ONCOSTATIN M REGULATION OF THE TISSUE INHIBITOR OF MATRIX
METALLOPROTEINASES-1 PROMOTER

By

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ABSTRACT

The progression of an inflammatory response is largely dictated by soluble factors termed cytokines reknown for their redundant and pleiotropic nature in modulation of both immune and stromal cells. Individual members of the interleukin-6 (IL-6)-type cytokine family possess both unique and shared biological activities. These cytokines may participate in tissue remodelling by promoting reconstruction of extracellular matrix (ECM) following nonspecific tissue damage by inflammatory cells. Consistent with this view, these cytokines upregulate expression of an ECM protease inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP-1) and thus may alter net enzymatic degradation of ECM. The overall goal of this thesis is to examine mechanisms by which TIMP-1 is regulated by IL-6-type cytokines, especially by the cytokine oncostatin M (OSM). The promoter of TIMP-1 has therefore been studied in detail to address the mechanisms by which OSM (and IL-6 type cytokines) regulate the transcription of the TIMP-1 gene. The approaches undertaken have included deletion analysis of the TIMP-1 gene nucleotide sequences proximal to the start of transcription to define DNA sequences necessary/sufficient for cytokine-induced TIMP-1 promoter activity. In addition, the binding of nuclear factors to these DNA elements and cytokine-response elements, their expression and involvement in the regulation of TIMP-1 transcription have

been explored.

We have identified sequences proximal to the start of TIMP-1 transcription that are necessary for maximal responsiveness to OSM and IL-6. Deletion analysis of the proximal TIMP-1 promoter (-95 to +47 TIMP-1 sequences) has identified a nucleotide sequence within -59 to -53 of the murine TIMP-1 promoter that harbours an AP-1 consensus DNA binding element. This element is necessary for maximal OSM or IL-6 induced promoter activity of TIMP1-CAT reporter gene constructs transfected into human hepatoma HepG2 cells. OSM is the most potent stimulus (approx. 11-fold for OSM, and 4-fold for IL-6) of this response and additional sequences 3-prime to +1 of the TIMP-1 gene are also necessary for maximal OSM responsiveness.

Electrophoretic mobility shift assays demonstrated two gel-shifted complexes which bind the TIMP-1 AP-1 site. An AP-1 gel-shifted complex is present in the absence of cytokine stimulation ("complex 1"), while OSM and not other IL-6-type cytokines, stimulated the formation of a second AP-1 gel shifted complex. Nuclear factors binding to TIMP-1 AP-1 complex 1 include junB, junD and fos-related antigens. However, unlike complex1, c-fos is present and necessary for the formation of the OSM-induced TIMP-1 AP-1 complex2. Consistent with this, OSM is a potent inducer of c-fos protein expression among IL-6-type cytokines. Both the formation of complex2 and c-fos expression require new protein synthesis. JunB and junD are constitutively expressed, while the expression of fos-related antigens

are induced in response to OSM. In addition, although PMA was also a potent inducer of c-fos expression, induction of TIMP-1 promoter activity by the combination of PMA and IL-6 was comparable to IL-6 alone and did not equal the significantly higher induction by OSM. Within the same cells, OSM and IL-6 equally induced STAT DNA-binding activity. An Ets-consensus site (nucleotides -45 to -40) flanking the 3-prime end of the AP-1 site is a weak binding site for Ets-related nuclear factors, and an SP-1 site near +1 (-11 to -6) is a strong binding site for SP1 nuclear factors and related SP-1 site binding proteins. No STAT nuclear factor binding to the proximal TIMP-1 promoter was detected. Taken together, the TIMP-1 AP-1 site and c-fos represent a unique target of OSM signalling and activation of AP-1 complexes (possibly containing c-fos) by OSM as well as sequences downstream of TIMP-1 +1 contribute to maximal responsiveness of the promoter to this cytokine among IL-6 family members.

The contribution of c-fos to OSM-induced TIMP-1 expression was further explored in murine cells. OSM stimulates the expression of c-fos and activates STATs 1, 3 and 5 DNA-binding activity in murine fibroblasts. As observed in human cells, OSM but not other IL-6-type cytokines upregulated c-fos expression which participated in complexes binding the TIMP-1 AP-1 site. OSM was also unique among IL-6 family members in activating STAT5 DNA-binding activity in murine fibroblasts. However, in contrast to observations in human HepG2 cells, deletion analysis of the TIMP-1 promoter showed that the AP-1 site (-59/-53) was

not necessary for OSM-mediated upregulation of the TIMP-1 proximal promoter activity over basal levels in murine NIH3T3 fibroblasts and co-transfection of a dominant-negative of AP-1 had no effect. However, transfection of dominant-negative STATs 1, 3 or 5 (especially STAT3) could diminish cytokine-induced TIMP-1 promoter activity. In addition, c-Fos was dispensable for OSM-mediated upregulation of TIMP-1 mRNA levels as TIMP-1 expression was detected in wild-type and c-fos knockout murine lung fibroblasts. Consistent with deletion analysis of the TIMP-1 promoter in human cells, dominant-negative AP-1 expression vectors abrogated OSM-mediated TIMP-1 promoter activity, while Stat-dominant negative expression vectors did not. Taken together, the examination of murine and human systems suggests that AP-1 and STAT nuclear factors can contribute to the regulation of the TIMP-1 promoter.

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LIST OF ABBREVIATIONS

a.a.	amino acid
A-Fos	acidic amphipathic c-Fos (dominant-negative c-Fos)
AP-1	activator protein-1
CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal regulated kinase
FRK	fos-regulating kinase
hOSM	human oncostatin M
IL-	interleukin-
JAK	janus kinase
JNK	jun amino-terminal kinase
kd	kilodalton
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
mOSM	murine oncostatin M
mRNA	messanger ribonucleic acid
OSM or OM	oncostatin M
PCR	polymerase chain reaction
RNA	ribonucleic acid
SH2	src-homology 2
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
STAT1d	dominant-negative STAT1
STAT3d	dominant-negative STAT3
STAT5d	dominant-negative STAT5
TIMP	tissue inhibitor of matrix metalloproteinases

CHAPTER 1

GENERAL INTRODUCTION

1.0 Cytokines and Inflammation

Cytokines are small molecular weight secreted proteins with growth/differentiation factor activity and function as modulators of immunity and inflammation. Their presence within the local microenviroment directs a spectrum of biological activities that communicate cellular and humoral responses and manifest the progression/resolution of immuno-inflammatory reactions.

Inflammation can be defined as the body's response to tissue injury (reviewed by Janeway and Travers, 1997). A number of factors precipitate in this response including microbial infections, physical and chemical agents (such as burns and toxins, respectively), necrotic tissue and many types of immunological reactions. The hallmarks of inflammation are swelling, pain, redness and fever. These effects result from vasodilation and increased vascular permeability and facilitate the infiltration of effector cells (leukocytes) and plasma proteins to sites of tissue injury. These events can be regulated by the secretion of cytokines from resident stromal cells (eg. endothelial cells, fibroblasts) as well as infiltrating leukocytes (monocytes and neutrophils). Monocytes and neutrophils digest invading microbes, secrete cytokines and stimulate the adaptive immune response.

Cytokines mediate a variety of effects for coordinating the inflammatory response and the course of tissue repair. Key inflammatory cytokines include interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-alpha), chemokines, interferons (IFNs) and interleukin-6 (IL-6). The majority of effects observed are initiated by the cytokines IL-1 and TNF-alpha. The primary cellular source of IL-1 and TNF is activated macrophages, although resident stromal cells such as endothelial cells and fibroblasts also produce these cytokines. IL-1 and TNF-alpha increase the adhesion of leukocytes to the endothelium, induce fever, stimulate intravascular coagulation and activate leukocytes to secrete chemokines. Chemokines, such as IL-8, are chemoattractant cytokines which promote the recruitment of peripheral blood mononuclear and polymorphonuclear cells to the site of inflammation. IL-1 and TNF also induce the expression of extracellular matrix (ECM) proteases, such as collagenases, which play a role in tissue remodelling during early stages of wound healing. The type I interferons (IFNs), IFN-alpha and IFN-beta, activate natural killer cells and potently suppress the replication of viruses in infected cells.

Resolution of the inflammatory response is paramount to recovery from tissue injury and re-establishing homeostasis. IL-6 is central to this process and is secreted by mononuclear phagocytes and endothelial cells in response to IL-1 or TNF. IL-6 is a potent stimulator of acute phase plasma protein synthesis by liver cells. Many of these acute phase proteins are protease inhibitors and function to

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downregulate proteases during the end stages of tissue repair and ECM remodelling. Thus, cytokines within the local microenviroment function together to coordinate the inflammatory response and are key to ECM remodelling and tissue repair.

1.1 Tissue Inhibitors of Matrix Metalloproteinases and Extracellular Matrix metabolism.

The regulation of extracellular matrix (ECM) catabolism is dependent upon the balance of matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs), within the microenviroment. The extent to which each of these is actively expressed (i.e, the MMPs versus TIMPs) will dictate whether tissue destruction or reconstruction occurs under conditions of tissue remodelling. Delineating the underlying molecular mechanisms of ECM metabolism, may lead not only to a better understanding of normal physiological processes such as embryonic development and wound healing, but also to therapeutic strategies for combating pathological ECM metabolism such as chronic inflammatory fibrosis, tissue destruction and metastasis.

1.1.1 The Matrix Metalloproteinases

The matrix metalloproteinases comprise a family of proteases that function to degrade components of the extracellular matrix (ECM) and participate in normal remodelling of tissues as well as contribute to the pathologies noted above. The MMPs have been categorized into several classes based on their relative specificity for different ECM components. These include collagenases, gelatinases, stromelysins, membrane-type MMPs and adamalysins (reviewed in Woessner, 1994; Killar et al., 1999). Collagenases digest type I, II and III interstitial collagens, while stromelysins degrade type IV collagen better than gelatinases. MMPs bind zinc within their catalytic site, and are secreted as proenzymes which require specific amino-terminal cleavage events to become proteolytically active (Woessner, 1994). Inflammatory cytokines such as IL-1 and TNF upregulate the expression of MMPs, and the activity of MMPs is largely dictated by the presence of TIMPs within the microenviroment (Takahashi et.al., 1993; Ito et al., 1990).

1.1.2 The Tissue Inhibitors of Matrix Metalloproteinases

Inhibitors of these extracellular zinc-dependent endopeptidases (the MMPs) can be grouped into a family of several tissue inhibitors of metalloproteinases (TIMPs). These include TIMP-1, -2, -3 and -4 and act to modulate extracellular matrix metabolism by the MMPs (Docherty et al., 1985; Stetler-Stevenson et al., 1990; Leco et al., 1994; Greene et al., 1996). All TIMPs share several structural

features (Gomez et al., 1997). They are all single polypeptide chains with 12 cysteine residues within conserved regions of the molecule that form 6 disulfide bonds. Amino-terminal a.a. sequences are essential for binding and inhibiting MMPs. Also, each is synthesized as a precursor protein from which a 29 amino acid leader sequence is cleaved to yield the mature form. All TIMPs are small molecular weight secreted proteins (20-34kilodaltons) that inactivate MMPs through non-covalent binding to either the catalytically active forms of MMPs or in some instances, to the precursor forms. Although, TIMPs appear to inhibit all MMPs, TIMP-1 and -2 display separate affinities for collagenase, gelatinase and stromelysin MMPs.

Originally described as human collagenase inhibitor (HCI) (Stricklin et al., 1983), TIMP-1 remains the best characterized of all TIMPs (being the first described) and has served as a model for the characterization and comparison with additional members of the TIMP family. TIMP-1 was first cloned from human skin fibroblasts (Carmichael et al., 1986) and is predominantly expressed in connective tissues, such as, fibroblasts and osteoclasts, as well as other tissues of mesodermal origin such as smooth muscle cells (Welgus et al., 1983). TIMP-1 is a 29kD secreted polypeptide, stericly inhibiting MMPs through binding in a 1:1 ratio (Stricklin et al., 1983). The tertiary structure of the human TIMP-1 molecule can be dissected into six loops held together by six disulfide bonds arranged into three knot-like structures (Bodden et al., 1994). The region encompassing the second disulfide

knot which requires cysteines13 and 124, and cysteines 127 and 174, is necessary for TIMP-1 inhibition of fibroblast-derived collagenase (MMP-1) activity (Bodden et al., 1994). Mutants of TIMP-1 which retain the amino-terminal three loops (Murphy et al., 1991; O'Shea et al., 1992) or tryptic peptides of TIMP-1 lacking 54 carboxy-terminal amino acids (Williamson et al.,1993) effectively inhibit MMP activity. However, the three N-terminal loops were not sufficient for forming a stable complex with the precursor form of the MMP 92 kDa gelatinase (Murphy et al., 1991). The TIMP-1 protein also harbors at least two glycosylation sites (Carmichael et al., 1986) and as a result, its molecular weight ranges from 20kilodaltons (unglycosylated) to 28.5 kDa (glycosylated form), although glycosylation is not necessary for MMP inhibitory activity as E.coli derived recombinant TIMP-1 (which lacks glycosylation) is sufficient for inhibiting MMP activity (Schultz et al., 1988).

In addition to binding directly to the active forms of MMPs, TIMP-1 has been demonstrated to interact with the proform of the MMP 92 kDa gelatinase (MMP-9) (Goldger et al., 1992). Interaction of TIMP-1 with MMP-9 leads to the formation of a stable ternary complex with the MMP interstitial collagenase and thus prevent the proteolytic activation of either MMP-9 or interstitial collagenase. TIMP-2 parallels TIMP-1 in its capacity to form a complex with the proform of MMP 72 kDa gelatinase, leading to a stable complex that can interact with additional gelatinases and collagenases, inhibiting their activity (Kolkenbrock et al., 1991).

1.1.3 ECM remodeling by TIMPs

The MMPs and TIMPs are instrumental in the destruction and remodelling of the extracellular matrix under normal physiological conditions and in precipitating pathological circumstances. Expression of TIMPs have been proposed to coordinate tissue remodelling during embryonic development. MMPs and TIMPs appear highly expressed during blastocyst implantation and TIMPs are observed to retard decidual remodelling and growth (Alexander et al., 1996). Other normal developmental and physiological repair processes that have been described for TIMPs include organization of the basement membrane for mammary gland development (Talhouk et al., 1992) and ECM maintenance during peripheral nerve repair (Lafleur et al., 1996).

The imbalance between MMPs and TIMPs also appears to contribute to the pathology of connective tissue diseases, such as arthritis. Cartilage joint tissue that is subject to elevated levels of MMP expression without equivalent changes in TIMP expression succumb to osteoarthritis (Dean et al., 1989) and rheumatoid arthritis (McCachren, 1991). Increased expression of MMPs in the absence of TIMP expression in atherosclerotic lesions degrade collagen of arterial walls and can exacerbate vascular disease (Vine and Powell, 1994). Thus, TIMPs may have therapeutic potential in reversing pathological destruction of tissues, as demonstrated by the inhibition of bone resorption by TIMPs (Hill et al., 1993).

The balance between MMP and TIMP expression has also been a topic of

interest in studying the invasive potential of tumors. Hicks et al.(1984) demonstrated that the invasive behaviour of tumor cells in vitro directly correlated with a 10- to 20-fold reduction in TIMP-1 expression. TIMP levels also appear diminished in metastatic tumors ex vivo, and higher levels of MMPs are present (Halaka et al., 1983). It is noteworthy however that the expression of TIMPs does not always correlate with the metastatic potential of tumor cells (Leco et al., 1995) and additional factors are likely involved.

TIMP-1 has other functions in addition to inhibition of MMPs. TIMP-1 is an erythrocyte growth factor (Gewert et al., 1987) and separate domains within TIMP-1 are responsible for its MMP inhibitory activity and its growth potentiating activity. N-terminal mutants of TIMP-1 unable to inactivate MMPs still maintained growth factor activity (Chesler et al., 1995). TIMP-2 was subsequently also demonstrated to promote erythrocyte proliferation (Hayakawa et al., 1994).

A complex of TIMP-1 and the proenzyme form of cathepsin L isolated from testis was also demonstrated to stimulate steroid hormone synthesis and thus, is a locally produced regulator of germ cell development (Boujrad et al., 1995). Finally, TIMP-1 has been demonstrated to inhibit angiogenesis (Moses et al., 1990). Moses et al. (1990) showed that TIMP-1 inhibits vascularization of cartilage by blocking capillary endothelial cell proliferation and migration. Thus, TIMP-1 is a multi-functional soluble factor best characterized in its ability to modulate the

remodelling of extracellular matrix.

TIMP-1 expression is upregulated in connective tissue cells by a variety of soluble factors and in many cases the expression of TIMPs and MMPs is inversely related. The inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF) both upregulate secreted TIMP-1 protein levels in human fibroblasts although TNF was also capable of upregulating MMP expression (Ito et al., 1990). Soluble factors, such as retinoids (Clark et al., 1987) and IL-10 (Lacraz et al., 1995) that upregulate the expression of TIMPs and downregulate MMPs favor the remodelling of the ECM rather than its catabolism. In contrast, downregulation of TIMP-1 expression and having no effect on or upregulation of MMPs, such as by dexamethasone (Clark et al., 1987; Roeb, et al., 1993), concanavalin A (Overall and Sodek, 1990) and TNF respectively, may contribute to increased metabolism of ECM.

Additional mediators have also been described to upregulate the expression of TIMP-1 include transforming growth factor (TGF)-beta (Gunther et al., 1994), lipopolysaccharide (Roeb et al., 1995), phorbol ester (Doyle et al., 1997), and by IL-6-type cytokines (Roeb, et al., 1993; Nemoto et al., 1996). Intracellular second messengers that have been described to signal TIMP-1 expression include protein kinase C and cAMP. Protein kinase C has been implicated in mediating IL-1 induction of TIMP-1 expression in cervical fibroblasts (Takahashi et al., 1993) while cAMP has been demonstrated to upregulate the expression of TIMP-1 in the

fibrosarcoma cell line HT1080 (Tanaka et al., 1995). Regulation of TIMP-1 expression is not exclusive to transcriptional control, as the expression of TIMP-1 may also be subject to post-transcriptional events. For instance, Doyle et. al. (1997) have suggested that phorbol ester-mediated de novo transcription of TIMP-1 follows increased stability of TIMP-1 mRNA in response to phorbol ester.

1.1.4 TIMP-1 Regulation by the IL-6 cytokine family

OSM and IL-6 (to a lesser extent) induce tissue inhibitor of metalloproteinases-1 (TIMP-1) mRNA expression in hepatocyte cell lines and primary fibroblasts (Richards et al., 1993). These cytokines appear to modulate inflammation by inducing acute phase protein (APP) synthesis in hepatocytes that participates in inactivating proteases during the process of tissue repair (Richards et al., 1992; Gauldie et al., 1987; Baumann and Gauldie, 1994). TIMP-1 regulation by OSM and IL-6 at local sites of inflammation would be supportive of these observations.

OSM potently induced TIMP-1 mRNA expression in the hepatoma cell line HepG2 where basal levels of TIMP-1 mRNA are much lower compared to basal levels in fibroblast cells. However, LIF and IL-11 poorly induced TIMP-1 transcription in these cells. Conversely, IL-11 stimulates TIMP-1 mRNA levels in human articular chondrocytes and synoviocytes, while IL-6 and LIF only weakly

induce TIMP-1 (Maier et al., 1993). The 2-receptor signal transducing subunit, gp130, common to the unique receptor complexes of IL-6-cytokine family members, likely accounts for their redundancy in activity (Kishimoto et al., 1994).

1.1.5 Transcriptional regulation by the TIMP-1 promoter.

Most work examining the mechanism of TIMP-1 transcription extends from studies of the murine TIMP-1 promoter. The TIMP-1 promoter contains several putative regulatory motifs. Work by Edwards et. al.(1992) has suggested that the -95 to +47 region of the murine TIMP-1 promoter is sufficient to confer serum responsiveness. The putative AP1, PEA3 and SP1 binding sites were all found to constitutively bind nuclear factors. The TIMP-1 AP1 and PEA3 elements were capable of binding their cognate recombinant proteins. Although serum treatment of murine fibroblasts only marginally increased nuclear factor binding to these sites in these studies, the AP1 site has been described as a key enhancer element for upregulating TIMP-1 transcription. Campbell et al.(1991) had previously characterized this site as a phorbol-ester and serum-responsive element that represented a putative site for binding AP1 nuclear factors. This AP1 site was also necessary for TGF-beta-mediated reponsiveness of chimeric TIMP-1 promoter/reporter gene constructs (Campell et al., 1991). Recently, this AP1 site has been shown to contribute to basal levels of transcription and bind single-stranded DNA-binding proteins (Phillips et al., 1999).

Multiple AP1 sites have also been described in the promoters of TIMP-2 and TIMP-3. The responsiveness of these elements to stimuli is largely dependent upon additional mechanisms that modulate the contribution of these sites. Evidence for this is demonstrated by the fact that TIMP-2 promoter AP-1 sites are primarily unresponsive to stimuli (Hamman et al., 1996), while TIMP-3 (and TIMP-1) AP-1 responsive elements have been identified (Wick et al., 1995). One potential mechanism for modulating AP-1 binding is through direct interaction with an adjacent Ets site binding factors in the murine TIMP-1 promoter (Logan et al., 1996).

It has been previously shown in our laboratory (unpublished) that the TIMP-1 -95/+47 promoter region is markedly responsive to OSM in a time- and dose-dependent manner as demonstrated by chloramphenicol acetyltransferase (CAT) assays using transfected human hepatoma (HepG2) cells or primary human lung fibroblasts(HLF) with CAT indicator plasmids. Responsiveness to OSM appears to be specific since this promoter fragment was poorly responsive to other growth factors and IL-6-type cytokines that were examined.

Previous experiments (C. Langdon and C. Richards, unpublished) showed that mutations within the AP1 or Ets site of the putative AP1-Ets element in the -223 to +47 TIMP-1 /CAT construct reduces OSM induction of CAT activity (a reduction by 75% and 25% of wild type for mutation within the AP1 or Ets site respectively). Thus, the AP1(and Ets) element may be involved in the mechanism by which the

CAT constructs (and the TIMP-1 gene) respond to OSM. The mechanism by which OSM activates TIMP-1 transcription may be investigated through examining signal transduction through the gp130 subunit of IL-6-type cytokine receptors and is a topic of this thesis.

1.2 The IL-6 Family of Cytokines.

1.2.1 IL-6-type Cytokines and their Receptors

The interleukin (IL)-6 family of cytokines possess both unique and shared biological activities and have been classified based on their ability to bind various receptor complexes that contain the gp130 signal transducing subunit. In addition to IL-6, cytokine family members include leukemia inhibitory factor (LIF), oncostatin M (OSM), IL-11, ciliary neurotrophic factor (CNTF) and cardiotropin-1 (CT-1) (Heinrich et al., 1998). The polypeptide primary structure of LIF displays homology with OSM, and to a lesser extent with IL-6, CNTF and granulocyte-colony stimulating factor(G-CSF), supporting both a functional and structural relationship between these cytokines (Rose and Bruce, 1991). Moreover, at least IL-6, LIF and CNTF have similar tertiary structures and each is predicted to assemble with a four-alpha-helix-bundle topology (Robinson et al., 1994; McDonald et al., 1995; Xu et al., 1997). These cytokines are small molecular weight proteins ranging in size between 20 to 45 kD, and are typical secreted factors with an amino-terminal signal peptide except for CNTF and CT-1 which are membrane bound and must be

cleaved to be released from cells (Heinrich et al., 1998). Except for CNTF, IL-6-type cytokines bind receptors consisting of type I membrane polypeptide chains, that is, having an extracellular amino-terminus and single transmembrane domain, that form multimeric complexes. The CNTF-binding receptor subunit is linked to the cell surface via a phospholipid anchor (Davis et al., 1991). The gp130 subunit, common to all IL-6-type cytokines, is essential for intracellular signalling. Cytokine-specific binding subunits are postulated to contribute unique physiological effects to individual cytokine family members (see Figure 1). However, for the IL-6-specific receptor subunit, the cytoplasmic domain is very short and biological effects have been attributed to homodimerization of gp130 subunits for the IL-6 receptor complex (Fourcin et al., 1996; Murakami et al., 1993).

The cytokines IL-6, LIF and IL-11 are expressed by cells of haematopoeitic, neuronal and connective tissue origin (reviewed by Heinrich et al., 1998). Other members are more restricted in their expression. OSM is produced by lymphoid and myeloid cells, and also detected in testis. CNTF is expressed within the nervous system and CT-1 is highly expressed by heart and skeletal muscle cells.

1.2.2 Cytokine Biology

The IL-6 family of cytokines collectively elicit a multitude of biological activities many of which are restricted by the expression of their receptors. Several

Figure 1. The Interleukin(IL)-6-type Cytokine Receptor Family

All members of this cytokine family utilize a signal transducing 2-subunit(gp130) that likely accounts for the biological activities they share. The K-subunit of each receptor complex may act to confer specific activities unique within the cytokine family. IL-6R: IL-6 receptor, LIF-R: leukemia inhibitory factor receptor, OSM-R: oncostatin M receptor, IL-11R: IL-11-receptor, CNTF-R: ciliary neurotrophic factor receptor, CT-1R: cardiotrophin-1 receptor. The IL-6R, CNTF-R and CT-1R are trimers, whereas as the LIF-R, OSM-R and IL-11R are dimers.

The IL-6-type cytokine receptor family

IL-6R   **LIF-R**   **OSM-R**   **IL-11R**   **CNTF-R**   **CT-1R**

gp130   LIF-Rα   OM-Rα   IL-11Rα   CNTF-Rα   CT-1Rα

IL-6Rα   LIF-Rα   LIF-Rα

Cytoplasm
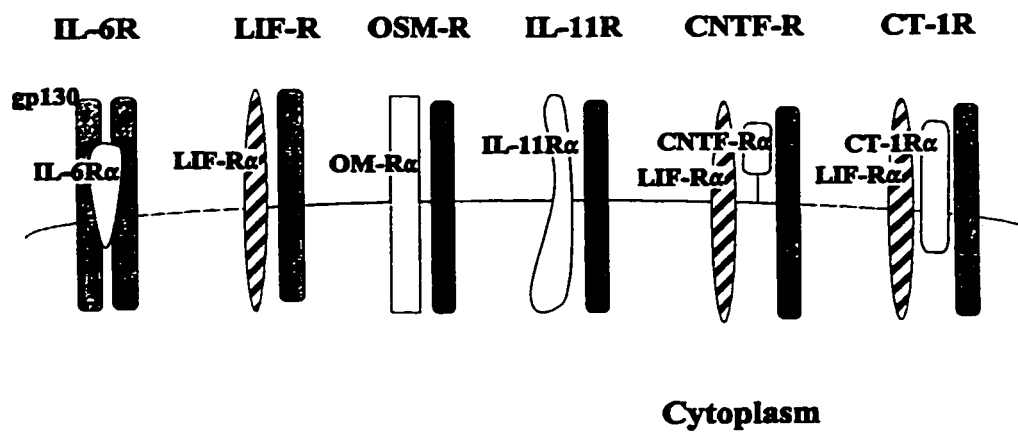
features are common among these cytokines. Most notable is their capacity to function as modulators of inflammation by inducing the synthesis of acute phase proteins, many of which are protease inhibitors that act to re-establish homeostasis (Baumann and Gauldie, 1994). In addition, IL-6, IL-11, LIF and OSM have been demonstrated to induce the proliferation and/or differentiation of lymphoid or myeloid cells, stimulate hematopoesis and effect neuronal cells (Taga, 1997).

Activities specific to individual IL-6 cytokine family members have also been established. IL-6 is unique in its capacity as a B cell differentiation factor (Poupart et al., 1987). IL-11 inhibits the differentiation of adipocytes (Heinrich et al., 1998). LIF is necessary for blastocyst implantation, while OSM promotes the survival of sertoli cells and gonocytes (Stewart et al., 1992; De Miguel et al., 1997). Finally, CNTF is an important mediator of nerve repair and CT-1 potentiates the survival of motorneurons and cardiac muscle cells (Ip and Yancopoulos, 1992; Pennica et al., 1995). Many of these unique biological responses are likely attributed to the tissue specific expression of cytokine receptors and/or the contribution of the cytokine-specific receptor-binding subunit to intracellular signalling events that manifest the biological effects observed.

1.2.3 Transgene and Knockout Models

Cytokine transgenic and knockout murine models have been used to explore the relative contribution of each of the IL-6-type cytokines in vivo and many of the findings reflect the observations described in vitro. Overexpression of an IL-6 transgene in mice induces plasmacytomas (massive proliferation of plasma B cells) (Suematsu et al., 1989). IL-6 knockout mice display normal development but elicit an increase rate of bone metabolism and B cells are incapable of differentiating into immunoglobulin A secreting cells (Kopf et al., 1994). Consistent with in vitro studies, transgenic CNTF mice prevent neuronal degeneration while CNTF knockouts lack motor neurons in adult mice (Tolosano et al., 1996; DeChiara, et al., 1995). Knockout CNTF receptor mice suffer from defective motor neuron development and die within the first day of birth. Mice with a null mutation for the LIF receptor also have significantly reduced levels of motorneurons as well as astrocytes (Rao et al., 1993). LIF knockouts have reduced survival of sympathetic neurons and females are infertile due to a defect in blastocyst implantation (Stewart et al., 1992). Transgenic IL-11 mice elicit inflammatory and fibrotic responses and female infertility is observed in IL-11 receptor knockouts due to abberant decidualization (Heinrich et al., 1998). IL-11, OSM, OSM receptor, CT-1 and CT-1 receptor knockout mice have not yet been characterized thus far. As might be predicted given its broad tissue expression, nullifying expression of the gp130 receptor subunit is lethal (Yoshida et al., 1996). Heart and haematopoetic

disorders are observed and embryos die between day 12.5 and term. Thus, the

IL-6 type cytokines collectively elicit a myriad of responses which include effects on

lymphoid/myeloid, neuronal, muscle, reproductive tissue and connective tissue

cells.

1.3 Signal Transduction By the gp130-utilizing IL-6-type Cytokine Family.

The IL-6 cytokine family bind receptor complexes that share in common the

gp130 cell surface molecule that is essential for signal transduction by this cytokine

family, and thought to confer biological redundancy within this cytokine family.

Receptor-alpha subunits specific to each cytokine receptor are necessary for cell

responses unique to each cytokine family member. Receptor-alpha subunits for

at least LIF and OSM are highly homologous within their cytoplasmic domain and

heterodimerization of receptor-alpha subunit and gp130 following ligand-receptor

interaction initiates signal transduction. However, in the case of the IL-6 receptor

complex, the IL-6R-alpha subunit cytoplasmic region is very short. Ligation of the

alpha subunit with IL-6 and subsequent gp130 dimerization is necessary and

sufficient to induce IL-6-mediated signal transduction (Fourcin et al., 1996;

Murakami et al., 1993). Thus, IL-6 signalling may be representative of signalling

events mediated by the gp130 subunit of IL-6-type receptors.

1.3.1 The JAK-STAT Signal Transduction Pathway.

Over the past few years, the JAK-STAT signalling pathway has emerged as a key intracellular signalling network that conducts the expression of target genes in response to the activation of cytokine receptors. The Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) were originally defined as being required for the expression of interferon-inducible genes (Pellegrini and Schindler, 1993) and subsequently demonstrated to be activated by most cytokines and some growth factors. To date, four mammalian JAK kinases (JAK1, JAK2, JAK3 and Tyk2) and six STAT family members (STATs1 through 6) have been identified (Heinrich et al., 1998). The response of JAK and STAT proteins is immediate as these molecules are resident within the cytoplasm prior to the ligand-induced receptor activation.

The simplest paradigm of the JAK-STAT pathway described involves a linear cascade of events from the cell membrane to the nucleus that lead to regulated gene transcription. Cytokine receptors lacking intrinsic kinase activity, such as the IL-6 and IFN-gamma receptors, require associated kinases in order to elicit their biological effects. Cytokine binding to and subsequent oligomerization of the receptor subunits results in autophosphorylation and activation of constitutively associated JAK kinases bound to the receptor (Briscoe et al., 1996).

The gp130-signal transducing subunit has become a well established model for JAK-STAT signalling (Heinrich et al., 1998). Activated JAK kinases phosphorylate

gp130 at tyrosine residues that provide docking sites for cytosolically resident STAT factors. Monomers of STAT interact with receptor phosphorylated tyrosines via the STAT src-homology 2 (SH2) domain and in turn become tyrosine-phosphorylated within this SH2 region by the JAKs. Phosphorylated monomers of STAT then dissociate from the receptor complex and form STAT homo- or heterodimers that translocate into the nucleus and bind unique DNA elements of varying specificities for different STAT dimers. The STAT consensus DNA binding sequence revolves around an element of partial dyad symmetry. For both STAT1 and 3, which are markedly activated in response to gp130-mediated signal transduction, the consensus DNA binding sequence is TTCCNNGAA and is found in the promoters of many acute phase protein genes and a variety of immediate early genes, such as the sis-inducible element (SIE) in the c-fos gene (Sadowski et al., 1993).

The specificity of signalling by the JAK-STAT pathway remains unclear as a variety of cytokine or growth factors activate more than one STAT signal transducer. Cytokines and growth factors such as IL-6, epidermal growth factor (EGF) and growth hormone (GH) confound the relative contribution of different STAT proteins to unique biological effects. IL-6 induces STAT1 and STAT3 (also known as the acute phase response factor; APRF) and the epidermal growth factor and growth hormone activate STATs 1, 3 and 5 (Darnell et al., 1994). Thus, STAT and JAK proteins may impart biological redundancies to a variety of cytokine and growth factor systems.

It appears that a key mechanism for JAKs/STATs contributing to a specific biological effect begins at the receptor itself. The IFN-gamma receptor utilizes only STAT1, while the gp130 subunits of the IL-6 receptor preferentially bind STAT3 over STAT1 (Pellegrini and Schindler, 1993; Boulton et al., 1995). For the gp130 receptors, STATs 1 and 3 bind separate phospho-tyrosine docking sites and swapping of the STAT 1 and 3 SH2 domain can confer STAT1 binding specificity to a chimeric STAT3-STAT1(SH2) protein (Stahl et al., 1995). Using JAK1 knockout mice, one independent study has suggested that JAK1 is essential for IL-6 signalling (Guschin et al., 1995). However, JAKs 1, 2 and Tyk2 have been repeatedly shown to phosphorylate the gp130 receptor subunit in a variety of cell types (Ernst et al., 1996; Auguste et al., 1997). Thus, the specificity of signalling is primarily dependent on receptor binding motifs than which JAK kinases are utilized by the receptor.

Dimerization of STAT factors and cross-talk with other signalling pathways may confer an additional level of specificity (and complexity) by cytokines and growth factors. Gp130-utilizing cytokines can activate STATs 1, 3 and 5. STAT1 and 3 can dimerize to form STAT1 or 3 homodimers and STAT1/3 heterodimers (reviewed by, Darnell et al., 1994). In response to IL-6, STAT3 homodimers appear to prevail over STAT1 and STAT1/3 dimers when binding the sis-inducible element of the c-fos promoter. Although both STAT5 and STAT1 homodimers can bind the same gamma-interferon activation sequence (GAS) in the 2-casein kinase

promoter, their relative affinity and tissue specific expression may impart unique responses for different cell types (Lai et al., 1995). Additional studies have also demonstrated preferential usage of STAT DNA binding sequences. Mutational analysis of a prototypical STAT binding site demonstrated selective binding by STAT1 and STAT3 (Lamb et al., 1995).

Since it is common for receptors to stimulate multiple signalling pathways, cross-talk with other pathways with JAKs and STATs may effect the participation of STAT transcription factors in cytokine and growth factor gene expression. Stable complex formation of STAT3 homodimers (but not STAT1 or STAT1/3 dimers) requires serine phosphorylation by an H7-sensitive serine/threonine kinase (Zhang et al., 1995). The C-terminal region of STATs, including STATs 1 and 3, contain the transcriptional activation domain, the domain necessary for participating in upregulation of target gene expression. In addition to tyrosine phosphorylation, serine phosphorylation is necessary for maximal activation of transcription by STATs 1 and 3 (Wen et al., 1995). A carboxy-terminal serine phosphorylation site, resembling a MAP-kinase (MAPK) phosphorylation site, is essential for STAT1 and STAT3-mediated transcription. STAT1-beta, which lacks this site due to alternative RNA splicing, is unable to activate transcription of a reporter gene (Wen et al., 1995). The MAPK signalling pathway has been further implicated in facilitating STAT-mediated gene expression as the the Erk2 MAPK has been demonstrated to associate with IFN-K and -2 receptors as well as directly interact with STAT1 (David

et al., 1995). STAT1 and 3 have also been shown to cooperate with additional transcription factors such as SP1 or c-Jun in regulating gene transcription (Look et al., 1995; Cantwell et al., 1998; Zhang et al., 1999). Thus, both DNA sequence specificities and interaction of additional signalling pathways add to the complexity of STAT-mediated gene regulation.

Recently a family of cytokine-induced inhibitors of JAK and STAT activation, collectively termed suppressors of cytokine signalling (SOCS) or STAT-induced STAT- inhibitors (SSI) have also been shown to participate in the regulation of these molecules (Starr et al., 1997; Naka et al., 1997). mRNA expression of SOCS has been demonstrated to be upregulated following IL-6 or LIF treatment after 30 minutes, and at least SOCS-1(SSI-1), physically interacts with JAK2 and Tyk2 (Starr et al., 1997) suggesting that direct interaction of SOCS with JAK kinases inhibits STAT-mediated signal transduction. Ultimately, the tissue specific expression of JAK/STAT factors, and the regulatory molecules that effect their function will determine their contribution to unique and/or overlapping biological effects of cytokines and growth factors.

1.3.2 The Ras-MAP-kinase pathway.

Receptor tyrosine kinases were the earliest model for studying how signals are transduced by growth factors. One signalling network which has been extensively

investigated is the ras-map-kinase pathway. Given the overlapping biological activities of the IL-6-type-cytokines, IL-6 has been used as the prototype for examining signal transduction through gp130. Several studies suggest a role for the ras-map-kinase pathway in gp130-mediated signaling. An involvement of ras, a guanine-nucleotide exchange factor, in mediating gp130 signals is supported by the finding of an increase in ras activity upon IL-6 binding to its receptor (Satoh et al., 1992). Akira et al.(1990) identified a C/EBP (CAAT/enhancer binding protein) transcription factor family member, coined nuclear factor-IL-6 (NF-IL6), which is activated by exogenous IL-6. Subsequent studies showed that functional NF-IL-6 required phosphorylation by map-kinase.

Biochemical studies on signalling by the gp130-utilizing cytokines have demonstrated the tyrosine phosphorylation of a variety of components of the ras-map-kinase cascade (Boulton et al., 1994; Kumar et al., 1994). The intercellular adapter molecule Shc has been demonstrated to physically interact with the gp130-receptor subunit and implicates gp130 in signalling via the ras-MAP-kinase pathway (Giordano et al., 1997). The Shc molecule contains two phospho-tyrosine binding domains: a carboxy-terminal SH2 domain and a phospho-tyrosine interaction (PI) domain with differing specificity compared to the SH2 domain (Pelicci et al., 1992; Kavanaough and Williams, 1994; Batzer et al., 1995). Upon phosphorylation of receptor associated Shc proteins, intracellular Grb2/SoS complexes bind Shc via interaction of the Grb2 SH2 domain and tyrosine phosphorylated Shc. Recruitment

of Grb/Sos to the receptor complex leads to re-localization of Sos within the membrane, an event sufficient to induce ras activation (Cutler et al., 1993; Rozakis-Adcock et al., 1992). Giordano et al. (1997) reported Shc to interact with the gp130-receptor subunit of the IL-6 receptor as well as the JAK2 kinase. IL-6 stimulation also led to the phosphorylation of Grb2 and a downstream target of ras activation, the Erk-1 and -2 MAP-kinases. SHP-2, a ubiquitously expressed intracellular tyrosine phosphatase, contains two SH2 domains at its N-terminus and interacts with JAK1 and 2 (Yin et al., 1997) and is necessary for gp130-mediated proliferative signals via MAP kinase activation (Fukada et al., 1996). Thus, IL-6 signalling induces events leading to activation of the ras-MAP-kinase pathway which may impart be facilitated by interaction with JAK kinases.

IL-6-type cytokine activation of the intracellular serine-/threonine phosphorylating MAP kinases and src-related tyrosine kinases has also been implicated by others. Ernst et al. (1996) showed that LIF and IL-6 could mediate tyrosine phosphorylation of Erk MAP kinases, a necessary event for the activation of the MAP kinases (Anderson et al., 1990). The IL-6-type cytokines IL-6, LIF, OSM and IL-11 are all capable of activating the Erk MAP kinases and a related MAPK, ribosomal S6 protein kinase in murine fibroblasts (Yin and Yang, 1994). The src-related tyrosine kinase Hck has been demonstrated to be activated by IL-6 and LIF stimulation and directly interacts with the gp130 receptor subunit (Ernst et al., 1994). Also, nonreceptor tyrosine kinases in addition to Hck; Fes, Btk and Tec,

have been shown to associate with the gp130 receptor (Matsuda et al., 1995; Matsuda et al., 1995).

Recently, it has been demonstrated in a prostate cancer cell line that the gp130 receptor subunit can also interact with the receptor tyrosine kinase erbB2, a member of the EGF receptor family (Qiu et al., 1998). IL-6 stimulated the association and phosphorylation of the erbB2 receptor. This event was also necessary for MAP kinase activation in these cells. Thus IL-6 can induce the association of additional receptor for stimulating intracellular signalling events. One in vitro target of MAP kinases include the AP1 transcription factor family (Karin et al., 1997). Thus, the ras-map-kinase pathway may participate in cytokine-induced signals that lead to TIMP-1 transcription.

1.4 Activator Protein-1 (AP-1) and Regulation by MAP-kinases

1.4.1 The fos/jun AP-1 family.

AP-1 transcription factors collectively describe a family of jun and fos nuclear proteins which homo- or heterodimerize and bind a common nucleotide sequence for regulating gene expression. AP-1 was originally characterized as a transcription factor binding to an essential promoter element of the human metallothionein IIa (hMTIIa) gene (Lee et al., 1987) and its DNA binding activity was subsequently found to be induced by the phorbol ester TPA (12-O-tetradecanolyphorbol 13-

acetate; also referred to as PMA; phorbol myristol acetate). As a result, AP-1 DNA binding elements are often referred to as TPA-responsive elements (TREs) with the nucleotide consensus sequence 5' TGACTCA 3' (Angel et al., 1987). AP-1 activity was subsequently found modulated in response to a vareity of stimuli including growth factors and cytokines (Angel and Karin, 1991). Fos and jun family members act as immediate early genes as induction of their transcription is independent of new protein synthesis and is transient. In the case of c-fos, mRNA levels are upregulated for up to 60 minutes in response to stimulation with epidermal growth factor, then rapidly reduced (Treisman et al., 1985).

In general, several factors influence AP-1 regulation of transcription. These include the abundance of AP-1 proteins, the partners utilized in dimeric AP-1 complexes and post-translational modifications, particularly serine/threonine phosphorylation. It should be noted that the capacity for AP-1 factors to upregulate transcription is not always reflected by the level of DNA-binding activity. Post-translational modifications, primarily serine/threonine phosphorylation of jun and/or fos proteins significantly dictates their activity as transcriptional activators. Thus, it is important to address AP-1 transcriptional activity by utilizing a chimeric AP-1 dependent promoter-reporter gene expression system. The human collagenase promoter, which is dependent on AP-1, is useful for such studies (Angel et al., 1987).

Jun and Fos family members constitute a subset of basic-leucine zipper

DNA-binding proteins that dimerize and interact with AP-1 DNA elements (see Angel and Karin, 1991 for review). The basic region of these dimers bind DNA. The zipper motif consists of leucine side chains protruding from an alpha-helix secondary structure that form a hydrophobic surface to facilitate dimerization. The Jun family of factors include, c-jun, junB and junD, and four fos-related proteins have been described: c-fos, fosB, fra-1 and fra-2. Jun nuclear factors can homodimerize or heterodimerize with each other or with Fos proteins, while Fos members do not homodimerize but must heterodimerize with Jun proteins in order to bind AP-1 DNA elements.

The nature of the AP-1 dimeric complex can influence its capacity to regulate gene transcription. Pre-existing Jun proteins occupy AP-1 elements in the absence of any cell stimuli (Smeal et al., 1989). Upon treatment of cells with phorbol esters, growth factors or cytokines, Jun protein and Fos protein synthesis is upregulated and Fos proteins can translocate into the nucleus and bind Jun to form more stable AP-1 complexes for interacting with their cognate DNA element (Smeal et al., 1989). The usage of fos proteins in fos/jun heterodimers may also affect the level of transcription acheived. For example, the strong activation domain of c-fos is absent in the smaller fra-1 protein and although c-fos/jun and fra-1/jun dimers are equally capable of interacting with similar AP-1 DNA binding sites, c-fos/jun complexes are more effective than fra-1/jun dimers in upregulating transcription (Suzuki et al., 1992). The expression of fos and jun proteins will therefore affect

which AP1 factors have the potential to regulate genes. AP-1 can also directly interact and cooperate with additional nuclear factors in regulating gene transcription. Binding sites for AP-1 and NFAT (nuclear factor of activated T cells) or AP-1 and Ets are often found closely linked and AP-1 nuclear factors cooperate with NFAT in the regulation of cytokine genes and with Ets proteins in regulating many of the matrix metalloproteinases (Wasylyk et al., 1998; Rao, 1994).

1.4.2 Regulation of fos and jun transcription.

Regulation of fos and jun transcription has been best described for c-fos and c-jun. For the c-fos promoter, there are three principle regulatory DNA elements. These are a cAMP-response element (CRE), a serum response element (SRE), and the sis-inducible element (SIE) (reviewed by Karin, 1995). The CRE utilizes cAMP or Ca2+ as second messengers, while the SIE and SRE respond to growth factor and cytokine stimuli. STAT nuclear factors 1 and 3 bind the SIE following activation of the JAK-STAT signalling cascade. The SRE is a target of the Ras-MAP-kinase pathway. Although the SIE element contributes to c-fos induction, it is dispensable and the SRE appears to be the most important element for growth factor induced c-fos transcription (Leaman et al., 1996). The SRE is constitutively occupied by a homodimer of the serum response factor (SRF) and upon activation of the Ras-MAP-kinase pathway a second nuclear factor, the ternary complex factor

(TCF), is recruited to the SRE element, to form what has been termed the ternary complex: assembly of the SRF and TCF with the SRE element (Treisman, 1992).

The c-jun promoter is simpler in comparison and regulated by mitogenic stimuli primarily through a single AP-1-like element containing a 1 base-pair insertion (Angel et al., 1988). This element, termed the c-jun TRE, is efficiently bound by heterodimerization of c-jun with ATF-2, a basic-leucine zipper protein (van Dam et al., 1993). c-jun/ATF-2 heterodimers constitutively occupy the c-jun TRE and activate transcription upon phosphorylation by the JNK family of MAP-kinases (Devary et al., 1992; Gupta et al., 1995). Thus, an increase in synthesis of c-jun and AP-1 genes dictates which family members can dimerize and contributes to the regulation of AP-1 dependent genes.

1.4.3 Post-translational Regulation of fos and jun.

A second mechanism of primary importance in controlling AP-1 activity is via serine/threonine phosphorylation of jun and fos nuclear factors. This is mediated through activation of the Ras/MAP-kinase pathway. Three types of MAP-kinases have been characterized that activate AP-1: the ERK, JNK and FRK MAP-kinases.

Phosphorylation of jun nuclear factors is best described for c-jun. The jun-N-terminal kinases (JNK) were characterized based on their capacity to bind and phosphorylate the N-terminus of c-jun (reviewed by Karin, 1995). Phosphorylation

of c-jun at serine-63 and serine-73 by JNK stimulates its ability to activate transcription as a homodimer or heterodimer with c-fos. This phosphorylation event is independent of any effect on DNA binding. JNKs appear to be the only kinases which efficiently phosphorylate the N-terminal positive regulatory sites of c-jun. Neither Erks or Frk are capable of phosphorylating these sites. Instead, Erks can phosphorylate an inhibitory site located next to the C-terminal basic DNA-binding domain of c-Jun.

Transcriptional activation motifs found within the N-terminus of c-jun, collectively termed homology box1 and 2 domains (HOB1 and HOB2), are also highly conserved within the C-terminal activation region of c-fos (Sutherland et al., 1992). However, phosphorylation of a positive regulatory threonine residue (at position 232) is mediated by the c-fos specific kinase FRK and not by JNKs (Deng and Karin, 1994). In these studies, TPA, a phorbol ester which is a potent activator of ERK kinases, also did not stimulate phosphorylation of c-fos at threonine-232. The contribution of ERKs to c-fos effects are primarily mediated indirectly by phosphorylation of the ternary complex factor Elk-1 for potentiating c-fos transcription (Marias et al., 1993). The c-fos protein also harbors a transrepression domain within its carboxy terminus modulated by phosphorylation at the serine-362 residue and phosphorylation of c-fos by a growth factor regulated kinase, termed c-Fos kinase, negatively regulates the potential of c-fos to increase AP1 activity (Taylor et al., 1993).

1.4.4 The Ras/MAP kinase pathway.

Activation of the Ras/MAP-kinase pathway begins with the recruitment of Ras by Sos/Grb2 complexes that are activated by receptor tyrosine kinases or cytokine receptors. Upon recruitment of membrane localized Ras, Sos facilitates the exchange of GDP for GTP on Ras (Cutler et al., 1993; Rozakis-Adcock et al., 1992). GTP-bound Ras is active, and can stimulate the localization of Raf-1 to the plasma membrane which can then initiate a cascade of events leading to the activation of MAP kinases (Avruch et al., 1994). Ras-independent signalling of MAP kinases has also been described such as protein kinase C (PKC) activation of Raf-1 (Ueda et al, 1996). PKC is activated by the binding of the phorbol ester TPA, or in vivo, by binding diacylglycerol (Newton, 1995).

The key pathway of ERK activation by Ras is through recruitment of Raf-1 to the plasma membrane (reviewed by Karin, 1995). Raf-1, a serine/threonine kinase, once activated phosphorylates MEK1 and 2 which then activates ERK kinases. Ras also induces MEKK, a serine/threonine kinase unrelated to raf-1. MEKK1 efficiently activates MEK1 as well as kinases which phosphorylate JNKs, such as JNKK1. Neither MEK1 or Raf-1 are efficient activators of JNKK1. Thus, JNKK1 doesn't lead to activation of ERKs, and disparate pathways with little cross-talk exist for activation of JNKs and ERKs and provides an additional mechanism of specificity for growth factors and cytokines of various MAP-related kinases.

1.5   Human OSM: Biology and Signal transduction.

1.5.1   Biology

OSM is secreted as a 28kD polypeptide by mitogen-activated T cells and endotoxin-stimulated macrophages (Brown et al., 1989; Grove et al., 1991). It is synthesized as a 252 amino acid (a.a.) precursor molecule and undergoes several post-translational events (Linsley et al., 1989). Cleavage of a 25 a.a. hydrophobic signal peptide and the carboxy-terminal 31 a.a. leads to the secretion of a 196 a.a. mature form of the protein. Human OSM possesses 4 conserved cysteine residues that form two intramolecular disulfide bridges, one of which (Cys49-Cys167) is essential for bioactivity of the molecule (Kallestad et al., 1991). In addition, there are several N-linked and O-linked glycosylation sites. Both the mature form and precursor form equally bind its receptor, however, the precursor form is 5 to 60 fold less bioactive in growth inhibition assays (Linsley et al., 1989).

Human OSM was first characterized by its ability to specifically inhibit the growth of the A375 melanoma cell line (Zarling et al., 1986). Subsequently, both positive and negative growth and differentiation effects have been observed. OSM stimulates growth of fibroblasts (Horn et al., 1990), aortic endothelial cells (Brown et al., 1990), hematopoietic cells (Taga et al., 1992) and promotes leukemic cell differentiation (Rose and Bruce, 1991), while suppressing embryonic stem cell differentiation (Gearing and Bruce, 1992) and several tumor cell lines including the HTB10 lung carcinoma (Lui et al., 1992). In vivo, circulating levels of OSM have

been detected in the sera of patients suffering from septic shock (Guillet et al., 1995), in breast cyst fluid of women (Lai et al., 1994), in normal human testis (De Miguel et al., 1999) and in the synovial fluid of individuals with rheumatoid arthritis (Okamoto et al., 1997).

OSM has been described as a key effector molecule for regulating the responses of many connective tissue cell types and has therefore been implicated in modulating extracellular matrix metabolism (ECM) and inflammatory processes. OSM stimulates the proliferation of fibroblasts (Horn et al., 1990) and osteoblasts (Jay et al., 1996) and regulates the secretion of ECM proteases and ECM protease inhibitors (Richards et al., 1992). The extracellular protease inhibitors alpha1-anti-chymotrypsin (Richards et al., 1992), alpha1-proteinase inhibitor (Sallenave et al., 1997), TIMP-1 (Richards et al., 1993), and TIMP-3 (Li and Zafarullah, 1998) have all been shown to be upregulated by OSM in fibroblasts or articular chondrocytes.

The OSM-mediated synthesis of collagen by dermal fibroblasts (Duncan et al., 1995), in addition to its effects on proteinase inhibitors, suggests a role for OSM in connective tissue remodelling in inflammatory tissue damage.

Evidence for OSM in tissue remodelling has in part stemmed from data of a transgenic OSM model implicating OSM in wound repair and chronic tissue repair resulting in fibrosis. Mice harbouring a bovine transgene of OSM displayed abnormal bone growth, fibrotic lesions surrounding the pancreas, and an accumulation of ECM components (Malik et al., 1995; Bamber et al., 1998). OSM

also activates articular osteoblast cell proliferation and inhibits bone resorption, consisent with its role as a cytokine mediating ECM remodelling (Jay et al., 1996).

In two separate murine models of inflammation, LPS-induced arthritis and experimental autoimmune encephalomyelitis models, Wallace et al. (1999) showed that systemic administration of recombinant human OSM could significantly down-regulate inflammation and tissue destruction. OSM decreased the expression of the inflammatory cytokine tumor necrosis factor-alpha (TNF-alpha), reduced arthritic joint inflammation (reduced swelling, redness), and decreased mononuclear infiltration in the EAE model.

In addition to its effects as an anti-inflammatory cytokine, OSM has also been suggested by others to mediate inflammation under certain conditions. Cawston et al. (1998) showed that in combination with the inflammatory cytokine IL-1, OSM increased the turnover of collagen and could be detected within the rheumatoid arthritic joint. Others have also demonstrated OSM to be secreted by inflammatory polymorphonuclear cells (Grenier et al., 1999) and to upregulate the expression of chemotactic cytokines (Langdon et al., 1997) with the potential to stimulate inflammatory cell infiltation. Thus, the effects of OSM in modulating tissue remodelling are complex and its participation in inflammatory processes is under investigation.

Aside from its effects on connective tissue cells, human OSM has been

described as a growth and maturation factor for other cell types. OSM is detected in conditioned media of human immunodeficiency virus (HIV)-infected T lymphocytes and functions as a growth factor for the most common cancer associated with AIDS; Kaposi's sarcoma (Radka et al., 1993). This cytokine can inhibit the proliferation of breast cancer cells by stimulating them to differentiate (Douglas et al., 1998). OSM has moreover been implicated in affecting the growth and/or differentiation of neuronal, hematopoietic, liver and germinal cells. Neuronal cell survival can be enhanced for some cell lines by this cytokine, and OSM can upregulate gene expression and neurotransmitter release from neuronal cells (Vos et al., 1996; Rao et al., 1992), although to a lesser extent than related IL-6 family members. OSM stimulates extra-thymic T lymphocyte development in an OSM-transgenic mouse model (Clegg et al., 1999) and lymphocytes in the fetal liver, a major site of hematopoiesis during embryonic development, expresses OSM that can also induce the differentiation of hepatocytes (Kamiya et al., 1999). OSM may in addition play a role in spermatogenesis and testicular development. It is detected in normal human testis (DeMiguel et al., 1999), is secreted by sertoli cells, and is a survival factor for sertoli cells and surrounding germ cells undergoing maturation (DeMiguel et al., 1997). In summary, human OSM is pleiotropic in nature and effects connective tissue, hematopoeitic, neuronal and germ cells.

1.5.2 Human Oncostatin M Receptor

The IL-6, LIF, IL-11 and CNTF receptor complexes all possess a low affinity binding K-receptor subunit and form high affinity receptor complexes in conjunction with the 2-signal transducing gp130 polypeptide (Taga, 1997). Both low and high affinity human OSM receptors have been characterized. Unlike other family members, OSM can directly bind gp130 alone with low affinity (Duncan et al., 1995). However, this binding is not sufficient to induce OSM-mediated signalling. Although OSM can bind to the LIF-receptor complex (Type-I OSM receptor), cells deficient in LIF-K-receptor are still responsive to OSM. An OSM-specific receptor subunit was recently cloned by Mosley et al. (1996) that heterodimerizes with gp130 to form the Type-II OSM-specific receptor. It shares 32% homology with the LIF receptor and 23% homology with gp130 at the amino acid level. The OSM-specific subunit is considered as the beta-subunit (gp130 the alpha-subunit) since OSM, in contrast to other IL-6-type cytokines, first binds gp130 prior to heterodimerization with the OSM-specific (beta-) subunit. The OSM-receptor beta-subunit is postulated to contribute biological effects unique to OSM within its cytokine family. Although OSM also binds the LIF receptor, LIF does not bind to the Type-II OSM receptor. Like other IL-6-type cytokine family members, OSM also upregulated acute phase protein gene expression through the OSM-specific receptor that was independent of the LIF receptor (Mosley et al., 1996). The detection of the OSM-specific receptor-beta subunit in connective tissue cell types, liver and bone marrow

stromal cells by these investigators (Mosley et al., 1996) is consistent with responses previously described that are mediated by OSM. Additional effects of OSM are likely attributable to its binding to the LIF receptor.

1.5.3 Intracellular signalling events and OSM-specific signalling

The transduction of signals leading to biological activities unique to OSM but not other IL-6-family members for the most part remain elusive and have only recently been carefully explored with the cloning of the OSM-specific receptor-beta subunit. Initial reports by Liu et al. (1992) showed that OSM upregulated the transcription of the immediate early genes egr-1, c-jun and c-myc. Unlike other IL-6-cytokines, OSM also specifically upregulates low density lipoprotein receptor (LDL-R) (Grove et al., 1991). Subsequently, evidence for the involvement of egr-1 in the OSM induction of LDL-R mRNA expression was demonstrated (Lui et al., 1993).

OSM, like all IL-6-type cytokines, activates the JAK-STAT pathway characterized for many cytokine and several growth factor receptors. JAK1, JAK2 and Tyk2 are activated by Type I (LIF receptor) and Type II (OSM-specific) receptors and there is precedence for JAK1 as the primary mediator of OSM-specific signalling among JAK kinases (Auguste et al., 1997; Burfoot et al., 1997).

STATs 1, and 3 are activated by OSM and STAT5 has been reported to be specifically induced by the Type II OSM receptor and not by interaction of OSM with

the Type I (LIF-binding) receptor in human cells in vitro (Auguste et al., 1997).

Others have implicated LIF and human OSM, but not IL-6, with the capacity to

stimulate STAT3 and 5 activity in murine cells (Kuropatwinski et al., 1997). The

binding of STATs to the OSM-specific beta-receptor subunit is dependent on a

carboxy terminal box3 motif while the LIF receptor does not share the same

restriction (Kuropatwinski et al., 1997).

Cross-talk of the JAK-STAT pathway with other intacellular signalling

pathways has also been demonstrated. OSM stimulates the association of JAK2

with Grb2 (Chauhan et al., 1995). Grb2 is an adaptor protein that binds Sos leading

to the activation of the Ras/MAP-kinase pathway (Cutler et al., 1993; Rozakis-

Adcock et al., 1992). The OSM (and IFN-beta) has been shown to trigger Erk2

MAP kinase (and Raf-1) activity in a JAK1-dependent manner (Stancato et al.,

1997) and JAK1 is dominant among JAK kinases in tyrosine phosphorylating the

cytosolic adaptor protein insulin receptor substrate (IRS)-1 in response to OSM

(Burfoot et al., 1997). IRS-1 contains several tyrosine phosphorylation motifs which

when phosphorylated provide docking sites for SH2-domain containing proteins

including PI-3' kinase, Shp-2 and Grb2 (Sun et al., 1993; Shoelson et al, 1992).

Moreover, stimulation of a lipid effector pathway in Kaposi's sarcoma cells

via the phosphatidylinositol(PI) 3' kinase as well as induction of src kinase, a

canonical nonreceptor tyrosine kinase has been demonstrated in Kaposi's sarcoma

(KS) cells in response to OSM (Soldi et al., 1994).

The MAP kinase pathway is activated by OSM in a variety of cell types including KS cells. OSM stimulates the growth of AIDS-related Kaposi's sarcoma cells and in contrast to other IL-6-type cytokines, is found to potently activate the MAP-kinase ERK2(p42) in these cells (Amaral et al., 1993). The Jun-kinases (JNK), a family of MAP-related kinases, are also markedly induced in response to OSM in KS cells (Faris et al., 1996). The fos/jun AP1 family of transcription factors is one target of the Ras/MAP kinase pathway. Consistent with the induction of ERK and JNK MAP kinases, OSM upregulated the expression of a reporter gene driven by an AP1-dependent promoter (Faris et al., 1996). In addition to STAT and AP1 DNA binding elements as targets of OSM signalling, an SP1 DNA binding motif (nucleotides 5' TCCTCC 3') has been characterized as an OSM-responsive element (Ihn et al., 1997).

Functional redundancy within cytokine families presents a challenge to delineate specific biological functions unique to a single family member such as OSM in the IL-6-type family. To date, targets of OSM signalling include the egr-1, p42 MAP-kinase, low density lipoprotein receptor, plasminogen activator inhibitor-1(PAI-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1) genes. An overview of signalling pathways acting through OSM are summarized in Figure 2.

Figure 2.   Oncostatin M Signal Transduction Pathways.

A summary of intracellular signalling molecules activated by oncostatin M (OM) is illustrated.   The OM receptor consists of at least two subunits: an OM-specific subunit (OM-RK) and the gp130 subunit.   The receptor is depicted here as being composed of two gp130 subunits to simply illustrating the interaction of various intracellular factors with both OM receptor subunits.   Induction of the JAK-STAT pathway leads to dimerization of STAT nuclear factors which then translocate to the nucleus and bind gamma-interferon activated sequences (GAS) such as the sis-inducible element in the promoter of the c-fos gene.   Components of the Ras-mitogen-activated kinase (MAPK) pathway have been shown to be activated by OSM.   Participants in the Ras-MAPK pathway demonstrated to interact with the OSM receptor or shown to be activated by OSM include shc, grb2,sos ras, raf-1, JNK (jun-N-terminal kinase) and ERK (extracellular-activated response kinase). One target of the JNK and ERK MAP-kinases are AP-1(jun) and NF-IL6 nuclear factors, respectively. OSM has also been shown to activate the intracellular factors Phosphatidyl-inositol 3' kinase (Phtdl 3' kinase), src and IRS-1. The phosphatase shp-1 interacts with the gp130 subunit of IL-6-type receptors and is likely candidate of OSM signalling.   (PLC3 = Phospholipase C).
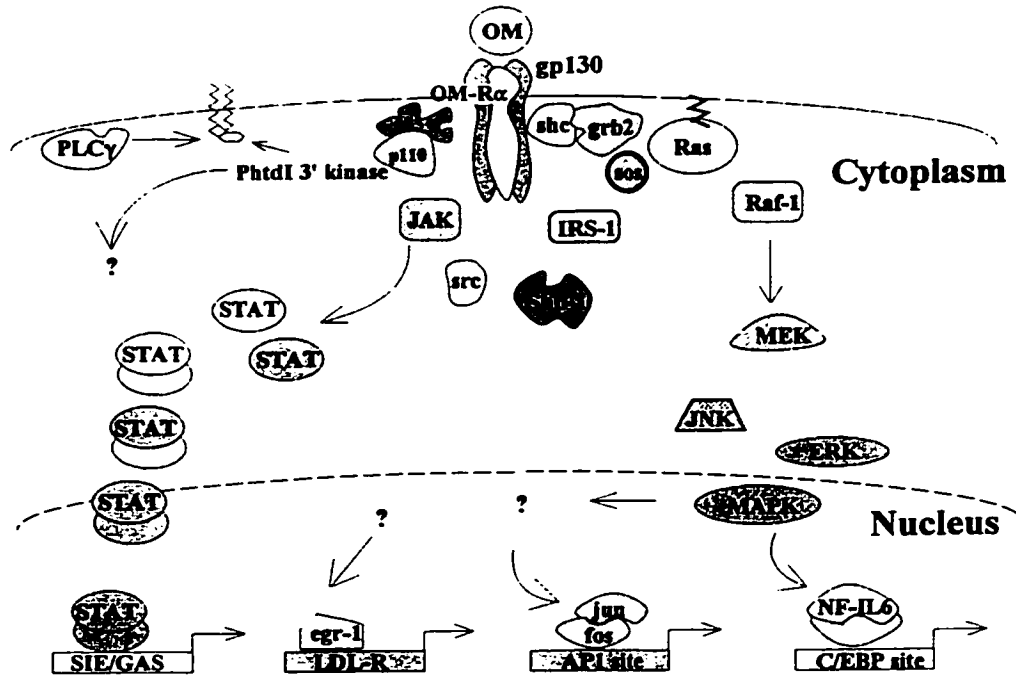
1.6   Murine Oncostatin M: Biology and Receptor Expression.

1.6.1   Biology

The biological activities of murine Oncostatin M (mOSM) have recently been explored following its cloning as an interleukin-2, -3 and erythropoetin (EPO)-responsive gene expressed by myeloid and lymphoid cells (Yoshimura et al., 1996). It shares 48% amino acid identity with the human cytokine, and responds as an immediate early gene: its expression is transient occurring within 30 minutes to 1 hour of cytokine/growth factor treatment in certain cell lines.   Promoter regulation of the murine OSM gene is dependent on STAT5 activation by IL-2, IL-3 and the EPO receptor (Yoshimura et al., 1996) and mRNA transcripts are detected at sites of myeloid and/or lympoid cell accumulation in normal mice.   mOSM mRNA is highly expressed in bone marrow and to a lesser extent in spleen and the thymus. mOSM is also found expressed in the developing testis by sertoli cells and enhances proliferation of these cells (Hara et al., 1998).

To date, few biological activities have been characterized for murine OSM, and the prediction that it mimics human OSM requires careful examination now that the murine cytokine has been cloned.   Recently our laboratory has shown that like human OSM, murine OSM upregulates acute phase protein synthesis and TIMP-1 gene expression in fibroblasts (Richards et al., 1997).   TIMP-1 mRNA and promoter activity was potently upregulated by mOSM among murine IL-6-type cytokines in fibroblast cell lines.   mOSM also stimulates the proliferation of DA-1 myeloid cells

(Yoshimura et al., 1996), Ba/F3 lymphoid cells (Tanaka et al., 1999), a subline of NIH3T3 fibroblasts (Ichihara et al., 1997) and the expansion of hematopoietic progenitor cells during embryonic development (Mukouyama et al., 1998). More recently, murine OSM expression has been detected in the fetal brain and observed to stimulate the differentiation of astrocytes from cultured fetal mouse neuroepithelial cells (Yanagisawa et al., 1999). Astrocyte differentiation was proposed to be mediated by STAT3 (Yanagisawa et al., 1999).

1.6.2 mOSM Receptor

The murine OSM receptor (mOSM-R)has recently been cloned and shares 55.5% and 27.8% amino acid homology with the human OSM receptor-beta subunit and LIF receptor-alpha subunit, respectively (Tanaka et al., 1999). Like the human OSM-specific receptor, the mOSM-R complex is composed of gp130 and the mOSM-specific beta subunit of approximately 200kD and shares the same intracytoplasmic binding motifs for JAKs and STATs. Data on signalling by murine OSM is currently limited to our laboratory's demonstration of STAT-1 and 3 activation in mouse and rat cell lines (Richards et al., 1997).

However, unlike human OSM, mOSM does not interact with the mouse LIF receptor, and has only been demonstrated to interact with a single OSM-specific receptor. Murine OSM binds the receptor-beta subunit with low affinity and the

functional gp130-OSM-beta-subunit complex with high affinity. Activities attributed to both human OSM and human LIF such as differentiation of murine M1 cells and suppressed differentiation of embryonic stem (ES) cells are not observed by mOSM (Ichihara et al., 1997). Consistent with this, the mOSM-R beta subunit is not expressed on ES cells (Tanaka et al., 1999). Thus, mOSM provides a model for delineating activites unique to OSM and LIF. The mOSM-receptor beta subunit is highly expressed in lung and is also detected in brain, heart, muscle, thymus, and spleen and kidney. It will be of interest to determine additional unique biological activities OSM displays in vivo and in vitro within the IL-6 family of cytokines. This may now be examined in detail by the use of transgene and knock-out mouse models.

1.7 Thesis Proposal

The TIMP-1 gene represents a unique target for signals induced by OSM but not other members of the IL-6-type cytokine family. Preliminary work showed that promoter sequences within -95 to +47 of TIMP-1 respond strongly to OSM signals. We hypothesize that the putative AP1 binding site within -95/+47 of the TIMP-1 promoter is a target for nuclear factors activated by an Oncostatin M-dependent signal transduction pathway and that AP1 factors bind to this site and modulate the transcription of the TIMP-1 gene. In addition, we postulate that the mechanism by

which oncostatin M potently upregulates TIMP-1 mRNA expression is unique to only this member of the IL-6-type cytokine family. Our proposed approach in addressing this hypothesis is to identify the minimal promoter element(s) necessary(/sufficient) for OSM-responsiveness, to characterize the nuclear factors binding to these elements, and to explore the mechanism(s) by which OSM modulates DNA binding (and transactivation) by these factors. Analysis of responses in human and mouse sytems is utilized.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture and reagents

2.1.1 Cell lines

The human hepatoma cell line HepG2 (purchased from ATCC) cultured and passaged by standard techniques in K-MEM medium (supplemented with 10% fetal bovine serum (FBS) and 1% penecillin/streptomycin (P/S)). The human primary lung and synovial fibroblast cells (HLF and HSF, respectively) and the SV-40 transformed HLF cell line (ATCC) were cultured in F15 media supplemented with 10% FBS and 1% P/S. The neuroblastoma cell line SY5Y (ATCC) was cultured in RPMI supplemented with 10%FBS, 1% P/S and .1% glutamine.

The murine embryonic fibroblast cell line NIH3T3 was subcultured in Dulbecco's modified medium supplemented with 10% calf serum (CS) and 1% P/S. Primary murine lung and synovial fibroblasts were cultured in F15 media supplemented with 10% FBS and 1% P/S. Stocks of cultured cells were maintained by freezing cells from a 100mm diameter dish in appropriate media containing 20% FBS and 10% dimethylsulfonylhydroxide (DMSO) in a 1 ml final volume.

2.1.2 Derivation of Primary Murine lung and skin fibroblasts

Primary murine lung fibroblasts and skin fibroblasts were derived from B6/129

wild-type (B6,129F2/J stock#101045) and c-fos knockout mice (B6,129-Fos tm1Pa

mutant homozyogotes) purchased from Jackson Laboratories. For the preparation

of lung fibroblasts, lungs were removed from mice, dissected into small fragments,

spread onto 100mm diameter culture dishes, and allowed to attached onto dishes

for 30 minutes at 37C. F15 culture media (supplemented with 20% FBS, 1%

penicillin/streptomycin and 0.3% l-glutamine) was then carefully added to allow the

outgrowth of fibroblasts from lung tissues. Cultures were supplemented with fresh

F15 media (as described above) every three days and after 1-2 weeks of culturing

tissues fibroblasts growing out from tissues was detected. Explanted tissue was

then removed from dishes with a sterile 200ul micro-pipette tip and fibroblast cells

were trypsinized (1X trypsin) and subcultured by standard methods in F15 media

containing 10% FBS.

Cultured skin fibroblasts were derived by first shaving mice and dissecting the

skin with minimal attachment of fat tissue. Explanted skin tissue followed the same

procedure for deriving cultured primary lung fibroblasts with the exception that the

hairy surface of skin tissue faced upwards on dishes and new skin fibroblasts could

be detected growing out of explants after 3 days.

2.1.3 Cytokines, Antibodies and Miscellaneous reagents

Cytokines used were: purified human recombinant OSM, expressed in CHO

cells, provided by M. Hanson (Bristol-Myers Sqibb Research Institute, Seattle, WA);

purified recombinant human IL-6, IL12 and LIF, kindly provided by Dr. M. Widmer (Immunex Corp., Seattle, WA); human IFN-3, purchased from Genzyme Corp. (Cambridge, MA). Purified murine recombinant oncostatin M, expressed in E. coli, was a generous gift of Takahiko Hara. Murine IL-6 and LIF cytokines were purchased from R&D and epidermal growth factor (EGF) was purchased from Sigma. Unless otherwise stated, all cytokines and were used at 50ng/ml, except for IFN-3 which was used at 500unit/ml. EGF was used at a concentration of 20ng/ml.

Rabbit polyclonal antibodies against fos/jun (anti-pan-Fos(K25), anti-c-fos(fos(4)), anti-c-jun(AP-1(N)), anti-junB(N-17), anti-junD(329) and SP1(PEP-2), STAT3(H-190), STAT5B(C-17) and Ets-1/Ets-2(C-275) nuclear factors, and specific peptides were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All Santa Cruz antibodies were purchased as the concentrated (1ug/ul) TransCruz supershift product. Anti-STAT1 (ISGF3) was purchased from Transduction Laboratories (Lexington, KY).

Phenyl-methylsulfonylfloride (PMSF), pepstatin A, phorbol-13-myristate-12-acetate (PMA), puromycin and emitine were purchased from Sigma (St. Louis, MI). When added, puromycin and emitine were used at a concentration of 20ug/ml. Leupeptin was purchased from Boehringer Mannheim.

2.2 RNA Analysis

2.2.1 Extraction of Total RNA

For extraction of total RNA, subconfluent cell cultures were washed and replenished in medium containing 0% FBS. Cytokines (at indicated concentrations) were then added and cultures were incubated for indicated times prior to RNA isolation. Total RNA was then prepared from cultured cells according to Chomczynski and Sacchi (1987), with several modification. In general, 2 mls of 1X trypsin was added to detach adherent cells grown in 150mm^2 flasks. Cells were then pelleted in 15ml polypropylene tubes at 1200RPM for 5 minutes, the supernatant (media) removed and kept on ice. 1ml of guanidium isothiocyanate (GITC) containing 10% 2-merceptoethanol was added and cells immediately resuspended by vortexing. 100ul of 2M sodium acetate (pH4) and an equal volume of phenol (water saturated) and chloroform were added and tubes were inverted 15 times and placed on ice for 15 minutes. Samples were then centrifuged at 2700 RPM for 30 minutes at 4C. The aqueous (top) layer was then collected and an equal volume of isopropanol (approximately 1ml) was added. RNA was allowed to precipitate overnight by storage of samples at -70C and pelleted the following day by centrifugation at 2700 RPM for 30 minutes at 4C. Supernatants were removed and the RNA pellet washed by the addition of 500ul of 70% ethanol. Pellets were resuspended in the 70% ethanol and transferred to a 1.5ml microcentrifuge tube and centrifuged at maximum speed (12000 RPM) for 5 minutes. Supernatant was

decanted and the RNA pellet partially dried in a speed vac for 5 minutes. 50ul of

autoclaved water was then added to samples. Samples were then stored at -70C

overnight and RNA was resuspended the following day and stored at -70C until

further use.

Total RNA was quantitated by spectrophotometry. Briefly, 2ul of sample was

diluted in 998ul of water and the RNA concentration was deteremined by dividing

the optical density reading by 2 in order to calculate the concentration in

micrograms per microliter, where 1 optical density unit represents 40ug of RNA.

2.2.2 RNA Gel Electrophoresis

RNA gel electrophoresis was carried out by standard techniques. In general,

2.5X volumes of 5X RNA denaturing buffer (13% 5X RNA Running buffer (0.2M 3-

[N-Morpholino] propane-sulfonic acid (MOPS), 50mM sodium acetate pH 4, 5mM

ethylene-diaminetetra-acetic acid disodium salt (EDTA) pH 8), 23% formaldehyde

and 65% deionized formamide) was added to RNA samples containing 1X sample

buffer (5% glycerol, .04% bromophenol blue) and 1ul of 10mg/ml ethidium bromide.

RNA samples were then denatured by heating to 56C for 10 minutes and loaded

onto a 1.2% agarose gel containing 1X RNA Running buffer and 17.6%

formaldehyde. (Agarose and 1X RNA Running buffer were preheated to dissolve

agarose before the addition of formaldehyde.)   Samples were electrophoresed in 1X RNA Running buffer overnight (approximately 10 hours) at 22 volts.   Gels were then soaked in water to remove excess formaldehyde and photographed under ultraviolet light to detect and assess the quality of 28S and 18S ribosomal RNA as a representative measure of the total RNA.

2.2.3 Northern Blotting

Northern blots were prepared by standard methods and probed with human TIMP-1 cDNA (gift of DR. A.J.P. Docherty, Celltech, Slough, UK), rat c-fos cDNA (kindly provided by Dr. Tony Cruz, Mount Sinai, Toronto, Canada) or a murine TIMP-1 oligonucleotide (5' CTTATAACGCTGGTATAAGGTGGTCTCG 3'). Briefly, gel electrophoresed RNA was transferred overnight onto a BioTrans (NEN Dupont) nylon membrane by capillary action in 10 mM sodium phosphate pH 7 as recommended by NEN Dupont.   Blotted RNA was then crosslinked to the membrane by ultraviolet irradiation for 2 minutes.   The intensity of ethidium bromide stained 18S and 28S bands on the blots was used to estimate loading of RNA.

Blots were then sealed in plastic bags containing 10 mls of hybridization buffer (0.1M sodium chloride, 50mM piperazine-N,N'-bis[2-ethanesulfoni acid] (PIPES) pH 6.8, 50mM sodium dihydrogen phosphate monobasic pH 4, 1mM EDTA pH 8

and 5% sodium dodecyl sulphate(SDS)) and incubated with radioactively labelled DNA probe overnight at 55C in a waterbath shaker. Blots were washed several times to remove nonspecific binding of probe to RNA. Blots were incubated at 55C in 0.1% SDS, and 4X SSC (sodium chloride, sodium citrate) for 15 minutes. Subsequent washes were for 30 minutes with a decreasing amount of SSC; 2X, 1X and .1X (if necessary), to increase stringency of washes. Blots were checked with a Giger Counter to estimate the strength of the specific probe signal and nonspecific binding of radioactivity. Membranes were exposed to film overnight and autoradiographs developed to detect probe bound to RNA of interest.

2.2.4 Radioactive labelling of cDNA and oligonucleotide probes for Northern Blots

cDNA probes were labelled by the random priming method using the T7 or Klenow polymerase random priming kit from Pharmacia. 50 ng of denatured cDNA (100C for 2 minutes) was incubated with 15 ul of labelling mix (Pharmacia), 50 microcuries (uCi) of alpha-^{32}phosphate-deoxycytosine triphosphate and 1 unit of T7 DNA polymerase or Klenow enzyme in a 20ul reaction volume at 37C for 1 hour. Radioactively labelled probes were then purified through Sephadex-G50 spin columns (Pharmacia) and denatured by boiling for 5 minutes prior to addition to blots.

For oligonucleotide probes, 50 ng of oligonucleotide was incubated with 1X T4 polynucleotide kinase buffer (New England Biolabs (NEB)), 50uCi of gamma-^{32}phosphate-adenosine triphosphate and 20 units of T4 polynucleotide kinase (NEB or Pharmacia) in a 20ul reaction volume. Labelling mixes were added directly to blots without any further purification.

2.3 General Cloning Techniques

2.3.1 DNA Restriction Digests

Restriction digests were carried out as recommended by the restriction enzyme supplier (New England Biolabs or Pharmacia) with the exception that DNA was restriction cut overnight (at least 12 hours) to maximize the amount of digested DNA. For cloning purposes, vector DNA (6ug) or gel purified polymerase chain reaction (PCR) products were digested with 3 ul (a minimum of 30 units) of HindIII and BgIII enzyme in a 60 ul reaction volume at 37C overnight. Restriction digested DNA was subsequently gel purified and ligated prior to transformation of competent bacteria.

2.3.2 Gel Purification of DNA Restriction Fragments

2.3.2.i Purification of DNA from 1% agarose gels

DNA fragments were purified from agarose gels using the Gene Clean kit (BIO 101 Inc.) as recommended by the supplier with special attention to the following steps. Gel slices (no larger than 300 mg) were melted in 3 volumes of sodium iodide stock solution at 52 to 55C in a 1.5ml microcentrifuge tube. Glassmilk (a minimum of 5ul) was added for 10 minutes at room temperature, pelleted for 5 seconds at maximum speed (12000 RPM), and washed 3 times in 400ul of ice cold New Wash Buffer. DNA was eluted twice from pelleted Glassmilk (12000 RPM for 2 minutes) in 10ul of autoclaved water at 52 to 55C. Eluates were pooled for a final volume of 20 ul and re-centrifuged for 5 minutes at maximum speed to remove residual Glassmilk. Eluted DNA was then ready for use in radioactive labelling reactions or as substrate for DNA ligations.

2.3.2.ii Purification of DNA molecules from 8% polyacrylamide gels

Double stranded oligonucleotides (30 base pairs or less) or DNA fragments less than 200 base pairs were purified from 8% polyacrylamide gels by the crush and soak method described in Sambrook et al. (1989) with the following modifications. In general, excised gel slices were crushed in a 1.5ml microfuge tube, 500ul of elution buffer (0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA and 0.1% SDS) added and samples incubated overnight in a 37C

shaker. Samples were then centrifuged at maximum speed (12000 RPM) for 5 minutes and eluate (approximately 400ul) removed to a second tube. Crushed gel was resuspended in elution buffer (by inverting tubes gently) twice more with 500ul and 200ul of elution buffer, respectively, with centrifugation at maximum speed for 5 minutes to pellet crushed gel. Eluate was pooled for a total volume of approximately 1 ml and re-centrifuged at maximum speed to pellet residual crushed gel. Eluate was then collected into two separate tubes (approximately 500ul in each tube), 1ml of 100% ethanol added and DNA was precipitated overnight at -20C. Precipitated DNA was then pelleted for 30 minutes at 12000 RPM. All but 20 ul of ethanol was withdrawn, and DNA samples were dryed in a speed vac for 15 minutes. Gel purified DNA samples were then resuspended in 20ul of autoclaved water and stored at -20C until further use.

2.3.3 DNA Ligation Reactions

pBLCAT3 vector and gel purified PCR products were restriction digested with HindIII and BglII enzymes (as described above) and 1 ul of vector (50ng) was ligated to an excess of PCR product (7 ul; approximately 200ng, 12:1 of PCR product insert to vector DNA) with 1ul of 10X T4 DNA ligase buffer (NEB) and 1ul of T4 DNA ligase (200 units; NEB) (10ul final reaction volume) at 14C overnight. 90 ul of autoclaved water was then added to ligation reactions that were then

purified using the Gene Clean kit (Bio 101, Inc.), beginning with the addition of 3 volumes of sodium iodide. 10 ul (out of the 20ul final volume of purified eluted DNA) was then used in transformation of competent bacteria. Purification of DNA ligation reactions by the above method yielded at least a 10-fold increase in transformation efficiency.

2.3.4 Preparation of Competent Bacteria

Competent DH5K E. coli bacteria were prepared by standard methods. In general, DH5K E. coli scraped from a frozen stock with a sterile pipette tip was added to 10 mls of Luria Broth (LB) media (1% bacto-tryptone, 1% sodium chloride, 0.5% bacto-yeast and 5mM Tris pH 7.5) containing 0.2% D-glucose, and cultured overnight in a 37C shaker. The following day, 400ul of overnight culture was added to 40ml of LB media containing 1% D-glucose and bacteria were grown for 2 hours in a 37C shaker (OD(optical density)$_{550}$ of approximately 0.4). Cells were then chilled on ice for 10minutes and centrifuged at 2700 RPM for 5 minutes. Supernatant was discarded and the pellet gently resuspended by pipetting in 20mls of Solution A (10mM MOPS pH 7, 10 mM rubidium chloride). Cells were pelleted by centrifugation at 2700 RPM for 10 minutes and supernatant discarded. Bacteria were then gently resuspended in 20 mls of Solution B (100mM MOPS pH 6.5, 50mM calcium chloride and 10mM rubidium chloride). Cells were then incubated on ice for 15 minutes and then centrifuged at 2700 RPM for 10 minutes.

Supernatant was removed (as much as possible) and competent bacteria were resuspended in 4mls of Solution B. Cells (competent bacteria) were kept on ice or refrigerated and used within 24 hours.

2.3.5 Bacterial Transformation

200 ul of competent DH5K E. coli bacteria (as prepared above) were incubated on ice for 30 minutes with 3 ul of dimethylsulfoxide (DMSO) and 1-100 ng of plasmid DNA or 10ul of Gene Clean (Bio 101, Inc.) purified ligation reaction mix. Samples were then heat shocked for 50 seconds at 42C. 1 ml of LB media was then added and cells were incubated for 1 hour at 37C. Bacteria were then plated at varying amounts (50ul, 100ul and 200ul) on LB agar plates and appropriate antibiotic (LB media containing 1.5% agar and 100ug/ml ampicillin). Plates were incubated overnight at 37C, and bacterial colonies were picked and grown in 2 mls of LB media containing 100ug/ml ampicillin for purification of DNA from bacterial clones of interest.

2.3.6 Colony Hybridization Screening

Bacterial colonies positive for harbouring recombinant DNA plasmid of interest, following transformation of competent bacterial cultures with purified ligation

reactions, were identified by colony hybridization screening as recommended by New England Nuclear (NEN; Boston, USA) with several modifications described below.

2.3.6.i Bacterial Colony DNA transfer on membrane discs

Following storage of bacterial plates at 4C, side A of Colony/Plaque Screen discs (NEN) were placed in contact with bacterial colonies on agar plates and allowed to sit for 5 minutes. Discs were needle pricked into the agar plate to mark orientation of discs on agar plates and then removed onto a pool of 0.5M sodium hydroxide (750ul) with bacterial colonies facing upwards. After 2 minutes discs were removed and dried on blotting paper. Discs were placed onto 0.5M sodium hydroxide a second time and dried prior to being placed onto 750ul of 1M Tris pH7.5 twice followed by drying on blotting paper each time. Colony/Plaque Screen discs were then ready for hybridization with radioactively labelled DNA probes to detect bacterial clones containing recombinant plasmids harboring inserted DNAs of interest.

2.3.6.ii Hybridization of bacterial plasmid DNA

Hybridization of bacterial plasmid DNA on Colony/Plaque Screen discs (NEN) was carried out according to the preferred method (method A) as described by the manufacturer with the following modifications. Discs were sealed in plastic bags

(maximum of two per bag) containing 10 mls of hybridization solution (0.1M sodium chloride, 50mM PIPES pH 6.8, 50mM sodium dihydrogen phosphate monobasic pH 4, 1mM EDTA pH 8 and 5% sodium dodecyl sulphate), preincubated for 30 minutes at 55C, and then incubated with radioactively labelled DNA probe (from entire labelling reaction) overnight at 55C. Membrane discs were then washed of nonspecific hybridized probe as described above for Northern blotting.

2.3.7 DNA purification of Plasmid DNA

2.3.7.i Small scale purification of plasmid DNA

Small scale preparation of plasmid DNA (5-10 ug) from selected DH5K E. coli bacterial transformed clones was carried out verify clones harboring plasmid DNA of interest. Bacterial clones were grown overnight in 2 mls of LB media containing 100 ug/ml ampicillin and plasmid DNA harvested following centrifugation of bacteria using the Qiaprep Spin Miniprep Kit protocol as recommended by Qiagen. Purified DNA was resuspended in 100 ul of autoclaved water and 20ul was used in DNA restriction digest reactions to identify clones harboring DNA inserts of interest.

2.3.7.ii Large scale purification of plasmid DNA

Large scale preparation of plasmid DNA (300-500ug) of DH5K E.coli transformants was preformed using the Qiagen Plasmid Maxi kit according to the

manufacturer's protocol with several modifications. 500 mls of bacterial cultures grown in LB media (containing 100ug/ml ampicillin) overnight in a 37C shaker were divided into two 250ml centrifuge bottles and bacteria pelleted at 6000 RPM for 10 minutes. Supernatant was removed and 5 mls of P1 buffer was added to each bottle. Pellets were scraped with a 10ml pipette off the inside of bottles and completely resuspended in P1 buffer by gentle mixing and pipetting. Resuspended bacterial clones initially divided into two separate 250ml bottles were then repooled into a single bottle and 10 mls of P3 buffer was added, immediately and gently mixed, and incubated on ice for 30 minutes with intermittent mixing. The precipitate should appear as a white flaking solution. Samples were centrifuged at 5000 RPM for 45 minutes, removed promptly, and filtered through two layers of 125 mm diameter filter discs (#1 Qualitative; Whatman). Filtered sample was then purified through a Qiagen-tip 500 according to the manufacturer's protocol and purified DNA eluate was precipitated with 0.7 volumes of isopropanol for 15 minutes at room temperature and centrifuged for 1 hour at 10 000 RPM. Pelleted DNA was then was once with 500 ul of 70% ethanol, allowed to dry, resuspended in 250-400ul of autoclaved water and stored at -20C. DNA concentration was quantitated at an optical density of 260, where 1 O.D. represents 50ug/ml. The purity of plasmid DNA was visually examined following electrophoresis of uncut and restriction digested DNA on a 1% agarose gel.

2.4 Polymerase Chain Reaction (PCR) Deletion of the TIMP-1 promoter and cloning

Deletions of the TIMP-1 promoter were carried out by generating truncated PCR products within -95 to +47. PCR reactions consisted of Vent Polymerase (5 units; New England Biolabs (NEB)), 100ng of TIMP-1 -223 to +47/pBLCAT3 (27) template DNA, 150uM of sense and antisense oligonucleotide primers, 5mM dNTPs and 1× Vent Polymerase NEB buffer in a 50ul final reaction volume. Reaction mixtures were overlayed with 30ul of mineral oil and denatured for 1 minute at 95°C, followed by annealing of primers at 55°C for 2 minutes and primer extension at 72°C for 2 minutes in a Perkin-Elmer PCR Thermal Cycler for 35 cycles.

Primers used to generate 5' deletions of the TIMP-1 promoter included: -62/-38 for construct B which contains AP1 and Ets sites, [5' GAGGCTAAGCT TGGATGAGTAATGCGTCCAGGAAGCC 3']; -52/-31 for construct C which retains Ets site, [5'GAGGCTAAGCTTTGCGTCC AGGAAGCCTGGAGGC3']; and -39/-16 for construct D [5' GAGGCTAAGCTTCCTGGAGG CAGTGATTTCCCCGCC3']. 3' PCR primers (for constructs B,C,D) included a sequence 3' to the pBLCAT3 multiple cloning site (+470/+447), [5' CTGAAAATTCGCCAAGCTCCTCG 3]'.

3' PCR primers (for constructs A,B,C) included a sequence 3' to the pBLCAT3 multiple cloning site (+470/+447), [5' CTGAAAATTCGCCAAGCTCCTCG 3]'. Primers used to construct 3' deletions downstream of -62 were: +23/+2 [5'

TCGTAC<u>AGATCT</u>GCACA GTGGAGAATAAATGTCC 3'];   +13/-6   [5'

TCGTAC<u>AGATCT</u>GAATAAAT GTCCATGCGAAGG 3']; +1/-18 for construct E

which contains a wild-type SP1 site, [5'TCGTACAGATCTGCGA AGGGCGGAGTT

GGCG 3'] and +1/ -18 with a mutated SP1 site for construct F [(GC to TT), 5'

TCGTACAGATCTGCGAAGG<u>TT</u>GGAGTTGGCG 3'].

Restriction sites for HindIII in 5' primers (AGCTTT) and BglII in 3' primers

(AGATCT), as well as 6 nucleotide residues 5' to each site (for efficient restriction

enzyme digestion of DNA termini), were included in the designed primers. PCR

products were purified from an 8% polyacrylamide gel, restriction digested with

HindIII/BglII, re-purified and ligated into linearized pBLCAT3 vector (HindIII/BglII

digested). Colony hybridization (Colony/Plaque Screen, NEN Research Products)

with end-labelled PCR primers as probes were then used to screen for DH5K

bacterial transformants. DNA sequencing (Mobix Molecular Biology Facility) was

used to verify the correct constructs.

2.5 Chloramphenicol Acetyltransferase (CAT) Assays

HepG2 or NIH3T3 cells in 100mm dishes were transfected with 10ug of CAT

reporter plasmid DNA (co-transfected with 1.4ug of pSV-2Gal plasmid, Promega)

by the calcium phosphate coprecipitation method. Cells were allowed to recover

overnight and then replated into 6-well tissue culture plates. Prior to cytokine stimulation, cells were serum-starved in serum-free K-MEM media for 6 hours. Cytokines were then added for 18 hours. CAT assays were carried out using cell lysates according to standard protocols (Sambrook et al., 1989) and ^{14}C-labelled chloramphenicol products quantified using a Molecular Dynamics phosphorimager and ImageQuant software. Values were normalized to 2-galactosidase activity in lysates (Sambrook et al., 1989).

2.6 Preparation of Nuclear Extracts

HepG2 cells were stimulated with the indicated cytokines for various time periods. Nuclear extracts were prepared according to Andrews and Faller (1991) with the following modifications. Buffers A and C contained 0.5mM PMSF, 10ug/ml aprotonin, and 2ug/ml of pepstatin A and leupeptin. Cells were resuspended in 400ul of buffer A and nuclear proteins were extracted in 100ul of buffer C, frozen in liquid nitrogen, and stored at -70°C.

2.7 Electrophoretic Mobility Shift Assays (EMSAs)

Nuclear extract (15ug) was incubated with 2ug of poly(dI:dC) and 5ug of calf thymus DNA in binding buffer (50mM Tris-Cl (pH 7.5), 50mM NaCl, 2mM EDTA, 2mM DTT, 1mM spermidine and 5% glycerol) for 15min. on ice. 10^5 cpm of ^{32}P-labelled probe was then added and the binding reaction (20ul) was incubated at room temperature for 20 minutes. Excess of unlabelled oligonucleotide (50-100 fold) was added to the reaction for competition assays. Following the reaction, samples were electrophoresed on 5% polyacrylamide gels (40:1) containing 1.25% glycerol in 0.25× TBE (1× TBE: 89mM Tris-borate, 2mM EDTA) at 95V for 3½ hours and dried prior to autoradiography. For supershift analysis, antibodies were added following the binding reaction for 1 hour at 4°C. Sequences of oligonucleotides used for mobility shift assays are shown in Table 1, and their location in the TIMP-1 promoter for probes #1 to 6 are illustrated in Figure 4. AP1 and SP1 consensus oligonucleotides were purchased from Santa Cruz. Oligonucleotides were annealed by heating to 100°C for 5 minutes in 100mM $MgCl_2$ and 400mM Tris-Cl (pH8) followed by gradual cooling to 20°C. TIMP-1 DNA probes were end-labelled using polynucleotide kinase and the hSIE labelled by the fill-in reaction using K^{32}P-dCTP and the Klenow enzyme. Probes were then gel-purified or purified using sephadex-G50 spin columns from Pharmacia.

Table 1. Oligonucleotides used in EMSA. Oligonucleotides were synthesized, annealed, and purified as in methods. The sequences correspond to the TIMP-1 promoter regions as indicated. All TIMP-1 sequences are human except for probes #5 and #6 or where noted. Defined nuclear factor binding sites for SP1, AP-1 and Ets have been underlined. Probe 3A contains a mutated AP-1 site (uAP-1). Probe 4A contains a G at position 49 corresponding to the mouse TIMP-1 sequence, whereas probe 4 has A in that position corresponding to the human TIMP-1 sequence. The 2-caseine kinase (2-CK) probe is derived from bovine promoter sequences and both the 2-CK and SIE probes used are high affinity binding sites for STAT nuclear factors.

probe #1
SP1(-95/-66)

5'AGCTGC<u>CCGCCCC</u>TAGGCTTTGACTCCAGCGGTG3'
3'CGGGCGGGGATCCGAAACTGAGGTCGCCACCTAG5'

probe #2
AP1(-66/-52)

5' AGCTATGGTGGGTGGA<u>TGAGTAA</u>TGCA 3'
3'TACCACCCACCTACTCATTACGTCTAG5'

probe #3
AP-1/Ets (-61/-34)

5' AGCTGA<u>TGAGTAA</u>TGCATCC<u>AGGAAG</u>CCTGGA 3'
3'CTACTCATTACGTAGGTCCTTCGGACCTCTAG5'

probe # 3A
uAP-1/Ets(-61/-34)

5' AGCTGA<u>GGAGTGA</u>TGCATCC<u>AGGAAG</u>CCTGGA 3'
3'CTACTCACTACGTAGGTCCTTCGGACCTCTAG5'

probe #4
Ets(-59/-36)

5' AGCTGCATCC<u>AGGAAG</u>CCTGGAGGCCTG 3'
3'CGTAGGTCCTTCGGACCTCCGGACCTAG 5'

probe #4A
Ets(-59/-36)

5' AGCTGCGTCC<u>AGGAAG</u>CCTGGAGGCCTG 3'
3'CGCAGGTCCTTCGGACCTCCGGACCTAG 5'

probe #5
TIMP-1 (-34/-11)

5' AGCTAGGCAGTGATTTCCCCGCCAACTC 3'
3'TCCGT CACTAAAGGGGCGGTTGAGCTAG5'

probe #6
SP1(-19/+2)

5'AGCTCGCCAACT <u>CCGCCC</u> TTCGCATG 3'
3'GCGGTTGAGGCGGGAAGCGTACCTAG5'

probe #7
murine AP-1(-64/-48)

5' AGCTGTGGA<u>TGAGTAA</u>TGCGT 3'
3' CACCTACTCATTACGCACTAG 5'

hSIE

5' GTCGACA<u>TTTCCCGTAAA</u>TC 3'
3'GTAAAGGGCATTTAGCAGCT5'

2-caseine kinase probe

5' AGATTTCTAGGAATTCAAATC 3'
3' TCTAAAGATCCTTAAGTTTAG 5'

AP1 consensus

5' CGCTTTGA <u>TGACTCA</u> GCCGGAA 3'
3' GCGAAACTACTGAGTCGGCCTT 5'

SP1 consensus

5' ATTCGATCGGGGCGGGGCGAGC 3'
3' TAAGCTAGCCCCGCCCCGCTCG 5'

2.8 Western Blots

Nuclear extract was electrophoresed on a 8% sodium dodecylsulfate (SDS) polyacrylamide gel prior to transfer of proteins onto an Immobilon nitrocellulose membrane. Membranes were then blocked overnight in 1× PBS and 5% milk. Following a 10 minute incubation in wash buffer (1×PBS, 0.5% Tween20), membranes were incubated with primary rabbit polyclonal antibody; (anti-c-fos, anti-pan-fos, anti-c-jun anti-junB or anti-junD, Santa Cruz) at between 1:1000 to 1:1333 dilution for 1 hour at room temperature. To assess specificity of antibody binding, 2 ug of peptide was preincubated with antibody for 1 hour at 4°C (as recommended by Santa Cruz). Membranes were then washed 3 times for 10 minutes each. Goat-anti-rabbit horse-radish peroxidase (Sigma) was added (1:7500 dilution) for an additional hour and Immobilon filters were developed by Enhanced Chemiluminescence Renaissance, (Dupont, NEN).

2-9 Whole Cell Extraction and Immunoprecipitations

Subconfluent NIH3T3 cells were incubated in 0% calf serum overnight and whole cell extracts were prepared following treatment of cells for 15 minutes with murine Oncostatin M or without cytokine treatment. For whole cell protein extraction, media was removed and cells washed twice with cold 1x phosphate-buffered saline (PBS). Lysis buffer as described by Look et al. (1995) (50mM Tris

pH8, 150mM NaCl, 0.5% Nonidet P-40, 1mM EDTA, 1mM PMSF, 750 uM DTT, 1mM sodium orthovanadate, 10 mM sodium flouride, 2 mM sodium pyrophosphate, 10ug/ml leupeptin and 10ug/ml aprotinin) was then added to cells for 1 hour. Cell lysates were scraped from dishes with a rubber policeman and pelleted by centrifugation for 15 minutes at 3000 RPM. Supernatant was then collected for immunoprecipitation of SP1 and STAT proteins.

Two micrograms of anti-SP1 polyclonal antibody (SP1(PEP2-X), Santa Cruz Biotechnology) was incubated with collected supernatant with constant rocking for 1 hour at 4C. 20 ul of Protein G-agarose beads (Santa Cruz Biotechnology) were then added and incubation continued at 4C for 12 hours. Immunoprecipitates (protein-antibody-bead complexes) were then collected by centrifugation for 5 minutes at 2500 RPM. Supernatant was removed and beads were resuspended in 500 ul of lysis buffer and re-centrifuged (at 2500 RPM) 5 more times to wash immunoprecipitates of nonspecific binding proteins. Pelleted immunoprecipitates were then resuspended in 50ul of 4X SDS-PAGE loading buffer (0.25M Tris-Cl pH6.8, 20% 2-merceptoenthanol, .008% bromophenol blue, 8% SDS, 2.9% glycerol), boiled for 3 minutes, centrifuged for 5 minutes at 12000 RPM and supernatant (containing dissociated proteins and antibody) loaded onto an 8% SDS-PAGE gel. Gels were then Western Blotted. Individual lanes binding the same immunoprecipitates on the membrance were cut apart and membrane slices probed using anti-SP1 or anti-STAT antibodies to detect proteins bound.

CHAPTER 3

REGULATION OF TIMP-1 PROMOTER ACTIVITY BY HUMAN ONCOSTATIN M

3.1   Oncostatin M Upregulation of TIMP-1 and c-Fos mRNA Expression

OSM has been previously found to elevate TIMP-1 mRNA levels after overnight stimulation in a variety of cell types including HepG2 (Richards et al., 1993). Examination of TIMP-1 mRNA expression in HepG2 cells over a time course of OSM stimulation (Figure 3) revealed a 5 fold increase in TIMP-1 message by 1 hour, near maximal at 2 hours (40 fold) and maximal mRNA levels at 6 hours (60 fold). OSM also transiently stimulated mRNA levels of the AP1 immediate-early gene c-fos (Figure 3).   As previously demonstrated in response to other stimuli (Treisman, 1985), the upregulation of c-fos mRNA occurred early (maximal induction at 30 minutes (20 fold)) and decreased thereafter. Thus, c-fos mRNA levels peaked slightly before the marked increase in TIMP-1 mRNA.

3.2   Deletion Analysis of the TIMP-1 promoter: Mapping of OSM (and IL-6) responsive elements

The proximal promoter of the TIMP-1 gene (-95/+47 sequence) can regulate downstream CAT gene expression in pBLCAT3 chimeric reporter constructs

Figure 3.   OSM upregulates TIMP-1 and c-Fos mRNA levels.

HepG2 cells were treated with OSM from 15 minutes to 18 hours and RNA extracted and probed by Northern analysis for TIMP-1 or c-fos mRNA. Ethidium bromide (EtBr) staining of 28S and 18S ribosomal RNA is shown in the bottom panel to show equivalent loading of RNA samples.

-TIMP-1

- c-Fos
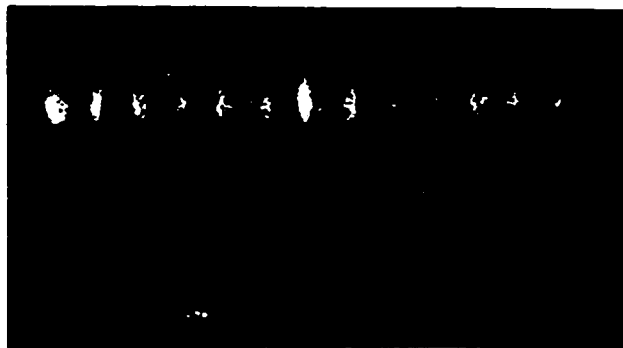
EtBr

0 .25 .5 1 2 6 18

(Edwards et al., 1992). Several putative DNA elements can be identified within -95 to +47 region of TIMP-1 including those for SP1, AP1 (fos/jun) and Ets proteins.

To examine the potential role of these sites in transcription, 5' deletions of this region were generated by PCR-directed deletion of TIMP-1/CAT chimeric constructs. HepG2 cells were cotransfected with constructs A to F (Figure 4) and pSV-2Gal to normalize for transfection efficiency. When transiently transfected into HepG2 cells, the expression of -95/+47 /CAT could be elevated upon OSM (5.2-fold) or IL-6 (3.8-fold) stimulation (Figure 4, construct A). Basal levels of CAT transcription were reduced in the plasmid lacking -95 to -63 sequences (construct B), and further reduced in the plasmid that also lacked the -62 to -53 sequences which contained the AP-1 site (construct C).

The -62/+47/CAT chimera (construct B) demonstrated maximal responsiveness to OSM with a 11.4-fold increase in CAT activity over unstimulated cells, whereas IL-6 induced 4.1-fold increases. Deletion of the AP1 site within this region (construct C) markedly reduced responsiveness to OSM (from 11.4 to 2.7 fold), while responses to IL-6 decreased from 4.1 to 2-fold. Thus, a promoter region from -62 to -53 of TIMP-1, containing a putative AP-1 binding site (at -59/-53), contributes to basal transcription and to induction of transcription by OSM. Deletion of this sequence removed any significant difference between OSM and IL-6 activity in this assay. Interestingly, deletion of sequences from +1 to +47 (construct E versus B) markedly abrogated the responsiveness to OSM (from 11.4 to 3.7 fold) and also

Figure 4. **Deletion analysis of the TIMP-1 promoter.**

Deletions of the TIMP-1 promoter spanning -95 to +47 were generated by PCR and cloned into pBLCAT3 to examine their capacity to regulate CAT reporter gene expression. HepG2 cells were co-transfected with each of the constructs (schematically illustrated at left) and pSV-2Gal and treated with either OSM (50 ng/ml) or IL-6 (100ng/ml) for 18 hours. Cellular extracts were then prepared and CAT activity was measured by standard methods and normalized to 2-galactosidase activity. Results are shown as percent conversion and fold induction upon cytokine stimulation. Values represent the mean of 3 separate experiments (standard deviation in parenthesis), each experiment done in duplicate.
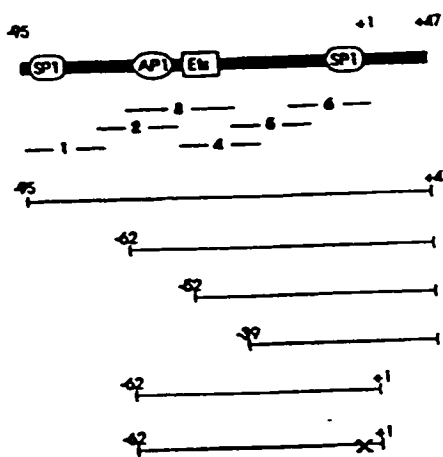
	CAT Activity				
	% Conversion			**Fold Induction**	
	Control	OSM	IL-6	OSM	IL-6
A	9.0	44.6	32.6	5.2(.8)	3.6(.4)
B	1.1	12.4	4.4	11.4(1.1)	4.1(.7)
C	0.4	1.1	0.8	2.7(.7)	2.0(.4)
D	0.2	0.2	0.3	1.1(.3)	1.6(.6)
E	1.7	6.9	3.9	3.7(1.1)	2.3(.5)
F	0.4	1.0	0.6	2.7(1.4)	1.4(.3)

reduced IL-6 responses (4.1 to 2.3 fold). Thus, sequences within +1/+47 may cooperate with the TIMP-1 AP1 element within -62/-53 for maximal responsiveness to OSM. SP1 elements are common within TATA-less promoters and have been demonstrated to participate in basal transcription (Dynan and Tjian, 1983). A chimeric plasmid with mutation of the SP1 site (construct F) showed reduced basal transcription (4-fold) but was still inducible by OSM (construct E versus F).

3.3 Nuclear factors bound to the TIMP-1 promoter

3.3.1 Analysis of AP1, Ets and STAT nuclear factor binding

We have examined a variety of overlapping oligonucleotide probes (#1-6, location shown in Figure 4) spanning -95 to +1 of the TIMP-1 promoter in EMSA analysis to identify elements which bind nuclear factors in HepG2 cells. A specific bandshift with either AP1 or AP1-Ets (probes 2 and 3) was seen in unstimulated cell nuclear extracts that we termed "complex 1" (Figure 5, panels A and B). At 12 hours of OSM treatment, much higher amounts of complex 1 are apparent. OSM induced the assembly of a second complex after 30 minutes of OSM stimulation with decreased mobility ("complex 2") that persisted for several hours. The mobility of complex 2 appeared to increase following two hours of OSM stimulation. The level of complex 2 increased in a dose-dependent manner (relative to complex 1) after 1 hour of OSM treatment (Figure 6). Concentrations of OSM between 20 and

Figure 5. OSM Induces binding of TIMP-1 AP1 probe complexes in a Time-Dependent Fashion.

Binding of nuclear factors to oligonucleotides (table 1) spanning the TIMP-1 promoter were tested by EMSA. The probes were: (A), AP1 [probe#2] , (B) AP1-Ets [probe #3], (C) Ets [probe #4] as well as the hSIE (D). HepG2 cells were treated with OSM from zero to 12 hours and nuclear extracts were prepared and stored at -70°C until analysis. EMSA gels were dried and subjected to autoradiography. Longer gel profiles (as seen in Fig. 4) showed no other specific bands, thus only the top of the gels are shown here.

0   .25   .5   1   2   4   8   12

TIMP-1 AP-1
complex 2 ⌐
complex 1 ⌐   A

TIMP-1 AP1-Ets
complex 2 ⌐
complex 1 ⌐   B

TIMP-1 Ets   C

hSIE
STAT 3/3 ⌐
STAT 1/3 ⌐   D
STAT 1/1 ⌐
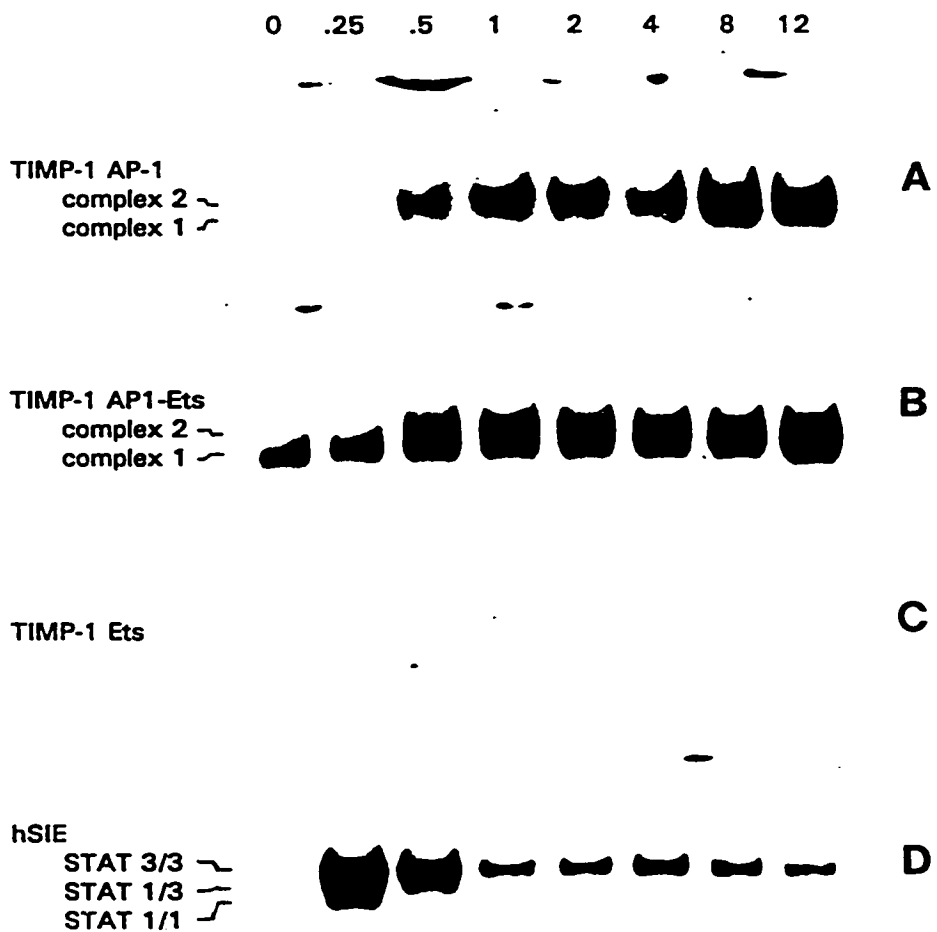
Figure 6.   OSM-dependent Dose Response of TIMP-1 AP1 Complex

Formation.

Nuclear extracts were prepared from HepG2 cells treated for 1 hour with varying doses of human OSM (0-50ng/ml) and probed for AP1 complex formation by EMSA analysis with a TIMP-1 AP1 DNA sequence. Both a constitutive (complex 1) and OSM-induced (complex 2) AP1 gel-shifts are observed.   Complex 2 formation is maximally induced within 10-50ng/ml of OSM after 1 hour of treatment. o = nonspecific complexes.

OSM(ng/ml): 0 .1 1 10 20 50

o
Complex 2 -
Complex 1 -

o

free probe -
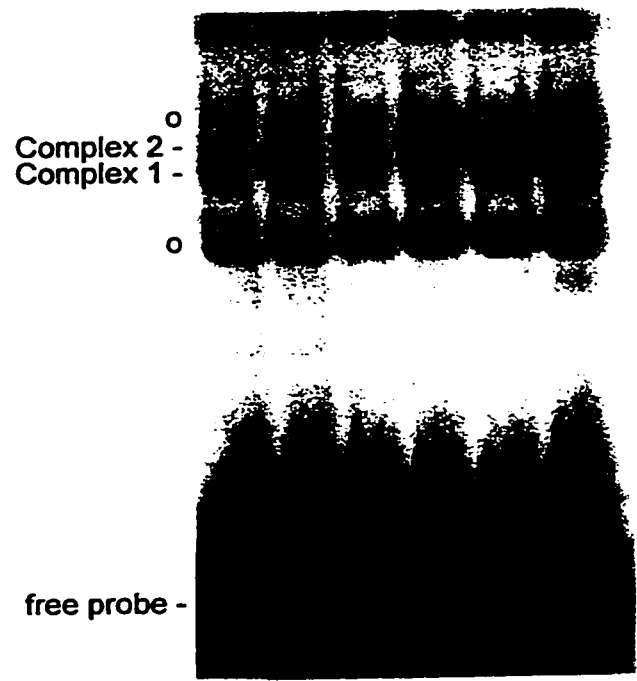
50ng/ml appeared maximally effective at this time point in inducing complex 2 formation.

Promoter sequences at -49 to -40 displayed an Ets core binding element as well as homology to STAT DNA binding sites (consensus TTCCNNNAA). Using a probe spanning this region (probe 4), a very weak binding activity was detected that was not altered upon OSM stimulation (Figure 5, panel C), and similar results were noted using the mouse TIMP-1 sequence (probe 4A) that has one base pair difference (data not shown). The mobility of this complex appeared similar to that of the AP1-complex 1 and no other complexes were detectable. Nuclear factors binding to probe 4 were specifically competed by cold Ets probe but supershift experiments using an anti-Ets-1/Ets-2 polyclonal antibody reactive against a highly conserved DNA binding domain of Ets proteins did not identify Ets-1 or Ets-2 binding to this probe (data not shown). STAT-1 and 3 activation can be detected using the high affinity sis-inducible element (hSIE) (Sadowski et al, 1993). Using the hSIE and the same HepG2 extracts as above, we noted that treatment with OSM resulted in rapid nuclear factor binding with gel shift mobilities consistent with those observed for homodimers of STAT3 or STAT1 and the heterodimer of STAT3 with STAT1 (Figure 5, panel D). Each of these complexes were verified using antibodies to STAT3 or STAT1 in EMSA supershift assays as previously established (Figure 7). STAT1 was detected at early time points, while STAT3 persisted from 15 minutes to 12 hours of OSM treatment. However, neither the AP1-Ets or Ets

Figure 7. STAT1 and 3 EMSA Supershifts of the hSIE probe.

Binding of STAT nuclear factors to the high affinity sis-inducible element (hSIE) using untreated or human OSM treated (for 15 minutes) HepG2 cell nuclear extracts in binding reactions for EMSAs was examined by supershift analysis. 1ug of anti-STAT1 or 2ug of anti-STAT3 antibody was added to binding reactions following the incubation of nuclear factors with hSIE DNA probe, and ternary nuclear factor-DNA-antibody complexes detected by their reduced electrophoretic mobility (supershift) relative to the absence of antibody on EMSA gels. STAT1 and 3 is readily supershifted from hSIE probe complexes. No complexes are detected in the absence of nuclear extract in DNA probe binding reactions (NE = no extract). Three complexes bind the hSIE probe from OSM-treated cells: homodimers of STAT3 (slowest migrating complex), heterodimers of STAT3 and 1 (intermediate gel-shifted complex), and homodimers of STAT1 (faint but fastest detected mobility gel shift). (o = nonspecific complex.)

OSM: -   +   +   +   NE   NE

Anti-STAT1:   -   -   +   -   +   -

Anti-STAT3:   -   -   -   +   -   +
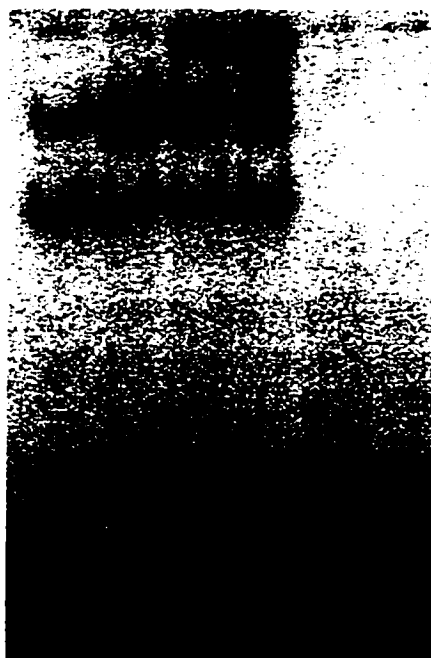
-STAT1 or 3 supershift

STAT   3/3 -   3/1 -   1/1 -

o

free probe -

sequences (probes 3 and 4) detected complexes with the same mobility and kinetics as compared to the hSIE in response to OSM.

The TIMP-1 AP-1/Ets (probe #3) complexes and STAT mobility shifts were specifically competed by the 50 fold addition of unlabelled probe #3 or hSIE probe, respectively (Figure 8). Neither AP-1 complexes 1 or 2 could be eliminated by competition with unlabelled hSIE probe (Figure 8) nor supershifted with anti-STAT1 or anti-STAT3 (data not shown). EMSA analysis with probe 3A, containing mutation of nucleotides in the AP-1 site, dramatically reduced complexes 1 and 2, and other specific complexes were not detectable (Figure 9) over the time course of OSM stimulation. Thus, neither STAT nuclear factors associated with the AP-1 complexes 1 and 2, nor binding to sequences immediately flanking the TIMP-1 AP1-like element that show partial homology to a STAT DNA binding site could be detected. Further downstream sequences showed weak homology to GAS (differing from the consensus GAS site by a two nucleotide insertion), however probe 5 (-38/-15) elicited no early or late induced gels shifts similar to STAT complexes (data not shown). Thus, we could not detect STAT-1/3 nuclear protein binding to TIMP-1 promoter sequences in these cells.

Figure 8. Cold Competition for AP-1 and STAT Nuclear Factor Binding.

Nuclear extracts from HepG2 cells stimulated for 0.25 (15 minutes) or 1 hour were probed with AP-1/Ets (probe #3) or the SIE probe in EMSA analysis. A 50 fold excess of unlabelled cold competitor-oligonucleotide was added as indicated. Complexes1 and 2 were competed by cold probe #3 but not by SIE, whereas cold SIE competed for labelled SIE. The open circle (o) indicates the position of non-specific complexes.

PROBE	AP1-Ets			hSIE				
IFN-γ stimulus	-	-	-	-	-	.25	-	.25
OSM stimulus	1	1	1	-	.25	-	.25	-
50 x cold comp.	-	AP1	SIE	-	-	-	hSIE	hSIE

complex 2 -
complex 1 -

STAT
complexes

free AP1
probe -

- free hSIE
probe
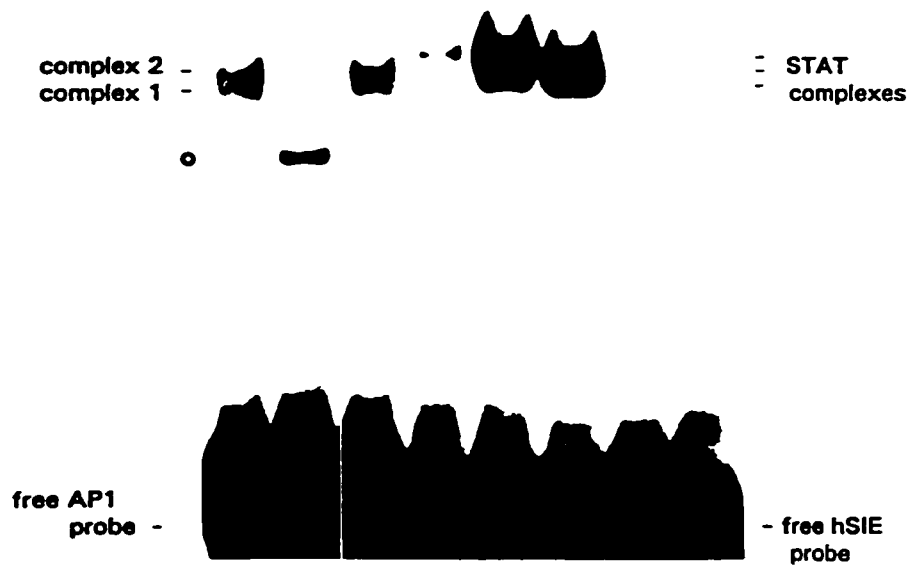
Figure 9. Mutation of the AP1 site in the AP1-Ets EMSA probe.

HepG2 cell nuclear extracts prepared from cells treated for 0, .25, 1 and 12 hours were probed with either a wildtype AP-1/Ets probe (probe #3) or mut AP-1/Ets probe containing mutations in the AP-1 site (probe #3A). The open circle (o) represents nonspecific binding. Mutation of the AP-1 site removed any detectable binding of OSM-inducible nuclear factors to this probe. STAT factor activation in nuclear extracts was confirmed using the hSIE probe at 0 and .25 time points after OSM stimulation.

3.3.2 EMSA supershift analysis of factors binding the TIMP-1 AP-1 site:

Detection of Fos and Jun nuclear binding factors

Supershift analysis of TIMP-1 AP-1 bands were carried out to identify fos and jun components of these complexes. Nuclear extracts were prepared from HepG2 cells treated with OSM for 0, 1 and 12 hours and incubated with polyclonal antibodies specific for AP-1 proteins prior to EMSA analysis. Using an antibody reactive against all fos-related antigens (anti-pan-fos), complex 1 was obliterated at zero hours and a fos-supershift is clearly visible at the 12 hour time point (Figure 10, panel A). The anti-pan fos antibody also supershifted complex 2 from HepG2 cells treated with OSM for 1 hour (Figure 10, panel A). However, when an anti-c-fos specific antibody was used, only complex 2 was supershifted and complex 1 remained unaffected, suggesting that c-fos is a prerequisite for the OSM-stimulated assembly of complex 2. Of the anti-jun antibodies used in EMSA binding reactions, anti-junD and anti-junB supershifted TIMP-1 AP1 probe complexes (Figure 10, panel B). JunD was detected at time 0, 1 and 12 hours of OSM treatment. JunB could also be detected in these complexes while c-jun could not be detected at any time. Together, this data suggest that while complex 1 consists of fos and jun, complex 2 specifically contains c-fos, which likely complexes with junD or junB.

Figure 10. Detection of c-fos and Jun nuclear factors bound to the Timp-1 AP-1 element by supershift analysis.

Cells were stimulated with OSM for 0, 1 or 12 hours and nuclear extracts prepared. (A) Anti-pan-fos (reactive against all fos-related antigens) or anti-c-fos antibody was used in supershift analysis of EMSA using the TIMP-1 AP-1 (probe #2) in binding reactions. EMSAs were then carried out to resolve gel-shifted and supershifted complexes on a 5% polyacrylamide nondenaturing gel. (B) Supershift analysis was carried out using antibodies to c-jun, jun B, jun D in binding reactions with nuclear extract of 1 hour OSM-stimulated cells and the AP-1 probe#2. Antibody was added to the reactions without extract (right 3 lanes, no extract) to show no nonspecific binding to probe. The mobility of nonspecific proteins binding probe #2 is indicated by an open circle (o).

A

OSM (hours): 0 1 12

| α-pan-Fos | - | + | - | - | + | - | - | + | - |
| α-c-Fos | - | - | + | - | - | + | - | - | + |

complex 2
complex 1

- Fos
supershift

B

Con +OSM No extract

α-c-jun	-	-	+	-	-	+	-	-
α-junB	-	-	-	+	-	-	+	-
α-junD	-	-	-	-	+	-	-	+

- junD supershift
- junB supershift
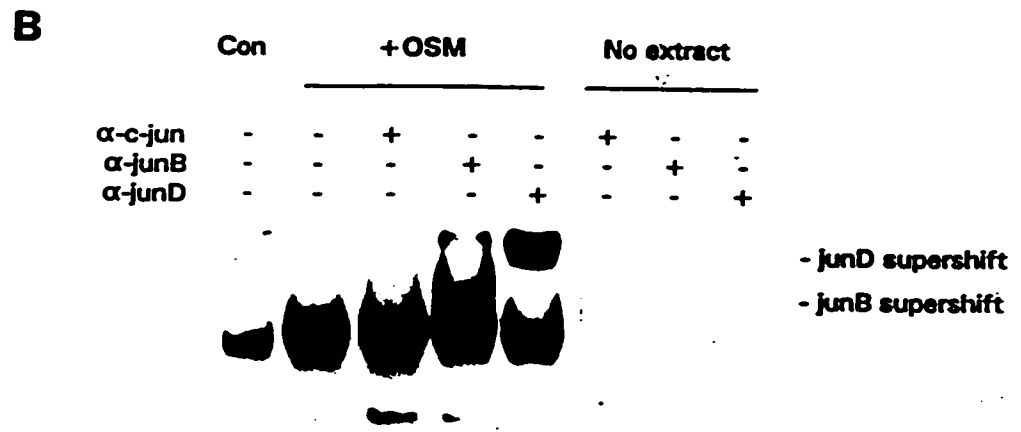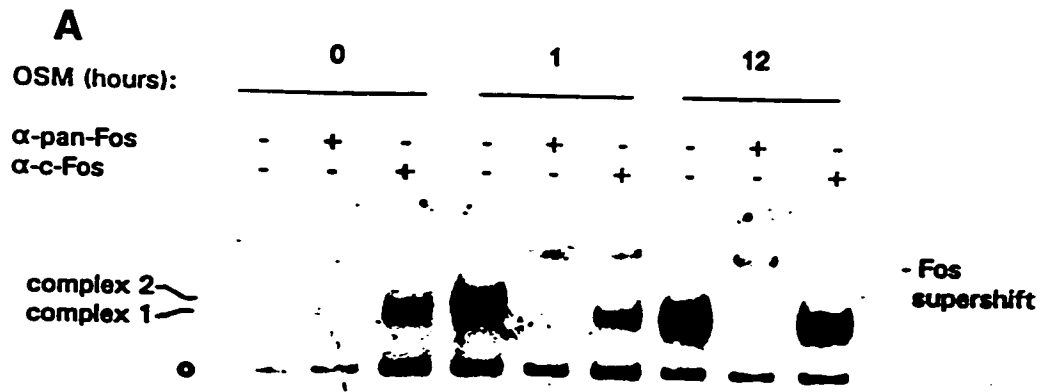
3.3.3 Binding of SP1 nuclear factors to the TIMP-1 promoter

Since sequences within -95 to +1 contained two putative SP1 binding sites, both of these were examined for the binding of HepG2 nuclear factors. Oligonucleotides spanning -95 to -66 (probe 1) or -19 to +2 (probe 6), constitutively bound two specific complexes which remained unchanged in response to OSM treatment (Figure 11A and B). Both complexes were competed by cold SP1 probe (Figure 11A) and are consistent with the mobility of SP1 nuclear factor binding to a consensus SP1 DNA element (data not shown). Anti-SP1 antibody supershifted the slower-migrating complex but did not appear to affect the faster-migrating specific complex (Figure 11C). This may indicate the binding of other SP1 family members (Hagen et al., 1992). Binding of SP1 to the -95/-66 oligonucleotide was only detectable following a one week exposure of EMSA gels (data not shown) although, weak binding to this probe may be due to insufficient sequence flanking the 5' end of the SP1 site. Thus, SP1 nuclear factors constitutively occupy binding sites within the TIMP-1 promoter and OSM does not appear to affect the SP1 DNA binding.

Figure 11. SP1 nuclear factor binding to the TIMP-1 promoter.

(A) An excess of cold specific unlabelled competitor oligonucleotides (cold SP1) or irrelevant competitor (cold AP-1/Ets, probe #3) were used to identify specific binding of SP1 (probe #6) gel-shifted complexes. Free probe migrates at the bottom of the gel.   (B) Nuclear extracts from cells stimulated with OSM for 0, 0.25, 1 or 12 hours were incubated with a TIMP-1 putative SP1 DNA-binding element (probe #6) binding examined by EMSA analysis. (C) Anti-SP1 antibody was used to supershift SP1 binding to probe #6. Supershifts were inhibited by preincubation of anti-SP1 antibody with SP1 peptide but not by an irrelevant peptide (fos peptide).   The open circle (o) represents the position of nonspecific complexes.

A

+ cold SP1   + cold AP1-Ets

0   500   250   100   500   250   100

SP1-

o

B

+OSM

0   .25   1   12

-SP1

C

+   +   +   anti-SP1
            +   fos peptide
                  +   SP1 peptide

- SP1 supershift
- SP1

o
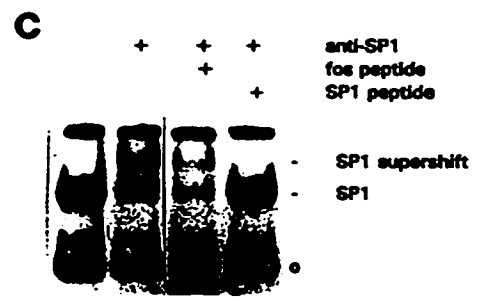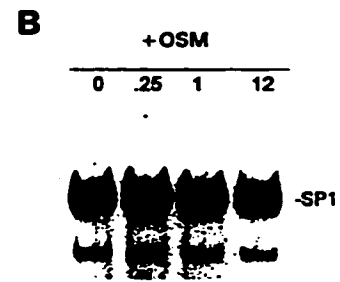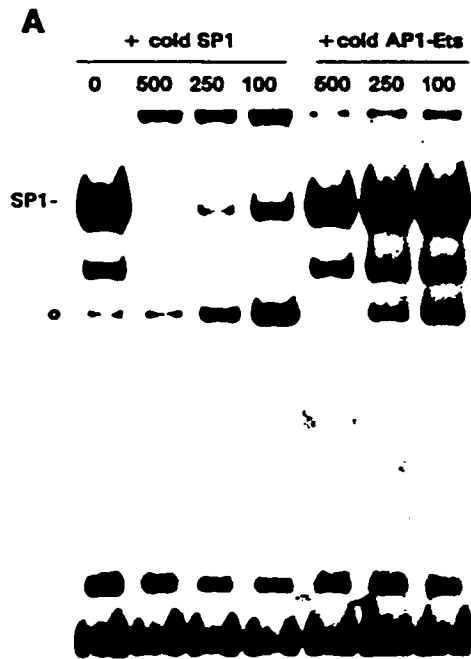
3.4 OSM is unique among cytokines tested in effecting the TIMP-1 promoter

3.4.1 OSM but not other cytokines induces TIMP-1 AP-1 Complex 2

Given the overlapping biological functions of OSM and IL-6, these and other cytokines were examined together for their ability to induce DNA binding of complexes to the TIMP-1 AP-1 probes in EMSAs. Interestingly, OSM was the only cytokine examined that strongly induced complex 2 (Figure 12, panel A) whereas both OSM and IL-6 markedly induced activation of STAT binding to the hSIE probe (panel B). Thus, within the same assay, OSM and IL-6 utilize shared (STAT) and distinct (AP-1-complex 2) nuclear signalling pathways leading to protein-DNA interactions.

3.4.2 OSM but not IL-6 or PMA markedly regulates TIMP-1 promoter activity

Phorbol-13-myristate-12-acetate (PMA) has been shown to activate AP-1 (Karin et al., 1997) and stimulate TIMP-1 expression (Murphy et al., 1985). When tested in HepG2 cells, PMA potently stimulated complex 2 formation (AP-1 probe 2) and to a greater extent than OSM (Figure 12, panel A). To examine the activity of PMA on transcription, we compared the effectiveness of PMA to OSM or IL-6 in stimulating CAT activity of the TIMP-1 -62/+47 reporter gene construct B. Table 2

Figure 12. Selective induction of complex 2 formation and c-fos upregulation by OSM and PMA.

Induction of complex 2 formation (and STAT activity) by OSM (50ng/ml), was compared with IL-6 (100ng/ml), IFN-3 (500units/ml), IL-12 (5ng/ml) and PMA (50nM). Stimulation of DNA-binding activity was tested using (A) probe #3 that contains the TIMP-1 AP-1 sequence, or (B) the hSIE probe. Protein levels of c-fos in HepG2 nuclear extracts (15 ug) of cells treated with the above cytokines or PMA was examined by Western blotting using an anti-c-fos specific primary antibody and a goat anti-rabbit horse-radish peroxidase secondary antibody (C). c-fos protein was detected by enhanced chemiluminescence followed by autoradiography.

time(h)	0	OSM .25	IL-6 .25	OSM 1	IL-6 1	IFNγ 1	IL-1β 1	PMA 1

TIMP-1 AP-1 probe
(EMSA)

complex 2 ⌐
complex 1 —

A

hSIE probe
(EMSA)

STAT 3/3 ⌐
STAT 3/1 ⌐
STAT 1/1 ⌐

B

c-fos protein
(Western)

c-fos ⌐

C
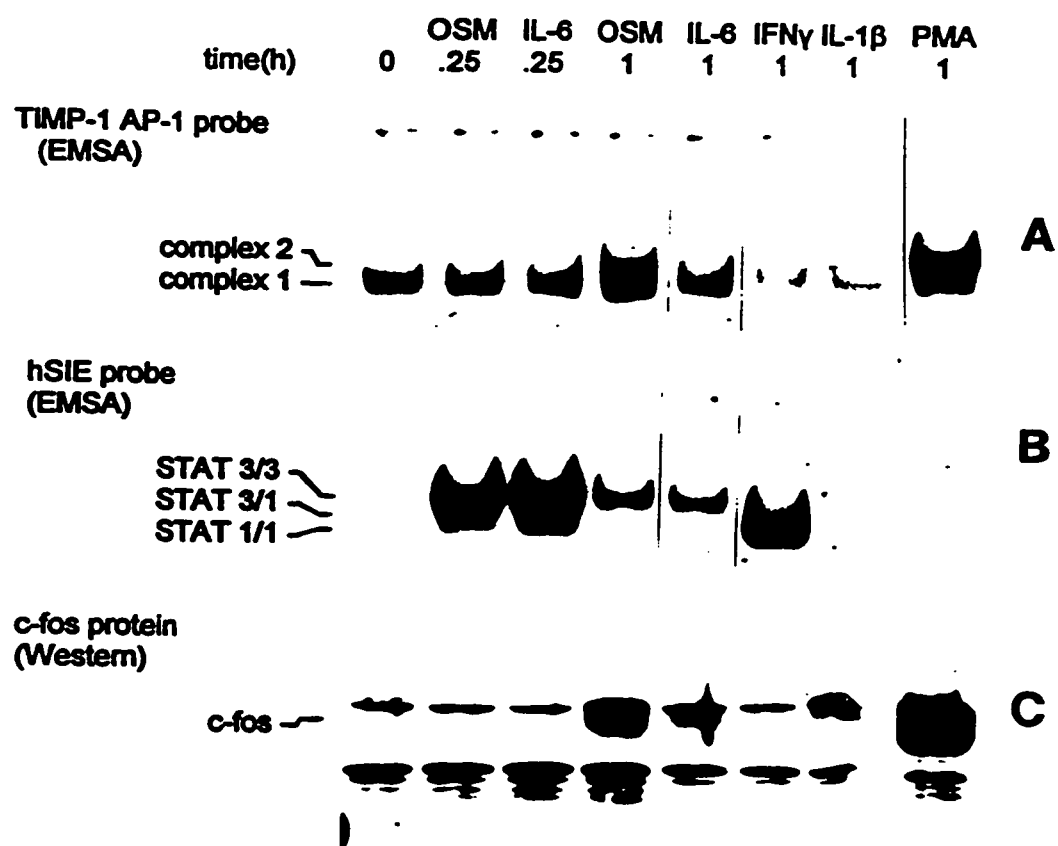
Table 2. **Regulation of -62/+47CAT Promoter Activity by OSM and IL-6 but not PMA.**

HepG2 cells were transfected with 10 ug of -62/+47CAT, allowed to recover overnight and replated in 6-well Costar plates. The cultures were then serum starved for 6 hours and stimulated with OSM, IL-6, PMA, IL-6 and PMA, or 20% fetal bovine serum (FBS) for 18 hours in serum-free conditions. CAT activity was assayed as described in methods. Basal levels of percent converted chloramphenicol were 1.59% ± 0.03%. Fold change was calculated from phosphorimagery results and averaged from at least 3 separate experiments ± the standard deviation.

Stimulus	Fold change over control
OSM (20 ng/ml)	10.5 ± 3.6
IL-6 (20 ng/ml)	4.2 ± 1.0
PMA (50 nM)	0.9 ± 0.2
20% FBS	0.8 ± 0.2
IL-6 (20 ng/ml) + PMA (50nM)	3.6 ± 0.6

shows that PMA alone (or 20% FBS) was unable to upregulate CAT activity through the TIMP-1 -62/+47 promoter element whereas OSM induced 10-fold increases and IL-6 induced 4-fold changes. When used in combination with IL-6, PMA also failed to stimulate CAT activity beyond that of IL-6 alone. Thus, despite equivalent STAT activation and c-fos induction as in OSM-treated cells, the combination of IL-6 and PMA was not sufficient to induce similar levels of transcriptional activation. OSM may induce qualitative differences in AP1 factors distinct from that by PMA and/or requires additional regulatory elements for the pronounced induction of the TIMP-1 -62/+47 reporter gene construct beyond that induced by IL-6.

3.4.3 Co-transfection of the OSM-specific type-II receptor in Hep3B cells regulates the TIMP-1 promoter

Human OSM binds a receptor complex originally characterized as the LIF receptor, an OSM-type I receptor, and a second OSM-specific receptor (type II receptor) that consists of gp130 and the OSM-2 receptor subunt. Biological effects unique to OSM are due to the OSM-specific type II receptor complex. In collaboration with Heinz Baumann (Roswell Park Cancer Institute, Buffalo) we examined whether the OSM type II receptor can signal TIMP-1 transcription independent of the LIF receptor, the Hep3B hepatoma cell line (which lacks endogenous LIF receptor and OSM-2 receptor expression) was cotransfected with

an OSM-2 receptor over-expression vector and the TIMP-1(-62/+47)/CAT chimeric construct, and CAT reporter gene expression was measured to assess TIMP-1 promoter activation. Cotransfection of these vectors led to a marked induction (3.6-fold) of CAT activity in OSM-stimulated Hep3B cells (Figure 13). Transfection of Hep3B cells with OSM-2 receptor with TIMP-1/CAT chimeras lacking the TIMP-1 AP-1 site effectively abolished activation of CAT activity in response to OSM (Figure 13). Deleting part of the cytoplasmic region of the OSM-2 receptor (amino acids 142 to 214) also eliminated regulation of AP-1-containing TIMP-1 promoter/CAT chimeras (Figure 13). Thus, in Hep3B cells the OSM-specific type II receptor is capable of eliciting signals for regulating the TIMP-1 promoter independent of LIF (OSM-type I) receptor expression.

3.5 Oncostatin M-mediated stimulation of TIMP-1 mRNA and c-fos protein expresssion is dependent upon de novo protein synthesis

3.5.1 Upregulation of TIMP-1 mRNA by OSM is dependent upon new protein synthesis

To assess whether OSM-mediated expression of TIMP-1 mRNA is protein synthesis dependent, HepG2 cells were treated with OSM for 0, 1 and 6 hours in the absence or presence of the protein synthesis inhibitors puromycin and emitine and TIMP-1 mRNA expression examined by Northern Blotting. As demonstrated

Figure 13.   OSM-specific Type-II receptor activation of the TIMP-1 promoter.

Hep3B cells (lack endogenous OSM Type I (LIF) and II receptors) were cotransfected with the OSM-specific Type-II receptor-2 subunit and the chimeric TIMP-1 -62/+47/CAT construct (contains AP1 site) or the TIMP-1 -52/+47/CAT construct (lacks AP1 site) and cells remained unstimulated or were treated with human OSM for 12 hours. Cells were also transfected with either of the two TIMP-1/CAT constructs and a truncated form of the OSM receptor-2 subunit which lacks 72 amino acids of the carboxy-terminal cytoplasmic domain. Whole cell extracts were prepared and CAT reporter gene expression was assessed by standard methods.   OSM upregulated reporter gene expression of OSM-2 receptor/TIMP-1 AP1/CAT cotransfected cells while truncation of the OSM-2 receptor cytoplasmic domain or deletion of the TIMP-1 AP1 site of TIMP-1/CAT chimeric constructs abolished OSM induction of TIMP-1 promoter activity.
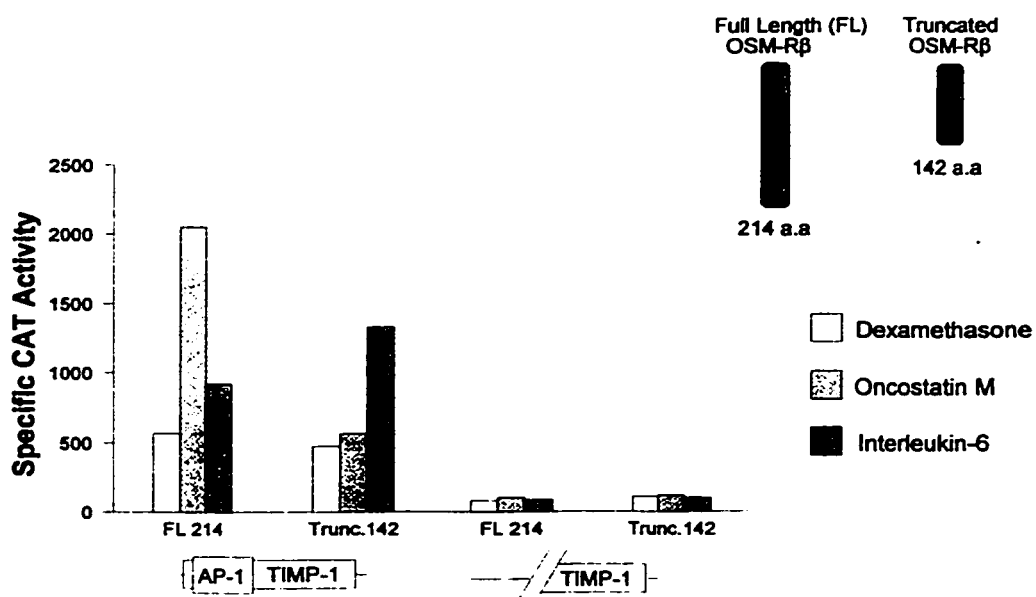
above, TIMP-1 mRNA levels increased after 1 and 6 hours of OSM treatment (5-fold and 60-fold, respectively; Figure 14). However, in the presence of the protein synthesis inhibitors puromycin and emitine, OSM-stimulated TIMP-1 mRNA expression was abolished (Figure 14). Thus, new protein synthesis is required for OSM-mediated increases in TIMP-1 mRNA.

3.5.2 Regulation of AP-1 (fos/jun) protein expression levels by OSM. c-Fos nuclear protein expression is dependent on new protein synthesis

As the data implicate c-fos in gel-shifted TIMP-1 AP1 complex 2, and OSM markedly stimulated early transient expression of c-fos mRNA (Figure 3), c-fos protein levels were examined in nuclear extracts from HepG2 cells by Western blots (Figure 12, panel C). Also, since OSM-mediated TIMP-1 mRNA expression is dependent upon new protein synthesis, the effect of protein synthesis inhibitors were tested on c-fos and additional AP-1 factors.

In the same extracts used for EMSA, OSM and PMA markedly upregulated c-fos protein levels in HepG2 cell nuclei. Expression of c-fos protein was abrogated in the presence of protein synthesis inhibitors (puromycin and emitine) (Figure 15, panel B), and in addition, these agents blocked complex 2 formation in response to OSM (Figure 15, panel A). Complex 1 formation was not affected at 0.25 or 1 hour, however, the inhibitors did reduce complex 1 at 12 hours which suggested

Figure 14. Protein Synthesis-Dependent Upregulation of TIMP-1 mRNA in Response to human OSM.

Total RNA was extracted from HepG2 cells untreated or treated with OSM and/or the protein synthesis inhibitors puromycin and emitine (P+E) for 0.5, 2 or 6 hours. The levels of human TIMP-1 mRNA was examined by Northern analysis using standard methods. Equivalent loading and transfer of RNA samples was assessed by ethidium bromide (EtBr) staining for 28S and 18S ribosomal RNA on Northern blot membranes.

| OSM (hrs): | 0 | .5 | 2 | 6 | .5 | 2 | 6 | - | - | - |
| P+E (hrs): | - | - | - | - | .5 | 2 | 6 | .5 | 2 | 6 |

A Northern

TIMP-1 -

B EtBr

28S -

18S -
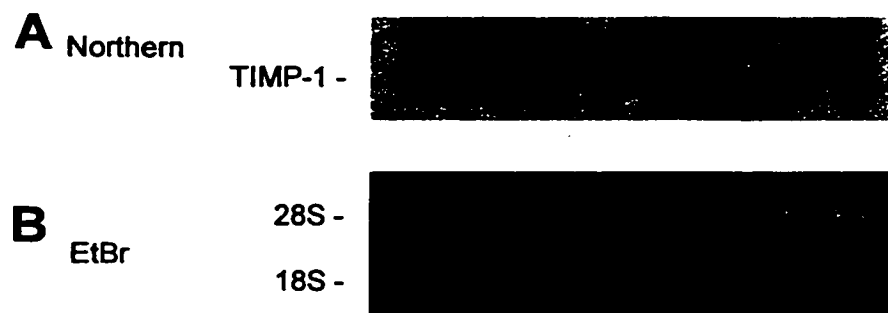
Figure 15. **Effect of OSM on TIMP-1 AP-1 complexes and nuclear c-Fos, JunB and JunD protein accumulation and the influence of protein synthesis inhibitors.**

AP-1 complexes (probe #2) were examined by EMSA analysis (A) and the same extracts assessed by Western blots for c-fos (B), JunB (C), and JunD (D) protein levels from HepG2 nuclear extracts of cells treated with OSM (50ng/ml) for the indicated times above. A requirement for new protein synthesis of TIMP-1 gel-shift complexes and nuclear AP1 protein levels was assessed by the addition of the protein synthesis inhibitors puromycin and emitine (PE), each used at 20ug/ml. Antibody specificity in Westerns was assessed by using relevant peptide competitors as noted in methods. Stars denote the position of bands that were competed by relevant peptide but not irrelevent peptides for c-fos, JunB and JunD.

| | | | - P/E | | | +P/E | |
| +OSM (h) | 0 | .25 | 1 | 12 | .25 | 1 | 12 |

EMSA (AP1 probe)

complex 2
complex 1

A

Westerns

c-fos

B

JunD

C

JunB

D
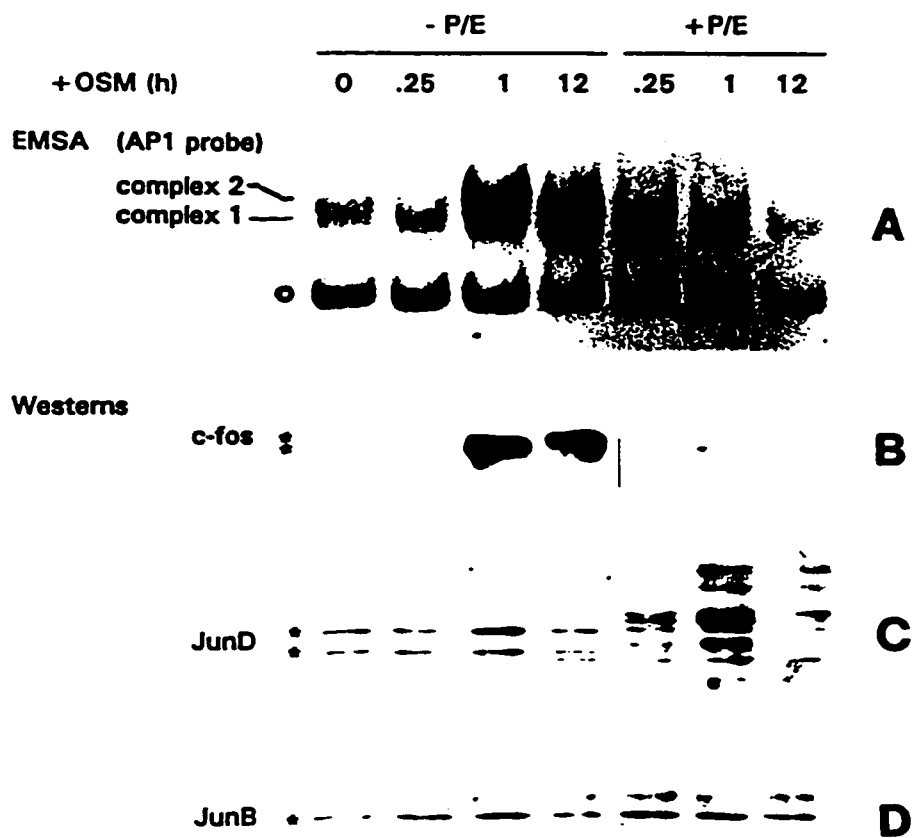
newly synthesized proteins are involved in complex 1 at this later time point. OSM stimulated nuclear accumulation of c-fos by 1 hour which persisted to 12 hours of OSM treatment (panel B). Interestingly, the electrophoretic mobility of c-fos was reduced at 12 hours when compared to 1 hour of OSM stimulation. This difference in mobility is first observed after 2 hours of OSM treatment (Figure 16), persists for up to 12 hours, and may represent a change in the phosphorylation status of c-fos in these cells.

In contrast to c-fos, and consistent with their presence in AP1 gel-shifted complexes, both junB and junD were constituitively present and junD was moderately upregulated at the 1 hour time point of OSM treatment (Figure 15, panels C and D). Puromycin and emitine had no effect on junB at early time points of OSM stimulation and only affected junD levels after 12 hours of treatment. Detection of c-fos, junB and junD in Westerns was confirmed by specific competition of relevant peptides for each of the antibodies used and c-jun was undetectable from the same nuclear protein extracts (data not shown). Thus, OSM stimulates the nuclear accumulation of c-fos protein while junB and junD nuclear factors are constitutively resident within the nucleus and appear largely unaffected by OSM at the level of new protein synthesis.

Figure 16. Time Course of c-Fos induction in Response to OSM.

HepG2 cell nuclear extracts were prepared following a time course of treatment with OSM for zero to 12 hours. 15ug of extract was loaded onto an 8% SDS-PAGE gel and protein Western blotted and probe for c-Fos with a polyclonal anti-c-fos antibody. C-Fos (3 bands) is detectable after 30 minutes of cytokine treatment, its mobility decreases after 1 hour and persists for 12 hours. o = nonspecific bands.

OSM(hrs):   0   .25   .5   1   2   4   8   12
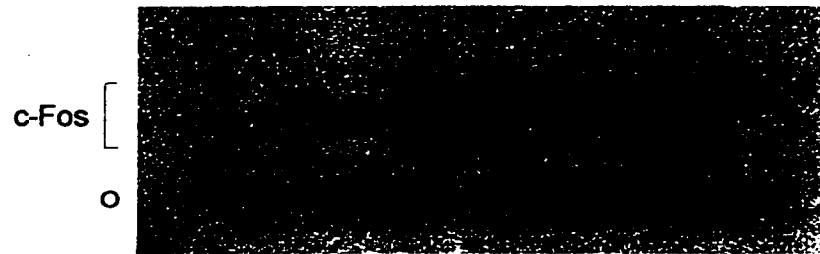
3.6   OSM-mediated TIMP-1 AP1-Complex 2 formation and c-Fos expression in Fibroblast and Neuronal cells

3.6.1 AP-1 complex 2 and c-Fos expression in primary human lung fibroblasts

TIMP-1 mRNA levels are upregulated by OSM in a variety of connective tissue cells, including primary human lung fibroblasts (HLF) (Richards et al., 1993). The presence of TIMP-1 AP-1-complex 2 formation and c-Fos expression was therefore examined following the treatment of primary HLF cells with human OSM. Consistent with findings in the heptatoma cell line HepG2, OSM stimulated the formation of a second complex ("complex 2") of slower mobility (Figure 17, panel A) after 1 hour in HLF cells that was concurrent with the upregulated expression of c-Fos protein levels (Figure 17, panel C).   STAT binding to the hSIE probe was also detected in these cells over a time course of OSM treatment (Figure 17, panel B). However, in contrast to HepG2 cells, STAT1 homodimers (as opposed to STAT3) remained the predominant species at early time points of OSM treatment.   Thus, similar to observations in human hepatocytes (HepG2 cells), OSM-inducible TIMP-1 AP-1 and STAT complexes are detectable from nuclei of HLF cells.

Figure 17. OSM induction of AP1 complexes, STAT and c-Fos in primary
human lung fibroblasts.

Nuclear extracts were prepared from primary human lung fibroblasts treated
with human OSM for 0, 0.5, 1, 2 and 8 hours, and probed for AP1 or STAT binding
activity (A and B, respectively) by EMSA analysis. A TIMP-1 AP-1 DNA probe was
used to detected AP1 complexes and the hSIE DNA probe was used to detected
homodimers of STAT 1 and 3, and STAT 1/3 heterodimers. A constitutive AP1
complex is detected in the absence of OSM treatment and a second complex
(complex 2) is transiently activated following 1 and 2 hours of OSM treatment.
STAT activation is observed after 30 minutes, STAT3 dimers persist for 8 hours
while STAT1 dimers are absent at this time point. C. Western analysis for c-fos
was carried out using the same extracts described above. C-Fos is detected
following 2 hours of OSM treatment and its mobility is increased at 8 hours.

OSM(hrs): 0 .5 1 2 8

A

Complex 2 -
Complex 1 -

B

STAT 3/3 -
3/1 -
1/1 -

C
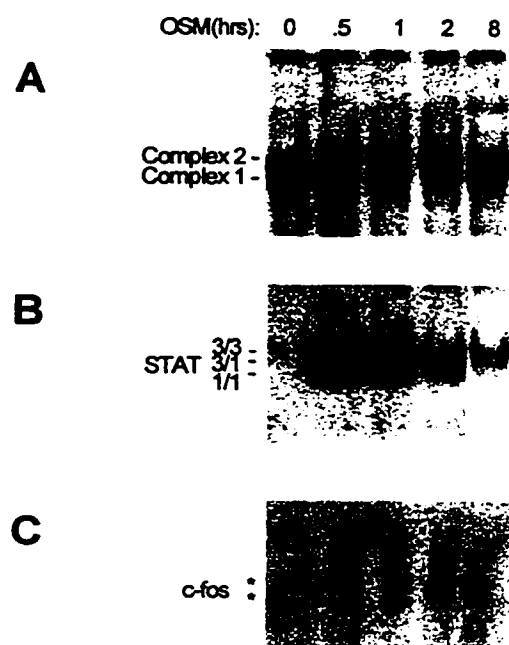
c-fos :

3.6.2 AP-1/c-Fos regulation in synovial fibroblasts, WI-26 lung fibroblasts and the SY5Y neuronal cell line

Several human cell lines, in addition to HepG2 and primary HLF cells, were tested for the induction of AP-1 complex 2 formation and c-Fos expression in response to OSM. Among these were SV-40 transformed lung fibroblasts (WI-26) and the neuroblastoma cell line SY5Y. TIMP-1 AP-1 complex 1 was readily detected in unstimulated and OSM-treated (for 1 hour) WI-26 (Figure 18) and SY5Y cells (Figure 19). However, both cell lines lacked the presence of OSM-induced AP-1 mobility shifts. This disparity may reflect the in vitro nature of these cell lines (primary HLF cells versus immortal WI-26 cells) and/or tissue specific differences between neuronal and fibroblast/hepatocyte cells.

Consistent with HepG2 and HLF cells, the WI-26 and SY5Y cell lines were capable of upregulating the expression of c-Fos protein in response to OSM (Figure 18 and 19, panel C). The expression of c-Fos protein was transient and maximal after 1 hour of OSM treatment.

OSM and members of this cytokine family; LIF, IL-6, IL-11, CT-1, as well as the phorbol ester PMA were all tested on SY5Y cells for c-Fos expression following 1 hour of treatment. As observed in HepG2 cells, OSM and PMA remained the most potent inducers of c-Fos expression in SY5Y cells. This is unlikely to represent differences in the surface expression levels of OSM-related cytokine

Figure 18. AP1, STAT and c-Fos in WI-26 human lung fibroblasts.

Nuclear extracts were prepared from WI-26 human lung fibroblasts treated with human OSM for 0, .25, 1 and 8 hours and probed for AP1 and STAT DNA binding activity and c-fos expression as described in Figure 15. 100-fold excess of cold unlabelled competitor (CC) DNA (TIMP-1 AP1 DNA competitor for AP1 complexes and cold hSIE competitor against STAT complexes, for part A and B, respectively) was used to confirm specificity of DNA-protein complexes. o = nonspecific complexes. A single specific AP1 complex is constitutively detected and no inducible AP1 complex(es) are observed. STAT activation is seen after 15 minutes and c-fos expression is transiently detected after 1 hour of OSM treatment.

CC
⌐¬
OSM(hrs): 0   .25   1   8   1

A

AP1 -

o

B

STAT 3/3 =
     3/1 :
     1/1 =

o

C
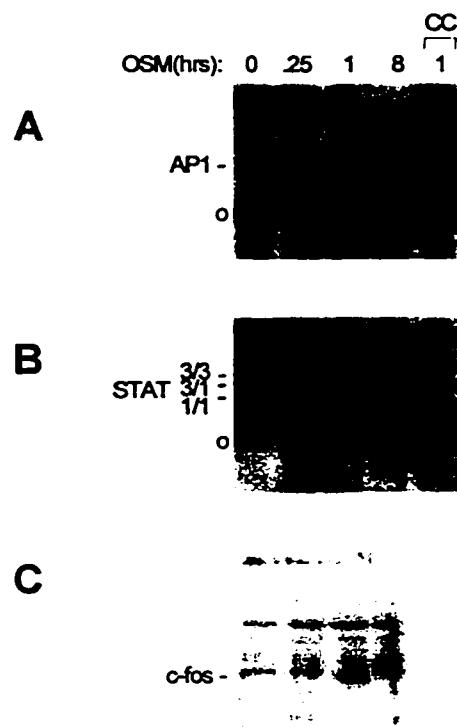
c-fos -

Figure 19. **Induction of c-Fos in Neuroblastoma SY5Y cells is unique to OSM among IL-6-type cytokines.**

SY5Y cells remained untreated or were treated with OSM, IL-6, LIF, CT-1 IL-11 or PMA for 1 hour. Nuclear extracts were prepared and probed for AP-1 and STAT DNA binding activity by EMSA analysis using the TIMP-1 AP-1 or hSIE probe, respectively (part A and B, respectively). Constitutive AP-1 binding is detected and no marked changes are observed following cytokine or PMA treatment (part A). OSM and LIF markedly induce STAT DNA binding activity to the hSIE probe, especially STAT1 homodimers (part B). Specific AP1 (A) and STAT (B) complexes were competed by 100X cold competition (CC) with relevant (rel.) double stranded oligonucleotide (TIMP-1 AP1 DNA for AP1 complexes (part A) or hSIE for STAT complexes (part B)) to demonstrate specific binding or with irrelevant (irrel.) oligonucleotides as a negative control (unlabelled hSIE competitor for AP1 complexes (part A) and unlabelled TIMP-1 AP1 competitor for hSIE probe (STAT) complexes). o = nonspecific complexes. C. c-Fos expression, from nuclear extracts prepared in part A and B, was detected by Western analysis using a polyclonal anti-c-fos antibody. Among IL-6-type cytokines tested, only OSM induced c-fos expression. PMA could also upregulate c-fos protein levels.
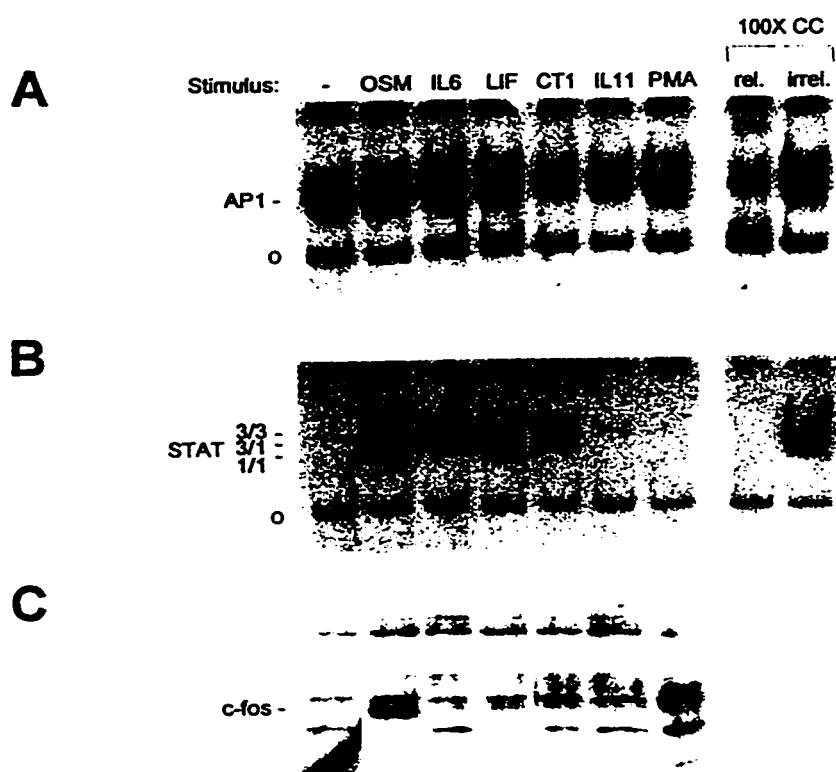
receptors, as OSM-related cytokine family members; notably, OSM, LIF and IL-6, were equally capable of inducing STAT complexes (predominately STAT1 dimers) onto an SIE element DNA probe (Figure 19, panel B). This data suggests that OSM primarily utilizes the OSM type- II specific receptor as opposed to the type-I receptor, the LIF receptor, in the induction of c-Fos expression.

3.7 OSM stimulates Fos-related antigens in addition to c-Fos

Nuclear extracts from unstimulated or OSM for 0.25, 1 and 12 hours (and PMA for 1 hour) treated HepG2 cells were prepared and Western Blotted for the presence of fos-related proteins in addition to c-fos using a pan-fos antibody. Protein with a molecular weight of approximately 62 kD, consistent with the mobility of c-fos, was the only molecular species detected following 1 hour of OSM treatment (Figure 20). Interestingly, a second molecular species at approximately 40 kD, consistent with the mobility of fos-related antigens (fra)-1 and -2, were exclusively detected at earlier (15 minutes) and later (12 hour) time points of OSM treatment (Figure 20). c-Fos could also be detected after 12 hours of OSM treatment but to a lesser degree and with varying mobility than observed after 1 hour. The phorbol ester PMA is a potent inducer of AP1-activation and dramatically stimulated the expression of fos-related antigens consistent with the mobility of c-Fos, FosB and Fra-1/-2 after 1 hour (Figure 20). Thus, OSM stimulates the protein expression of

Figure 20. OSM stimulates multiple Fos-related family members.

HepG2 cell nuclear extracts from cells treated with OSM for 0, .25, 1, and 12 hours or with PMA for 1 hour were examined for expression of fos-related antigens by Western analysis and probing protein transfered to a nylon membrane with polyclonal anti-pan-fos, an antibody reactive against all fos family members. OSM-induction of c-fos is markedly observed after 1 hour of treatment, while additional fos-related antigens are detected at earlier (after 15 minutes) and later (12 hours) time points of OSM treatment. PMA dramatically upregulates the expression of both c-fos and additional fos-related antigens after 1 hour of treatment (lane 5: 5 minute exposure, lane 6: 30 second exposure).

Stimulus:	OSM				PMA	PMA
time(hrs):	0	.25	1	12	1	1

fos family members in a time-dependent fashion with the preferential upregulation

of c-Fos at 1 hour of cytokine treatment. Moreover, the expression of each of these

factors is significantly upregulated in response to 1 hour treatment with PMA.

Chapter 4

REGULATION OF TIMP-1 PROMOTER ACTIVITY BY MURINE ONCOSTATIN M

We have previously established in our laboratory the induction of TIMP-1 mRNA expression by the recently cloned murine oncostatin M (mOSM) (Richards et al., 1997). Similar to human OSM, mOSM was unique among IL-6-type cytokines in its capacity to upregulate TIMP-1 mRNA. Northern Blot analysis demonstrated that this cytokine stimulated an increase in TIMP-1 mRNA levels by 7-fold after 18 hours. IL-6 stimulated a 2-fold increase and CT-1 had no effect on upregulating TIMP-1 mRNA levels. We therefore pursued studies of TIMP-1 promoter regulation in murine cells to examine whether intracellular signalling by the mOSM receptor paralleled observations of its human counterpart.

4.1 Deletion Analysis of TIMP-1 promoter constructs in NIH3T3 murine fibroblasts

The proximal promoter region of TIMP-1 was examined for OSM-response elements by PCR-directed deletion analysis of sequences spanning -62 to +47 of the TIMP-1 gene upstream of a CAT reporter gene. NIH3T3 cells were transfected with TIMP-1/CAT chimeric constructs containing one of several deletions of TIMP-1 and assessed for responsiveness to murine OSM in CAT assays.

TIMP-1/CAT chimeric constructs demonstrated high levels of basal activity and an overall 5-fold induction in CAT activity in response to oncostatin M (Figure 21). Deletion of the TIMP-1 AP-1 site (-62/-53) markedly reduced the basal and OSM-inducible levels of acetylated chloramphenicol (approximately an 8-fold and 10-fold decrease, respectively). Sequences could be deleted from -62 to -39 without significantly effecting the fold induction in reponse to OSM, while the basal levels of CAT activity remained dramatically reduced. 3' deletion of sequences from +47 to +1 showed a gradual reduction in the percent conversion of chloramphenicol (both basal and inducible levels) as well as the overall decline in the fold-induction in CAT activity in response to OSM. Percent conversion of basal and OSM-inducible levels of chloramphenicol decreased by 3-fold and 7-fold respectively, while an overall 2.4- fold reduction in OSM-inducible CAT activity was observed. Thus, deletion of the TIMP-1 AP-1 site (-62/-53) dramatically affects the basal and OSM-induced levels of CAT activity. However, the AP-1 site is not necessary for maximal fold-induction over basal levels in response to OSM in these constructs. Sequences downstream of +1 also play a role in the activity of the TIMP-1 proximal promoter.

Figure 21. **Deletion Analysis of the proximal TIMP-1 promoter in NIH3T3**

murine fibroblasts. **Characterization of OSM-responsive**

elements.

NIH3T3 cells were transfected with TIMP-1 promoter 5' and 3' deletion constructs upstream of the CAT reporter gene. Deletions span -62 to +47 of TIMP-1 promoter. Transfected cells were subcultured and triplicate cultures either remained untreated or were stimulated with OSM for 18 hours. Whole cell extracts were then prepared and assayed for CAT activity by standard methods to assessed reponsiveness of the various TIMP-1 promoter sequences. Cells were co-transfected with pSV-betaGal and assayed for beta-galactosidase activity to normalize for CAT activity. Open bars represent basal levels and hatch bars, murine OSM(mOSM) stimulated levels of percent converted chloramphenicol as a measure of CAT activity. Black bars represent overall fold induction over basal levels. Values represent the mean of triplicate cell cultures that were untreated or cytokine-treated (error bars indicate standard deviation). Represents one of two separate experiments.
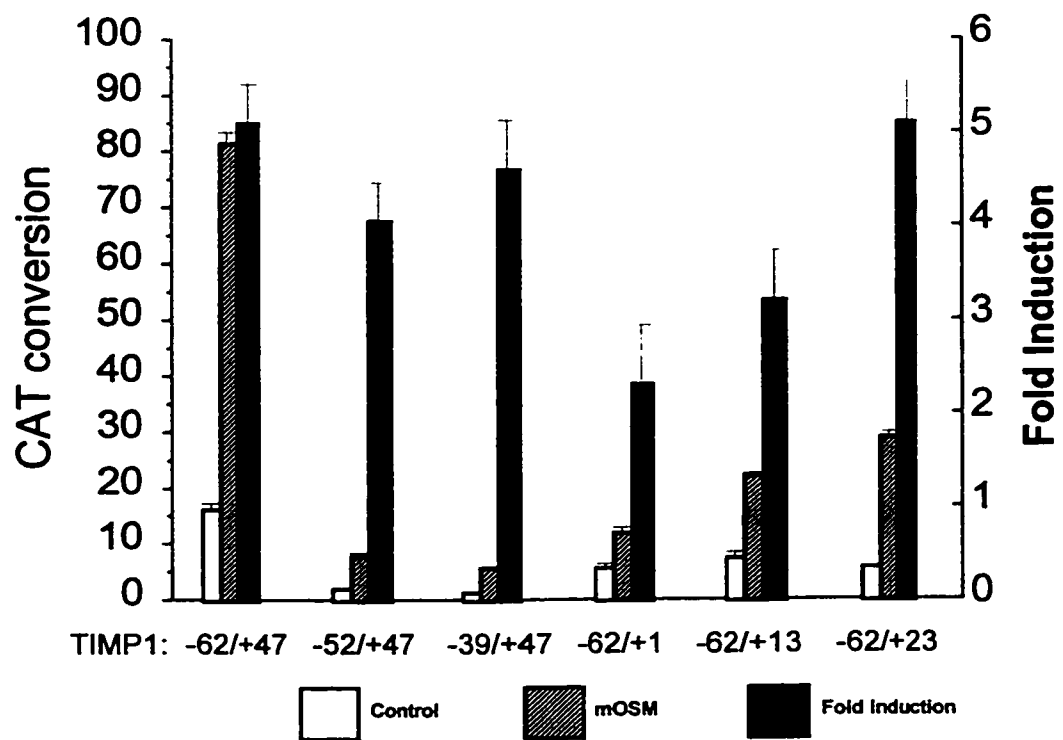
4.2 Regulation of TIMP-1 promoter activity is unique to OSM in NIH3T3 cells

OSM, IL-6 and EGF were compared in their ability to stimulate TIMP-1 promoter activity as assessed by transfection of NIH3T3 cells with the TIMP-1 -62/+23/CAT chimeric construct and assaying for CAT reporter gene expression. OSM was the most potent inducer of CAT activity, approximately 20 fold, while the related cytokine family member IL-6 only upregulated CAT activity to a lesser extent (10-fold) (Figure 22). EGF did not regulate TIMP-1 promotor activity. Thus, OSM but not IL-6, which both utilize a common receptor subunit, could effectively modulate TIMP-1 promoter activity.

4.3 Upregulation of c-Fos protein expression by OSM in murine fibroblasts

The upregulation of c-fos protein levels by mOSM was compared to LIF and IL-6, two cytokine family members which utilize the common signal-transducing receptor subunit gp130, in nuclear extracts from NIH3T3 cells and primary mouse lung fibroblasts (MLF). mOSM markedly elevated the amount of c-fos nuclear protein in these cells, while LIF and IL-6 demonstrated only a modest upregulation of c-fos in comparison (Figure 23). EGF, an inducer of both AP-1 and STAT, increased c-fos nuclear protein levels in MLF cells (Figure 23). Thus, as demonstrated for human in vitro models, OSM was a potent inducer of c-fos protein expression

Figure 22.   Regulation of TIMP-1 promoter activity is unique to OSM in

   NIH3T3 cells.

NIH3T3 cells were co-transfected with 10ug of TIMP-1 -62/+47/CAT chimeric construct and 3 ug of pSV-betaGal and subcultured for stimulation in triplicate with OSM, IL-6 or EGF (C = control, untreated transfected cells). CAT activity (percent conversion of chloramphenicol) is represented by open bars for basal levels; black bars, OSM treatment, hatch bars; IL6 treatment, and double hatched bars; EGF treatment. CAT activity was normalized to beta-galactosidase levels by standard methods. Values represent the mean of triplicate control of stimulated cells. Represents one of two separate experiments.
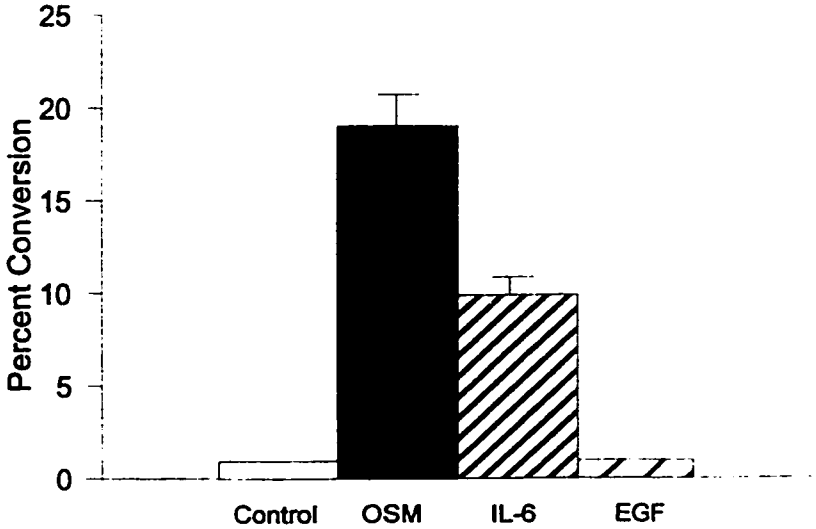
Figure 23. **c-Fos protein expression by OSM and IL-6-type cytokines in**

murine fibroblasts.

Nuclear extracts were prepared from NIH3T3 (A) or primary murine lung

fibroblasts (B) following treatment of cells for 1 hour with OSM, LIF, IL-6 or EGF

(C = control; unstimulated). Western blot analysis was carried out using an anti-

pan-fos antibody for the detection of c-fos and related family members. C-Fos is

readily detectable in response to OSM in NIH3T3 and MLF cells, and EGF in MLF

cells. Upregulation of c-Fos is also observed in response to LIF in NIH3T3 cells but

to a lesser extent compared to OSM.

NIH 3T3

A

— c-fos

C   OSM   LIF   IL-6   EGF

MLF

B

— c-fos

C   OSM   LIF   IL-6   EGF
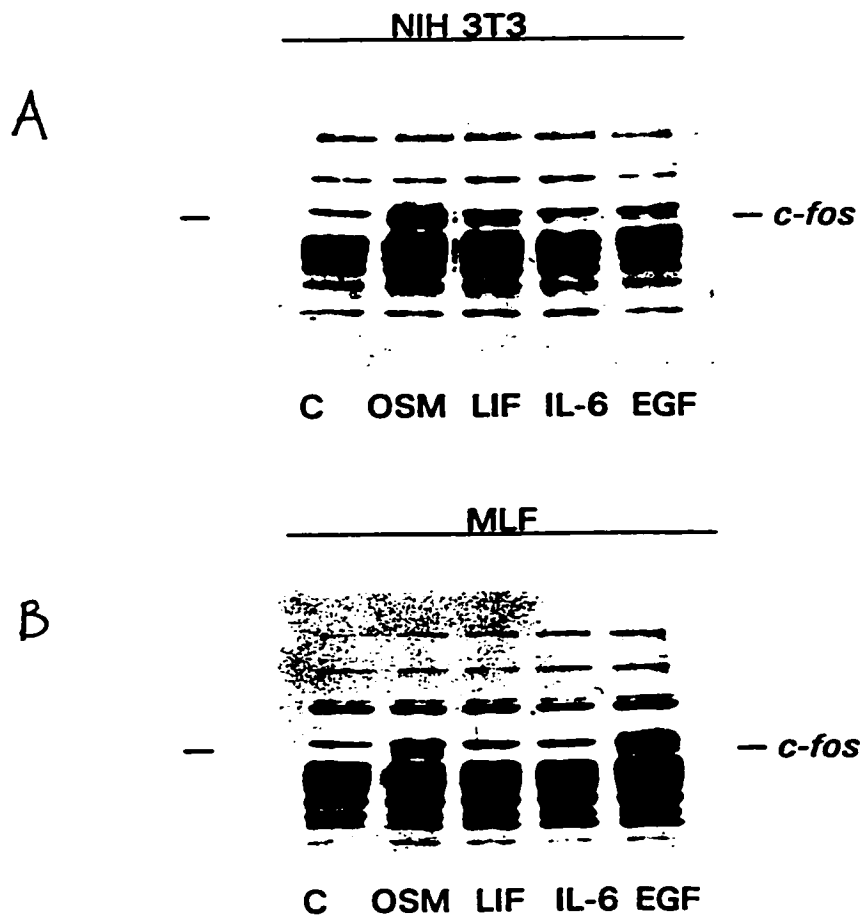
among IL-6-type cytokines.

4.4 TIMP-1 promoter protein-DNA complexes in murine fibroblasts

4.4.1 Analysis of TIMP-1 AP1, Ets, and SP1 nuclear protein-DNA complexes in murine fibroblasts

Double-stranded oligonucleotide probes of TIMP-1 promoter sequences that encompass putative AP1, Ets and SP1 binding sites (within -62 to +1 of TIMP-1) were examined by EMSA analysis for binding of nuclear factors.

Formation of TIMP-1 AP-1 protein-DNA complexes was examined using nuclear extracts prepared from NIH3T3 cells stimulated with OSM over a time course of 0 to 8 hours. A single mobility shift representing AP-1 complex formation was detected in untreated cells and cells treated with mOSM for .25 to 8 hours and the specificity of this gel-shifted complex was confirmed by competition with cold AP-1 oligonucleotides but not an irrelevant cold competitor (Figure 24B). The amount of AP-1 complex appeared to remain constant over time and likely represents a population of jun/jun and fos/jun complexes.

Using the high affinity sis-inducible element (hSIE) as a probe for STAT DNA-binding and the same NIH3T3 nuclear extracts from cells treated with mOSM

Figure 24. Time course of STAT DNA binding and TIMP-1 AP-1 probe

complex formation in NIH 3T3 murine fibroblasts treated with

OSM.

NIH 3T3 cells were treated with OSM from zero to 12 hours and nuclear

extracts incubated with the hSIE DNA probe for detection of STAT DNA binding, or

the TIMP-1 AP-1 DNA probe. Binding reactions were run on 5% non-denaturing

polyacrylamide gels for 3.5 hours and gels were dried and subjected to

autoradiography. A 100-fold excess of unlabelled relevant and irrelevant cold

competitor-oligonucleotide was added to demonstrate specific binding. Hash

marks indicate the position of specific complexes. An immediate and prolonged

activation of STAT DNA-binding is detected in response to mOSM while a single

gel-shifted mobility for AP1-DNA complexes is detected in untreated and mOSM

treated cells.

A

time (hr)	0	.25	.5	1	2	4	8	12	hSIE .25	AP-1 .25	-100X c.c.

hSIE probe

B

time (hr)	0	.25	.5	1	2	4	8	12	AP-1 1	hSIE 1	-100X c.c.

AP-1 probe
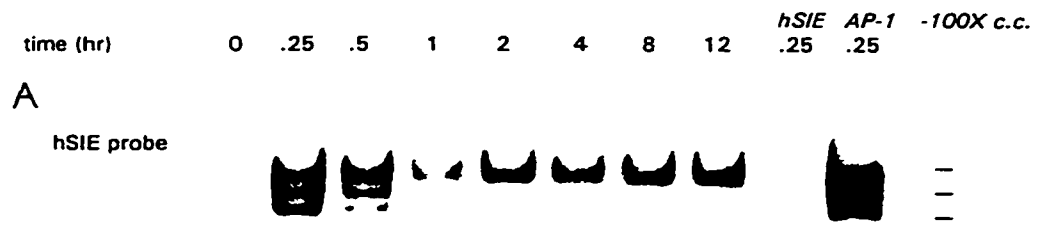
for 0 to 8 hours in EMSAs, hSIE probe complexes demonstrated mobility shifts with similar kenetics to that previously shown for STAT1 homodimers, STAT1/STAT3 heterodimers and STAT3 homodimers (Darnell et al., 1994) (Figure 24A). The immediate and transient induction of STAT1 homodimers and STAT1/3 heterodimers were detected within 15 and 60 minutes, while STAT3 homodimers were observed to persist for up to 8 hours of mOSM treatment. Cold competition with excess hSIE oligonucleotides and not by irrelevant oligonucleotide confirmed the specificity of these complexes (Figure 24A). Thus, although no change in AP1 DNA binding could be detected, murine OSM (like its human counterpart) could induce STAT DNA binding activity.

Sequences encompassing a putative Ets/STAT (-49 to -40), and SP1 (-11 to -6) binding site were also tested for their capacity to bind cognate nuclear factors. Over a OSM time course of zero to 8 hours treatment of NIH3T3 cells, no relevant DNA-binding activity to the TIMP-1 Ets probe could be observed with nuclear extracts from these cells (Figure 25A). Thus, neither Ets or STAT nuclear factors may significantly interact with this sequence as examined by EMSA analysis. However, consistent with observations in human HepG2 cells specific nuclear factor binding to sequences encompassing an SP1 binding site was detectable using the same nuclear extracts (Figure 25B). The level of SP1 binding was not modulated in response to OSM. Competition with an excess of cold TIMP-1 SP-1 sequence,

Figure 25.   SP1 DNA binding to the TIMP-1 promoter in NIH3T3 cells.

NIH3T3 cells were stimulated with OSM for 0, .25, 1 and 8 hours, and nuclear extracts prepared for EMSA analysis with the TIMP-1 SP1 or Ets DNA probes. 100X cold competition (CC) with unlabelled DNA probe was carried out to assess specifity of complexes detected.   No specific complex were observed binding the Ets probe although a single DNA-binding activity was detected with the SP1 probe which was not modulated in response to OSM.   * denotes the position of non-specific bands.
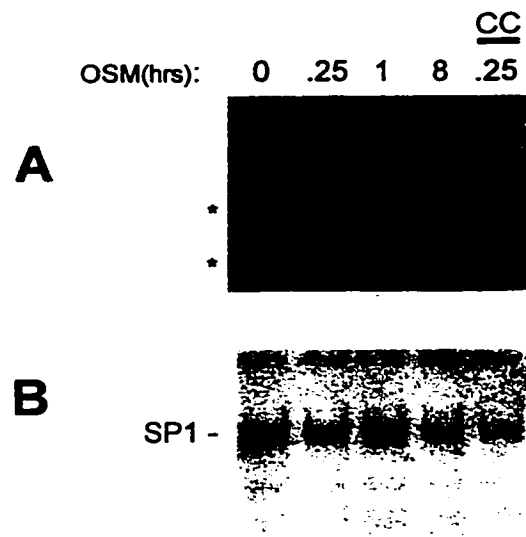
reduced the level of nuclear factor binding to radioactively labelled probe. Thus, AP-1 and SP-1 nuclear factor binding to TIMP-1 promoter sequences is observed although the levels of DNA-protein complex formation is not modulated in response to murine OSM.

4.4.2 Detection of c-Fos and Fos-related proteins in TIMP-1 AP1 complexes

C-Fos is prerequisite to the formation of TIMP-1 AP1 complex 2 in HepG2 cells in response to human OSM, and constitutively present TIMP-1 AP1 complex(es) in these cells displaying increased mobility in EMSAs (complex 1) contain alternative fos-related antigens (see Chapter 3). Since a single gel-shifted TIMP-1 AP1 complex is observed by EMSA with nuclear extracts from untreated or OSM-treated NIH3T3 cells, the participation of c-fos and additional fos-related antigens within these complexes was tested. Nuclear extracts prepared from NIH3T3 cells or primary murine lung fibroblasts (MLF) were treated with OSM for 0, 1 and 8 hours and c-fos or pan-fos antibodies included in TIMP-1 AP1 probe-nuclear protein binding reactions to detect DNA-protein-antibody complexes (supershifts) by EMSA analysis.

Anti-pan fos antibody (reactive against all fos-related antigens) supershifted all of the gel-shifted TIMP-1 AP1 probe complex from untreated or OSM-treated NIH3T3 or MLF cells (Figure 26, panel A and B). C-fos, which is transiently

Figure 26. Recruitment of c-Fos in AP1 Complexes following OSM treatment of Murine Fibroblasts.

NIH3T3 (A) or primary murine lung fibroblasts (MLF) (B) were stimulated with murine OSM for 0, 1 or 8 hours and nuclear extracts prepared for EMSA analysis with a murine TIMP-1 AP-1 double stranded oligonucleotide probe. TIMP-1 AP1 complex(es) (single mobility shift) from each time point were super-shifted (i.e., a greater reduction in mobility) by incubation of antibodies reactive against c-fos (anti-c-fos) or all fos-related antigens (anti-pan-fos) in DNA-protein binding reactions. Fos-related antigens are present in AP1 complexes of unstimulated or OSM-treated cells, while c-Fos is detected only at the 1 hour time point of OSM treatment.

OSM(hrs): 0   1   8
anti-c-fos: - + -   - + -   - + -
anti-pan-fos: - - +   - - +   - - +

A (NIH3T3 cells)

AP1 -

o

- Fos supershift

B (primary MLF cells)

AP1 -

o
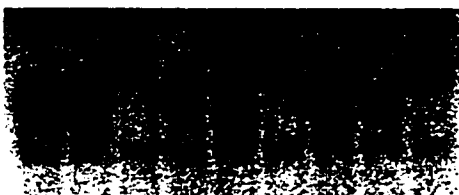
- Fos supershift

upregulated in response to OSM, was detected in TIMP-1 AP1-probe complexes following 1 hour OSM treatment. However, in contrast to the pan-fos antibody, the TIMP-1 AP1 complex was not significantly decreased and c-fos was not present in constitutively formed AP1 complexes or after 8 hours of OSM treatment of cells (Figure 26, panel A and B). Thus, fos-related antigens appear to be necessary for the majority of TIMP-1 AP1 complexes (likely heterodimers of fos and jun) and c-fos can participate in AP1 complex formation upon stimulation of its expression.

4.5 STAT activation in NIH3T3 fibroblasts by OSM, LIF and IL-6

Electrophoretic mobility shift assays were carried out to test STAT DNA binding activity from NIH3T3 cells treated with the IL-6 type cytokines OSM, leukemia inhibitory factor (LIF) or interleukin(IL)-6, and also with epidermal growth factor (EGF), all of which have been previously shown to induce STAT3 DNA binding activity.

OSM, LIF and IL-6 could all induce STAT3 and STAT1 DNA binding to the SIE (sis-inducible element) DNA probe following treatment of cells with cytokine for 15 minutes. EGF could not induce STAT3/STAT1 DNA binding activity in these cells (Figure 27A, left panel). Interestingly, only OSM and not LIF or IL-6 was capable of sustaining STAT3 activation after 1 hour of cytokine treatment (Figure 27A, right panel).

Figure 27. STAT activation in NIH3T3 fibroblasts by OSM, LIF and IL-6.

Nuclear extracts were prepared from NIH3T3 cells following treatment with OSM (OSM in figure), LIF, IL6 or EGF (C= control, untreated) for 15 minutes (Left panels) or 1 hour (right panels). EMSA analysis was performed to examine nuclear factor binding to the hSIE (A), beta-casein kinase (B), or murine TIMP-1 AP-1 (C) DNA probes. OSM, LIF and IL6 readily induced STAT1 and 3 activation after 15minutes, while OSM was most potent in sustaining STAT1/3 activation (particularly STAT3) after 1 hour of cytokine treatment. EGF did not induce STATs, likely due to the low expression of its receptor. Activation of STAT5 binding to the beta-casein kinase probe was preferentially induced by OSM following 15 minutes of treatment. AP1 complex DNA-binding levels were not significantly altered upon stimulation of cells with the IL-6-type cytokines or EGF. o = position of nonspecific complexes

Time: __15 min.__ __1 hour__

Stimulus: - OM LIF IL6 EGF - OM LIF IL6 EGF

A

STAT 3/3 -
3/1 -
1/1 -

B

STAT 5 -

C

AP1 -
o
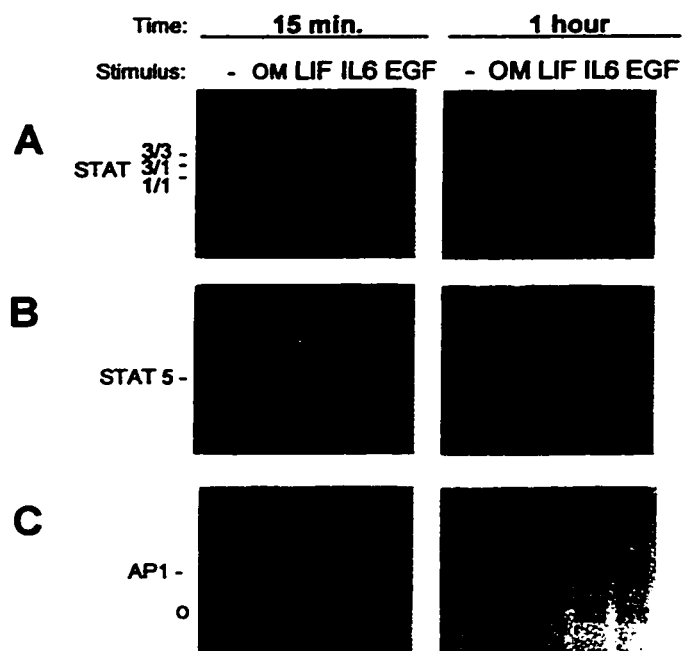
Utilizing a STAT5 binding DNA probe, (contains the STAT5-binding beta-caseine kinase promoter element), OSM but not LIF or IL-6 could induce STAT5-binding activity following 15 minutes treatment of cells (Figure 27B, left panel). STAT5 activation by OSM was markedly reduced following 1 hour of OSM treatment (Figure 28 and 27B, right panel). To confirm the binding of STAT5 to the beta-caseine kinase probe, antibodies against STAT nuclear factors were included in EMSA binding reactions to detect supershifted complexes. Nuclear extracts of cells treated for 15 minutes with OSM were used as they showed the highest levels of STAT activation. Anti-STAT1 and anti-STAT5 (but not anti-STAT3) antibodies supershifted beta-caseine kinase probe complexes (Figure 29). However, the complexes binding this probe consisted mostly of STAT1 as anti-STAT1 supershifted a large fraction of complexes compared to anti-STAT5.

Thus, the sustained activation of STAT3 and immediate activation of STATs 1 and 5 by murine OSM demonstrates that signalling events, in addition to those leading to c-fos/AP-1 activation, may contribute to OSM specific gene regulation within the IL-6 family of cytokines. TIMP-1 AP-1 DNA-nuclear protein complexes were also examined using the same extracts described for STAT binding activity (Figure 27C). No significant change in DNA-binding activity was detectable in EMSAs following 15 minutes or 1 hour treatment of NIH3T3 cells with OSM, LIF, IL6 or EGF.

Figure 28. OSM time-course of STAT1, 3 and 5 activation in NIH3T3 cells.

NIH3T3 cells were stimulated with OSM for 0, .25, 1 and 8 hours and nuclear extracts prepared for examining hSIE probe (left panel) and beta-casein kinase probe (right panel) nuclear factor binding in electrophoretic mobility shift assays (STATs 1 and 3 bind the hSIE probe while STAT5 binds with high affinity to the beta-casein kinase probe). OSM markedly stimulates binding of STAT 1 and 3 homo- and heterodimers (STAT 3/3, 1/3, and 1/1) after 15 minutes of treatment. STAT3 binding persists for at least 8 hours and STAT1 is transiently activated and is absent after 1 hour. STAT5 binding to the beta-casein kinase probe is readily detected following 15minutes (fastest migrating complex), and binding dramatically decreases thereafter. Additional mobility shifts binding the beta-casein kinase probe at 1 and 8 hours likely represent STAT3 and STAT5 dimers as previously characterized by others using different stimuli. 100X cold competition with unlabelled hSIE (left panel) or unlabelled beta-casein kinase probe in binding reactions was performed to confirm the specificity of complexes observed. An uncharacterized strong binding activity to the beta-casein kinase probe migrating immediately below STAT5 and present in unstimulated cells also appears specific for the beta-casein kinase probe as assessed by cold competion analysis.

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Probe: hSIE   b-casein kinase (b-CK)

Stimulus (hrs):	0	.25	1	9	.25	0	.25	1	9	.25
100X Cold Comp.:	-	-	-	-	hSIE	-	-	-	-	b-CK

STAT 3/3- 3/1- 1/1-

-STAT5

free probe -
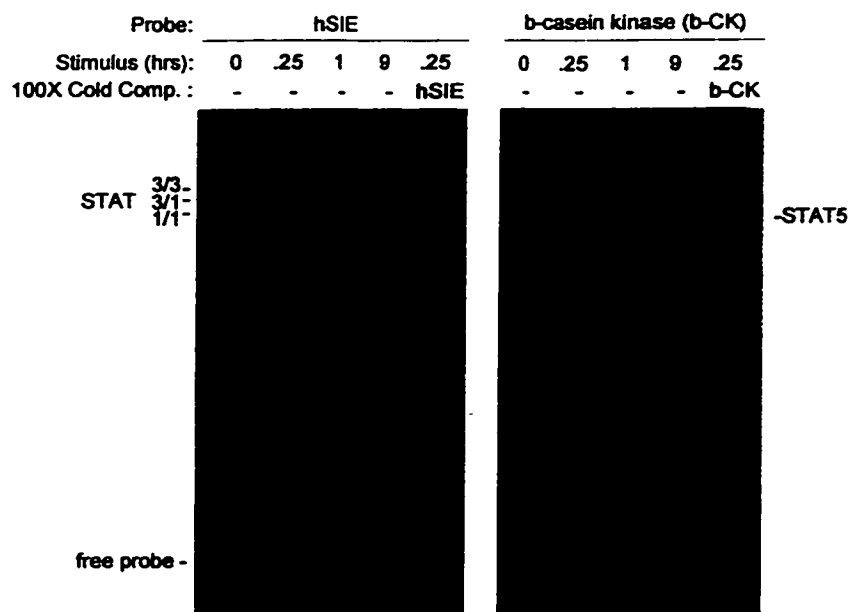
Figure 29. EMSA Supershifts with the 2-caseine kinase probe.

Nuclear extracts prepared from NIH3T3 cells stimulated with murineOSM for 15 minutes were incubated with the 2-caseine kinase probe in EMSA binding. Antibodies against various STAT nuclear factors (anti-STAT1(KST1), anti-STAT3(KST1) or anti-STAT5(KST1)) were included in binding reactions to detect the presence of STAT nuclear factors. A c-fos-reactive antibody (Kcfos) was used as a negative control. Both STAT1 and STAT5 were detected to bind the 2-caseine kinase probe.

Antibody: — αST1 αST3 αST5 αcfos

β-CK supershifts

β-CK complexes

free probe -
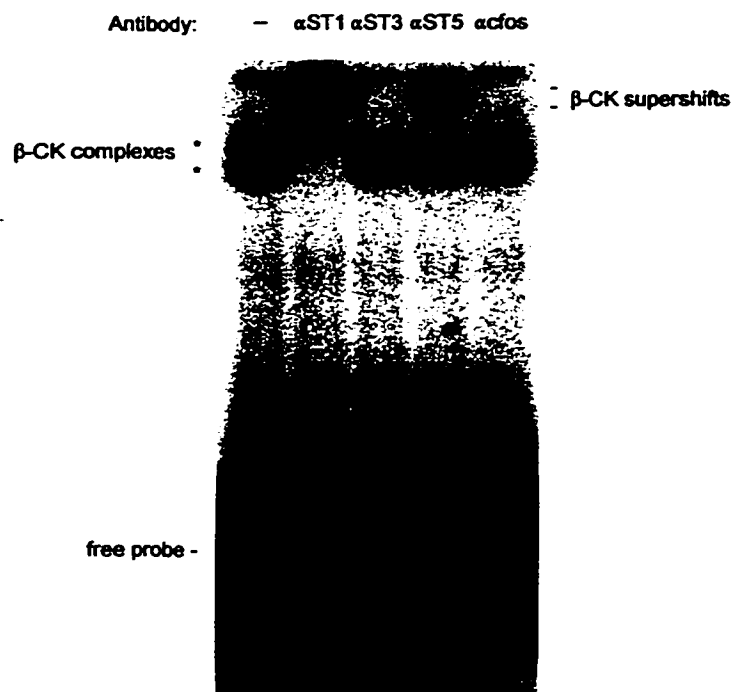
4.6   Direct Protein-Protein Interaction of SP1 with STAT3

It has previously been demonstrated by others (Look et al., 1995) that the transcription factors SP1 and STAT1 can directly interact to form a complex which participates in gene regulation.   Since SP1 binding to the TIMP-1 promoter is detectable by EMSA analysis and murine OSM markedly activates STAT3, a direct interaction between SP1 and STAT3 was tested by co-immunoprecipitation and western blot analysis.   Whole cell extracts were prepared from unstimulated or OSM-treated NIH3T3 cells and SP1 immunoprecipitated from these extracts. Western blot analysis showed detectable levels of STAT3 from SP-1 immunoprecipitates (Figure 30).   No significant difference in the level of co-precipitated STAT3 was observed from extracts of unstimulated and OSM-treated NIH3T3 cells and STAT3 could not be detected when a negative control antibody was used for immunoprecipitation of SP1 protein.

EMSA supershift analysis of TIMP-1 SP1 probe complexes was carried out to detect binding of SP1 and STAT (via binding to SP1) nuclear factors to this probe. Although, anti-SP1 antibody could supershift SP1 probe bound complexes, none of the STAT antibodies (anti-STATs 1, 3 and 5) tested could reduce the mobility of these complexes (Figure 31).   Thus, although a direct interaction between SP1 and STAT3 could be observed, assembly of STAT factors with SP1 on an SP1 probe could not be detected in EMSAs   The demonstration of direct binding of SP1 and STAT3 in co-immunoprecipitation however suggests that recruitment of STAT3 (and

Figure 30. Direct Protein-Protein Interaction of SP1 and STAT3.

NIH3T3 cells remained untreated (control, left panel) or were stimulated (right panel) with OSM for 15 minutes and whole cell extracts prepared for immunoprecipitation (I.P.) of SP1 with 2ug of anti-SP1 antibody. Antibody against OSM (2ug) was used as a negative control (NC) for immunoprecipitations. Immunoprecipitates were electrophoresed on an 8% SDS-PAGE gel and western blotted for the presence of SP1 and STAT3. SP1 and STAT3 migrate with similar mobilities and co-immunoprecipitate independent of OSM stimulation. Equal loading of immunoprecipitates was assessed by comparing the amount of free antibody (Ab) heavy chain from immunoprecipitation reactions.
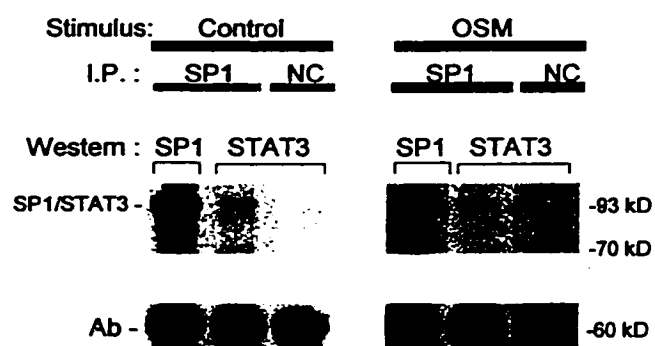
Figure 31. EMSA supershifts of complexes binding the TIMP-1 SP1 probe.

NIH3T3 nuclear extracts from cells stimulated with murine OSM for 15 minutes were incubated with TIMP-1 SP1 probe in EMSA binding. Antibodies against SP1 (anti-SP1) or various STAT nuclear factors (anti-STAT1(KST1), anti-STAT3(KST1) or anti-STAT5(KST1)) were included in binding reactions to detect the presence of SP1 or STAT nuclear factors. A c-fos-reactive antibody (Kcfos) was used as a negative control. SP1 but not STAT factors could be detected binding to the TIMP-1 SP1 probe.

Antibody: −   αSP1 αST1 αST3 αST5 αcfos

SP1 supershift -

SP1 -

free probe -
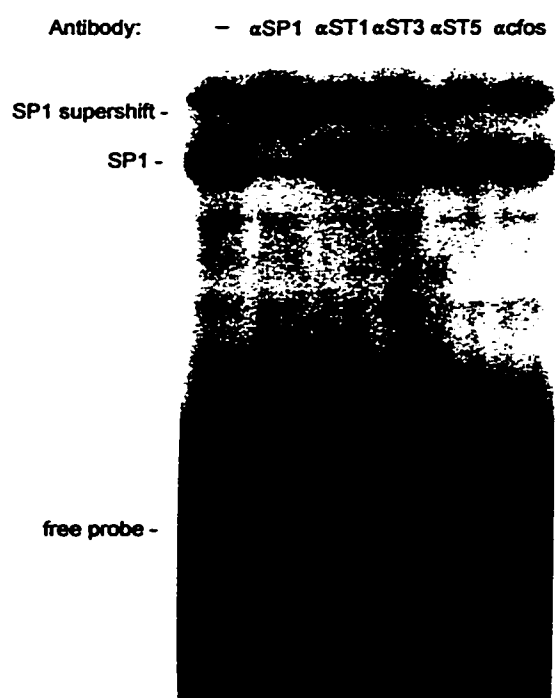
additional STATs) to the promoters of genes harboring SP1 binding sites, such as TIMP-1, may contribute to transcriptional regulation of these genes.

4.7 Regulation of TIMP-1 expression in wild-type and c-fos knockout murine fibroblasts

4.7.1 C-Fos is not necessary for OSM-mediated upregulation of TIMP-1 mRNA levels

As noted above, c-fos has been demonstrated to participate in AP1 complexes that are necessary for induction of a mininal TIMP-1 promoter construct by oncostatin M in human HepG2 cells. Murine c-fos wild-type and knockout cell lines were derived and tested whether c-fos is necessary for regulation of TIMP-1 transcription by OSM, LIF or IL-6. TIMP-1 mRNA levels were found to be upregulated by OSM, and not LIF or IL-6, in both wild-type and knockout cells following 6 hours of cytokine treatment. Thus, c-fos is not necessary for upregulating TIMP-1 in response to murine OSM (Figure 32).

Figure 32.   Murine OSM upregulates TIMP-1 mRNA levels in Wild-Type and

c-Fos knockout murine lung fibroblasts.

Wild-type (*c-fos* +/+) and c-Fos (*c-fos* -/-) knockout cells were stimulated with

OSM, LIF and IL-6 for 6 hours and total RNA extracted from treated and untreated

(control (C)) cells and probed by Northern analysis for murine TIMP-1 mRNA. OSM

and not LIF or IL-6 was capable of upregulating TIMP-1 mRNA levels in both wild-

type and knockout cells.   Ethidium bromide (EtBr) staining of 28S ribosomal RNA

is shown in the bottom panel to show equivalent loading of RNA samples.

TIMP-1 —

28S —

C OSM LIF IL-6   C OSM LIF IL-6

c-fos+/+   *c-fos+*
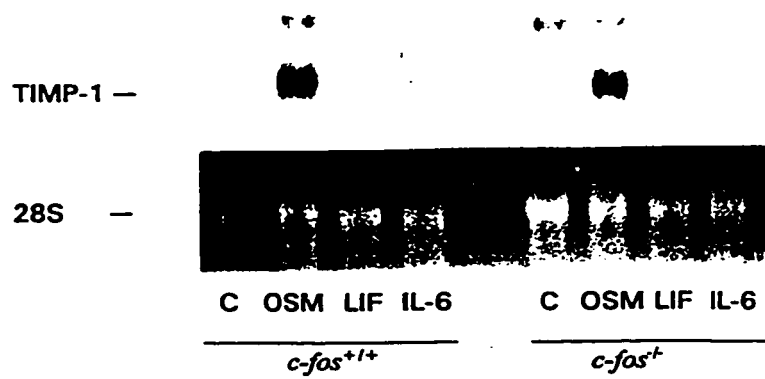
4.7.2 OSM induction of the proximal TIMP-1 promoter in murine fibroblasts is independent of c-Fos

Murine OSM was also equally effective in upregulating CAT activity from c-fos wild-type and knockout cells transfected with the TIMP-1 -62/+23/pBLCAT3 chimeric construct (Figure 33). OSM induced approximately a 3.5-fold and 4.3-fold increase in CAT activity for wild-type and mutant cells, repectively. Percent converted basal and OSM-induced chloramphenicol levels were notably higher in transfected knockout cells. Thus, in murine cells, c-fos is not necessary for OSM-mediated increases in TIMP-1 minimal promoter activity.

4.8 OSM time-course of TIMP-1 mRNA levels in wild-type and c-fos knockout murine fibroblasts

Utilizing wild-type and knockout murine lung fibroblasts, OSM has been shown to upregulate TIMP-1 mRNA levels in both these cell types with only a marginal reduction in the knockout cells. This is in contrast to our hypothesis which suggested that c-fos was not only unique to OSM signalling among IL-6-type cytokines but was also required for an AP1 complex that was shown to be necessary in regulating TIMP1 promoter/CAT chimeric constructs.

Since c-fos is an immediate early gene that is expressed transiently within 30 to 60 minutes in response to OSM, a time course of OSM treatment, from zero to 8 hours, of wild-type and knockout murine lung fibroblasts was carried out to

Figure 33.   Induction of the proximal TIMP-1 promoter by OSM does not require c-Fos.

Wildtype (c-$fos^{+/+}$) and c-Fos (c-$fos^{-/-}$) knockout murine lung fibroblasts were transfected (using Effectene (Qiagen)) with 10ug of TIMP-1 -62/+23/pBLCAT3, subcultured into a 6 well co-star and triplicates remained untreated or were stimulated with 50ng/ml of murine OSM.   Whole cell extracts were prepared and CAT assays performed by standard methods to assess responsiveness of TIMP-1 promoter sequences to OSM.   The TIMP-1 proximal promoter sequences (include TIMP-1 AP1 site) appear equally responsive to OSM in wildtype and c-$fos^{-/-}$ cells.
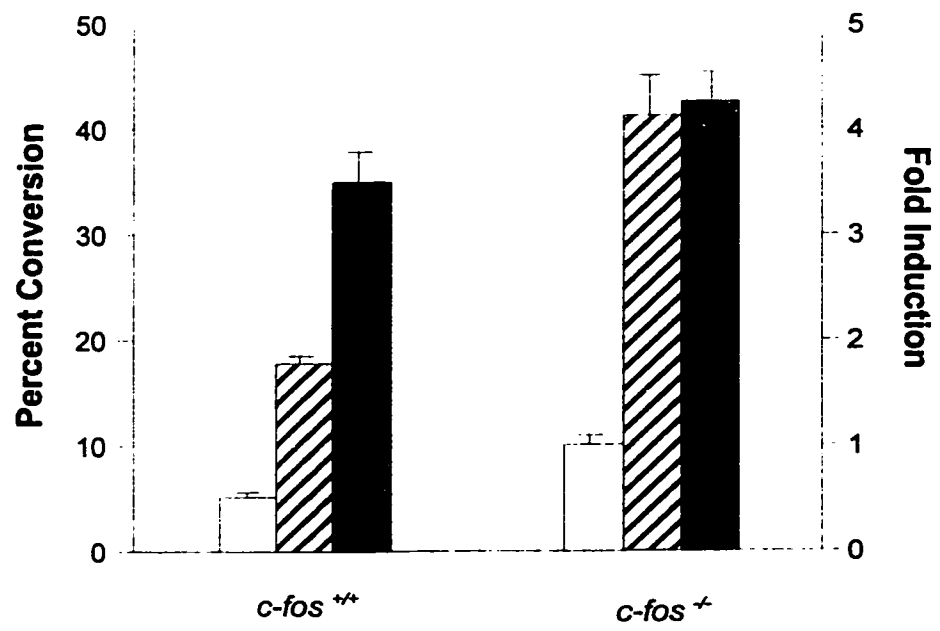
assess if there was any marked difference in the kinetics of TIMP-1 mRNA upregulation. However, consistent with earlier findings, only a marginal difference was observed. (A 4-fold increase in TIMP-1 mRNA versus a 3-fold increase, after 8 hours of OSM treatment.) (Figure 34A).

C-fos is expressed transiently (within 30 minutes to 2 hours) in response to OSM in wild-type murine lung fibroblasts, as assessed by Western blotting with a pan-fos antibody (reactive against c-fos and fos-related antigens(fra)) (Figure 34C). The levels of TIMP-1 AP1 DNA binding remain unchanged in reponse to OSM and STAT1 and 3 DNA binding activity (using the SIE probe) is detected in both wildtype and knockout murine lung fibroblasts in response to OSM (Figure 34D and E). Thus, over a time-course of OSM treatment, c-fos is not an essential participant in the regulation of TIMP-1 mRNA expression by OSM. Additional fos family members may participate in TIMP-1 promoter AP1 complexes and/or alternative signalling events may contribute to OSM-mediated TIMP-1 transcription.

Figure 34. OSM time-course of TIMP-1 mRNA levels in wild-type and c-fos

knockout murine fibroblasts.

(A) Total RNA was prepared from wild-type($fos^{+/+}$) and c-Fos ($fos^{-/-}$) knockout

murine lung fibroblasts stimulated with murine OSM for zero to 8 hours, or EGF for

4 hours.   15ug of RNA was subsequently electrophoresesed on a 1.2% agarose

gel, northern blotted and probed with an antisense oligonucleotide to TIMP-1 mRNA

(mTIMP-1). TIMP-1 mRNA is upregulated with similar kinetics in response to OSM

in wild-type and knockout cells. (B) Ethidium bromide staining of RNA blotted onto

the membrane was carried out to assess equal loading and membrane transfer of

RNA. (C) Nuclear extracts were prepared from c-Fos wild-type and knockout cells

following the same time course of stimulation described in (A).   Western Blot

analysis of nuclear extracts using an antibody reactive against all fos family

members (anti-pan fos) was performed to detect c-fos and additional fos-related

antigens (fra).   C-fos is transiently upregulated by OSM (at 0.5 and 1 hour) in

wildtype cells, while being absent in knockout cells. Additional fos-related antigens

(fra) are observed in unstimulated and OSM/EGF stimulated cells and are not

significantly upregulated in response to OSM.   EMSA analysis on the above

nuclear extracts was performed for the detection of TIMP-1 AP1 (D) and

hSIE(STAT1 and 3) complexes. STAT 1 and 3 activation showed similar kinetics

in wildtype and c-Fos knockout primary murine lung fibroblasts to earlier findings in other cell lines. OSM did not modulate the levels of total AP1 DNA binding complexes in either wildtype or c-Fos knockout cells. Specificity of DNA binding was confirmed by 100-fold excess of cold competitor (C.C.) DNAs using either relevant (rel.) competitor (i.e., cold AP1 for competing AP1 probe and cold hSIE for competing hSIE probe) or irrelevant (irrel.) competitor (i.e, cold SIE for competing AP1 probe and cold AP1 for competing hSIE probe).
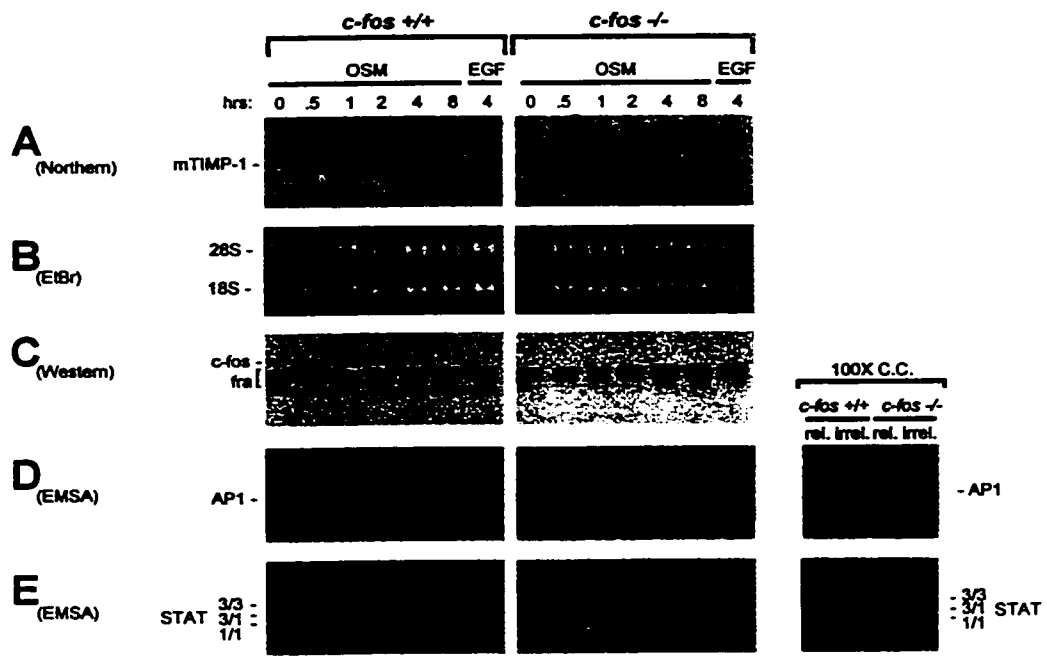
CHAPTER 5

REGULATION OF TIMP-1 PROMOTER ACTIVITY BY AP-1 AND STAT DOMINANT-NEGATIVE OVEREXPRESSION VECTORS

5.1 Murine Oncostatin M regulation of the TIMP-1 promoter

5.1.1 STAT Dominant-Negative cotransfection with TIMP1/CAT in murine fibroblasts

Transfection of NIH3T3 cells with TIMP-1 -62/+23/CAT and dominant negative over-expression vectors of STAT was carried out to examine the importance of STAT nuclear factors in the regulation of minimal TIMP-1 promoter activity by murine OSM. These dominant negatives have a deletion of their carboxy-terminal transactivation domain and thus can dimerize with native STAT factors and/or competitively inhibit the DNA-binding of endogenous STAT factors thereby effectively blocking endogenous STAT participation in the regulation of gene transcription. When cotransfected with the TIMP-1 (-62/+23)/CAT chimeric construct, dominant negatives of STAT1, STAT3 or STAT5 could markedly reduce the overall fold-induction of CAT reporter gene expression by OSM (reduced from 5.3-fold to approximately 2-fold by each of the dominant-negative STATs) (Figure 35). Dominant-negative STAT3 appeared to be the most effective in decreasing OSM-induced CAT activity in these cells (3-fold reduction in OSM-induced CAT activity, with comparable basal levels to mock (bluescript plasmid) transfected).

Figure 35. Co-transfection of STAT and TIMP-1 -62/+23/CAT in Murine NIH3T3 fibroblasts. Mouse OSM-regulation of proximal TIMP-1 promoter sequences by STAT.

NIH3T3 cells were cotransfected with TIMP-1 -62/+23/CAT (TIMP1/CAT) and overexpression vectors of dominant negative STAT and OSM-stimulated TIMP-1 promoter activity assessed in CAT assays. Whole cell lysates of untreated or cytokine-stimulated cells were prepared in triplicate from subcultured transfected cells for CAT analysis. NIH3T3 cells were transfected with dominant-negatives of STAT factors (STAT1(Stat1d), STAT3 (Stat3d), or STAT5 (Stat5d)) or expression vectors for Ets2 or PEA3 with the TIMP-1 -62/+23/CAT reporter gene construct. Bluescript plasmid was co-transfected with TIMP-1/CAT to control for total amount of transfected DNA. CAT gene expression (percent conversion of chloramphenicol) was measured following stimulation of transfected cells (hatched bars) and compared to unstimulated cells (control; open bars) to calculated overall fold induction (black bars). Dominant-negatives for each of the STATs markedly reduced the overall fold-induction of TIMP-1 promoter activity. Values represent the mean of triplicate contol or cytokine-stimulated cells. Represents one of two separate experiments.
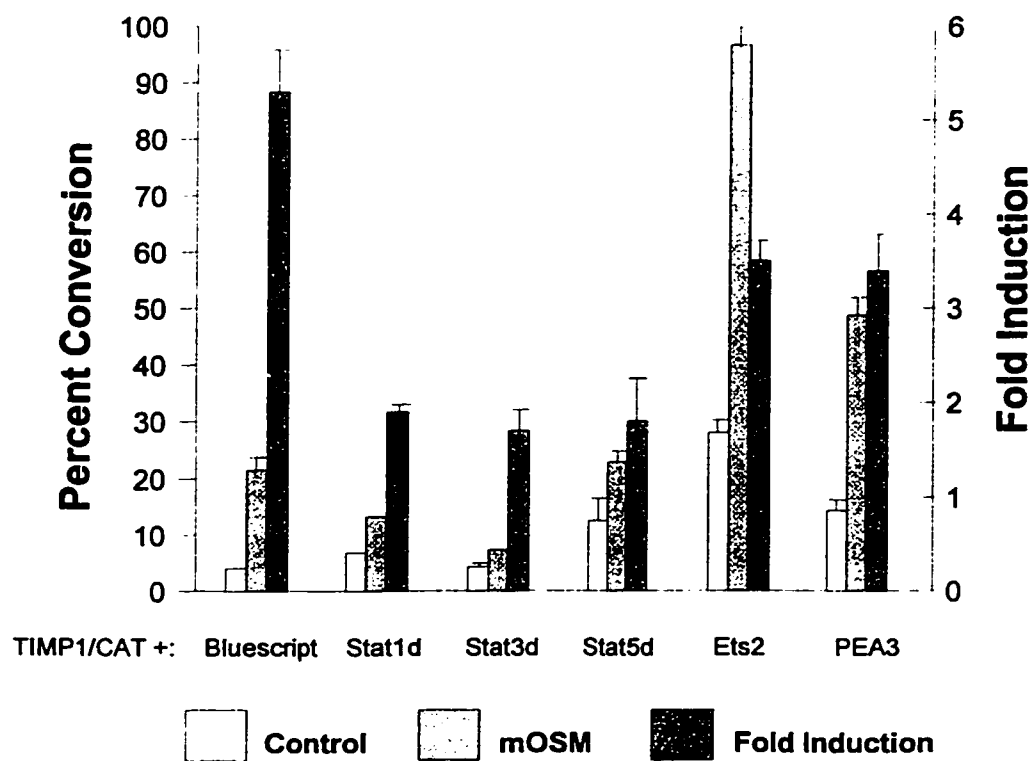
Cotransfection with Ets factors, Ets2 or PEA3, could also reduce the fold-induction in response to OSM, although the basal and OSM-induced levels of CAT conversion were greater.

5.1.2 AP1 Dominant-Negative cotransfection with TIMP1/CAT in murine fibroblasts

A dominant-negative of c-Fos (A-Fos: essentially an AP1 inhibitor, as c-fos must hetero-dimerize with jun AP1 family members) was obtained through collaboration (Dr. C. Vinson, National Institute of Health, Bethesda, Maryland, USA) to test the effect of this AP1 inhibitor on the OSM-responsiveness of the TIMP-1 -62 to +23 region. This inhibitor consists of an acidic amphipathic sequence appended onto the polypeptide sequence of the c-Fos leucine zipper. The wildtype c-Fos leucine zipper was used as a control vectors, as was vector lacking any gene, CMV500, and a second dominant-negative, A-CREB (an acidic amphipathic sequence appended onto a leucine-zipper polypeptide sequence of CREB).

Expression vectors for dominant-negative wildtype forms of c-Fos were co-transfected with the -62/+23TIMP-1/CAT chimeric construct into NIH3T3 cells and OSM-responsiveness of the TIMP-1 promoter was assessed in CAT reporter gene assays. Transfection with A-Fos and c-Fos vectors dramatically reduced basal and OSM-induced CAT activity (Figure 36). However, consistent with AP-1 deletion analysis studies, fold-induction over basal levels were not maintained.

Figure 36. Co-transfection of Dominant-negative AP-1 and TIMP-1/CAT in

NIH3T3 cells.

NIH3T3 cells were co-transfected with TIMP-1 -62/+23/CAT (TIMP1/CAT) and either backbone overexpression vector (CMV500) containing no overexpressed polypeptide, or with the following inserts for leucine-zipper domains of c-Fos, dominant-negative c-Fos (A-Fos; an amphipathic polypeptide is appended onto the leucine-zipper domain of c-Fos) or dominant-negative Creb (A-Creb; an amphipathic polypeptide is appended onto the leucine-zipper sequence of the CREB nuclear factor).   Transfected cells were subcultured into 6-well costars and stimulated in triplicate with human OSM or remained untreated.   Open bars represented CAT activity (percent conversion of chloramphenicol) of basal levels (mean of unstimulated cells), hatch bars OSM-stimulated levels of CAT activity, and black bars overall OSM-induced fold-induction of CAT activity. The AP-1 repressor A-Fos had no significant effect in modulating OSM-mediated TIMP-1 promoter activity. Values represent the mean of triplicate well cultures (errors indicate standard deviation).   Represents one of two separate experiments.
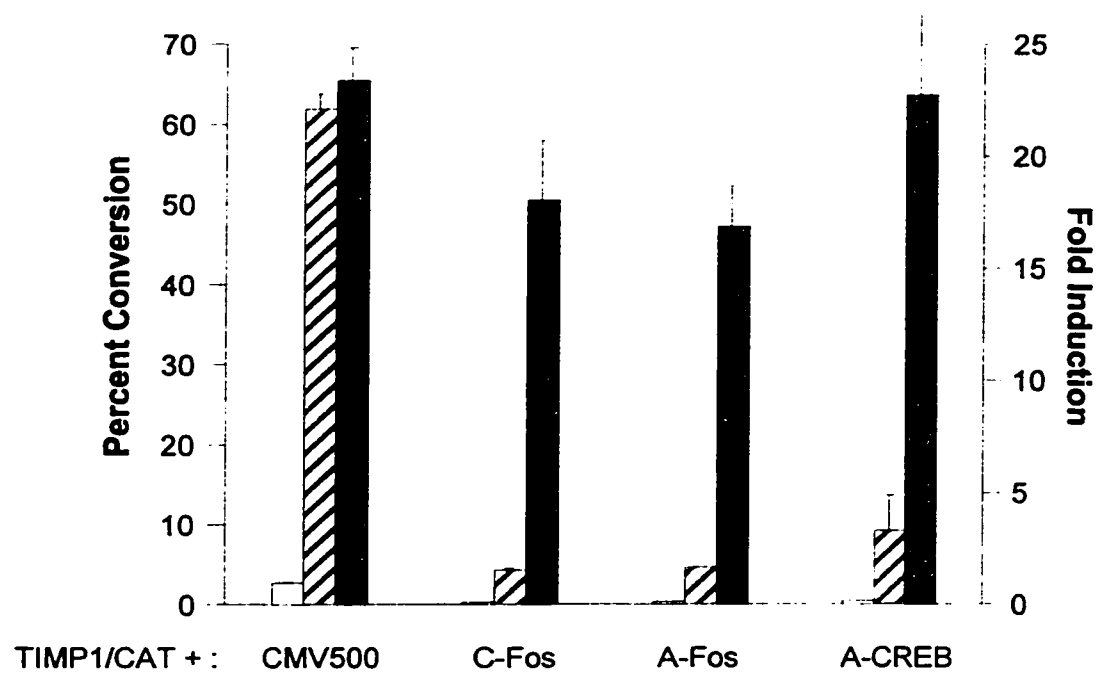
Thus, although the TIMP-1 AP-1 site contributes to higher basal and OSM-inducable levels of reporter gene expression (as observed in deletion analysis studies), AP-1 is not nessary for increases in the overall fold-induction of TIMP-1 promoter activity in response to mOSM in murine fibroblasts.

5.2 Human Oncostatim M regulation of the TIMP-1 promoter

5.2.1 STAT Dominant-Negative cotransfection with TIMP1/CAT in human HepG2 cells

Dominant negatives of STAT1 or STAT3 were cotransfected with TIMP-1 -62/+23/CAT into HepG2 cells and responsiveness of the TIMP-1 promoter to OSM was assessed by CAT assays (Figure 37A). When compared to cotransfection with a bluescript plasmid (controlling for amount of DNA transfected), the percent CAT conversion from OSM-treated cells did not decline in the presence of either STAT dominant-negative, instead the levels of converted chloramphenicol appearred to increase. Basal levels of CAT activity were relatively higher when dominant-negative STAT3 was used and the fold-induction in response to OSM showed a modest reduction.

Figure 37. **Co-transfection of STAT and AP1 dominant-negatives with TIMP-1 -62/+23/CAT in Human HepG2 cells. AP1 predominants over STAT in OSM-regulation of proximal TIMP-1 promoter sequences.**

HepG2 cells were cotransfected with TIMP-1 -62/+23/CAT (TIMP1/CAT) and overexpression vectors of dominant negative STAT (A) or leucine-zipper polypeptides of c-Fos or a dominant negative of c-Fos/AP1 (A-Fos) (B) to examine the contribution of each overexpressed protein to OSM-stimulated TIMP-1 promoter activity. CAT assays were performed following preparation of whole cell lysates in triplicate from subcultured transfected cells. A. HepG2 cells were co-transfected with dominant-negatives of STAT1(Stat1d) or STAT3 (Stat3d) and TIMP-1 -62/ +23/CAT. Bluescript plasmid was co-transfected with TIMP-1/CAT to control for total amount of transfected DNA. CAT reporter gene expression (percent conversion of chloramphenicol) was measured following stimulation of transfected cells (hatched bars) and compared to unstimulated cells (control; open bars) to calculated overall fold induction (black bars). B. HepG2 cells were co-transfected TIMP-1 -62/+23/CAT (TIMP1/CAT) and either backbone overexpression vector (CMV500) containing no overexpressed gene, or with inserts for the c-Fos leucine-zipper or a dominant-negative c-Fos (A-Fos). Transfected cells were subcultured

into 6-well costars and stimulated in triplicate with human OSM or remained untreated. Open bars represented CAT activity (percent conversion of chloramphenicol) of basal levels (mean of unstimulated cells), hatch bars OSM-stimulated levels of CAT activity, and black bars overall OSM-induced fold-induction of CAT activity. Dominant-negative c-Fos markedly down-regulates OSM-mediated TIMP-1 promoter activity while dominant-negative STAT3 has a minor affect on overall levels of fold-induction. Values represent the mean of triplicate well cultures (errors indicate standard deviation). Represents one of two separate experiments.

A

Percent Conversion / Fold Induction

TIMP1/CAT + : Bluescript Stat1d Stat3d

B

Percent Conversion / Fold Induction

TIMP1/CAT + : CMV500 C-FOS A-FOS
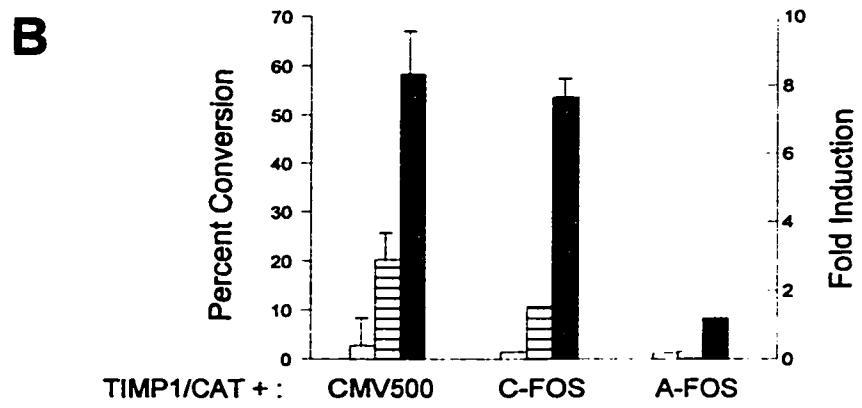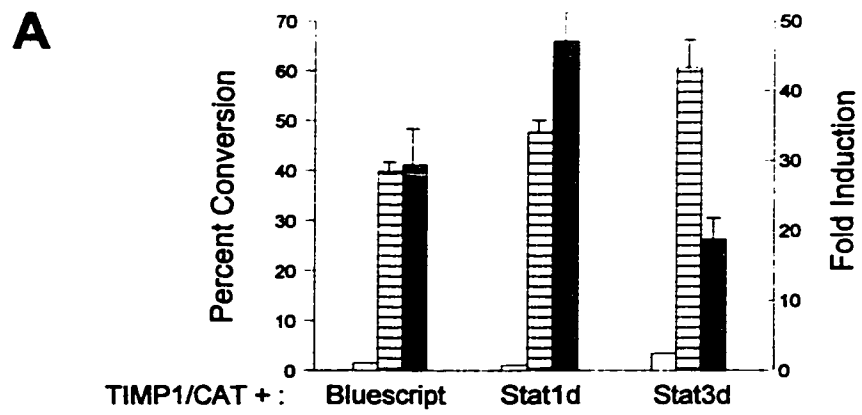
5.2.2 AP1 Dominant-Negative cotransfection with TIMP1/CAT in HepG2 cells

When compared to the negative control and wildtype c-Fos expression vectors, consistent with deletion of the TIMP-1 promoter AP1 site, A-Fos significantly reduced both the OSM-induced levels of CAT activity and the fold-induction over basal levels in HepG2 cells (Figure 37B). Thus, AP-1 appears key to the fold-induction and total activity of the TIMP-1 promoter by hOSM in human HepG2 cells.

CHAPTER 6

DISCUSSION

REGULATION OF TIMP-1 BY HUMAN AND MURINE ONCOSTATIN M

Our overall goal has been to identify and examine mechanisms by which OSM regulates the transcription of the TIMP-1 gene. We have examined the contribution of various TIMP-1 DNA promoter elements, nuclear factors binding these elements and their participation in mediating OSM signalling of TIMP-1 transcription. Throughout the progress of this thesis a number of independent findings in other laboratories have aided in acheiving these goals. The family of STAT nuclear factors, murine OSM, and murine and human OSM receptor-beta subunits were cloned during the course of these studies. Through collaborations, obtaining wildtype and/or mutant versions of several of these clones has allowed our laboratory to carefully examine OSM signalling events that initiate TIMP-1 expression in human and murine systems, and contributed to novel findings.

In the human HepG2 hepatoma cell line, we have demonstrated the necessity of the TIMP-1 proximal AP-1 DNA element for upregulating OSM-induced TIMP-1 promoter activity. PCR deletion analysis of the proximal murine TIMP-1 promoter (from -95 to +47) has shown theTIMP-1 -59/-53 AP-1 site to be essential for maximal OSM-induced CAT reporter gene expression. This element bound fos

and jun nuclear factors and mobility shift assays identified two separate gel shifted bands when HepG2 nuclear extracts from OSM treated cells were incubated with radiolabelled TIMP-1 AP-1 probe. A constitutively present complex (complex 1) and an OSM-induced complex (complex 2) were detected and represent populations of fos/jun and jun/jun dimers. Complex 1 was observed to include fos-related antigens (but not c-fos), junB and junD, while OSM (and PMA) but not IL-6 stimulated the formation of complex 2 which requires c-fos. This induction of complex 2/c-fos was transient (peaking at 1-2 hours). OSM also stimulated an increase in c-fos protein in fibroblast and neuronal cells and upregulated c-fos mRNA levels in HepG2 cells consistent with the kinetics of immediate early genes. Both the induction of c-fos protein and TIMP-1 mRNA expression in response to OSM was blocked by the protein synthesis inhibitors puromycin and emitine while the expression of jun factors was independent of new protein synthesis. In addition, OSM was unique among IL-6-type cytokines (notably IL-6 and LIF) in hepatocyte and neuronal cell lines in upregulating c-fos protein expression. This was despite the fact that all IL-6-type cytokines could still activate STAT1 and 3 DNA-binding activity. Transfection of the OSM-beta receptor specific subunit demonstrated the type II human OSM receptor to stimulate TIMP-1 promoter activity independent of the promiscuous human OSM and LIF-binding type I receptor. Human OSM (c-fos and STAT activation) was unique in its capacity to regulate expression of the TIMP-1 promoter activity when compared to IL6 (STAT activation)

or the combination of IL6 (STAT activation) and PMA (c-fos activation). Thus, qualitative differences in c-fos and/or sequences in addition to the AP1 site likely account for the pronounced and maximal induction by OSM.

These findings implicate AP1 complex 2/c-fos participation in a signalling pathway which is potently activated by OSM and contributes to the activation of genes unique to this cytokine family member. In addition to c-fos, OSM was also a potent inducer of additional fos-related antigens as examined using a pan-fos antibody in Western blot analysis. Utilization of antibodies reactive against individual fos-related antigens will be necessary to clarify which family members in addition to fos are activated by OSM and whether this activation is exclusive to OSM among other IL-6-type cytokines. Previous work by others has shown OSM-mediated induction of other immediate early genes such as egr-1, c-jun and c-myc in fibroblasts (Lui et al., 1992). Taken together, this suggests that a broad range of gene products could be regulated by OSM stimulation and manifest biological effects unique to this IL-6-type cytokine.

Interestingly, OSM induced AP-1 complex 2 formation in HepG2 and human lung fibroblasts (HLF) but not in SV-40 transformed HLF cells (WI-26 cells) or the SY5Y neuroblastoma cell line, despite the induction of c-fos by all these cell types. This suggests either qualitative differences in the c-fos protein (i.e., phosphorylation) which are necessary for the formation of complex 2 or in the jun dimerization partners that interact with c-fos. It is also possible that an additional

factors may interact with the AP1 dimer in order to cause the reduced mobility of gel-shifted complex 2. Possible factors include Ets and NF-AT proteins which have been previously established to directly interact with AP1 nuclear factors (Bassuk and Leiden, 1995; Rao, 1994). Biotinylated double-stranded AP1 and mutant AP1 oligonucleotide probes are now commercially available and can be readily used to address proteins binding the AP1 site in untreated and OSM-treated cells. This involves incubation of nuclear extracts with biotinylated probes in binding reactions described for the EMSA studies, followed by centrifugation with bound streptavidin for separating out AP1-probe binding complexes. Proteins bound to these probes are then denatured and run on an SDS-PAGE gel followed by Western blotting with various antibodies such as those for jun-,fos-, ets- and NFAT-related nuclear factors. A comparison in parallel studies with the mutant AP1 probe (that can not bind AP1 factors) would be necessary to assess the specificity of proteins binding the wildtype AP-1 probe.

Sequences in addition to the TIMP-1 -59/-53 AP-1 site are also necessary for maximal OSM-induced TIMP-1 promoter activity. Deletion of +1 to +47 (construct E, Figure 4) markedly reduced the OSM-inducible CAT activity compared to AP1 containing construct (construct B, Figure 4) from 11-fold to 3-fold. There are several putative DNA-binding elements within this region. Logan et al. (1996) characterized a weak AP-1 binding site in this region and deletion of this element

would be consistent with the marked induction of AP-1 activity by OSM.

The TIMP-1 promoter is a TATA box-less promoter. As is the case for many of these types of gene promoters, the TIMP-1 promoter harbors SP1 binding sites proximal to the start of transcription and contains a putative pyrimidine-rich initiator DNA element (Inr) (Smale and Baltimore, 1989) immediately 3' to +1. SP1 and Inr elements have been described as important elements for promoting the assembly of a functional apparatus for initiating transcription in the absence of a TATA box element (Smale and Baltimore, 1989; Dynan and Tjian, 1983). Recently a novel OSM-inducible SP1 binding element defined by the nucleotide sequence 5' TTCTCC 3' has been characterized and stimulated a 2-3 fold increase in reporter gene expression (Ihn et al., 1997). An element with close homology is also present at +11 to +16 of the TIMP-1 promoter with the sequence 5' TCCTTC 3' and may function as an OSM-responsive element. The SP1 site proximal to the start of TIMP-1 transcription at -11/-6 was observed by mutational analysis to contribute to basal levels of CAT reporter gene expression (compare constructs E and F, Figure 4). This SP1 site was strongly bound by the SP1 nuclear factor although nuclear factor binding was not modulated by OSM. An upstream SP1 site at -93 to -88 was however depensable for regulation by OSM and weakly bound SP1 factors.

In addition to the AP1 and SP1 sites, the proximal TIMP-1 promoter contains a putative Ets element (at -45 to -40; 5' AGGAAG 3') sharing homology with a STAT binding site (within -49 to -40; 5' GTCCAGGAA 3') as well as a downstream STAT-

like element (-24 to -14; TTCCCCGCCAA) with a two base insertion. Neither of these elements contribute to OSM or IL6-induction although deletion of the Ets/STAT element (constructs B versus C, Figure 4) modestly reduces basal levels of reporter gene expression. STAT nuclear factor binding was not detected to either of these sites as assessed by EMSA and supershift analysis with STAT antibodies. The Ets element did however display a weak binding activity in nuclear extracts independent of cytokine treatment of cells. The Ets element is closely juxtaposed to the AP1 OSM-responsive element and AP1 and Ets nuclear factors have been demonstrated to cooperate in regulating gene transcription (Wasylyk et al., 1998). As described by others, factors binding the Ets site may help to tether AP1 nuclear factors to its DNA binding site (Logan et al., 1996).

Others have shown that STATs can bind AP-1/Ets sequences of the rat TIMP-1 in HepG2 cells (Bugno et al., 1995), and human TIMP-1 promoter in astrocytes (Korzus et al., 1997) and that this site also contributes to transcription by OSM. Our results in HepG2 cells did not detect binding of STATs to this sequence despite the presence of activated STAT-1 and STAT-3 (hSIE binding) in the nuclear extracts and long exposures of gels. In addition, mutation of the AP-1 site in the AP-1-Ets probe completely eliminated detection of any OSM-inducible nuclear factors capable of binding this probe (Figure 9). Differences between the levels of STAT proteins expressed in HepG2 cells and astrocytes could account for this result. The prominence of AP1 nuclear factor binding to this sequence that we

observe is consistant with previous data (Korzus et al., 1997) where AP-1 binding appeared dramatically greater than STAT binding in EMSA assays with an equivalent of an AP-1/Ets probe. We suggest that this abundance of AP-1 binding reflects a physiological importance amongst other factors participating in the regulation of this proximal TIMP-1 promoter.

The significance of AP-1 in the regulation of TIMP-1 promoter activity of TIMP-1/CAT reporter gene chimeric constructs and the potent induction of c-fos by OSM which leads to the formation of an OSM-specific AP1 complex binding the TIMP-1 promoter prompted us to address the role of cytokine-induced c-fos expression for regulating TIMP-1 gene expression in vivo. The dependence of both c-fos and TIMP-1 gene expression on de novo protein synthesis also supported a role for OSM-induced c-fos activation in the regulation of TIMP1 transcription. Studies with murine cells and mouse OSM were therefore carried out to address whether intracellular signalling events were consistent with that observed in human cells and to genetically ascertain the role of c-fos in cytokine-mediated TIMP-1 transcription by examining cells from wildtype and c-fos deficient mice.

Mouse OSM (mOSM) does not possess similar promiscuity in receptor binding as does human OSM (hOSM). While mOSM only binds the mOSM-specific receptor, hOSM interacts with both the type I OSM-/LIF-binding receptor and type II OSM-specific receptor (and not the mOSM receptor). Thus, activities unique to mOSM can be examined independent of LIF-receptor initiated events in murine

cells.

Our laboratory has established mOSM as a potent upregulator of TIMP-1 mRNA levels among IL-6-type cytokines (Richards et al., 1997). mOSM, but not IL-6 or CT-1, stimulated TIMP-1 mRNA expression in murine fibroblasts. We have extended these studies by examining intracellular signalling events previously determined for human OSM. Consistent with studies using hOSM, mOSM was a potent upregulator of c-fos protein expression in murine fibroblasts among IL-6 type cytokines (Figure 23). Mouse OSM also activated STAT DNA-binding activity in murine fibroblasts and was unique among IL-6-type cytokines in the prolonged activation of STAT3 (after 1 hour; Figure 27) and the marked activation of STATs 1 and 5 at earlier time points of stimulation (Figure 27).

Murine OSM upregulated CAT activity of -62/+47 TIMP-1/CAT reporter gene chimeric constructs (which contains the -59/-53 AP1 site) transfected into NIH3T3 murine fibroblasts cells. IL-6 could also markedly regulate this construct although to a lesser extent than mOSM (20-fold by mOSM versus 10-fold by IL-6). 5' and 3' deletion analysis of the -62 to +47 TIMP-1 sequences, as described for studies in HepG2 cells, was carried out to define minimal sequences necessary for responsiveness to OSM. Both common and disparate findings with studies using human OSM and human HepG2 cells were observed. In contrast to observations with human OSM (in human cells), deletion of the AP-1 site at -59/-53 did not affect the OSM fold-induction over basal levels in TIMP-1 promoter activity in response to

OSM (compare -62/+47TIMP1 versus -52/+47TIMP1 constructs). Deletion of the AP-1 site however dramatically reduced basal and OSM-inducible CAT reporter gene expression indicating that this element is an active participant in mediating expression by proximal TIMP-1 promoter sequences.

Comparison of basal and inducible levels of CAT activity to the fold induction over basal levels merits careful interpretation. Basal and cytokine-induced increases in percent conversion of chloramphenicol each express total promoter activity affecting CAT reporter gene expression. However, calculated increases in fold induction over basal levels expresses the contribution of both basal and cytokine-stimulated mechanisms to the activity of the promoter.

Nucleotide sequences from -62 to -39 (harbors AP1 and Ets/STAT-like (-49/-40) elements) could be deleted without any notable change in fold-induction of CAT activity. In agreement with the hOSM studies, deletion of sequences 3' to +1 of the TIMP-1 gene significantly reduced the overall levels of CAT activity in response to mOSM. Deletion of sequences within +13 to +23 reduced the fold-induction by OSM of CAT activity from approximately 5-fold to 3-fold. Further deletion to +1 reduced the overall OSM-stimulated levels of CAT activity down to 2-fold. Thus, sequences within this region contribute to maximal murine OSM-inducibility of the TIMP-1 promoter in murine fibroblasts. As discussed above for hOSM studies, several putative DNA binding motifs exist within this region and include an AP-1-like element, a putative OSM-responsive SP1 binding site and a initiator element,

previously described in other promoters as essential for the assembly of a competent transcriptional initiation apparatus.

DNA binding activity from murine fibroblast nuclear extracts was defined for sequences proximal to the start of TIMP-1. AP-1 (-59/-53) and SP1 sites (-11/-6) constitutively bound nuclear factors as examined by EMSA analysis which was not significantly modulated in response to IL-6 type cytokines. Murine OSM appears to elicit a small change (approximately 2-fold) in AP-1 DNA binding activity which requires constitutively present fos-related antigens (likely dimerizing with jun nuclear factors) and OSM stimulates the recruitment of c-fos (at 1 hour of cytokine treatment) for the formation of AP-1 complexes. No separate OSM-specific AP1 complexes were detected and suggests that qualitative changes in c-fos and/or additional factors are necessary for complex 2 formation in murine cells in response to mOSM. c-fos was also not essential for the formation of AP-1 complexes at the 1 hour time point of OSM treatment. Recruitment of c-fos to bind the TIMP-1 promoter AP-1 element may however contribute a potent c-fos transcriptional activation domain which is absent in the smaller c-fos-related fra-1/-2 proteins (Suzuki et al., 1991). As neither IL-6 or LIF stimulates c-fos nuclear accumulation to the same degree as OSM, this may in part explain the selective upregulation of TIMP-1 mRNA by OSM and not other IL-6-type cytokines. Analysis of mechanisms regulating c-fos expression will further clarify how OSM is a potent inducer of this gene among IL-6-type cytokines. The c-fos promoter is under the

control of several regulatory elements including the SIE element (which binds STAT factors) as well as SRE and CRE elements (Karin, 1995). In addition to STAT binding to the SIE an examination of factors binding the SRE and/or CRE elements as well as signalling pathways activating these nuclear factors may define signal transduction pathways novel to the OSM among IL-6-type cytokines.

The role of c-fos in OSM-mediated TIMP-1 transcription was examined by utilizing murine lung fibroblasts derived from wildtype and c-fos-deficient mice. TIMP-1 mRNA levels were upregulated in mOSM treated murine lung fibroblasts but not by IL-6, LIF and EGF confirming previous observations demonstrating mOSM as a unique activator of TIMP-1 transcription among IL-6-type cytokines. However, upregulation of TIMP-1 mRNA was independent of c-fos expression as c-fos-deficient cells treated with OSM also increased TIMP-1 mRNA levels and showed similar kinetics in TIMP-1 mRNA expression over a time course of 8 hours. Moreover, no difference in the upregulation of CAT activity was observed from wildtype or c-fos-deficient cells transfected with the -62 /+23TIMP-1/CAT construct. Both wildtype and c-fos knockout cells equally activated STATs 1 and 3, and elicited AP1 complex formation. Constitutively expressed fos-related antigens in addition to c-fos were also present in wildtype and c-fos-deficient cells over a time course of OSM treatment. Although there is a modest reduction in OSM-induced TIMP-1 expression in c-fos-deficient cells (from 4-fold in wildtype cells to 3-fold in

c-fos knockout cells), alternative mechanism(s) likely account for the OSM-specific upregulation of TIMP-1 mRNA. Additional fos-related antigens which are present c-fos-deficient cells may compensate for c-fos in regulating the expression of the TIMP-1 gene. As described above, human OSM is capable of upregulating the expression of fos-related antigens other than c-fos. Although in murine lung fibroblasts, mOSM did not significantly alter the levels of fos-related antigens, mOSM may stimulate post-translational modifications that have a positive effect on TIMP-1 gene transcription. Moreover, the c-fos-independent regulation of OSM-induced TIMP-1 transcription in c-fos-deficient murine lung fibroblasts does not preclude c-fos involvement in upregulating TIMP-1 mRNA levels. Others have demonstrated upregulation of TIMP-1 promoter activity in murine F9 cells co-transfected with c-fos and junD expression vectors and over-expression of an estrogen-inducible c-fos transgene stimulates an increase in TIMP-1 mRNA levels (Logan et al., 1996, Reichmann et al., 1992). Thus, although c-fos is not necessary for murine OSM-regulation of the TIMP-1 transcription, its participation contributes to the expression of a variety of target genes which include TIMP-1.

STAT nuclear factors are markedly activated by mOSM. STATs 1 and 5 are preferentially induced by OSM after 15minutes and STAT3 after 1 hour when compared to other IL-6-type cytokines. Although no STAT DNA binding sites within the proximal TIMP-1 promoter region could be detected at least STATs 1 and 3 can directly bind and cooperate with SP1 in regulating gene transcription (Look

et al., 1995; Cantwell et al., 1998). We have shown by co-immunoprecipitation experiments that STAT3 and SP1 from murine fibroblast nuclear extracts can directly interact independent of any DNA binding activity. Supershift analysis of a TIMP-1 SP1 probe complexes could not however detect STAT nuclear factor binding to this probe. The proportion of STAT-SP1 complexes may however be relatively lower in comparison to the amount of SP1 and make detection difficult by EMSA analysis. This would be in agreement with higher levels of SP1 than STAT3 immunoprecipitated from nuclear extracts (Figure 30).

The relative contribution of STATs and AP-1 factors in the regulation of TIMP-1 promoter activity was assessed by utilizing dominant-negative STAT and AP-1 over-expression vectors in murine and human systems. Dominant-negatives of STAT1, 3 and 5 lack a carboxy-terminal transactivation domain and repress transcriptional activation by endogenous STATs through a variety of mechanisms (Kim and Baumann, 1997; Heinz Baumann, personal communication). Dominant-negative STATs may dimerize with native STAT factors thereby generating an inefficient transcriptional activator. These repressors may also competitively inhibit the interaction of endogenous STAT factors to binding sites on receptors or promoter DNA elements. A dominant-negative of AP-1 (A-Fos) consisting of an acidic amphipathic peptide sequence appended onto the leucine-zipper dimerization motif of c-fos has been previously demonstrated to function as an efficient repressor of AP-1 activity (Olive et al., 1997). We utilized this AP-1 repressor in concurrent

experiments to assess the contribution of AP-1 to TIMP-1 promoter activity and compared this with results obtained from deleting the TIMP-1 AP-1 element of TIMP-1/CAT reporter gene chimeric constructs.

Deletion studies demonstrated an important role for AP-1 TIMP-1 promoter activity in response to human OSM in HepG2 cells and murine OSM in NIH3T3 cells. Despite reduction of basal and OSM-induced CAT activity the fold-induction by murine OSM over basal levels w3as not affected by deletion of the AP-1 site in the murine NIH3T3 fibroblast studies. This suggests a role for AP1 in the mechanism of OSM upregulation of TIMP-1 promoter activity in HepG2 cells that may not exist in NIH3T3 fibroblasts.

A-Fos abrogated hOSM-induced TIMP-1 promoter activity in human HepG2 cells (Figure 37B). Similar experiments were done in NIH3T3 cells (Figure 36). Since c-Fos and A-Fos similarly reduced basal levels of promoter activity the result could not be interpreted (A-Fos has been previously described as a 3000-fold more effective inhibitor of AP-1 activity (Olive et al., 1997)). Further experiments utilizing an AP-1-dependent promoter linked to a reporter gene may help to clarify the effectiveness of the c-Fos and A-Fos vectors in these cells.

Thus, AP-1 appears to play an important role in the regulation of the TIMP-1 promoter in human cells stimulated with hOSM. The levels of c-fos protein are greater in human HepG2 cells (Figure 12) than in the mouse NIH3T3 cells (Figure 23) while levels of STAT activity (such as, STAT binding to the SIE probe) are

relatively equivalent in both cell types (compare Figures 5 and Figure 24). This difference in c-fos protein levels may significantly affect the mechanism by which OSM regulates the TIMP-1 promoter and explain the predominant role of c-fos in HepG2 but not NIH3T3 cells.

In separate experiments dominant-negatives of STATs 1, 3, and 5 markedly suppressed mouse OSM-induced TIMP-1 promoter activity in murine NIH3T3 cells. Ets overexpression vectors (Ets2 and PEA3) also had a repressive effect but to a lesser extent in comparison to STAT dominant-negatives. Ets nuclear factors have been characterized as repressors of transcription (Goldberg et al., 1994) and overexpression of Ets factors may promote its interaction with a putative Ets-DNA binding motif that flanks the TIMP-1 AP-1 site (at -59/-53) (Edwards et al., 1992). Dominant-negatives of the STATs may be preventing the direct or indirect interaction of endogenous activated STAT proteins with a previously described putative TIMP-1 STAT DNA element (at -49 to -41) (Bugno et al., 1995). Alternatively, and consistent with observations by us and others, STAT factors may participate by binding the SP1 nuclear factor and facilitate increases in TIMP-1 transcription (Look et al., 1995; Cantwell et al., 1998).

Dominant-negative STAT3 (STAT3d), but not dominant-negative STAT1 (STAT1d), could also reduce human OSM-stimulated upregulation of CAT reporter gene expression of TIMP-1 promoter/CAT chimeric constructs transfected into human HepG2 cells. However, this reduction was significantly less than that

observed by A-Fos (a 9-fold to 1.5-fold decrease with A-Fos versus a 30-fold to 20-fold reduction with dominant-negative STAT3). In addition, basal levels of CAT activity are higher following TIMP-1/CAT and STAT3d cotransfection and account for reduced fold-induction over basal. OSM-stimulated CAT activity is not diminished but also increased in this cotransfection experiment. Thus, AP-1 appears to predominate over STAT in the upregulation of the TIMP-1 promoter by human OSM in human HepG2 cells. However, STAT may still contribute to TIMP-1 promoter activation as described above for mOSM and murine cells. Moreover, STATs have been characterized to upregulate AP-1 factor gene expression, and binding of STATs to the SIE DNA element of the c-fos promoter is consistent with the de novo expression of c-fos protein and TIMP-1 mRNA in response to human OSM.

As c-fos-deficient human HepG2 cells have not been described, developing a cell line which lacks the expression of c-fos will aid in determining the relative contribution of c-fos to OSM-induced TIMP-1 mRNA upregulation. An anti-sense c-fos gene stably introduced into a HepG2 cell line could be utilized to address this issue. Anti-sense expression of c-fos RNA has been previously demonstrated to inhibit the expression of c-fos mRNA (Ledwith et al., 1990). Anti-sense c-fos could be cloned into a commercially available expression vector that is under the control of an inducible promoter (such as by estradiol). HepG2 cells could then be transfected with this vector and HepG2 cell clones selected (i.e., for neomycin

resistance) that stably integrate into the genome a chimeric estradiol-regulated antisense c-fos gene. Isolated clones could then be stimulated with human OSM or remain untreated in the absence or presence of the transgenes inducer. The expression of c-fos and TIMP-1 mRNA would then be tested. One possible outcome would be that the expression of anti-sense c-fos ablates human OSM-inducible TIMP-1 mRNA expression. This would be consistent with the above observations that demonstrate the necessity of AP-1 in cytokine regulation of TIMP-1/CAT chimeric reporter gene expression and the OSM-stimulated recruitment of c-fos to the TIMP-1 AP-1 promoter element.

In summary, OSM is the most potent stimulator of TIMP-1 expression among IL-6-type cytokines and specific regulation of this gene by OSM is acheived through preferential activation of intracellular signalling pathways. Human OSM is unique in its capacity to stimulate the nuclear accumulation c-fos protein which participates in AP-1 complexes that interact with a proximal TIMP-1 promoter AP-1 DNA element necessary for maximal OSM-induction of TIMP-1 promoter activity. Sequences downstream of TIMP-1 +1 also contribute to the maximal induction of promoter activity in response to OSM. Both the activation of c-fos, AP-1 complexes containing c-fos and TIMP-1 expression are sensitive to protein synthesis inhibitors. SP1 and Ets-DNA binding factors also interact with the TIMP-1 promoter and contribute to basal levels of CAT reporter gene expression of TIMP-1 promoter/CAT

chimeric plasmids. Human OSM also stimulates the expression of additional fos-related antigens. Human OSM is unique among IL-6 type cytokines in stimulating c-fos expression. AP-1 nuclear factors appear to be important in the regulation of the TIMP-1 promoter in response to OSM. A proposed series of signalling events illustrating the participation of AP-1/c-Fos and STAT nuclear factors in mediating OSM-induced TIMP-1 transcription are depicted in Figure 38.

Murine OSM is also unique among IL-6-type cytokines in upregulating c-fos and TIMP-1 expression as well as STAT nuclear factors. C-fos is not necessary for mOSM-mediated induction of TIMP-1 transcription as assessed in c-fos-deficient murine cells and dominant-negative STAT factors effectively repress the activity of the TIMP-1 promoter. Additional factors such as SP1 interact with the sequences proximal to the start of TIMP-1 transcription and may be instrumental in interacting with STAT3 for mediating the upregulation of TIMP-1 transcription.

Thus, human and murine OSM can utilize alternate mechanisms for acheiving the specific upregulation of TIMP-1 gene expression among IL-6-type cytokines and the distinctive induction AP-1/c-fos and STAT nuclear factors suggests their participation in a variety of biological responses unique to this cytokine.

Figure 38. Model of Oncostatin M Signalling of TIMP-1 Transcription.

A model of Oncostatin M(OSM) specific induction of TIMP-1 transcription is illustrated. OSM interacts with a dimeric receptor complex composed of gp130 and an OSM-specific receptor subunit in order to manifest the potent induction of TIMP-1 transcription among IL-6-type cytokines. The JAK-STAT pathway is activated by OSM and STAT nuclear factors dimerize, translocate into the nucleus and may directly interact with the TIMP-1 promoter through binding to SP1 nuclear factors or interacting with a putative Ets/STAT-like binding site. Alternatively, and likely the predominant mechanism in human HepG2 cells, OSM-induced STAT activation contributes to increases in c-fos protein. Upregulated levels of c-fos then participates with jun nuclear factors in forming AP-1 complexes that bind the TIMP-1 promoter and markedly increase transcription of the TIMP-1 gene. Fos-related (fosB, fra-1, fra-2) and jun factors also constitutively bind the TIMP-1 AP-1 site and may contribute to basal levels of TIMP-1 transcription. MAP-kinases (MAPK) may also contribute to the activation of STAT and AP-1/c-fos nuclear factors. Additional elements within the TIMP-1 promoter including the putative Ets/STAT-like site, an SP-1 element and sequences downstream of TIMP-1 +1 also contribute to TIMP-1 gene expression.
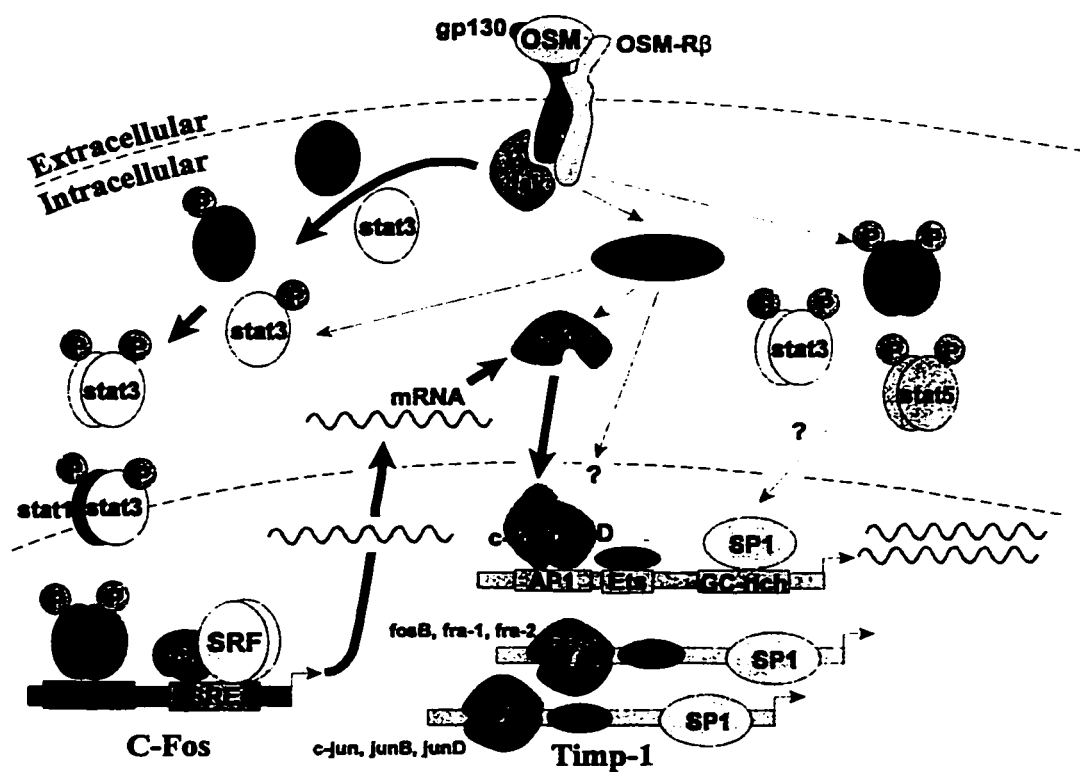
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