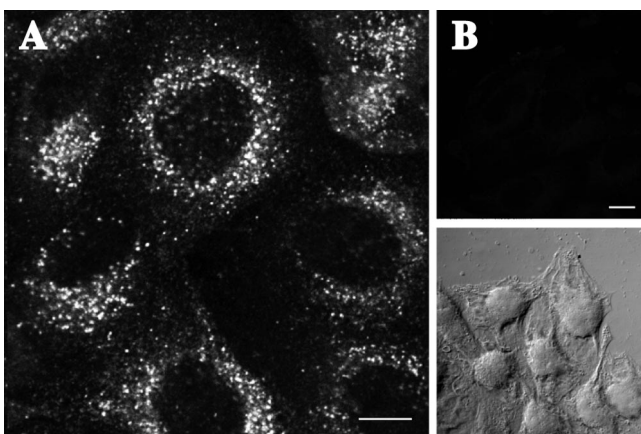

Figure 5. Internalization of US28. HeLa cells stably expressing CD4-US28 were incubated with an antibody against CD4, Q4120, at 37°C for 1 h to allow internalization. After acid washing to remove the antibody bound to the cell surface the cells were fixed. Cells permeabilized with saponin (A) or intact (B, top and bottom show the same field) were stained with a rhodamine-conjugated secondary antibody to detect Q4120. Scale bars, 10 μm.
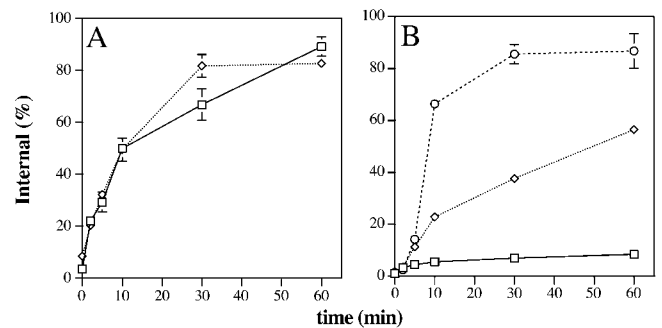

Figure 6. Endocytosis of US28. (A) HeLa CD4-US28 cells were labeled at 4°C with ^{125}I-Q4120 (□) or ^{125}I-RANTES (◇), washed, and warmed to 37°C to allow endocytosis. The amount of internalized ligand was determined by acid washing as described in MATERIALS AND METHODS. (B) Mv-1-Lu cells expressing the hybrid CD4(2D)CXCR4 were labeled at 4°C with ^{125}I-Q4120 for 2 h and then warmed to 37°C in BM (□), BM containing 100 ng/ml phorbol myristate acetate (◇), or 250 nM SDF-1 (○). At the indicated times the cells were cooled and the internalized antibody was determined. The plots show the acid-resistant radioactivity (internal) as a proportion of the total cell-associated activity. All data points show means and SDs for duplicate samples from a representative experiment.
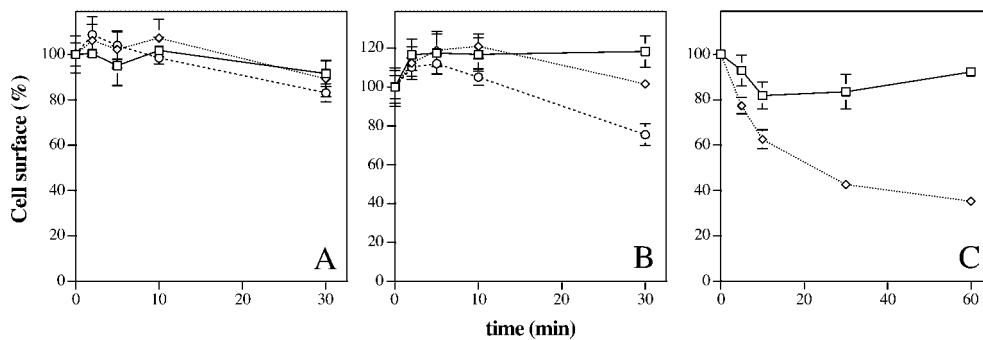
Figure 7. Down-modulation of US28 and CCR5. (A) HeLa CD4-US28 cells were incubated in medium (□), or in medium containing 0.3 nM (◇), or 2.5 nM (○) unlabeled Q4120 antibody at 37°C for the indicated times. The cells were acid washed and the cell surface CD4-US28 molecules were then detected by labeling with ^{125}I-Q4120 antibody for 2 h at 4°C. (B) HeLa CD4-US28 cells were incubated in medium (□), medium containing 100 nM RANTES (◇), or 100 nM fractalkine (○) for up to 30 min at 37°C. At the indicated times the cells were cooled on ice, washed with cold medium, and labeled as in A with ^{125}I-Q4120 to detect cell surface CD4-US28. (C) CHO-CCR5 cells were incubated in medium (□) or medium containing 100 nM RANTES (◇) for the indicated times at 37°C. The cells were cooled on ice, washed with cold medium, and then labeled with ^{125}I-MC-5 at 4°C to determine the cell surface levels of CCR5. Each point shows the mean and SD of triplicate samples from a representative experiment.

the cell surface ^{125}I-RANTES. In HeLa CD4-US28 cells, ^{125}I-RANTES underwent a rapid endocytosis (~6% of the cell surface pool/min) that reached steady state between 30 and 60 min, when ~80% of the initial cell surface pool was inside the cells. The kinetics of US28 endocytosis measured with ^{125}I-RANTES were very similar to those observed for ^{125}I-Q4120 (Figure 6A).

The observation that both ^{125}I-Q4120 and ^{125}I-RANTES were internalized at similar rates suggests that US28 may undergo endocytosis constitutively. To exclude the possibility that the rapid endocytosis of US28 was induced by antibody binding or by the ligand, HeLa CD4-US28 cells were incubated with unlabeled Q4120 or RANTES for up to 30 min at 37°C; at the indicated times the cells were transferred to ice. For cells treated with unlabeled Q4120, antibody remaining at the cell surface was removed by incubating the cells in cold media adjusted to pH 3.0. The cells were then labeled with ^{125}I-Q4120 for 2h at 4°C to determine the level of CD4-US28 remaining at the cell surface. Figure 7A shows that incubation with 0.3 or 2.5 nM Q4120 at 37°C did not induce down-modulation of cell surface US28. Similarly, 100 nM RANTES induced very little down-modulation of US28 (Figure 7B). To ensure that ligand and antibody did not compete for binding to US28, HeLa CD4-US28 cells were incubated with 0.5 nM ^{125}I-Q4120 in the presence or absence of 100 nM RANTES and with 125 pM ^{125}I-RANTES in the presence or absence of 2.5 nM Q4120. Similar levels of Q4120 and RANTES binding, respectively, were observed on both treated and untreated cells (Fraile-Ramos and Marsh, unpublished results). The preparation of RANTES used for these experiments was tested for its ability to down-modulate the human chemokine receptor CCR5. RANTES induced a rapid down-modulation of cell surface CCR5 expressed in CHO cells (Figure 7C).

Together these results indicate that the HCMV chemokine receptor US28 is rapidly endocytosed. This endocytosis is not induced by the presence of the ligand RANTES or by antibody binding, but represents constitutive endocytosis. The kinetics of endocytosis of US28 are similar to those of an activated chemokine receptor. In addition, the failure of RANTES or Q4120 to down-modulate cell surface US28 levels suggests that internalized receptors are recycled to the cell surface.

US28 Recycles to the Plasma Membrane

To observe recycling of internalized US28 we carried out an antibody-feeding experiment in the presence of cycloheximide. HeLa CD4-US28 cells were treated with cycloheximide for up to 2 h to stop the synthesis of new proteins and then incubated with medium containing cycloheximide and ^{125}I-Q4120 for 1 h at 37°C to determine the levels of antibody uptake. Antibody remaining at the cell surface was removed by washing the cells in cold media adjusted to pH 2.0. A second set of cells was treated similarly but without cycloheximide. Treatment with cycloheximide had no significant effect on antibody uptake (Fraile-Ramos and Marsh, unpublished results), suggesting that the steady-state levels of cell surface US28 are maintained through recycling rather than delivery of newly synthesized US28 to the plasma membrane.

Recycling of US28 could also be demonstrated directly. HeLa CD4-US28 cells were incubated with Q4120 for 1 h at 37°C, to allow antibody uptake, and then cooled on ice and acid washed to remove antibody remaining at the cell surface. Figure 8A shows that the antibody was internalized, as indicated by the intracellular staining, and that there was no antibody remaining at the cell surface after the acid wash. A second set of cells was treated similarly but then reincubated with a biotin-coupled secondary antibody against mouse IgG for 1 h at 37°C. If the US28-antibody complex recycled to the cell surface, the secondary antibody would be internalized. After the second incubation, the cells were transferred to ice, acid washed, and stained with fluorescein-streptavidin. As shown in Figure 8B, intracellular vesicular staining indicated that the secondary antibody was internalized, supporting the notion that US28 undergoes constitutive endocytosis and recycling even when conjugated with an anti-CD4 antibody. Cells that were not incubated with primary antibodies showed no fluorescence, indicating that the labeling was dependent on US28-antibody complex–mediated uptake of the secondary antibody.

Fractalkine Down-Modulates US28

Fractalkine has been identified as the preferred ligand for US28 and can modulate the constitutive signaling of this molecule (Casarosa *et al.*, 2001; Kledal *et al.*, 1998; T. Kledal
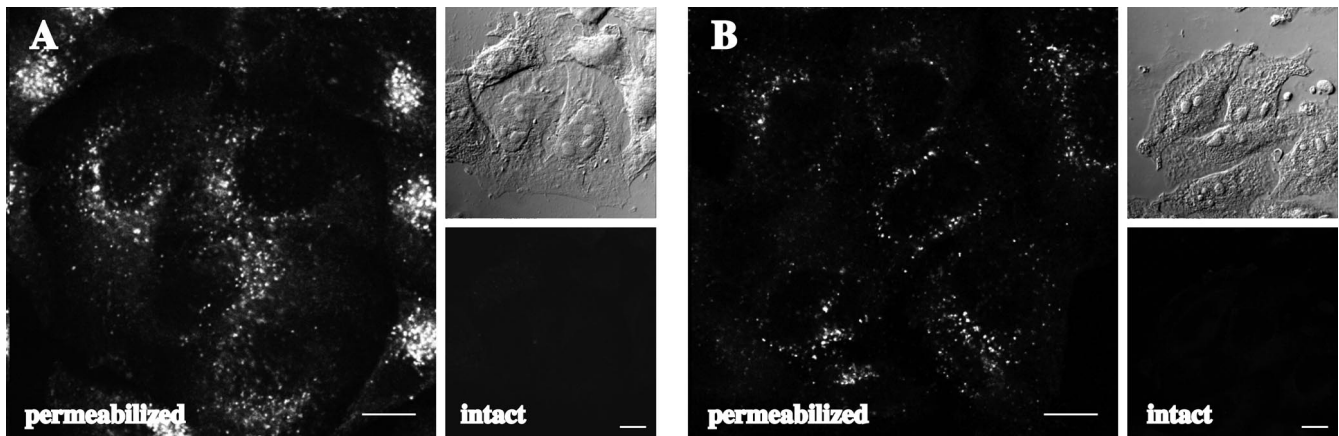
Figure 8. Recycling of US28. HeLa CD4-US28 cells were incubated with Q4120 antibody at 37°C for 1 h to allow antibody uptake, cooled on ice, and washed in acid medium to remove the Q4120 bound to the cell surface. (A) The cells were fixed, left intact or permeabilized with saponin (right and left, respectively), and then stained with a biotin-conjugated secondary antibody to detect Q4120 followed by fluorescein-streptavidin. (B) The cells were reincubated with a biotin-conjugated secondary antibody for 1 h at 37°C, cooled on ice, acid washed, fixed, and stained with fluorescein-streptavidin as in A. Scale bars, 10 μm. The righthand fields for each panel show Nomarski and fluorescence images of the same field.

and T. Schwartz, unpublished data). We investigated whether fractalkine could induce endocytosis of its receptor by using CD4-US28, ^{125}I-Q4120, and prebound unlabeled fractalkine. To test the preparation of fractalkine we first determined its ability to compete with ^{125}I-RANTES for binding. HeLa CD4-US28 cells were incubated with 125 pM ^{125}I-RANTES in the presence of increasing concentrations of fractalkine for 2 h at 4°C and washed, and the associated radioactivity was determined. ^{125}I-RANTES binding was reduced ~50% with 0.5 nM fractalkine and ~90% with 100 nM fractalkine, as previously observed (Kledal *et al.*, 1998). In addition, control experiments demonstrated that fractalkine did not compete with ^{125}I-Q4120 for binding to CD4-US28. Similar levels of Q4120 binding were observed on HeLa CD4-US28 cells treated with 100 nM fractalkine or on untreated cells (Fraile-Ramos and Marsh, unpublished results).

To determine whether fractalkine induced endocytosis of US28, HeLa CD4-US28 cells were labeled with ^{125}I-Q4120 and 100 nM fractalkine for 2 h at 4°C, washed, and then warmed to 37°C. The amount of internalized ligand was determined by acid washing, as described above. CD4-US28 underwent rapid endocytosis in the presence of fractalkine. However, the kinetics of uptake were identical to those observed in its absence, and they were almost identical to those observed in the presence of RANTES (Figure 9), indicating that prebinding of fractalkine did not influence the constitutive endocytosis of US28.

Finally, we investigated whether fractalkine can induce down-modulation of its receptor. HeLa CD4-US28 cells were incubated in 100 nM fractalkine for up to 30 min, transferred to ice, washed, and labeled with ^{125}I-Q4120 to measure the level of receptor at the cell surface. Figure 7B shows that incubation in fractalkine induced some down-modulation of cell surface US28 (25% in 30 min). The levels of cell surface US28 continued to decrease with prolonged incubations, with 60% of the initial levels remaining by 2 h of treatment. Together these results indicate that fractalkine does not in-

fluence the rapid endocytosis of US28 and that the observed down-modulation of US28 by this ligand may be due to an inhibition of recycling.

DISCUSSION

The HCMV genome contains four ORFs with the potential to encode proteins with homology to 7TM GPCRs, namely, *UL33*, *UL78*, *US27*, and *US28* (Chee *et al.*, 1990; Gompels *et al.*, 1995). UL33 and US28 transcripts can be detected at early
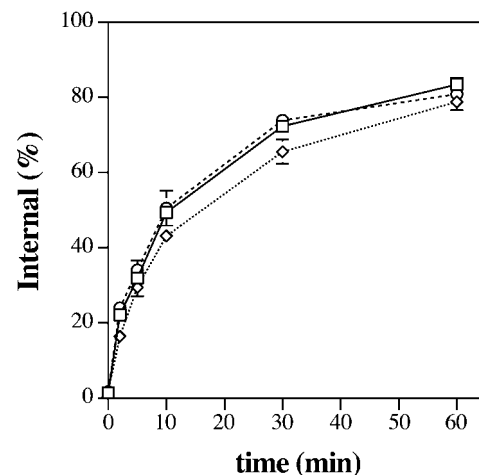

Figure 9. Endocytosis of US28 in the presence of ligands. HeLa CD4-US28 cells were labeled with ^{125}I-Q4120 in medium ($\square$), medium containing 100 nM RANTES ($\diamond$), or 100 nM fractalkine ($\bigcirc$) for 2 h at 4°C, washed, and then warmed to 37°C. The amount of internalized antibody was determined by acid wash. All data points show means and SDs for duplicate samples from a representative experiment.
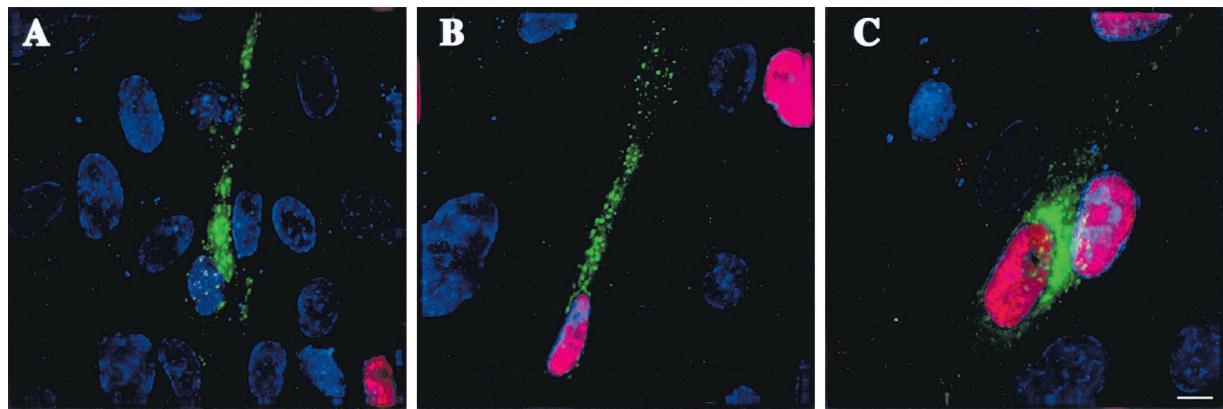
Figure 10. Intracellular localization of US28 in HCMV-infected fibroblasts. Localization of US28-YFP in transiently transfected human foreskin fibroblasts, infected with the Towne strain of HCMV. Antibody against HCMV IE1 used to detect HCMV infected cells is seen as red nuclear staining. Hoechst staining of all nuclei is seen in blue. (A–C) US28-YFP (green) is seen as mainly punctate intracellular staining in both uninfected (A) and HCMV-infected fibroblasts at both 24 h (B) and 48 h (C) postinfection. Scale bar, 10 μm.

and late times after infection, but US27 is detected only at late times postinfection (Welch *et al.*, 1991; Bodaghi *et al.*, 1998; Vieira *et al.*, 1998; Zipeto *et al.*, 1999). Very little is known about *UL78*. Although homologues have been found in both mouse and rat CMVs, the function of these proteins remains unclear. Knockout studies indicate that these proteins are not essential for viral replication in culture but may have some role in viral tropism in vivo (Davis-Poynter *et al.*, 1997; Beisser *et al.*, 1998). US28 binds chemokines and has been proposed to modulate anti-CMV immune responses by clearing host chemokines from the environment around infected cells (Bodaghi *et al.*, 1998; Vieira *et al.*, 1998). In addition, the suggestion that these proteins are incorporated into the viral membrane may also indicate some role in cell-cell transmission (Margulies *et al.*, 1996; Kledal *et al.*, 1998). Here we have studied the cellular distribution and trafficking of US28, with a view to better understanding the function of this protein in HCMV replication.

When expressed in the absence of other HCMV proteins, tagged US28 molecules were located mostly intracellularly. We attempted to identify this localization with the use of markers for various cellular compartments. This demonstrated a significant overlap with markers for early endosomes and late endosomes/lysosomes, suggesting that this viral protein is located at least in part in the endocytic pathway. We also studied the distribution of US28-GFP by EM of immunogold labeled ultrathin frozen sections. The protein was seen to be associated with multivesicular bodies that have the characteristics of late endosomes and can be labeled with antibodies to the late endosome markers CD63 and lyso bis-phosphatidic acid (Kobayashi *et al.*, 1998). Labeling of US28-GFP was seen on both the limiting membrane of these structures and on the internal vesicles. In addition, US28-GFP was located to some extent at the plasma membrane and in small tubules and vesicles that could correspond to early endosomes. Although we have not yet been able to observe native US28, we have no evidence that the tags applied to the molecules used here significantly affect the distribution of the protein. Molecules tagged in both the cytoplasmic C-terminal domain and in the extracellular N-terminal domain showed an identical cellular distribution.

Moreover, both long (CD4 domains 1 and 2) and short (HA-epitope) N-terminally tagged US28 molecules have indistinguishable properties. Together, these observations indicate that US28 is located primarily in endosomal organelles. This distribution is markedly different from most cellular chemokine receptors, which frequently appear at the cell surface (Amara *et al.*, 1997; Signoret *et al.*, 1997). Redistribution of these receptors to endosomes occurs only when expressing cells are bound by agonists (Amara *et al.*, 1997; Signoret *et al.*, 1997). In addition, the distribution of US28 is distinct from that of another viral chemokine receptor, ORF74 from human herpesvirus 8, which is located at the cell surface (A. Fraile-Ramos, T.W. Schwartz and M. Marsh, unpublished observation).

The distribution we describe for US28 is based on transient and stable expression of tagged-proteins in the absence of other viral transcripts and illustrates the intrinsic trafficking properties of the protein. Whether this reflects the properties of US28 in HCMV-infected cells is currently unclear. Infection by HCMV, like that of a number of other viruses, may interfere with trafficking of cellular proteins or with the organization of cellular compartments (Fish *et al.*, 1996; Sanchez *et al.*, 2000a). Moreover, the interaction with tegument components might influence the sorting properties of viral envelope proteins (Sanchez *et al.*, 2000b). However, when US28-YFP was expressed in human foreskin fibroblasts and these cells were then infected with HCMV, the distribution of US28-YFP at 24 and 48 h postinfection was similar to that seen in uninfected cells (Figure 10). Thus, US28 may also be located in endocytic compartments in HCMV-infected cells.

Although US28 is mostly intracellular, it is also found on the surface of both transfected (Pleskoff *et al.*, 1998; Streblow *et al.*, 1999; Ohagen *et al.*, 2000) and HCMV-infected cells (Michelson *et al.*, 1997; Billstrom *et al.*, 1998; Bodaghi *et al.*, 1998; Vieira *et al.*, 1998). We found that this cell surface US28 undergoes rapid constitutive endocytosis and recycling. The rate of internalization was ~7% of the cell surface pool per minute, and after 60 min up to 90% of the initial surface pool was intracellular. These internalization properties are similar to those of activated chemokine receptors. We previously

demonstrated that both phorbol esters and SDF-1 induce endocytosis of the cellular chemokine receptor CXCR4 (Signoret *et al.*, 1997). The kinetics of US28 endocytosis were similar to those seen for SDF-1–induced internalization of CXCR4 and significantly faster than phorbol ester-induced uptake. The rapid endocytosis of US28 was not affected by the ligand RANTES or by binding of the bivalent tracer antibody Q4120, which has the potential to cross-link US28 receptors. The fact that cells maintain a constant level of US28 at the cell surface, while constitutive endocytosis occurs, suggests that internalized US28 is recycled. Cycloheximide treatment did not significantly deplete cell surface US28. Moreover, recycling could be demonstrated directly in antibody-feeding experiments. Interestingly, antibody molecules internalized on CD4-US28 can be seen in multivesicular endosomes by immunolabeling cryosections of antibody-treated CD4-US28 cells (A. Fraile-Ramos, A. Pelchen-Matthews and M. Marsh, unpublished observations), suggesting that the multivesicular body pool of US28 may be part of the recycling itinerary. In this respect the recycling pathway of US28 may be similar to that described for the lysosomal tetraspanin CD63, which is also found on the internal membranes of multivesicular bodies but is able to recycle via the plasma membrane (Arribas and Cutler, 2000; Kobayashi *et al.*, 2000). Whether most of the newly synthesized US28 is delivered to the plasma membrane and then internalized, or whether the protein is directly targeted to endosomal compartments and then cycles to the cell surface, is not yet established.

We have previously shown that US28 binds fractalkine with high affinity (Kledal *et al.*, 1998) and that fractalkine partially inhibits the constitutive activity of US28 (Casarosa *et al.*, 2001; T. Kledal and T. Schwartz, unpublished data). Here we found that the isolated extracellular domain of fractalkine can induce some down-modulation of cell surface US28. Fractalkine did not affect the kinetics of US28 endocytosis, suggesting that down-modulation may be due to an inhibition of US28 recycling. The fact that fractalkine only partially inhibits US28 signaling may be a consequence of the cellular distribution of the protein, with only a portion of the receptor being available for ligand binding.

It was previously shown that US28 internalizes extracellular chemokines, suggesting that this viral chemokine receptor may be able to sequester CC-chemokines from the environment of HCMV-infected cells (Bodaghi *et al.*, 1998; Vieira *et al.*, 1998). Here we demonstrated directly that ^{125}I-RANTES is also rapidly internalized. Constitutive endocytosis will probably occur for any ligand that binds US28. Once internalized, the ligand may dissociate from the receptor in early or late endosomes and eventually be degraded. The Duffy antigen receptor for chemokines (DARC) has been shown to be a promiscuous receptor that binds both CXC and CC-chemokines with high affinity. DARC is expressed on red blood cells, endothelial cells of postcapillary venules, and Purkinje cells of the cerebellum (Hadley and Peiper, 1997). Although DARC on red blood cells does not internalize, there is evidence that DARC-transfected nonerythroid cells can internalize chemokines (Peiper *et al.*, 1995). The physiological role of this chemokine receptor is not clearly defined, but it has been postulated that DARC also acts as a sink or clearance mechanism for the chemokines that it binds (Darbonne *et al.*, 1991). Whether DARC is internalized

and recycled in a manner similar to that described here for US28 remains to be determined.

The molecular mechanisms of US28 endocytosis and intracellular trafficking remain to be elucidated. Clathrin-mediated endocytosis has been implicated in the internalization of some cellular GPCRs (Lin *et al.*, 1998). This internalization is dependent on phosphorylation of serine residues in the cytoplasmic tail of the receptor and at least in some cases involves interaction with nonvisual arrestins (Ferguson *et al.*, 1996; Goodman *et al.*, 1996). US28 has a Ser-rich C-terminal domain, and we have occasionally seen US28 in coated pits on immunolabeled cryosections. Our kinetic data indicate that the properties of US28 endocytosis are similar to those of an agonist-treated GPCR; however, it remains unclear whether the clathrin-mediated route is the principal pathway for US28 uptake, whether arrestins play a role, or whether US28 is phosphorylated.

The assembly of HCMV particles is a complex and poorly understood process. One model proposes that the viral core is assembled in the nucleus of the infected cell and is then released into the cytoplasm. This release involves budding through the inner nuclear membrane and fusion with the outer nuclear/endoplasmic reticulum membrane. In the cytoplasm the core associates with proteins that form the tegument and then buds through a membrane system into the lumen of an as yet poorly characterized compartment. Various locations have been proposed for the final step in herpes virus envelopment including Golgi and post-Golgi membranes (Browne *et al.*, 1996; Whiteley *et al.*, 1999). Previous EM studies of HCMV assembly showed viral particles budding into cytoplasmic vacuoles including multivesicular bodies (Smith and De Harven, 1973), and it has been proposed that the HCMV particles can be wrapped by endosomal membranes (Tooze *et al.*, 1993). Importantly, the HCMV 7TM protein UL33 has also been found to accumulate intracellularly in the perinuclear region of the infected cell (Margulies *et al.*, 1996), and both UL33 and US27 have been found to be incorporated into the viral envelope (Margulies *et al.*, 1996; H. Browne perssonal communication). Because there are as yet no antibodies available against US28 it has not been possible to demonstrate that this protein is incorporated into the viral envelope. However, we have located UL33 and US27 in endosomal membranes by immunofluorescence. When US27 and US28 were coexpressed, their distributions showed a significant overlap and UL33 was also seen to localize to multivesicular endosomes by EM (A. Fraile-Ramos, A. Pelchen-Matthews, T. W. Schwartz and M. Marsh, unpublished observations). Together these observations suggest that the localization of UL33, US27, and US28 to endosomes may allow these proteins to be incorporated into the viral membrane during the final stages of HCMV assembly. This viral US28 may have some propensity to bind fractalkine on target cells (Kledal *et al.*, 1998) and would be inserted into the plasma membrane of the target cell after fusion. Whether this protein is responsible for cellular changes associated with G protein signaling (Albrecht *et al.*, 1990) remains to be established. The possibility that other proteins, including the viral glycoproteins involved in fusion and entry, are located on endocytic membranes also remains to be established.

ACKNOWLEDGMENTS

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