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Regulation of Gene Expression and Survival in Cellular Stress

A thesis submitted to the University of London

By Sadia Janjua

Institute of Child Health
University College London

In fulfilment of the requirements for the degree of Doctor of Philosophy

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
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Abstract

All organisms have developed regulated mechanisms to maintain homeostasis. At the cellular level, this normal functioning of cells is regulated by expression of regulatory genes that are required for normal cell function. Most cells in multicellular organisms are capable of altering gene expression in response to extracellular signals such as elevated temperature, ischaemia/reperfusion, inflammation, infection, cytokines, and amino acid analogues.

In this thesis the effects of cellular stresses in the form of elevated temperature or simulated ischaemia have been investigated. Previous studies show that elevated temperature or simulated ischaemia can induce expression of heat shock proteins (Hsps) in order to prevent misfolding of cellular proteins. Moreover, it has been shown that the stress responsive transcription factor heat shock factor-1 (HSF-1) is phosphorylated and translocates to the nucleus to bind to heat shock elements within hsp gene promoters. In addition, HSF-1 can interact with other transcription factors such as the signal transducer and activator of transcription-1 (STAT-1), which is a latent cytoplasmic transcription factor activated in response to regulatory cytokines such as interferon γ (IFNγ).

Preliminary data shows that elevated temperature can induce expression of Hsp90 in the STAT-1 deficient cell line (U3A) treated with IFNα (activates STAT-1 and STAT-2), but reduces the levels of Hsp90 expression in the U3A cell line treated with IFNα and IFNγin combination. These findings suggest that there may be competition between STAT-1 homodimers and STAT-1/STAT-2 heterodimers and will require further investigation

The STAT-1 transcription factor has previously been demonstrated to play a role in stress- induced apoptosis. In this study, STAT-1 is shown to be required for stress-induced apoptosis using the STAT-1 deficient U3A cell line. Cells lacking STAT-1 show reduced cell death/apoptosis in response to elevated temperature or simulated ischaemia. However, expression of STAT-1 in these cells restores

sensitivity to stress-induced death. The C-terminal domain alone of STAT-1 is also able to enhance stress-induced cell death, and may be acting via a novel co-activator-type mechanism.

Many protective agents have been identified that are able to reduce cell death due to ischaemic injury. Cardiotrophin-1 (CT-1), a member of the IL-6 family of cytokines, has been shown to protect rat neonatal cardiomyocytes subjected to simulated ischaemia via the p42/p44 MAPkinase and PI-3 Kinase pathways. In addition, the unrelated peptide urocortin (Ucn) also protects cardiomyocytes via the same pathway as CT-1 in response to simulated ischaemia and both CT-1 and Ucn induce Hsp expression. In this study, Ucn has been shown to be able to induce enhanced expression of CT-1 at mRNA and protein levels in response to simulated ischaemia. Moreover, the effect is mediated by activation of the CT-1 promoter and requires the transcription factor C/EBPβ /NFIL-6. This finding indicates that a common pathway exists for these two protective agents with Ucn inducing CT-1 synthesis.

Overall, the work performed indicates that multiple interacting pathways modulate the cellular stress response with either protective or damaging effects.

Aims

The aim of this thesis is to provide an insight into various mechanisms in different cell types in which protective or damaging effects are induced in response to stresses including heat shock, hypoxia, and ischaemia.

Firstly, the STAT-1 transcription factor will be investigated for its role in stress-induced apoptosis. This study will demonstrate that the C-terminal activation domain is required for stress-induced apoptosis. This would show that STAT-1 can be targeted in order to protect cells from stress.

In the second study, STAT-1 will be investigated for its role in Hsp induction. This study will demonstrate that STAT-1 is able to activate the Hsp90 promoter in response to heat shock.

In the third study, the aim is to investigate whether the cardioprotective agent Ucn can induce the expression of CT-1 at the promoter and protein levels. Both these factors induce hsp expression. This study would demonstrate that Ucn is able to induce direct expression of CT-1 in response to hypoxic stress, and that activation of the CT-1 promoter is mediated in part by the C/EPBβtranscription factor.

One of the purposes of the study is to investigate whether urocortin, a protective agent, can enhance the expression of the CT-1 promoter and the possible pathway of activation that may be involved since both these different molecules signal via a common pathway and protection by Ucn requires protein synthesis whereas CT-1 does not.

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Declaration

The work presented in this thesis is the work of Sadia Janjua. Contributions by other researchers are acknowledged in the text.

Publications

Janjua S., Stephanou A., and Latchman D.S. (2002). The C-terminal activation domain of the STAT-1 transcription factor is necessary and sufficient for stress-induced apoptosis. *Cell Death and Differentiation* 9:1140-1146.

Janjua S., Lawrence K., Ng L.L., and Latchman D.S. (2003). A common pathway for two cardioprotective agents:Urocortin induces expression of CT-1. Cardiovascular Toxicology 3(3):255-261.

The STAT-1 paper was presented as a poster at the Keystone symposium for Jaks and STATs in Utah, USA, in January 2003.

Table of Contents

Abbreviations

STAT	Signal Transducer and Activator of Transcription
MAPK	Mitogen Activated Protein Kinase
JAK	Janus Kinase
GAS	Gamma Activated Sequence
IL-6	Interleukin 6
Th2	T Helper 2
SH2	Src Homology Domain
CBP	CREB Binding Protein
TNF-α	Tumour Necrosis Factor α
MCM5	Microchromosome Remodelling Factor
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
cDNA	Complementary Deoxyribonucleic Acid
CT-1	Cardiotrophin-1
PBS	Phosphate Buffered Saline
FCS	Fetal Calf Serum
DMEM	Dulbecco's Modified Eagle Medium
HSP	Heat Shock Protein
HSF	Heat Shock Factor
CRF	Corticotrophin Releasing Factor

CHAPTER 1.

INTRODUCTION

INTRODUCTION

1.0 Signal Transduction and Control of Gene Expression

The ability of organisms to respond to the extra-cellular environment requires highly sophisticated responses at the intracellular level, and is maintained by the control of gene expression as well as by signal transduction. Moreover, the control of gene expression, when it occurs, where and how, is critically dependent on signal transduction by extra-cellular molecules and the intensity of the signal, which may tip the cell towards death or survival.

In eukaryotes, RNA polymerase II initiates the start of the transcription process to produce messenger RNAs (mRNAs). However, before RNA polymerase initiates, other proteins are recruited such as general transcription factors, co-activators, chromatin remodellers, histone acetylases, kinases, deacetylases and methylases (Malik and Roeder, 2000; Naar et al., 2001). These proteins are crucial components of the transcription initiation complex and are required in addition to RNA polymerase to produce the primary messenger RNA transcript.

Genes are expressed at widely varying levels. There are two modes of expression of a gene. Constitutively expressed genes (housekeeping genes) are expressed at all times and the corresponding protein is essential for all cells. Alternatively, some genes are expressed in specific tissues, or in response to a particular stimulus, and are not required by all cells.

Regulation of these genes is highly dependent on a large number of proteins, known as transcription factors, which have a DNA binding domain to recognize and bind gene specific sequences directly, and a transcriptional activation domain. Transcription factors also control the transcription of house keeping genes also, but do not require regulation.

Subsequently, co-activator proteins are recruited by these site-specific transcription factors to initiate gene-specific transcription. As many as 2000 proteins may be involved in such transcriptional responses, and having such a large variety of proteins means that the activation of genes is specific and can be activated at the required time. It has been shown in various studies that more than six proteins can act in concert to activate specific transcription of a gene, and can act on one enhancer to regulate gene specific transcription (Grosschedl, 1995).

Transcription of a gene can be repressed by factors either indirectly or directly. Indirect repression involves an inhibitor interfering with the action of an activator. Inhibition of an activator can occur in several ways: (1) Inhibition of an activator can occur by the inhibitor masking the DNA binding site of the activator. The negatively acting factor effectively inhibits gene activation, which can be achieved by the negatively acting factor binding to the same site as the positively acting factor but failing to activate transcription.

The inhibitor can also act indirectly by reorganizing the nucleosome arrangement of the chromatin, therefore the binding site is masked by a nucleosome, which effectively represses gene activation; (2) Activator binding can also be inhibited by formation of a

non-DNA-binding complex with the activating factor; (3) Inhibition of the activator can be achieved by quenching the activator. This involves the inhibitor interfering with transcriptional activation by a DNA bound factor and neutralizing the effect of the activator (4) The action of the repressor can promote the degradation of the activator following repressor binding.

An inhibitory effect of a repressor can also inhibit transcription directly by interacting with the basal transcription complex to reduce its activity. Some factors that are direct repressors have been shown to bind specific DNA binding sites within their target genes and reduce the activity of the basal transcriptional complex. Inhibitory factors can also bind to the basal complex itself by protein-protein interaction therefore interfering with its activity or assembly (Latchman, 1998; 2001).

A large group of positive-acting eukaryotic transcription factors have recently been identified and can be either constitutive nuclear factors or regulatory factors depending upon their function. A general tree diagram can be seen in figure 1.0

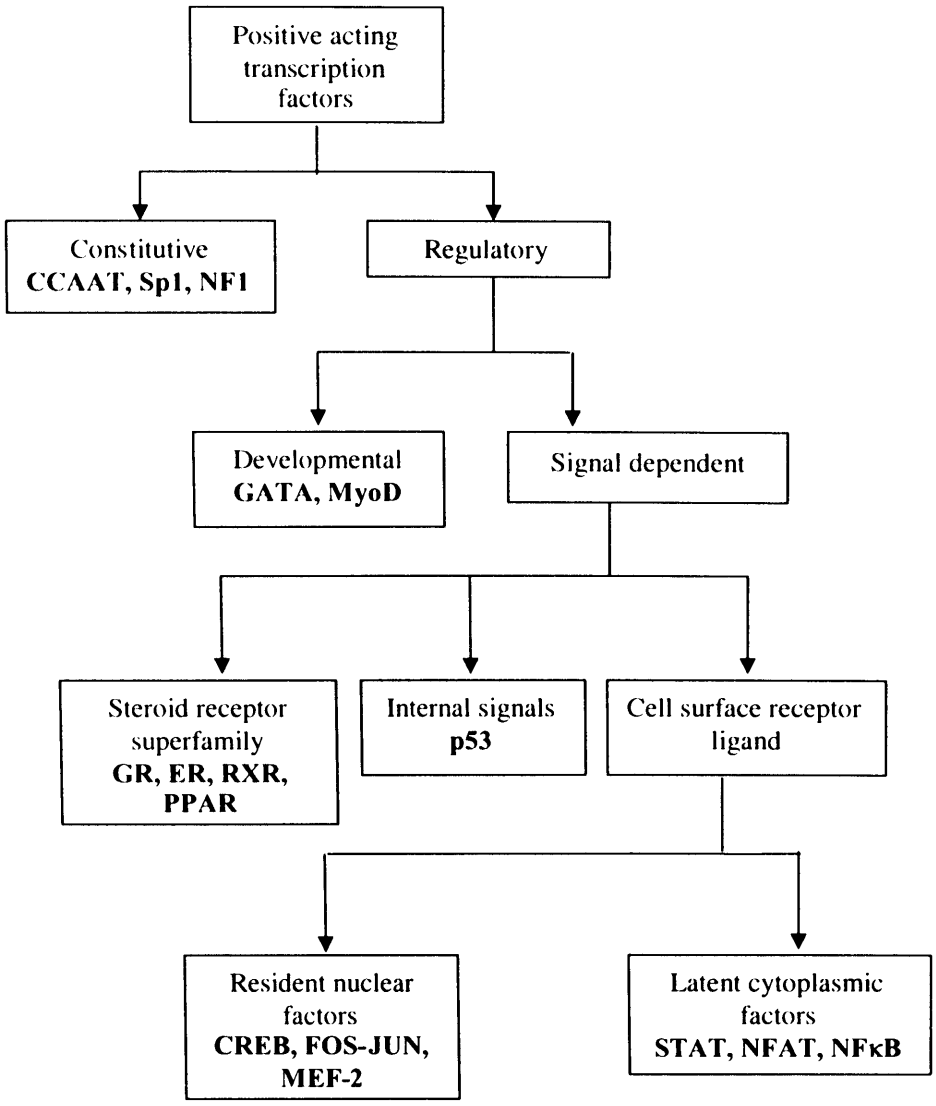


Figure 1.0 Classification of Positive Acting Transcription Factors

1.0.1 Constitutive Transcription Factors

The constitutively active nuclear factors for example, CCAAT binding protein, Sp1, and NF1 are DNA binding proteins and are present in the nucleus all the time (Chodosh et al., 1988; Briggs et al., 1986; Johnson and McKnight, 1989; Rosenfeld and Kelly, 1986) These factors are known to facilitate transcription of house keeping genes including actin, tubulin, ubiquitous metabolic enzymes and GAPDH (Brivanlou and Darnell, 2002). These binding proteins can also participate in enhancing transcription of genes by acting in concert with regulatory transcription factors (Johnson and McKnight, 1989; Rosenfeld and Kelly, 1986).

1.0.2 Regulatory Transcription Factors

Regulatory transcription factors encompass two classes of transcription factors: developmentally active and signal dependent transcription factors respectively. The developmental class of transcription factors includes MyoD (required for muscle differentiation) (Yun and Wold, 1996). These factors are normally expressed in a cell type-specific manner, and require extra-cellular signals to be activated themselves, after which they enter the nucleus without being post-translationally modified (Xanthopoulos et al., 1989; Johnson and Nusslein- Volhard, 1992).

The regulatory transcription factors play roles in development, or can be activated in a signal dependent manner. The signal dependent transcription factors are usually present in the cells in inactive or precursor forms until the cells are exposed to the required signal and

consist of three major groups: (1) Transcription factors activated by cell surface receptor-ligand interactions; (2) Transcription factors activated by internal signals (3) Steroid receptor superfamily. These groups will be discussed in turn.

1.0.3 Transcription Factors activated by cell surface-receptor ligand interactions

Cell surface receptor-ligand interactions can lead to two routes to the nucleus. Firstly, intracellular serine phosphorylation cascades that end at hundreds of resident nuclear protein substrates occurs. Secondly, a more limited number of latent cytoplasmic transcription factors are activated after cell surface receptor-ligand interaction and then accumulate in the nucleus to drive transcription (Brivanlou and Darnell, 2001).

1.0.3.1 Latent Cytoplasmic Factors

These transcription factors are inactive under normal conditions and are found in the cytoplasm. However, when a particular ligand binds to its cell surface receptor, dimerisation of the receptor leads to phosphorylation of the cytoplasmic tail of the receptor. Phosphorylation of the receptor creates docking sites for the latent factors, where they are also phosphorylated. The phosphorylated factors dissociate from the receptor to translocate to the nucleus to bind to promoters of ligand specific genes. Many transcription factors of this type have been identified and include SMADs, STATs, NFkB, NFAT and catenins (Masyuma et al., 1999; Darnell et al., 1994; Karin et al., 1999; Crabtree et al., 1999).

The STATs and SMADs are proteins that are activated at the cell surface receptor and both transcription factors require phosphorylation of serine and/or tyrosine residues. The STAT family of transcription factors will not be discussed any further in this section, but will be discussed in subsequent sections since they are relevant to this thesis.

Those factors that require serine phosphorylation and subsequent serine phosphorylation and proteolysis include NFkB /Rel family of transcription factors. This group is highly conserved in vertebrates (Baeuerle and Baltimore, 1996; Perkins, 2000). In mammals five proteins have been identified and are NFkB1, NFkB2, c-Rel, Rel-a and Rel-b. They are activated by various stimuli including oxidative stress, viral and bacterial infections, cytokines (Perkins, 2000). Two proteolytic events occur in the final translocation of NFkB to the nucleus. NFkB is a heterodimer, which is post-translationally activated. NFkB is bound to an inhibitor protein called IkB, which binds to NFkB and also binding to the actin cytoskeleton thus keeping NFkB in the cytoplasm. However, phosphorylation of IkB at two serine residues causes dissociation and proteosomal degradation of IkB and hence release of NFkB. NFkB then translocates to the nucleus (Karin, 1999).

Fluctuations in secondary messenger proteins also cause activation of latent transcription factors such as NFAT (nuclear factor of activated T cells) (Crabtree, 1999; Rao et al., 1997). In resting cells, NFAT is phosphorylated. However, when immunoglobulins bind to their receptors, the internal concentration of Ca^{2+} ions is increased, which leads to activation of calcineurin (a phosphatase) and activation of NFAT. De-phosphorylated

NFAT moves to the nucleus to bind to proteins such as AP-1 which is necessary since NFAT binds weakly to DNA (Rao et al., 1997; Okamura et al., 2000).

1.0.3.2 Resident nuclear factors

This group of factors is constitutively expressed and is activated by serine phosphorylation. Phosphorylation occurs when peptides such as thyroid stimulating hormone or molecules such as epinephrine bind to their cognate G-protein coupled receptors (GPCRs) leading to the increase of secondary messengers such as cyclic AMP, calcium ions, and phosphoinositides. Increases in secondary messengers triggers serine kinase cascades and ultimately phosphorylation of resident nuclear factors such as CREB, Jun-Fos. The target proteins in the nucleus are already bound to DNA (Gille et al., 1995; Janknecht, 1995). Resident factors are also phosphorylated through receptor tyrosine kinases (RTKs) as a result of ligands binding to cell surface receptors containing intrinsic tyrosine kinases (Pawson, 1997). Serine phosphorylation of resident factors also allows their binding to co-activators such as CBP/p300 and is catalysed by multiple MAPKinases (Janknecht, 1993).

1.0.4 Transcription factors activated by internal signals

Various internal signalling molecules regulate the activation of pre-existing transcription factors in response to intracellular signals. For example, DNA damage causes an increase in activation of the p53 transcription factor (Levine, 1997).

In yeast, internal fatty acid concentration leads to cleavage of precursor molecules Spt23 and MGA2, which on release are able to translocate to the nucleus to regulate genes for

fatty acid synthesis (Foreman et al., 1995). This group is not relevant to this thesis therefore it will not be discussed any further.

1.0.5 Steroid receptor superfamily

Steroid molecules enter cells by diffusing through the lipid bilayer. Once inside the cell, steroids can bind to various receptors, which are activated and then participate in activating transcription of responsive genes (Manglesdorf et al., 1995). All steroid receptors are in the nucleus before being activated by their appropriate hormone except for the glucocorticoid receptor (GR), which is in the cytoplasm as a complex with heat shock protein (Hsp) 90, and is released when glucocorticoids bind, allowing GR dimers to enter the nucleus (Htun et al., 1996; Mackem et al., 2001).

1.1 The Signal Transducers and Activators of Transcription

1.1.1 Introduction

Cytokines and interferons are molecules that play roles in the regulation of a variety of cellular functions in the lympho-haematopoietic system and stimulate proliferation, differentiation, and survival signals as well as specialized functions in host resistance to pathogens. Cytokines are known to activate many signaling pathways, of which the JAK-STAT pathway is one. This pathway is activated in response to cytokines and interferons. It allows the activation of latent cytoplasmic transcription factors known as the Signal Transducers and Activators of Transcription (STATs), which can modulate expression of specific genes. These transcription factors are also known to display specific functions that are vital to mammals. In this section, an overview of the signaling pathways, structure, activation and roles of the STATs will be given.

1.1.2 The STAT Proteins

There are seven mammalian STATs that have been identified and are denoted as STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Darnell, 1997). They are a group of transcription factors that are approximately 750 to 800 amino acids in length. Splice variant forms also exist, which lack a portion of the C-terminal domain. These variants are natural dominant negative forms since they bind DNA but are unable to activate gene transcription.

The genes encoding STATs exist in clusters. STAT1 and STAT4 genes are found on mouse chromosome 1 and STAT2 and STAT6 genes are found on mouse chromosome 11 (Copeland et al., 1995). Such clustering suggests that a primordial ancestral gene duplication event occurred. STAT genes are structurally conserved therefore suggesting essential roles in multiple cellular functions.

1.1.3 Biological Roles of STATs

Although the first STATs were identified on the basis of their activation by interferons (IFNs), much more important roles have been deciphered from mouse knockout models of each of the STATs and are shown in table 1.1

Table 1.1 Features of STAT Proteins Required for Gene Regulation

STAT	Knockout Defect	Phenotype	Phosphorylation Site (serine (S) or tyrosine (Y))	Transcriptional Activation Domain	Interaction With Other Proteins in Transcription
1	Type I and II IFN Signalling	No response to Viral or bacterial infection	Y701, S727	Aa 711-750	CBP/p300, SP1, TFII-I, Nmi-1, MCM5, BRCA1
2	Type I IFN Signalling	Early embryonic lethal	Y689	Human: 736-851 Murine: 699-749	CBP, p48, STAT-1
3	Unknown	Early embryonic lethal	Y705, S727	716-770	TF-II-1, c-Jun, Nmi-1, CBP
4	IL-12 Signalling	No T_H1 cell function	Y694, S722	699-749	Nmi-1
5a	Prolactin Signalling	No breast development or Lactation	Y694, S726	722-794	GR, Nmi-1
5b	Growth Hormone Signalling	No breast development or lactation	Y699, S731	727-787	GR, Nmi-1
6	IL-4 Signalling	No T_H2 cell function	Y641	661-847	C/EBPβ, Nmi-1

1.1.3.1 STAT-1

STAT1 is activated by various cytokines and growth factors. STAT1 knockout mice display selective signaling defects in their response to both type I IFNs (IFNα/β) and type II IFNs (IFNγ) (Meraz et al., 1996; Durbin et al., 1996). These mice are highly sensitive to bacterial and viral infection. In addition, expression of the major histocompatibility complex II (MHC class II) protein, complement protein C3, IFN-regulatory factor-1 (IRF-1) and guanylate binding protein 1 (GBP-1) was also diminished in these mice. STAT1 knockout mice respond normally to other cytokines, which shows that STAT1 is necessary for mediating IFN-dependent responses but not for other responses.

1.1.3.2 STAT-2

STAT2 is activated in response to IFNα and IFNβ and is unique in that it is the only STAT protein that has not been found to bind to GAS element DNA as a homodimer. Instead, after IFN activation STAT-2 forms a heterodimer with STAT-1, which must associate with p48 to form the interferon-α-induced interferon-stimulated gene factor-3(ISGF-3) transcription factor complex (Horvath et al., 1996). Mice that lack STAT-2 are viable and develop normally. However, STAT-2 null mice are susceptible to viral infections, and cells from these mice are unresponsive to IFNα/β. In addition, the absence of STAT-2 results in reduced tyrosine phosphorylation and reduced activation of STAT-1 (Park et al., 2000).

1.1.3.3 STAT-3

STAT3 is activated in response to the IL-6 family of cytokines, which mediate their signal via the gp130 transmembrane receptor subunit. STAT3 activity is detected during early embryonic development in the mouse (Duncan et al., 1997; Takeda et al., 1997). Mice deficient for STAT3 die early in embryogenesis prior to gastrulation and the phenotype is unknown (Takeda et al., 1997). In myeloid M1 cells, dominant negative STAT3 overexpression abolishes any differentiating response to IL-6 or LIF therefore, STAT3 is required for IL-6 mediated growth arrest and differentiation of M1 cells (Minami et al., 1996).

1.1.3.4 STAT-4

STAT-4 plays an important role in immune responses. STAT4 expression is restricted to myeloid cells, thymus and testes and it is phosphorylated in response to IL-12 (Jacobson et al., 1995). Knockout mice lacking STAT4 are viable, but are impaired in their response to IL-12. Moreover, an increase in Th2 cells is also observed (Kaplan et al., 1996).

1.1.3.5 STAT-5a and STAT-5b

STAT5 function was originally identified in mammary gland tissue by gene targeting and was called a prolactin-induced transcription factor (MGF) (Wakao et al., 1994). This transcription factor was activated by a variety of cytokines including IL-2, IL-3, IL-5, IL-7, IL-9, IL-15 and GM-CSF, erythropoietin, and growth hormone.

STAT5 consists of two highly related genes encoding STAT5a and STAT5b proteins. These proteins display 96% similarity at the amino acid level (Lui et al., 1998), and differ

mainly at their C-terminal domains. A single amino acid difference in the DNA binding domain results in distinct DNA binding specificities of STAT5a and STAT5b (Boucheron et al., 1998).

STAT5a knockout mice displayed normal development and were indistinguishable from the wild type mice. However, female mice failed to lactate, and mammary lobuloalveolar out growth was inhibited (Lui et al., 1997). These mice are also defective in GM-CSF induced proliferation of macrophages (Feldman et al., 1997)

Disruption of the STAT5b gene led to the loss of multiple responses linked to the sexually dimorphic pattern of pituitary growth hormone secretion. Body growth rate and male specific liver gene expression were decreased to levels observed in wild type female mice, whereas female specific liver gene expression was increased to levels observed in wild type male mice (Udy et al., 1997). STAT5b knockout mice were also defective in augmentation of natural killer (NK) cytotoxic activity mediated by IL-2 or IL-15 and proliferation of these cells was also stunted (Imada et al., 1998). In a double knockout of both STAT5a and STAT5b, female mice were found to be infertile (Teglund et al., 1998).

1.1.3.6 STAT-6

STAT6 is activated in response to IL-4, and knockout mice have defects in IL-4 mediated functions including MHC II expression, induction of CD23, immunoglobulin class switching to IgE, B and T cell differentiation, and Th2 development (Takeda et al., 1996;

Shimoda et al., 1996; Kaplan et al., 1996). These knockout mice have also shown that STAT6 is involved in allergy induced airway inflammation (Kuperman et al., 1998).

1.1.4 STAT Signalling and the JAK-STAT Pathway

Investigations into the transcriptional response to interferons have led to the identification of the Janus Kinases (JAKs) as being required for cytokine mediated STAT activation (Darnell et al., 1994; Darnell JE Jr., 1997; Ihle, 1996). Thus the common pathway activating the STATs is known as the JAK-STAT pathway, and is activated by a variety of other cytokines.

The JAK family currently consists of the proteins kinases JAK1, JAK2, JAK3 and TYK2, which vary in their size from 120-135 kDa. They are composed of Jak homology domains (JH), JH1 to JH7 (Figure 1.1). However, for the majority of these regions, a clear function has not been determined. JH1 is the most studied of all the domains. This region confers the catalytic activity of the kinase protein. Evidence shows that mutation in a single lysine residue renders the kinase inactive (Gushin et al., 1995). Structural studies of JH1 show that an activation loop is necessary for kinase activity, and mutations within the activation loop at a tyrosine residue have effects on catalytic activity ranging from reduced activity to abrogation of activity (Feng et al., 1997). The N-terminal domain of JAK2 has been found to be involved in binding to cytokine receptors (Frank et al., 1994).

The JAKs are essential for cytokine signaling as mutations in JAK3 lead to severe combined immunodeficiency (SCID) in humans (Machi et al., 1995). Similarly, knockout

mice for JAK3 display defects in B lymphocyte maturation and T lymphocyte activation (Thomis et al., 1995).

Figure 1.1 Structure of JAKs
JAKs are structurally unique in having tandem kinase and kinase-like domains. The C-terminal domain is the catalytic domain.

Once IFNs (or other cytokines) are bound to cognate receptors, the receptor components dimerise, allowing phosphorylation by the JAKs at the cytoplasmic tail of the receptor at tyrosine residues. The tyrosine residues become docking sites for latent cytoplasmic STAT monomers by means of the Src Homology 2 (SH2) domains. Following recruitment to the receptor, STATs become phosphorylated at tyrosine residues in the C-terminus. Phosphorylated STATs dissociate from the receptor to form homo or hetero-dimers that can translocate to the nucleus to bind STAT responsive elements and subsequently activate transcription of STAT responsive genes (Darnell et al., 1994) (Figure 1.2)

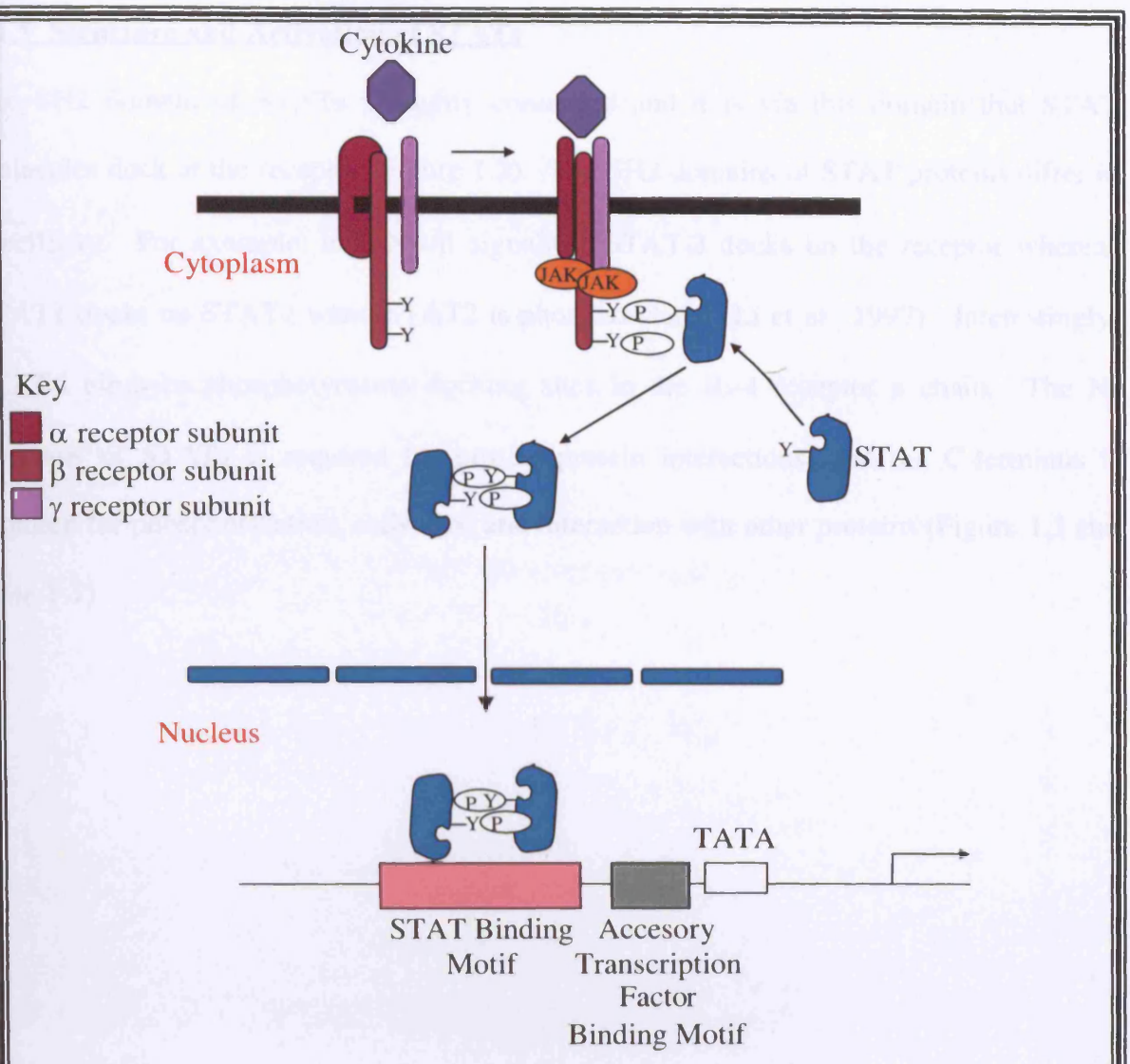


Figure 1.2 Cytokine Induced STAT Activation and Transcriptional Regulation
Latent cytoplasmic STAT monomers are recruited and phosphorylated at the receptor upon cytokine interaction and dimerisation of the receptor. Phosphorylated STATs can form homo or heterodimers which translocate to the nucleus where they bind STAT binding motifs to activate transcription of STAT responsive genes.

1.1.5 Structure and Activation of STATs

The SH2 domain of STATs is highly conserved and it is via this domain that STAT molecules dock at the receptor (Figure 1.2). The SH2 domains of STAT proteins differ in specificity. For example, in IFNα/β signaling, STAT-2 docks on the receptor whereas STAT1 docks on STAT2 when STAT2 is phosphorylated (Li et al., 1997). Interestingly, STAT6 binds to phosphotyrosine docking sites in the IL-4 receptor a chain. The N-terminus of STATs is required for protein-protein interactions, and the C-terminus is required for phosphorylation, activation and interaction with other proteins (Figure 1.3 and table 1.1).

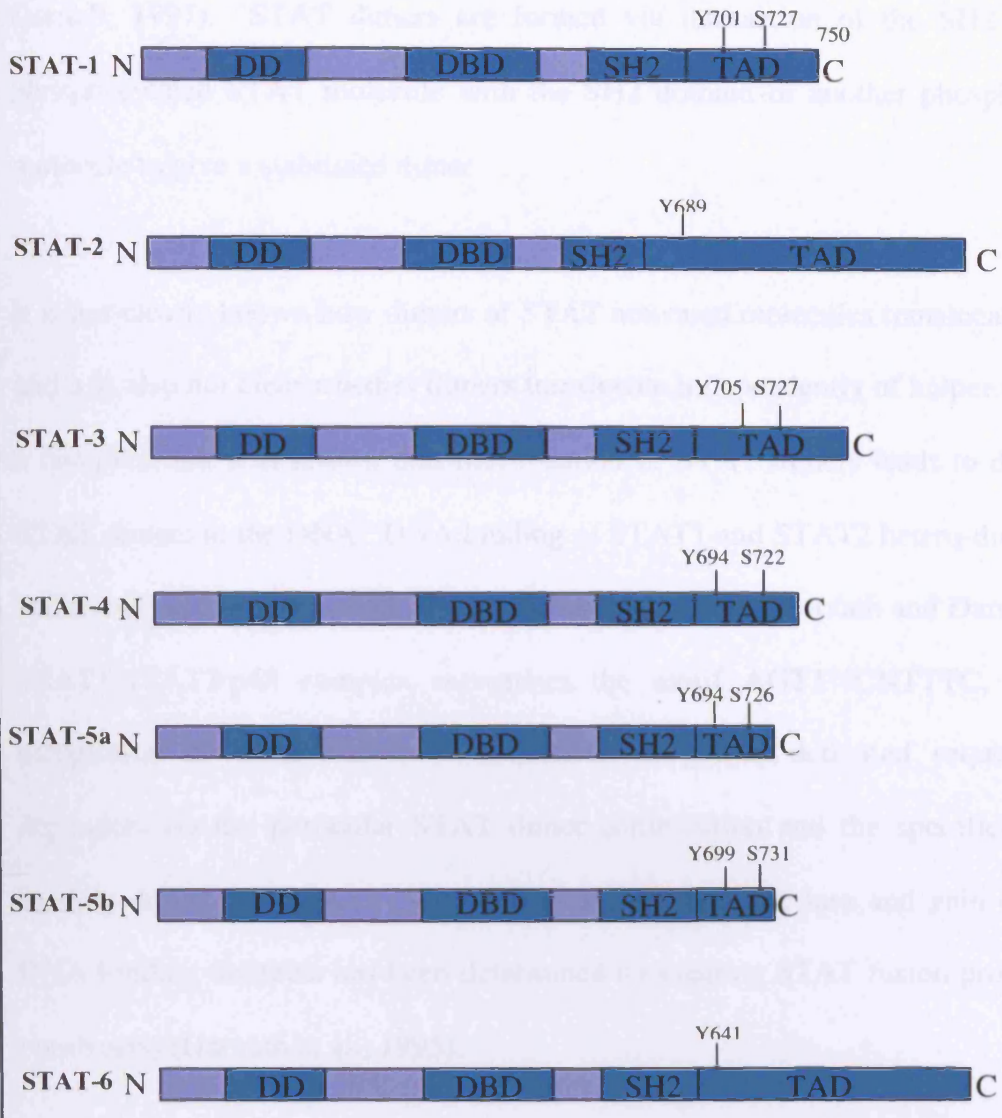


Figure 1.3 Structures of STATs. The figure shows domains common to the STAT proteins. DD: dimerisation domain; DB: DNA binding domain; SH2: Src Homology 2 domain; TAD: trans-activation domain.

All STATs have conserved tyrosine residues at approximately 700 residues from the N terminus which become phosphorylated following receptor activation (Horvath and Darnell, 1997). STAT dimers are formed via interaction of the SH2 domain of one phosphorylated STAT molecule with the SH2 domain of another phosphorylated STAT molecule to give a stabilized dimer.

It is not clearly known how dimers of STAT activated molecules translocate to the nucleus and it is also not clear whether dimers translocate independently of helper chaperones or in a complex, but it is known that translocation of STAT dimers leads to direct binding of STAT dimers to the DNA. DNA binding of STAT1 and STAT2 hetero-dimers requires an additional protein p48, which is a DNA binding protein (Horvath and Darnell, 1997). The STAT1/STAT2/p48 complex recognizes the motif AGTTNCNTTTC. However, the recognition of other consensus sequences or gamma-activated sequences (GAS) is dependent on the particular STAT dimer combination and the specificity of the DNA binding domain of STATs (Schindler et al., 1995). The loss and gain of specificity of DNA binding domains has been determined by creating STAT fusion proteins (chimaeric constructs) (Horvath et al., 1995).

Activation of STATs occurs at the C-terminus of all the proteins. Studies on STAT-1 for example, show that STAT-1β, the splice variant of STAT-1α, lacks the terminal 38 amino acids and does not activate gene transcription in response to IFNγ (Muller et al., 1993). In addition, the 38 amino acids at the C-terminus are essential for STAT-1 activation. STAT-

1α, 3, 4, and 5a are about the same length, and the C-terminus of these proteins is required for full gene activation (Moriggl et al., 1996; Caldenhoven et al., 1996; Wang et al., 1996).

The C-terminal amino acids are not highly conserved in these proteins, except for Pro-met-ser-pro in the C-terminal domain. STAT-1 and STAT-3 are phosphorylated on a single serine residue (serine 727) and is one of the conserved amino acids in the C-terminal domain (Wen et al., 1995; Wen et al., 1997). Serine 727 phosphorylation is required for maximal gene activation by STAT-1 and STAT-3 in cells stimulated with IFNγ. For transcriptional activation of STAT-1 and STAT-3, tyrosine phosphorylation is necessary and obligatory. However, serine phosphorylation is supplementary.

Tyrosine phosphorylation of STAT-1 is independent of serine phosphorylation and vice versa (Zhu et al., 1996), since mutant serine to alanine mutants can be phosphorylated on tyrosine, and a tyrosine to phenylalanine mutant can be phosphorylated on serine. Tyrosine phosphorylation induced by IFNγ occurs within 5 minutes, whereas serine phosphorylation takes 10 minutes (Zhu et al., 1996). STAT-1 tyrosine phosphorylation occurs at the plasma membrane, whereas serine phosphorylation is likely to occur at the cytoplasmic tail of the receptor. Therefore, although tyrosine and serine phosphorylation is essential, it seems that they are controlled by distinct pathways (Darnell, 1997).

In addition to tyrosine phosphorylation and serine phosphorylation, physical interactions of STATs with other proteins have been identified. STAT1 can associate with the activator Sp-1 to bind to the ICAM-1 promoter (Look et al., 1995), and with CBP/p300, which

interacts with the N-terminal domain of STAT-1 as well as the C-terminal domain via the E1A binding region of CBP/p300 (Zhang et al., 1996). The N-terminal domain of STAT1 is also involved in interactions with N-terminal domains of other STATs to mediate dimerisation (Xu et al., 1996) and stabilization of DNA binding (Vinkemeir et al., 1996). Interestingly, STAT3β can associate with c-jun in a co-operative manner (Schaefer et al., 1993).

1.1.6 Inhibition of STATs

Inactivation and subsequent loss of DNA binding occurs by de-phosphorylation of the tyrosine residues (Haspel et al., 1996). The N-terminal domain of STAT1 can bind a phosphatase (Shuai and Song, 1996) thus reversing tyrosine phosphorylation. However, there is not one single mechanism of deactivation. Thus, for example, it has been shown that STAT1 is degraded via the ubiquitin-proteasome pathway (Kim and Maniatis, 1996). Other mechanisms involve binding of STAT splice variants to DNA. For example, STAT1β lacks 38 amino acids at the C-terminus, and does not activate gene transcription.

However, cytokine activated STATs are also negatively regulated by inhibitor proteins at various stages of the JAK-STAT pathway. These include protein inhibitor of activated STAT (PIAS) family (Liu et al., 1998; Shuai, 1999), the cytokine-inducible SH2 containing protein (CIS) (Yoshimura et al., 1995), the suppressor of cytokine signaling (SOC) family, also known as JAB (JAK binding protein), and SSI-1 (STAT induced STAT inhibitor-1) (Endo et al., 1997; Starr et al., 1997; Naka et al., 1997). These groups will be described in turn.

1.1.7 The PIAS family of proteins

PIAS was originally isolated as a partial cDNA clone encoding the C terminus of an unknown protein which was later termed as PIAS1 as it was able to interact with STAT1β (Liu et al., 1998; Shuai, 1999). Additional related proteins were identified subsequently: PIAS3, PIASy, PIASxα, PIASxβ (Chung et al., 1997; Liu et al., 1998). The structure of all PIAS members consists of a conserved LXXLL motif in the N terminal domain, which is known as the nuclear receptor box (Heery et al., 1997) and mediates ligand-dependent co-activator-nuclear receptor interactions. Also present is a zinc-binding motif in the middle of the protein, and serine/threonine residues in the C-terminal region.

The potential functions of the PIAS proteins in signaling were identified by co-immunoprecipitations using antibodies to STAT1 and STAT3 (Chung et al., 1997; Liu et al., 1998). PIAS3 has been shown to associate with STAT-3 but not STAT-1, and inhibits signal transduction by suppressing STAT3 activity by blocking the DNA binding activity of STAT3. Furthermore, PIAS3 only associates with STAT3 when cells are stimulated with IL-6, CNTF or OM. PIAS3 also inhibits DNA binding of the STAT1/STAT3 heterodimer (Chung et al., 1997). Deletion of amino acids 1-191 of STAT1 results in loss of PIAS1 association with STAT1 (Liao et al., 2000). The N terminal domain of STAT-3 is also likely to be necessary for STAT3 and PIAS3 interaction.

1.1.8 The SOCs Family of Cytokine Inhibitors

The SOCS family consists of eight proteins, SOCS-1 to SOCS-7 and CIS (cytokine inducible SH2 containing protein), and they all have similar structures (Masuhara et al., 1997; Minamoto et al., 1997).

The first member of this family was identified as an immediate early gene induced in response to cytokines and was named CIS (Yoshimura et al., 1995). This protein was able to bind to tyrosine- phosphorylated receptors and competes with STAT5 for binding to the receptor (Matsumoto et al., 1999; Yoshimura et al., 1995). CIS expression is induced by IL-3 and erythropoietin by activation of STAT-5. In addition, the CIS gene promoter contains STAT5 binding sites (Matsumoto et al, 1997). Mice over-expressing CIS display defects in growth hormone signaling as well as defects in mammary gland development, and similar defects are observed in STAT5 knockout mice (Matsumoto et al., 1999). Therefore, it is possible that cytokine induced STAT-5 activation leads to CIS expression, which in turn, binds to phosphorylated tyrosine within the receptor to prevent further STAT-5 recruitment in a negative feedback loop.

SOCS-1 was identified as an inhibitor of IL-6 induced differentiation of myeloid leukaemic M1 cells that was able to bind to JAKs (Endo et al., 1997; Starr et al., 1997; Naka et al., 1997) through its SH2 domain. SOCS-1 knockout mice were retarded in their growth and were not able to survive more than 3 weeks after birth (Naka et al., 1998). It was also shown that these mice had lost maturation function of B-lymphocytes in bone marrow, spleen and peripheral blood cells (Naka et al., 1998). Interestingly, embryonic fibroblasts lacking SOCS-1 were sensitive to TNF-α induced apoptosis (Morita et al.,

2000). Other studies show that SOCS-1 may be involved in modulating responses to IFNγ (Alexander et al., 1999).

SOCS (also known as JAB, SSI-1) factors function by binding JAK kinases. SOCS-1 binds to the phosphorylated tyrosine residue in the activated JAK via its SH2 domain to inhibit JAK catalytic activity, thus preventing access of STAT and ATP to the catalytic pocket of JAKs. A kinase inhibitory region within the SH2 domain of SOCS-1 is required for high affinity binding of SOCS-1 to the kinase domain (Nicholson et al., 1999; Yasukawa et al., 1999). Mutation of 12 amino acids within the SH2 domain of SOCS-1 prevents binding to phospho-tyrosine.

1.1.9 Other Roles of STATs

It is known that STATs are required for normal functioning of cells, primarily in response to cytokines to trigger growth or differentiation of cells. STATs are versatile since they can take part in early signaling in ligand binding and receptor dimerisation and activation, and they also execute the final steps by translocating to the nucleus to bind DNA and activate transcription of STAT responsive genes.

Recent studies have shown that STATs are part of a larger multi-step signaling pathway that results in programmed cell death. STAT-1 has been shown to be activated by the TNFα death ligand (Kumar et al., 1997). Although it is not clear at what stage in the apoptotic pathway STAT-1 is activated, Chin and colleagues have implicated STAT-1 in transcriptional activation of some caspases (Chin et al., 1997). Apoptosis is initiated by activation of a caspase cascade of caspase proteases that cleave cellular proteins (Fraser

and Evans, 1995). Studies have shown that the serine 727residue is required for apoptotic activity after ischaemia/reperfusion (Stephanou et al., 2001) and mutation of serine 727 reduces apoptotic activity of STAT-1.

It is important to further investigate the involvement of STAT-1 in apoptosis and that STAT-1 could be a potential target in minimizing cell death especially when cells are subjected to stress such as elevated temperature and ischaemia/reperfusion injury. In the following section, a background introduction to apoptosis will be given, and the role of STAT-1 in apoptosis will be addressed.

1.2 Cell Death Pathways

1.2.1 Introduction

Cell death is a physiological process that is required for maintenance of cellular homeostasis and development in organisms. Death of a cell can occur when exposed to a serious physical or chemical insult (predominantly necrosis) or during development and other normal biological processes (predominantly apoptosis or programmed cell death). Features of necrosis and apoptosis can be seen in table 1.2.

Table 1.2 Differential Features and Significance of Necrosis and Apoptosis

Features	Necrosis	Apoptosis
Morphology	Loss of membraine intregrity	Membrane blebbing, no loss of integrity
	Swelling of cytoplasm and mitochondria	Shrinking of cytoplasm
	Total cell lysis	Fragmentation of cell into smaller bodies
	No vesicle formation, complete lysis	Formation of membrane bound vesicles
	Disintegration (swelling) of organelles	Mitochondria become leaky due to pore formation
		DNA fragmentation
		Nuclear envelope shows characteristic clustering of nuclear pores
Biochemistry	Loss of regulation of ion homeostasis	Tightly regulated process involving activation and enzymatic steps
	No energy requirement (passive process)	Energy (ATP)-dependent
	Random DNA digestion	Non-random mono and oligonucleosomal length fragmentation of DNA
	Post-lytic DNA fragmentation (late event of death)	Prelytic DNA fragmentation
		Release of various factors into cytoplasm by mitochondria Activation of caspase cascade Alterations in membrane asymmetry (ie, translocation of phosphatidyl serine from the cytoplasmic region to the extracellular side of the cell membrane)

Physiology	Affects groups of contigious cells	Affects individual cells
	Evoked by non-physiological disturbances	Induced by physiological stimuli
	Phagocytosis by macrophages	Phagocytosis by adjacent cells or macrophages
	Significant inflammatory response	No inflammatory response

Necrosis involves uncontrolled bursting of cells caused by for example, stroke, trauma, heat, radiation, lack of oxygen, or toxins, leading to damaging immune responses (Valance et al., 1997). This mechanism is rapid and leads to leakage of cellular contents, as the integrity of the cell membrane is lost.

Cell death by apoptosis occurs in many physiological and pathological processes such as development, differentiation, tumorigenesis and infections (Kerr et al., 1972; Martins and Earnshaw, 1997; Thompson, 1995). Apoptosis is a slow process and can last up to 24 hours (Collins et al., 1997). A diagrammatic representation of the apoptotic pathways are shown in figure 1.4

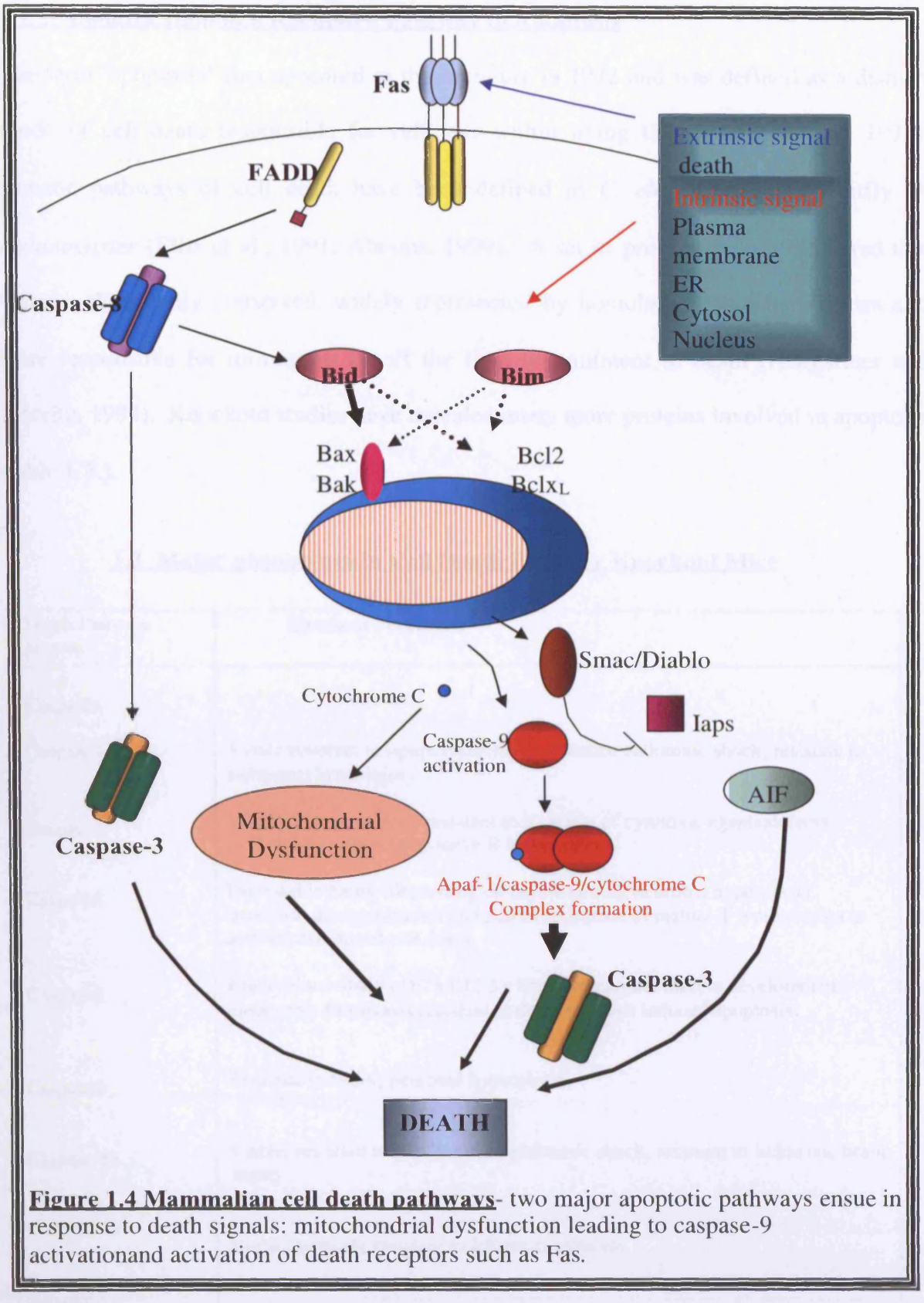


Figure 1.4 Mammalian cell death pathways- two major apoptotic pathways ensue in response to death signals: mitochondrial dysfunction leading to caspase-9 activationand activation of death receptors such as Fas.

1.2.2 Identification of a Regulated Pathway to Apoptosis

The term 'apoptosis' first appeared in the literature in 1972 and was defined as a distinct mode of cell death responsible for cell loss within living tissues (Kerr et al., 1972). Genetic pathways of cell death have been defined in *C. elegans* and the fruitfly *D. melanogaster* (Ellis et al., 1991; Abrams, 1999). A set of proteins were discovered that were evolutionarily conserved, widely represented by homologues in other species and were responsible for turning on or off the final commitment to death (Hengartner and Horvitz. 1994). Knockout studies have revealed many more proteins involved in apoptosis (table 1.3.).

1.3 Major phenotypes in Cell Death Pathway Knockout Mice

Death Pathway protein	Knockout Phenotype
Caspases	
Caspase1	Viable:resistant to lipopolysaccharide induced endotoxic shock; resistant to ischaemic brain injury.
Caspase2	Viable; excess oocytes resistant to a variety of cytotoxic agents;defects in B cell apoptosis by granzye B and perforin
Caspase3	Perinatal lethality (depending on mouse strain), neuronal hyperplasia, structural disorganization and partial resistance of mature T lymphocytes to activation-induced cell death
Caspase8	Embryonic lethality (E11-E12.5); impaired cardiac muscle development; embryonic fibroblasts resistant to death receptor induced apoptosis
Caspase9	Perinatal lethality, neuronal hyperplasia
Caspase 11	Viable; resistant to LPS induced endotoxic shock; resistant to ischaemic brain injury
Caspase 12	Viable; partially resistant to ER stress inducers
Adaptors	

Apaf1	Embryonic lethality (E16.5); severe craniofacial abnormalities; infertility in surviving males
Fadd	Embryonic lethality (E9-E12.5); impaired cardiomyogenesis and abdominal hemorrhage; impaired proliferation of thymocytes
Anti-apoptotic	
Bcl2	Death within few months of birth; renal failure; neuronal death in postnatal period
BclX	Embryonic lethality; extensive neuronal death; death of hematopoietic cells of the liver
Mcl1	Peri-implantation embryonic lethality
A1	Viable; accelerated neutrophil apoptosis
BclW	Viable; male infertility; no production of mature sperm
Pro-apoptotic	
Bax	Viable; male infertility; increased oocyte lifespan in females
Bak	Viable; fertile
Bax/Bak	Accumulation of neurons in the central nervous system; adults resistant to anti-Fas-induced hepatocellular apoptosis; 90% perinatal lethality
BH3 domain only	
Bid	Viable; resistance to anti-Fas induced hepatocellular apoptosis
Mitochondrial genes	
Cytochrome c	Embryonic lethality (E8.5)
Aif	Defective embryoid body cavitation; Escells resistant to growth factor withdrawal

1.2.3 The Cell Death Pathway and its Components

The identified proteins were called the cell death abnormal (ced) (Yuan et al., 1993). The proteins resulting from ced 3 and ced 4 genes were identified as the initiators of apoptosis. Ced 9 was found to prevent cell death. In addition, seven more genes were identified, and were involved in the recognition and phagocytosis of the cell.

Ced 3 protein is a protease that also resembles at least twelve mammalian proteases (Alnemri et al., 1996) called caspases (so called because of the obligatory cysteine in their active site and their tendency to cut adjacent to aspartate residues). The ced genes were found to be highly homologous to the human genes for interleukin-1 converting enzyme (ICE) (also known as caspase 1), which is a protease that cuts IL-1 from its precursor protein (Yuan et al., 1993).

Caspases can be divided into two functional subfamilies: initiator caspases, which are involved in upstream regulatory events, and effector caspases, which are directly responsible for cell disassembly events.

Initiator pro-caspases 8 and 9 are capable of trans or auto activation after they are aggregated with the adaptor molecules Fadd and Apaf-1 (Li et al., 1997). The downstream effector caspases 3, 6 and 7 are substrates of the initiators, which result in a protease cascade that ensures widespread cleavage of multiple substrates, and ultimately death.

Caspase 3 carries out the final irreversible commitment to death in many situations. Cytoskeletal proteins such as actin and nuclear laminins, regulatory and chaperone-like

proteins, are functionally altered by cleavage (Thornberry, 1997). One such example is cleavage of apoptotic nuclease which is thereby activated and is responsible for chromatin cleavage to oligonucleosome fragments (Ehari et al., 1998). Caspases are present in most cells in an inactive pro-enzyme form, which, when active, are able to cleave target substrates.

Ced4 was found predominantly in the mitochondria, and the mammalian equivalent was named Apaf-1 (Zou et al., 1997). Under conditions of cellular shutdown, Ced 4 was found to initiate apoptotic signals by changes in cellular energy metabolism. Thus, Ced 4 may play the linking role between mitochondrial injury associated agents such as calcium and reactive oxygen species in the initiation of apoptosis.

1.2.4 Death Ligands

Many death and survival signals stimulate extrinsic or intrinsic pathways composed of several adaptors, regulators, caspases and members of the Bcl-2 family. Extrinsic signals involve Fas and TNFα, which induce apoptosis by means of their receptors. The intracellular domains of the death receptors TNF receptor 1 (TNFR1) and Fas/Apo-1/CD95 receptors are known as the death domains (DD). Oligomerisation of Fas causes binding of a Fas associated death domain protein (FADD). Oligomerisation of TNFR1 requires a TNFR1 associated death domain (TRADD) to recruit FADD. Upon association to both receptors, FADD interacts with the death effector domain (DED) of the initiator procaspase-8 thereby causing proteolytic autoactivation to generate caspase-8 (Martin et al., 1998).

In addition to activating the downstream effector caspase-3, caspase-8 also cleaves Bid (Budihardjo et al., 1999) to an active form, which translocates to the mitochondria to initiate cytochrome c release into the cytoplasm by Bcl2 family- mediated membrane permeabilisation (Li et al., 1998) (see section 1.2.3.1). Cytochrome c is a component of the mitochondrial electron transport chain necessary for mitochondrial respiration and is a well- characterized component of the mitochondrial driven death pathway (Li et al., 1997). In addition to cytochrome c release, Smac/Diablo (pro-apoptotic) and apoptosis inducing factor (AIF) are also released. The release of cytochrome c into the cytoplasm results in oligomerisation of the Apaf-1 adaptor protein by binding to procaspase 9. The binding of Apaf-1 to procaspase 9 leads to activation of caspase 9 without cleavage (Zou et al., 1997). Caspase 9/cytochrome C/Apaf-1 complex promotes activation of the effector caspase 3 and mediate the final death signal (Li et al., 1997).

1.2.4.1 Death regulation by the Bcl-2 Family of Proteins

The Bcl-2 family of proteins is also involved in the regulation of mitochondrial outer membrane permeabilisation (MOMP) (Martinou and Green, 2001). The Bcl-2 family consists of anti-apoptotic, and pro-apoptotic proteins. The anti-apoptotic members include Bcl-2, $BclX_L$, Mcl-1, Bcl-W and A1. Pro-apoptotic members include Bax, Bak and Bok. All members are BH (Bcl-2 Homology) 1, 2, 3 proteins. BH3 only proteins include Bid, Bim, Bik, Bmf, Bad, Hrk, BNIP3, Noxa, and Puma (Martinou and Green, 2001).

Bcl-2, $BclX_L$, Bax, and Bid share 3D structures that resemble pore-forming chains of some bacterial toxins (Martinou and Green, 2001). Bcl-2 and $BclX_L$ block mitochondrial outer membrane permeabilisation, while Bax and Bak promote it (Lindsten et al., 2000).

Pro-apoptotic Bax can oligomerise and migrate to the mitochondria (Eskes et al., 2000). Oligomerised Bax can then generate a pore, or can alter the integrity of the outer mitochondrial membrane. Bcl-2 can block Bax activity (Cheng et al., 2001).

1.2.5 STATs as Activators of Apoptosis

Increasing evidence suggests that STATs are not restricted to roles in modulating gene expression induced by cytokine stimulation, and that STATs can display specific roles in modulating gene expression in response to specific cellular processes such as apoptotic cell death.

Recent studies have shown that STAT-1 and STAT-3 have distinctively opposing effects on apoptotic cell death (Battle and Frank, 2002). For example, cell lacking STAT-1 are more resistant to apoptotic cell death induced by TNFα (Kumar et al., 1998) whereas STAT-3 has been shown to have oncogenic properties and its activity is enhanced in several tumours (Garcia and Jove, 1998). Anti-apoptotic effect for STAT-3 has also been demonstrated in breast cancer (Bromberg et al., 1999). In our laboratory, it has been shown that overexpression of STAT-1 in cardiac myocytes leads to enhanced apoptosis upon ischaemia reperfusion. In contrast, over-expression of STAT-3 leads to a reduction in apoptotic cell death induced by STAT-1 (Stephanou et al., 2000). In addition, ischaemia

reperfusion or IFNγ leads to induction of caspase-1, Fas, and FasL in a STAT-1 dependent manner. This effect is abolished with the presence of anti-sense STAT-1 (Stephanou et al., 2000; Stephanou et al., 2001, Stephanou et al., 2002). STAT-1 is also able to inhibit the anti-apoptotic genes $BclX_L$ and Bcl-2 whereas STAT-3 enhances expression of $BclX_L$ and Bcl-2 genes (Stephanou et al., 2000a).

STAT-1 pro-apoptotic activity is dependent upon phosphorylation of its serine 727 residue within the C-terminal domain, and this has been shown to be critical for its ability to interact with other regulatory proteins including MCM5 and BRCA1 (Da Fonesca et al., 2001; Ouchi et al., 2000).

In these circumstances, STAT-1 can potentially be a therapeutic target in terms of reducing cell death after lethal stresses. However, it is vital that the biological activity of STAT-1 in stress-induced apoptosis is understood, and at what stage of the apoptotic pathway STAT-1 is required. In a previous study by King and Goodbourn, STAT-1 is shown to be cleaved at position 694 by caspase-3, therefore it is possible that STAT-1 may be involved in amplifying the apoptotic loop following caspase activation (King and Goodbourn, 1998). It is equally important to understand how STAT-1 may interact with other proteins to exert its effects, and which genes are activated or repressed. For example, the heat shock proteins are commonly known for their cell protective roles in response to various stresses such as heat shock and ischaemia/reperfusion. Hsp70 and Hsp90 can bind to Apaf-1 thus inhibiting apoptosome formation (Saleh et al., 2000; Beere et al., 2000; Pandey et al., 2000). Hsp70 can also inhibit caspase-3 activity further downstream of the

apoptotic pathway (Jaattela et al., 1998). In our laboratory, it has been shown that STAT-1 can interact with heat shock factor 1 (HSF-1) (Stephanou et al., 1999). Therefore, it is important to determine whether this interaction between STAT-1 and HSF-1 is also required for Hsp inhibition in stress-induced apoptosis. The Hsps will be discussed in the next section.

1.3 The Stress Response

1.3.1 Introduction

Cells are equipped to ensure correct folding of proteins. Under stressed conditions, proteins can become denatured, and as a result, can misfold, leading to proteins that fail to function and/or give rise to diseases. Molecular chaperones assist in the correct folding of other proteins so that they can fulfill their proper functions under normal conditions (Gething and Sambrook, 1992; Hightower et al., 1991; Lidquist and Craig, 1988; Welch, 1992). This function is essential for normal cells, but when cells are subjected to elevated temperature protein misfolding is enhanced and prevention of aggregation of other proteins with each other is of even more importance.

The stress response was first discovered by Ritossa (1962), who reported the formation of a new puffing pattern, in the salivary gland polytene chromosomes of the fruit fly *Drosophila buskii* on exposure to elevated temperature. It was subsequently found that the pattern of gene transcription had been reprogrammed (Ashbourner, 1970). The pattern observed was related to the rapid synthesis of a novel group of proteins known as the heat shock proteins (Hsps) (Tissiers et al., 1974), which were named simply because of their inducibility by elevated temperature. However, other stresses such as cytokines, microbial and viral infections, ischaemia, hypoxia, and amino acid analogues also enhanced the synthesis of the Hsps. The Hsps were found to be a phenomenon common to all prokaryotes and eukaryotes (Lindquist, 1986) and since the Hsps were found to constitute 2% of total cellular proteins in the unstressed cell, this implied that these proteins play important roles in the general maintenance of the cell under normal conditions as well as

stress conditions. For example, the 70 kilodalton stress protein has two forms, a constitutive and an inducible form. The constitutive form is present in the unstressed cell whereas inducible Hsp70 is expressed at high levels when the cell is stressed to refold misfolded proteins, and in addition, proteins that are damaged permanently as a result of stress and cannot be refolded are recognized and subsequently degraded.

The hsp family of proteins is a large family consisting of Hsp 110, 100, 90, 70, 65, 56, 47, and the small Hsps. All Hsps are classified according to their molecular weights and members of this family can be seen in table 1.4.

Table 1.4 The Heat Shock Proteins in Eukaryotes and their Prokaryotic homologues

HSP	Members	Other Homologues	Roles
Hsp110		-	Glucose regulated, predominant in nucleoli
Hsp100	Hsp104, Hsp100	ClpA, ClpB	Glucose regulated, predominant in Golgi
Hsp90	Hsp90, Grp94	TRAP1 (eukaryotic), C62.5(E. coli) Hsp83(yeast, Drosophila) mitochondrial	Keeps steroid receptors inactive. α and β forms from distinct genes
Hsp70	Grp78 (BiP), Hsp70 Hsc70	DnaK	Involved in cell survival, protein Folding and unfolding, involved in thermotolerance Involved in binding to nascent polypeptide chains, keeping them from forming misfolded proteins
Hsp60	Hsp60	GroEL	Involved in mitochondrial protein folding
Hsp56	Hsp56 (FKBP59)	-	Can associate with Hsp70 and Hsp90 in steroid receptor complexes and is a target of immunosuppressive drugs
Hsp47	Hsp47	-	Involved in collagen protein folding, homology to protease inhibitors
Hsp27	Hsp27, Hsp26, Hsp25	Hsp26(yeast) Hsp25(mouse)	Can bind to actin filaments, involved in protein folding, thermotolerance and cell survival
Ubiquitin	Ubiquitin	Hsp22,Hsp23,Hsp26, Hsp28 (Drosophila)	Involved in protein degradation and can associate with H2A histone in nucleus

In this section, the roles of the Hsps will be discussed in detail individually, especially those Hsps that are directly relevant to this thesis.

1.3.2 Hsp90

Hsp90 protein is constitutively expressed in unstressed cells and is one of the most abundant proteins in eukaryotic cells, constituting 1-2% of total cellular protein. Hsp90 is

highly conserved since homologues are found in bacteria yeast and humans. Two cytoplasmic isoforms of the human Hsp90 protein have been identified: Hsp90α and Hsp90β (Gupta et al., 1995). These are encoded by separate genes and have resulted from a gene duplication event (Hickey et al., 1989). Hsp90α shares 76% amino acid homology with Hsp90β (Gupta et al., 1995). Hsp90α is more inducible than Hps90β (which is constitutively expressed).

For both isoforms dimerisation and phosphorylation are essential for chaperone activity (Minami et al., 1994). Hsp90β and Hsp90α are situated in the cytoplasm, and a large majority of these proteins is localized in the nuclear envelope region (Perdew et al., (1993). Hsp90 exists predominantly as phosphorylated homodimers and forms oligomers, with 2-3 covalently bound phosphate molecules per monomer (Ianotti et al., 1988). In cases when cells are stressed due to elevated temperature, Hsp90 chaperone activity (Yonehara et al., 1996) and hydrophobicity increases, thus enhancing binding to other unfolded proteins. Hsp90 protects the cell by associating with actin filaments (Kellemayer and Csemely, 1995) as a result of lower levels of ATP in the cell (Kabakov and Gabai, 1997).

Hsp90 also contains a hinge-domain between the C and N termini, which is highly charged and another highly charged domain is located within the C-terminal domain, thus giving Hsp90 its binding activity to other proteins. The N-terminal domain of Hsp90 contains a binding site for ATP/ADP (Prodromou et al., 1997) and the antitumourigenic drug

geldanamycin (Stebbins et al., 1997). This domain is also involved in target protein binding (Young et al., 1997).

The highly charged central domain is thought to have possible regulatory functions since putative phosphorylation sites are present in this domain, and can be phosphorylated by protein kinases (Dougherty et al., 1987). The C-terminal domain is the site at which dimerisation occurs (Minami, 1994) and is also the site for calmodulin binding (Mimami, 1993). This domain is also involved in binding with actin filaments as the binding of the antiHsp90 antibody AC88 (which recognises an epitope in the C-terminal domain) to Hsp90 interferes with actin filament binding (Schlatter et al., 1992).

Functions of the Hsp90 family involve the prevention of aggregation of unstable proteins, as well as partially re-natured and heat denatured proteins since purified Hsp90 can prevent aggregation of unstable proteins. Hsp90 can bind protein kinases to enable proper function and correct cellular localization (table 1.5). The binding of Hsp90 with actin filaments suggests that Hsp90 plays a vital role in cell protection due to heat stress (Williams and Nelson, 1997).

Table 1.5. Complex formation of Hsp90 with various protein kinases

Protein Kinases	Examples	Reference
Tyrosine Kinases	VSrc, cSrc	Brugge et al., 1981; Oppermann, 1981; Blakenship and Matsumura, 1997
	Insulin receptor	
Serine/Threonine Kinases	V-Raf, c-Raf, b-Raf	Stancato et al., 1993; Wartmann and Davis, 1994; Jaiswal et al., 1996
	MEK	Stancato et al., 1997
	EIF-2-α kinase	Rose et al., 1987
	Protein Kinase CK-II	Dougherty et al., 1987

Roles of Hsp90 in modulation of transcription factors such as MyoD1 and hypoxia induced factor 1α in the nucleus by conformational changes have been suggested as a low affinity complex formation of Hsp90 with these transcription factors enhances DNA binding (Shaknovich et al., 1992; Shue and Kohtz, 1994). In addition, an important role of Hsp90 has been observed in regulation of the heat activated heat shock factor 1 (HSF1) transcription factor (See Section 1.3.6). The highly charged connecting loop of Hsp90 resembles DNA and can be bound by many factors (Nadeau et al., 1993).

Finally, one of the most studied roles of Hsp90 is the interaction with steroid hormone receptors and in vivo Hsp90 is essential for steroid action (Picard et al., 1990). Interaction with steroid receptors requires formation of a complex with other co-chaperones such as hsc70, Hop, p23. This complex keeps the receptor in a partially unfolded state and allows high affinity binding of the steroid. Steroid binding destabilizes the steroid receptor/Hsp90 complex, leading to dissociation of Hsp90 followed by nuclear translocation of the receptor and gene activation.

1.3.3 Hsp70

The Hsp70 family of chaperones is one of the most studied protein families. This family of proteins is most abundant and highly conserved in eukaryotic cells compared to other Hsps (Tavaria et al., 1996). The Hsp70 family includes Hsc70 (constitutive form) (present in the cytoplasm and nucleus in the unstressed cell), Hsp70 (inducible form; produced in response to stress), Grp78 (present only in the endoplasmic reticulum), and mitochondrial Hsp70. The different isoforms are encoded by multiple gene copies in eukaryotes (Becker and Craig, 1994). In prokaryotes (*E. coli*) there is one copy of the dnaK gene (Hsp70 homologue), whereas in yeast several hsp70 genes are present (Becker and Craig, 1994).

Hsp70 assists in folding of newly synthesized proteins and facilitates degradation of unstable proteins. Other roles involve guiding translocating proteins across organellar membranes, disassembly of oligomeric protein structures, controlling biological activity of folded regulatory proteins (such as transcription factors), and enabling proteolytic degradation of unstable proteins (Flynn et al., 1989; Rüdger et al., 1997).

Hsp70 proteins consist of a highly conserved N- terminal domain, which confers ATPase activity (44 kDa) and a C-terminal domain (25kDa), which is also highly conserved. The function of this domain is not known. Both termini are separated by a linker domain and substrate- binding domain. ATP binding to the N- terminal domain drives changes in the C-terminal substrate -binding domain. Thus, weak ATP binding affinity with Hsp70 is formed, which allows high affinity for the substrate as a binding pocket of Hsp70 opens (Palleros et al., 1993; Pierpaoli et al., 1997). Hsp70 in its ADP-bound state has high affinity binding and low substrate affinity, thus closing the binding pocket of Hsp70 (Theyson et al., 1996). This two- step process for association/dissociation is extremely important for Hsp70 activity. Mutations in the substrate binding domain of Hsp70 result in failure to bind to its substrate (Ha et al., 1997).

1.3.4 Hsp47

Hsp47 is a collagen binding glycoprotein and displays characteristics of serine protease inhibitors. This protein is located in the endoplasmic reticulum (ER) and assists in protein folding and assembly. An ER retention signal is found in the C-terminal domain of the protein (Nakai et al., 1992).

Hsp47 plays a major role in the maturation of pro-collagen into collagen types I to IV (Nagata et al., 1996). Moreover, hsp47-/- mice clearly lack the ability to process pro-collagen into mature collagen, and embryos only survive until day 11.7 (Nagai et al., 2000). No other roles of Hsp47 have yet been identified.

1.3.5 Hsp27

Hsp27 or the small Hsps (sHsps) consist of proteins upto 40kDa, which are less conserved compared to Hsp70 or Hsp90. The number of sHsps varies in different organisms. For example, in Drosophila, four small Hsps have been identified and in mammals three sHsps have been identified (αB-Crystallin, Hsp20 and Hsp27). In yeast, three sHsps have been identified (Hsp12, 26 and 42) (Wotton et al., 1996). Common to all sHsps is the highly conserved α-Crystallin core (100 amino acids in length) located at the C-terminal domain of the protein (Boelens et al., 1998). The N- terminal domain is variable in sequence and length. Under non- stressful conditions, the sHsps are located in the cytoplasm (Lavoie et al., 1993a) and also in the perinuclear zone (Preville et al., 1996). sHsps are also found in mitochondria as observed in Drosophila (Plesofsky Vig and Bramble, 1990). Under stress conditions, aggregates of sHsps translocate to the nucleus (Arrigo et al., 1988).

All sHsps have the ability to form oligomers or aggregates, with molecular weights of upto 800kDa depending on the cell type and conditions (Seizen et al., 1978a). sHSps aggregates are also found in prion disease (Radford et al., 1999), and neurodegenerative diseases including Parkinson's disease (Renkawek et al., 1999), Huntington's disease (Reddy et al., 1999), Creutzfeld-Jakob disease and Alzheimer's disease Like other Hsps, expression of the sHsps increases in response to elevated temperature (Landry et al., 1989). In vitro, sHsps display chaperone activity (Jakob et al., 1993) as they can induce re-naturation of denatured proteins in an ATP-independent manner. Non-native proteins interact with the large sHsp oligomers, which accumulate during heat shock, and may

create a reservoir of folding intermediates that could prevent further aggregation of non-native proteins (Ehrnsperger et al., 1997).

Both phosphorylation and oligomerisation are induced by stress, TNFα, Il-6, IL-3, IL-1, and retinoic acid (Kim et al., 1984; Welch, 1985; Kaur and Satlatvala, 1988; Mehlen et al., 1995b). The N- terminal domain contains MAPKAPK2 phosphorylation sites that are highly conserved (Stokoe et al., 1992). Human Hsp27 is phosphorylated at serine residues 15, 78, and 82 (Landry et al., 1992), whereas murine Hsp25 is phosphorylated at serine residues 15 and 86 (Gaestel et al., 1991). Oligomerisation has been studied in unphosphorylatable mutants of human Hsp27 in which serine 15, 78 and 82 were replaced with alanine, glycine or aspartic acid. Different patterns of Hsp27 oligomers were observed with large aggregates being generated with alanine substitution, and small aggregates formed with glycine and aspartic acid substitutions (Mehlen et al., 1997a).

1.3.6 Mechanism of Hsp gene regulation

The mechanism of Hsp gene regulation in response to stress such as heat shock has been well documented. The response is regulated by transcription factors known as Heat shock factors (HSFs). The mammalian family of HSFs consists of four members: HSF-1, HSF-2, HSF-3 and HSF-4. HSF-1 and HSF-3 acquire DNA binding activity in response to heat shock, ischaemia/reperfusion, hypoxia, and exposure of cells to heavy metal and amino acid analogues (Baller et al., 1993; Sarge et al., 1993). Moreover, cells in which HSF-1 is disrupted are not tolerant to mild stress and are not protected against heat-induced apoptosis (McMillan et al., 1998).

HSF-2 is constitutively expressed in mouse embryonic stem cells (Murphy et al., 1994). HSF-3 has been cloned only from chicken cells (Nakai and Morimoto, 1993) and is activated by various stresses that activate HSF-1. However, the thresholds of HSF-1 and HSF-3 are dissimilar (Tanabe et al., 1997) as HSF-3 activation is slow, whereas HSF-1 activation is rapid. HSF-3, however, is activated in conditions of severe stress.

HSF-4 is expressed in the heart, brain, pancreas and skeletal muscles. It lacks a carboxy terminal domain and is thought to be a negative regulator of the heat shock response, since it also lacks transactivation properties (Nakai et al., 1997).

The structure of HSFs is conserved. All HSFs have a DNA binding domain (DBD), which resembles the prokaryotic helix-turn-helix motif and is thought to be involved in intramolecular interactions (for sequestering monomers that are inactive) and intermolecular interactions (to form homotrimers that are capable of binding DNA) with high affinity. Further downstream is an oligomerisation domain, which forms a three-stranded α helical coiled coil and controls protein-protein interactions (Sorger and Nelson, 1989). The C-terminal domain is required for transcriptional activation. Also within this domain is an internal repressive domain, which regulates the protein's activity (Wu et al., 1995). Phosphorylation of HSF-1 occurs in the cytoplasm when HSF-1 monomers form a homo-trimer that is active, which then translocates to the nucleus to bind heat shock elements (HSEs) within the promoter of Hsp genes (Figure 1.5).

It was first suggested from experiments in yeast that the products of HSF target genes within the Hsp90 chaperone complex were directly involved in the negative regulation of HSF, and deletion of Hsp70 showed that there was an increase in abundance of other Hsps under HSF control in non-stressful conditions. Such studies have shown that Hsp70 is not sufficient alone to negatively regulate HSF (Abravaya et al., 1992).

Hsp90 is also another component, which interacts with HSF1 and negatively regulates its activity. Depletion of Hsp90 from the complex results in activation of the monomer to the trimeric state (Ali et al., 1998) whereas depletion of other chaperone proteins including Hsp70 does not activate trimerization and this suggests that the most important contacts of HSF1 in the complex are with Hsp90 itself (Zou et al., 1998). Furthermore, under non-stressful conditions, the association of HSP90 and HSF1 keeps HSF1 in an inactive form, however, under stressed conditions trimerisation of HSF1 occurs as a result of loss of Hsp90 from the chaperone complex.

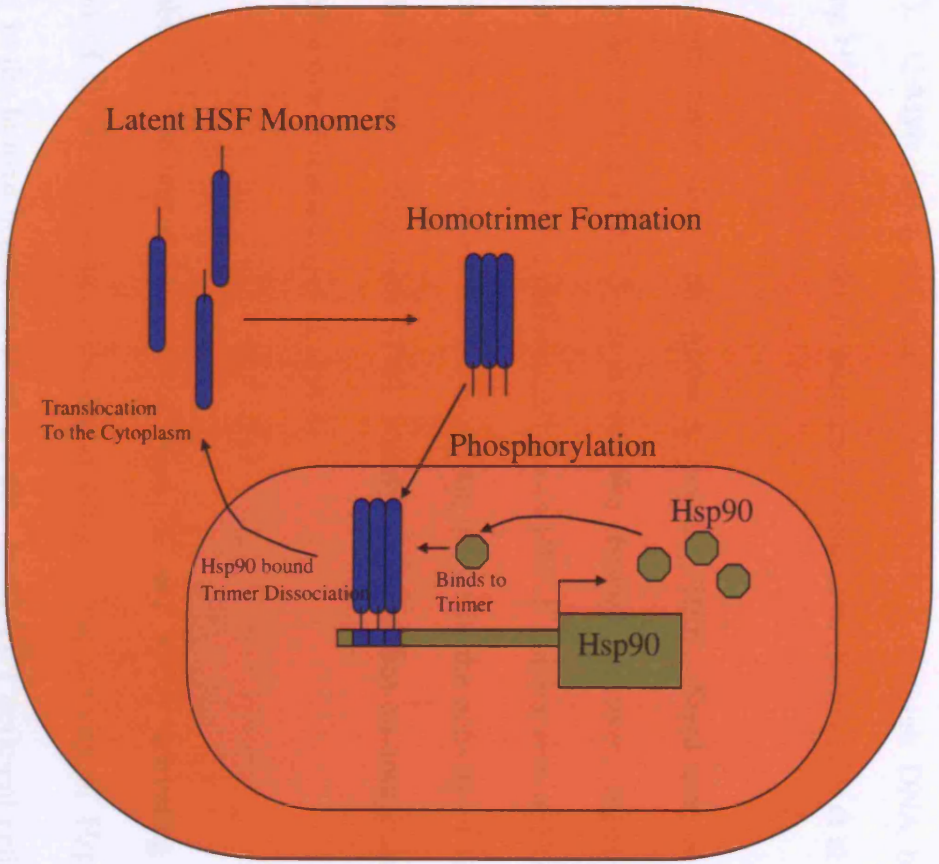
Other growth signals also promote activity of HSF1 through phosphorylation sites in the C-terminal trans-activation domain in response to MAPK (Chu et al., 1996). Serine to alanine mutations in the C-terminal domain results in lack of activation under normal conditions (Kline and Morimoto, 1997). HSF activity is also influenced by protein kinases at the trans-activation or DNA binding stages (see review by Morano and Thiele (1999)).

Figure 1.5 Mechanism of HSF-1 Activation

Environmental Stress
Inhibitors of Energy Metabolism
Transition Heavy Metals
Amino Acid Analogues
Heat Shock
Drugs and Toxic Chemicals

Non-Stressful Conditions
Cell Cycle
Development and Differentiation
Growth Factors

Pathophysiological State
Viral and Bacterial
Infection
Fever and Inflammation
Neuronal Injury
Hypertrophy
Ischaemia
Ageing
Cancer



HSF-1 has been shown to be required for roles in functions other than stress. Over-expression of cDNA of HSF-1 can block cell growth (Espinet et al., 1995) in yeast. In mammalian cells under non-stressful conditions, HSF can interact with c-Myb to stimulate the expression of hsp70 expression in cell proliferation and the cell cycle (Kanei-Ishii et al., 1997). C-Myb can form a complex with HSF-3 through DNA binding domains, stimulating HSF-3 nuclear entry in unstressed conditions (Kanei-Ishii et al., 1997).

Other proteins have also been shown to regulate HSF. Stephanou et al (1998) have previously shown that STAT-3 can activate the Hsp90β promoter. It has been shown that NFIL-6 and STAT-3 interact differentially with HSF-1. Interaction of HSF-1 and NFIL-6 in the presence of heat shock has a synergistic effect on the activity of Hsp90β promoter whereas HSF-1 interacting with STAT-3 and heat shock has an antagonistic effect on the Hsp90β promoter (Stephanou et al., 1998).

IFNγ is able to activate expression of Hsps 70 and 90 by activating HSF-1. Over-expression of STAT-1 has been shown to enhance the activity of Hsp70 and Hsp90β promoters in the human hepatoma HepG2 cells. In STAT-1 deficient cells (U3A) IFNγ is unable to activate Hsp70 and 90β promoters. However, when STAT-1 is re-introduced into this cell line, activation of Hsp70 and 90β is re-established in response to IFNγ. Interaction studies show that STAT-1 and HSF-1 co-operate in activating Hsp70 and Hsp90β promoters (Stephanou et al., 1999). Protein-protein interaction studies also show that HSF-1 interacts with STAT-1 but not with STAT-3.

1.3.7 Thermotolerance and Cell Protection

One of the most important roles of the Hsps is observed in stressful conditions such as heat shock, when proteins misaggregate due to intolerance to high temperature. One such phenomenon in which cells confer greater tolerance to heat stress is known as thermotolerance and has been studied extensively.

A state of tolerance is achieved when cells are subjected to a mild heat stress, thereby inducing the synthesis of the Hsps and protecting against a more severe stress. Thermotolerance is also known as preconditioning (Gerner and Schneider, 1975) and has been observed in various conditions ranging from developmental stages of organisms to cultured cells, whole mice, and Drosophila adults, but is not observed in all organisms such as aged animals. Heat treatments, which induce Hsps can be replaced by other conditioning treatments including ethanol, hypoxia and ischaemia. Such conditioning is usually transient, lasting 24 hours in cultured cells. Examination of tissues and organs subjected to various metabolic insults such as ischaemia has revealed that Hsp synthesis is increased for example in rat heart (Delcayre et al., 1988). Moreover, rats that were subjected to whole body hypothermia were found to suffer less myocardial damage in response to a subsequent ischaemia/reperfusion treatment (Donnelly et al., 1992).

High levels of Hsps can also protect cells from apoptosis as has been observed with Hsp70. In transient transfections, Hsp70 has been shown to block caspase activation and suppress mitochondrial damage as well as nuclear fragmentation (Mosser et al., 1997; Buzzard et al., 1998). Hsp70 has also been shown to inhibit apoptosis at particular points in the apoptotic pathway by preventing the recruitment of pro-caspase 3 and 9, and also JNK

activation (Beere et al., 2000; Merrin et al., 1999). Similarly, Hsp27 can also block apoptosis induced by heat shock, fas ligand, and anticancer drugs when overexpressed (Richards et al., 1996). However, this effect has not been observed in response to other stresses such as UV radiation (Trautinger et al., 1997).

It has been shown that Hsps can protect against ischaemic damage (Martin et al., 1997). For example, dorsal root ganglion (DRG) neurons can be protected against heat or ischaemic stress by overexpressing hsp70 and hsp90 using disabled herpes simplex viruses (Amin et al., 1996; Wyatt et al., 1996). In contrast, only Hsp27 has been shown to protect DRG neurons from apoptosis induced by nerve growth factor withdrawal (Wagstaff et al., 1999).

The sHsps are thus negative regulators in apoptosis (Samali and Cotter, 1996). For example, many studies have shown that expression of Hsp27 interferes with apoptotic death mediated by staurosporine (Mehlen et al., 1996b) in vitro.

In cultured cardiomyoctyes Hsp70 and Hsp90 gene transcription is induced by heat shock or ischaemia (Nayeem et al., 1997). In the intact heart, cardiac function is improved after a whole body heat shock followed by a global ischaemic insult applied 24 hours later compared to hearts that are given an ischaemic insult alone (Currie et al., 1988). Overexpression of Hsp27 and Hsp70 in viral vectors can protect cardiac cells from heat shock or ischaemia since they are able to survive better compared to control cells (Brar et al., 1998b). Transgenic mice over-expressing hsp70 have also been shown to exhibit

improved recovery of the myocardium after ischaemic injury (Marber et al., 1995; Plumier et al., 1995).

Hsps play a vital role in cell survival. Elucidation of new non-stressful Hsp inducers and their mechanisms are of importance since they could provide protection against stresses such as myocardial ischaemic injury. Cardiotrophin-1 (CT-1) and Urocortin (Ucn) are such inducers of Hsps that have been identified as protective factors which will be discussed in the next section.

1.4 Cardiotrophin-1 (CT-1) – A Member of The Interleukin-6 Family of Cytokines

1.4.1 Introduction

Cytokines are essential mediators of normal and pathological responses such as tissue injury and infection. Cytokines mediate signals into the cell via different cytokine receptors and intracellular signalling pathways. Responses to infection lead to activation of inflammatory and immune derived cytokine genes that also act on various organs in the body to maintain homeostasis (Dinarello, 1996). Cytokines show a wide variety of biological functions on various tissues and cells, and several different cytokines can exert similar and overlapping functions on a certain cell type. The IL-6 family of cytokines display overlapping functions and include interleukin 6 (IL-6), leukaemia inhibitory factor (LIF), oncostatin M (OM), interleukin 11 (IL-11), ciliary neurotrophic factor(CNTF) and cardiotrophin-1 (CT-1) (Gearing et al., 1992; Pennica et al., 1995b). This section will commence with a brief introduction about the IL-6 family of cytokines, followed by a detailed discussion about CT-1 signalling pathways, and finally, sections on CT-1- induced hypertrophy and cell protection. The final part of this introduction will discuss urocortin, signalling pathways activated by Ucn, and Ucn in cell protection.

1.4.2 The IL-6 Family of Cytokines

The IL-6 family of cytokines is pleiotropic and play varied roles in various tissues including haematopoietic, nervous and cardiovascular systems (Hilton, 1992); Yang, 1993; Kishimoto et al., 1995; Ip and Yancopoulos, 1996). IL-6 is involved in B cell differentiation (Miyaura et al., 1988) and is elevated in patients with congestive heart failure and myocardial infarction (Miyao et al., 1999) and in the myocardium of the canine model of ischaemia/reperfusion (Gwechenberger *et al.*, 1999; Chandrasekar *et al.*, 1999).

LIF is an inhibitor of M1 cell growth (Hilton and Gough, 1991), and OM has similar effects to LIF (Rose and Bruce, 1991). CNTF is involved in survival of ciliary neurons as well as motor neurons, and induces differentiation of oligodendrocytes into astrocytes (Stokli et al., 1989). IL-11 has similar activites to IL-6, whereas CT-1 is an inducer of cardiac hypertrophy (Pennica et al., 1995a).

1.4.3 IL-6 family receptor components

All members of the IL-6 family of cytokines share the common receptor component gp130. Gp130 expression is ubiquitous and is involved in signal transduction, but does not bind to the cytokine itself. The receptor subunit gp130 is widely expressed in other organs including the heart (Saito et al., 1992), Kishimoto et al., 1995) and has been identified to be a signal transducing protein The various receptor components of the IL-6 family can be seen in figure 1.6. All IL-6 and IL-6 –like cytokines can recruit gp130 alone (Hibi et al., 1990) or in combination with the LIF receptor (LIFR), or the OM receptor (OMR) (Gearing et al., 1991; Mosley et al., 1996). OM signals via gp130-LIFR heterodimerisation (Gearing et al., 1992).

IL-6, IL-11 and CNTF bind to specific α-receptor subunits: IL-6R, IL-11R, and CNTFR (Yamasaki et al., 1988; Hilton et al., 1994; Davis et al., 1991). Ligand binding, ligand/receptor-α complexes recruit the corresponding signal transducing receptor components. Receptor-α subunit expression, or, the presence of soluble receptors can determine the responsiveness of a cell to the cytokine. For example, LIF directly binds to the signal-transducing receptor components, as does OM. CT-1, however, requires an α-receptor subunit in addition to LIFR and gp130 (Robledo et al., 1997).

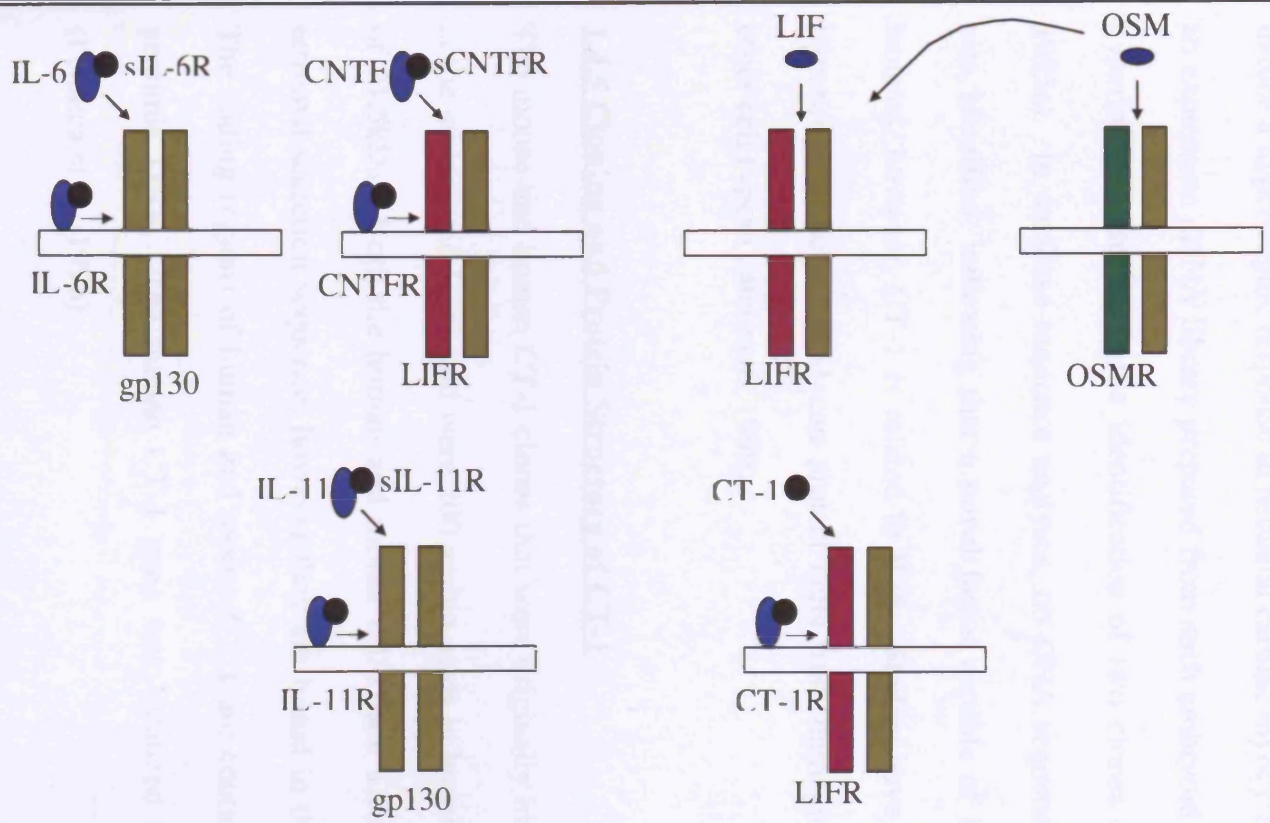


Figure 1.6 Receptor complex sharing gp130 as a signal transducer

IL-6 or IL-11 Signalling is triggered by homodimerisaion of gp130 as does IL-11. LIF, CNTF, OM, and CT-1 signaling requires heterodimerisation of gp130 with LIFR. However, OM can also signal directly through an OM specific receptor component with gp130, whereas CNTF, IL-11, and IL-6 associate with receptor components CNTFR, IL-11R, and IL-6R with then binds to cause dimerisation of gp130.

1.4.4 Identification of CT-1 as a hypertrophic factor

CT-1 was originally identified in a screen based on the finding that conditioned medium from differentiated embryoid bodies derived from mouse embryonic stem cells, was able to induce a hypertrophic response in neonatal cardiac myocytes. The subsequent screening of an expression cDNA library prepared from such embryoid bodies for the ability to induce hypertrophy resulted in the identification of two clones encoding CT-1 (Pennica et al, 1995a). In database sequence analyses, no DNA sequence matching these cDNA clone was identified, indicating that a novel factor capable of inducing hypertrophy had been isolated, however, CT-1 is related to IL-6. Studies have shown that as well as being a hypertrophic factor, CT-1 can protect cells from damaging stimuli in cardiac cells and other cell types (Latchman, 1999).

1.4.5 Cloning and Protein Structure of CT-1

The mouse and human CT-1 clones that were originally identified were 80% homologous at the amino acid level, and were 200 amino acids in length, thus giving a molecular mass of 21.5kDa. Both the human and mouse CT-1 lack a conventional hydrophobic amino terminal secretion sequence, however they are found in the medium of transfected cells. The coding regions of human and mouse CT-1 are contained in three separate exons of genomic DNA. The human CT-1 gene was localized to chromosome 16p11.1 p11.2 (Pennica et al., 1996).

Expression of both human and mouse CT-1 have been shown to occur during different stages of development and different tissues. For example, CT-1 is expressed in the heart at

day 8.5 in the mouse embryo whereas it is not expressed in the other tissues at this stage (Sheng *et al.*, 1996). However, in later developmental stages CT-1 is expressed in other organs but is still predominant in the heart.

The structure of CT-1 has been established. Its structure contains four amphipathic helices that are also observed in structures of other cytokines and growth hormones (Abdel-Meguid et al., 1987; Bazan et al., 1991). Although the cytokines share biological activities and receptor subunits, the amino acid sequences of human CT-1 and other members of the IL-6 family members reveal that they are only distantly related in primary sequences (15-25%). The cysteine residues are not conserved between but individual family members are more conserved across species (Gearing et al., 1992).

1.4.6 CT-1 signalling in hypertrophy

1.4.6.1 Overview to hypertrophy

Hypertrophy is observed in many pathological conditions and is an important mechanism in order to compensate to maintain cardiac function in response to increased work load, where increased workload is defined as an increase in wall tension in the heart and can occur as a result of hypertension, myocardial infarction or abnormalities in the valves. Wall tension is inversely proportional to thickness of the heart wall, therefore thickening of the heart as in hypertrophy reduces the wall tension and thereby reduces the oxygen demand of the myocardium (Force et al., 1999). Hypertrophy is characterised by an increase in cell size without division (Neyes and Pelzer, 1995).

In vivo, hypertrophy is measured by an increased heart to body ratio. In vitro, hypertrophy can be assessed by measurement of cell size, protein synthesis, sarcomeric organisation, and altered gene expression (Force et al., 1999). Contractile proteins such as myosin light chain 2 (MLC-2) are upregulated in hypertrophy and are involved in the formation of additional sarcomeric units in the hypertrophied cell (Lee et al., 1998). Skeletal α- actinin and β myosin heavy chain (β-MHC) embryonic contractile protein isoforms are also reactivated (Schwartz et al., 1986; Izumo et al., 1987) after 2 days of hypertrophic stimulus. Other non contractile foetal genes are also reactivated and include atrial natriuretic peptide (ANP- a characteristic marker of hypertrophy) and brain natriuretic peptide (BNP) (Knowlton et al., Lee et al., 1988; Harada et al., 1998; Hanford et al., 1994). These peptides are vasodilators and diuretics that lower blood pressure and enhance excretion of excess fluid, so reducing cardiac wall tension.

Within one hour of hypertrophy, expression of the immediate early genes c-fos, c-myc, c-jun and egr-1 is observed (Izumo et al., 1988). Moreover, changes in the expression of proteins required to maintain calcium levels are also observed during hypertrophy. Elevated intracellular calcium is associated with heart failure and cardiac hypertrophy (Richard et al., 1998).

1.4.6.2 Hypertrophic Stimuli

CT-1 and LIF are able to induce cardiac hypertrophy (Pennica et al., 1995a). Other stimuli are also able to induce cardiac hypertrophy and include physical stress such as cell stretch and mechanical overload (Komura et al., 1990; Kira et al., 1984; Cooper et al., 1985).

Angiotensin II (Ang II) and endothelin-1 (ET-1) and α-adrenergic agonists noradrenaline, adrenaline, and phenylephrine also induce hypertrophy (Dostal and Baker, 1998; Force et al., 1999).

1.4.6.3 The p42/p4 MAPK and STAT-3 Pathways in Hypertrophy

As mentioned above, CT-1 is able to induce hypertrophy in the heart and involvement of the p42/p44 MAPKinase and STAT-3 pathways have been implicated in CT-1 induced hypertrophy. In this section, these two pathways will be addressed.

The activation of the p42/p44 MAPKinase pathway requires the activation of Ras, an upstream activator of the p42/p44 MAPKinase pathway. Transgenic mice that express constitutively active ras have been shown to develop ventricular hypertrophy (Hunter et al., 1995). Constitutively active ras is able to stimulate increase in size and a hypertophic pattern of gene expression is observed in cardiac myocytes (Fuller et al.,1998). Raf is a kinase that is activated by Ras, and activates MEK-1 and the p42/p44 MAPKinase pathway. Transfection of constitutively active MEK-1 is able to increase ANP promoter activity in cultured cardiomyocytes (Gillespie-Brown et al., 1995). Transfection of a dominant negative MEK-1 reduces the induction of the ANP promoter activity by phenylephrine but does not effect sarcomeric organisation (Gillespie-Brown et al., 1995; Thorburn et al., 1994). Therefore, the p42/p44 MAPK pathway is required for induction of ANP by phenylephrine but not for other effects of phenylephrine. Inhibition of MEK-1 with PD98059 has no effect on ANP promoter activity by phenylephrine, but inhibits activation by AngII (Aoki et al., 2000). Therefore, the p42/p44 MAPK pathway is

involved in some aspects of the hypertrophic response, but it is not known which hypertrophic agents utilise this pathway to mediate their hypertrophic effects. In a study by Sheng et al. (1997) it is shown that the p42/p44 MAPK pathway is not involved in the hypertrophic response mediated by CT-1 since the hypertrophic effect in cardiomyocytes was not blocked by either PD98059 or a dominant negative mutant of MEK-1 (Sheng et al., 1997), and STAT-3 phosphorylation was not affected by inhibition of the MAPK pathway and is therefore likely to mediate the hypertrophic effect by CT-1 (Sheng et al., 1997).

Evidence to suggest that STAT-3 may be required for CT-1 induced hypertophy has been shown in studies in transgenic mice over expressing cardiac specific STAT-3. These mice developed hypertrophy with increased expression of ANP, b-MHC and CT-1 genes (Kunisada et al., 2000). Mechanical stretch and pressure overload also lead to phosphorylation of STAT-3 (Pan et al., 1997) which is mediated via gp130 (Pan et al., 1998). CT-1 has also been shown to activate angiotesinogen promoter via STAT-3 in cardiac cells and it has been suggested that this is a cellular signal for hypertrophy in cardiac muscle (Fukazawa et al., 2000). It is likely that the p42/p44 MAPK pathway is required for protective effects of CT-1 and STAT-3 is required for hypertrophic effects of CT-1.

1.4.7 CT-1 signalling in cell protection

Other than its ability to cause hypertrophy, it has been shown that CT-1 plays a role in cell survival (Sheng et al., 1996). In this study, treatment of rat neonatal cells with CT-1 was able to enhance survival in serum free media. Later studies have shown that pretreatment

of cardiac neonatal myocytes with CT-1 is also able to protect against heat shock or simulated ischaemia (Stephanou et al., 1998). Such protection by CT-1 in cardiac cells has been shown to be due to the ability of CT-1 to induce elevated levels of the heat shock proteins Hsp70 and Hsp90, which are primarily involved in the stress response, and as previously described (see section on the stress response) over expression of these proteins have shown to protect cardiac cells against ischaemia and heat shock (Heads et al., 1994; Cummings et al., 1996).

The protective effect observed in cultured cardiac cells is of great importance and it would be valuable to observe whether CT-1 is able to protect against ischaemia in the human heart. Sheng et al (1997) have shown that CT-1 is able to promote survival effects by minimizing the degree of apoptosis induced by serum removal. This has also been observed when cells are exposed to heat shock or ischaemia (Stephanou et al., 1998). Severe stresses such as heat shock or ischaemia have shown that more cells are prone to die due to apoptosis during the period of reperfusion (Gottlieb et al., 1994). However, studies have shown that CT-1 can protect when given after ischaemia at reperfusion in cardiac cells (Brar et al., 2001) and as well as before ischaemia. In the isolated rat heart, CT-1 is also able to protect whether added before ischaemia or at reperfusion (Brar et al., 2001). This result is desired since in a clinical setting the therapeutic effects of CT-1 would be required to operate when given at reperfusion after ischaemia.

Like many other cytokines, CT-1 has multiple effects. However, these effects are probably not separate, and there may be a fine- tuning as to when CT-1 should induce hypertrophy,

or when it should be protective to the cell. This fine balance can be understood by investigating the signaling pathways that CT-1 may activate to exert its effects. It is commonly understood that the IL-6 family of cytokines transmit signals via the gp130 subunit. Binding of CT-1 to its receptor results in the activation of the MAPkinase enzymes p42 (ERK1) and p44 (ERK2). In turn, threonine phosphorylation of nuclear factor IL-6 (NFIL-6) (or C/EBPβ) allows it to activate gene transcription (Nakajima et al., 1993).

In addition to activating the MAPKinases, the JAK/STAT pathway is also activated by CT-1 via dimerisation of STAT3 (Akira et al., 1994; Horvath and Darnell, 1997). Therefore, these two separate pathways are activated for cell survival or proliferation of the signal to cause hypertrophy. Thus Sheng et al (1997) have shown that the protective effect of CT-1 can be blocked by using the MAPKinase inhibitor PD98059. In cardiac myocytes, similar results have been demonstrated when exposed to ischaemia. The protective effects of CT-1 (whether added before or after an ischaemic episode) was abrogated in the presence of the PD98059 inhibitor, and similar results were observed in the intact heart (Brar et al., 2001). The JAK/STAT pathway is not affected by the inhibitor PD98059 in Sheng's study (1997), therefore, it could be concluded that the MAPKinase pathway is involved in the protective effect of CT-1 whereas the JAK/STAT pathway maybe involved in the hypertrophic effect of CT-1. Therefore, it may be possible to design CT-1 analogues which stimulate only the protective pathway or block the hypertrophic effect of CT-1 whilst maintaining its protective effect.

Another possibility would be to examine the inducibility of CT-1 gene expression by using various stimuli to enhance CT-1 levels. Recently, mouse CT-1 promoter studies have shown that norepinephrine can enhance the activity of the promoter. Various binding sites have also been mapped, one of which is a C/EBPβ binding site.

1.5 Urocortin

1.5.1 Introduction

Urocortin is a 40 amino acid peptide that exists endogenously in mammals and belongs to the family of corticotropin releasing factors (CRF). This family was originally isolated from ovine brain (Vale et al., 1981). CRF is known to coordinate endocrine responses to stress through its neurohormonal action as the major physiologic regulator of the hypothalamic pituitary adrenal axis (HPAA), and evidence supports the idea that CRF can function as a neurotransmitter within the central nervous system (CNS), along with coordinating autonomic, behavioural and immunological responses to stress (Owens and Nemeroff, 1991). Homologues to CRF have been isolated from fish as well as some amphibians (Okawara et al., 1988; Montecucchi and Henschen, 1981; Anastasi et al., 1980) and eventually from vertebrates (Stenzel et al., 1992).

It was thought that many other members of the CRF family would exist in mammals, which may be closely related to urotensin I, which was also isolated as a relative of CRF from fish. UCN was isolated by using urotensin I cDNA probes as well as antisera, and was found to be a peptide of 40 kDa, similar to CRF. This novel peptide was isolated from the rat brain, and showed at least 45% homology to rat CRF and 63% homology to carp urotensin I (Vaughan et al., 1995). Mouse and human UCN were isolated and characterized (Zhao et al., 1998). The human homologue for UCN was identified from partial genomic sequences (Donaldson et al., 1996). Nucleotide cDNA homology between human and mouse amounts to 88% and 97% respectively and 95% and 100% homology at the mature peptide level. UcnII and UcnIII have also been isolated.

At the genetic level, the UCN gene is very similar to the CRF gene, with 2 exons and the coding region being contained within the second exon (Zhao et al., 1998). In transient transfections, promoter activity of urocortin was significantly reduced in response to forskolin stimulation when mutations were introduced in the CRE sequence, suggesting that CRE is involved, but other factors may be involved in regulation of the urocortin promoter. The CRE has also been shown previously to mediate activation of the CRF promoter by cAMP (Dorin et al., 1993; Seasholtz et al., 1998).

Messenger RNA for Ucn is predominantly located in the Edinger-Westphal nucleus (EWN) and the lateral superior olive in the central nervous system (Bittencourt et al., 1999; Kozicz et al., 1998). Reasonable levels of mRNA of Ucn are found in other regions of the brain including cerebellum, hippocampus, neocortex, olfactory system, basal ganglia, supra optic nuclei (Bittencourt et al., 1999).

1.5.2 CRF receptors

High affinity binding sites for CRF were identified by autoradiographic techniques (De Souza et al., 1985) and the first receptor was cloned in 1993 (Chen et al., 1993; Perrin et al., 1993). This receptor, CRF1R, is 415 amino acids in length, and is a member of the seven transmembrane spanning G-coupled protein receptor family. CRF receptors are coupled to adenylate cyclase (Vaughan et al., 1995). Binding of the ligand to the receptor causes phosphorylation of PKA by cyclic AMP release. In turn, phosphorylated PKA phosphorylates calcium channels to allow calcium ions to enter the cell (Ikeda et al., 1998).

There is a splice variant of Ucn receptor which is found in humans, and has 29 amino acids extra in the extracellular domain (Xiong et al., 1995).

The second receptor, CRF2R, was later cloned from mouse, rat, and human in 1995 in three separate studies (Kishimoto et al., 1995; Lovenberg et al., 1995; Liaw et al., 1996). The CRF2R shows 70% homology to CRF1R. CRF2R exists as two splice variant forms α and β, and the distribution of these variants is different. The CRF2Rα is 411 amino acids in length and is the primary isoform found in the brain, whereas CRF2Rβ is only expressed in the heart. In addition, a third splice variant has been identified as CRF2Rc (Kostick et al., 1998). Ucn appears to be the preferred endogenous ligand for the CRF2R as it has high affinity for CRF2R. CRFR1 receptors show little specificity between CRF family members (Gottowik et al., 1997). Synthetic UCN has much higher affinity binding to the CRFR2 than CRF itself (Zhao et al., 1998).

Ucn has been implicated in various biological effects. For example, as mentioned earlier, it is responsible for many behavioural and physiological effects that are in fact, similar to the effects of CRF. Action of synthetic UCN on the CRFR1 leads to stimulation of pituitary ACTH release, increase in anxiety, and action on the CRFR2 leads to vasodilation, cardiac ionotropism, reduction of vascular permeability and suppression of appetite (Vaughan et al., 1995; Parkes et al., 1997; Turnbull et al., 1996; Spina et al., 1996).

CRF1R antisense, agonists, and knock out mice have shown that CRF1R is predominantly required for stress responses (Skelton et al., 2000). Interestingly, mice that lack a CRF2R exhibit effects on the co-ordination of food and appetite intake (Coste et al., 2000), and lack of cardiovascular effects of Ucn.

The CRF2Rα message is found in arteries and arterioles (Chalmers et al., 1995)and is the predominant isoform in the human heart (Chen et al., 1993). CRF2Rβ is highly expressed in the rat heart (Stenzel et al., 1995). CRF/Ucn affect cardiovascular and respiratory functions indicated by hyperventilation and hypotension (Kubler et al., 1994). Ucn can also induce relaxation of blood vessels in vitro (Lei et al., 1993), and has recently been shown to have relaxant actions in the isolated rat basilar artery (Schilling et al., 1998). Evidence is also increasing showing that Ucn can affect mechanisms in the heart predominantly via the CRF2β receptor. The first report to find that Ucn mRNA was present in rat cardiac myocytes suggested that Ucn may play roles in protecting these cells from hypoxia induced apoptosis (Okasi et al., 1998) and it also stimulates the secretion of atrial natriuretic peptide (ANP) (Ikeda et al., 1998).

1.5.3 Urocortin in the Heart

Many studies have shown that Ucn has an important role in the heart. By injecting Ucn intravenously, cardiac contractility increases in a dose-dependent manner, as does heart rate, cardiac output and coronary blood flow (Parkes et al., 1997).

Ucn levels are increased in cardiac myocytes in response to simulated ischaemia.

Ucn can protect cardiac myocytes from lethal ischaemic injury and subsequent apoptosis. Cardiac myocytes that are given preconditioned media exposed to 2 hour simulated

ischaemia were protected from cell death induced by 6 hours ischaemia. This protective effect is blocked by α helical CRF, which is a competitive inhibitor of CRF family peptides. Ischaemia increases Ucn mRNA through the CCAAT enhancer binding protein (C/EBPβ) and that mature peptide protected via the CRF2R (Brar et al., 1999a). Ucn causes rapid phosphorylation of the ERK 1/2-p42/p44 MAPkinase pathway, and the effect is abrogated by blocking MEK1-ERK1/2 p42/p44 cascade using a specific inhibitor PD98059 in the intact heart (Brar et al., 2000). Ucn/hypoxia reoxygenation leads to cardioprotection by MEK1 and MEK2, which is an upstream component of the p42/p44 MAPK pathway (Brar et al., 2002).

Ucn is also able to protect cardiac cells by activating Akt (Brar et al., 2001). Akt is known to be activated by growth factors and also PI-3 Kinase (PI-3K is crucial for cell survival of non cardiac and cardiac cells (Yao et al., 1995; Kaufmann et al., 1997; Kuwahara et al., 2000)). Akt phosphorylation inhibits caspase 9 activity (Cardone et al.,1998). Inhibitors of PI-3K, Akt block the cardioprotective effect of Ucn. CT-1 is also known to activate Akt and PI-3K to mediate cell protection (Brar et al., 2001), therefore, signaling pathways of CT-1 and Ucn are identical. In addition, the protective effect of Ucn requires de novo protein synthesis whereas that of CT-1 does not (Brar et al., 2002). Ucn has also been shown to increase expression of hsp90 in cardiac myocytes in a MEK1/2-dependent manner (Brar et al., 2002).

CHAPTER 2.

MATERIALS AND METHODS

2.0 Consumables and Conditions

Bacterial culture manipulations were carried out under sterile conditions using media and glassware that were autoclaved prior to use at 120°C, 10lb/square inch for 20 minutes. All plastics also used were sterile. Aseptic conditions were used to prevent contamination.

2.1 Propagation and purification of plasmid DNA

2.1.1 Preparation of competent cells

XL1Blue *Escherichia coli* (*E. coli*) were used to propagate plasmid DNA. XL1Blue cells were streaked on a Luria Bertani (LB) agar plate (1% (w/v) NaCl, 1% (w/v) Bacto®-tryptone, 0.5% Bacto®-yeast extract, and 2% Bacto®-agar) and incubated at 37°C for 18 hours. A colony was picked from the plate to inoculate 5ml LB broth (1% (w/v) NaCl, 1% (w/v) Bacto®-tryptone, 0.5% Bacto®-yeast extract). The culture was grown at 37°C at 220 rpm in an orbital shaker for 18 hours.

200µl of the culture was used to inoculate 200ml LB in a sterile conical flask and incubated at 37°C 220rpm for 4-6 hours until opaque. The culture was centrifuged at 3000g in 50ml Falcon tubes for 10 minutes in a Sorvall centrifuge at 4°C to obtain a pellet. The pellet was washed in 500µl of ice- cold 100mM $CaCl_2$ by re-suspending the cells carefully and centrifuged for 2 minutes at 13000rpm at 4°C. After discarding the supernatant, the pellet was re-suspended in 2ml of ice cold $CaCl_2$ (100mM) and stored on ice for 30 minutes. 100µl aliquots were made in sterile 1.5ml eppendorfs for immediate use.

2.1.2 Transformation of plasmid DNA

For transformation, 1μg of plasmid DNA was added to 50μl of competent cells and incubated on ice for 15 minutes. Cells were heat shocked for 1 minute at 42°C and placed on ice immediately after for a further 15 minutes. 900μl of LB was added to the cells and incubated at 37°C for 1 hour in an orbital shaker at 220rpm. The cells were centrifuged for 30 seconds at 145g at 20°C. The supernatant was discarded and the cell pellet was re-suspended in 100μl LB and spread on a LB agar plate containing 100μg/ml Ampicillin. The plate was kept in a 37°C incubator for 18 hours and then stored at 4°C for at least a month.

2.1.3 Extraction of plasmid DNA from *E. coli* XL1Blue bacterial cells (Large Scale)

For a large- scale DNA preparation, a single colony of the transformed *E. coli* cells containing the DNA plasmid of interest was picked to inoculate 250ml of LB medium containing 100μg/ml of Ampicillin and grown for 18-24 hours at 37°C in an orbital shaker at 220rpm. Plasmid DNA was isolated by alkaline lysis of cells, purification of DNA using a Qiatip100 (Qiagen Ltd, UK), followed by elution and precipitation in cold isopropanol. Precipitated DNA was centrifuged at 12000g for 30 minutes at 20°C, and then washed in 70% ethanol. Purified DNA was dried and re-suspended in 100μl of sterile water.

Concentration of DNA was determined by spectrophotometry by reading the absorbance wavelengths at A_{260} and A_{280}. The average yield of DNA obtained from a Qiatip 100 preparation was 100μg per preparation. DNA concentration was calculated by the following equation:

Purity of DNA = OD_{260}/OD_{280} = 1.7 in a pure sample of DNA.

1 OD at A_{260} =50μg/ml of double stranded DNA

Concentration of DNA = $\frac{OD_{260} \times 50 \times 100}{1000}$

2.1.4 Extraction of plasmid DNA (small scale)

Small- scale DNA preparations were carried out to screen several colonies for the correct plasmid. A single colony of transformed XL1Blue cells was picked from a plate to inoculate 5ml of LB containing Ampicillin at 100μg/ml and grown for 18 hours at 37°C in an orbital shaker set at 220rpm. 1.5ml of the resulting culture was transferred to a sterile 1.5ml eppendorf tube and centrifuged for 1 minute at 12000g. The supernatant was discarded and the pellet was re-suspended in 100μl of re-suspension buffer (100mg/ml RNAse-A, 50mM Tris-HCl pH 7.5, 10mM EDTA pH 8.8). Cells were lysed in 200μl of lysis buffer (200mM NaOH, 1% Triton X-100) and neutralized in 150μl 3M NaOAc, pH 5.5. Tubes were vortexed and centrifuged at 12000g for 2 minutes. The pellet (cell debris) was removed with a bent hypodermic needle. To precipitate DNA, 500μl of isopropanol was added and mixed. The supernatant/isopropanol was centrifuged at 12000g for 5 minutes. The supernatant was discarded and the pellet washed with 500μl of 70% ethanol. The ethanol was removed carefully and air-dried. The DNA pellet was re-suspended in 25μl distilled H_2O.

2.1.5 Identification of plasmid DNA by Restriction digestion

Plasmid DNA was digested with the appropriate restriction enzymes (Promega) to identify plasmid and insert DNA. 1μg of DNA was incubated with 10 units of restriction enzyme. 1μl of restriction buffer was added and the final volume was made up to 10μl with ddH_20. DNA digestion was carried out at 37°C for 2 hours, or as recommended by Promega, depending upon the enzyme being used.

Digested DNA products were examined on a 1% agarose gel (1% agarose (w/v) dissolved in 1xTAE (0.4 M Tris, 0.2M NaAc, pH8.3 adjusted with HCl). Ethidium bromide (0.5mg/ml) was added upon cooling. The gel was cast in a tray containing a suitable comb. Once set, the comb was removed and the gel was placed in an electrophoresis tank containing 1xTAE. DNA was mixed with 1xDNA loading buffer (0.025% (w/v) bromophenol blue, 50% glycerol (w/v), 1xTAE). A 1kb ladder (Gibco) was loaded along side the samples and run at 70-100 volts depending on the size of the gel. DNA bands were observed on a trans-illuminator and a photograph was taken on the Syngene gel analyzer).

2.2 Mammalian Cell Culture

All media, balanced salt, trypsin, versene, penicillin/streptomycin, and foetal calf serum were purchased from Life Technologies, UK. All plastics used were supplied by Nunc and Falcon.

All cell culture work was carried out under sterile conditions in a class II microbiological safety cabinet. Media and reagents were either filter sterilized (0.2μm filter) or autoclaved before use.

2.2.1 Cell Lines

2fTGH and U3A human fibrosarcoma cell lines were obtained from the Imperial Cancer Research Fund (ICRF, London, U.K.). They were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS (w/v), 100 units/ml penicillin and streptomycin (all Life Technologies, U.K.) and 250μg/ml Hygromycin B (Sigma).

For 2fTGH and U3A cells, 2ml of 10% trypsin (w/v) in versene was used to wash cells. A further 2ml was added, making sure that all cells were covered and incubated at room temperature for 60 seconds. The side of the flask was tapped to detach cells from the flask.

Cells were transferred to a 15 ml Falcon tube and centrifuged at 145g for 5 minutes. PBS was removed carefully and the cell pellet re-suspended in fresh growth medium. Cells were plated at a density of $1x10^3$ cells/ml for transfections, and $1x10^6$ cells/ml for Western blotting. Freezing Cells for maintenance of stock cells

Stocks of cells were maintained by freezing and storing them at $-80^{o}C$. Cells were trypsinised and centrifuged as previously described. The cell pellet was re-suspended in media I (60%DMEM and 40% FCS in a total of 2.1ml). 0.9ml of cells was aliquoted into

cryo tubes and made up to 1.8ml with media II (5.4ml DMEM, 3.0ml FCS, 1.6ml DMSO). Tubes were placed on ice for 30 minutes before transferring to –80°C for 24 hours. Cells were placed in liquid nitrogen until required.

To recover cells, the vial was placed in 37°C incubator. Cells were transferred to $25cm^3$ flask containing media with 20% FCS. After 24 hours of incubation, any dead cells were removed and replenished with fresh media (20% FCS).

2.2.2 Preparation of primary neonatal cultured cells

Primary rat neonatal cardiac cells were prepared according to the method described by Simpson and Savion (1982b). Hearts were tri-sected from 2-3 litters of neonatal rats into ADS buffer (6.8g NaCl, 4.76g HEPES, 0.12g NaH_2PO_4, 1.0g, 0.4g KCl, 0.1g $MgSO_4$). The heart tissue was then incubated in an enzyme solution to digest the tissue (30mg collagenase and 500µl pancreatin in 100ml ADS buffer). 5mls of the enzyme containing ADS buffer was added to wash the tissue. Subsequent digestions of the tissue were carried out by digesting for 15 minutes in an incubator at 37°C with 5%CO_2, 95%O_2. Cells were then centrifuged for 5 minutes at 500g. The supernatant was discarded, and the pelleted cells re-suspended in 2ml of FCS. The cell suspension was kept in the incubator at 37°C. Digestions were repeated 5 more times, and the cell suspension was pooled, and centrifuged. The pellet was re-suspended in DMEM containing 15% (v/v) FCS, 1% Penicillin and Streptomycin. Cells were pre-plated in a $175cm^3$ flask so that fibroblasts adhere to the plate, leaving cardiac myocytes in suspension. Medium containing mostly cardiac cells were then plated on gelatin- coated dishes (1% gelatin in PBS) at 10^6 cells per

ml. The cells were allowed to adhere to the plates by incubating for 24 hours, after which they were ready to either transfect or treat otherwise. When stimulating cells, media was replenished with DMEM containing 1% FCS and 1%Penicillin-Streptomycin).

2.2.3 Cell stimulation treatments

Recombinant Murine Interferon γ (Sigma) and recombinant human Interferon γ (Sigma) were dissolved in sterile 1x PBS to a final concentration of 25ng/μl. For experiments, a final concentration of 50ng/μl was used.

Synthetic Rat Urocortin (Sigma) was dissolved in ethanol and used at a dose of $1x10^{-7}$M.

Heat stress was applied to cells by wrapping the plate containing cells with parafilm and floating in a water bath for 30 minutes at 43°C. Since pre-warming the medium did not have any affect when small amounts of media were used, medium on cells was not changed prior to or after heat shock. After heat shock, parafilm was removed and the plate dried of excess water and placed back in the incubator at 37°C.

Simulated hypoxic stress was administered by using a hypoxic chamber with a constant flow of 5%CO_2, 95%Argon (BOC gases) at 37°C. Prior to hypoxic stress, cells were replenished with media (either 500μl of media for a 6 well plate, or 200μl for a 24 well plate. With low or nil oxygen conditions, hypoxic conditions are achieved, and any small amount of molecular oxidative phosphorylation will dissipate within minutes and the cells will no longer have any molecular O_2 to respire). The plate was placed in the chamber in a sealed environment to prevent escape of any gas and stress was given for 4 hours, after

which 1.5ml of fresh media was added to each of the wells and the plate returned to the incubator at 37°C, 95%O_2, 5%CO_2.

Simulated ischaemia/re-oxygenation was applied by using the hypoxic chamber as previously described. However, cells were replenished with ischaemic buffer (137mM NaCl, 12mM KCl, 0.49mM $MgCl_2$, 0.9mM $CaCl_2.2H_2O$, 4mM Hepes, 10mM deoxyglucose, and 20mM sodium lactate pH 6.7) (Esumi et al., 1991) and placed in the chamber for 4 hours.

2.2.4 Transient transfections

All transfections were carried out by the calcium phosphate method. Cell lines and primary neonatal rat cardiac myocyte cells were transfected 24 hours after plating. In order to determine transfection efficiency, 5μg of β-gal expression vector was transfected overnight into cardiac myocytes. The cells were allowed to express the gene for 24 hours. The cells were then fixed with 1% (w/v) paraformaldehyde solution in PBS and stained with X-gal (Stephanou et al. 2000). The number of blue stained β-gal expressing cells is expressed over the total number of cells. The transfection efficiency for myocytes was 5% and for cell lines 10%. Plasmid DNA used for transfections are shown in table 2.1.

The DNA precipitate was prepared by aliquoting DNA and making up the remainder up to 250μl with sterile distilled water. 31μl of 2M $CaCl_2$ was added to the DNA and mixed. The DNA/$CaCl_2$ mix was added dropwise to 220μl of 2xHepes buffered saline (HBS) (50mM HEPES, 280mM NaCl, 1.5mM Na_2HPO_4 made up in water to pH 7.1). This mixture was left at room temperature for 20-30 minutes so that a fine white precipitate had

formed. 501μl of the precipitate was added to either a 6 well plate or 24 well plates and incubated at 37°C for 18 hours. Cells were washed with fresh DMEM containing 1% FCS, followed by a final 1ml of the same media per well. Cells were then treated as required.

Table 2.1. A list of DNA plasmids used in transient transfections

Plasmid	Details	Reference
Hsp90β-A	-1044 to +36 region of the hsp90β promoter upstream of the CAT reporter gene	Rebbe et al., 1989
Rccmv	Empty vector containing the cmv promoter only	Invitrogen, 9351 NV Netherlands
cmv-β-gal	Lac-z cDNA driven by the cmv promoter	Liu et al. (1998)
pR19-27	Chinese hamster hsp27 cDNA driven by cmv promoter	Wagstaff et al (1997)
CT-1d	-99 to +19 region of the ct-1 promoter upstream of the luciferase reporter gene	Funamoto et al.,1998
TK- *Renilla* luciferase	Viral thymidine kinase (TK) promoter driving expression of the *Renilla* luciferase gene	Promega
STAT-1	Full length cDNA of STAT-1	Curt Horvath, Mount Sinai Hospital, USA
STAT-1 390-750	Amino acids 390-750 of STAT-1	
STAT-1 691- 750	Amino acids 691-750 of STAT-1	
STAT-3	Full length cDNA of STAT-3	Akira et al. (1994)

	cloned in expression vector	
[1,3]297-514[1]	Aa 1-297 of STAT-1 fused with aa 293-514 of STAT-3, and 509-750 of STAT-1 at the C-terminus	Darnell et al. (1995)
[3,1]293-508[3]	Aa 1-296 of STAT-3 fused with aa293-508 of STAT1, and 515-781 of STAT-3	Darnell et al. (1995)
[3]296[1]	Aa 1-296 of STAT-3 fused with 293-750 of STAT-1	Darnell et al (1995)
pEF STAT-1α D694/E	Mutation at position 694 of asapartic acid residue to glutamic acid in STAT1	King and Goodbourn (1998)
pEF STAT-1α D694/A	Mutation at position 694 of aspartic acid to alanine	King and Goodbourn (1998)
pEFSTAT-1α G695/STOP	C-terminal amino acids at glycine 695 replaced with STOP codon	King and Goodbourn, (1998)

2.3 Assessment of promoter activity

Treated cells were washed in 1xPBS to remove excess media with an aspirator. 1 x reporter lysis buffer was added to the cells (100µl to 6 well dish) and incubated at room temperature for 10 minutes to allow lysis of cells. Cells were scraped using a cell scraper and transferred to a sterile1.5ml eppendorf. The cell suspension was centrifuged at 13000g for 5 minutes at 4°C to pellet the cell debris. The supernatant containing total cell contents was transferred to a sterile eppendorf and stored at –20°C until required.

2.3.1 Luciferase assay

In order to measure the amount of luciferase produced by the reporter constructs, the dual luciferase system™ (Promega) was used. This system allows quantification of the firefly luciferase and sea pansy (*Renilla reniformis*) luciferase in the same assay, because their enzyme structures are dissimilar, therefore the bioluminescent reactions of both can be distinguished. The *Renilla* luciferase is driven by the constitutively active thymidine kinase promoter and is co-transfected with the firefly luciferase construct and is an internal control for the assay.

The general method that has been adopted is according to the manufacturers instructions. Briefly, 10μl of the lysate is mixed with 100μl of the Luciferase assay Reagent (LARII). The amount of luciferase was measured by using a delay time of 5 seconds, and an integration time of 20 seconds (actual reading) on the luminometer (Turner). In order to read the Renilla, 100μl of Stop and Glow® was added to the tube and mixed and the Renilla reading was taken from the luminometer. For standardized transfection efficiency, the firefly luciferase reading was divided by the Renilla luciferase reading.

2.3.2 Chloramphenicol acetyl transferase (CAT) assay

As previously described, protein was extracted from cells that were transfected with CAT reporter constructs. In addition, 3 cycles of freeze-thaw were also carried out to increase the amount of protein for the assay. CAT catalyses the transfer of the acetyl group from acetyl-CoA to the substrate, chloramphenicol. The enzyme reaction can be quantified by incubating the lysates with [^{14}C]chloramphenicol and following product formation by

physical separation with thin layer chromatography (TLC). The components for the assay were as below per tube:

50µl cell extract

20µl 40mM acetyl CoA

2µl 200uCi/ml [^{14}C] chloramphenicol

32.5µl 1M Tris-Cl, pH 7.5

45.5µl ddH_2O

The reaction was incubated at 37°C for 2 hours. To stop the reaction, 1ml of ethyl acetate was added to the tube and vortexed for 1 minute. The top layer (organic phase) was transferred to a sterile 1.5ml eppendorf tube and dessicated in a Speedvac for 45 minutes, after which the sample was re-suspended in 15-20µl of ethyl acetate. The sample was spotted 5ul at a time onto a dot (marked by pencil) on a thin layer chromatography plate 2 cm from the bottom. The plate was run for 55 minutes in a tank containing 100ml of 19:1 chloroform:methanol. The tank was covered with a glass lid, and sealed with Vaseline to prevent evaporation of the solvent. The TLC plate was allowed to dry, and then exposed to autoradiographic film for 2-3 days. For quantification, spots on the film were measured by using a GS-800 denistometer (BioRad), and CAT activity was calculated as:

$$\% \text{ Acetylated} = \frac{\text{Acetylated product}}{\text{Acetylated product} + \text{Non-Acetylated product}}$$

2.3.3 β-galactosidase assay

The β-galactosidase reporter vector was used in co-transfections with the CAT constructs as a control vectors for normalizing transfection efficiency. The lac-z reporter gene is driven by the RSV promoter, and the amount of β-galactosidase enzyme produced was measured with the Galacto-Light™ kit (Tropix Inc. Massachusetts, USA) according to the manufacturer's instructions.

The Galacton substrate was diluted with Galacto-Light reaction buffer diluent (1/100 dilution). 20μl of cell lysate was mixed with 50μl of reaction buffer in an eppendorf tube and incubated at room temperature for 30 minutes. 30μl of accelerator was added to the tube, mixed and read on a luminometer with a 5 second delay and 45 seconds reading time. A negative control was used where cells were not transfected with the lac-z reporter construct. The amount of enzyme in the lysate was proportional to the luminometer reading, which represented the amount of light produced by the β-gal reaction.

2.4 Preparation of complementary DNA (cDNA) probes

Complementary DNA probes were designed and supplied by (company). The probes were labeled by random priming (Feinberg and Vogelstein, 1983). All solutions and water were treated with diethyl pyrocarbonate (DEPC) to prevent any RNAse contamination.

In order to label DNA probes, 2μl of the DNA fragment was denatured at 97°C for 10 minutes in 20μl of ddH2O and snap cooled on ice. 10μl of oligolabelling buffer, 2μl of bovine serum albumen, 50μCi α-^{32}P-dATP and 5 units of DNA polymerase large fragment

1 (Klenow) were added to the single strand DNA and incubated at 37°C for 1 hour. The labeled probe was then filtered through a G50 sephadex column by centrifugation for 5 minutes at 400g.

2.4.1 Analysis of RNA levels

Gloves were worn at all times to prevent contamination from RNAses and all solutions were DEPC treated and autoclaved. All plastics were sterile, and all equipment was treated with RNAse Zap (Invitrogen) and washed with DEPC treated water.

2.4.2 RNA extraction

Cells were washed twice in sterile 1xPBS, and all traces of PBS were removed. To cells plated on a 6 well plate, 500µl of RNAsol (Genyosys) was added per well. Cells were scraped using a cell scraper, and transferred to a sterile 1.5ml eppendorf tube. Cells were incubated at room temperature for 15-20 minutes and then centrifuged at 13000g for 1 minute at 4°C. The aqueous layer (upper) was transferred to a sterile 1.5ml eppendorf tube. 500µl of chloroform was added and vortexed. This mixture was incubated for 2 minutes on ice before centrifuging at 13000g for 1 minute at 4°C. The aqueous layer was transferred to a sterile 1.5ml eppendorf tube. 500µl of isopropanol was added and mixed. RNA precipitation was achieved by storing at –80°C for 24 hours. The RNA was pelleted by centrifugation at 13000g for 20 minutes at 4°C. The supernatant was carefully removed and the pellet re-suspended in 20µl of DEPC treated water, and stored at –80°C until required. 1µl of the RNA was used to run on an agarose gel to determine the quality of the RNA preparation.

2.4.3 RNA slot blot

10mg of RNA was incubated in 3 volumes of denaturing solution (500ml formamide, 163ml formaldehyde (37% solution), 100ml 10xMOPS buffer) at 65°C for 5 minutes. The denatured RNA was then chilled on ice and one volume of ice -cold 20x SSC (150mM NaCl, 15mM sodium citrate, pH 8) was added. RNA was spotted on to Hybond N+ membrane using a slot blotting apparatus, and fixed to the membrane by UV cross linking.

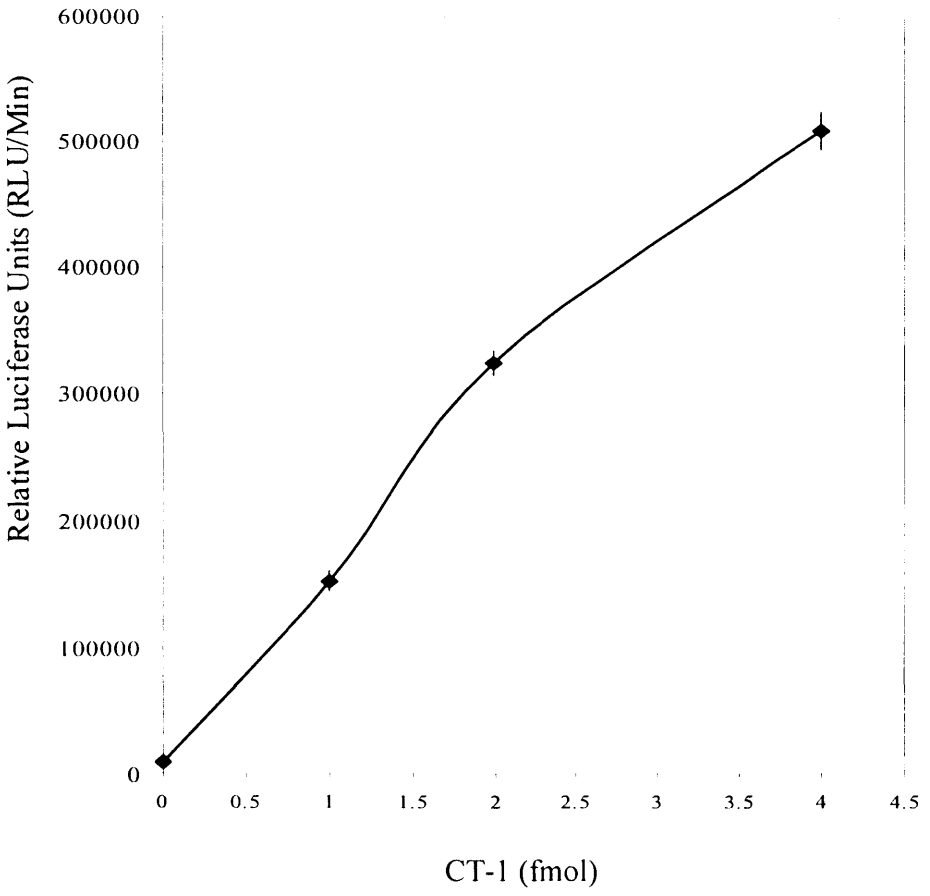
The membrane to be probed was pre-hybridised in hybridization solution (5x SSC, 5x Denhardts reagent, 0.5% (w/v) SDS, 100μg/ml denatured herring sperm DNA, made up to volume with ddH_2O) for 1 hour at 65°C. The hybridization solution was replaced with fresh pre-warmed hybridization solution. The radiolabeled probe was denatured at 97°C for 5 minutes and snap- cooled on ice. The denatured radiolabeled probe was added to the hybridization solution with the membrane and incubated overnight at 65°C in a rotating hybridization oven for 20 hours. The membrane was washed in 2 x SSC, 0.1% SDS in ddH_2O at 65°C for 15 minutes and wrapped in cling film to be exposed to film for 20 hours. If the membrane was not clean, additional washes were performed and subsequently exposed to film.

2.4.4 Non competitive Immunoluminometric Assay for CT-1

Medium supernatants from cardiac myocytes subjected to hypoxia, urocortin or both were recovered and EDTA and aprotinin added to a final concentration of 4 mmol/L and 1mg/L, respectively, before freezing at -70°C until assayed.

The assay was devised and performed by Professor Leong L. Ng (Leicester Royal Infirmary, University of Leicester). Enzyme-linked immunosorbent (ELISA) plates were coated with 100ng per well of CT-1 antibody directed toward the mid-section (amino acids 105-120) of human CT-1 followed by blocking in 10% foetal calf serum. The affinity purified antibody directed towards that C-terminal (amino acids 186-199) of CT-1 was biotinylated and added to the wells with 100 μL of standards (ranging from 0-20 fmol per well) or media supernatants. After incubation for 24 hours and washes, detection was with streptavidin labelled with 4-(2-succinimidyl oxycarbonylethyl) phenyl-10-methylacridinium 9-carboxylate fluorosulfonate, with sequential injections of H_2O_2 in HNO3 followed by NaOH and acetyl ammonium bromide. Chemiluminescence was measured as relative light units on a Dynex MLX microplate luminometer. The lower limit of detection in this assay was 2.9fmol/mL and can be seen in figure 2.1.

Figure 2.1 Standard curve to show CT-1 levels



2.5 Analysis of protein levels

After treatments, cells were washed in 1ml 1xPBS. Cells were then harvested by scraping in 80μl of 2xSDS sample loading buffer (10% glycerol, 0.1% bromophenol blue, 2%SDS, 50mM Tris, 100mM dithiotheitol). Samples were then heated at 80°C for 4 –5 minutes.

Samples were loaded and run on a 10% polyacrylamide SDS gel ((made from 30% acrylamide (w/v), 0.8% bisacrylamide) (Amersham, UK), 375mM Tris-HCl pH8.8, 0.1% SDS (w/v), 0.05% ammonium persulfate, 0.05% NNNN-tetraethylethalinediamine (TEMED)) with a 5% stacking gel (5% acrylamide, 125mM Tris-HCl pH 6.8, 0.1%SDS (w/v), 0.1% ammonium persulfate, 0.1% TEMED). Rainbow molecular weight marker (Amersham, UK) was also run to identify protein size in samples. The gel was run in tris-glycine running buffer (25mM Tris, 250mM glycine (pH8.3), 0.1% SDS) at 30 milliamps for 4 –6 hours, or until the dye was 2 cm from the bottom of the gel. Proteins were transferred onto Hybond C membrane (Amersham, UK) for 18 hours (Medium size gel) or for 2 hours at 4°C (Biorad Protean III) in a transblotter (Biorad) in transfer buffer (192mM glycine, 20% (v/v) methanol, 25mM Tris-HCl pH 8).

The membrane was placed in block buffer (5% Marvel (skimmed milk powder), 0.1% Tween-20 in PBS) for 1 hour to block non-specific sites. The primary antibody was the added (antibody was diluted in 0.1% Tween 20 in PBS, 4% Marvel) and incubated for 1 hour at room temperature. The membrane was then washed with wash buffer (1% Marvel, 1% Tween 20 in PBS) for 5 minutes twice. The secondary antibody was then incubated for 1 hour (HrP conjugate in 4%marvel, 0.1% Tween 20 in PBS). The membrane was then washed 3 times for 5 minutes with wash buffer and the final wash in wash buffer without marvel.

In order to visualize bands, enhanced chemiluminescence (ECL, Amersham, UK) was applied and used according to manufacturer's instructions followed by exposure to

photographic film. The membrane was also probed with an actin antibody to check for equal loading of protein. Bands were quantified by using a GS-800 densitometer.

The membrane was stripped in 1M glycine pH 2.8 for 15 minutes so that it could be re-probed with other antibodies. The antibodies used are shown in table 2.2.

Table 2.2 List of Antibodies Used in Western Blotting

Antibody	Species raised against	Species raised in	Species recognised	Dilution	Secondary Antibody	Company
Actin (46 kDa)	Human	Goat polyclonal	Most Mammalian species	1:1000	Anti-goat HrP (DAKO)	Santa Cruz Biotechnology USA
Hsp90 (90kDa)	Achlyia ambisexualis	Mouse polyclonal	Most mammalian species	1:500	Anti-mouse HrP (DAKO)	Kind gift from D.O. Toft, Rochester, USA
Hsp70 (inducible 70kDa)	Human	Mouse monoclonal	Most species	1:1000	Anti-mouse HrP (DAKO)	StressGen Biotechnologies, York, UK
STAT-1 (91kDa)	Human	Rabbit polyclonal	Most mammalian species	1:1000	Anti-rabbit HrP (DAKO)	StressGen Biotechnologies, York, UK

2.6 Identification of β-galactosidase positive cells by X-gal staining

This technique would identify those cells that were successfully co- transfected with the β-galactosidase expression vector along with the expression vector being studied. After stress treatments, cells were washed with PBS and fixed with 1 ml 0.5% gluteraldehyde in PBS for 10 minutes. The fixative was removed and cells were washed twice with PBS. X-

gal stain (12mM $MgCl_2$, 200mM potassium ferrocyanide, 200mM potassium ferricyanide, and 0.4mg/ml 5-Bromo-4-Chloro-3-tridoyl-β-D-galactopyranoside in dimethyl sulfoxide (DMSO, Sigma)) was added to each well (1ml) and incubated for 16 hours at 37°C. Cells were washed twice in 1xPBS.

2.7 Assessment of cell death

Cell death was measured by Annexin V staining, TUNEL and trypan blue exclusion

2.7.1 Trypan blue exclusion

Cells were washed in phosphate buffered saline (PBS) and trypsinised for 1 minute in 0.25μ/ml trypsin in versene (Gibco, Life Technologies, UK). The cell suspension was centrifuged for 5 minutes at 1000 rpm, and the supernatant aspirated. The cell pellet was resuspended in 100μl PBS and stored on ice. Cell counts were made, using a haemocytometer, by addition of 0.4% trypan blue (Sigma) to equal volume of cell suspension. The percentage of blue cells/total cells was counted by scoring 100 cells per well 3 times.

2.7.2 Assessment of apoptosis by TUNEL labelling

Cells were stained with Terminal deoxynucleotidyl transferase (3'–OH labelling of DNA) and 2mM Fluorescein-12-2'-deoxy-uridine-5'-triphosphate (Roche, Germany) and incubated for 1 hour 30 minutes in a humidified incubator at 37°C in the dark. Cells were washed with PBS and subsequently viewed under a phase contrast microscope (Zeiss) for the total number β-galactosidase (β-gal) positive cells and fluorescent microscopy for β-

gal and TUNEL positive cells. The percentage of apoptotic cells was expressed as the percentage of total β-gal positive cells, which were TUNEL positive.

2.7.3 Assessment of apoptosis by Annexin V staining

To assess early apoptosis and the changes that occur on the cell surface, Annexin-V-Fluos (Roche, Germany) staining was applied after heat shock or ischaemic stress. Incubation buffer (10mM Hepes/NaOH, pH 7.4, 140mM NaCl, 5mM $CaCl_2$) was prepared. Annexin-V-Fluos-labelling solution was prepared by pre-diluting 20μl of Annexin-V-Fluos labelling reagent in 1000μl of incubation buffer and 20μl of propidium iodide (PI) (50μg/ml stock). PI was included to differentiate necrotic cells from apoptotic cells. 200μl of labelling solution was added per well and incubated for 15 minutes in dark at room temperature. Annexin-V binding cells were observed under fluorescent light (Zeiss). Cells from 3 separate fields of view (200 cells per view) from the same well were counted and apoptotic cells were expressed as a percentage of total cells.

2.8 Site directed mutagenesis of murine CT-1 –99 to +19 plasmid

Mutagenesis of plasmids was carried out using the QuickChange™ Site-Directed Mutagenesis Kit (Stragagene) and was followed according to manufacturer instructions. Prior to using the kit, primers were designed to the C/EBPβ sequence from the CT-1 –99 to +19 promoter (Figure 2.2).

-99 **ctgaactatg attggccgag cccgagccac gcccctagcc ctttcccccc ttttcccccc**
gacttgatac taaccggctc gggctcggtg cggggatcgg gaaaggggga aaaaggggga

gg *cc*
ttttcccccc cccctcctcc tccccccggag ggg*tgtg*<u>*tt*</u>*g agg*<u>*aa*</u>*cctgg
aaaaggggga ggggaggagg agggggcc tc ccc*acacaac tcctt g gacc*

***ataagcct*gg ggccagcatg ag** +19
***tattcgg*acc ccggtcgtac tc**

Figure 2.2 DNA sequence of the CT-1 minimal promoter (-99 to +19) region showing the C/EBPβ transcription factor binding site (italics) and mutations (underlined)

This region also contained a C/EBPβ binding site and was of interest since previous studies have demonstrated that the ucn promoter also contains a C/EBP binding site and that the expression of C/EBP transcription factors is increased in response to simulated ischaemia (Brar et al., 1999). Four base mutations were included in the forward and reverse primers: *5'- GGAGGGGTGTGTTGAGGAACCTGGATAAGCCTG-3'* and *5'-CCAGGCTTATCCAGGTTCCTCAACACACCCCTCC-3'*. In addition, the DNA that was to be mutated was prepared from a Qiagen plasmid DNA purification kit, and the concentration was determined by spectophotometry. A control reaction was set up with pWhitescript (comes with kit), and a sample reaction was set up as follows:

5μl of 10x reaction buffer

X (5-50ng) of dsDNA template

Xμl (125ng) of oligonucleotide primer #1

Xμl (125ng) of oligonucleotide primer #2

1μl of dNTP mix

ddH_2O to a final concentration of 50μl

-Then 1μl of Pfu Turbo DNA polymerase was added (2.5U/μl)

Cycling parameters for mutagenesis by the polymerase chain reaction (PCR) were modified depending upon the size of the template DNA and also the number of mutations to be introduced into the generated template.

The reaction was placed on ion reaction was then digested with DpnI restriction enzyme (10U/μl) and mixed before centrifuging and incubating the digestion for 1 hour at 37℃. This digestion allowed the parental (non-mutated) DNA to be digested, leaving only the mutated DNA.

Mutated DNA was then transformed into XL1-Blue super-competent cells and plated on agar-Ampicillin plates for 16 hours at 37°C. The resulting mutant plasmid was sent for sequencing to confirm that the mutation was successful.

2.9 *In vitro* protein-protein interaction

HSF1 expression vector were kindly given by I Benjamin, USA

Brn3b and p53 vectors were kindly provided by A Nissam, MRC Centre for protein engineering, Cambridge, UK

2.9.1 Transformation of plasmid DNA containing protein of interest

Expression vectors were transformed into *Epicurian coli* competent cells (BL-21 PlysS) provided by Stratagene. Cells were kept on ice for 30 minutes before heat shocking for 20 seconds at 42°C. Transformants were returned to ice for 30 minutes, after which they were incubated in 800μl LB at 37°C in an orbital shaker for 1 hour. Cells were centrifuged for

20 seconds at 13000rpm. The supernatant was discarded, but leaving 100ul in the eppendorf to resuspend the bacterial pellet. The cell suspension was spread on agar plates containing chloramphenicol and ampicillin antibiotics. Plates were incubated for 16 hours in a 37°C incubator.

2.9.2 Sub-culturing transformed colonies (large scale)

A colony was picked from the plate and inoculated in 5ml LB containing the appropriate antibiotics and incubated at 37°C in an orbital shaker for 16 hours. 250ml of sterilized LB/Ampicillin was inoculated with 5ml sub-culture. The LB culture was incubated at 37°C in an orbital shaker until the OD600nm reached 0.5 (an aliquot of 1ml was taken to run on SDS gel). Depending on the type of vector (pET or GST), proteins were over-expressed by inducing with the appropriate concentrations of IPTG (1M stock) for 2-4 hours at 37°C in an orbital shaker until the OD at 600nm reached 1.0 (an aliquot of 1ml was taken to run on SDS gel).

The bacterial culture was centrifuged at 13000rpm. The supernatant was discarded and the pellet re-suspended in SDS gel loading buffer with β mercaptoethanol. Samples were boiled for 2-3 minutes and proteins were separated on 10% polyacrylamide gel in the presence of Rainbow marker (Amersham, UK). Electrophoresis was carried out at 60 volts on a mini-gel. Un-induced and induced proteins were detected by staining the gel with Coomassie blue stain for 1 hour at room temperature and de-stained until bands were observed. The gel was dried in a gel dryer for 20 minutes and kept as a reference to identify over-expressed proteins.

2.9.3 Purification of Histidine tagged fusion proteins

The cell pellets (see section 2.9.2) were lysed by 3 cycles of freeze thaw from –20°C to room temperature in 1xextraction/wash buffer (Clontech,USA). To remove cell debris the suspension was centrifuged at 13000rpm for 5 minutes. The supernatant was carefully transferred to a clean sterile eppendorf.

Talon Resin (Clontech, USA) was completely resuspended and 200µl of resin transferred to a 15ml falcon tube so that it would accommodate 10-20x the resin bed volume. The resin was equilibriated by centrifugation at 700xg for 2 minutes. The pellet was re-suspended in 1ml of 1x extraction/wash buffer (triton X-100 in PBS). This step was repeated 3 times before the clarified protein sample was added. The protein/resin complex reaction was carried out at room temperature for 20 minutes with gentle agitation (rotating wheel). A final centrifugation at 700g for 5 minutes was carried out and the supernatant removed carefully not disturbing the pelleted resin. To wash the pelleted resin, two washes of 1.5ml of 1x extraction wash buffer was added and mixed gently on a rotating wheel for 10minutes at room temperature. Resin was centrifuged at 700g for 5 minutes, and the supernatant removed.

The resin was re-suspended in 1ml of 1 x extraction wash buffer by vortexing, and stored at –20°C until required for interaction studies.

2.9.4 Purification of GST tagged fusion proteins

Cells were lysed in 1 x PBS/Triton 1% (37ml) by three cycles of freeze thaw from –20°C to room temperature. For 1litre cultures, 500µl of Glutathione Sepharose 4B beads (Amersham, UK) was used.

2.9.4.1 Preparation of Glutathione Sepharose 4B medium

Glutathione Sepharose 4B (supplied as 75% slurry) medium was transferred to a new tube. The transferred medium was sedimented by centrifuging at 500x g for 5 minutes. The supernatant was discarded and the Glutathione Sepharose 4B washed by adding 3.76ml of cold (4°C) 1xPBS (Manufacturer protocol suggests 10ml of 1xPBS per 1.33ml of 75% slurry of Glutathione Sepharose 4B). The tube was inverted to mix. The medium was sedimented again by centrifuging at 500 xg for 5 minutes. For 500µl of original slurry used, 0.376ml of 1x PBS was added (Manufacturer protocol suggests 1ml of 1xPBS per 1.33ml of 75% slurry of Glutathione Sepharose 4B), resulting in 50% slurry. The medium was mixed well before subsequent steps.

2.9.4.2 Purification of Protein of interest

The bed volume is equal to 0.5 x the volume of the 50% slurry used.

0.7446 ml of the prepared glutathione sepharose 4B (50% slurry) was equilibriated with 1 xPBS to 37ml of bacterial lysate. This mixture was incubated at room temperature for 30 minutes, by gentle agitation. The medium was sedimented by centrifuging at 500 x g for 5 minutes. The supernatant was carefully decanted. The medium was washed with 10 bed volumes of 1xPBS and inverted to mix. The medium was then sedimented by centrifuging

at 500g for 5 minutes. The supernatant was carefully removed. This washing step was repeated twice. The Glutathione Sepharose 4B-bound protein was eluted from the sedimented medium by adding 1ml of elution buffer (50mM Tris-HCl, 10mM reduced glutathione, pH 8.0) per 1 ml bed volume of the original slurry. The medium was mixed gently to resuspend and incubated at room temperature for 10 minutes to elute the fusion protein from the medium by gentle agitation of the tube. The medium was sedimented by centrifuging at 500 x g for 5 minutes. The supernatant containing eluted protein was carefully transferred to a new tube and used for *in vitro* protein-protein interaction studies.

2.9.5 *In vitro* translation of proteins

The appropriate expression vector containing cDNA of the transcription factor was in vitro translated to radiolabel methionine residues with ^{35}S. This was carried out using the in vitro transcription/translation kit from Promega, using T4 polymerase enzyme. 2µl of radiolabelled products were separated on an acrylamide gel containing SDS (10% w/v) and the gel dried on a gel dryer for 30 minutes at 80°C. Photographic film was then exposed for 24 hours so that the radiolabelled protein size would be visible.

2.9.6 Pull down method for protein-protein interaction

Fusion beads were washed in NENT buffer (100mM NaCl, 1mM EDTA, 20mM Tris pH8, 1% (v/v) NP-40 or Igepal) containing 0.5% milk powder to a final volume of 1ml. Beads were incubated at room temperature for 20-30 minutes in the wash buffer. The supernatant was carefully removed and NENT buffer containing 20% milk powder was added to the beads to a final volume of 0.5ml. Beads were incubated at room temperature on a rotating

wheel for 15 minutes. A further 1ml of NENT buffer (without milk) was added to the beads in order to make it easier to see the pellet. After mixing the suspension, beads were centrifuged for 1 minute at 13000rpm. The supernatant was removed and beads were washed twice with 1ml of NENT alone, followed by one wash with transcription buffer (20mM Hepes pH7.9, 60mM NaCl, 1mM DTT, 6mM $MgCl_2$, 8.2% Glycine, 0.1mM of 0.5 M EDTA). Beads were stored in this buffer.

For protein-protein interaction, 30µl of beads were equalized to 100µl with transcription buffer. 5µl of the in vitro translated radio-labelled product was mixed with the fusion beads and incubated at room temperature on a rotating wheel for 1 hour. The beads were then washed five times with 1ml of NENT buffer. Beads were centrifuged for 5 minutes on a microcentrifuge. The supernatant was removed, and SDS loading buffer (with β-mercaptoethanol) was added to beads to separate on a SDS polyacrylamide gel. 10% of the in vitro translated product was also loaded on the same gel in order to identify interacting protein. The gel was dried for 30 minutes to 1 hour at 80°C and a film was exposed for 24 hours.

2.10 Statistical analysis

In results where two comparisons were made in a data set, the Students t-test was performed to test for significance between the means. A p value of less than 0.05 was considered significant. T-tests were performed using Microsoft Excel Analyse It.

For data sets where multiple comparisons were made between treatment groups, one -way analysis of variance (ANOVA) was performed. This test determines whether there are any

differences between the treatments and a p value of less than 0.05 was considered significant.

When an ANOVA test showed significant differences between treatment groups, the post hoc Bonferroni's test was performed to test for significant differences between specific treatments, and a p value of less than 0.05 was considered significant.

For comparisons being made from scanned Western blots, the Wilcoxon signed ranked test was used as this test determines whether there is an increase or decrease in pairs of data, and is appropriate when n numbers are low. Microsoft Excel Analyse It was used.

CHAPTER 3.

The C-terminal Domain of STAT-1 is Necessary and Sufficient for Stress-Induced Apoptosis

3.0 INRODUCTION

The STATs are known to play key roles in mediating transcriptional responses to specific interferons and cytokines. It is known, for example, that treatment with IFNγ results in phosphorylation of STAT-1 at tyrosine 701 and serine 727 residues within the C-terminal domain, leading to the formation of STAT-1 dimers which translocate to the nucleus to bind DNA and activate transcription of STAT-1 responsive genes (Horvath and Darnell, 1997; Darnell, 1997; Chatterjee-Kishore, van der Akker, and Stark, 2000; Horvath, 2000).

Other STATs are activated by specific cytokines (see Introduction section). For example, STAT-3 is activated by cytokines belonging to the IL-6 family via a common receptor subunit known as gp130. STAT-3 activation has been linked to enhanced cell proliferation. In addition, when over-expressed, a constitutively active form of STAT-3 can behave as an oncogene that is able to transform cells either alone or in co-operation with other oncogenes (Bromberg et al., 1999; Ram, Horvath and Iyengar, 2000).

In contrast to this role for STAT-3 in cell proliferation (Bromberg et al, 2000; Ram et al., 2000), STAT-1 has been shown to play a role in the induction of programmed cell death or apoptosis. Treatment of various cell lines with IFNγ induces apoptotic cell death and activation of a proteolytic enzyme called caspase 1 (Chin et al., 1997). Moreover, cells that lack either functional STAT-1 protein or Jak1 kinase are not sensitive to apoptotic cell death, and caspase 1 is not activated in response to IFNγ in these cells (Chin et al., 1997). Similarly, cells that lack functional STAT-1 protein show reduced caspase expression and

are also less sensitive to TNF-α induced apoptosis compared to parental cells that do express STAT-1 (Kumar et al., 1997).

These studies demonstrate that STAT-1 plays a key role in inducing apoptotic cell death in response to regulatory factors such as IFNγ or TNF-α. Interestingly, recent studies have also shown that STAT-1 plays a role in apoptosis induced by simulated ischaemia/re-oxygenation in cardiac cells. Moreover, STAT-1 is shown to be expressed at enhanced levels following simulated ischaemia/re-oxygenation and phosphorylation of STAT-1 occurs on both tyrosine 701 and serine 727 residues respectively (Stephanou et al., 2000; Stephanou et al., 2001). In addition, introduction of an antisense STAT-1 construct into cardiac cells protects them from apoptosis induced by simulated ischaemia/re-oxygenation (Stephanou et al., 2000).

Therefore, this study aims to investigate further the features of STAT-1 required for stress-induced apoptosis. The U3A cell line which lacks a functional STAT-1 protein is a fibrosarcoma cell line (Darnell, Kerr and Stark, 1994) will be used and will be compared to the parental 2fTGH cell line, which does contain a functional STAT-1 protein (Darnell, Kerr and Stark, 1994). The U3A cells will be transfected with expression vectors encoding different forms of STAT-1 to determine which region(s) of the transcription factor are required for stress-induced apoptosis observed in previous studies.

3.1 Differences in Stress-Induced Cell Death In Parental 2fTGH Cells Expressing STAT-1 and Mutant U3A cells Lacking Functional STAT-1

In order to study the role of STAT-1 in stress-induced apoptosis, the 2fTGH and U3A cell lines were used. 2fTGH is a human cell line containing the selectable marker guanine - phosporibosyltransferase regulated by IFNα. 2fTGH cells contain a functional STAT-1 protein, whereas U3A cell line does not contain functional STAT-1 (Figure 3.1). In addition, U3A cells are unresponsive to IFNα and also responsive to IFNγ (McKendry et al., 1991). For this reason, the introduction of various expression vectors was possible into the mutant cell line to compare with the parental cell line.

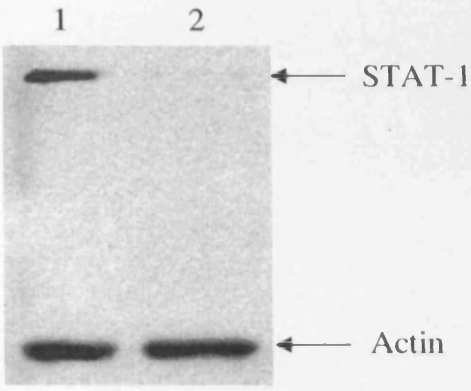


Figure 3.1 Western blot analysis of 2fTGH and U3A cell lines. 2fTGH cells show band for STAT-1 (Lane 1), and U3A with no band for STAT-1 (Lane 2).

Previous studies have demonstrated that STAT-1 is involved in, for example, apoptosis induced by simulated ischaemia (Stephanou et al., 2000; Stephanou et al., 2001). Thus, in primary experiments, the effect of stress such as simulated ischaemia and elevated temperature was investigated in 2fTGH and U3A cell lines. The effect of simulated ischaemia was investigated to determine whether any differences in cell death could be observed between 2fTGH and U3A cell lines. Cells to be subjected to stress were placed them in a simulated ischaemic chamber for 2 hours, 4 hours or 8 hours. Control cells were kept under normoxic conditions. The percentage of cell death was measured by trypan blue exclusion, which would identify live cells (white) and dead cells (blue). The mean percentage cell death was calculated as the number of live cells divided by the total number of cells counted. 200 cells per sample were scored (Figure 3.2a).

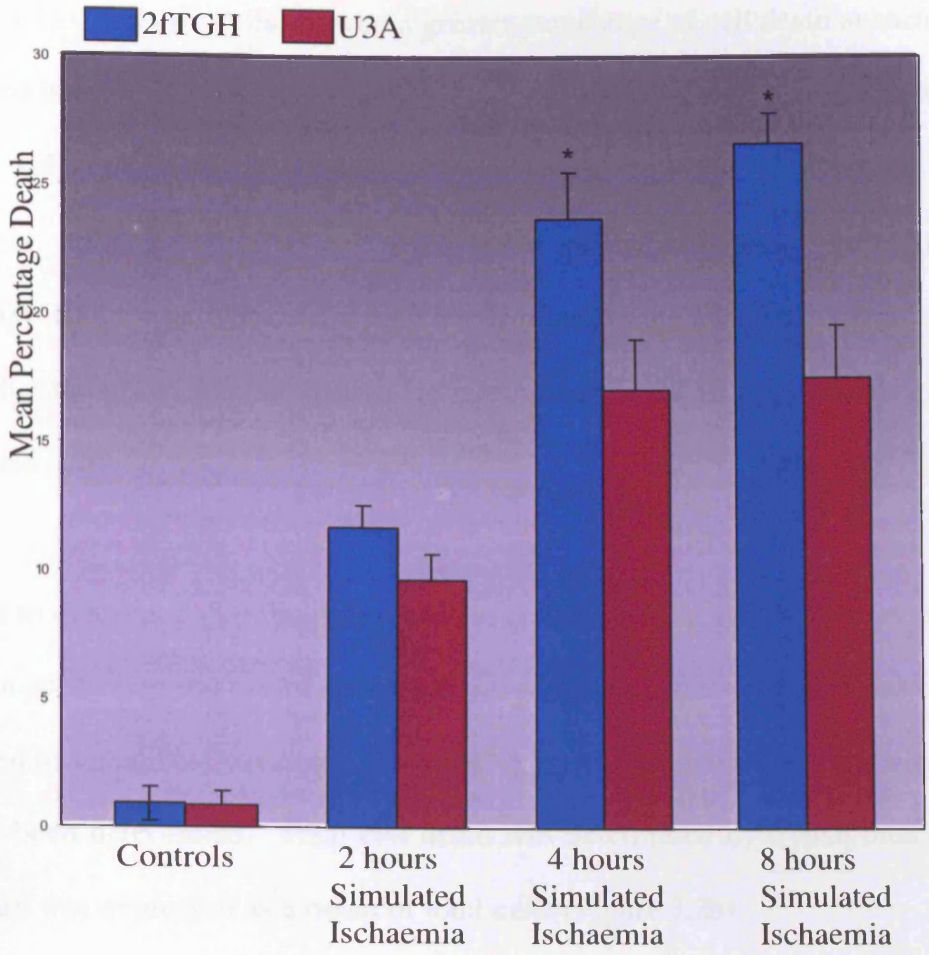


Figure 3.2 a. Cell Death in Parental 2fTGH cells (expressing STAT-1) or in U3A derivative (lacking STAT-1) following exposure to either simulated ischaemia for the period indicated. Values indicate percentage dead cells unable to exclude trypan blue and are the means of three determinations each performed in duplicate whose standard error is shown by the bars. *$p \leq 0.05$ U3A versus 2fTGH determined by Student *t* test

The experiment showed differences in cell death between 2fTGH cells and U3A cells (Figure 3.1a). 2fTGH cells showed a greater percentage of cell death at each time point of simulated ischaemic treatment compared to the U3A cells. The percentage of cell death in 2fTGH cells increased significantly with increasing time of exposure to simulated ischaemia, with the level of cell death being greater at 4 hours ($p \leq 0.05$) and 8 hours ($p \leq 0.05$). These results show that the levels of cell death observed is regulated by STAT-1, and that the effect may be specific to stress since no difference in death was observed in unstressed cells.

In order to determine that the differences in cell death observed in the previous experiment were not specific to the use of simulated ischaemia as a stress. 2fTGH and U3A cells were subjected to lethal heat stress at either 43°C or 44°C for 2 hours as sensitivity to such stress had not been determined. Total cell death was determined by trypan blue exclusion, and cell death was expressed as a mean of total cells (Figure 3.2b).

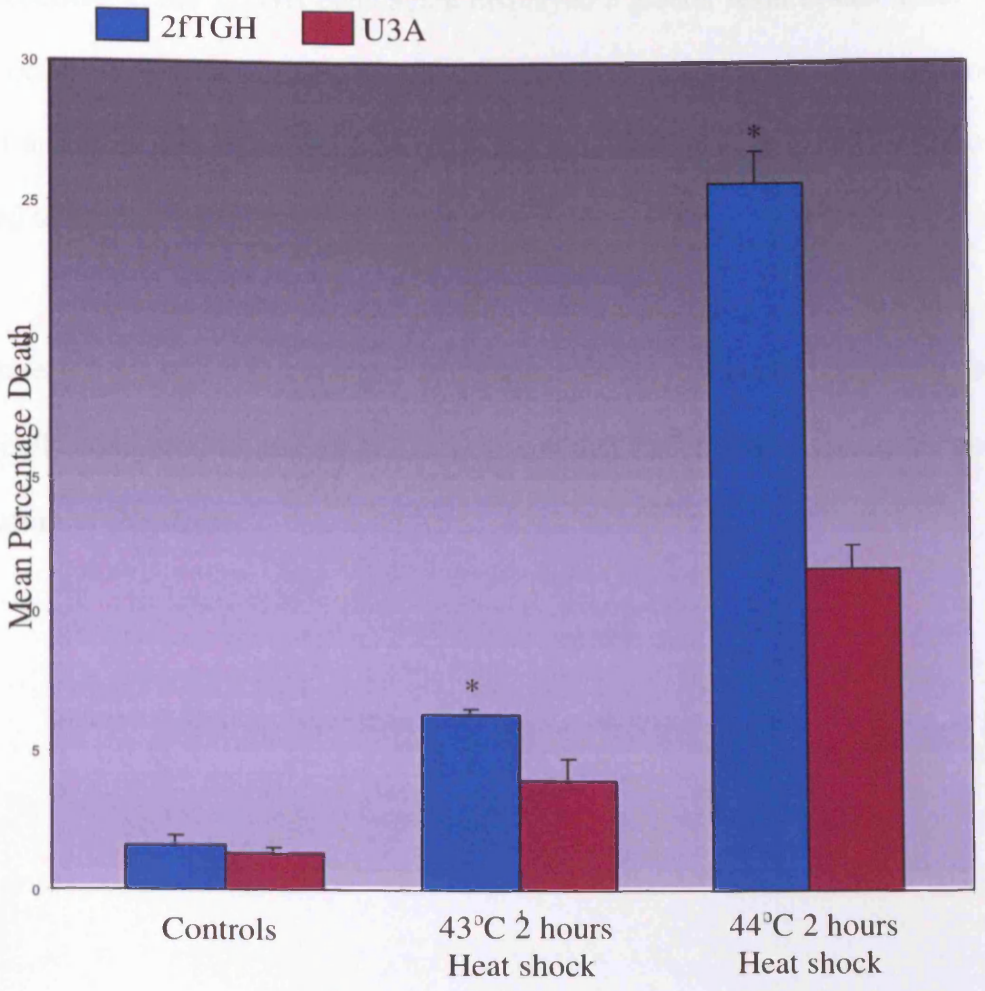


Figure 3.2b. Cell death in parental 2fTGH cells (expressing STAT-1) or their U3A derivative (lacking STAT-1) following exposure to heat shock for 2 hours at 43°C or 44°C. Values indicate the percentage of dead cells unable to exclude trypan blue and are the mean of three determinations each performed in duplicate whose standard error is shown by the bars. $p \leq 0.05$ versus 2fTGH determined by Student *t* test

In this experiment, the 2fTGH cells again displayed a greater level of cell death compared to U3A cells. The difference in cell death between both cell types was apparent at 43°C ($p \leq 0.05$) and was more severe at 44°C ($p \leq 0.05$) as there was a 2-fold increase in death compared to U3A cells.

It is apparent from these experiments that greater sensitivity to either stress is observed in 2fTGH cells compared to mutant U3A cells, and that the effects observed are not specific to the nature of the stress.

3.2 Confirmation of Apoptosis by Annexin V and TUNEL Assays

The previous experiments showed that STAT-1 enhances cell death in response to two different stimuli. Although trypan blue can distinguish between those cells that are dead and those cells that are alive, this method cannot determine whether dead cells result from necrosis or apoptosis.

Since STAT-1 has been implicated in apoptotic cell death induced by simulated ischaemia/repersfusion, in other studies, it was important to determine whether the differences in sensitivity to stress observed in this study was due to enhanced apoptosis. Again, 2fTGH and U3A cells were subjected to heat stress at 43°C or simulated ischaemia for 4 hours. In order to measure cell death by apoptosis, two methods of detection were used. Annexin V surface staining is used to identify early stages of apoptosis by detecting phosphatidyl serine, which translocates to the surface of the cell. In normal cells, phosphatidyl serine is located on the inside of the membrane. TUNEL label detects cells that are at later stages of apoptosis, as it labels 3' OH ends of fragmented DNA.

In this experiment, those cells that were TUNEL positive or annexin V positive were scored and represented as a mean percentage of cells that were apoptotic (Figure 3.3).

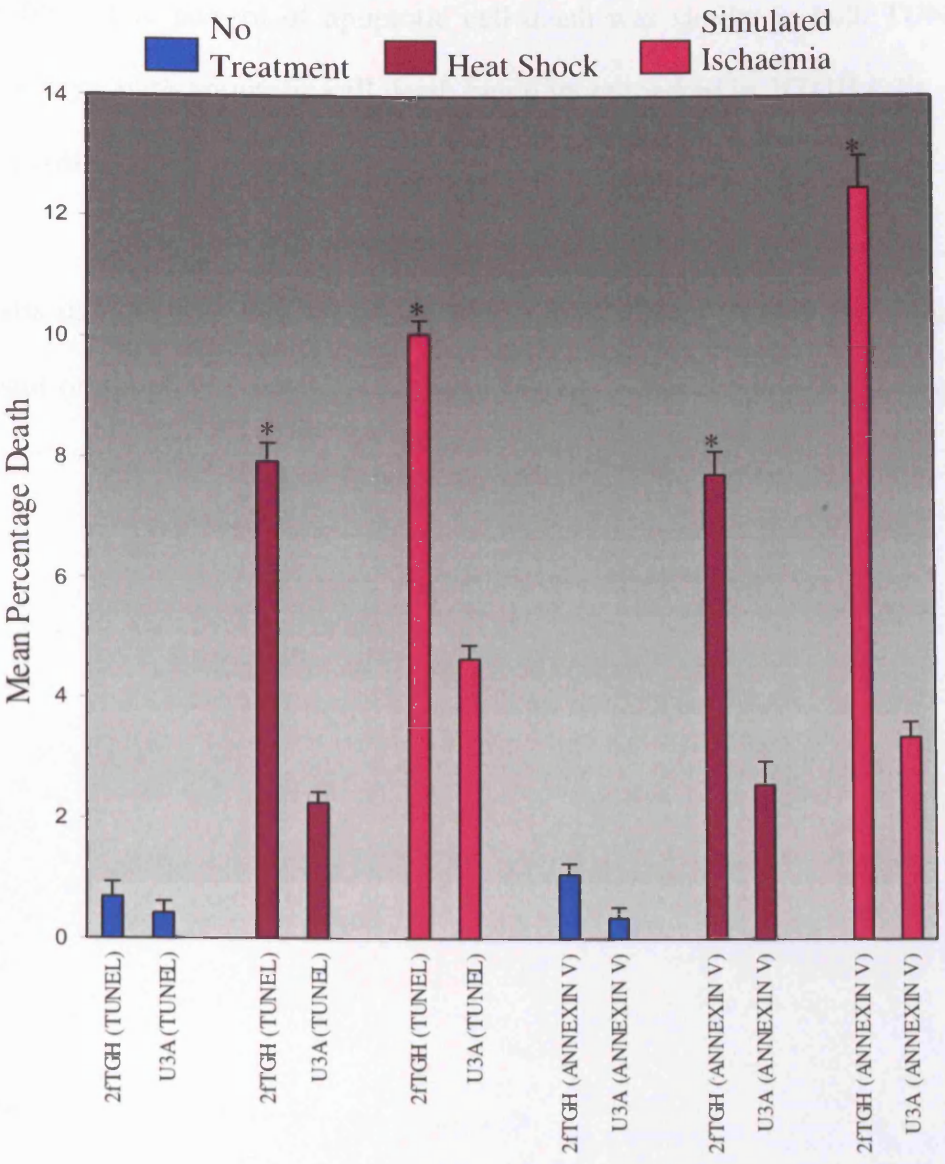


Figure 3.3. Apoptotic cell death in 2fTGH cells or their U3A derivative following exposure to heat shock or simulated ischaemia as assayed by TUNEL or Annexin V staining. Values are the mean of three determinations each performed in duplicate whose standard error is shown by the bars.
*$p \leq 0.05$ U3A versus 2fTGH determined by Student *t* test

2fTGH cells were more sensitive to both simulated ischaemic stress ($p \leq 0.05$) and heat stress ($p \leq 0.05$). The pattern of apoptotic cell death was similar in both TUNEL and in annexin V assays, with apoptotic cell death being more marked in 2fTGH cells. U3A cells were less sensitive to the treatments.

These results demonstrate that simulated ischaemia and heat stresses cause these cells to die as a result of apoptosis, and STAT-1 enhances the extent of apoptosis observed.

3.3 Enhanced Sensitivity of U3A cells to stress by introduction of STAT- 1

In the following experiments, an expression vector encoding full length STAT-1α was introduced into the U3A cell line to determine whether enhanced apoptosis in response to stress could be restored by expression of STAT-1. U3A cells were transfected with the STAT-1α full length expression vector and also a cmv β-galactosidase expression vector (Figure 3.4).

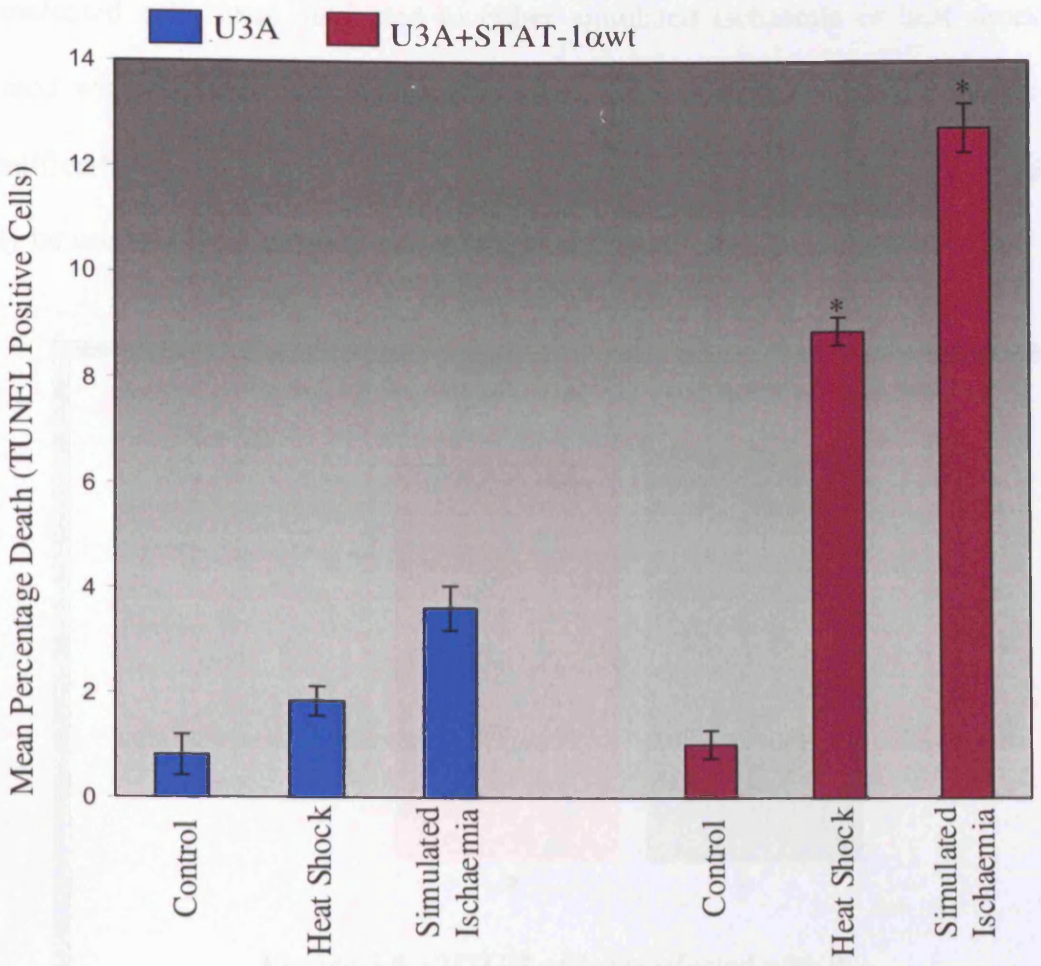


Figure 3.4. Apoptotic cell death in U3A cells transfected with expression vector lacking any insert or with STAT-1 expression vector and then either left untreated or exposed to simulated ischaemia for 4 hours or heat shock for 2 hours at 43°C. Values indicate percentage of successfully transfected (β-galactosidase positive, 10%) cells which are positive in TUNEL assay of apoptosis and are the mean of three determinations each performed in duplicate whose standard error is shown by the bars. *$p \leq 0.05$ U3A+STAT-1 cells versus control U3A cells determined by Student *t* test

The cmv β-galactosidase vector was included to identify successfully transfected cells. Transfected cells were subjected to either simulated ischaemia or heat shock, and then stained with X-gal to identify β-gal positive cells (Figure 3.5a). Apoptotic cells were identified by assaying with TUNEL (3.5b). Annexin V stain was not used as this stain can only be used on live cells and not cells that are fixed.

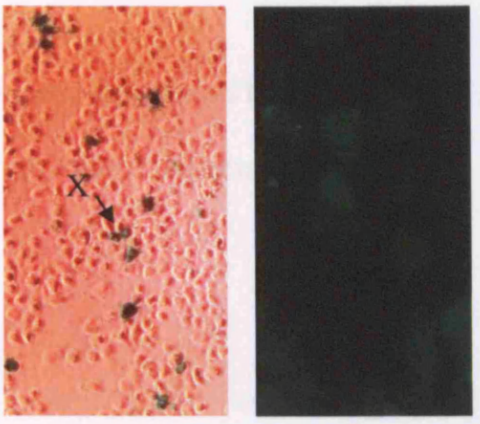


Figure 3.5 2fTGH cells transfected with β-galactosidase and stained with X-gal (X) (Magnification x 10, light microscope) (a); 2fTGH cells assayed for TUNEL (Magnification x 40, fluorescence microscope) (b). Brightly fluorescent cells were β-galactosidase and TUNEL positive (T).

In cells transfected with STAT-1 only a minimal effect of apoptotic cell death was observed in the absence of stress (Figure 3.4). When STAT-1 transfected cells were exposed to stress, the percentage of apoptotic cells was increased compared to the U3A cells transfected only with control vector. A four-fold increase was observed in U3A plus STAT-1 cells exposed to heat stress ($p \leq 0.05$) compared to control U3A cells. An approximately 3-fold increase in apoptotic cell death was observed in U3A plus STAT-1 cells exposed to simulated ischaemia ($p \leq 0.05$) compared to control U3A cells.

Therefore, this experiment demonstrates that STAT-1 mediates pro-apoptotic effects in cells that are exposed to heat or simulated ischaemic stress.

3.4 Opposing Effect on Apoptosis by STAT-3 in U3A Cells

The previous experiment demonstrated that by transfecting a functional STAT-1 protein into the mutant U3A cells, levels of apoptotic cell death were enhanced to levels observed in the parental 2fTGH stresses cells after heat or simulated ischaemic stress.

The next set of experiments involved testing whether this effect of STAT-1 could be reproduced with the related protein STAT-3 or with mutant or truncated derivatives of STAT-1. In other experiments, these factors have been shown to be expressed at similar levels in the transfected cells (Stephanou et al., 2000; Stephanou et al., 2001) and this was also confirmed in these experiments in a Western blot (Figure 3.6).

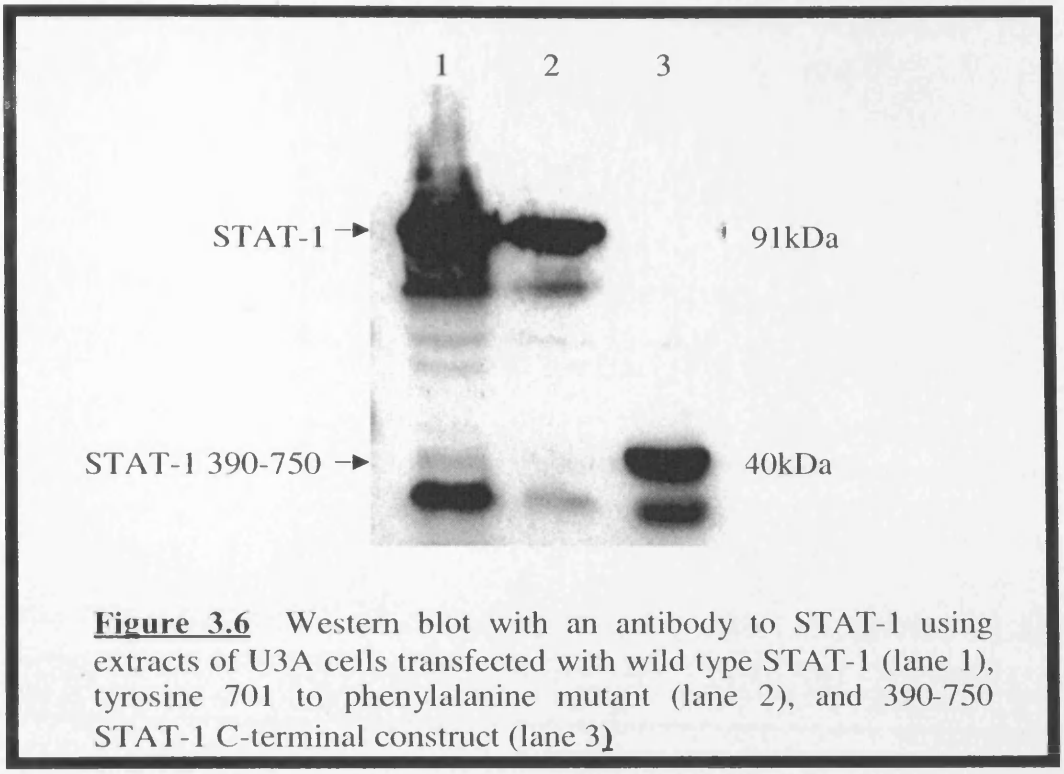


Figure 3.6 Western blot with an antibody to STAT-1 using extracts of U3A cells transfected with wild type STAT-1 (lane 1), tyrosine 701 to phenylalanine mutant (lane 2), and 390-750 STAT-1 C-terminal construct (lane 3)

A STAT-3 expression vector was transfected into the mutant U3A cells to compare levels of enhanced apoptosis with those cells transfected with STAT-1(Figure 3.7). In other studies STAT-3 has been shown to have an anti-apoptotic role in enhanced cell proliferation and transformation (Bromberg et al., 1999; Ram et al., 2000).

In addition, both STAT-1 and STAT-3 expression vectors were also transfected together into U3A cells (figure 3.7). All transfected cells were subjected to either heat stress or simulated ischaemic stress, after which they were assayed for apoptosis by TUNEL labelling. Those cells that were β-gal positive and also TUNEL positive were scored as a mean percentage of apoptotic cells.

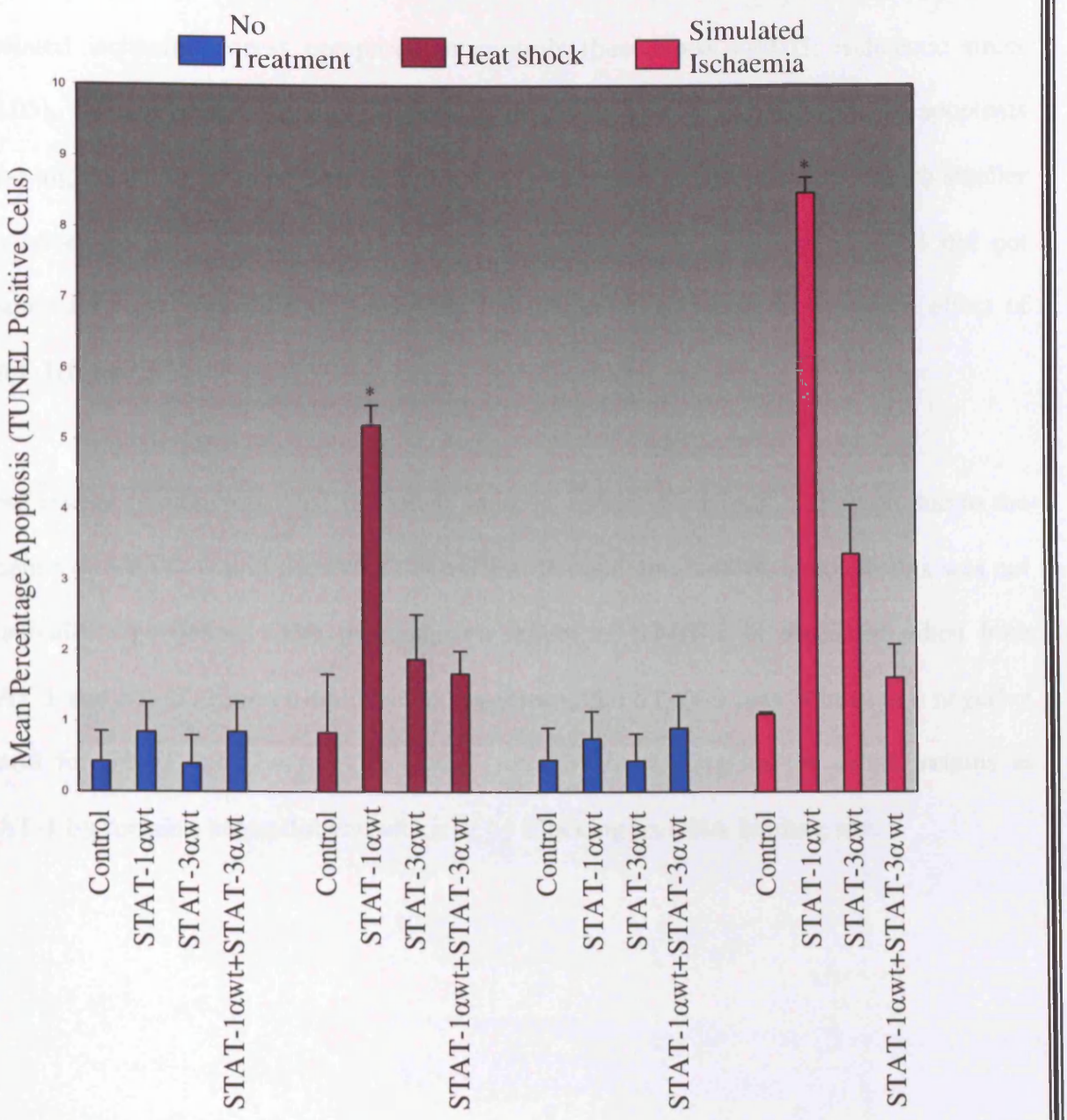


Figure 3.7. Apoptotic cell death in U3A cells transfected with empty expression or expression vectors encoding STAT-1 or STAT-3 singly or in combination and then either left untreated or exposed to heat shock at 43°C for 2 hours or simu.lated ischaemia for 4 hours. Values indicate the percentage of successfully transfected cells which are TUNEL positive and are the mean of three determinations each performed in duplicate whose standard error is shown by the bars. *$p \leq 0.05$ versus empty expression vector transfected cells determined by Student *t* test.

Transfection of STAT-1 alone significantly induced enhanced apoptosis after heat and simulated ischaemic stress compared to controls (heat stress $p \leq 0.05$; ischaemic stress $p \leq 0.05$). However, transfection of STAT-3 produced a small enhancement of apoptosis following exposure to either heat or simulated ischaemia, and this effect was much smaller than observed with STAT-1 alone. Co-transfection of STAT-1 and STAT-3 did not enhance apoptosis significantly indicating that STAT-3 can block the apoptotic effect of STAT-1(figure 3.7).

These results demonstrate that the stress induced enhanced apoptotic effect is due to the presence of STAT-1, and that STAT-3 reduces the apoptotic effect, although this was not statistically significant. The pro-apoptotic effect of STAT-1 is abolished when both STAT-1 and STAT-3 are co-transfected suggesting that STAT-3 may behave as a negative control for STAT-1 activity. This could occur by competing for the same proteins as STAT-1 by forming heterodimers with it or by blocking its DNA binding site.

3.5 Identification of Region of STAT-1 Required for Apoptosis

Differences observed by introducing STAT-1 or STAT-3 into the mutant U3A cells showed that these two proteins had opposing effects on apoptotic cell death induced by simulated ischaemia or heat stress. In order to probe further the regions of STAT-1 required for apoptosis, chimaeric expression vectors encoding different regions of STAT-1 or STAT-3 were used (Figure 3.8):

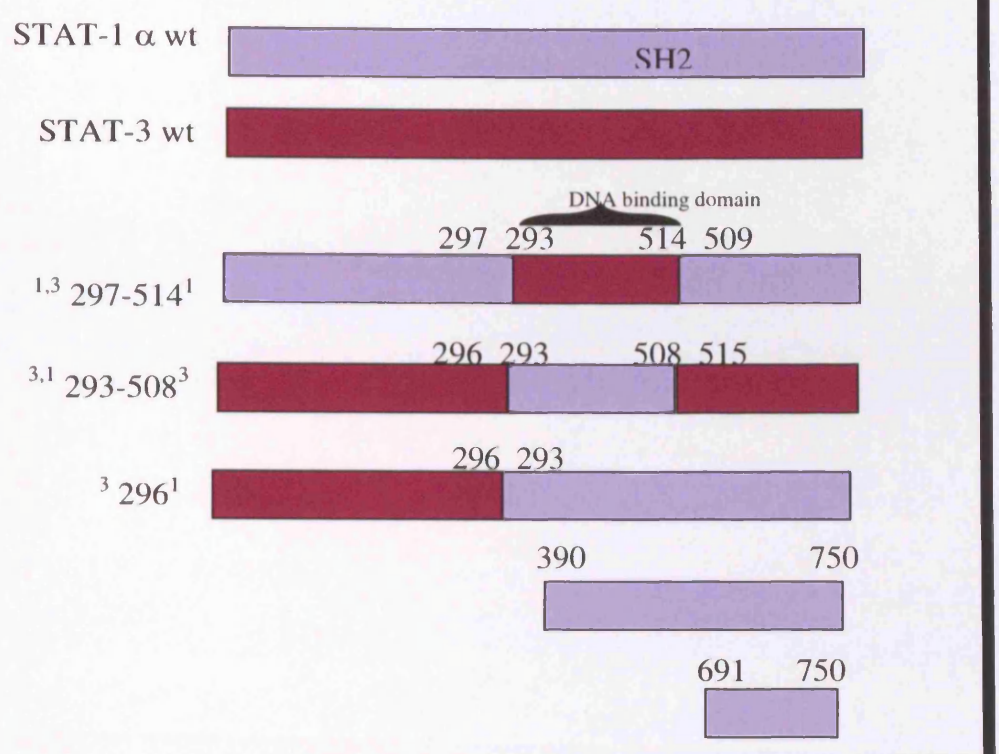


Figure 3.8 Structure of the STAT-1/STAT-3 chimaeric constructs, and truncated STAT-1 constructs

In these experiments, U3A cells were transfected with various chimaeric constructs. Cells were subjected to either simulated ischaemia or heat stress. Only those cells that were β gal positive and also TUNEL positive were scored and represented as a mean percentage of apoptotic cell death (Figure 3.9)

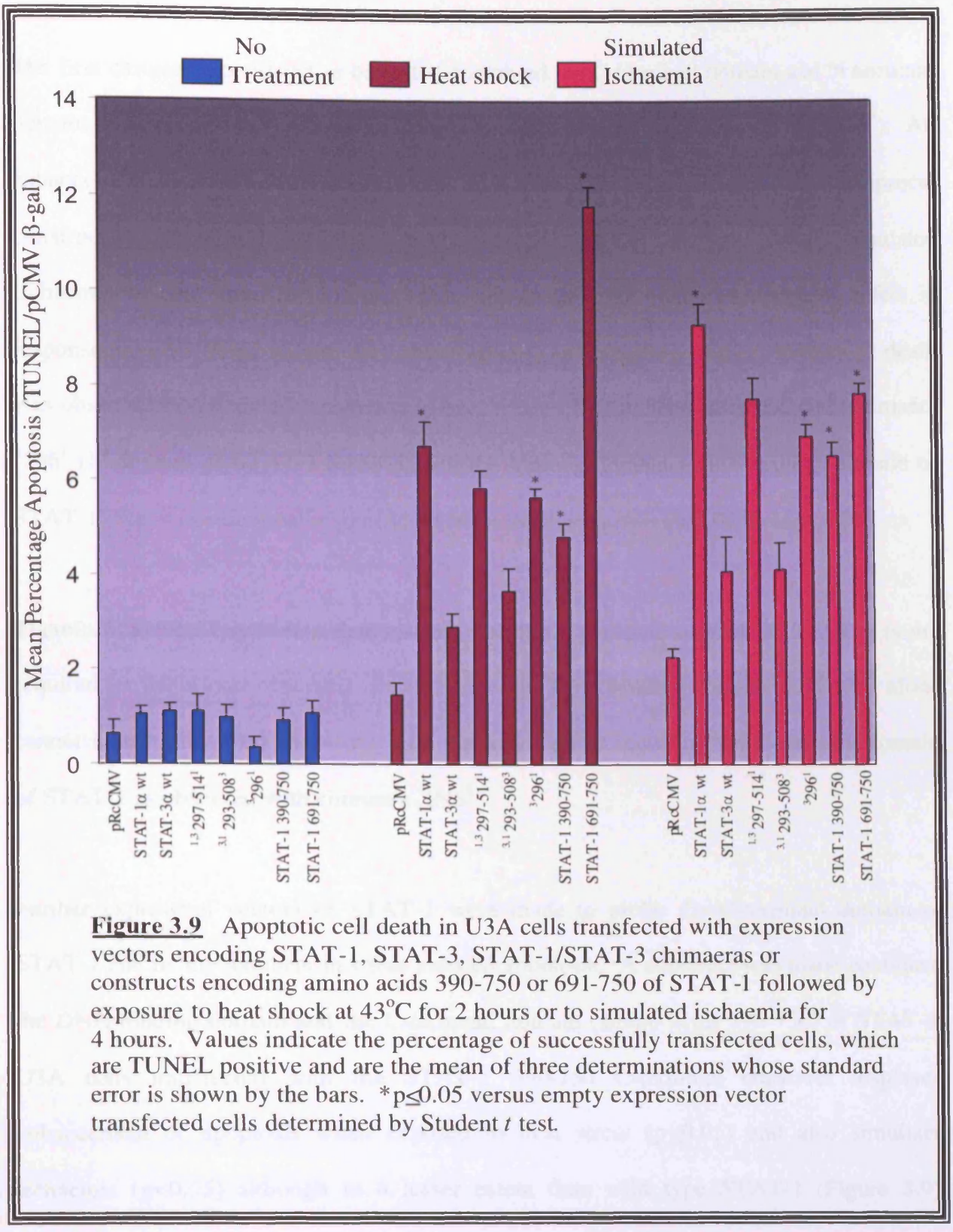


Figure 3.9 Apoptotic cell death in U3A cells transfected with expression vectors encoding STAT-1, STAT-3, STAT-1/STAT-3 chimaeras or constructs encoding amino acids 390-750 or 691-750 of STAT-1 followed by exposure to heat shock at 43°C for 2 hours or to simulated ischaemia for 4 hours. Values indicate the percentage of successfully transfected cells, which are TUNEL positive and are the mean of three determinations whose standard error is shown by the bars. *$p \leq 0.05$ versus empty expression vector transfected cells determined by Student *t* test.

The first chimaeric construct to be tested contained the C-terminal domain and N terminal domain of STAT-1 and the DNA binding domain of STAT-3 (construct ^{1,3} 297-514^{3}). An enhancement of apoptotic cell death was observed with this construct. The reciprocal construct was transfected in U3A cells (construct ^{3,1}293-508^{3}) and subjected to simulated ischaemia or heat stress. This chimaera did not have an enhanced apoptotic effect in response to either stress (figure 3.9). Interestingly, an enhancement of apoptotic cell death was observed in simulated ischaemia or heat treated U3A cells transfected with chimaera ^{3}296^{1} (N domain of STAT-3 fused with DNA binding domain and C-terminal domain of STAT-1) that was statistically significant (simulated ischaemia $p \leq 0.05$; heat $p \leq 0.05$).

Therefore, these experiments demonstrate that the N-terminal domain of STAT-1 is not required for the effects observed. Furthermore, the DNA binding domain of STAT-1 alone cannot induce enhanced apoptosis, with the major effect requiring the C-terminal domain of STAT-1 as observed with chimaera ^{3}296^{1}.

Further expression vectors of STAT-1 were made to probe the C-terminal domain of STAT-1 and its involvement in stress induced apoptosis. A construct was made contained the DNA binding domain and the C-terminal domain (amino acids 390-750) of STAT-1. U3A cells transfected with the STAT-1 390-750 C-terminal construct displayed enhancement of apoptosis when exposed to heat stress ($p \leq 0.05$) and also simulated ischaemia ($p < 0.05$) although to a lesser extent than wild type STAT-1 (Figure 3.9). Therefore, this result demonstrates further that the effect observed with the chimaeric

construct is not due to the N-terminal domain of STAT-3 substituting for that of STAT-1. Rather, enhanced apoptosis can still be observed in the absence of the N-terminal domain.

To determine whether the isolated C-terminal domain without the DNA binding domain is not only necessary but also sufficient for enhanced stress-induced apoptosis, a construct expressing only amino acids 691-750 of STAT-1 was also prepared. In transfected U3A cells, this construct was able to induce enhanced apoptosis exposed to heat stress ($p \leq 0.05$), and similar levels of enhancement to STAT-1 wild type when exposed to simulated ischaemia ($p \leq 0.05$) (Figure 3.7). In addition, a truncated STAT-1 construct in which the 694-750 region had been replaced with a STOP codon was not able to induce enhanced apoptosis, suggesting that the C-terminal domain of STAT-1 is essential for stress-induced apoptosis (Figure 3.9). Hence, the C-terminal domain of STAT-1 is not only necessary for enhanced stress-induced apoptosis but is also sufficient for this effect, producing it in the absence of the adjacent DNA binding domain or other regions of STAT-1.

3.6 Requirement of Caspase Cleavage of STAT-1 in Stress Induced Apoptosis

The previous results demonstrated that the isolated 691-750 domain of STAT-1 was able to induce enhanced apoptosis significantly. STAT-1 in other studies has been shown to be cleaved by caspase 3 at amino acid position 694 to release a C-terminal fragment (King and Goodbourn, 1998).

Therefore, in order to determine whether this cleaved C-terminal fragment was essential for stress induced apoptosis, two mutant constructs in which an aspartic acid at position 694 had been replaced by either an alanine or glutamic acid residue were used . In another study, a cleavage site for caspases at aspartic acid residue 694 in the STAT-1 protein was identified (King and Goodbourn, 1998). To test whether this residue was a target for cleavage, the aspartic acid residue was changed to alanine or glutamic acid. Upon treatment with double stranded RNA and cyclohexamide, these mutant STAT-1 proteins were not cleaved, but endogenous STAT-1α and STAT-1β was cleaved (King and Goodbourn, 1998).

Therefore, in this study, using these two mutants would determine whether cleavage of STAT-1 to release a C-terminal fragment is necessary for stress- induced apoptosis (Figure 3.10).

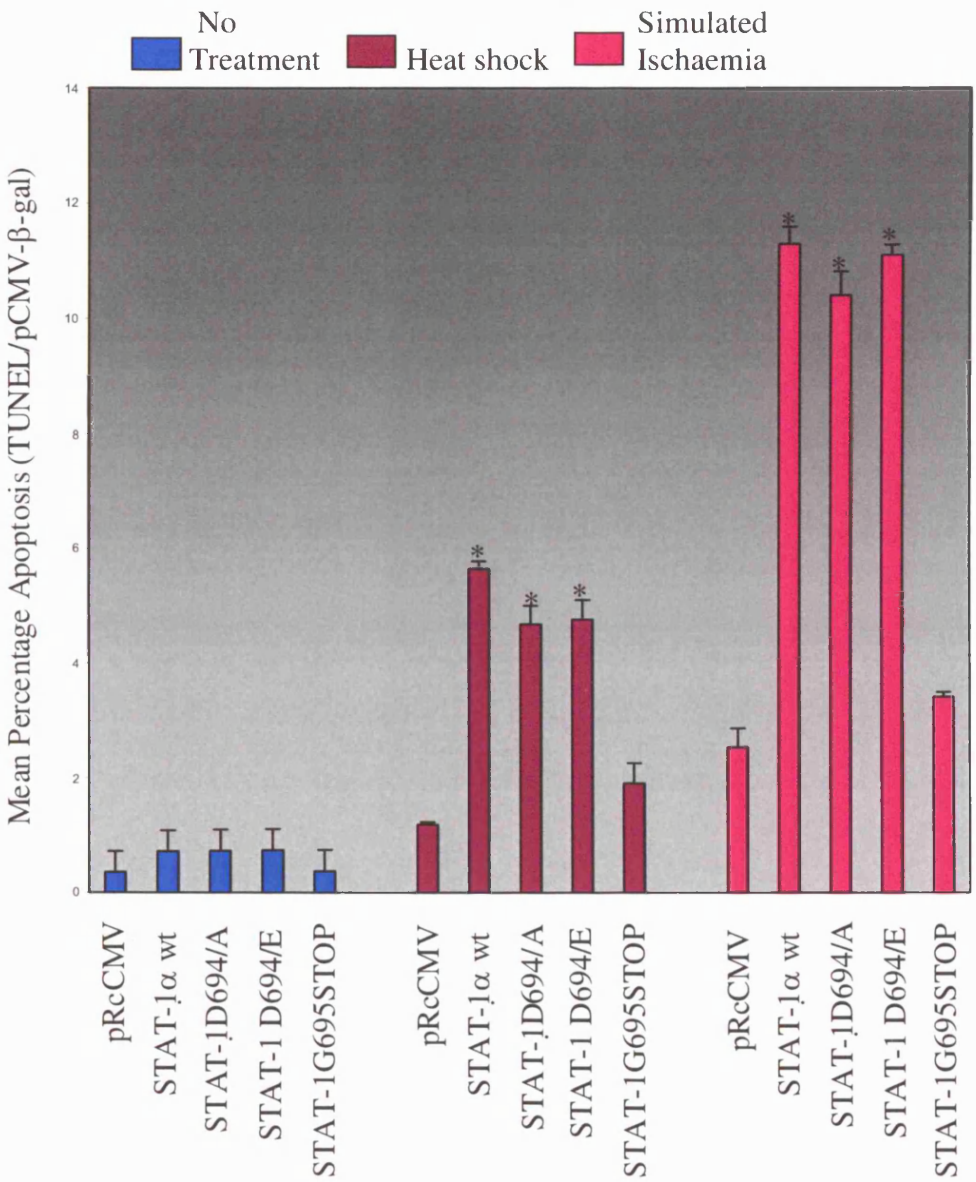


Figure 3.10. Apoptotic cell death in U3A cells transfected with expression vectors encoding full length STAT-1, a construct encoding amino acids 1-694 of STAT-1 with a STOP codon at position 695, and two constructs (D694A and D694E) in which amino acid 694 has been replaced with a residue to prevent caspase cleavage. Transfected cells were either left untreated or exposed to heat stress at 43°C for 2 hours or simulated ischaemia for 4 hours. Values indicate the percentage of successfully transfected cells, which are TUNEL positive and are the mean of three determinations each performed in duplicate whose standard error is shown by the bars. *$p \leq 0.05$versus empty expression vector transfected cells determined by Student *t* test.

When cells were transfected with these two vectors separately both constructs were able to induce enhanced apoptosis significantly (D694A, $p \leq 0.05$; D694E, $p \leq 0.05$).

Therefore, these results demonstrate that a cleaved C-terminal fragment is not essential for stress-induced apoptosis, and that the apoptotic effect can be induced by STAT-1 as an intact molecule as well as an isolated C-terminal fragment.

3.7 Determination of phosphorylation sites of STAT-1 required for stress-induced apoptosis

The previous results show that an enhanced apoptotic effect can be produced when STAT-1 is an intact molecule as well as by a truncated fragment containing only the C-terminal domain (residues 691-750).

The C-terminal domain of STAT-1 contains two phosphorylation sites; a tyrosine residue at position 701 and a serine residue at position 727 (Horvath and Darnell, 1997; Darnell, 1997; Chatterjee-Kishore et al., 2000; Horvath, 2000). Previous studies have shown that phosphorylation on serine 727 residue is required for STAT-1 activation (Zhang et al., 1995). Moreover, it has been shown that phosphorylation of serine 727 is not coupled to prior tyrosine 701 phosphorylation following stress (Kovarik et al., 2001). Therefore, in order to determine whether tyrosine 701 and/or serine 727 phosphorylation was important in apoptosis induced by stress, U3A cells were transfected with either a tyrosine 701 mutant (tyrosine changed to phenylalanine) or a serine 727 mutant (serine changed to alanine). These cells were exposed to heat and simulated ischaemic stress (figure 3.11).

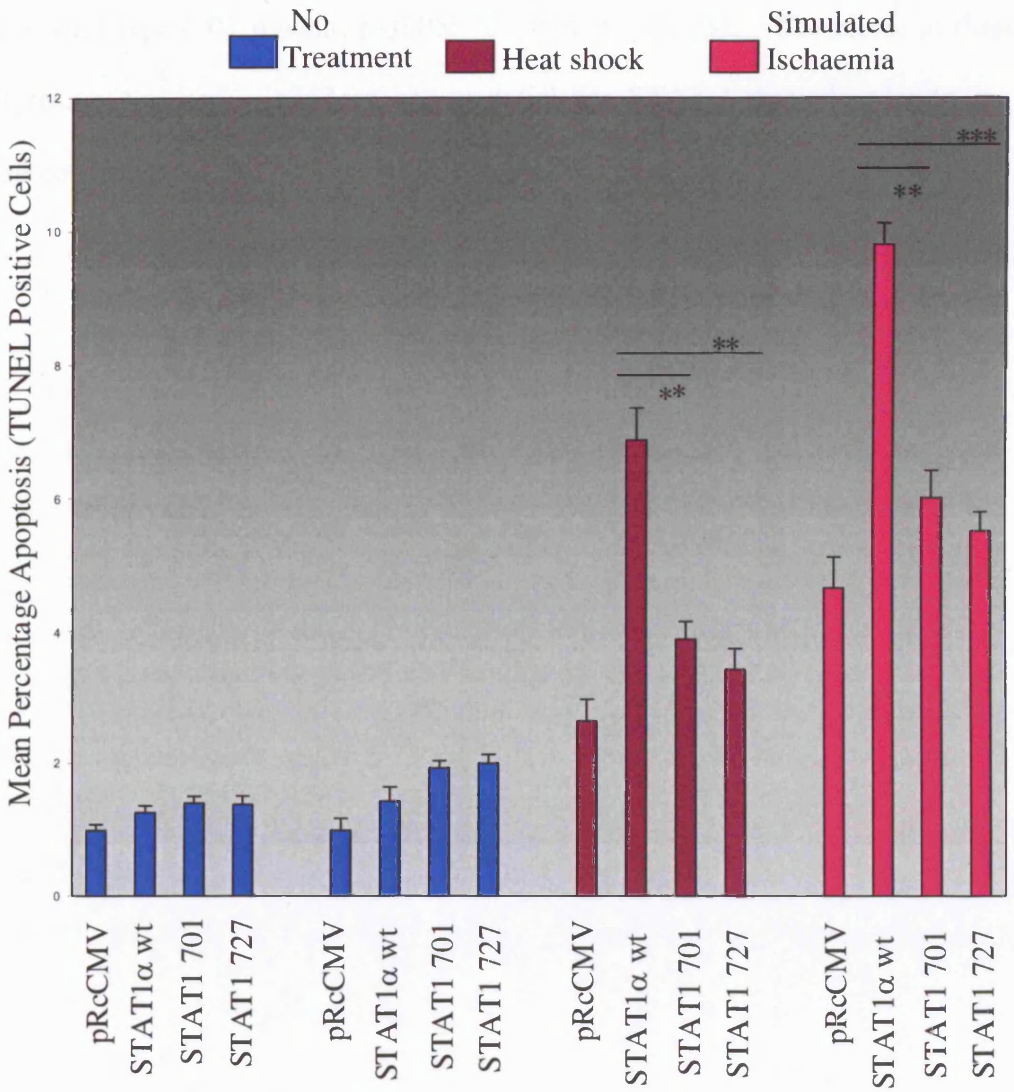


Figure 3.11 Apoptotic cell death in U3A cells transfected with expression vectors encoding wild type STAT-1 or mutants and then exposed to heat stress at 43°C for 2 hours or simulated ischaemia for 4 hours. Values indicate the percentage of successfully transfected cells, which are TUNEL positive and are the mean of three determinations performed in duplicate whose standard error is shown by the bars. Single factor ANOVA was carried out and showed significant differences between treatment groups ($p<0.0001$). Post- hoc Bonferroni test was carried out and significant levels compared to STAT-1 wt are depicted as follows: * <0.05 **<0.01 ***<0.0001

Both STAT-1 mutants significantly reduced the effect on apoptotic cell death compared to STAT-1 wild type (701 mutant, $p \leq 0.05$; 727 mutant $p \leq 0.05$). Therefore, in these cells, both tyrosine and serine residues are essential for STAT-1 phosphorylation in stress-induced conditions.

3.8 Discussion

The aim of this study was to investigate the role of STAT-1 transcription factor in stress-induced apoptosis. By using the U3A mutant cell line, which lacked a functional STAT-1 protein, the region of STAT-1 required for stress-induced apoptosis was also identified.

This study demonstrates that differences in sensitivity to stress occur in the presence and absence of STAT-1 in 2fTGH and U3A cells respectively. U3A cells are resistant to simulated ischaemia or heat stress. This could be due to the loss of STAT-1 or the other factors such as increased number of passages of cells in culture (Darnell et al., 1994).

However, confirmation that the sensitivity of 2fTGH cells to stress is due to STAT-1 has been obtained when STAT-1 is re-introduced into the U3A cell line. The level of apoptosis is enhanced to levels observed in the parental 2fTGH cell line, which demonstrates that STAT-1 is behaving as a pro-apoptotic factor. It is also apparent that the pattern of cell death induced by both simulated ischaemia and heat stress is similar, which suggests that death occurs via a common pathway, and that STAT-1 is required for this process.

Transfecting STAT-3 into U3A cells did not induce enhanced apoptosis but had similar characteristics to the wild type U3A cells. Co-transfection of STAT-1 and STAT-3 resulted in abolition of the enhanced apoptotic effect observed with STAT-1 alone. In this case, the apoptotic characteristic of STAT-1 may be hindered by STAT-3 interference by forming a STAT-1/STAT-3 heterodimer, which is unable to induce enhanced cell death, or

by competing with STAT-1 for binding to a cellular protein or DNA binding site required for death induction.

By transiently transfecting various expression vectors encoding chimaeras and mutants of STAT-1, the region responsible for stress-induced apoptosis was identified. Chimaeric STAT-1 and STAT-3 expression vectors used to probe regions of STAT-1 that may be required for the apoptotic nature of STAT-1 revealed that the N- terminal domain was dispensable for the apoptotic effect observed since the $^{3}296^{1}$ construct was still able to induce enhanced apoptosis. This was confirmed by transfecting the C-terminal STAT-1 construct containing part of the DNA binding domain (390-750) as it also induced enhanced apoptosis. Therefore the effect observed with the chimaeric construct was not due to the N-terminal domain of STAT-3 substituting for that of STAT-1 but rather enhanced apoptosis could still be observed in the absence of the N-terminal domain.

Further experiments confirmed that indeed the N terminal domain was not required for the enhanced apoptotic effect. A STAT-1 construct containing a STOP codon at position 695 showed that the C-terminal domain of STAT-1 downstream of amino acid 695 was essential for the apoptotic effects observed. In addition, the C-terminal domain alone induces apoptosis without the DNA binding domain (STAT-1 691-750 construct).

Other studies have shown that the C-terminal fragment of STAT-1 is released as a result of cleavage by caspase 3 at position 694 (King and Goodbourn, 1998). However, in this study, mutation of the 694 amino acid residue to either alanine or glutamic acid, which

blocks cleavage, still significantly induced apoptosis. Therefore, cleavage of STAT-1 to release a free C-terminal fragment is not essential for stress-induced apoptosis and hence the C-terminal domain can produce apoptotic effects either within an intact molecule or as an isolated domain produced artificially or naturally by caspase cleavage.

Some transcriptional regulators have been shown to interact directly with STAT-1. These proteins include CBP (Zhang et al., 1996), BRCA-1 (Ouchi et al., 2000) and MCM5, a chromatin re-modeling factor (Zhang et al., 1998). Interaction of the C-terminal domain of STAT-1 with DNA-bound transcription factors such as CBP may lead to recruitment independently of the DNA binding domain of STAT-1. This co-activator type role for STAT-1 would allow the C-terminal domain to enhance cell death by activating specific target genes when present alone, or linked to the remainder of the STAT-1 molecule. The ability of STAT-3 to prevent induction of cell death by STAT-1 suggests that STAT-3 may negatively regulate STAT-1 by binding to the same DNA-bound factor as STAT-1 but such that binding does not induce enhanced cell death, as observed with STAT-1.

Recent data from the Chatterjee-Kishore study (Chatterjee-Kishore *et al.*, 2000) has shown that some STAT-1 dependent genes are still activated when the tyrosine 701amino acid residue is mutated to a phenylalanine that cannot be phosphorylated. In other studies, phosphorylation on the serine 727 amino acid residue is essential for interaction of STAT-1 with MCM5 or BRCA-1 (Zhang *et al.*, 1998; Ouchi *et al.*, 2000). Following stress, phosphorylation of serine 727 is not coupled to prior tyrosine 701 phosphorylation (Kovarik *et al.*, 2001). Therefore, testing this effect in the U3A cells showed that

phosphorylation of both tyrosine 701 and serine 727 is essential for stress-induced apoptosis. In contrast, in U3A cells that are treated with TNF-α the tyrosine 701 to phenylalanine mutant is still able to induce enhanced apoptosis whereas the serine 727 to alanine mutant is not able to induce enhanced apoptosis (Kumar *et al.*, 1997). In addition, cardiac cells that are exposed to simulated ischaemia/re-oxygenation following transfection of wild type STAT-1 or the tyrosine 701 to phenylalanine mutant displayed enhanced apoptosis. However, this was not observed when cardiac cells were exposed to simulated ischaemia/re-oxygenation following transfection of the serine 727 to alanine mutant (Stephanou *et al.*, 2001).

The pattern of STAT-1 phosphorylation observed in our study and in the studies by Kumar *et al.* (1997) and Stephanou *et al.* (2001) may be due to use of TNFα rather than thermal or simulated ischaemic stress, and also the cells that were used in the other studies were primary cardiac neonatal cardiac myocytes, that become terminally differentiated rapidly and stop dividing. In contrast, the fibroblast cells that were used in our study were stably transformed cell lines. Therefore, the differences in phosphorylation of residues within the C-terminal domain of STAT-1 could be due to differences in cell type, which would also involve differences in signals that lead to STAT-1 phosphorylation.

Therefore, this data shows that the presence of STAT-1 results in enhanced sensitivity upon exposure to thermal or simulated ischaemic stress compared to cells where it is absent, and also demonstrates that the C-terminal activation domain of STAT-1 is necessary and sufficient for the enhanced apoptotic effect observed.

CHAPTER 4.

REGULATION OF HEAT SHOCK PROTEINS BY THE STAT FAMILY OF TRANSCRIPTION FACTORS

Regulation of Heat Shock Proteins by the STAT Family of Transcription Factors

4.0 INTRODUCTION

Extracellular stresses such as elevated temperature and ischaemia /reperfusion are known to induce the heat shock proteins (Hsps) (Lindquist, 1988). Other stimuli such as cytokines including Interferon γ (IFN γ) and Cardiotrophin-1 (CT-1) are also able to induce Hsp expression via Jak-STAT-1 or Jak-STAT-3 pathways respectively (Stephanou et al., 1998; Stephanou et al., 1999).

The mechanism of Hsp induction has been well established for example, exposure of cells to elevated temperature leads to activation of the latent monomeric transcription factor called Heat Shock Factor (HSF)-1, leading to homo-trimerisation followed by translocation to the nucleus to bind specific HSE sequences within Hsp gene promoters (Morimoto, 1993). Thus, during stress, normally expressed genes are down regulated, and an increase in Hsp gene activity occurs and this mechanism is generally accepted for many other stress conditions of the cell.

Other mechanisms of Hsp induction also exist but are not activated in response to stresses such as elevated temperature. For example, recent studies have shown that Hsps are induced in response to cytokine stimulation, and that different transcription factors are also involved.

Studies have shown that IL-6, a pleiotropic cytokine, is able to stimulate the liver to induce synthesis of acute-phase proteins. IL-6 stimulation of cells leads to activation of two separate signalling pathways namely the MAPKinase pathway, and the Jak/STAT pathway (Akira and Kishimoto, 1992; Akira et al., 1994). Further studies on IL-6 have shown that this cytokine can induce Hsps. IL-6 has been shown to be elevated in systemic lupus erythematosis (SLE), rheumatoid arthritis and juvenile chronic arthritis (Eastgate et al., 1988; De Benedetti et al., 1991; Linker-Israeli et al., 1991). Interestingly, Hsp90 levels have been shown to be elevated in peripheral blood mononuclear cells (PBMCs) from a specific subset of SLE patients (Latchman and Isenberg, 1994). Stephanou et al (1997) demonstrated that IL-6 was able to induce accumulation of Hsp90 in both liver cells and PBMCs, and that it was the Hsp90β promoter that was activated in response to IL-6 (Stephanou et al., 1997). Morover, this effect was mediated by activation of the NFIL-6 transcription factor (Stephanou et al., 1998a).

CT-1 is also able to induce expression of Hsp90 and Hsp70 in neonatal cardiac myocytes and protects against exposure to severe thermal or ischaemic stress (Stephanou et al., 1998b). Furthermore, STAT-3 (which is activated by CT-1 or IL-6) is able to induce activation of Hsp90β promoter, and NFIL-6 and STAT-3 synergize in activating the Hsp90β promoter (Stephanou et al., 1998a). In addition, it has been demonstrated by Stephanou et al. (1998) that both NFIL-6 and STAT-3 are able to interact differently with HSF-1 or heat shock. Over-expression of NF-IL6 and HSF-1(or heat shock) co-operate and enhance activity of Hsp90β promoter, while over-expressed STAT-3 and/ HSF-1(or heat shock) antagonise each other (Stephanou et al., 1998).

Other cyokines have also been shown to produce induction of Hsps in different cell types, for example IL-1β can induce expression of Hsp90 and Hsp70 in rat islet pancreatic cells (Helquest et al., 1991).

IFNγ is a multi-functional cytokine that has anti-viral, anti-tumour properties (Darnell et al., 1994), and specifically activates STAT-1(Schindler et al., 1995). IFNγ has been shown to enhance Hsp70 levels in granulosa-luteal cells and HepG2 cells (Kim et al., 1996). Over-expressed STAT-1 enhances Hsp90β and Hsp70 promoter activity. IFNγ was not able to induce expression of Hsp90β or Hsp70 promoters in the STAT-1 deficient cell line U3A. However, when STAT-1 was re-introduced, Hsp90β and Hsp70 promoter activity was stimulated (Stephanou et al., 1999). Stephanou et al (1999) demonstrated that both STAT-1 and HSF-1 were able to produce an additive effect on Hsp90β and Hsp70 promoter activity, and that a direct protein-protein interaction occurs between STAT-1 and HSF-1. Moreover, a composite response element was also identified in this study that was able to integrate HSF-mediated heat shock response with IL-6 and IFNγ signalling to mediate differential regulation of HSPs.

In previous studies it has been shown that various cytokines can activate and enhance Hsp promoter activity. For example, CT-1 (an IL-6 like cytokine) can induce Hsp expression via the JAK/STAT-3 pathway. IFNγ can induce expression of Hsps via the JAK/STAT-1 pathway specifically (Darnell et al., 1994). IFNα can activate STAT-1 and STAT-2 and hence mediate expression of IFNα specific genes. IFNα and IFNγ both display

overlapping transcriptional signalling pathways (Darnell et al., 1994; Schindler et al., 1995). Induction of Hsps by IFNα has not been investigated previously, and it is possible that IFNα can also induce expression of Hsps like IFNγ in the absence and presence of heat shock (Stephanou et al., 1999). Therefore, in this chapter, the protein-protein interaction of STAT-1 and HSF-1, and the effect of STATs on Hsp90 induction by IFNα, IFNγ and heat stress were investigated. The effect on hsp protein induction was also investigated.

4.1 In vitro protein-protein interaction of STAT-1 and HSF-1

Stephanou *et al* (1999) have previously demonstrated that a direct protein-protein interaction occurs between STAT-1 and HSF-1 *in vivo*. Therefore, the *in vitro* protein-protein interaction of STAT-1 and HSF-1 was investigated by *in vitro* translating STAT-1 (and incorporating S^{35} as a label) and expressing HSF-1 in bacterial cells (for methods, see Chapter 2). The autoradiograph can be seen in figure 4.1:

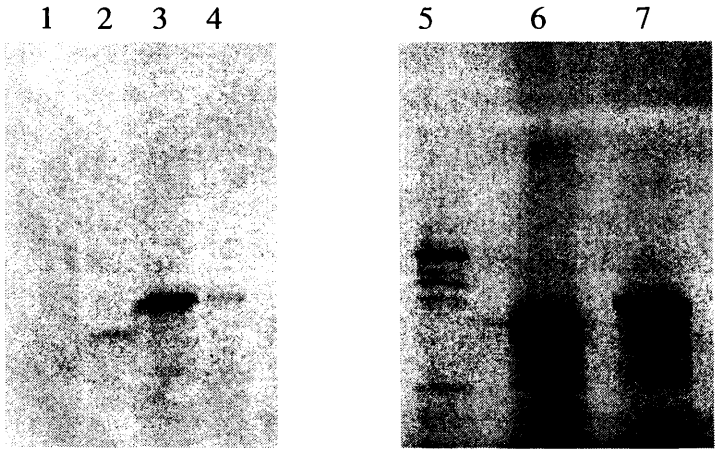


Figure 4.1 *In vitro* protein-protein interaction of STAT-1 and HSF-1. Lane: STAT-1+HSF-1; Lane 2: T7 Luciferase control+HSF-1; Lane 3:ERα+Brn3b (+ve control); Lane 4:ERα+GST; Lane 5: STAT-1 radiolabelled in vitro translated product; Lane 6: T7 Luciferase radiolabelled in vitro translated product (control); Lane 7: ERα radiolabelled in vitro translated product. The experiment was repeated 3 times.

In this experiment a protein-protein interaction was not observed between STAT-1 and HSF-1.

4.2 Effect of IFNs on the Hsp90 promoter and Hsp90 protein

The Hsp90β full length promoter (figure 4.2) was transfected into 2fTGH and U3A cell lines. Cells were then left untreated or stimulated with IFNα, IFNγ or IFNα+IFNγ (50ng/ml of IFNα or IFNγ) for four hours, and then maintained at 37°C or heat shocked at 43°C for 30 minutes. 18 hours later, cells were harvested and CAT assays were performed to measure promoter activity. Cells were co-transfected with an RSVpromoter driven β−galactosidase expression vector as a control for transfection efficiency. The results presented as CAT assays are representatives from 3 experiments with similar results.

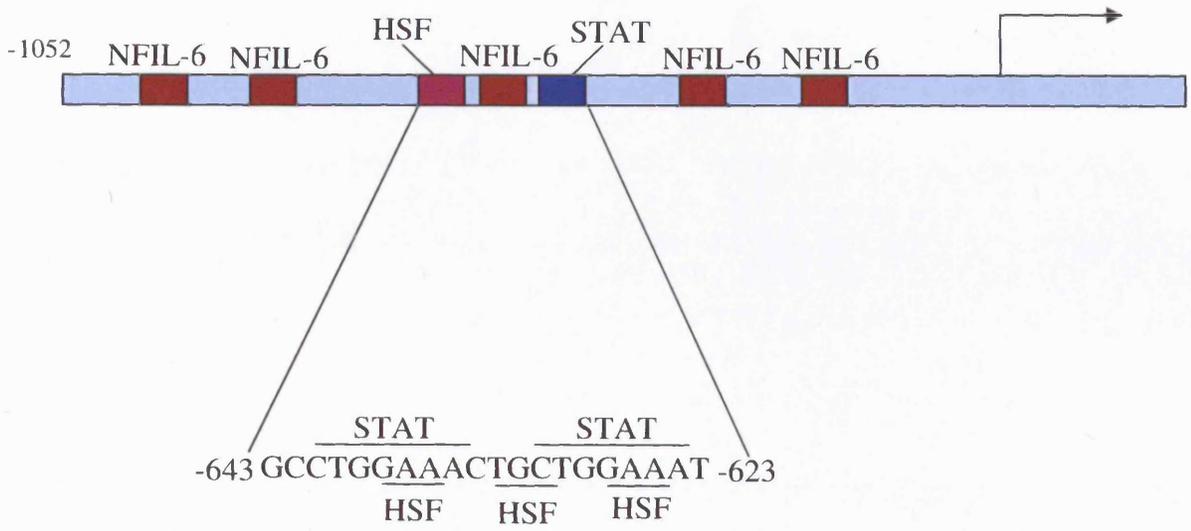


Figure 4.2. A diagrammatic representation of the transcription factor binding sites within the Hsp90β promoter. The sequence shows overlap of transcription factor binding sites for STAT and also HSF.

Western blots presented here are representative of 3 experiments showing similar results and were quantified by densitometry and equalised for actin. The results are presented as bar graphs with standard error bars. Wilcoxon signed rank test was used to test for statistical significance.

4.3 IFNα and IFNγ have different effects in 2fTGH and U3A cells which are likely to be due to STAT-1

In the next set of experiments, the effects of IFNα and IFNγ were investigated in 2fTGH and U3A cell lines. 2fTGH is the parental cell line in which STAT-1 is functional, whereas in U3A cells STAT-1 is knocked out (Darnell et al., 1992).

IFNα treatment was able to activate Hsp90β promoter activity in the absence of heat stress in 2fTGH cells. However, very slight Hsp90β promoter activity was observed when 2fTGH cells were treated with IFNγ (Figure 4.3). Both IFNα and IFNγ together did not have any effect on Hsp90β promoter activity, therefore, both IFNα and IFNγ together had an antagonistic effect on the promoter.

Under conditions of heat stress, both IFNs did not have any effect on Hsp90β promoter activity since the heat shock treatment alone had the same effect. IFNα and IFNγ treatments together did not have any effect on Hsp90β promoter activity and this was reduced compared to either IFNα/HS or IFNγ/HS treatment alone.

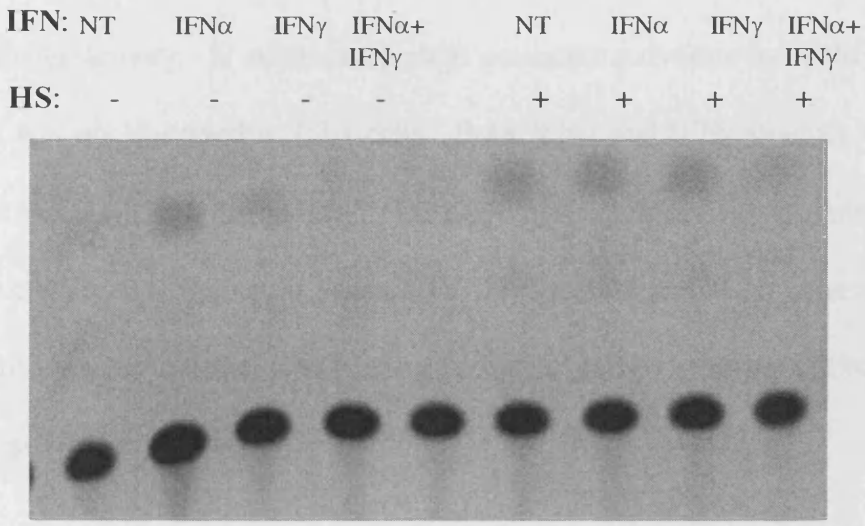


Figure 4.3 Assay of CAT activity of the Hsp90β promoter in 2fTGH cells. Cells were treated with IFNα or IFNγ, or both. Some cells were subjected to heat shock at 43°C for 30 minutes, or left in an incubator at 37°C. A β-galactosidase assay was performed to check for transfection efficiency. β-galactosidase values were constant in each experiment. The experiment was repeated 3 times. Western blots were performed on the lysate for each treatment.

In U3A cells, the pattern of Hsp90β promoter activation was found to be distinct from that of 2fTGH cells (figure 4.4). Treatment with IFNα did not have any effect on Hsp90β promoter activity. In addition, Hsp90β promoter activation by IFNα observed in 2fTGH cells was not observed in U3A cells. Both IFNα and IFNγ together did not have any effect on Hsp90β promoter activity. No Hsp90β promoter activity was observed when U3A cells were given a mild heat stress after IFN treatment, and no increased Hsp90β promoter activity was observed when heat stress was applied after both IFNα and IFNγ treatments together (figure 4.4).

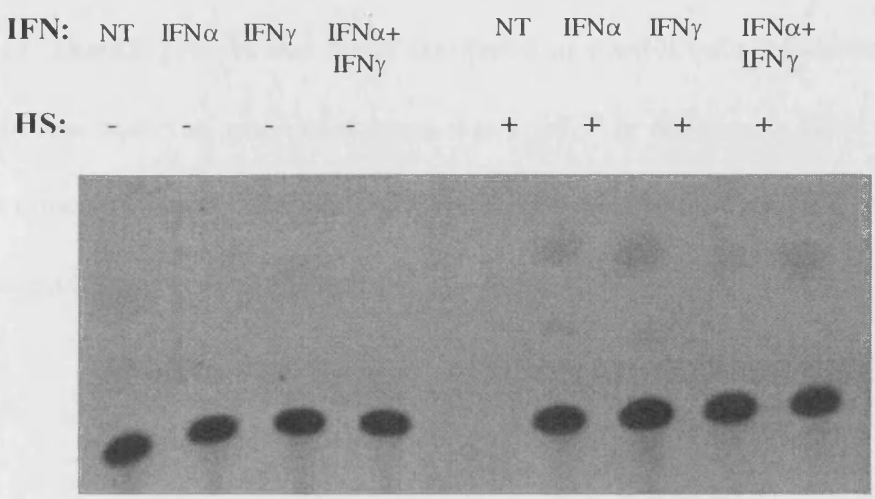


Figure 4.4 Assay of CAT activity of the Hsp90β promoter in U3A cells. Cells were treated with IFNα or IFNγ, or both. Some cells were subjected to heat shock at 43°C for 30 minutes, or left in an incubator at 37°C. A β-galactosidase assay was performed to check for transfection efficiency. β-galactosidase values were constant in each experiment. The experiment was repeated 3 times. Western analysis was performed on the lysate for each treatment.

At the protein level, in 2fTGH cells IFN treatments had little effect on Hsp90 protein levels in the absence of heat stress (Figure 4.5). However, with a mild heat stress in addition to IFNα treatment, Hsp90β protein was lower compared to control cells. Enhancement of Hsp90β protein was observed when heat stress was applied in addition to IFNγ treatment ($p<0.05$), and enhancement of Hsp90β protein was also observed when heat stress was applied in addition to IFNα and IFNγ treatments together ($p<0.05$) (figure 4.6).

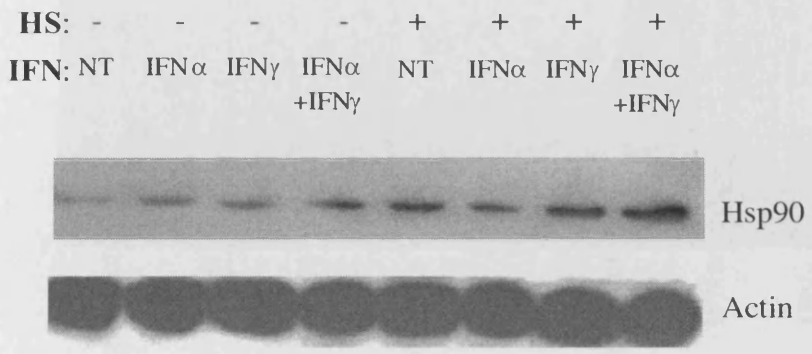


Figure 4.5 Western blot analysis of Hsp90β protein in 2fTGH cells. Blots were stripped and re-probed with actin antibody.

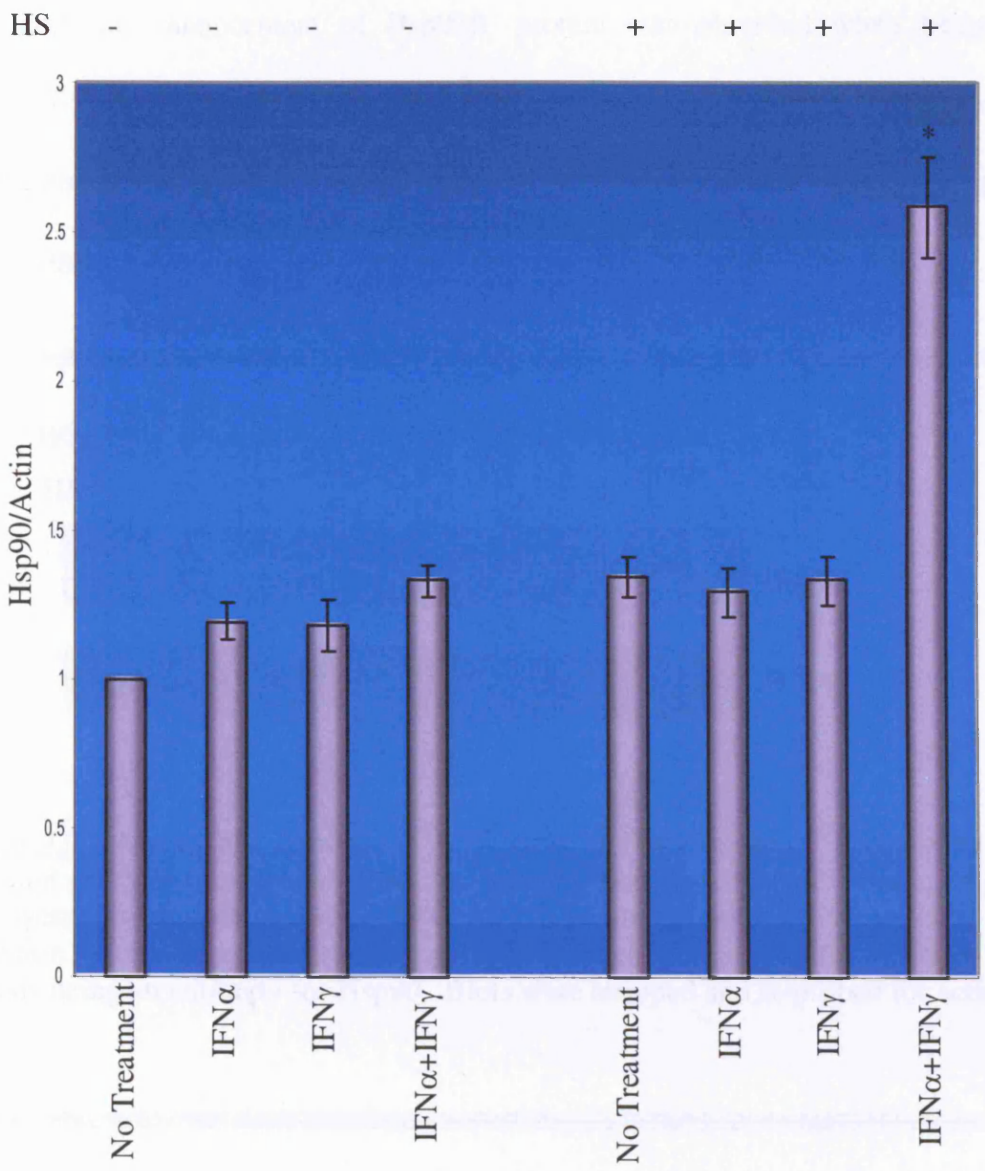


Figure 4.6 Hsp90β protein levels in 2fTGH cells. The values for Hsp90β protein were obtained from densitometric analysis, and were divided by densitometric values for actin. The values indicate the mean of 3 determinations for each treatment (n=3) whose standard error is shown by the bars. *$p \leq 0.05$ treatment vs control, determined by Wilcoxon signed rank test.

In U3A cells an enhancement of Hsp90β protein was observed when treated with IFNα ($p<0.05$) or IFNγ ($p<0.05$), or both IFNα and IFNγ together (figure 4.7). However, when heat stress was applied, Hsp90β protein levels were reduced after IFNα ($p<0.05$) treatment (figure 4.8). This effect was also observed in IFNγ treated U3A cells.

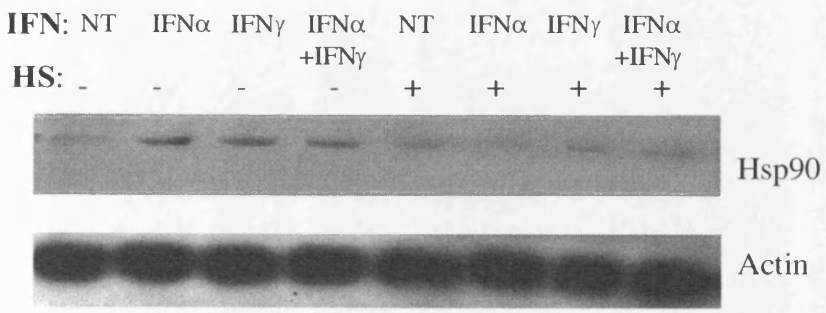


Figure 4.7 Western blot analysis in U3A cells of Hsp90 protein. Cells were left untreated (NT) or treated with IFNα or IFNγ, or both together for 4 hours. Some cells were given heat shock at 43°C for 30 minutes, and then returned to the incubator. Cells were harvested after 18 hours and lysates used for Western blot analysis using an antibody for Hsp90. Blots were stripped and re-probed for actin.

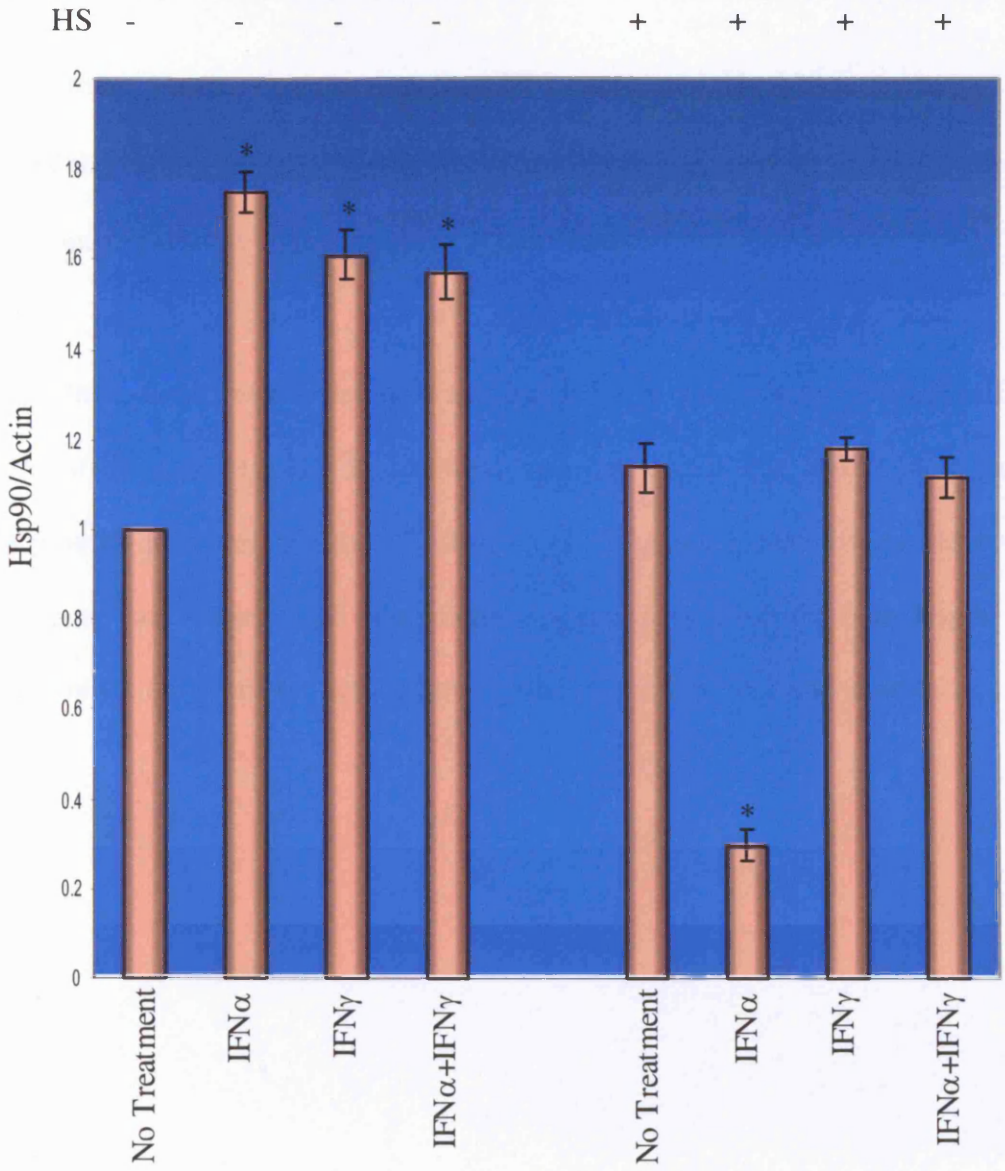


Figure 4.8 Hsp90β protein levels in U3A cells. The values for Hsp90β following various treatments were obtained from densitometric analysis, and were divided by densitometric values for actin. The values indicate the mean of 3 determinations for each treatment, whose standard error is shown by the bars. *$p \leq 0.05$ treatment vs control determined by Wilcoxon signed rank test.

Therefore, these results demonstrate that IFNγ activated Hsp90β promoter activity is affected by the lack of STAT-1 in U3A cells and IFNα treatment has a negative effect on Hsp90β promoter activity in the presence of heat stress.

Taken together, these results demonstrate that IFNs α and γ have different effects on Hsp90 in different cell types. In addition, by using a cell line that lacks a functional STAT-1 protein, it is possible that STAT-1 may be required for the effects observed both in the absence and presence of heat shock. A summary table for both Hsp90 protein induction and Hsp90 promoter activity are represented in table 4.1 and table 4.2.

Table 4.1 Summary table to show the effect of IFNα and IFNγ and heat shock on Hsp90 levels

Cell Type	No Treatment	IFNα	IFNγ	IFNα+ IFNγ	Heat shock	IFNα+ Heat shock	IFNγ+ Heat shock	IFNα+IFNγ +Heat shock
2fTGH (n=3)	+	++	++	++	++	++	+++	++++
U3A (n=3)	+	+++	++	++	++	++	+++	++++

+ = Hsp90 levels of protein induction
- = No Hsp90 protein induction

Table 4.2 Summary table to show the effect of IFNα and IFNγ and heat shock on Hsp90 promoter activity

Cell Type	No Treatment	IFNα	IFNγ	IFNα+IFNγ	Heat shock	IFNα+ Heat shock	IFNγ+ Heat shock	IFNα+IFNγ +Heat shock
2fTGH (n=3)	+	+	+	-	+	+	+	+
U3A (n=3)	-	-	-	-	+	+	-	+

+ = Hsp90 promoter activity
- = No Hsp90 promoter activity

4.4 Discussion

Previous studies have demonstrated that IFNγ has antiviral and anti-tumour properties by inducing specific IFNγ responsive genes (Pestfa et al., 1987; Darnell et al., 1994). INFγ activates the STAT-1 signalling pathway via the JAKs (Schindler et al., 1995). IFNγ is also able to induce expression of Hsp90 in HepG2 cells (Stephanou et al., 1999). In addition, over-expression of STAT-1 enhanced the activity of the Hsp90β promoter whereas in U3A cells, IFNγ was not able to activate the Hsp90β promoter. However, re-introduction of STAT-1 into the cell line restored Hsp90β promoter responsiveness to IFNγ (Stephanou et al., 1999). In the same study, HSF-1 and STAT-1 protein-protein interaction was also demonstrated, however, in this thesis, a protein-protein interaction in vitro was not found. Therefore, the effect of IFNα as well as IFNγ was studied on the Hsp90β promoter since this had not been demonstrated previously. IFNα is known to activate the JAK/STAT pathway via STAT-1 and STAT-2, which form heterodimers. Thus, using the 2fTGH and U3A cell lines, the responsiveness of Hsp90 to IFNα was investigated.

A different effect on Hsp90β promoter and protein levels was observed in the 2fTGH and U3A cell types. The U3A cell type lacks a functional STAT-1 protein whereas the 2fTGH cells are the parental cell line in which a functional STAT-1 protein is present (Darnell et al., 1992). This cell line is ideal to study the effect of STAT-1 in response to the stimulatory treatments given by IFNα and IFNγ. In these two cell lines, the effects of

IFNα or IFNγ were more apparent when a heat stress was applied subsequent to IFN stimulation.

In U3A cells, lack of STAT-1 reduced levels of Hsp90β in all IFN treatments at the promoter level. This effect would suggest that STAT-1 is likely to be required for IFN mediated Hsp90β expression. STAT-1 is not only essential for activation by IFNγ, but also for activation by IFNα. The lack of STAT-1 reduces Hsp90β activity and IFNα stimulation, suggesting that STAT-1 is required for the formation of the ISGF3 DNA complex. The effects of STAT-1 on the ISGF3 binding site were not studied due to time limitations.

Other studies have shown that in the absence of STAT-1, STAT-2 phosphorylation is abolished, and that STAT-1 phosphorylation is required for STAT-1/STAT-2 dimer formation. Therefore, in this case IFNα has a negative regulatory effect on Hsp90β promoter and protein levels, whereas IFNγ has a synergistic effect with heat stress.

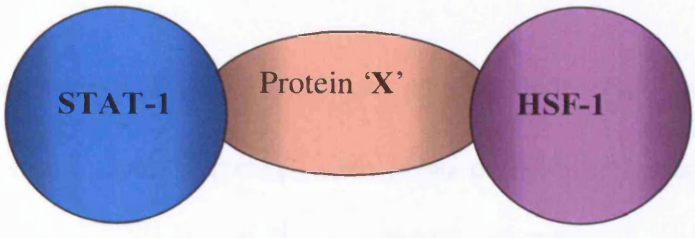
IFNγ stimulation of all cell types leads to potent activation of Hsp90β at both promoter and protein levels. In this chapter it can be inferred that the effects observed on IFNγ stimulation is due to STAT-1. This effect is also observed after heat shock treatment of IFNγ treated cells, which further suggests that an interaction between HSF-1 and STAT-1 may occur. STAT-1 and HSF-1 do not form a protein-protein interaction in vitro. This effect does not complement the results observed by Stephanou et al (1999). The study by Stephanou (1999) has demonstrated that a HSF-1 binding site is contained within the

STAT binding site in the short region of the Hsp70 and Hsp90β promoters. In addition, IFNγ treatment induces the expression of Hsp70 and Hsp90 in a STAT-1-dependent manner. HSF-1 and STAT-1 expression vectors have also been used in this study to show an interaction of STAT-1 and HSF-1 in transfection studies, which show that presence of both STAT-1 and HSF-1 have an additive effect in activating Hsp70 and Hsp90β promoters. In co-immunoprecipitation experiments, Stephanou et al. (1999) have shown a direct protein-protein interaction between STAT-1 and HSF-1 by using an anti-STAT-1 polyclonal antibody endogenous STAT-1 immunoprecipitate followed by a specific anti-HSF-1 polyclonal antibody on the same blot.

In this thesis, an in vitro approach to observing a protein –protein interaction between STAT-1 and HSF-1 was taken. The STAT-1 mammalian expression vector was added to a rabbit reticulocyte lysate, S^{35}, and T7 polymerase mix for translation of STAT-1. HSF-1 was expressed by ITPG induction in bacterial cells. HSF-1 protein was purified from bacterial lysate by using His tagged beads which bind to His residues fused to the HSF-1 insert. Both STAT-1 and HSF-1 proteins were incubated together in order for the interaction to take place. The interaction was visualised on SDS PAGE gel for autoradiography. Unfortunately, a band was not observed for the two proteins, which shows that in vitro STAT-1 and HSF-1 do not interact under these conditions.

The main difference between these two methods is that the Stephanou study uses whole cell lysate in which endogenous STAT-1 is already present, therefore, by using an antibody specific to STAT-1 it is easier to identify interaction with HSF-1. However, in an in vitro

condition, all components are separate, ie STAT-1 is expressed in a different system, whereas HSF-1 is expressed in a bacterial system and it may be possible that a "co-chaperone" exists in vivo that aids the interaction of STAT-1 and HSF-1 and this is probably not present in the in vitro reticulocyte lysate system.



In addition, if the concentration of HSF-1 protein is extremely low, then it may not be possible to observe an interaction. An alternative would be to use a different bacterial expression vector to enhance HSF-1 protein levels after IPTG induction. Unfortunately, due to time limitations this has not been possible.

Reduction of Hsp90β by IFNα and IFNγ together upon heat shock treatment could be due to the presence of STAT-1/STAT-2 dimers. It is known that STAT-1β can also form a dimer with STAT-2 (Qureshi et al., 1996). Therefore, STAT-1β/STAT-2 may behave as a negative regulator in Hsp90β expression by IFNα. These effects are more clearly observed in U3A cells. Lack of STAT- 1 in this cell type reduces Hsp90β levels in all treatments, showing that STAT-1 is required for Hsp90β stimulation by IFNγ. In addition, lack of induction by IFNα suggests that STAT-1 is likely to be required for formation of

the ISGF3 DNA complex. Absence of STAT-1 does not promote phosphorylation of STAT-2 thus preventing heterodimer formation (Qureshi et al., 1996). Therefore, in order to prove that indeed STAT-1 is required for the effects observed, it is essential that further transfection experiments are carried out in U3A cells by re-introducing STAT-1 into the cell line and subject STAT-1 transfected cells with IFNs in the absence or presence of heat shock.

In ND7 cells when IFNα or IFNγ are administered, upon heat stress Hsp90β promoter activity is significantly increased with IFNα or IFNγ treatment alone. Interestingly, heat stress causes a synergistic effect when IFNα and IFNγ are administered together followed by heat stress. It has been shown by Stephanou et al (1999), that STAT-1 and HSF-1 together do have an additive effect on the HSp90β promoter, and that this effect is specific to IFNγ treatment (Stephanou et al., 1999). It is not known, however, if the STAT-1/ STAT-2 dimer, interacts with HSF-1, or whether an interaction occurs between STAT-1/STAT-1 and HSF-1. STAT-1 homodimers can be formed after IFNα treatment, but not as readily as after IFNγ treatment. IFNγ treatment also leads to formation of STAT-1α or STAT-1β and STAT-2 heterodimers (Schindler et al., 1992; Qureshi et al., 1995; Shuai et al., 1994). Therefore, it is a possibility that there are more STAT-1 homodimers present after IFNα and IFNγ treatment, therefore, enhancing activation of the Hsp90β promoter or there is a preference for STAT-1/STAT-1 dimers over STAT-1/STAT-2 dimers. This would need to be studied further.

At the protein level, no distinct changes in levels of Hsp90β were discovered with IFN treatments alone, however, an increase in protein levels was observed in combined treatment with IFNα and IFNγ together without heat stress, which was also observed at the promoter level. It is not known why a synergistic effect is observed but it is possible that both STAT-1/STAT-1 dimers and STAT-1/STAT-2 dimers could contribute to the effect, or the effect is specific to the cell type.

Hsp90β activity was not enhanced in HepG2 cells treated with IFNα but was stimulated with IFNγ treatment alone, which is in accordance with previous published data (Stephanou et al., 1999). Upon heat stress, IFNα was able to stimulate Hsp90β promoter activity, and IFNγ strongly enhanced Hsp90β promoter activity in the presence of heat stress, suggesting that the presence of STAT-1 and HSF-1 had an effect on activation of the Hsp90β promoter. Interestingly, when both IFNα and IFNγ were administered, followed by heat stress, activity of the Hsp90β promoter was significantly reduced compared to either IFNα or IFNγ treatment alone.

At the protein level, similar results were also observed, where IFNγ treatment increased Hsp90 protein, in the absence of heat stress and also in the presence of heat stress. In addition, protein levels were reduced when IFNα and IFNγ were administered together followed by heat stress, suggesting that IFNγ strongly stimulates Hsp90β promoter activity, with activity being enhanced when heat stress is given whereas IFNα does not. IFNα seems to have a negative regulatory effect on the Hsp90β promoter in the presence of heat stress and reduces the effect of IFNγ on the promoter and also protein levels. This

could be due to competition between STAT-1/STAT-2 dimers and STAT-1/STAT-1 dimers for DNA binding.

To conclude, the results demonstrated in this chapter are preliminary. STATs modulate genes depending upon the stimulus. It is known that regulatory sequences in eukaryotic genes contain multiple transcription factor binding sites which provide different combinations of interactions between many transcription factors to mediate integration of multiple signals. It is therefore, apparent that the Hsp90β promoter contains composite response elements that are able to integrate multiple signals and allow interaction of transcription factors with each other to have either a stimulatory or inhibitory effect on geneexpression.

CHAPTER 5.

The Cardioprotective Agent

Urocortin Induces

Expression of Cardiotrophin-1

The Cardioprotective Agent Urocortin Induces Expression of CT-1

5.0 Introduction

In the previous two chapters, extracellular stress such as simulated ischaemia or elevated temperature were used to investigate the role of the STAT-1 transcription factor and Hsp90 respectively in order to understand the mechanisms and importantly to consider them as important factors in cell survival. In this chapter, simulated hypoxic stress has been used to investigate the induction of expression of cardiotrophin-1 (CT-1), which is known to be cardioprotective, by urocortin (an unrelated peptide that is also cardioprotective via the same signaling pathway as CT-1). The identification of cardioprotective agents and an understanding of the mechanism by which they act is likely to be of considerable importance in understanding the processes which occur during cardiac stress and in their ultimate modulation for therapeutic benefit.

We previously identified two agents, cardiotrophin-1 (CT-1) and Urocortin (Ucn), both of which are able to protect the heart against the damaging effects of cardiac ischaemia followed by reperfusion by reducing the levels of apoptotic cell death (Latchman, 1999; Latchman, 2000; Latchman, 2001; Latchman, 2002). In particular, the protective effect of both these agents in either cultured cardiac cells or in the intact heart can be demonstrated when they are added following the ischaemic episode prior to reperfusion, as well as when they are added before the ischaemic event itself (Brar et al, 2001; Liao et al, 2002; Brar et al, 2000). Hence, these agents or their derivatives could potentially be utilised therapeutically at reperfusion following an ischaemic episode.

Despite the differences in the nature of these two factors, common signaling pathways appear to mediate their protective effects. Thus, both CT-1 and Ucn have been shown to activate the p42/p44 mitogen activated protein kinase (MAPK) pathway and inhibition of this pathway either by chemical inhibitors or by dominant negative mutants of the enzymes involved inhibits the protective effect of CT-1 or Ucn (Brar et al, 2001, Liao et al, 2002; Brar et al, 2000 Sheng et al, 1997). In contrast, neither of these agents activates the p38 MAPK or JNK pathway and inhibition of these pathways has no effect on the protective abilities of Ucn or CT-1. Interestingly, however, both CT-1 and Ucn are also able to activate the PI-3 kinase/Akt pathway and inhibition of this pathway also prevents the cardioprotective effect of both these agents (Brar et al, 2001; Brar et al, 2002).

Ucn and CT-1 do differ however, in their requirement for protein synthesis to produce their protective effects. Thus, while new protein synthesis is necessary for the protective effect of Ucn, which can be blocked by the protein synthesis inhibitor cylohexamide (Brar et al, 2002), this is not the case for CT-1 whose protective effect is extremely rapid and is insensitive to inhibitors of protein synthesis (Brar et al, 2001, Railson et al, 2000). In addition, both CT-1 and Ucn are able to induce Hsps (Brar et al., 2002). In view of this, and the similarities between the pathways activated by these two diverse protective agents, it has been hypothesized here that Ucn induces the expression of CT-1.

5.1 Urocortin induces expression of CT-1 at the messenger RNA level

Previous studies by Brar et al (1999) have shown that Ucn can induce cardio-protection in cardiac myocytes against lethal ischaemic injury. In this study, the aim was to investigate whether Ucn can induce the expression of CT-1, another cardio-protective agent. Therefore, initial experiments involved exposing cardiac neonatal myocytes to Ucn for various periods of time in order to test the possibility that Ucn could induce the expression of CT-1 by detecting changes in the CT-1 messenger RNA (mRNA) levels. Cells were treated for 4 hours, 16 hours, or 24 hours with Ucn, and then harvested to prepare mRNA to be hybridised with a CT-1 complementary DNA (cDNA) probe (figure 5.1a).

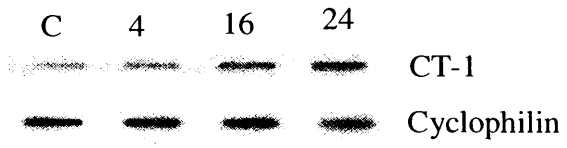


Figure 5.1a. RNA slot blot to show time course (hours) of CT-1 mRNA levels and control Cyclophilin in cardiac cells treated with Urocortin (10^{-7}M)

Hybridisation of CT-1 probes to total mRNA showed that CT-1 expression was induced by treatment with Ucn, and the induction of CT-1 increased with time, with induction being greatest at 24 hours of Ucn treatment, which was approximately 3.5-fold compared to control cells.

In the next set of experiments, cardiac myocytes were also exposed to hypoxia or hypoxia/ re-oxygenation, which was also able to clearly enhance induction of CT-1 expression by 2.5-fold compared to control (figure 5.1b).

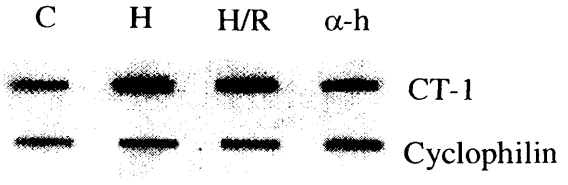


Figure 5.1b. RNA slot blot to show levels of CT-1 mRNA and control cyclophilin mRNA in cardiac cells in control cells (C), cells exposed to simulated hypoxia (H) for 4 hours, or cells exposed to simulated hypoxia followed by re-oxygenation (H/R), or to hypoxia in the presence of α-helical CRH (α-h) (10^{-7}M). α-helical CRH was added 10 minutes before simulated hypoxic treatment.

Enhancement of induction of CT-1 expression by Ucn was blocked when α-helical CRH, which blocks Ucn binding to its receptor, was added (figure 5.1c).

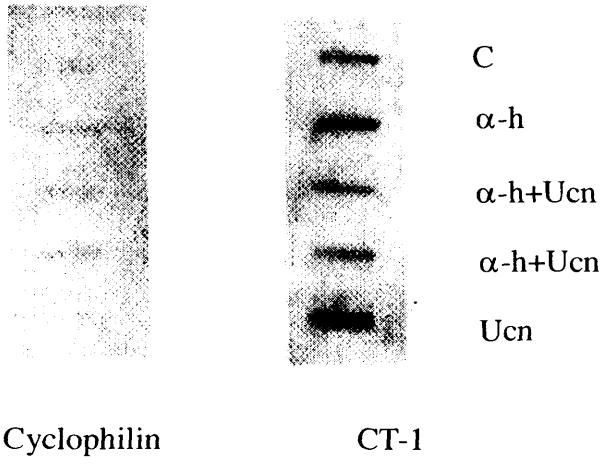


Figure 5.1c. RNA slot blot to show levels of CT-1 mRNA in cardiac cells in control cells (C), or in cellstreated with Urocortin (Ucn) and/or α-helical CRH (α-h) (10^{-7}M). α-helical CRH was added 10 minutes before Ucn (10^{-7}M)

Interestingly, a similar inhibition of CT-1 induction was also observed when α-helical CRH was added during simulated hypoxia (figure 5.1b). These results suggest that Ucn may mediate induction of CT-1 by hypoxia. Ucn mRNA expression increases during simulated hypoxia or simulated ischaemia (Okosi et al, 1998; Brar et al, 1999) and may mediate the induction of CT-1 by simulated hypoxia.

5.2Urocortin induces enhancement of CT-1 protein

In order to confirm that the enhanced CT-1 mRNA levels induced by Ucn were paralleled at the protein level, assays of CT-1 were carried out to detect levels of CT-1 protein in the medium of cells treated with Ucn or left untreated (figure 5.2).

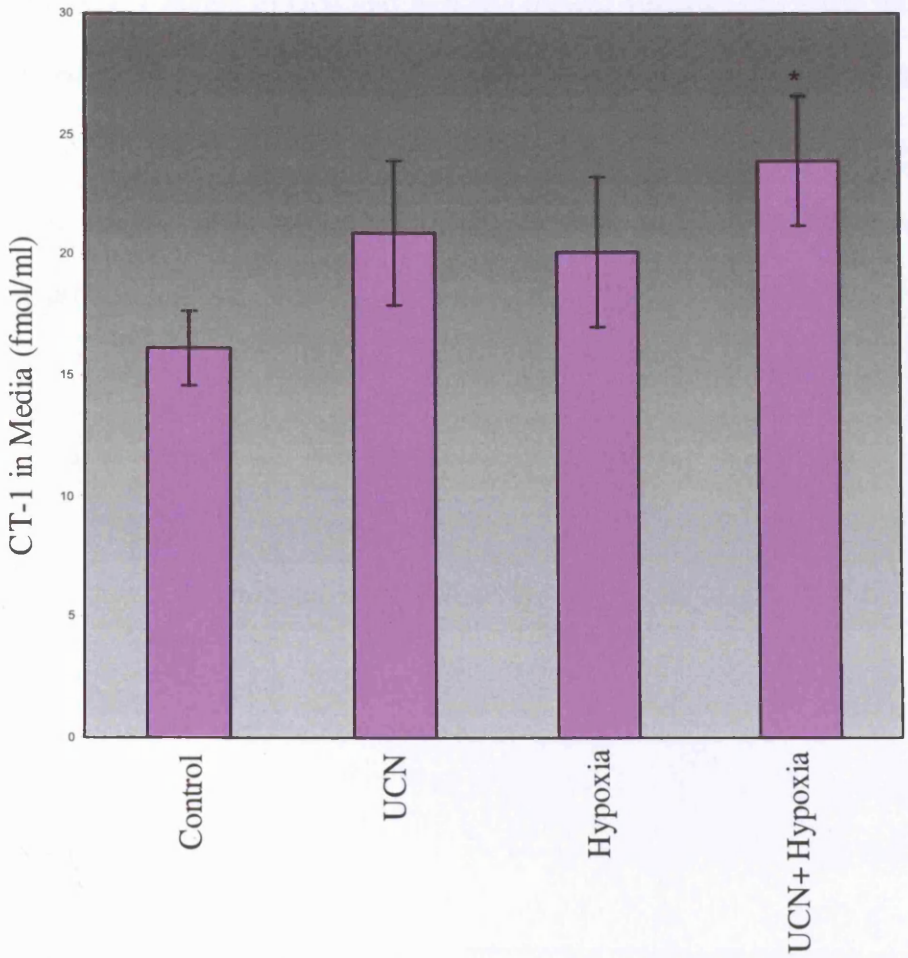


Figure 5.2 CT-1 level in the medium of cardiac myocytes exposed to indicated treatment. Values are the mean of 5 determinations carried out in duplicate ,whose standard error is shown by the bars. Single Factor ANOVA showedsignificant differences between groups, P<0.0001. Post-hoc Bonferroni tests were performed between pairs of data, with a significance level of P<0.05 Control.

In these experiments, cells that were treated with Ucn clearly showed increase in CT-1 protein, although this effect was not as dramatic as observed at the mRNA level. Interestingly, an increase in CT-1 protein was also observed when cells were exposed to hypoxia/re-oxygenation. Moreover, the CT-1 levels in cells exposed to hypoxia and also treated with Ucn were higher than those in cells exposed to either treatment alone. The CT-1 protein detected in supernatants was significantly different across the four conditions tested ($P<0.008$). CT-1 levels in Ucn and hypoxia treated supernatants were significantly elevated compared to control cells ($P<0.003$), although the elevation with either Ucn or hypoxia alone did not reach significance ($P<0.06$). These results therefore demonstrate that treatment of cardiac cells with Ucn results in enhanced CT-1 mRNA levels and increased CT-1 protein levels.

5.3 Ucn activates the CT-1 gene promoter

In the following experiments, it was investigated whether the enhanced CT-1 mRNA and protein levels induced by Ucn were produced by activation of the CT-1 gene promoter by Ucn. In order to do this, a reporter construct in which the CT-1 promoter (from -99 to +19) relative to the transcriptional start site) was fused to the luciferase reporter gene was used (Funamoto et al., 2000). This construct was transfected into neonatal cardiac myocytes and the response to various stimuli of this promoter construct was tested, for example, with CT-1 or Ucn in the presence or absence of hypoxia (figure 5.3).

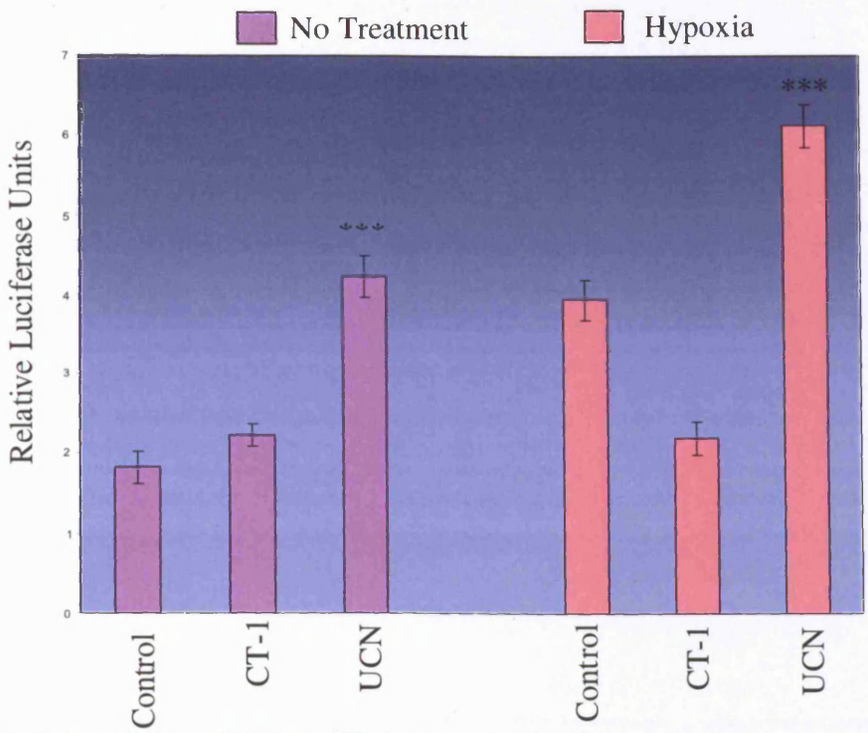


Figure 5.3 Activity of the wild type CT-1 promoter-luciferase reporter construct following transfection into cardiac myocytes and subsequent exposure to indicated treatment. Values are the mean of 6 determinations (corrected to Renilla) whose standard error is shown by the bars. Single Factor ANOVA was carried out and showed significant differences between treatment groups ($P<0.0001$). Post-hoc Bonferroni tests were carried out and significance levels compared to control are depicted as follows: *<0.05, **<0.01, ***<0.0001.

This construct showed a two-fold response to hypoxia, paralleling the response at the mRNA level and protein level. Interestingly, this promoter was not responsive to CT-1 itself, however, it showed a two-fold activation upon treatment with Ucn. In addition, a stronger induction of approximately three-fold relative to control was observed in cells that were treated with Ucn and also exposed to hypoxia. These results demonstrate, therefore, that the response of CT-1 mRNA and protein levels to Ucn are paralleled by activation of the promoter, indicating that Ucn treatment has a direct effect on the CT-1 promoter activity rather than, for example, stabilising the CT-1 mRNA, leading to enhanced mRNA and protein levels.

5.4 The C/EBPβ transcription factor binding site is required for CT-1 promoter activity induced by Ucn

Previous studies have localised a number of transcription factor binding sites within the CT-1 promoter, one of which mediates its induction by norepinephrine for example (Funamoto et al, 2000;Erdmann et al, 1998). However, all of these binding sites are located upstream of the region of the promoter which has been shown to confer responsiveness to Ucn onto a heterologous gene. Upon examination of this sequence (Figure 5.4a) using the Transfac 4.0 program, a potential binding site for the C/EBPβ/NF-IL6 transcription factor was identified (Figure 5.4b).

-99 ctgaactatg attggccgag cccgagccac gcccctagcc ctttcccccc ttttcccccc
gacttgatac taaccggctc gggctcggtg cggggatcgg gaaaggggga aaaaggggga

ttttcccccc ccccteetcc tccccccggag gggt*gtgttg aggaacct*gg
aaaaggggga ggggaggagg agggggcc tc ccc*a cacaac tcctt* g gacc

ataagcctgg ggccagcatg ag +19
tat t cggacc ccggtcgtac tc

Figure 5.4a CT-1 minimal promoter sequence with the C/EBPβ transcription factor binding site in italics.

V$TCF11_01	1 (-)	0.807	0.873	ATCAtagttcaga
V$NFY_01	6 (-)	1.000	0.965	ctcggCCAAtcatagt
V$NFY_C	8 (+)	1.000	0.875	tatgATTGGccgag
V$NFY_Q6	9 (-)	1.000	0.940	cggCCAAtcat
V$CAAT_01	10 (-)	1.000	0.972	ctcggCCAAtca
V$NF1_Q6	11 (+)	1.000	0.931	gatTGGCcgagcccgagc
V$NF1_Q6	16 (-)	1.000	0.854	gcgTGGCtcgggctcggc
V$GC_01	25 (-)	1.000	0.900	taggGGCGtggctc
V$AHRARNT_01	26 (-)	1.000	0.869	ggctaggggCGTGgct
V$SP1_Q6	26 (-)	1.000	0.854	taggGGCGtggct
V$NFAT_Q6	40 (-)	1.000	0.907	aggggGAAAggg
V$IK1_01	40 (-)	1.000	0.863	aaggGGGAaaggg
V$GKLF_01	40 (-)	1.000	0.939	aaaggggggaaAGGG
V$GKLF_01	41 (-)	0.937	0.918	aaaaggggggaAAGG
V$IK2_01	41 (-)	1.000	0.906	aaggGGGAaagg
V$MZF1_01	45 (-)	1.000	0.969	aagGGGGa
V$GKLF_01	46 (-)	0.951	0.879	ggggaaaaagGGGG
V$GKLF_01	47 (-)	0.951	0.888	ggggggaaaaaGGGG
V$GKLF_01	48 (-)	1.000	0.890	agggggaaaaAGGG
V$GKLF_01	49 (-)	0.937	0.863	gagggggaaaAAGG
V$NFAT_Q6	50 (-)	1.000	0.948	agggggAAAAaag
V$IK1_01	50 (-)	1.000	0.871	gaggGGGAaaaaag
V$IK2_01	51 (-)	1.000	0.918	gaggGGGAaaaa
V$GC_01	54 (-)	0.877	0.890	gaggGGAGggggaa
V$MZF1_01	55 (-)	1.000	0.965	gagGGGGa
V$SP1_Q6	55 (-)	0.845	0.902	gaggGGAGgggga
V$GKLF_01	57 (-)	0.951	0.897	gaggaggggaGGGG
V$IK2_01	57 (-)	1.000	0.863	ggagGGGAgggg
V$GKLF_01	58 (-)	1.000	0.892	ggaggaggggAGGG
V$MZF1_01	61 (-)	1.000	0.982	ggaGGGGa
V$GKLF_01	62 (-)	0.951	0.864	gggaggaggaGGGG
V$GKLF_01	63 (-)	1.000	0.853	ggggaggaggAGGG
V$GC_01	65 (-)	0.877	0.872	cgggGGAGgaggag
V$SP1_Q6	66 (-)	0.845	0.870	cgggGGAGgagga
V$IK2_01	68 (-)	1.000	0.865	ccggGGGAggag
V$MZF1_01	72 (-)	1.000	0.953	ccgGGGGa
V$AP2_Q6	73 (+)	1.000	0.856	ccCCCGgagggg
V$CETS1P54_01	75 (+)	1.000	0.893	ccCGGAgggg
V$CEBPB_01	86 (+)	0.873	0.924	gtgttgaGGAAcct
V$GATA1_03	96 (+)	1.000	0.944	acctgGATAagcct
V$GATA1_02	96 (+)	1.000	0.927	acctgGATAagcct
V$GATA1_04	97 (+)	1.000	0.917	cctgGATAagcct
V$GATA3_02	98 (+)	1.000	0.859	ctgGATAagc
V$GATA2_02	98 (+)	1.000	0.919	ctgGATAagc
V$GATA1_06	98 (+)	1.000	0.887	ctgGATAagc
V$GATA1_05	98 (+)	1.000	0.892	ctgGATAagc
V$LMO2COM_02	99 (+)	1.000	0.927	tgGATAagc
V$GATA_C	100 (+)	1.000	0.969	gGATAAgcctg
V$NF1_Q6	102 (-)	1.000	0.875	tgcTGGCcccaggcttat
V$CP2_01	108 (-)	1.000	0.853	gctggccCCAG

Figure 5.4b Analysis of the CT-1 minimal promoter sequence (-99 to +19) for potential transcription factor binding sites using the MatInspector V2.2 based on TRANSFAC 4.0

The C/EBPβ transcription factor binding site was chosen in this study since previous studies have shown that simulated ischaemia can increase C/EBP transcription factor expression in cardiomyocytes (Yan et al., 1997). Moreover, cardioprotective effects of CT-1 are mediated via MAPKinases and C/EBPβ/NFIL-6 (Sheng et al., 1997). In addition, NFIL-6/C/EBPβ is a potent transactivator of the CRH gene in neuronal cells and lymphoblastoid cells (Stephanou et al., 1997). Brar et al (1999) have shown that simulated ischaemia increases expression of urocortin mRNA partly through increased expression of C/EBP transactivators (Brar et al., 1999). Therefore, in order to investigate whether the C/EBPβ/NF-IL6 transcription factor binding site is required, the CT-1 promoter/luciferase reported construct was transfected together with expression vector encoding the wild type C/EBPβ/NF-IL6 or a dominant negative mutant of C/EBPβ/NF-IL6 (Figure 5.5).

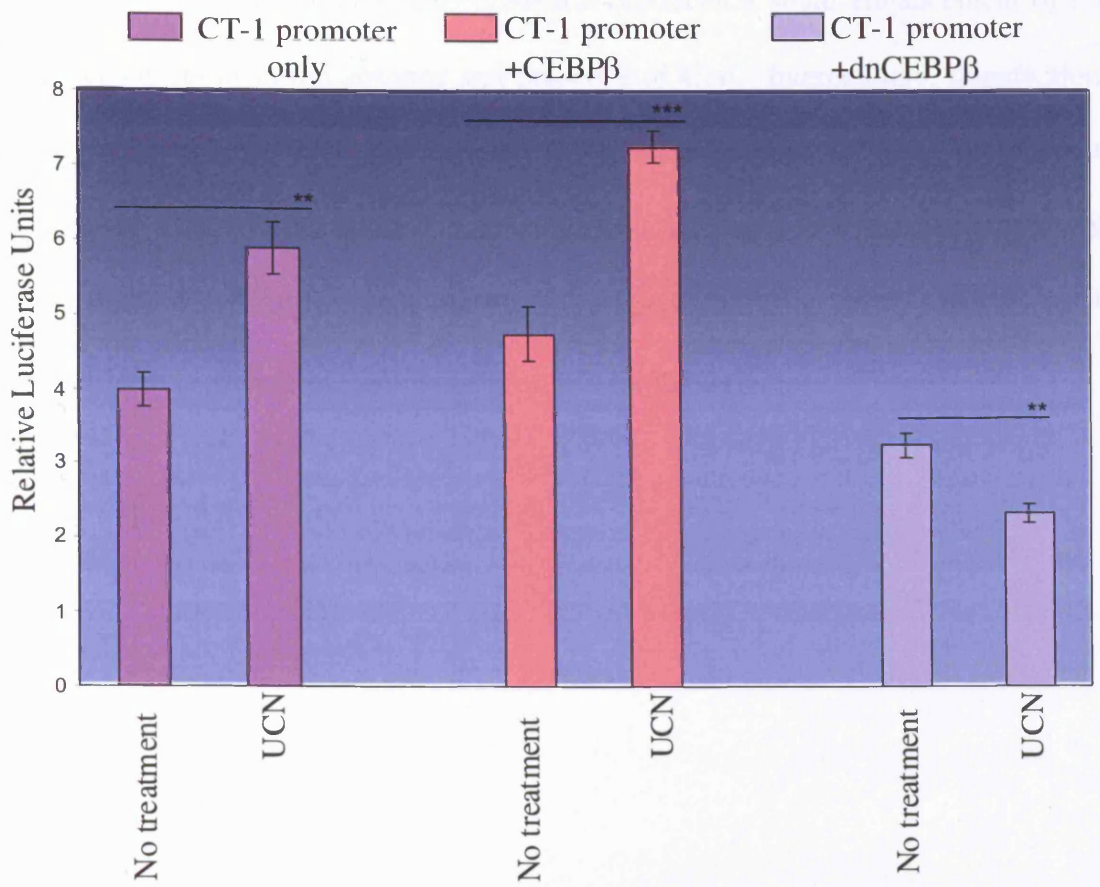


Figure 5.5 Activity of the wild type CT-1 promoter-luciferase reporter construct following co-transfection into cardiac myocytes with empty expression vector or the same vector expressing wild type or dominant negative (dn) C/EBPβ and exposure to the indicated treatment. Values are the mean of 6 determinations whose standard error is shown by the bars. Single Factor ANOVA was carried out and showed significant differences between treatment groups ($P<0.0001$). Post-hoc Bonferroni tests were carried out and significance levels compared to control are depicted as follows: *<0.05, **<0.01, ***<0.0001.

In these experiments, wild type C/EBPβ/NF-IL6 produced a small enhancement of CT-1 promoter activity both in the absence and presence of Ucn. Interestingly, transfection of dominant negative C/EBPβ/NF-IL6 reduced the basal activity of the CT-1 promoter, and its induction by Ucn. These results demonstrate that inhibition of C/EBPβ/NF-IL6 with a dominant negative C/EBPβ mutant can block the response of the CT-1 promoter to Ucn, and that C/EBPβ is therefore likely to be required for this effect.

5.5 Inactivation of the C/EBPβ/NF-IL6 transcription factor binding site does not reduce CT-1 promoter activity in response to Ucn

Since the previous experiments demonstrated that the C/EBPβ/NF-IL6 transcription factor binding site was important for activation of the CT-1 promoter, the next set of experiments were carried out to investigate whether mutation of this binding site would reduce the activity of the CT-1 promoter in response to Ucn. Therefore, four mutations were introduced into the C/EBPβ binding site by site directed mutagenesis (figure 5.6), and this promoter construct was co-transfected into cardiac myocytes with C/EBPβ/NF-IL6, or dominant negative C/EBPβ/NF-IL6 expression vectors in the presence or absence of Ucn (figure 5.7).

-99 **ctgaactatg attggccgag cccgagccac gcccctagcc ctttcccccc ttttcccccc**
gacttgatac taaccggctc gggctcggtg cggggatcgg gaaaggggga aaaaggggga

gg cc
ttttcccccc ccccteetcc tccccccggag ggg*tgtgttg aggaacctgg*
aaaaggggga ggggaggagg agggggcc tc ccc*acacaac tcctt g gacc*

ataagcctgg **ggccagcatg ag +19**
***tat tcgg*acc ccggtcgtac tc**

Figure 5.6 Sequence of CT-1 -99 to + 19 promoter region showing the C/EBPβ transcription factor binding site (italics) and mutations (red)

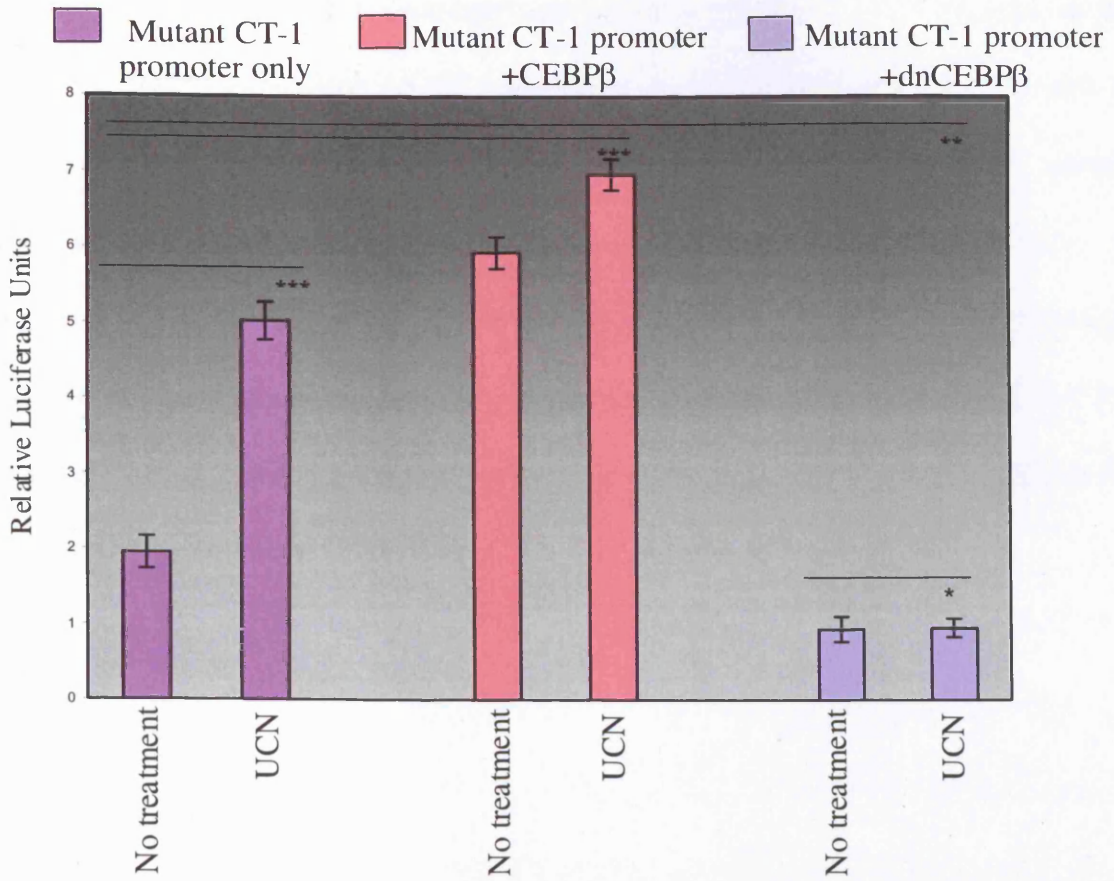


Figure 5.7 Activity of a mutant CT-1 promoter-luciferase reporter construct following co-transfection into cardiac myocytes with empty expression vector or the same vector expressing wild type or dominant negative (dn) C/EBPβ and exposure to the indicated treatment. Values are the mean of 6 determinations whose standard error is shown by the bars. Single Factor ANOVA was carried out and showed significant differences between treatment groups ($P<0.0001$). Post-hoc Bonferroni tests were carried out and significance levels compared to control are depicted as follows: *<0.05, **<0.01, ***<0.0001.

Surprisingly, this mutant CT-1 promoter was still able to respond to Ucn even in the absence of C/EBPβ expression vector. Moreover, the CT-1 promoter was still able to respond to Ucn in the presence of C/EBPβ. However, the response of this mutant promoter to Ucn could still be inhibited by dominant negative C/EBPβ/NF-IL6. In addition, the mutant promoter was able to strongly respond to functional C/EBPβ even in the absence of Ucn. These results demonstrate that C/EBPβ/NF-IL6 is involved in CT-1 promoter activation, but the potential binding site in the promoter is not essential for the response to Ucn or to C/EBPβ/NF-IL6.

5.6 Discussion

This study was carried out to investigate the induction of CT-1 expression by the cardioprotective agent Ucn. Findings from this study indicate that Ucn, acting via the C/EBPβ transcription factor, is able to activate the CT-1 promoter resulting in enhanced CT-1 mRNA and CT-1 protein levels. In addition, C/EBPβ is important in the response of the CT-1 promoter to Ucn because blocking its activity on both the wild-type and mutant promoters blocks Ucn induction, while its over-expression induces the promoter.

It is likely, therefore, that other binding sites for C/EBPβ must exist in the minimal promoter in addition to the single C/EBPβ site that was mutated because mutation of this site does not abolish the responsiveness to either Ucn or C/EBPβ. Indeed, the stronger activation of the mutant compared to the wild type promoter by Ucn suggests that the single site that was mutated may have an inhibitory effect rather than an activating effect.

Ucn may mediate its effect on cardioprotection, at least in part, via inducing the synthesis of the cardioprotective CT-1 protein. This would explain the similar signaling pathways that are involved in cardioprotection mediated by Ucn and CT-1 despite the distinct protein families to which they belong (Brar et al, 2001; Liao et al, 2002; Brar et al, 2000,Sheng et al., 1997;Brar et al., 2001). Similarly, the need to induce CT-1 synthesis would explain why Ucn requires protein synthesis for its cardioprotective effect (Brar et al., 2002). In contrast, CT-1 itself may act by inducing, for example, post-translational modifications of other proteins, therefore explaining why new protein synthesis is not necessary for its cardioprotective effect.

It should be noted however, that we have also identified a number of other genes whose expression is induced by Ucn (Lawrence et al., 2002) and some of these are likely to also play a role in its protective effects, which are not therefore likely to be mediated exclusively by CT-1. Interestingly, one of the induced genes is that for the epsilon isoform of PKC and this enzyme is also activated by Ucn. It is possible therefore that PKCε or other Ucn-activated kinases may modify C/EBPβ and thereby activate CT-1 gene expression. Clearly, it would be of interest to determine the effect on the protective effect of Ucn, of specifically blocking activation of CT-1 by Ucn. The mechanisms discussed here were not investigated further due to time limitations to finish the project.

As well as responding to Ucn, the data presented here also indicates that the CT-1 is activated in cardiac cells exposed to simulated hypoxia/reoxygenation resulting in enhanced mRNA and protein levels. α-helical CRH is able to block the effect of simulated hypoxia on the CT-1 promoter. It is possible therefore, that Ucn also plays a role in this effect.

Thus, previously it has been demonstrated that Ucn is released from cardiac cells exposed to simulated hypoxia (Okosi et al., 1998; Brar et al., 1999). Hence, endogenous Ucn released in response to simulated hypoxia could be responsible for the effect of simulated hypoxia on the CT-1 promoter and CT-1 mRNA/protein levels.

Although further studies will be required to determine whether Ucn is indeed involved in the response of CT-1 to simulated hypoxia, it is clear that Ucn treatment is able to directly

activate the CT-1 promoter in cardiac myocytes resulting in enhanced mRNA and protein levels of the cardioprotective agent CT-1.

CHAPTER 6.

DISCUSSION

CHAPTER 6. DISCUSSION

The aim of this thesis was to investigate the regulation of gene expression and survival in cellular stress. The results presented in Chapter 3 show that the C-terminal domain of the STAT-1 transcription factor is required for apoptosis induced by lethal stresses including heat shock and simulated ischemia.

In this study, lethal heat stress or ischemic stress caused significant cell death, with the effects being more severe after ischemic stress. Parental 2fTGH cells were more sensitive compared to their mutant counterpart U3A cells. Re-introduction of STAT-1 into the U3A cell line confirmed that STAT-1 was causing the sensitivity to stress since STAT-1 transfected U3A cells became more sensitive to stress compared to untransfected U3A cells. This effect is in agreement with other work in our laboratory (Stephanou et al., 2002).

The U3A cell line was ideal to study effects of STAT-1 since no functional STAT-1 was present in this cell line. Thus, comparison of STAT-1 with STAT-3 showed that indeed the enhanced apoptotic effects that were observed were due to the presence of STAT-1. STAT-3 transfected U3A cells were more resistant to stress, suggesting that STAT-3 has an anti-apoptotic function compared to STAT-1 (Bromberg et al., 1999; Ram et al., 2000). Effects of chimeras containing different regions of STAT-1 and STAT-3 indicated that the N-terminal domain of STAT-1 was dispensable for the apoptotic effects observed. Other studies have shown that the N-terminal domain is required for interactions with other proteins (Zhang et al., 1996); however, in this case, stress-induced apoptosis does not

require an N-terminal domain. The DNA binding domain of STAT-1 was also dispensable for the apoptotic effects observed as demonstrated using STAT-1/STAT-3 chimeras. Moreover, this is the first demonstration that even the isolated C-terminal domain of STAT-1 can cause apoptosis in the absence of the DNA binding domain.

STAT-1 is known to interact with other regulatory proteins such as CBP, BRCA1, and the chromatin remodelling factor MCM5 (Zhang et al., 1996; Ouchi et al., 2000; Zhang et al 1998). Moreover, serine phosphorylation of STAT-1 has been shown to be critical for its ability to interact with such molecules (Da Fonesca et al., 2001; Ouchi et al., 2000) and it is possible that the C-terminal domain of STAT-1 requires interaction with other pro-apoptotic regulatory factors to mediate its apoptotic effects.

Recent studies have shown that STAT-1 is cleaved at position 694 by caspase 3, leading to release of the C-terminal fragment of STAT-1, which may be required for down-regulating cellular responses to cell death (King and Goodbourn, 1998). However, in this thesis, a mutation at the caspase cleavage site of STAT-1 did not have any preventative effect on apoptosis, and would suggest that cleavage of STAT-1 to release a free C-terminal fragment is not essential for stress-induced apoptosis and hence the C-terminal domain can produce apoptotic effects either within an intact molecule or as an isolated domain produced artificially or naturally by caspase cleavage in the cell type studied.

IFNγ or ischemic treatment of cardiac cells leads to induction of caspase-1 and fas/fasL gene expression, and this is dependent upon STAT-1 activation. Also, over expression of STAT-1 inhibits expression of antiapoptotic BCl2 and BCLX, whereas STAT-3 enhances expression of these anti-apoptotic genes (Stephanou et al., 2000a). It is possible that if pro-apoptotic signals outweigh anti-apoptotic signals, then STAT-1 may intervene to make sure that the cell undergoes apoptosis, and also to make sure that the commitment phase of apoptosis is irreversible.

In past studies it has been shown that IFNγ activated apoptosis and caspase activation is dependent upon STAT-1and is defective in STAT-1 deficient U3A cells (Chin et al., 1997). It is possible that a full length STAT-1 molecule is involved in upstream caspase activation, resulting in cleavage of STAT-1 at the same time as caspase cleavage. Cleavage of STAT-1 results in the release of the C-terminal trans-activating domain (TAD) and also the N-terminal domain containing the DNA binding domain. Release of the STAT-1 TAD could lead to its interaction with other factors containing a DNA binding domain required for activation of pro-apoptotic genes and perpetuation and amplification of the apoptotic signal. Such an explanation is plausible, since STAT-1 is known to have its potent apoptotic effect in later stages of apoptosis in cardiac cells exposed to ischaemia/reperfusion (Stephanou et al., 2002). The remaining N-terminal domain could behave as a negative regulator of full length STAT-1 or even anti-apoptotic genes or survival pathways that may be down-regulated during apoptosis (Figure 6.1).

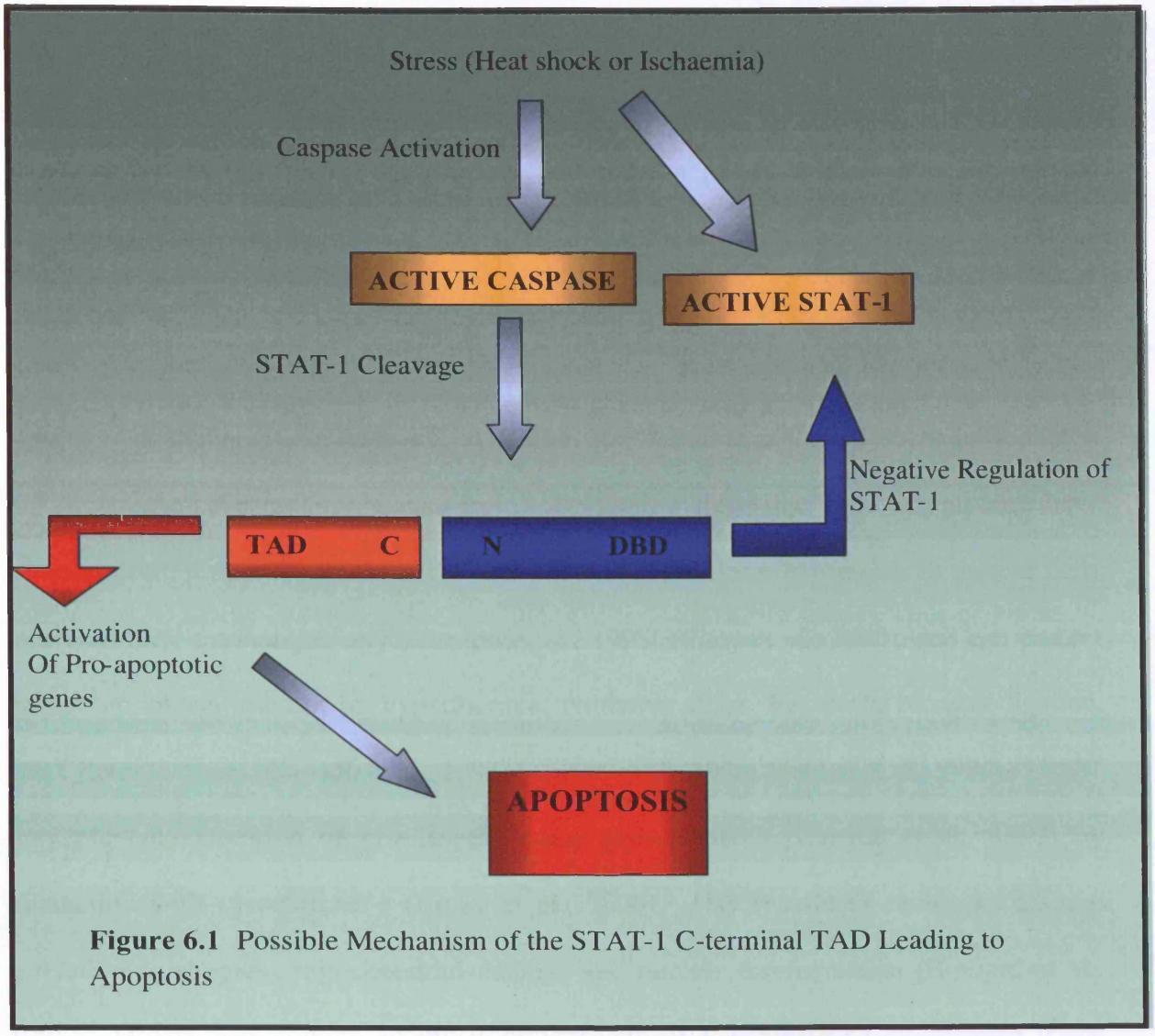


Figure 6.1 Possible Mechanism of the STAT-1 C-terminal TAD Leading to Apoptosis

It is likely that STAT-1 exerts its effects at various levels of the apoptotic pathway to ensure that cell survival is not possible. In a recent study by Townsend (2003) STAT-1 has also been shown to interact with the important tumour suppressor p53. Interestingly, this interaction requires the C-terminal domain of STAT-1, which is critical for its apoptotic effect in response to stress (Townsend et al., 2003). Therefore, STAT-1 behaves as a negative regulator of cell proliferation and displays tumour suppressor qualities like p53.

The results presented in chapter 3 therefore, show a novel mechanism for stress-induced apoptosis by STAT-1, and demonstrates that the C-terminal domain is sufficient and necessary for the effects observed.

Hsps are required for cell protection against stress, and predominantly act as negative regulators of the apoptotic pathway. Although Hsp27 and Hsp70 have not been studied in this thesis, the information obtained from other studies show that these two proteins have anti-apoptotic properties in the cell death pathway, since over expression in tumour cells increases their tumourigenicity (Garrido et al., 1998l Bruey et al., 2000), and can protect against apoptosis induced by hyperthermia, oxidative stress, Fas death receptor ligation, and cytotoxic drugs (Garrido et al., 1998; Garrido et al., 1996; Mehlen et al., 1996). Hsp27 may prevent activation of pro-caspase-9 and pro-caspase-3 (Garrido et al., 1997) by interaction with cytochrome c (Bruey et al., 2000). Hsp70 reduces or blocks caspase activation to suppress mitochondrial damage and nuclear fragmentation (Buzzard et al., 1998). Hsp70 also inhibits apoptosis downstream of the release of cytochrome c and upstream of caspase-3 activation (Li et al., 2000). In addition, Hsp70 also prevents recruitment of procaspase- 9 to the apoptosome by binding directly to APAF-1 (Saleh et al., 2000; Beere et al., 2000).

Hsp90 appears to display pro-apoptotic as well as anti-apoptotic characteristics. Over - expression of Hsp90 in U937 cells increases the rate of apoptosis upon TNFα induction (Galea-Lauri et al., 1996). However, Hsp90 can directly bind to APAF-1 to inhibit its

oligomerisation and further recruitment of procaspase-9 (Pandey et al., 2000). Therefore, it appears that the role of Hsp90 in the apoptotic pathway could be dependent on the type of stimulus that the cell is subjected to. In this thesis it seems that if STAT-1 is activated then the likely path that Hsp90 will activate would be that of a pro-apoptotic nature rather than an anti-apoptotic one. It is possible that the STAT-1/HSF-1interaction observed by Stephanou et al. (1999) could be required for this effect. If this is the case, then it would be interesting to know whether the C-terminal construct of STAT-1 used in chapter 3 (691-750) could also activate Hsp90 promoter activity.

Previous work in our laboratory has shown that cytokines as well as stress can induce expression of Hsps. For example, IL-6 can induce expression of HSp90β in hepatoma cells and also in peripheral blood mononuclear cells (Stephanou et al., 1997; Stephanou et al., 1998). IL-6 is a multifunctional cytokine with pleiotropic activities on various cell types (Akira et al., 1992). This property of IL-6 is dependent upon the IL-6 receptor and the receptor subunit gp130 that is shared among other cytokine receptors belonging to the IL-6 receptor super family (LIF, IL-11, OM, CT-1) (Kishimoto et al., 1995).IL-6 stimulation leads to activation of STAT-3 and also C/EBPβ (Stephanou et al., 1998). STAT-3 and C/EBPβ have been shown to interact differentially with HSF-1 (Stephanou et al., 1998).

IFNγ has antiviral and antitumour properties by inducing specific INF-γ dependent genes, one of which is Hsp90β (Darnell et al.,, 1994; Schindler and Darnell, 1994). IFN-γ induces expression of Hsp90β directly via activation of STAT-1 (Stephanou et al., 1999).

Interestingly, in this study STAT-1 and HSF-1 were found to interact directly via protein-protein interaction (Stephanou et al., 1999). Therefore, the effects of IFNγ and also IFNα on the Hsp90β promoter were investigated in chapter 4.

IFNγ alone can activate Hsp90β gene expression, but in the presence of heat shock, Hsp90β promoter activity is enhanced further. IFNα was also able to activate the Hsp90β promoter but the effect was much lower compared to IFNγ. These results suggest that although differences in activation of STATs are apparent, both IFNs are able to activate the Hsp90β promoter directly with the effect of IFNγ being more potent than IFNα and that in some cell types the pattern of Hsp90β expression is different to other cell types. Hypothetically, STAT-1/STAT-2 dimers activated by IFNα may have a negative effect on the Hsp90β promoter in the presence of IFNγ in HepG2 cells. It has been demonstrated in other studies that STAT-2 contains a highly potent transcriptional activation domain at the C-terminal region which could lead to STAT-2 being more dominant over STAT-1 in interferon-a-induced interferon stimulated gene factor-3 (ISGF-3) mediated antiviral and antiproliferative effects of IFNα (Bromberg et al., 1996; Horvath and Darnell, 1996). Therefore it is possible that for this reason, STAT-1/STAT-2 dimers predominantly reduce activity of Hsp90β by competing for STAT binding sites within Hsp90β promoter, and also interaction with HSF-1. It would be interesting to investigate the heterodimer interaction with HSF-1.

Both IFNα and IFNγ are multifunctional and elicit many biological responses from target cells, and are also capable of inducing many unique responses (Stark et al., 1998). The

IFNs display overlapping pathways leading to transcriptional regulation of different sets of genes with overlap of functions (Der et al., 1998). Further work would need to be carried out to investigate similar responses with other Hsps.

Other cytokines and hormones have been shown to induce Hsps and also activate STATs for their effects on cells. CT-1, a member of the IL-6 family of cytokines, induces Hsp70 and Hsp90 in cardiac cells (Latchman, 1998). Hypertrophic effects of CT-1 are mediated via activation of STAT-3 (Railson et al., 2002). CT-1 protection is achieved via the p42/p44 MAPKinase pathway, and this has also been observed for Ucn, a peptide hormone which mediates the endocrine response to stress (Sheng et al., 1997; Brar et al., 2000; Chen et al.,1993).

The work presented in chapter 5 showed that the cardioprotective agent Ucn was able to induce expression of another cardioprotective agent CT-1. Such studies are of considerable importance in understanding protective mechanisms that are activated due to hypoxic stress followed by reperfusion and an ultimate modulation for therapeutic benefit.

CT-1 and Ucn have been previously identified to be protective agents and can protect the heart against damaging effects of cardiac ischemia/reperfusion (Brar et al., 2001; Liao et al., 2002; Brar et al., 2000). Both agents protect by activating the p42/p44 MAPK pathway. Dominant negative mutants of the p42/p44 enzymes inhibit the protective effects by these two proteins (Sheng et al., 1997).

Although CT-1 and Ucn belong to different protein families, in this chapter, the results demonstrated that CT-1 expression was induced by Ucn. Time course experiments of CT-1 expression in Ucn treated cells showed that CT-1 expression was more apparent at 16 hours-24 hours. This suggests that CT-1 expression is not instant and rapid and it is probable that at this time point CT-1 is more important in exerting its protective effects.

The results in chapter 5 also indicate that CT-1 is activated in cardiac cells exposed to hypoxia/reoxygenation, resulting in enhanced CT-1 mRNA and protein levels. It is possible; however, that Ucn plays a role in mediating this effect. Previous studies in our laboratory have demonstrated that Ucn is released from cardiac cells exposed to hypoxia (Okosi et al., 1998; Brar et al., 1999). Endogenous Ucn released in response to hypoxia could be responsible for the effect of hypoxia on the CT-1 promoter and CT-1 mRNA and protein levels. It is clear that Ucn treatment is able to directly activate the CT-1 promoter, resulting in enhanced mRNA and protein levels of cardioprotective CT-1. Further studies would require determining whether Ucn is involved in the response to hypoxia since exposure of cells to hypoxia/reoxygenation leads to enhanced CT-1 levels which could be blocked by α helical CRH. Furthermore, α helical CRH also blocked enhanced CT-1 levels in Ucn treated cells under hypoxic conditions (Okosi et al., 1998; Brar et al., 1999).

An enhanced level of CT-1 was also detected in the media of Ucn and hypoxia treated cells, suggesting that Ucn enhances mRNA levels and protein levels of CT-1. To our knowledge, this is a novel effect by which Ucn is able to induce expression of CT-1. Ucn also has a direct effect on the CT-1 promoter rather than stabilising CT-1 mRNA. In

addition, Ucn induced CT-1 expression is mediated, in part, by C/EBPβ. C/EBPβ is known to be activated by CT-1 and other members of the IL-6 family of cytokines (Nakajima et al., 1993). Dominant negative C/EBPβ has a negative effect on the CT-1 promoter, suggesting that the effect of Ucn on the CT-1 promoter requires activation of C/EBPβ.

Mutation of the C/EBPβ site does not block activation of CT-1 by Ucn therefore, is likely that other transcription factor binding sites must exist in the minimal CT-1 promoter that are required for the effect observed. Cardio-protective effects of Ucn may be mediated by inducing synthesis of CT-1, which would explain the similar signalling pathways involved in cardio-protection by these different factors (Sheng et al., 1997; Brar et al., 2001). Induction of CT-1 by Ucn could lead to induction of other cell protective proteins; for example, Ucn can increase expression of Hsp90 protein. Synthesis required for protection of cardiac cells by Ucn, which could be due to a need to induce CT-1 protein (Brar et al., 2002). Interestingly, Ucn also induces expression of the K_{ATP} channel gene to mediate its cardioprotective effect (Lawrence et al., 2002). The gene for PKCε, also activated by Ucn, could modify C/EBPβ and activate CT-1 expression. It would be interesting to determine whether blocking PKCε can block the effect of Ucn on CT-1.

The work presented in this thesis opens up new and interesting avenues for research. In particular, an investigation of the interaction of other proteins with STAT-1 could be of interest since the mechanism could be targeted for therapeutic purposes. The effect of the

C-terminal domain of STAT-1 presented here highlights a potentially important role for this protein in understanding the mechanism of apoptotic cell death.

6.1 Future Work

The work presented here opens up many questions about the events that take place when cells are exposed to stress. In chapter 3, the role of the STAT-1 transcription factor in stress-induced apoptosis led to the conclusion that other factors that contain DNA binding domains are likely to be involved in apoptotic processes that require the C-terminal activation domain of STAT-1. With these factors acting by recruiting STAT-1 to the DNA the next step in this project is to unravel which factors may interact with STAT-1 in a pro-apoptotic manner, and can be achieved by mapping the candidate promoter region needed for a STAT-1 response and ideally the DNA binding function. Protein-protein interaction studies (in vitro) can also be carried out to see whether STAT-1 interacts with candidate pro-apoptotic transcription factors.

In the IFN study, there was no conclusive evidence that STAT-1 was required for Hsp90 induction, and this could not be studied further due to time restrictions. However, further experiments would involve transfection studies ie, to re-introduce STAT-1 into the U3A cell line (which lacks functional STAT-1) with Hsp90 promoter constructs and subject transfected cells to IFN and heat shock treatments as carried out in this thesis. In addition, the effect at the protein level in these cells would be tested by Western blotting. It would also be of importance to extend the study to other Hsps including Hsp70, Hsp47 and Hsp27 as this has not been tested so far. The interaction of the STAT-1/STAT-2 heterodimer with HSF-1 needs to be investigated, and can be achieved by protein protein interaction in vitro.

Further experiments for Ucn induced CT-1 activity would also be required. This thesis demonstrated that other transcription factor binding sites are present in the minimal CT-1 promoter as well as the binding site for C/EBPβ. It would be necessary to further investigate the C/EBPβ transcription factor binding site within the CT-1 minimal promoter and to test whether C/EBPβ binds elsewhere in the promoter. In this thesis only four mutations were made in this site (2x thymine to guanine, and 2x adenine to cytosine). Therefore, further site directed mutagenesis of this binding site is required. In addition, it would be of importance to also mutate amino acid residues of other transcription factor binding sites in the CT-1 promoter by site directed mutagenesis to determine whether they are also involved in activating the CT-1 promoter in response to Ucn.

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