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**Characterization of Tissue Inhibitor of Metalloproteinases-3 (TIMP-3) from
the Extracellular Matrices of Cultured Human and Avian Cells**

by

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B.Pharm., M.Pharm., M.S., M.B.A.

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

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Dedication

To my family,
Barkha and Nikhaar,
whose unwavering love and support made this dissertation
a pleasant experience

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Characterization of Tissue Inhibitor of Metalloproteinases-3 (TIMP-3) from the Extracellular Matrices of Cultured Human and Avian Cells

Narendra Sugno Kishnani

Abstract

A transient stimulation in the synthesis and deposition of an extracellular matrix (ECM), 21-kDa protein occurs during the early stages of oncogenic transformation of chicken embryo fibroblasts (CEF) or after treatment of cells with phorbol myristate acetate (Blenis and Hawkes, 1983; 1984). This protein not only exhibits metalloproteinase inhibitory activity (Staskus *et al.*, 1991) but also accelerates morphological transformation and stimulates proliferation of growth-retarded CEF (Yang and Hawkes, 1992). Collectively, these observations implicate the 21-kDa protein in the development of transformation.

In this thesis, the function of the 21-kDa protein was probed by specifically inhibiting its synthesis using antisense oligodeoxynucleotides (ODNs). A segment of its cDNA was amplified by PCR, cloned and sequenced. Based primarily on the complete cDNA sequence, the 21-kDa protein was re-named ChIMP-3 (Chicken Inhibitor of MetalloProteinases-3) or chicken TIMP-3 (Pavloff *et al.*, 1992). Synthesis of this protein could be inhibited by as much as 75% by ODNs targeted at the 5' region of its mRNA. However, they

appeared to interact with ChIMP-3 and ChIMP-a (another ECM-specific inhibitor) in a sequence independent but a concentration dependent manner and caused these proteins to be released from the ECM. This unexpected result ruled out this approach to determining the role of matrix-bound ChIMP-3 in cell transformation.

Interactions of ChIMP-3 and ChIMP-a with various charged polymers indicated that polycations were more effective in eliciting a change in location than polyanions. The inhibitors bound heparin but not poly(lysine), indicating different mechanisms of interaction. Polycations probably displace the inhibitors from the ECM whereas polyanions may competitively bind to them and release them from their matrix binding sites.

Finally, human TIMP-3 was identified exclusively in the ECM of cultured cells by protease-substrate gels and by Western blots using anti-ChIMP-3 antibody. It is not N-glycosylated, appears to be resistant to trypsin digestion and exhibits a characteristic shift in mobility upon reduction. For those residues determined, the N-terminal sequence of the isolated protein is identical to that of ChIMP-3.

Susan P. Hawkes

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List of Abbreviations

2.0.0+	Media 199 with 2% tryptose phosphate broth, 0.1% glucose
2.0.0.1+	Media 199 with 2% tryptose phosphate broth, 0.1% chicken serum, 0.1% glucose
2.2.1+	Media 199 with 2% tryptose phosphate broth, 2% calf serum, 1% chicken serum, 0.1% glucose
APMA	p-Aminophenylmercuric acetate
AS-ODN	Antisense oligodeoxynucleotides
bisTris	{[bis-(2-Hydroxyethyl)-imino]-tris-(hydroxymethyl) methane}
BSA	Bovine serum albumin
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CEF	Chicken embryo fibroblasts
CHAPS	(3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate)
ChIMP	Chicken inhibitor of metalloproteinases
CM	Conditioned media
CMF-PBS	Phosphate buffered saline, Ca ²⁺ -, Mg ²⁺ -free
CTAB	Cetyltrimethylammonium bromide; Hexadecyltrimethylammonium bromide; Cetrimide, USP
Da	Daltons
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DTT	Dithiotheritol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EPA	Erythroid potentiating activity
ESAF	Endothelial cell stimulating angiogenic factor
F12	Ham's nutrient mixture
FBS	Fetal bovine serum
HgCl ₂	Mercuric chloride
IL	Interleukin
IMP	Inhibitor of metalloproteinases
kDa	Kilodaltons
KLH	Keyhole limpet hemocyanin
MEM α	Minimum essential media with ribonucleosides and deoxyribonucleosides
MMP	Matrix metalloproteinases
M _r	Molecular weight
MW	Molecular weight
MWCO	Molecular weight cutoff
ODN	Oligodeoxynucleotides
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PMN	Polynuclear neutrophils
PMNL	Polymorphonuclear leucocytes
PMSF	Phenylmethylsulfonyl fluoride
PN-1	Protease nexin-1
PVDF	Polyvinylidene difluoride

RSV	Rous sarcoma virus
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
t-PA	Tissue plasminogen activator
TBS	Tris buffered saline
TD	Tris diluent
TES	(2-[[<i>tris</i> -(Hydroxymethyl)-methyl]-amino]-ethanesulfonic acid)
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
TPA	12-O-Tetradecanoylphorbol-13-acetate; phorbol myristate acetate
Tris	Tris(hydroxymethyl)aminomethane; Trizma®
tsRSV	Rous sarcoma virus, temperature-sensitive mutant
tsRSV-LA24	Rous sarcoma virus, temperature-sensitive mutant, LA-24
u-PA	Urokinase plasminogen activator
°C	Degree Centigrade

Chapter 1

Introduction

1.1 Synopsis

The synthesis of an extracellular matrix-specific 21-kDa protein is stimulated during oncogenic transformation of chicken embryo fibroblasts (CEF) or by treatment of these cells with phorbol myristate acetate, a potent tumor promoter. This protein is a multifunctional protein: it is a metalloproteinase inhibitor which is capable of stimulating proliferation of non-transformed CEF, promoting detachment of transforming cells from their extracellular matrix (ECM) and accelerating morphological transformation. This protein has now been established as a third member of the TIMP (Tissue Inhibitor of MetalloProteinases) family. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are secreted as zymogens and upon activation can degrade the ECM. Degradation occurs in a highly controlled manner in normal physiological processes such as wound repair, ovulation and morphogenesis, but appears to be accelerated in some pathological conditions such as tumor invasion and metastasis in cancers, arthritis and periodontitis. This may be due, in part, to an imbalance between zymogen MMPs, activated MMPs and their naturally occurring inhibitors, TIMPs. In the following sections, characteristics of transformed cells and the involvement of MMPs and TIMPs in ECM remodelling in normal and diseased states have been described. The apparent role of the 21-kDa protein in oncogenic transformation implicates this protein during the development of metastatic disease. As a consequence, particular emphasis has been placed on various aspects of cancer and characteristics of tumor cells.

1.2 The 21-kDa Protein

Rous sarcoma virus (RSV) is a retrovirus that infects chickens and results in development of sarcomas in infected birds, primarily due to increased expression of the *src* gene of RSV. Mutants of RSV that are temperature sensitive with respect to manifestation of the transformed phenotype have been invaluable in identification of cellular events associated with oncogenic transformation, such as the effect of the *src* gene product pp60^{src} (a tyrosine protein kinase) on phosphorylation of cellular proteins and on the expression of other novel proteins. A major advantage of the temperature-sensitive RSV mutants is that they allow synchronization of transformation, and hence such a system has been adopted by this laboratory to investigate changes that occur in the extracellular matrix of cultured chicken cells *during* the process of transformation by RSV.

Studies with cultured chicken embryo fibroblasts (CEF) infected with a temperature sensitive mutant of RSV, LA24-clone G2, indicated a transient 3-fold increase in the expression and deposition of a 21-kDa protein in the extracellular matrix of transforming CEF. Maximal synthesis occurred between 8 and 12 hours after shift to a permissive temperature of 35 °C in comparison to infected CEF maintained at a non-permissive temperature of 41 °C (Blenis and Hawkes, 1983; Blenis and Hawkes, 1984). Similar stimulation of the 21-kDa protein deposition was also observed when uninfected CEF were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter, indicating that the 21-kDa protein may be playing a role in the manifestation of the transformed phenotype. N-terminal sequencing of the purified protein (Staskus *et al.*, 1991) revealed similarities to the two members of a family of proteins designated TIMP-1 (Tissue Inhibitor

of MetalloProteinases-1) and TIMP-2. Indeed, reverse zymographic analysis showed that the 21-kDa protein was a metalloproteinase inhibitor (Staskus *et al.*, 1991).

The 21-kDa protein has now been clearly established as a new member of the TIMP family, a TIMP-3, following cloning and sequencing of its cDNA (partially presented in this thesis) (Pavloff *et al.*, 1992). In addition, *in vitro* experiments involving the culture of uninfected nontransforming CEF on dishes coated with purified 21-kDa protein revealed that the protein elicited phenotypic changes (as assessed by morphology or by a functional assay such as cell detachment) similar to those observed in transforming CEF (Yang and Hawkes, 1992). Also, the 21-kDa protein was found to stimulate the proliferation of growth retarded, nontransforming CEF maintained under low serum conditions. These cells divided at the same rate as exponentially growing transforming CEF cultured on dishes with or without a matrix of 21-kDa protein. However, the fact that these observations were made in *in vitro* experiments (where the experimental design required certain manipulations of both the cells and the 21-kDa protein) necessitated that the results be confirmed by a more direct and independent method. For example, the effect of decreased amount of the 21-kDa protein on the characteristics of the transformed phenotype could be determined by specifically inhibiting the biosynthesis of the 21-kDa protein during transformation in cultured cells.

Collectively, these studies indicate that the 21-kDa protein plays a role during the early stages of oncogenic transformation. In addition, the fact that the 21-kDa protein is exclusively located in the extracellular matrix of cells and exhibits metalloproteinase inhibitory activity may be a significant property of this protein. Its proximity to extracellular matrix-degrading metalloproteinases may be a critical factor in controlling matrix degradation

under both normal physiological events as well as in pathological conditions such as cartilage destruction in arthritis or tumor invasion and metastasis in cancers.

1.3 Cancer

Cancer is considered to be a genetic disease resulting from one or more genetic alterations caused by point mutations, mitotic recombination, gene amplification, chromosome loss, translocation, deletion and other mechanisms (Temin, 1990). Carcinogenesis or tumorigenesis is generally a multistage process which may involve the participation of multiple agents. These may exert their effects at various stages in the progression of the disease. The nature of these agents may be physical (e.g. radiation), chemical (genotoxic carcinogens e.g. polycyclic hydrocarbons, N-nitrosamines, heterocyclic amines or nongenotoxic carcinogens e.g. okadaic acid, TPA, teleocidin) or biological (e.g. DNA or RNA viruses). In the case of certain highly oncogenic retroviruses, such as Rous sarcoma virus and feline leukemia virus, infection is usually sufficient for establishment of carcinogenesis. Viral DNA may also randomly integrate in the host genome resulting in normal cellular proto-oncogenes being placed under the control of strong transcriptional signals with overexpression of oncogenic proteins (Temin, 1990).

1.4 Transformed Cells in Culture

Cells transformed in culture with a carcinogenic agent, such as a virus, exhibit characteristic behavior that is different from non-transformed cells.

Morphologically, transformed cells in culture grow in a disoriented pattern in contrast to normal cells that grow in a organized pattern. Proliferation of normal cells is typically contact-inhibited and is often constrained to a confluent monolayer. In contrast, transformed cells are not contact-inhibited and can grow "piled up" at high cell density in multiple layers, often with a decreased requirement for growth factors (which are typically provided by serum present in culture media). Transformed cells also may be "immortal" in culture and may be capable of growing in soft agar in an anchorage-independent manner. In addition, the characteristic uncontrolled proliferation of cells in tumors has been proposed to be related to loss of multiple controls on cell division as a result of overexpression of specific oncogene products. These are often growth factors, growth factor receptors or regulatory cell-division signaling proteins. Also, cells transformed in culture or tumor-derived cells are capable of forming new, biologically malignant tumors when injected into immunocompromised host animals. Overall, several properties of cells transformed in culture are similar to those of tumor cells derived from tumors excised from humans or other animals, indicating the usefulness and validity of cells transformed in cell culture as a reasonable tumor model.

1.5 Characteristics of Malignant Tumors

Generally in cancer, there appears to be activation of certain oncogenes and genes that accelerate cell proliferation and metastasis, while tumor suppressor genes appear to be inactivated (reviewed by Sugimura *et al.*, 1992). From a clinical point of view, tumors fall in one of two categories: benign or malignant. Benign tumors are easily treated and cured, often by surgical

removal, because they grow locally within tissue boundaries. In contrast, malignant tumors are capable of breaking away from the primary tumor mass after initial local growth, penetrating through basement membranes to enter the circulation, lodging at a distant vascular bed, extravasating into a target site and growing to form secondary tumors. Even though a vast majority (99.99%) of metastatic tumor cells are killed by the host immune system, the ability of the remaining cells to evade immune detection, invade and colonize other normal tissues makes malignant cancers life-threatening. Clinical treatment of most cancers often involves surgical removal of the tumor mass, when possible, and administration of anti-neoplastic chemotherapeutic agents and/or ionizing radiation to slow the proliferation of tumor cells either at the primary tumor site or distant metastasized locations. However, drug intervention at other stages of the disease, such as invasion of surrounding tissues and metastasis, is still in a developmental stage, although protease inhibitors such as TIMPs, synthetic inhibitors of metalloproteinases and cysteine protease inhibitors are being actively investigated to block invasion.

To date, events or factors involved in tumor invasion and metastasis are not fully understood although significant progress has been made since Hippocrates' (460-370 BC) theory that tumor cell invasion is caused by "diffusing toxic fluids" (Liotta, 1990). Interestingly, as pointed out by Liotta, Hippocrates' concept of a localized 'imbalance of the Four Humors' may be valid even today because the net proteolytic activity exhibited by invasive cells may be dependent on a critical balance between degradative (proteolytic and more recently, glycosidic) enzymes and their inhibitors (Nakajima *et al.*, 1988; Liotta and Stetler-Stevenson, 1991). However, there is increasing evidence of involvement of factors other than just degradative enzymes in

tumor cell invasion and metastasis. These include increased expression of oncogene products and factors affecting coagulation, cell adhesion, cell motility and angiogenesis.

An altered expression of cell adhesion molecules and their receptors has been observed in malignant phenotypes, e.g. a gene (termed DCC) for a cell adhesion protein has been shown to be deleted in colorectal cancer (Fearon *et al.*, 1990). Anticoagulant agents (e.g. heparin) or antithrombotic agents (e.g. nonsteroidal anti-inflammatory agents) have been shown to inhibit progression in those tumors that exhibit increased expression of coagulation factors, such as thrombin in small cell carcinoma of the lung. Increased expression of angiogenic proteins not only stimulates neovascularization for tumor growth, but also may signal the onset of metastasis. In addition, the abundance of angiogenic inhibitors in certain tissues, such as cartilage, has been thought to prevent invasion by tumors such as osteogenic sarcoma (Folkman, 1990a; Folkman, 1990b).

For malignant tumor cells to migrate, they typically would need to disengage their adhesive contacts with either the ECM or neighboring cells (Ruoslathi, 1991), and this would probably occur concomitantly with other events such as matrix degradation and increased production of autocrine motility or scatter factors. For example, when cell-cell adhesion molecules were blocked using antibodies, noninvasive cells were observed to be invasive, and when expression of a cell surface adhesion receptor was increased, tumorigenicity was decreased. These data suggest that metastatic potential of malignant cells can be decreased by 'gluing' them in place (Ruoslathi, 1991). Conversely, *ex vivo* pretreatment of highly metastatic mouse melanoma cells with fibronectin or laminin receptor binding peptides (RGDS or YIGSR, respectively) inhibited lung colonization, when injected

into mice via their tail veins (Humphries *et al.*, 1986; Gehlsen *et al.*, 1988). The mechanism of inhibition appears to involve an increased clearance rate of tumor cells from the pulmonary microcirculation. This is presumably due to fibronectin and laminin receptors on the tumor cell surface being blocked by the peptides which reduces the ability of these cells to attach to endothelial binding sites in the microcirculation. Hence it appears that these and other unknown factors act in concert, rather than in isolation, to enable a tumor cell to colonize at a location distant from the primary tumor.

Specific oncogenes, such as *ras*, have been shown to induce metastatic properties in some cells. This may be due to increased expression of degradative enzymes, such as metalloproteinases and cysteine proteinases, coupled with decreased expression or activity of their inhibitors (TIMPs and cystatins, respectively). Also, other *ras*-responsive genes showing increased expression are calyculin (a cytoplasmic calcium binding protein) and osteopontin (a secreted calcium-binding protein), which possibly may alter adhesive properties of metastatic cells (Chambers and Tuck, 1993). Su *et al.*, (1993) looked for changes in expression of specific putative tumor-suppressor and metastasis-promoting genes in Ha-*ras*-transformed cloned rat embryo fibroblasts. They found that the metastatic potential of these cells in nude mice correlated directly with reduction in nm23-H1 (a putative metastasis-suppressor gene product) and TIMP-1 transcription and mRNA levels and a concomitant increase in expression of cripto (a novel growth factor) and degradative enzymes such as the 92-kDa matrix metalloproteinase, osteopontin and transin. In contrast, expression of other tumor-suppressor genes such as retinoblastoma and p53, and tumor-promoting genes or genes "associated" with tumorigenesis such as the 72-kDa matrix metalloproteinase, fibronectin, major excreted glycoprotein (MEP) and intracellular adhesion

molecule (ICAM-1) were not altered, suggesting that specific combinations of tumor-suppressor and metastasis-promoting genes are coordinately regulated in a specific tumor progression.

Another fundamentally important observation has been the susceptibility of various tissues to invasion and colonization by metastatic malignant tumors, e.g. liver, lung and bone marrow are extremely susceptible, while spleen, heart valves, lens, cornea, uncalcified cartilage and cirrhotic liver are resistant. Although the reason for such resistance is unknown, it is noteworthy that these tissues, with the exception of cirrhotic liver, are devoid of an intrinsic blood supply, a medium through which tumor cells disseminate. In the case of cartilage, it has been proposed that resistance to tumor invasion may be due to the presence of specific angiogenesis inhibitors (that also have metalloproteinase inhibitory activity) (Moses *et al.*, 1990; Moses and Langer, 1991; Moses *et al.*, 1992) or to the presence of various types of other protease inhibitors (serine- and cysteine-protease inhibitors) in cartilage or to components of cartilage that are particularly resistant to degradation. Cartilage is primarily composed of collagen type II (a cartilage specific collagen which is more resistant to proteolytic degradation than collagen I or III). It also contains proteoglycans, which may exhibit resistance to invasion (Evans, 1991), as demonstrated by the unusual ability of the Yoshida rat sarcoma to invade cartilage, presumably due to its ability to degrade proteoglycans (Mikuni and Gross, 1984). On the other hand, myofibroblasts in cirrhotic livers have been shown to express high levels of a ~40-kDa metalloproteinase inhibitor, which is not expressed in normal livers. In *in vitro* invasion assays this inhibitor was shown to block invasion by malignant cells and may be a possible mechanism by which cirrhotic livers resist metastasis (Barsky and Gopalakrishna, 1988).

It is clear that the immediate environment of cancer cells influences their ability to metastasize. For example, human colon carcinoma tumor cells implanted in the cecal wall of nude mice produced high levels of ECM degrading enzymes and metastasized to lymph nodes and liver (Nakajima and Chop, 1991). In contrast, there was no metastasis when cells were implanted in the subcutis. In general, tumor cells and tumor-derived molecules interact with the host cells, their extracellular matrix components and host organ-derived paracrine and autocrine motility factors, which are probably differentially expressed in tumor and host cells, and collectively these factors determine the colonization ability of the tumor cells (Nicolson, 1991).

1.6 The Extracellular Matrix of Cells

The extracellular matrix (ECM), broadly defined, is composed of relatively stable and immobilized structural materials that either lie under cells (e.g. epithelial cells) or surround them (e.g. connective tissue cells). The ECM is a complex network of interacting macromolecules which usually includes various types of collagens, proteoglycans, elastin and structural glycoproteins, but under specific conditions it may be composed of primarily a single component. For example, when an oocyte resumes meiosis its ECM is mainly hyaluronic acid, a large glycosaminoglycan molecule, which is laid down by cumulus cells surrounding the oocyte (Dekel and Phillips, 1979). Until about a decade ago, the ECM was often considered to be an inert scaffolding for supporting cells within a tissue (Hay, 1991). However, there is a growing body of evidence suggesting that physiologically important macromolecules often exert their effects while residing in the ECM and

depending on the organization and composition of the ECM affect the metabolism and ultimate fate of the cell. The ECM is dynamic. Cells interact with their own ECM as well as with the ECM produced by other cells. In general, the ECM has a profound effect on cellular characteristics such as cell shape, cell migration, control of proliferation, morphological phenotype and differentiation.

1.7 Matrix Metalloproteinases

Collectively, components of the extracellular matrix, such as interstitial and basement membrane collagens, fibronectin, laminin and proteoglycans can be degraded by the various members of a growing family of proteolytic enzymes designated matrix metalloproteinases (MMPs) (Woessner, 1991). Some recent evidence indicates that members of other protease families, such as serine proteases and cysteine proteases, may also participate in matrix degradation, either directly, or indirectly by activating MMPs (Everts *et al.*, 1992; Palmgren *et al.*, 1992). In contrast, Wong *et al.*, (1992) used specific protease inhibitors and provided evidence that strongly suggested a role for MMPs and plasmin, but not cysteine and aspartic proteases, in degradation of the ECM. Also, Young and Spevacek, (1993) have observed that leupeptin, a thiol proteinase inhibitor, completely blocked invasion of murine B16F10 metastatic melanoma cells through reconstituted basement membrane (Matrigel), indicating that either secreted or cell-surface associated thiol proteinase(s) are also involved in ECM degradation. Hence, it appears that degradation of the ECM may involve various interacting enzymes, with the MMPs probably being the major degradative group of enzymes responsible for ECM breakdown.

MMPs were first discovered in 1962 when it was demonstrated that collagenase specifically cleaved native collagen during tadpole tail resorption (Gross, 1962). MMPs are one of the most important classes of enzymes involved in matrix remodeling (extensively reviewed by Woessner, 1991; Matrisian, 1992; Birkedal-Hansen, 1993; Birkedal-Hansen *et al.*, 1993). While MMP activity is tightly regulated under normal physiological conditions such as ovulation, wound repair, and early embryonic morphogenesis, it appears to be aberrant under pathological conditions such as tumor invasion and metastasis, arthritis and periodontitis. Within the past few years, the potential role of MMPs in normal and diseased states has expanded to include: 1) a role of MMPs during dilation of the cervix in women in labor (Rechberger and Woessner, 1993); 2) aberrant MMP activity, specifically MMP-2, that results in excessive deposition of amyloid protein in the extracellular matrix of neural tissues in Alzheimer's disease (Miyazaki *et al.*, 1993b); 3) an increased secretion of MMP-9 by HIV-1-infected human lymphocytes results in these cells being conferred with an enhanced ability to leave the circulation and localize in target tissues (Weeks *et al.*, 1993); and, 4) increased MMP activity in chronic wound fluid which has been proposed to be responsible for the failure of wound closure (Wysocki *et al.*, 1993).

To date, the MMP family consists of several well characterized members with molecular weights ranging between 28- and 92-kDa (see recent reviews: Woessner, 1991; Matrisian, 1992; Birkedal-Hansen *et al.*, 1993). These have been classified into 3 groups primarily based on their substrate specificity (Table 1.1). Also, comparisons of the primary amino acid sequences of MMPs reveal that they contain conserved regions. Based on these conserved sequences, the largest MMP (MMP-9) has been divided into 7 domains (Figure 1.1). In other MMPs, some of these domains have been deleted.

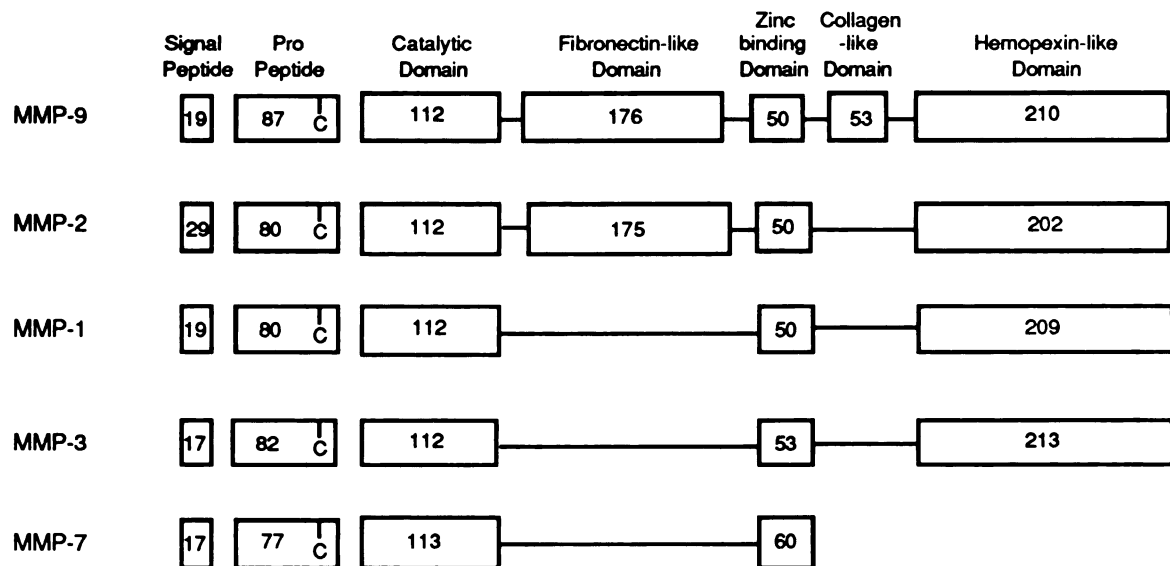


Figure 1.1. Domain structures of selected human MMPs. The cysteine residue involved in the "cysteine switch" mechanism is indicated in the pro-peptide domain

Although substrates for MMPs have been believed to be mainly ECM components, specific MMPs may act on other unusual substrates, as recently reported for MMP-1, which inactivated two major plasma serine protease inhibitors (serpins), α_1 -proteinase inhibitor and α_1 -antichymotrypsin, by cleaving peptide bonds at sites unrelated to those cleaved in collagenous molecules (Desrochers *et al.*, 1991). Also, α_1 antitrypsin, which is the main physiological inhibitor of neutrophil elastase, is cleaved by stromelysin, resulting in potentiation of neutrophil elastase activity (Winyard *et al.*, 1991). Recently, MMP-2 and MMP-9 have been shown to display elastase properties (Senior *et al.*, 1991).

A recent addition to the MMP family is stromelysin-3 (MMP-11) which has been cloned and sequenced by using a subtracted cDNA library from a surgical specimen (Basset *et al.*, 1990). It has been found to be overexpressed

Table 1.1**Selected Matrix Metalloproteinases and their Common Substrates**

Group	Name	Other names	Common Substrates
I	MMP-1	Interstitial collagenase Fibroblast collagenase	Collagen I, II, III, VII, X
	MMP-8	Neutrophil collagenase PMN collagenase	Collagen I, II, III
II	MMP-2	72-kDa gelatinase 72-kDa Type IV collagenase Gelatinase A	Gelatins, Collagen IV, V Elastin
	MMP-9	92-kDa gelatinase 92-kDa Type IV collagenase Gelatinase B	Gelatins, Collagen IV, V Elastin
III	MMP-3	Stromelysin, Stromelysin-1, Proteoglycanase, Transin, Procollagenase activator	Gelatins, Proteoglycans, Collagen III, IV, V, IX Fibronectin, Laminin
	MMP-10	Stromelysin-2 Transin-2	Gelatins, Proteoglycans, Collagen III, IV, V, IX Fibronectin, Laminin
	MMP-7	Uterine metalloproteinase Pump-1 Matrilysin	Gelatins, Proteoglycans, Elastin
?	MMP-11	Stromelysin-3	?
?	?	Metalloelastase	Elastin, fibronectin, casein, α_1 -antitrypsin

in stromal cells surrounding invasive breast carcinomas in comparison to normal tissues and several breast cancer cell lines (Basset *et al.*, 1990). However, its substrate specificity remains to be characterized. Another recent addition to the MMP family is a murine macrophage metalloelastase. This may represent a fourth group of the family based on its substrate specificity (Shapiro *et al.*, 1992).

Recently there have been an increasing number of reports of metalloproteinases that are not activated by p-aminophenylmercuric acetate (APMA), the most common activator of MMPs, and are not inhibited by TIMPs. A novel 150-kDa metalloproteinase that is not inhibited by TIMP-2 has been identified in the conditioned media of cultured rat glomerular epithelial cells and is distinct from MMP-2 and MMP-9 as determined by Northern blots (Johnson *et al.*, 1992). Similarly, a novel 80-kDa MMP has been identified in hormone-dependent breast cancer cell lines (Shi *et al.*, 1993). Again, like the 150-kDa metalloproteinase, it not activated by APMA and is not inhibited by TIMP-2. A similar, but higher molecular weight MMP, that is not activated by APMA and is not inhibited by TIMP, has been purified from human metastatic carcinoma tissue (Tsuda *et al.*, 1993). There is evidence for the presence of other less characterized MMPs, e.g. 13 proteins, ranging in molecular weights between 64 kDa and 163 kDa which display metalloproteinase activity in prostatic secretions from patients with either adenocarcinoma or benign hyperplasia (Wilson *et al.*, 1993). Also, a highly glycosylated MMP (95,000 kDa) has been isolated from the conditioned media of cultured rat mammary carcinoma cells, where it is constitutively expressed, in an inactive zymogen form and not complexed with any TIMP molecule(s) (Lyons *et al.*, 1991). Although it can be activated like other MMPs, by treatment with 1% SDS, organomercurials or trypsin and spontaneously

during storage, its relationship to MMP-9 (92-kDa MMP), especially considering its close resemblance in size, has not been reconciled.

MMPs are reversibly inhibited by members of a family of small natural protein inhibitors called TIMPs and irreversibly by α_2 -macroglobulin, usually by covalent modification of the activated MMP. To date, the TIMP family consists of three members: TIMP-1, TIMP-2 and TIMP-3. These inhibitors exhibit a fair degree of specificity in their ability to inhibit metalloproteinases, and usually bind in a 1:1 stoichiometry. TIMP-1 preferentially inhibits MMP-9, although it can inhibit MMP-2 at high inhibitor:enzyme ratios and TIMP-2 preferentially inhibits MMP-2. The binding of TIMP-3 to any specific MMP(s) has not been established as yet.

Genes of only three human MMPs (MMP-1, MMP-2 and MMP-9) have been completely sequenced (Huhtala *et al.*, 1990; Huhtala *et al.*, 1991). The human gene for MMP-9 spans almost 26 kb with a 7.7-kb structural region, 15-kb 5'-end and 3.5-kb 3'-end flanking regions. It contains 13 exons like the MMP-2 gene and all intron locations of these two genes coincide. The MMP-1 gene consists of 10 exons and 9 introns. Two putative TPA response elements and a TGF- β 1 inhibitory element have been found in the promoter region of MMP-2 (Huhtala *et al.*, 1991).

1.7.1 Activation of Matrix Metalloproteinases

MMPs are secreted as inactive zymogens and are maintained in this form by binding of Zn^{2+} to a cysteine (in the conserved sequence PRCGVPD) and three conserved histidine residues. This mechanism is referred to as the "cysteine switch" (Springman *et al.*, 1990; Park *et al.*, 1991). A widely accepted model of activation of MMPs is presented in Figure 1.2. Essentially,

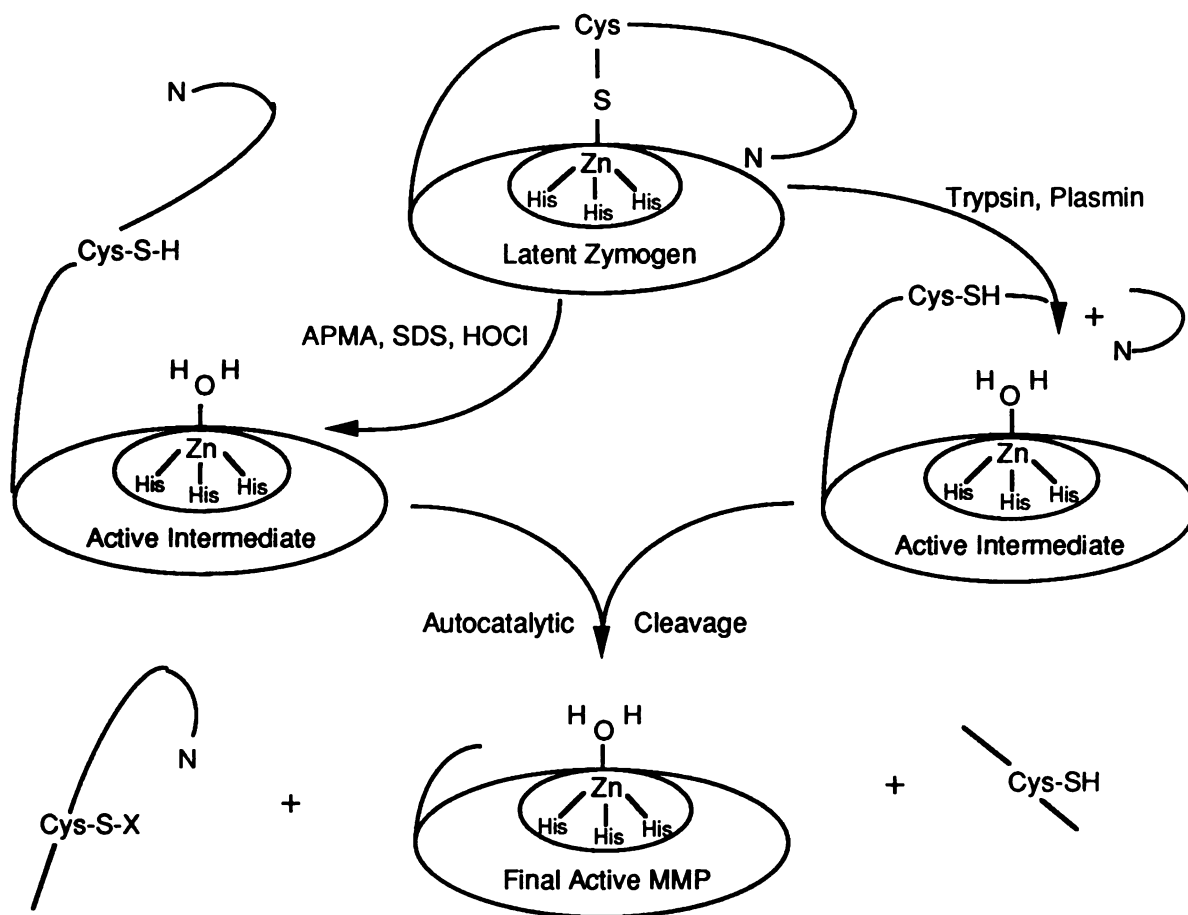


Figure 1.2. The "cysteine switch" model of activation of MMPs as proposed by Springman *et al.* (1990)

disruption of this "cysteine switch" at the N-terminal region of the zymogen either *in vivo* or *in vitro* by agents such as plasmin, stromelysin, trypsin, SDS and organomercurials is followed by autoproteolytic cleavage of the inhibitory pro-piece of the zymogen. This cleavage results in a permanently activated MMP, which is ~10-kDa shorter than the zymogen. Activated MMPs require Ca^{2+} for proteolytic activity.

1.7.1.1 Physiological Activation

The mechanisms of activation of zymogen MMPs have been studied extensively, with emphasis on a search for physiologically relevant factors. Essentially, activation of MMPs can occur either by cleavage of the inhibitory pro-piece of the zymogen MMP or by release of activated MMPs from inhibition by the breaking of the activated MMP:inhibitor complex. For example, a non-protein, low molecular weight molecule, designated endothelial cell stimulating angiogenic factor (ESAF) has been shown to be able to break the metalloproteinase-inhibitor complex to release the free inhibitor and an active metalloproteinase enzyme (McLaughlin *et al.*, 1991). Also ESAF released by chondrocytes during calcification has been shown to activate MMPs *in vitro* and has been proposed to be a physiological activator of MMPs, acting in concert with other initiators released by surrounding cells (Brown and McFarland, 1992). Keski *et al.* (1992) have demonstrated the ability of urokinase plasminogen activator (u-PA), but not tissue plasminogen activator (t-PA) to proteolytically activate MMP-2. This activation could be blocked by anti-u-PA antibodies, but was unaffected by aprotinin, a plasmin inhibitor, indicating a new potential role for u-PA as a direct regulator of MMP activation. Following the observation of the unusual ability of MMP-1 to hydrolytically inactivate serpins (Desrochers *et al.*, 1991), these authors investigated the mechanism of activation of MMP-1 and found that human neutrophils used the hydrogen peroxide-myeloperoxidase-chloride system to generate chlorinated oxidants that were capable of activating MMP zymogens (Desrochers *et al.*, 1992). Involvement of reactive oxygen species in activation of latent MMPs has been further substantiated by the observation that a latent 94-kDa MMP present in conditioned media from TPA-activated cells could be

activated with 35 to 70 mM hydrogen peroxide to release an active gelatinolytic 66-kDa species (Shaughnessy *et al.*, 1993). In contrast to these studies, Atkinson *et al.* (1992) showed that while plasmin played a key role in activation of MMP-9 and MMP-3 (stromelysin), the involvement of oxygen radicals was unlikely. Stromelysin has been suggested to be a specific activator of collagenase by Unemori *et al.* (1991). These authors observed restoration of collagenolytic activity after addition of purified, activated stromelysin (collagen is not a substrate for stromelysin) to the conditioned media of cells treated with interferon-gamma (IFN-gamma). The role of activated stromelysin as a physiologic activator of other MMPs is reinforced by the observation that although plasmin (another activator) could marginally activate procollagenase, addition of active stromelysin enhanced the activation of procollagenase 10-fold (Murphy *et al.*, 1992). Activation of MMPs can also be brought about by proteases produced by an infecting organism as in periodontitis, where several prevalent periodontopathogenic bacteria produce proteases that are capable of activating latent MMPs, thus causing periodontal tissue destruction (Sorsa *et al.*, 1992).

1.7.1.2 *In vitro* Activation of MMPs

In vitro activation of MMPs by both physiologically relevant agents (e.g. plasmin, u-PA, trypsin, stromelysin, oxidative species) and other non-physiological agents (e.g. SDS, organomercurials) has also been studied extensively. The various MMPs respond differently to the activating agents. MMP-9 can be activated by APMA, yielding two active species: an intermediate 83-kDa form which has lost its inhibitory pro-piece and a 67-kDa form which has lost a C-terminal portion in addition to the N-terminal piece.

TIMP-1 complexed with MMP-9 permits activation and formation of the intermediate form, but inhibits C-terminal cleavage to produce the 67-kDa form. Plasmin, leukocyte elastase, plasma kallikrein, thrombin or MMP-1 were unable to activate MMP-9, but trypsin, α -chymotrypsin, stromelysin and cathepsin G were able to activate MMP-9 (Okada *et al.*, 1992a). In contrast, these authors noted that since MMP-2 was not activated by serine proteases such as trypsin, the activation mechanisms of these two MMPs may be different. Plasma membranes from concanavalin A-stimulated human fibroblasts were found to be capable of activating MMP-2, but not MMP-9, and the activation was readily inhibited by TIMP-2 but poorly by TIMP-1. However, the activation capability of the membranes was destroyed by heat and trypsin, indicating the proteinaceous nature of the activator (Ward *et al.*, 1991). Strongin *et al.* (1993) reported similar activation of MMP-2, but not MMP-9, by isolated plasma membranes of TPA-stimulated HT1080 cells. Also this activation was competitively inhibited by a 26-kDa C-terminal fragment of MMP-2, suggesting that the C-terminal domain of MMP-2 interacts with a plasma membrane-associated component(s) that leads to its activation via autoproteolytic cleavage. Miyazaki *et al.* (1992) found that only 10% of the TIMP-2:MMP-2 complex could be activated by APMA. However, when stromelysin was added, the gelatinolytic activity increased 8-fold, indicating to these authors that stromelysin was a natural activator of TIMP-2-bound MMP-2 and also lending support to other studies (e.g. Unemori *et al.*, 1991) that implicated a role for stromelysin as an activator of MMPs. Activation of PMNL procollagenase (MMP-8) by HgCl_2 results in sequential autoproteolytic cleavage at specific intramolecular sites with formation of at least 2 intermediates before the formation of the fully activated MMP. Although TIMP-1 is capable of binding the zymogen procollagenase, it cannot suppress

the first intramolecular autoproteolytic cleavage (Blaser *et al.*, 1991). However, it does prevent further autoproteolytic decay and formation of the fully activated MMP due to its complexation with the MMP. Activation of matrilysin (MMP-7) also occurs in a similar manner (Crabbe *et al.*, 1992). These authors also proposed that the amino acid sequence L13-K33 was particularly important in protecting the E77-Y78 site from autocatalytic cleavage in order to maintain latency.

Howard *et al.* (1991b) have found that MMP-2 purified by HPLC under acidic conditions undergoes autoactivated degradation resulting in a mixture of active and inactive fragments. Kleiner *et al.* (1993) have confirmed similar autodegradation of free MMP-2 following similar HPLC purification, but they obtained essentially inactive fragments of MMP-2. The reason for this discrepancy is not known. Also, Kleiner *et al.* (1993) found that no autoactivation occurred in the presence of TIMP-2 and only 20% autoactivation occurred in the absence of TIMP-2 after a 24 h exposure at 37 °C. Hence, when MMP-2 exists as a free enzyme, it exhibits high activity but autodegrades rapidly compared to the TIMP-2-complexed form of the enzyme, which has low activity but is resistant to autodegradation.

Human polymorphonuclear-leucocyte collagenase (MMP-8) has been shown to autodegrade to two major fragments: a C-terminal inactive 27 kDa fragment and a N-terminal 40 kDa active fragment that was able to cleave all its normal substrates except collagen, indicating that the C-terminal region was important in binding with collagen substrates (Knauper *et al.*, 1993).

Fridman *et al.* (1992) have expressed and purified recombinant full length MMP-2 and two truncated forms of MMP-2 ending at residues 425 (delta 426-631) and 454 (delta 455-631) from the Vaccinia virus mammalian cell expression system. They have found that all these forms of MMP-2 were

activated by treatment with APMA and were capable of degrading collagen, but the truncated enzymes were almost 10-fold less sensitive to inhibition by TIMP-2. The activated, but not the latent, forms of the truncated MMP-2 formed a complex with TIMP-2. Also, the 205-amino acid carboxyl fragment of MMP-2 bound to TIMP-2, indicating that all forms of activated MMP-2 and the carboxyl-terminal fragment participated independently and cooperatively in TIMP-2 binding.

Some groups have found that not all MMPs are activated by proteases such as trypsin and plasmin. For example, MMP-2 from chicken embryo fibroblasts was poorly activated by trypsin and not activated by plasmin *in vitro* (Chen *et al.*, 1991), while purified human MMP-2 was not activated by either trypsin or plasmin (Hipps *et al.*, 1991). Also the APMA-activated chicken MMP-2 rapidly degraded denatured collagens, and although it was unable to degrade native collagens under identical conditions, it was active when high MMP-2 to substrate ratios were used.

1.8 Inhibitors of Metalloproteinases

Collagenase inhibitors were first discovered in 1970 when it was demonstrated that whole human serum could inhibit collagenase activity from a variety of tissues (Eisen *et al.*, 1970). This inhibitory activity was initially shown to be associated primarily with the α -globulin components of serum: α_1 -antitrypsin and α_2 -macroglobulin (Eisen *et al.*, 1970). However, it was soon recognized that α_1 -antitrypsin did not possess inhibitory activity (Bauer *et al.*, 1972), and in the same year, using immuno-adsorbed α_2 -macroglobulin, the collagenase inhibitory activity of α_2 -macroglobulin was clearly demonstrated (Abe and Nagai, 1972). However, α_2 -macroglobulin was

found to be a non-specific and irreversible inhibitor of proteases (Barrett and Starkey, 1973; Harpel, 1973; Starkey and Barrett, 1973; Werb *et al.*, 1974). Also due to its large size (~725-kDa) it was thought to be largely confined to the vascular system under normal conditions and hence probably inaccessible to tissues (Steinbach, 1974; Woolley *et al.*, 1976). In addition, the irreversible nature of inhibition by α_2 -macroglobulin was thought to limit its ability to be a physiologically relevant reversible "regulator" (Kuettnner *et al.*, 1976). These observations stimulated the search for a small specific collagenase inhibitor in serum.

Earlier, while characterizing collagenase extracted from rabbit tumors, McCroskery *et al.* (1975) had reported the presence of a smaller 40- to 50-kDa collagenase inhibitor which was distinct from α_1 -antitrypsin. The presence of a ~40-kDa specific collagenase inhibitor had also been detected during serum-free cell culture of human skin fibroblasts (Bauer *et al.*, 1975). Soon thereafter, Woolley *et al.* (1976) reported the identification of a similar small molecular weight protein in serum (~40-kDa, designated β_1 -AC), which specifically inhibited human collagenases derived from different tissues. During the following 5 years a large number of collagenase inhibitors were detected in tissue extracts (Kuettnner *et al.*, 1976; Roughley *et al.*, 1978), in media from cultured connective tissues (Shinkai *et al.*, 1977; Vater *et al.*, 1979; Nolan *et al.*, 1980; Cawston *et al.*, 1981; Murphy *et al.*, 1981; Yasui *et al.*, 1981) and in media from cell cultures derived from connective tissue (Nolan *et al.*, 1978; Kerwar *et al.*, 1980; Pettigrew *et al.*, 1980; Simpson and Mailman, 1981; Vater *et al.*, 1978; Welgus *et al.*, 1979).

These inhibitors primarily differed from each other in molecular size. The smaller inhibitors were 6- to 8-kDa in size while the larger inhibitors were 40- to 50-kDa in size. Several specific properties that were almost

universally exhibited by these collagenase inhibitors were as follows: they possessed intramolecular disulfide bonds, which when modified by alkylation or by thiol modifying agents resulted in an inactive inhibitor; they exhibited the ability to inhibit mammalian collagenases but not bacterial collagenases or proteases from other classes such as trypsin, a serine protease; the interaction between the protease and the inhibitor occurred with a 1:1 stoichiometry; and, the inhibitors were moderately stable to acid and heat. With the exception of a 28-kDa inhibitor (TIMP, see below), none of the other inhibitors has been further characterized.

1.9 Tissue Inhibitor of Metalloproteinases

In 1981, following purification of a collagenase inhibitor (~28,000 kDa) from rabbit bones in tissue culture, it was suggested that this low molecular weight inhibitor should be called 'TIMP' to distinguish it from other inhibitors that are not synthesized by tissues in culture (Cawston *et al.*, 1981). It is now clear that there is a family of TIMPs and to date the TIMP family consists of three members: TIMP-1, TIMP-2 and the most recently described TIMP-3 (Staskus *et al.*, 1991; Pavloff *et al.*, 1992). However, certain collagenase inhibitors have been designated names other than TIMP, often to reflect additional unique properties of such molecules. In 1985, Gasson *et al.* reported the cloning and sequencing of a novel protein designated EPA (erythroid potentiating activity), which, as the name suggests, stimulated growth of erythroid progenitor cells. Later in the same year, when TIMP was cloned and sequenced from a human source (Docherty *et al.*, 1985), it was found to be identical to EPA. TIMP has also been described as phorbin, to reflect its increased expression in rodent cells treated with TPA (Johnson *et*

al., 1987). Following identification and cloning of another metalloproteinase inhibitor, TIMP-2 (Goldberg *et al.*, 1989; Stetler-Stevenson *et al.*, 1989; Boone *et al.*, 1990; Stetler-Stevenson *et al.*, 1990), TIMP is now often referred to as TIMP-1. A bovine cartilage-derived collagenase inhibitor (CDI, 27,650 Da) (Moses *et al.*, 1990) and a chondrocyte-derived inhibitor (ChDI, 35,550 Da) (Moses *et al.*, 1992) have been shown to inhibit neovascularization and to inhibit proliferation and migration of capillary endothelial cells which have been stimulated with basic fibroblast growth factor. Both proteins are also collagenase inhibitors. Comparison of the N-termini sequences of CDI and bovine TIMP-1 (Freudenstein *et al.*, 1990) revealed that they are identical. The N-terminal sequence of ChDI has not yet been reported. Neither CDI or ChDI has been cloned and sequenced and hence their relationship with each other or with known bovine TIMPs remains to be established.

1.9.1 TIMP-1

The first member of the TIMP family, TIMP-1, has been cloned and sequenced from several sources, including human (Docherty *et al.*, 1985; Gasson *et al.*, 1985; Carmichael *et al.*, 1986), bovine (Freudenstein *et al.*, 1990), murine (Edwards *et al.*, 1986; Gewert *et al.*, 1987; Johnson *et al.*, 1987), porcine (Tanaka *et al.*, 1992), and rabbit (Horowitz *et al.*, 1989). TIMP-1 is also referred to as TIMP, EPA, phorbin and human collagenase inhibitor (HCI). Initial characterization of TIMP established its ability to inhibit several metalloproteinases (collagenase, gelatinase, neutral proteinase III, human leucocyte collagenase and gelatinase). However, it was not able to inhibit thermolysin, bacterial collagenase or serine proteases such as trypsin and plasmin. Also it was demonstrated that TIMP interacted with rabbit

collagenase with a 1:1 stoichiometry. TIMP-1 was originally identified as erythroid potentiating activity (EPA) that stimulated growth of erythroid progenitor cells (Gasson *et al.*, 1985). Subsequently, TIMP-1 has been shown to stimulate growth in a variety of non-hematopoietic cells. Bertaux *et al.* (1990) demonstrated the ability of rTIMP-1 (recombinant TIMP-1) to stimulate growth of normal human keratinocytes in primary culture when added at concentrations ranging between 0.2 and 4.6 μM , but rTIMP-1 had no effect on human fibroblasts. Using rTIMP-1 labeled with ^{125}I , they showed the presence of 135,000 TIMP-1 binding sites per cell with a K_D of 8.7 nM (Bertaux *et al.*, 1991). Growth-promoting activity of TIMP-1 has also been demonstrated in a wide range of human and bovine cells, including leukemia cell lines, fibroblasts, epithelial and endothelial cell types (Hayakawa *et al.*, 1992b). The EPA and growth-promoting activity of TIMP-1 has been re-examined and confirmed using a mouse erythroleukemia cell line (ELM-I-1-3) and several other erythroid and non-erythroid cell lines (Murate *et al.*, 1993). A recent study has shown a 2- to 3-fold increase in the expression of EPA mRNA when human monocytes and macrophages are immunologically induced to phagocytose erythrocytes (Bergamaschi *et al.*, 1993). This stimulation in EPA increased the number of cells responsive to erythropoietin and hence EPA has been proposed to act as a mediator of the rate of red cell production, although these authors did not measure the EPA protein levels.

TIMP-1 is capable of binding and inhibiting activation of MMP-9, but it does not form a complex with MMP-2 (72-kDa progelatinase) (Kolkenbrock *et al.*, 1991). However, TIMP-1 is capable of binding and inhibiting the activated form of MMP-2, although with lower affinity than TIMP-2 (Howard and Banda, 1991; Howard *et al.*, 1991b; Howard *et al.*, 1991a). Goldberg *et al.* (1992)

found that in the absence of TIMP-1, MMP-9 formed a covalent homodimer and a novel heterodimer complex with MMP-1, but the binding of TIMP-1 to MMP-9 inhibited the formation of these dimers. The MMP-9 homodimer was unable to bind TIMP-1. All TIMP-1-free MMP-9 forms could be activated by stromelysin.

Structural properties of TIMP-1 have been studied by Williamson *et al.* (1990) using rTIMP-1. Assignment of intrachain disulfide bonds formed by the 12 cysteine residues of TIMP-1 were made following peptide mapping, with 5 assignments being made from the experimental data while the sixth assignment was inferred because no evidence of free sulfhydryl groups was found. These assignments predict a two domain molecule containing 3 loops in each domain (Figure 1.3). Tryptic mapping of rTIMP-1 released numerous peptides following cleavage at exposed R or K residues but precipitated an insoluble hydrophobic core where peptides were held together by disulfide

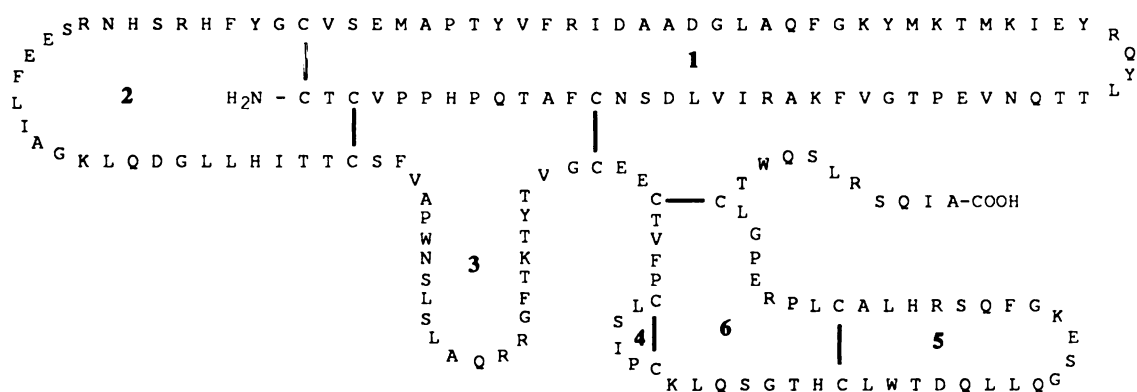


Figure 1.3. Primary structure of TIMP-1 based on disulfide bond assignments made by Williamson *et al.*, 1990.

bonds (Williamson *et al.*, 1993). Reduction with DTT solubilized the core with release of several peptides. The active metalloproteinase inhibitory region of the mature TIMP-1 appears to reside in the N-terminal domain (1-126) of the protein (Murphy *et al.*, 1991a). This region contains an even number of cysteine residues and two potential sites for N-glycosylation. Residues 127-184 of the mature TIMP-1 were deleted and the mutant was expressed in mammalian cells. Three forms expressed were 24, 19.5 and 13 kDa in size and corresponded to peptides with either both sites, one site or no site being glycosylated. All forms were recognized by anti-TIMP-1 antibodies and were active in their ability to inhibit activated MMPs, although the truncated forms were more susceptible to trypsin and did not form stable complexes with the zymogen form of MMP-2. Hence, these studies confirmed an earlier report that glycosylation was not essential for activity (Stricklin, 1986) and established that the N-terminal domain contained the inhibitory activity. In contrast, Coulombe *et al.* (1988) had observed no activity with a mutant TIMP-1 containing only amino acids 1-99. It is conceivable that the odd number of cysteines in the expressed protein could have resulted in incorrect folding and hence loss of inhibitory activity. The putative inhibitory region of TIMP-1 has been narrowed down to a short N-terminal, proline-rich region between Cys3 and Gys13. In site-directed mutagenesis experiments conversion of His7 and Gln9 to alanine residues resulted in weaker binding to MMP-7 than the binding of wild type TIMP-1 to MMP-7 (O'Shea *et al.*, 1992).

The TIMP-1 gene has been mapped to the proximal region of the X chromosome in both mouse and humans (Chapman *et al.*, 1991). TIMP-1 has been more recently mapped to the Xp21.1-p11 region on the human

chromosome and to the centromeric end A1-A5 of the mouse X chromosome (Derry and Barnard, 1992).

1.9.2 TIMP-2

TIMP-2, the second member of this family, was isolated as a complex with 72-kDa gelatinase (MMP-2) from the conditioned media of cultured human cells and the primary structure of the entire protein was determined by sequencing overlapping peptide fragments (Stetler-Stevenson *et al.*, 1989). TIMP-2 was independently discovered by 3 groups in 1989 (DeClerck *et al.*, 1989; Goldberg *et al.*, 1989; Stetler-Stevenson *et al.*, 1989) and was subsequently cloned and sequenced from human (Boone *et al.*, 1990; Stetler-Stevenson *et al.*, 1990), murine (Leco *et al.*, 1992; Shimizu *et al.*, 1992) and bovine (Boone *et al.*, 1990) sources. A long 3'-untranslated region has been found in the murine gene, which has not been observed in either human or bovine TIMP-2. TIMP-2 is ~21 kDa in molecular size and is not N-glycosylated. It was found to be 39% identical to TIMP-1 with all 12 cysteine residues conserved, which would assign TIMP-2 a similar two-domain structure like TIMP-1 if the pattern of disulfide bonding is the same. TIMP-2 binds and inhibits both pro-form and activated MMP-2 in a 1:1 stoichiometry. The inhibitory domain of TIMP-2, like TIMP-1, may reside in the N-terminal region of the protein as demonstrated recently when a 13.5 kDa purified fragment (1-132 aa) of a limited tryptic digest of TIMP-2 inhibited interstitial collagenase activity and blocked autocatalytic activation of procollagenase (DeClerck *et al.*, 1993).

TIMP-2 has invariably been found and isolated as a complex with MMP-2 in conditioned media of cultured cells. However, TIMP-2 released from macrophages, in contrast to human fibroblasts, has been found to be free

of any apparent complexed MMP, but is secreted at 30% of the basal level of fibroblasts (Shapiro *et al.*, 1992). Exogenously added lipopolysaccharides, denatured type I collagen and zymosan decreased TIMP-2 mRNA and synthesis, but increased MMP-1 and TIMP-1 synthesis. These authors also found that TIMP-2 levels in cultured human fibroblasts were unaffected by treatments with IL-1, TNF- α , PDGF or TPA. However, all of these agents stimulated the synthesis of MMP-1 and TIMP-1, indicating that TIMP-2 biosynthesis was regulated distinctly and in an opposite manner from TIMP-1 and MMP-1 (Shapiro *et al.*, 1992).

Native, purified human TIMP-2 has been shown to possess erythroid-potentiating activity similar to that of TIMP-1/EPA. This activity was inhibited by addition of antibodies to TIMP-1/EPA, indicating that TIMP-1 and TIMP-2 shared a common structural domain for this growth-promoting activity (Stetler-Stevenson *et al.*, 1992a). More recently, the growth stimulatory activity of TIMP-2 has been observed in non-erythroid cell types (Nemeth and Goolsby, 1993). Conditioned media from HSF4-T12 (a human fibroblast cell line transfected with Simian virus-40 genes) stimulated growth of normal human fibroblasts and when this activity was purified and sequenced, it was found to be TIMP-2. These results were confirmed by inhibition of growth when antibodies to TIMP-2 were added exogenously during culture (Nemeth and Goolsby, 1993).

Characterization of TIMP-2 binding to MMP-2 and its various autoactivated fragments has been investigated by Banda and his colleagues (Howard and Banda, 1991; Howard *et al.*, 1991b; Howard *et al.*, 1991a). Following purification of MMP-2 by HPLC in the presence of trifluoroacetic acid (which dissociates the TIMP-2:MMP-2 complex) and dialysis at neutral pH, MMP-2 was found to undergo autoproteolytic cleavage to form several

inactive fragments and two major active 62- and 43-kDa fragments. The 62-kDa fragment had lost an N-terminal pro-piece while the 43-kDa fragment had lost a C-terminal region in addition to the N-terminal pro-piece. The inactive fragments resulted from additional cleavage at the N-terminal region following removal of the pro-piece. Autoactivation and degradation could be prevented by both 1,10-phenanthroline (a metalloproteinase inhibitor) and TIMP-2. TIMP-2 is proposed to bind to a stabilization site that is independent of the active site since the TIMP-2:MMP-2 complex was still capable of gelatinolytic activity. TIMP-2 was also found to be more effective than TIMP-1 in its ability to inhibit activated MMPs: TIMP-2 was 10-fold, 7-fold and 2-fold more effective than TIMP-1 in inhibiting the 43 kDa fragment, activated MMP-9 and MMP-2, respectively. TIMP-2 also bound to these active enzymes with 33-, 4.4- and 10-fold greater affinity than TIMP-1. However, TIMP-1 inhibited interstitial collagenase (MMP-1) 2-fold more effectively than TIMP-2. TIMP-2 was also found to bind to a proteolytically inactive 29 kDa fragment from autoactivated MMP-2. The 29 kDa fragment was determined to be a 218 amino acid carboxyl-terminal piece of MMP-2 which did not contain the putative zinc- or gelatin-binding sites. This indicates that the TIMP-2:MMP-2 binding site probably lies in the C-terminal region of the MMP. In addition, these authors found that the formation of TIMP-2:MMP-2 complexes was not affected by 1,10-phenanthroline whereas the formation of TIMP-1:MMP-2 complexes was inhibited by this agent.

Kleiner *et al.* (1992) have detected a tetrameric complex of two molecules of TIMP-2 with two molecules of MMP-2 in addition to the more commonly observed bimolecular 1:1 complex, with the former structure constituting 21% of total complex as determined by native PAGE. Also an intermediate form consisting of two molecules of MMP-2 and 1 molecule of

TIMP-2 was observed when such tetrameric forms were activated with organomercurials. The physiological significance of these complexes is not known.

To date, detailed structural information of TIMP-2 protein and gene is not available. A truncated form of this protein lacking residues 128-194 has been crystallized, but better resolution may be required for the 3-D structure to be established (Tolley *et al.*, 1993). The human TIMP-2 gene has been mapped to the terminal region, 17q25 of chromosome 17 (De *et al.*, 1992) while the mouse TIMP-2 gene has been mapped to chromosome 11 (Stetler-Stevenson *et al.*, 1992b).

1.9.3 TIMP-3

The most recent addition to the TIMP family is chicken TIMP-3 or ChIMP-3 (Chicken Inhibitor of MetalloProteinases), a transformation-sensitive 21-kDa protein that is exclusively found in the ECM of cultured CEF. This protein has been purified from CEF and characterized (Staskus *et al.*, 1991), and has been cloned and sequenced (Pavloff *et al.*, 1992). TIMP-3 is a relatively insoluble component of the matrix and requires strong denaturants in order to be solubilized (Blenis and Hawkes, 1984). Nonionic detergents, such as NP40, are unable to solubilize the protein except in the presence of high salt concentrations or reducing agents, such as β -mercaptoethanol. Solubilization of this protein requires strong anionic detergents such as SDS or chaotropic agents such as urea and guanidine hydrochloride. Also this protein is resistant to digestion by TPCK-treated trypsin and does not appear to be phosphorylated or glycosylated, as determined by the lack incorporation of radiolabelled phosphate or mannose, respectively.

TIMP-3 differs from TIMP-1 and -2 in several respects: TIMP-3 is a relatively insoluble protein exclusively located in the ECM while TIMP-1 and -2 are soluble proteins isolated from conditioned media of cultured cells and are found in physiological fluids. TIMP-1 and -2 have been found to bind and inhibit MMP-9 and MMP-2 respectively, but TIMP-3 has not as yet been found associated with a specific MMP. TIMP-2 has usually been detected and isolated as a complex with zymogen MMP-2 from conditioned media of cultured cells and has been proposed to be secreted as a complex (Brown *et al.*, 1993). TIMP-1 is the only member of the family which is N-glycosylated (Murphy *et al.*, 1985; Murphy and Werb, 1985; Stricklin, 1986). Murphy and Werb (1985) had demonstrated the presence of several N-linked glycosylation sites based on the different sizes of TIMP-1 obtained by cell-free translation and by treatment of cultured cells with monensin and tunicamycin. At least two potential N-glycosylation sites were identified in the nucleotide sequence following sequencing of the TIMP-1 cDNA (Gasson *et al.*, 1985), which is in agreement with the observations of (Stricklin, 1986). However, these sites have still not established as glycosylation signals by mutagenesis studies. TIMP-3 protein isolated from cultured chicken cells is clearly not N-glycosylated, although the TIMP-3 cDNA sequence shows the presence of a single potential N-glycosylation site. It is possible that other higher molecular weight inhibitors found in this system (Staskus and Hawkes, 1989; Pavloff *et al.*, 1992) may include a glycosylated TIMP-3. The fact the glycosylation signal in the TIMP-3 is close to the C-terminus, unlike TIMP-1, may be a reason for the lack of glycosylation of TIMP-3 or as pointed out by (Beeley, 1985), not all such signals result in glycosylation.

1.9.4 Other Metalloproteinase Inhibitors

Several other metalloproteinase inhibitors have been reported, but they have not been sufficiently characterized to be classified as members of the TIMP family. Banda and co-investigators have reported the presence of at least 3 such inhibitors in secretions of mouse, rabbit, sheep and human cells, which are referred to as IMP-1, -2 and -3 with molecular sizes of 26, 21 and 18 kDa. These inhibitors are not proteolytic fragments or deglycosylated variants of TIMP-1 and are clearly distinct from TIMP-1 as demonstrated by their lack of cross reaction with other TIMP antibodies (Banda *et al.*, 1992). In addition to ChIMP-3, there are at least four other inhibitors ranging in size from 20 kDa to 28 kDa in the chicken system (Staskus and Hawkes, 1989; Staskus *et al.*, 1994). One of these inhibitors is the chicken TIMP-2, based on its N-terminal sequence and its association with a 70-kDa gelatinase (chicken MMP-2). The largest inhibitor in this system, designated ChIMP-a, is exclusively located in the ECM and is N-glycosylated (Pavloff *et al.*, 1992). Two novel forms of TIMPs have been identified recently by nonreducing SDS-PAGE: an 18-kDa inhibitor which is a two-chain form of TIMP-2 (tcTIMP-2) produced by proteolytic cleavage of the C-terminal portion, and a 22-kDa inhibitor that may be a partially glycosylated form of TIMP-1 (Miyazaki *et al.*, 1993a). The purified 18-kDa inhibitor resolved into a 17-kDa protein and a 2.5-kDa peptide on reducing SDS-PAGE and into 4 isoforms with pIs of 7.6, 7.3, 7.2 and 6.8 by isoelectric focusing. However, unlike TIMP-2, tcTIMP-2 does not bind zymogen MMP-2, indicating that the C-terminal region of TIMP-2 is required for binding to MMP-2.

Miyazaki *et al.* (1993b) have reported the presence of a domain resembling a metalloproteinase inhibitor in the C-terminal glycosylated

region of the secreted form of Alzheimer's amyloid precursor protein (APP). They have identified a novel 100-kDa gelatinase inhibitor in the conditioned medium of the EJ-1 bladder carcinoma cell line, which retains its inhibitory activity under reducing and nonreducing conditions. Its N-terminal sequence is identical to that of the mature secreted APP (molecular size ~100 kDa after cleavage from the membrane-bound precursor by secretase) and it has no sequence homology with either TIMP-1 or TIMP-2. Digestion of the purified 100-kDa inhibitor with Lys-C yielded two active fragments (28 and 31 kDa), where the N-terminal sequence of the 28-kDa fragment is identical to residues 439-446 of APP, indicating that this region may constitute the gelatinase inhibitory domain of APP. They also determined that MMP-2 cleaved a synthetic partial β -amyloid protein (β -AP₁₀₋₂₀) at the same site as did APP secretase, which suggested two possible functions for MMP-2: to produce secreted APP (like APP secretase does) and to degrade soluble β -amyloid protein to prevent its accumulation in the extracellular matrix.

1.9.5 Synthetic Metalloproteinase Inhibitors

Several synthetic collagenase inhibitors or synthetic inhibitors of metalloproteinases (SIMP) are being evaluated as potential therapeutic agents. SC 44463 (N4-hydroxy-N1-[1S[(4-methoxyphenyl)methyl]-2-(methylamino)-2-oxoethyl]-2R-(2-methylpropyl)butane-diamide) at concentrations of 25 nM and 250 nM inhibited ovulation in rats by 55% and 75%, respectively (Butler *et al.*, 1991). This compound was 100, 500, and 75 times more potent than a previous SIMP S 40827 in blocking ovulation, inhibiting action of ovarian collagenase and inhibiting rat MMP-7.

Another SIMP designated N-[D,L-2-isobutyl-3(N'-hydroxycarbonyl-amido)-propanoyl]-O-methyl-L-tyrosine methylamide has been shown to effectively block activity of MMP-9 *in vitro* (Fini *et al.*, 1991). A structurally similar compound, BB-94, [4-(N-hydroxyamino)-2R-isobutyl-3S-(thiopen-2-ylthiomethyl)-succinyl]-L-phenylalanine-N-methylamide, has been shown to increase survivability of nude mice receiving human ovarian carcinoma xenografts by 5-6 fold in a dose-dependant manner (Davies *et al.*, 1993). This effect has been attributed to inhibition of MMPs, specifically MMP-9, and is not observed with the diastereoisomer of BB-94, which is inactive. The authors propose that inhibition of MMP-9 causes transition of xenograft-created ascites with free-floating tumor clumps to solid tumors with development of tumor stroma, accompanied by slower tumor cell growth.

More recently, tetracycline analogues have been demonstrated to possess collagenase inhibitory activity which appears to be independent of the antimicrobial activity of these drugs (Arsenis *et al.*, 1992). Doxycycline has been found to be the most potent of all commercially available tetracyclines (Golub *et al.*, 1992). Other chemically modified tetracyclines without antimicrobial activity have also been shown to possess collagenase inhibitory activity *in vitro* and also have demonstrated reduced tissue destruction in several animal models of periodontal and other diseases (Golub *et al.*, 1992). Greenwald *et al.* (1992) showed that treatment of adjuvant arthritis with combined therapy using flurbiprofen and tetracycline completely inhibited collagenase activity, reduced gelatinase activity and significantly reduced joint damage, and hence the combination produced better results than administration of either drug alone.

A xenobiotic biochemical metalloproteinase inhibitor designated U27391 has been shown to reverse both loss of glycosaminoglycans and

inhibition of their synthesis induced by rIL-1 β in rat cartilage, indicating therapeutic benefits of this inhibitor in erosive cartilage disease in humans (Seed *et al.*, 1993). In addition, since plasmin may be a physiological activator of MMPs, Stevens *et al.* (1993) investigated the ability of protease nexin-1 (PN-1), an inhibitor of plasminogen, plasmin and urokinase, to prevent cartilage destruction. PN-1 inhibited IL-1 β -induced glycosaminoglycan loss, demonstrating its potential in preventing cartilage degradation.

1.10 Roles of MMPs and TIMPs in Normal and Diseased States

The involvement of MMPs and TIMPs in normal and diseased states has been extensively studied primarily by evaluating their relative abundance usually at the mRNA level by Northern analyses or at the protein level by measurement of activities. Also, their spatial distribution and abundance has been studied by localization techniques to correlate their expression with the observed physiological or pathological processes. Localization of the proteins or their mRNA either intracellularly or within a tissue is typically accomplished either by immunohistochemical methods or by *in situ* hybridization with specific DNA probes, respectively. The choice of the method is based on both the characteristics of the protein (i.e. secreted soluble proteins may be more difficult to localize than restrained proteins such as membrane proteins, receptors, extracellular matrix components, etc.) and/or availability of an antibody with high specificity. Although *in situ* hybridization may exhibit high specificity, it does not necessarily reflect the presence of the protein. The fact that all MMPs and TIMPs, with the sole exception of TIMP-3, are secreted soluble proteins may explain the paucity of good immunohistochemical data on the localization of these proteins in

tissues. Furthermore, the variability in specificities and affinities of antibodies raised by different laboratories makes comparisons of data and interpretations difficult (Woolley, 1984; Reynolds and Hembry, 1992). Hence, for this reason *in situ* hybridization has been the preferred technique in most localization studies.

1.10.1 Normal Physiological Processes

Expression of MMPs is tightly regulated as is evident under normal physiological processes such as post-partum uterine involution, ovulation and wound repair where extensive remodelling of the ECM is involved. There is also a large body of evidence to suggest involvement of MMPs in embryonic development and morphogenesis. Werb and her colleagues have demonstrated significant increases in expression of several metalloproteinases at the blastocyst stage which coincides with the migration of the blastocyte through the uterine stroma to its anchorage site on the endometrium (Brenner *et al.*, 1989). Evidence of MMP involvement in early morphogenesis of branched organs, such as lung development, was examined by using known inducers of collagenase expression (growth factors EGF or TGF- α) and purified collagenase during tissue culture of embryonic lung rudiments from mice. All treatments produced fewer branches with enlarged end buds, unlike controls that underwent extensive growth and repetitive branching. Also, TGF- α effects were reversed by addition of TIMP, indicating growth factor-controlled involvement of MMPs during morphogenesis (Ganser *et al.*, 1991) with TGF- β showing effects opposite to that of EGF and TGF- α (Matrisian *et al.*, 1992). Similar results were observed during salivary gland morphogenesis where cleft formation appeared to be regulated by

collagenase secretion, with a significant increase in clefts formed in the presence of exogenously added TIMP (Hayakawa *et al.*, 1992a). MMPs and TIMPs have also been shown to play a role in ECM changes of mammary glands during gestation, lactation and involution, along with t-PA and PAI-1 (Talhouk *et al.*, 1991; Talhouk *et al.*, 1992). The enzymes expressed by cultured mammary epithelial cells were similar to those expressed by the whole gland, with gelatinases being preferentially secreted in the basal direction, indicating that mammary epithelial cells secrete these enzymes in a vectorially defined manner.

Secretion of functional proteases by trophoblasts, including MMP-9 as the major gelatinolytic protease, has been shown to increase dramatically at the time of implantation of mouse embryos. Addition of TIMP-1 at 5-500 nM and antibodies against MMP-9 effectively inhibited ECM degradation by trophoblast outgrowths (Behrendtsen *et al.*, 1992). In addition to expression of metalloproteinases, the invasive cytotrophoblasts produce urokinase-type plasminogen activator, which may also be partly responsible for the invasive effect (Librach *et al.*, 1991). TIMP and antibody to MMP-9 completely inhibited cytotrophoblast invasion, but inhibitors of the plasminogen activator system inhibited invasion only partially (20-40%), indicating that MMP-9 was critical to cytotrophoblast invasion. Graham and Lala have studied the mechanism of control of trophoblast invasion *in situ*. TIMP-1 and TGF- β are two factors present locally in the microenvironment of invading trophoblasts. TGF- β induces the production of TIMP-1 in both trophoblasts and in decidual cells of the uterus, thus providing control of invasion, and TGF- β also promotes differentiation of invasive trophoblasts into multinucleated giant cells, which presumably are noninvasive (Graham and Lala, 1992).

MMPs have been proposed to play a role in producing dilatation of the cervix during active labor in pregnant women. Rechberger and Woessner (1993) looked at activities of collagenase, TIMP-1, α_2 -macroglobulin, elastase, hydroxyproline (collagen), and decorin in the lower uterine segments of women at term and during labor. They found that the collagenase concentration was 23-fold higher during labor than at term, and the combined TIMP-1 and α_2 -macroglobulin concentrations increased 2-fold. Elastase concentrations were also 2-fold higher. The ratio of decorin to collagen also doubled, probably due to increased degradation of collagen by collagenase, and this increased ratio supported their hypothesis of the importance of the interaction of decorin and collagen in dilatation.

Another novel role that MMPs have been suggested to play *in vivo* is in the formation of new capillaries. Schnaper *et al.* (1993) found that when human umbilical vein endothelial cells were cultured on Matrigel, a basement membrane preparation, the cells rapidly aligned and formed networks of tubes. The formation of capillaries was decreased by addition of either antibodies to MMP-2 or by TIMP-1 and TIMP-2. Conversely, addition of purified MMP-2 to the cultures increased the tube-forming activity. Also, protease activity appeared to be a prerequisite and essential in the early stages of tube formation because inhibition was greatest when either antibodies or TIMPs were added at the initiation of the cultures. Earlier, along similar lines, Southgate *et al.* (1992) had reported that two structurally unrelated MMP inhibitors, Ro 31-4724 and Ro 31-7467, inhibited proliferation of smooth muscle cells from rabbit aortic explants, indicating that constitutively secreted MMPs, MMP-2 and MMP-9, were likely to be involved in the initiation of smooth muscle proliferation.

1.10.2 Diseased States

1.10.2.1 Tumor Invasion and Metastasis. Over the last decade there have been several reports that indicate an inverse correlation between TIMP levels and invasive potential of tumorigenic cells. Among the first reports, Halaka *et al.* (1983) and Hicks *et al.* (1984) showed that invasive tumors produced less metalloproteinase inhibitors and increased amounts of collagenases. Khokha and her colleagues have demonstrated in a series of papers that TIMP-1 plays a role in tumor invasion and metastasis. When TIMP-1 expression was down-modulated in transfected Swiss 3T3 cells constitutively expressing antisense RNA, noninvasive and nontumorigenic cells were found to be not only invasive *in vitro* (as determined by a human amnion invasion assay), but were also tumorigenic and metastatic in athymic mice (Khokha *et al.*, 1989). Conversely, by inducibly increasing TIMP-1 levels in transfected mouse B16-F10 melanoma cells, the *in vitro* invasive ability of these cells was decreased relative to the parental cells using a Matrigel, trans-well invasion assay (Khokha *et al.*, 1992a). When stably transfected clones of these cells, constitutively expressing higher amounts of TIMP-1, were injected in the chorioallantoic membrane veins in chicken embryos, these cells were less metastatic and formed fewer and smaller metastatic tumors. Presumably the growth of these tumors was slower, as seen independently in cell culture experiments (Khokha *et al.*, 1992b).

Tsuchiya *et al.* (1993) prepared derivatives of KKLS cells (a human gastric cancer cell line capable of forming metastatic colonies in chick liver) transfected with human TIMP-1 cDNA and expressing TIMP-1 at varying levels. Metastatic ability was suppressed 70% in transfectants expressing high amounts of TIMP-1, indicating that TIMP-1 was a negative regulator of

metastasis and suggesting that MMP activity may be a prerequisite for metastasis. In a different approach Schultz *et al.* (1988) arrived at a similar conclusion. They showed that lung colonization by metastatic B16-F10 cells was inhibited 50% with periodic intraperitoneal injection of rTIMP-1. However, once colonization was established, it did not affect the growth or size of the tumors. They also demonstrated that TIMP-1-mediated inhibition of lung colonization was not due to alterations in the cell adhesion capability of the B16-F10 cells, unlike peptide YIGSR and RGD-containing peptides which inhibit experimental metastasis and invasion by blocking receptor sites on cell surfaces (Humphries *et al.*, 1986; Gehlsen *et al.*, 1988; Schultz *et al.*, 1988). On the basis of these studies, TIMP-1 and peptides such as GRGDS and YIGSR have been proposed to be used therapeutically during surgical removal of primary tumors or during chemotherapy, because these could potentially inhibit tumor cell dissemination and colonization (Humphries *et al.*, 1986; Schultz *et al.*, 1988). Har *et al.* (1992) have shown that lack of production of TIMP-1 and TIMP-2 was an important determinant of metastatic phenotype, lending support to several previous studies (Schultz *et al.*, 1988; Khokha *et al.*, 1989; Khokha *et al.*, 1992a; Khokha *et al.*, 1992b).

In contrast to all these studies indicating an inverse correlation between metastatic phenotype and TIMP levels, some investigators have observed elevated TIMP-1 or phorbol mRNA levels (Guillem *et al.*, 1990) and TIMP-1 protein (Lu *et al.*, 1991) levels to correlate with the extent of invasion in human colon tumors. A recent report by Carome *et al.* (1993) supports these observations. Both TIMP-1 and TIMP-2 mRNA levels (determined by reverse transcription of microdissected glomeruli followed by competitive PCR) were found to be elevated 4-fold and 3-fold, respectively, in glomeruli of patients with glomerulosclerosis associated with renal carcinoma. However,

the authors of these studies did not measure levels of MMPs. Guillem *et al.* (1990) have suggested that levels of TIMP-1 could serve as a biomarker for cancer rather than an indicator of progression of the disease.

Tumor cell invasion was shown to be inhibited by exogenously added TIMP-2 in *in vitro* human amnion invasion assays (Albini *et al.*, 1991). Furthermore, recombinant TIMP-2, at a concentration of 10 µg/ml, inhibited degradation of rat smooth muscle cell ECM by 70% and penetration of a multilayer of growing smooth muscle cells and their surrounding matrix by two invasive tumor cell lines, HT 1080 human fibrosarcoma and c-Ha-ras-1 transfected rat embryo cell line 4R (DeClerck *et al.*, 1991). Along similar lines, i.v. injection of stably transfected cells from a clone expressing TIMP-2, suppressed formation of lung colonies in nude mice (DeClerck *et al.*, 1992). These authors found that local tissue invasion was also completely inhibited and tumor growth rate was reduced *in vivo* in nude mice following s.c. injection of 4 clones transfected with TIMP-2 cDNA.

Studies have also been directed at competitively inhibiting the activation of MMPs using peptides. All MMPs contain a highly conserved peptide sequence, MRKPRCGNPDV, where a critical conserved cysteine binds to a zinc atom to maintain enzyme latency. This residue lies immediately upstream from the cleavage site that converts the 72-kDa zymogen into a 62-kDa active enzyme. For example, a synthetic peptide, TMRKPRCGNPDVAN, encompassing the conserved region has been shown in an *in vitro* cell invasion assay to inhibit HT1080 and A2058 cell invasion, when used at a concentration of 30 µM, while the control peptide, in which the conserved cysteine was substituted by serine, had no effect on invasion (Melchiori *et al.*, 1992).

Distribution of MMPs and TIMPs in tumors has been investigated by *in situ* hybridization. Pulmonary carcinomas examined for distribution of MMPs and TIMPs showed TIMP-1 and TIMP-2 transcripts predominantly over tumor stroma, while MMP-1, MMP-3 and MMP-9 were distributed evenly over both stromal and tumor cells (Urbanski *et al.*, 1992). Also, while TIMPs were expressed in non-neoplastic tissues and carcinomas, MMP-1, MMP-3 and MMP-9 were seen only in carcinoma tissues, with MMP-3, unlike MMP-1 and MMP-9, being present in all tissues examined. Samples of squamous cell carcinomas examined by *in situ* hybridization for gene expression of interstitial collagenase and TIMP showed high collagenase expression in stromal fibroblasts within the connective tissues adjacent to tumor masses but low in peripheral basaloid tumor cells as compared to normal, hyperplastic or dysplastic epithelial tissues (Gray *et al.*, 1992b). TIMP levels were negligible in all samples. A similar stromal expression pattern was also observed in colonic neoplasia in three cells types (fibroblasts, eosinophils and vascular endothelium) with high to moderate expression in 1-5% of eosinophils (Gray *et al.*, 1993). Poulson *et al.* (1992) also looked at colorectal carcinoma samples by *in situ* hybridization to locate MMP-2 and TIMP-2 mRNA and found stronger signals for both in the mesenchymal cells in the desmoplastic stroma than in the tumor epithelial cells. Collectively, these results indicate that stromal cells may be participating actively in concert with neoplastic cells in tumor invasion and metastasis.

Edwards and co-investigators have extensively examined the relationship between the clinical stage of malignant lymphomas and expression of TIMPs (TIMP-1 and TIMP-2) and MMPs including MMP-2, MMP-9, interstitial collagenase, MMP-7 and stromelysin-1 and -3, using *in situ* hybridization. When 28 non-Hodgkin's lymphomas were screened for

mRNA levels of TIMP-1, MMP-2 and interstitial collagenase, the most aggressive tumors contained the highest amount of TIMP-1 transcripts (localized to stromal cells of endothelial or fibroblastic origin), while MMP-2 and interstitial collagenase were expressed at very low levels, with collagenase transcripts being observed only in high grade tumors (Kossakowska *et al.*, 1991). However, in the following study with 18 high grade large cell immunoblastic lymphomas (Kossakowska *et al.*, 1992), levels of MMP-9 transcripts were useful predictors of survivability, although the clinical stage was the most important predictor. In their most comprehensive study, they evaluated mRNA expression of TIMP-1, TIMP-2, MMP-1, MMP-2, MMP-7, MMP-9 and stromelysin-1 and -3 in 42 cases of malignant lymphomas classified into 25 high grade, 15 low grade and 2 intermediate grade (Kossakowska *et al.*, 1993). TIMP-1 was expressed in 41 cases, while TIMP-2 and MMP-9 were expressed in 35 cases. Stromelysin-1 and -3 were not detected in any of the tumors, but MMP-1, MMP-2 and MMP-7 were detected in a few cases. However, high grade lymphomas showed a trend towards elevated TIMP-1 and MMP-9 transcripts, as seen earlier, and variable amounts of TIMP-2 and other MMPs. Low grade lymphomas displayed low and relatively constant levels of TIMP-1, TIMP-2 and MMP-9. In their conclusion, they suggest that levels of TIMP-1 and MMP-9 may play a role in controlling biologic aggressiveness of these tumors.

Immunolocalization of both MMPs and TIMP-1 has been performed by Clavel *et al.* (1992) on 30 benign and 79 malignant lesions of the breast. MMP-1, MMP-2 and MMP-3 were detected in 9, 16 and 12 cases, preferentially in tumor cells, with MMP-2 and MMP-3 localizing at the periphery of some non-invasive and well-differentiated clusters. Only a few stromal fibroblasts were immunopositive. In contrast, TIMP-1 was more frequently detected in 7

benign tumors and 55 carcinomas and was localized mainly at the periphery of endothelial cells and occasionally in cancer cells and fibroblasts.

Analysis of conditioned media from cell cultures of two highly invasive squamous cell carcinomas showed high amounts of MMP-9 which increased 20-fold on treatment with TPA, indicating a protein kinase C pathway. The involvement of this pathway was confirmed by treating cultures with a protein kinase C inhibitor, calphostin-C, which led to a decrease in MMP-9 production (Juarez *et al.*, 1993). In contrast, TPA stimulation of human neutrophils has been shown to release MMPs from secretory granules and also produce reactive oxidant HOCl, which could serve to activate MMPs (Michaelis *et al.*, 1992). Only a third of the total MMP-1 was activated by the coreleased HOCl, but most of the activity was destroyed after 1 h. MMP-1 was activated at HOCl/enzyme ratios between 10 and 40, but was totally inactivated at higher ratios, indicating that the HOCl/enzyme ratios must fall within a narrow range for activation to occur. Sato *et al.* (1992) found that MMP-2 and MMP-9 mRNA were more frequently expressed in mesenchymal tumor cells and in epithelial tumor cells, but MMP-9 expression in mesenchymal cells correlated well with tumorigenicity of cells. Only about 10% of the tumor cells expressed MMP-3 mRNA. TIMP-1 transcripts were detected in only 50% of the cells whereas TIMP-2 mRNA was expressed in the majority of the cells.

Ossowski investigated the loss of invasive phenotype of HEp3, an invasive malignant carcinoma cell line, following continuous *in vitro* culturing for 30 passages and found that the change in phenotype correlated with almost complete cessation of interstitial collagenase production and was not due to an increase in production of TIMPs (Ossowski, 1992). Similar observations have been made by Testa (1992), where HEp-3 cells lost their

metastatic ability by week 8 in culture. However, this preceded decreases in MMP-2 and MMP-9 activity, a 2-fold increase in TIMP-2 mRNA and no change in TIMP-1 levels. These data implicate the involvement of MMP(s) other than MMP-2 and MMP-9 in the metastatic ability of HEp-3 cells and lend support to observations made by Ossowski (1992). Conversely, a decrease in the amount of TIMP may result in higher net collagenase activity. For example, metastatic and nonmetastatic cell lines derived from murine mammary adenocarcinoma cells secreted similar amounts of collagenase, but the metastatic cells exhibited higher net collagenase activity due to decreased TIMP mRNA levels and transcription in these cells (Ponton *et al.*, 1991).

Korczak *et al.* (1991) investigated autocrine and paracrine regulation of TIMP, stromelysin and uPA in genetically related cells: nonmetastatic SP1 and metastatic A3a cell lines. They observed 15-20-fold higher stromelysin and 3-5-fold lower TIMP mRNA in A3a cells than in SP1 cells and comparable levels of uPA. Also, when conditioned medium from A3a cells was added to the nonmetastatic SP1 cells, a similar qualitative shift in expression of TIMP and stromelysin genes was observed in a short time of 4-8 h, indicating secretion of regulatory factors by the metastatic cells.

1.10.2.2 Arthritis. Arthritis is a pathological condition characterized by inflammation of the joints and cartilage destruction (reviewed by Murphy *et al.*, 1991b; Murphy and Hembry, 1992). Although a complex cascade of degradative events leads to manifestation of the diseased state, MMPs have been strongly implicated to playing a central role in arthritis (Martel *et al.*, 1988). Based on an experimental model, excessive cartilage destruction has been proposed to result from an imbalance of MMPs over TIMPs (Cambray *et al.*, 1981; Dean *et al.*, 1989). For example, stromelysin concentrations in knee

joint synovial fluid were found to be 15- to 45-fold greater in diseased than in normal control groups, while the ratio of stromelysin to free TIMP was 0.5 in the reference group and between 1.6 to 5.3 in the diseased group, indicating that high stromelysin concentration and high stromelysin to TIMP ratio may be indicators of cartilage destruction (Lohmander *et al.*, 1993). Walakovits *et al.* (1992) found that stromelysin concentration was 20-fold higher than collagenase in synovial fluid from patients with rheumatoid arthritis and 8-fold higher in patients with traumatic injury, suggesting that stromelysin played a role in joint destruction. However, in this study MMP concentrations, and not activities, were measured, and in light of data suggesting that stromelysin is a natural activator of collagenase, it is conceivable that increased stromelysin expression translates into increased collagenase activity, where stromelysin plays an indirect role in tissue destruction. Commonly prescribed anti-inflammatory drugs for the treatment of arthritis, such as indomethacin and naproxen, have been shown to suppress the production of MMPs. In particular, indomethacin has been found to reduce ConA-induced production of MMP-9 (Corcoran *et al.*, 1992). Similarly, a decrease in total MMP activity (activity of individual MMPs decreases ranged between 40% and 87%) has been obtained in dogs treated with naproxen for 4 weeks at steady-state plasma levels of the drug of 40-50 $\mu\text{g/ml}$ (Ratcliffe *et al.*, 1993). Also, analysis of articular cartilage explants indicated that proteoglycan synthesis rates were unaffected and proteoglycan release (i.e. degradation) was suppressed. Furthermore, they found that TIMP activity was unaffected.

Firestein and coworkers have studied MMP and TIMP gene expression in arthritic synovial tissues (Firestein *et al.*, 1991; Firestein and Paine, 1992). They reported that collagenase and TIMP mRNA were localized primarily in

the synovial lining layer with significantly more collagenase in rheumatoid than osteoarthritic tissues, but with similar amounts of TIMP levels. Both collagenase and TIMP mRNA, although abundant in untreated tissues, were nearly undetectable following steroid treatment with triamcinolone. They also observed that normal synoviocytes did not express the stromelysin gene but constitutively expressed TIMP. Cells stimulated with IL-1 β or TNF- α induced expression of stromelysin, but had no effect on TIMP expression. In double-labeled tissues they observed discordant expression of stromelysin and TIMP genes, which indicated that these genes were likely to have distinct transcriptional control. Zafarullah *et al.* (1993) looked at mRNA expression levels of collagenase, stromelysin and TIMP-1 and found them to be higher (40%, 55% and 80%, respectively) in osteoarthritic patients than in normal controls, where most controls expressed TIMP-1 at low levels and very few expressed any MMP.

Okada *et al.* (1989; 1992b) have looked for the distribution of MMPs and TIMP-1 in rheumatoid synovium by immunohistochemical techniques. They found MMP-1, MMP-3 and TIMP-1 to colocalize in hyperplastic synovial lining cells of rheumatoid synovia, but only after treatment of these tissues in culture with 1 μ M monensin for 3 hours to allow synthesis and intracellular accumulation of these proteins. In contrast, normal synovium did not show the presence of these proteins. Brown *et al.* (1989) looked at the distribution of MMP-2, MMP-3 and MMP-9 in the growth plate of the rabbit and found that these enzymes were not co-ordinately expressed, indicating that their regulation was perhaps independent. Although such immunolocalization studies have provided information on precise spatial and temporal expression of proteins of interest, at least in the case of MMPs, as pointed out by Murphy *et al.* (1991b), more useful interpretations could be made if the

nature of the MMP (i.e. zymogen, activated or complexed forms) could be defined by using specific antibodies to these forms.

1.11 Overview

The complexity of diseased states, such as cancer and arthritis, is evident from the current lack of complete understanding of factors or events involved in the manifestation of these diseases, although treatment with drugs has been successful in slowing their progression in many cases. From the MMPs' and TIMPs' involvement point of view, the delicate balance between the proteases and their inhibitors is maintained with great fidelity under normal physiological conditions, but appears to be altered in favor of the MMPs in conditions such as arthritis and tumor invasion and metastasis. Conceivably, neutralizing aberrant MMP activities with TIMPs could result in control of that disease at a specific stage. For example, evidence has been accumulating over the past 5 years indicating that TIMPs could inhibit tumor invasion and dissemination of tumors in malignant types of cancers.

1.12 Some Unanswered Questions for TIMP-3

One of the most unique and striking features of TIMP-3, that distinguishes it from TIMP-1 and TIMP-2, is its exclusive extracellular matrix location. TIMP-1 and TIMP-2 are found in various body fluids, most notably plasma, and in conditioned media of cultured cells or tissues. Why TIMP-3 is localized to the ECM, when very other similar TIMPs are not, is a major unanswered question. It is possible that it is bound to a specific receptor on the cell surface and/or component of the ECM. Alternatively, it may interact

non-specifically with other components of the ECM. A third possibility is that the protein is intrinsically relatively insoluble and precipitates or aggregates in an aqueous environment.

Another important question relates to the MMP partner for TIMP-3. It is clear that both TIMP-1 and TIMP-2 preferentially inhibit a particular MMP (MMP-9 and MMP-2, respectively), but does TIMP-3 inhibit a particular MMP, and if so, which one?

Although the cell culture experiments with purified ChIMP-3 had revealed that the protein stimulated proliferation of nontransformed cells and accelerated morphological transformation, these results needed to be confirmed by an independent method that circumvented the logistic and technical difficulties of performing these experiments. This would allow determination of a possible role of ChIMP-3 during transformation.

Finally, could TIMP-3 inhibit tumor invasion and metastasis in *in vitro* models, like TIMP-1 and TIMP-2 do? Could its exclusive ECM location be a significant property that could be instrumental in inhibiting the degradative actions of the MMPs within the matrix more effectively than either TIMP-1 or TIMP-2? Could this ECM location be a key factor in inhibiting cartilage destruction in arthritis? Also, thus far, the presence of TIMP-3 had been shown only in the chicken system. Is such a protein present in other systems, such as the mammalian system, especially in humans?

1.13 Specific Objectives

Some of the unanswered questions have been addressed in this thesis. The first objective was to test the hypothesis that ChIMP-3 plays a role during the transformation of RSV-infected CEF, as suggested by the cell culture

experiments with purified ChIMP-3 (Yang and Hawkes 1992). This hypothesis was tested by a transient inhibition of ChIMP-3 expression with antisense oligodeoxynucleotides. However, in order to achieve specific inhibition of ChIMP-3 synthesis using an antisense ODN-mediated approach, it was essential to determine the cDNA sequence encoding the protein. Data for the cloning and sequencing of a major portion of the ChIMP-3 cDNA are presented in Chapter 2. Results of the antisense ODN experiments directed at specifically inhibiting the synthesis of ChIMP-3 are presented and discussed in Chapter 3.

The second objective was to investigate the molecular interactions of ChIMP-3 and ChIMP-a (another ECM-specific inhibitor) with components of the ECM. Based on observations made during the antisense ODN-mediated inhibition studies, particular emphasis was placed on interaction of these inhibitors with charged polymeric compounds. The potential binding site(s) of ChIMP-3 and ChIMP-a in the ECM, which may be instrumental in the exclusive ECM localization of these proteins, were investigated. Results of these experiments are presented in Chapter 4.

The third and final objective was to identify and characterize the human counterpart of ChIMP-3. Results of these experiments are presented in Chapter 5.

Chapter 2

Cloning and Sequencing of cDNA Encoding the 21-kDa Protein

2.1 Introduction

The 21-kDa protein is a multi-functional ECM protein (Blenis and Hawkes, 1983; Blenis and Hawkes, 1984) with paradoxically diverse properties. It exhibits both metalloproteinase inhibitory activity (Staskus *et al.*, 1991) and the ability to stimulate growth of non-transformed chicken embryo fibroblasts (CEF) (Yang and Hawkes, 1992). *In vitro* experiments involving the culture of uninfected, non-transforming CEF on dishes coated with purified 21-kDa protein (Yang and Hawkes, 1992) were aimed at mimicking the transient increased expression of the 21-kDa protein in transforming CEF in order to determine its functional properties. This study revealed that the 21-kDa protein stimulated the proliferation of growth-retarded, non-transforming CEF maintained under low serum conditions. These cells divided at the same rate as exponentially growing transforming CEF cultured on dishes with or without a matrix of 21-kDa protein. Also, the 21-kDa protein elicited phenotypic changes, for example, acceleration of morphological transformation and promotion of cell detachment of transformed cells from the ECM. The design of these experiments involved the preparation of the purified 21-kDa protein-bound substrata and the trypsinization and seeding of tsRSV-LA24-infected CEF on these surfaces. This was followed by a period of up to 16 hours to allow the cells to attach to the substrata, to resynthesize those proteins degraded during trypsin treatment and to begin the process of transformation after transfer from 41 °C to 35 °C. Thus, for technical reasons, the effect of matrix-bound 21-kDa protein on these cells was by necessity indirectly measured. However, the results indicated that increased amounts

of 21-kDa protein in the ECM appeared to *promote* the development of the transformed phenotype.

Another, more direct, approach to determining the effect of 21-kDa protein on cell transformation would be to interfere with its synthesis and to ask the question: does a decreased amount of 21-kDa protein in the ECM prevent or slow down the development of cell transformation? Thus, the approach of using antisense oligodeoxynucleotides (ODNs) to specifically inhibit 21-kDa protein synthesis was pursued to address its role in transformation. Since the basis of antisense-ODN-mediated inhibition is hybridization of a specific ODN sequence to its complementary mRNA sequence for the protein, it is imperative to know the nucleotide sequence encoding the protein. Hence, it was necessary to clone and sequence the cDNA for the 21-kDa protein. Even a partial cDNA sequence could provide sufficient information for antisense ODN inhibition studies to be initiated.

Several methods are available and used routinely to identify and sequence genes and cDNAs (Glover, 1984-1987). These include expression cloning, cloning of purified DNA fragments into vectors like plasmids, cosmids, phage lambda or yeast artificial chromosomes and homology cloning. Typically, the cloning method used to sequence a gene is determined by criteria as diverse as the investigator's preference for a technique to elimination of a certain technique due to the lack of key information. For example, the ability to purify and obtain significant amino acid sequence, typically > 5 residues, of a novel protein is a prerequisite to using polymerase chain reaction (PCR). Difficulty in purifying a protein may necessitate the use of an alternate cloning method to sequence its gene, for example, expression cloning was used for sequencing the TGF- β receptor (Lin and Wang, 1992).

Recently, amplification of segments of genes by PCR using degenerate oligodeoxynucleotides spanning specific segments of the gene or genes of interest has been used with reasonable success. The degenerate oligodeoxynucleotide primers used in these PCRs are based on either partial N-terminal or internal sequences of the protein of interest or are based on consensus amino acid sequences of other similar proteins. The amplified DNA can be either cloned and sequenced or can serve as a probe to screen cDNA libraries to obtain the entire coding sequence of the gene of interest. Since the N-terminal sequence of the 21-kDa protein was known (Staskus *et al.*, 1991), the PCR approach was used to obtain cDNA sequence of a segment of the gene coding for the 21-kDa protein. This method was preferred over the alternative approach of screening cDNA libraries with short (15-24 nucleotide) degenerate oligonucleotides due to the relative speed of the former method.

2.2 Materials and Methods

2.2.1 Design of ODN Primers for PCR: The N-terminal sequences of purified 21-kDa protein (Staskus *et al.*, 1991) and human TIMP-2 obtained by automated Edman degradation (Stetler-Stevenson *et al.*, 1989) and human TIMP-1 (Gasson *et al.*, 1985), deduced from its cDNA sequence, are as follows:

21-kDa protein	CTCVP <u>IHPQDA</u> FCNSDIVIRAKVvgkkim
TIMP-1	CTCVPPHPQTAFCSNDLVIRAKFVGTPEV
TIMP-2	CSCSPVHPQQAFCNADVIRAKAVSEKEV

The two main criteria in designing primers were selection of a segment which was least similar to other reported TIMPs and which simultaneously

had the smallest amount of codon degeneracy. The selected segment IHPQDA (double underlined) was unique to the 21-kDa protein at residues I and D and had a 96-fold degeneracy when only the first two nucleotides were used for alanine. Although another unique sequence with lower degeneracy was present in the 21-kDa protein sequence (underlined), it was not used because these residues (in lowercase) were determined at less than full confidence by Edman sequencing. Primer 1 and 2 were 24-base oligodeoxynucleotides and their sequences are shown below. Primer 2 contained a 17-base degenerate sequence complementary to IHPQDA and was designed to amplify the first strand cDNA, while its companion Primer 1 included a 17-base oligo dT sequence designed to amplify the 2nd cDNA strand by binding to the poly(A) region. The latter primer was designed on the presumption that the 21-kDa protein mRNA contained a poly(A) tail. In each case, six of the seven remaining bases at the 5' end of the primers coded for restriction enzyme recognition sites (underlined) and an additional base was included to ensure PCR amplification of the entire restriction site and to facilitate recognition of the restriction enzyme site during subsequent cloning.

Primer 1: *Xba* I
 5' -C TCT AGA (T)₁₇-3'

Primer 2: *Sal* I
 5' -G GTC GAC ATA(or C or T) CAC(or T) CCA(or C or G or T)
 CAA(or G) GAC(or T) GC-3'

Primers 3 and 4, with two adjacent restriction enzyme sites, were also synthesized. These were identical to Primers 1 and 2 with the exception of having *Pst* I, *Bam* HI and *Eco* RI, *Sma* I as restriction enzyme sites (underlined) for Primers 3 and 4, respectively. Two extra nucleotides were also added to these primers at the 5' end.

Primer 3: 5' -CA ^{*Pst* I} GAA TTC ^{*Bam* HI} CCC GGG (T)₁₇-3'

Primer 4: 5' -CA ^{*Eco* RI} CTG CAG ^{*Sma* I} GGA TCC ATA(or C or T) CAC(or T) CCA(or C or G or T) CAA(or G) GAC(or T) GC-3'

2.2.2 ODN Syntheses and Purification: The ODNs were synthesized on an automated DNA synthesizer (Millipore/Biosearch, Model 8600) using standard phosphoramidite chemistry. At the completion of synthesis, ODN samples were eluted from the solid-phase support using concentrated ammonium hydroxide and concentrated (Speed-vac, Savant Instruments) following removal of protecting groups by heating at 55 °C for 6 h. ODNs were purified using denaturing polyacrylamide gels. Typically, 15% acrylamide-8 M urea slab gels (1.5-mm or 3.0-mm thick) were used. Concentrated ODN samples were reconstituted in DNA sample loading buffer (90% deionized formamide, 0.025% xylene cyanole FF, 0.025% bromophenol blue, 10% 10 x TBE) and electrophoresed in 1 x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) electrophoresis buffer at constant power of 35W until the bromophenol blue tracking dye had migrated to within 1 cm of the bottom of the gel. The ODNs were visualized by UV-shadow casting using a fluorophore-coated TLC plate (Whatman HP-KF). The ODNs appear as dark bands against a green fluorescent background when short wavelength UV light (254 nm) is used to illuminate the gel. ODN bands on a typical purification gel are shown in Figure 2.1. Gel bands containing ODNs were excised and the ODNs electroeluted using a commercial elution device (Elutrap™, Schleicher & Schull) using an elution buffer consisting of either 0.1 M ammonium bicarbonate or 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1% SDS and 0.1 mM EDTA. The eluted ODNs were

desalted using C₁₈ reverse phase SEP-PAK cartridges (Millipore) essentially according to the manufacturer's instructions, dried under vacuum, reconstituted in sterile water and a rough estimate of their concentration was assayed spectrophotometrically (Hewlett Packard, Model 8452A) at 260 nm using a standard conversion factor of 1 OD = 20 µg ODN (Sambrook, 1989).

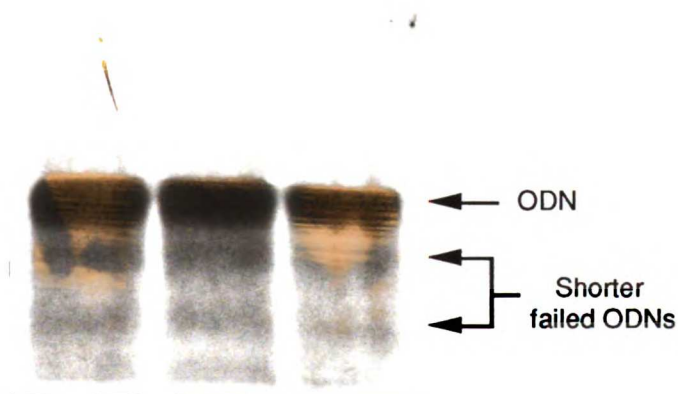


Figure 2.1. Purification of ODNs by gels. Approximately 200 µg (10 OD units) of unpurified Primer 1 was loaded in each lane of a 15% acrylamide-8M urea 3-mm preparative gel and visualized as described under "Materials and Methods". Note the presence of shorter failed ODN sequences and their relatively low abundance compared to the primary ODN product.

2.2.3 mRNA Isolation: Total RNA was isolated from transforming CEF, essentially as described (Chomczynski and Sacchi, 1987). Several precautions were taken to minimize RNA degradation. All disposable plasticware used for RNA preparations was sterile and from freshly opened packages which had been handled with gloves. All chemicals were also from freshly opened containers and were retained solely for RNA use. Clean glassware was baked overnight at 250 °C and reserved for RNA work only. Water was treated

with DEPC (final concentration 0.1% v/v) overnight with stirring, followed by autoclaving to remove traces of DEPC. Tertiary CEF infected with tsRSV-LA24 and transformed at 35 °C for 10 hours were used for isolation of RNA. The denaturant solution containing buffered 4 M guanidium isothiocyanate was added (1 ml per 100-mm dish) directly to approximately 4×10^6 cells cultured in 100-mm tissue culture dishes, gently mixed with disposable polypropylene cell lifters (Fisher), collected in 15 ml centrifuge tubes (Falcon #2059) and either placed on ice and treated further or frozen at -20 °C. To the solubilized cells, 0.1 volume of 2 M sodium acetate (pH 4.0), 1 volume of water-saturated phenol and 0.2 volume of chloroform:isoamyl alcohol (49:1) were added sequentially with gentle mixing after each addition. The mixture was vortexed after the last addition, cooled on ice for 15 minutes and centrifuged (Sorvall, Model RC-3) at $10,000 \times g$ at 4 °C for 20 minutes. The aqueous upper layer was transferred to a fresh tube, an equal volume of cold isopropanol (-20 °C) added and allowed to precipitate overnight at -20 °C. The precipitated RNA was centrifuged at $10,000 \times g$ at 4 °C for 20 minutes, redissolved in the denaturant solution and reprecipitated. The final RNA pellet was rinsed with 75% ethanol in DEPC-treated water, air dried and the RNA was reconstituted in DEPC-treated water. The concentration of RNA was determined spectrophotometrically (Hewlett Packard, Model 8452A) at 260 nm using a standard conversion of 1 OD = 40 µg (Sambrook, 1989). The quality and integrity of all RNA preparations was checked by electrophoresis through denaturing 1% agarose gels containing 2.2 M formaldehyde using 1 X MOPS buffer (20 mM 4-morpholinopropanesulfonic acid (MOPS), 1 mM EDTA, 5 mM sodium acetate, pH 7.0). Typically 1-10 µg of RNA was loaded per lane, stained with ethidium bromide (incorporated in the gel at a final

concentration of 0.5 µg/ml) and visualized over an UV transilluminator (Fotodyne, Model 3-3000, New Berlin, WI).

Poly(A)+ RNA was selected from the total RNA by binding to oligo(dT)-cellulose in a batch process. Briefly, 0.3 g oligo(dT)-cellulose (GIBCO BRL) was rinsed extensively with 0.1 N NaOH and binding buffer (0.01 M Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.5% SDS). The RNA (1 mg) was heated at 65 °C for 10 minutes in loading buffer (0.02 M Tris-HCl, pH 7.5, 1 M NaCl, 2 mM EDTA, 0.5% SDS) and then cooled to room temperature. The RNA and oligo(dT)-cellulose were mixed and allowed to bind at room temperature for 15 minutes. The supernatant was collected and the oligo(dT)-cellulose was rinsed 4 times with wash buffer (0.01 M Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA). The bound RNA was eluted with 3 rinses of elution buffer (0.01 M Tris-HCl, pH 7.5, 1 mM EDTA), mixed with 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol and precipitated at -20 °C overnight. The mRNA yield was determined spectrophotometrically (Hewlett Packard, Model 8452A) using 1 OD = 40 µg/ml as the basis for conversion (Sambrook, 1989). In addition, the integrity of the mRNA was indirectly tested by analyzing an aliquot of the non-binding RNA fraction following poly(A) selection with oligo(dT). This sample was electrophoresed through a non-denaturing 1% agarose gel cast in 1 × TAE buffer (40 mM Tris, 1 mM EDTA, pH adjusted to 8.0 with glacial acetic acid) at 80 V using 1 × TAE buffer. The presence of distinct ribosomal RNA bands indicated that the RNA, and hence presumably the mRNA, had not been degraded during the isolation procedure (Figure 2.3, lane 10).

2.2.4 Reverse Transcription: First strand cDNA synthesis was performed essentially according to the manufacturer's instructions using M-MLV H⁻ RT

(a cloned Moloney-Murine Leukemia Virus Reverse Transcriptase from which RNase H sequence has been deleted, Superscript™ RNase H⁻ Reverse Transcriptase, GIBCO BRL), with the exception of using Primer 1 instead of the recommended oligo(dT)₁₂₋₁₈ and using human placenta ribonuclease inhibitor (Collaborative Research, 5.5 units/10 ml reaction mix) as an additional precaution against possible RNA degradation. Approximately 10 µg mRNA was reverse transcribed by incubation of the reaction mix (80 µl) at 37 °C for 1 hour and was used without further treatment as a template for PCR.

2.2.5 Polymerase Chain Reactions: Initially, PCR was optimized using varying Mg²⁺ concentrations: 1.5, 2, 3, 4, 6, 8 and 10 mM. Primers 1 and 2 were used with 10 µl of reverse transcribed cDNA reaction mix in a final volume of 100 µl. After initial denaturation at 94 °C for 5 minutes, each PCR cycle consisted of denaturation at 94 °C for 1 minute, annealing at 45 °C for 2 minutes and extension at 72 °C for 90 seconds with auto-extension of this latter time segment by 10 seconds for each cycle. A typical PCR was performed for 30 cycles. A 10 µl sample, representing 10% of the reaction mix, showed bands ~500 bp and ~700 bp in size (Figure 2.3), when analyzed by ethidium bromide-stained non-denaturing 1% agarose gels in a horizontal 8-well mini-gel electrophoresis unit (BRL, Model H6), as described above. Preparative 1% agarose gels were cast similarly in a larger 12- or 30-well electrophoresis unit (Elutrap™, Schleicher and Schuell) and were used for purification of these PCR products. Gel slices were excised and the DNA was extracted either by electroelution using TAE in a commercial electroelution device (Elutrap™, Schleicher and Schuell) or by using a commercial kit based on selective adsorption of DNA on glass beads (GeneClean®, BIO 101 Inc., La Jolla, CA).

The purified fragments were designated P500 (500-bp fragment) and P700 (700-bp fragment) and were used for cloning or as templates in all subsequent PCRs. Also, in subsequent PCRs, the cycle was changed to: 98 °C for 1 second, 50 °C for 15 seconds and 60 °C for 4 minutes.

2.2.6 Cloning and Extraction of Plasmid DNA: The purified PCR products were treated with Klenow fragment of DNA polymerase, to fill in recessed ends, and blunt-end ligated into *Hinc* II-linearized pUC19 using standard techniques (Sambrook, 1989). DH5- α competent cells (GIBCO BRL) were transformed with the treated plasmids, essentially according to the manufacturer's instructions. The transformed cells were split into a one-fifth part and a four-fifth part and spread individually on 100-mm YT plates (0.8% bactotryptone, 0.5% bacto yeast extract, 0.5% sodium chloride and 1.5% agar in water, prepared according to standard protocols, (Sambrook, 1989), coated with 50 μ l 2% X-gal and 10 μ l 100 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The plates were incubated overnight in an inverted position at 37 °C to provide either blue or white colonies, which indicate either absence or presence of an inserted DNA fragment in the plasmid, respectively. Several individual white clones were selected and grown overnight at 37 °C in liquid YT media (0.8% bactotryptone, 0.5% bacto yeast extract and 0.5% sodium chloride in water) and their DNA extracted using standard protocols (Sambrook, 1989). Sufficient plasmid DNA was obtained for sequencing and for checking the presence of the insert by cutting with specific restriction enzymes i.e. *Xba* I and *Pst* I, sites which flank the cloning site. The released insert was confirmed by electrophoreses through non-denaturing 1% agarose gels stained with ethidium bromide. Alternatively, the presence of an insert in a clone was checked by re-amplifying the DNA sequence by PCR using the

cloned plasmid as the template and by using the same primers used in the initial amplification (Shaw, 1991-Winter, Suppl 13).

2.2.7 DNA Sequencing: The cloned DNA was sequenced by the dideoxy terminator method (Sanger *et al.*, 1977) using a commercial sequencing kit (^{T7}Sequencing™ kit, Pharmacia LKB Biotechnology) and [α -³⁵S]dATP (Amersham). The cloned plasmid DNA was extracted from the *E. coli* cells by resuspending the pelleted cells from a 1 ml culture suspension in 270 μ l TE buffer and treating the cells with 20 μ l SDS (10%) and 8 μ l NaOH (5 M) for 5 minutes on ice. This was followed by addition of 150 μ l sodium acetate (3 M), phenol-chloroform (1:1) extraction and precipitation with ethanol at -20 °C. The DNA was recovered by centrifugation at 10,000 $\times g$, rinsed with 75% ethanol in water, air-dried, resuspended in water and used for sequencing, according to the manufacturer's instructions (^{T7}Sequencing™ kit, Pharmacia LKB Biotechnology). M13 universal and reverse sequencing primers were used to sequence both strands of the cloned DNA. Sufficient sequence was obtained in overlapping regions for unambiguous confirmation. Typically, 6% acrylamide-7 M urea denaturing gels (0.4-mm thick) were cast using a set of plates where only the shorter plate was treated with Repel-Silane (LKB-Produkter AB, Bromma, Sweden) to allow easy separation and removal of this plate from the gel, while allowing the gel to remain adhered to the longer plate. Pre-electrophoresis and electrophoresis was carried out at constant power (60 W), typically for 30 minutes and 2 to 6 hours, respectively, using Model S2 electrophoresis apparatus (GIBCO BRL). After electrophoresis, the short plate was carefully removed and the larger plate with the attached gel was placed in a shallow aluminium foil tray. The gel was fixed by soaking in 10% acetic acid/10% methanol (deposited on gel surface by gentle squirting)

for at least 30 minutes. The surface of the gel was kept moist by repeated deposition of the fixing solution, typically 10-15 ml every 5 minutes. The gel was then transferred to a chromatographic filter paper (Whatman, 3 mm), dried at 60-65 °C for 1.5-2 hours and autoradiographed (Kodak XAR 2) at room temperature for at least 1 day.

2.2.8 PCR to Amplify the C-terminal Region of the 21-kDa Protein cDNA:

Primers 9 and 10 were designed to independently amplify the C-terminus of the 21-kDa protein cDNA when used individually in conjunction with oligo(dT) Primer 1. The sequences of the primers are as follows:

Primer 9: 5' ^{Eco RI} GAATTC ^{Eco RI} GAATTC ^{Sma I} CCCGGG GTA(or C or G or T) ATA(or C or
T) CGA(or C or G or T or AGA or AGG) GCA(or C or G or T)
AAA(or G) 3'

Primer 10: 5' TGC TCT CCA ACT TCG GCC ACT 3'

Primer 9 was a degenerate ODN encoding the VIRAK sequence of the 21-kDa protein and possibly other TIMPs since this sequence is conserved in all other sequenced TIMPs, TIMP-1 and TIMP-2. Two *Eco* RI and one *Sma* I restriction enzyme sites (underlined) were incorporated in succession in primer 9 to facilitate cloning at a later stage. Unlike Primer 9 however, Primer 10 was a specific primer closer to the C-terminus and was designed based on the sequence of the cloned PCR product shown in Figure 2.7 (underlined). No restriction sites were incorporated in this primer. P700 (purified 700 bp PCR product) was used as template for the PCR at a concentration of 50 pg/μl. PCR conditions were 98 °C 1 second, 50 °C 15 seconds and 60 °C 4 minutes for 30 cycles. PCR products were analyzed by

non-denaturing 1% agarose gels stained with ethidium bromide, as described earlier.

2.3 Results and Discussion

The relationship of all primers used in PCR to the cDNA of the 21-kDa protein are presented schematically in Figure 2.2.

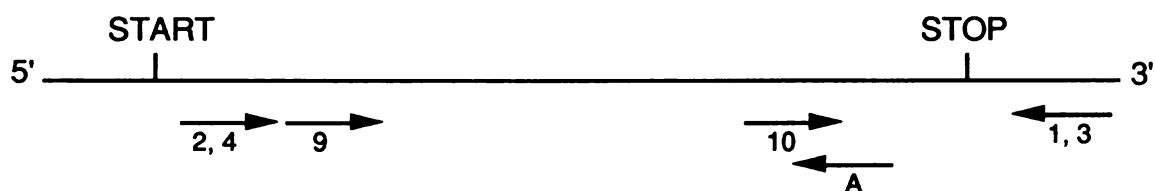


Figure 2.2. Schematic representation of positioning of the various primers used in PCRs relative to the 21-kDa protein cDNA. The arrow labelled A indicates participation of Primer 2 in certain PCRs, as discussed later in this section.

PCR with an internal degenerate primer based on a segment of the N-terminal sequence and an oligo d(T) primer as a pair (Primers 1 and 2), with reverse transcribed 1st strand cDNA as template, resulted in amplification of two fragments ~500 bp and ~700 bp in size (Figure 2.3, lanes 1-7). The 500 bp fragment appeared to be marginally more abundant than the 700 bp fragment. Also, there appeared to be no significant differences in the PCR products when the concentration of Mg^{2+} was varied between 1.5 mM to 10 mM

(Figure 2.3, lanes 1 - 7; due to poor photographic reproduction of the gel, the amplification may appear to be better in lanes 4 - 7). However, a better yield of PCR products was obtained when an alternate PCR cycle (98 °C for 1 second, 50 °C for 15 seconds and 60 °C for 4 minutes) instead of a standard PCR cycle (94 °C for 1 minute, 45 °C for 2 minutes and 72 °C for 90 seconds with auto-extension of the latter segment by 10 seconds for each cycle) was used (Figure 2.4, lanes 2 and 3). Hence for all subsequent PCRs a Mg^{2+} concentration of 1.5 mM and the alternate PCR cycle were used.

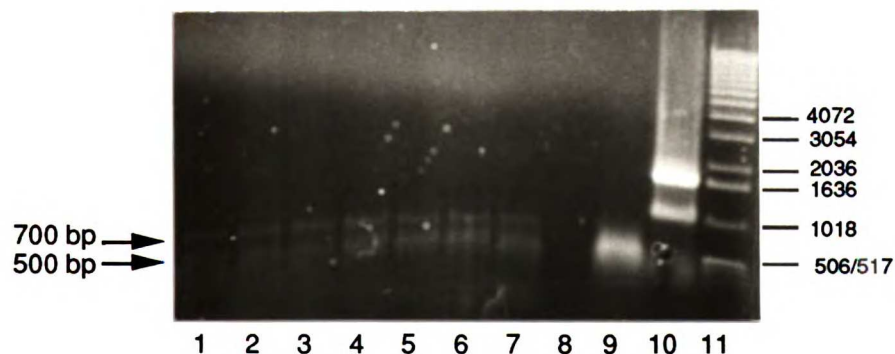


Figure 2.3. Effect of Mg^{2+} concentration on amplification of DNA fragments by PCR. Single stranded cDNA obtained by reverse transcription of mRNA from transforming CEF was used as template, as described under "Materials and Methods". Lanes 1 through 7 contain aliquots from PCRs with final Mg^{2+} concentrations of 1.5, 2, 3, 4, 6, 8 and 10 mM respectively. Note near equivalent amplification of a 500 bp and a 700 bp product at all Mg^{2+} concentrations. Lane 8 is blank, lane 9 contains a 500 bp PCR control amplified using whole bacteriophage lambda DNA as template and specific primers (provided in GeneAmp™ DNA Amplification Reagent Kit, Perkin Elmer Cetus), lane 10 contains an aliquot of residual non-poly(A) RNA following mRNA selection and lane 11 contains DNA molecular weight standards (1 Kb DNA Ladder, GIBCO BRL).

A subsequent PCR with Primers 3 and 4 and P500 as template amplified a 500 bp fragment as expected (Figure 2.4, lanes 4-7). However, PCR with P500 as template and Primers 1 and 2 amplified a 500-bp fragment as a major product along with a 700-bp fragment as a minor product (Figure 2.4, lanes 2, 3). This indicated that the P500 template may have been contaminated with the 700-bp fragment, especially since the relative amount of the amplified 700-bp fragment in this PCR (Figure 2.4, lanes 2, 3) is considerably lower than in

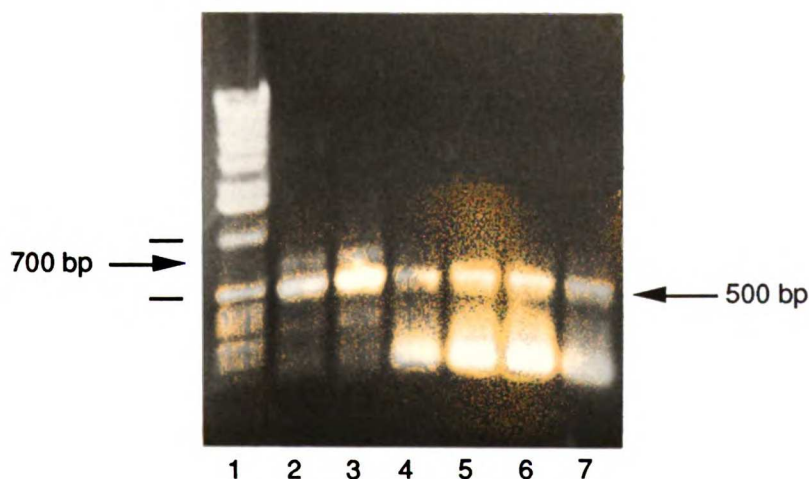


Figure 2.4. Analyses of PCR products obtained with purified 500 bp PCR-generated DNA fragment as template and either Primer 1 + Primer 2 (lanes 2, 3) or Primer 3 + Primer 4 (lanes 4 - 7) on an ethidium bromide-stained 1% agarose gel. The alternate PCR cycle (98 °C for 1 second, 50 °C for 15 seconds and 60 °C for 4 minutes) instead of a standard PCR cycle (94 °C for 1 minute, 45 °C for 2 minutes and 72 °C for 90 seconds with auto-extension of the latter segment by 10 seconds for each cycle) was used. Aliquots are from replicate PCRs. Note amplification of only a 500 bp product with the longer 31-base Primers 3 and 4, while the shorter 24-base Primers 1 and 2 amplified an additional 700 bp product, as discussed. Also note the presence of additional lower molecular weight DNA bands (lanes 4 - 7) amplified in PCRs with Primers 3 + 4 in comparison to PCR with Primer 1 + 2 (lanes 2, 3). These may be due to non-specific amplification. Lane 1 contains DNA molecular weight standards (1 Kb DNA Ladder, GIBCO BRL) The 1018-bp and 506/517-bp standards are indicated by dash marks.

the initial PCR using the reverse transcribed template (Figure 2.3, lanes 1-7). However, the 700-bp fragment was not amplified in the PCR with Primers 3 and 4. It is conceivable that the larger overhang in Primers 3 and 4 contribute to destabilization of the annealing of the primers. Hence the 500 bp fragment appears to be the predominant product.

The PCR products were purified by electrophoresis through agarose gels followed by extraction using Geneclean® or by electroelution. Both these extraction methods were comparable in quality and yields, as determined visually on ethidium bromide-stained agarose gels. Basically, two cloning experiments (each with 4 separate ligation reactions) were conducted and results for these are presented. Cloning of these PCR products into the multiple cloning site of pUC19 using cohesive-end ligation did not provide satisfactory transformation of the host organism (Library efficiency DH5α' competent *E. coli* cells, GIBCO BRL). The transformants were fewer in number (less than 50) than expected and all formed blue colonies. However, blunt-end ligation using *Hinc* II-linearized pUC19 and Klenow-treated PCR products yielded high transformation efficiencies. Typically several hundred transformants were generated in each 100-mm plate from each cloning and approximately 2-10% were white transformants, indicating the presence of a recombinant vector. The presence and size of the inserts was checked by treating the extracted DNA with restriction enzymes *Xba* I and *Pst* I (these sites flank the insertion site) and analyzing the products by ethidium bromide-stained agarose gels (Figure 2.5). Seven positive clones were identified from twelve selected clones. Due to the inadvertent addition of a restriction enzyme (*Hinc* II) instead of Klenow, all the positive clones showed shorter inserts (150 bp to 350 bp) than the expected 500 bp or 700 bp. For the subsequent cloning an alternate method of checking for inserts by PCR was

used. This technique has advantages of being faster, easier and more specific since the expected size of amplified DNA would be obtained only if the insert of choice was present in the recombinant clone (Figure 2.6). Using this technique, nine positive clones were identified from the fifteen clones that were selected. Four clones 1C1, 1C8, 1C10 and 1C11 from the first cloning and four clones 2P1, 2P3, 2P10 and 2C3 from the second cloning were sequenced.

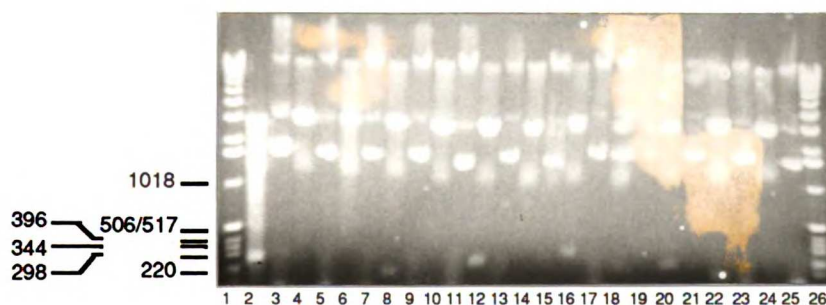


Figure 2.5. Analysis of 12 positive (white) clones by restriction enzyme digestion of isolated plasmids on an ethidium bromide-stained 1% agarose gel. Lanes 1 and 26 are DNA molecular weight standards. Even numbered lanes contain plasmid clones treated with restriction enzymes *Xba* I and *Pst* I, while the following odd numbered lanes contain corresponding undigested plasmid as a control, as described under “Materials and Methods”. Lanes 8, 12, 16 and 20 show DNA fragments approximately 200, 300, 350 and 300 bp, respectively, while the remaining clones either contained inserts shorter than 200 bp or were false positives.

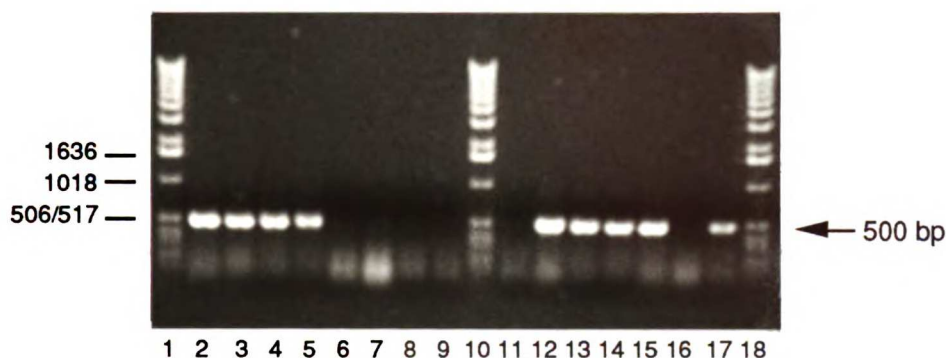


Figure 2.6. Analysis of 15 positive (white) clones by PCR using Primers 1 and 2, as described under "Materials and Methods". Lanes 1, 10 and 18 contain DNA molecular weight standards. An aliquot of the PCR mix from the 15 clones was electrophoresed through a 1% agarose gel. Amplification of a 500 bp product (lanes 2-5, 12-15 and 17) indicates presence of the insert while the remainder indicate false positives.

In the first cloning the PCR products were inadvertently subjected, albeit very briefly, to *Hinc* II (instead of Klenow). However, clones 1C10 and 1C11 were indeed fragments from the coding region for the 21-kDa protein when sequenced. Clone 1C10 contained a 189 bp sequence encoding the N-terminus of the 21-kDa protein immediately following Primer 2 whereas clone 1C11 contained an identical, but shorter sequence because it was missing an intermediate 68 bp section, (77-144 bp) (Figure 2.7). This may have been a result of simultaneous ligation of two *Hinc* II cleaved fragments. The tail of the 189 bp fragment contains a *Hinc* II site confirming it as a subclone.

Although clone 1C8 contained a 17 bp poly(A) segment corresponding to the 17 bp poly(dT) of Primer 1, the sequence showed no similarity or overlap with latter 21-kDa protein sequences and hence may have been an artifact. Although, clone 1C1 contained a 240-bp insert, its sequence did not match the sequence of clones 1C10 and 1C11, and hence was an artifact.

Clones 2P1, 2P3, 2P10 and 2C3 from the second cloning experiment were all identical in sequence. A 449 bp sequence was obtained from these clones after excluding the primer sequences (Figure 2.7). Surprisingly, a sequence complementary to that of Primer 2 was present on both strands of the PCR amplified DNA. Hence it appears that the degenerate Primer 2 was solely involved in the PCR and primed both strands of the cDNA (Figure 2.1, arrow labelled A). The clones were sequenced in both directions and had an extensive overlap region (minimum overlap of 154 bp). The sequence is shown in Figure 2.7 and a schematic representation of the sequencing of the clones is shown in Figure 2.8.

PCR using Primers 1 and 10 amplified a ~350 bp product (Figure 2.9), when the 700 bp fragment (P700) was used as the template. This 350 bp fragment appeared to be of the expected size. Approximately 150 bp could be accounted for as part of the C terminus open reading frame to the stop codon for the 21-kDa protein and the remaining 150 - 200 bp as part of the 3' non-coding segment upstream of the poly(A) region. Hence, successful amplification using oligo(dT) as one of the primers (Primer 1) indicated the probable presence of a poly(A) tail in the purified 700 bp template used in this PCR. In addition, PCR with Primer 1 and Primer 9 yielded 2 major products approximately 500 bp and 650 bp in size, which support the presence of a poly(A) region in the template. These fragments were not cloned or sequenced.

TTC TGC AAC TCC GAC ATC GTG ATC CGT GCT AAA GTT GTG GGG	42
AAG AAG CTC ATG AAA GAT GGA CCA TTT GGA ACA ATG CGA TAC	84
ACA GTC AAG CAG ATG AAG ATG TAC AGG GGC TTC CAG ATA ATG	126
CCA CAC GTT CAG TAC ATC TAC <u>ACA GAA GCC TCA GAG AGT CTT</u>	168
<u>TTG TGG TGT GAA ACT GGA GGT CAA CAA ATA CCA GTA TCT GAT</u>	210
<u>TAC AGG CCG CGT GTA CGA AGG GAA GGT TTA CAC TGG CCT GTG</u>	252
<u>CAA TTG GTA TGA GAA ATG GGA CCG ACT GAC TCT GTC CCA GCG</u>	294
<u>TAA AGG ACT</u> GAA TCA TCG TTA TCA TCT GGG CTG TGG ATG CAA	336
GAT TCG GCC CTG CTA CTA TTT GCC CTG CTT TGC CAC CTC CAA	378
GAA TGA GTG CAT TTG GAC AGA <u>CAT GCT CTC CAA CTT CGG CCA</u>	420
<u>CTC</u> AGG ACA CCA AGC GAA GCA CTA TGC CT	449

Figure 2.7. The cDNA sequence of clones 2P1, 2P3, 2P10 and 2C3 containing the 500 bp DNA fragment amplified by PCR. The flanking sequence of Primer 2 used in the PCR is not shown. Both strands of the clones were sequenced and the overlap region is underlined. An internal primer 10 (double underlined) was synthesized to aid in amplifying the C-terminal end by PCR.

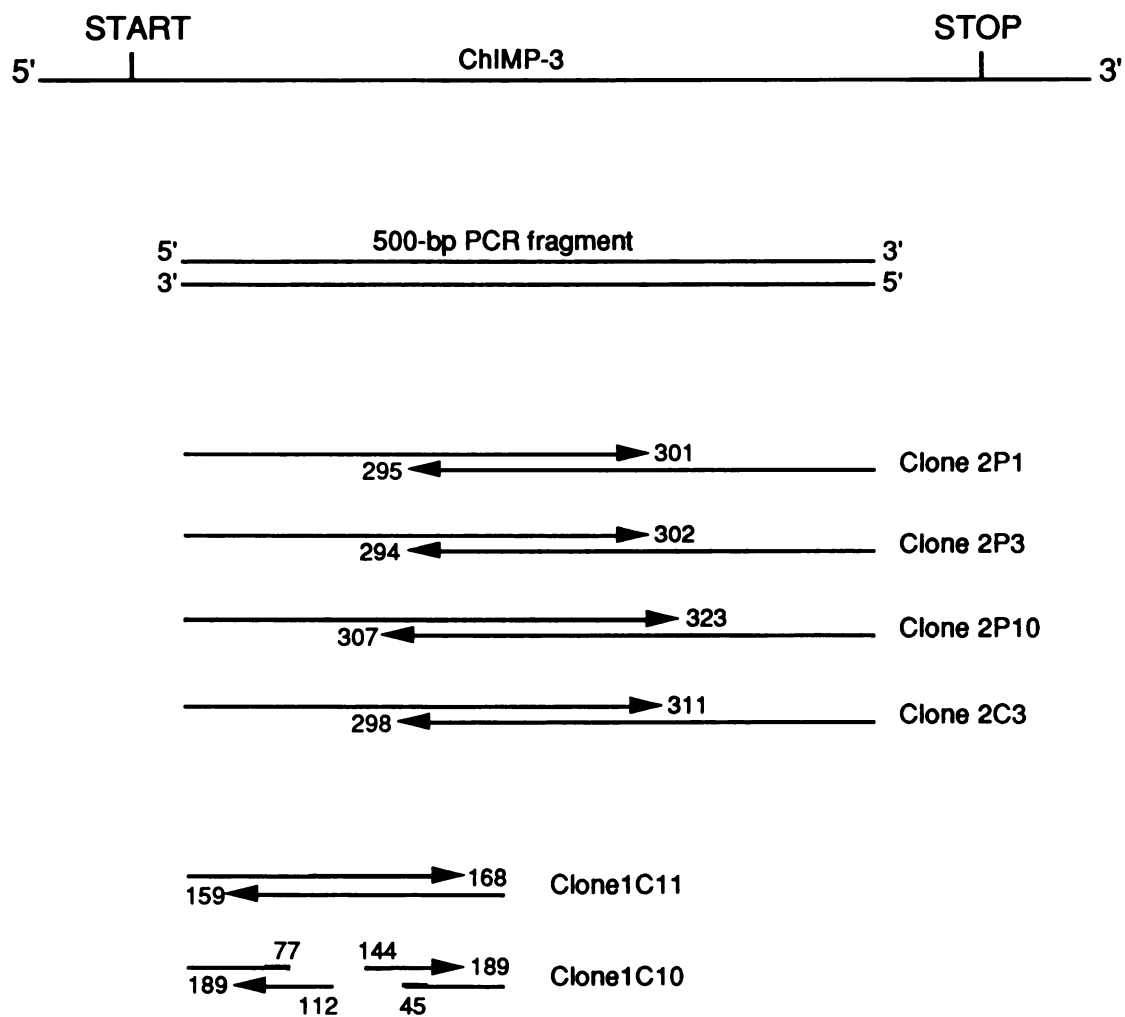


Figure 2.8. Schematic representation of DNA sequencing of the various clones.

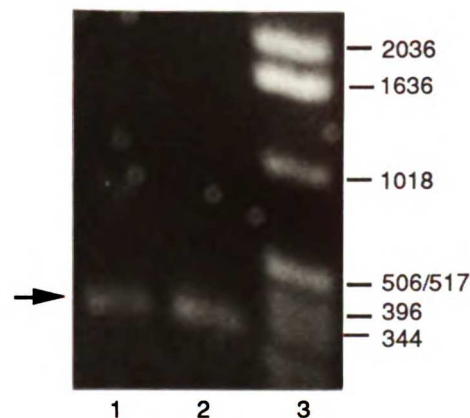


Figure 2.9. Agarose gel analysis of PCR products using Primers 1 and 10. Lanes 1 and 2 are duplicates while lane 3 contains DNA molecular weight standards. A single 350-bp product (arrow) indicates priming of a C-terminal poly(A) region with the oligo(dT)₁₇, as discussed under “Results and Discussion”.

2.4 Summary

A 449 bp cDNA sequence encoding the 21-kDa protein was obtained using RT-PCR and standard cloning and sequencing techniques. This sequence information was considered to be sufficient for designing antisense ODNs unique for the 21-kDa protein which could be used in an attempt to selectively inhibit expression of the 21-kDa protein in cell culture. In parallel studies in the laboratory, PCR with Primers 1 and 2 was used to amplify a similar ~500-bp fragment using a commercial chicken cDNA library (Clontech) as the template. This was in contrast to the reverse transcribed

cDNA prepared from transforming CEF and presented in this thesis. The DNA sequences of the cloned 500-bp PCR fragments obtained with either templates were identical. The complete cDNA sequence encoding the 21-kDa was subsequently obtained and based on its sequence and unique properties, the 21-kDa protein was established to be a new member of the TIMP family, designated ChIMP-3 (Chicken Inhibitor of MetalloProteinases-3) or the chicken TIMP-3 (Pavloff *et al.*, 1992).

Chapter 3

Antisense Oligodeoxynucleotide-Mediated Inhibition of ChIMP-3

3.1 Introduction

Over the past decade the popularity of antisense oligonucleotide-mediated inhibition of gene expression has soared along with other oligonucleotide-based procedures such as in antigene and in aptamer strategies. Antisense-mediated inhibition is based on sequence-specific binding of either short oligonucleotides (typically 12 to 24 nucleotides in length) or larger RNA fragments (generated in cells by transfection of the cDNA in a reverse orientation) to the mRNA encoding the target protein. Such binding often results in decreased expression of the protein by mechanisms that have been proposed to either disrupt the translation process by physical occlusion or to rapid degradation of the mRNA by RNase H, an enzyme that recognizes DNA:RNA complexes. The target for an antigene oligonucleotide is double-stranded DNA and involves the formation of stable triple-stranded structures that inhibit transcription by interfering with binding of transcription factors to DNA (Maher *et al.*, 1989). An aptamer (Ellington and Szostak, 1990) is a highly specific oligonucleotide sequence, selected by screening large pools ($\sim 10^{13}$) of degenerate sequences, on the basis of its ability to bind and inhibit the activity of a specific protein (Bock *et al.*, 1992). Various aspects of these approaches, including toxicity, stability, specificity, chemical modifications, *in vivo* and *in vitro* pharmacological activities and intracellular delivery of these compounds have been extensively reviewed (Goodchild, 1991; Hélène, 1991; Mirabelli *et al.*, 1991; Calabretta *et al.*, 1992; Gray *et al.*, 1992a; Neckers *et al.*, 1992; Stein, 1992;

Calabretta *et al.*, 1993; Milligan *et al.*, 1993; Neckers and Whitesell, 1993; Stein and Cheng, 1993).

Optimism about possible use of oligodeoxynucleotides (ODNs) as therapeutic agents can be attributed primarily to a large number of studies that have successfully inhibited the synthesis of target proteins and to automation and improvements in ODN synthesis procedures (Geiser, 1990) which have provided reasonable quantities of ODNs. Clinical trials with antisense-ODNs are currently underway in human volunteers for the treatment of infections with viruses such as papillomavirus and HIV-1 (Alper, 1993). In *in vitro* experiments, antisense-mediated inhibition offers an unique opportunity to specifically inhibit a target protein in order to evaluate its function(s). The functions of a host of growth factors and oncogene products have been confirmed by treatment of cultured cells with antisense ODNs. For example, by inhibiting *c-myc* expression it was demonstrated that the cells were blocked from entering into the S phase (Heikkila *et al.*, 1987; Harel *et al.*, 1988) and as a result growth was inhibited. The importance of proliferating cell nuclear antigen (PCNA or cyclin) has also been clearly demonstrated by suppressing DNA synthesis and mitosis of Balb/c3T3 cells with antisense ODNs to PCNA (Jaskulski *et al.*, 1988). Along similar lines, as discussed in section 2.1, antisense ODN-mediated inhibition of ChIMP-3 was attempted to determine the role of this protein during transformation of chicken embryo fibroblasts with Rous sarcoma virus.

3.2 Materials and Methods

3.2.1 Selection of Target Sequences for Antisense Oligodeoxynucleotide-Mediated Inhibition: Two regions of the ChIMP-3 mRNA were selected as

targets for antisense ODN-mediated inhibition, based primarily on maximal sequence differences between ChIMP-3 and the other known inhibitors, TIMP-1 and TIMP-2. Both antisense ODN sequences (AS1 and AS2) were at the 5' end of the ChIMP-3 mRNA and corresponded to residues 14 to 19 (AS1) and 24 to 30 (AS2) of the mature ChIMP-3 protein. AS1 and AS2 and their respective random sequences (R1 and R2), along with the corresponding protein and cDNA sequences, are presented below:

ChIMP-3		N	S	D	I	V	I	
cDNA	5 '	AAC	TCC	GAC	ATC	GTG	AT	3 '
AS1	3 '	TTG	AGG	CTG	TAG	CAC	TA	5 '
R1	5 '	ACG	TCG	ATG	AGT	TAG	CT	3 '

ChIMP-3		V	G	K	K	L	M	K
cDNA	5 '	TG	GGG	AAG	AAG	CTC	ATG	A 3 '
AS2	3 '	AC	CCC	TTC	TTC	GAG	TAC	T 5 '
R2	5 '	AC	GTT	CCT	ACT	CGT	CAC	T 3 '

The random sequences were checked with DNA sequences in the GenBank™ using EuGene (Molecular Biology Information Resource, Houston, TX) to ensure that these sequences were not complementary to known sequences.

3.2.2 Oligodeoxynucleotide Synthesis and Purification: ODNs were synthesized on an automated DNA synthesizer (Millipore-Biosearch, Model 8600) using standard phosphoramidite chemistry, and essentially following the protocol supplied by the manufacturer. The ODNs were purified by denaturing gel electrophoresis, as described earlier under section 2.2.2. For use in cell cultures, the purified ODNs were sterilized by aseptic filtration through a 0.2 micron filter (Acrodisc, Gelman Sciences) and aliquots were

mixed aseptically with appropriate volumes of 5 X methionine-free media 2.0.0.1+ (methionine-free media 199 with 2% tryptose phosphate broth, 0.1% chicken serum and 1 g/L glucose) and water to obtain the desired ODN concentrations. The chicken serum was heated at 55 °C for 2 hours to inactivate viruses and heat-sensitive nucleases, such as phosphodiesterases.

3.2.3 Modifications of Cell Culture Techniques for Antisense Experiments:

Synthesis of ODNs is expensive. In order to conserve available quantities of these reagents several routine assays were modified to obtain targeted concentrations of ODN (ranging between 1 and 50 μ M) with smaller amounts of ODNs. The modified procedures included a cell proliferation assay, a cell culture based functional assay and a metabolic-labeling technique.

The cell proliferation assay was adapted to 35-mm dishes instead of 60-mm dishes. Chicken embryo fibroblasts (CEF) infected with a temperature-sensitive mutant, LA24, of Rous sarcoma virus (tsRSV-LA24) were cultured as described (Blenis and Hawkes, 1983; Staskus *et al.*, 1991). At the tertiary stage, 4×10^4 cells were seeded in 35-mm culture dishes in 1 ml of media 2.0.0.1+, and transformed at 35 °C. At periodic time intervals, cells were removed from the dish surface by trypsinization, collected and counted in a Coulter Counter. Control non-transforming CEF maintained at 41 °C were also counted similarly. The average cell number from three dishes at each time point was plotted as a function of time.

The functional assay of EGTA-mediated cell detachment of transforming cells was performed in 60-mm culture dishes, essentially as described by Yang and Hawkes (1992). Briefly, transforming tsRSV-LA24-infected CEF were incubated with Ca^{2+} -, Mg^{2+} -free phosphate buffered saline containing 5 mM EGTA (pH 7.4) for 10 minutes, followed by a 5 minute

agitation on a rotary shaker (Braun Thermonix Shaker, Model 1460) set at 120 r.p.m.. The EGTA-released cells were collected and counted in a Coulter Counter. The remaining cells were removed by trypsinization, collected and counted. Cells released by EGTA treatment were expressed as a percentage of the total number of cells. Since the experimental logistics of this assay excluded varying culture dish size, the only modification of this assay for the antisense experiments would be to culture the cells in a reduced volume of media i.e. 1.5 ml of media instead of 5 ml.

Metabolic labeling of proteins with [³⁵S]methionine (Amersham, 5-10 μ Ci/dish) was also performed in 35-mm dishes, instead of 60- or 100-mm dishes. The cells were seeded in 2.0.0.1+, as described above, but at a higher density of 4×10^5 /dish in order to obtain sufficient quantities of labeled ChIMP-3 in the extracellular matrix to enable visualization on a autoradiogram. Details of the metabolic labeling method and analysis of the labeled proteins are given below.

3.2.4 Antisense ODN Treatment of CEF: In initial experiments only metabolic labeling was used as a technique to evaluate the efficacy of the ODNs, mainly because changes in the protein levels would be directly measurable. CEF infected with tsRSV-LA24 were seeded in 1 ml 2.0.0.1+ (4×10^5 /35-mm dish) and cultured at 41 °C for 12-15 hours. The media was aspirated and the cells rinsed carefully with methionine-free 2.0.0.1+. [³⁵S]methionine (5 μ Ci/dish) was added to each dish in 900 μ l methionine-free 2.0.0.1+. The ODNs were added in 100 μ l methionine-free 2.0.0.1+ at a final concentration of 0.7 μ M (AS1) and 5 μ M (AS2, R1 and R2) and the cells were then transformed at the permissive temperature of 35 °C. One set of cells received a second dose of ODNs 6 hours after the initial dose, while two

other sets of cells received only a single dose of ODNs. The conditioned media (CM) was collected and the extracellular matrix was harvested either 6 hours (single dose set) or 12 hours (single dose set and double dose set) after the initiation of transformation. ECM was prepared as described by Blenis and Hawkes (1983), with the exception of solubilizing the ECM in Laemmli sample buffer without any reducing agent. In an experiment to determine the effect of ODN concentration on ChIMP-3 synthesis, CEF were treated, as described above, for 12 hours with AS2 and R2 at final concentrations of 5, 15 and 50 μM .

3.2.5 Gel Electrophoresis: The ECM samples were analyzed by electrophoresis through a 15% polyacrylamide-SDS gel (1.5-mm thick), using the (Laemmli, 1970). Following electrophoresis, the gel was fixed for at least 1 hour in acetic acid-methanol-water mix (10:45:45), followed by a 30 minute incubation in an autoradiographic signal enhancer solution (Enlightening™, DuPont). The gel was dried and exposed to X-ray film (Kodak, XAR-2) at -70 °C for varying time periods to obtain bands of sufficient intensity.

3.2.6 Scintillation Counting: Following autoradiography, the autoradiogram was aligned with the dried gel and ChIMP-3 bands were excised. The gel slices were solubilized (depolymerized) by overnight treatment with 30% hydrogen peroxide (500 μL /slice) at 60 °C, mixed with a scintillation cocktail (Fisher, 7 ml/slice) and counted. Results were expressed as a percentage of the untreated control.

3.2.7 Protease-Substrate Gel Electrophoresis: Metalloproteinase inhibitory activities in ECM and CM samples were assayed by electrophoresis through

protease-substrate gels, essentially as described earlier (Staskus *et al.*, 1991). Briefly, CEF CM and gelatin (as a source of metalloproteinase and substrate, respectively) were incorporated into 15% acrylamide, 0.75-mm SDS-PAGE Laemmli gels at final concentrations of 23.5% v/v and 1 mg/ml, respectively, during polymerization. After electrophoresis, the gels were subjected to two 15 minute incubations in 2.5% v/v Triton X-100 and one 15 minute incubation in developing buffer (50 mM Tris, pH 8.0, 5 mM CaCl₂, 0.02% w/v sodium azide) at room temperature followed by incubation at 35 °C for at least 12 hours in the same developing buffer. The gels were then fixed and stained with either 0.1 % w/v Coomassie Blue R-250 (occasionally Coomassie Blue G-250 was used) in acetic acid/methanol/water (10:45:45) and destained in methanol/water (50:50) or alternatively stained in a colloidal suspension of Coomassie Blue G-250 in 2% v/v phosphoric acid and 6% w/v ammonium sulfate (Neuhoff *et al.*, 1985). For autoradiographic analysis, the gels were dried after staining and exposed to X-ray film (Kodak, XAR-2) at -70 °C.

3.3 Results and Discussion

Results of the modified cell proliferation assay (Figure 3.1) indicated that this assay could be satisfactorily performed in 35-mm dishes instead of 60-mm dishes commonly used for this assay in our laboratory. The transforming cells clearly demonstrated reduced serum requirement and their number increased more than 3-fold over 4 days in culture, though their number declined by the fifth day. The nontransforming control cells, in contrast, divided at a much slower rate, with their numbers increasing only 1.4-fold over the same time period. This assay clearly would be suitable for determining the effects of extended ODN treatments on cell proliferation.

Results of the cell detachment assay (Figure 3.2) demonstrated the propensity of transforming cells to exhibit reduced adhesion. The relatively large, almost 5-fold, difference in adhesive properties of normal and transforming cells would allow antisense-mediated changes in the amounts of the 21-kDa protein to be readily detected. However, results of an experiment (described below) precluded the use of these functional assays.

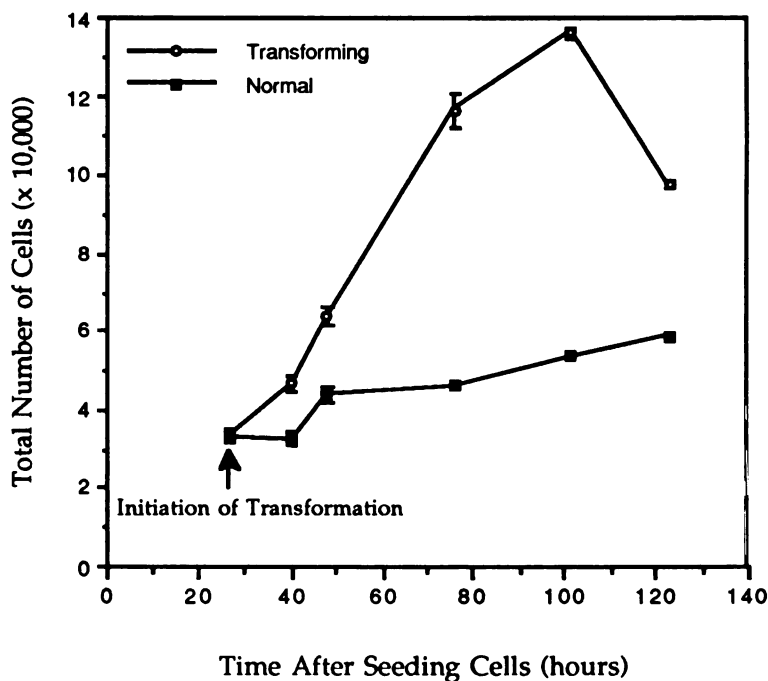


Figure 3.1. Cell proliferation assay. tsRSV-LA24-infected CEF were cultured in 2.0.0.1+ media in 35-mm dishes and maintained at 35 °C (transforming) and 41 °C (normal, control) and counted at the indicated times, as described under "Materials and Methods". Each time point represent average of three replicates.

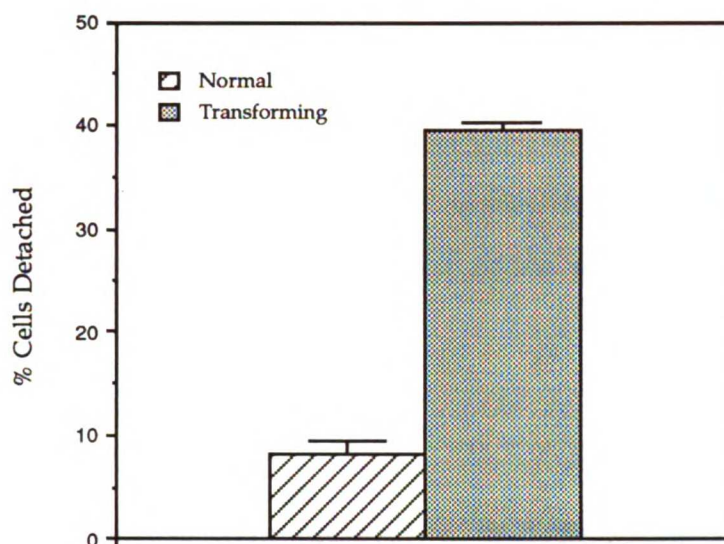
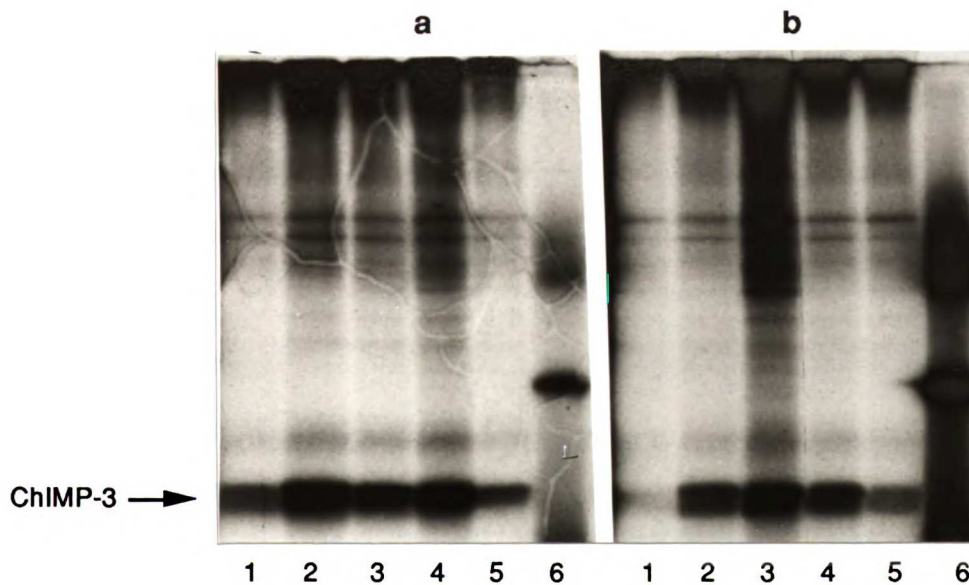


Figure 3.2. Cell detachment assay. tsRSV-LA24-infected CEF were cultured in 60-mm dishes and maintained at 35 °C (transforming) and 41 °C (control) for 16 hours before determining cells detachable by EGTA treatment, as described under "Materials and Methods". Each time point represent average of three replicates.

The antisense ODN-mediated inhibition of ChIMP-3 synthesis was variable in the experiments performed. In some experiments, the antisense ODNs elicited a marginal effect (as discussed below), while in at least one experiment the ODNs showed good inhibition of ChIMP-3 synthesis. In this experiment, treatment of CEF with a single dose of ODN for 6 hours did not show any significant inhibition of ChIMP-3 (data not shown), suggesting that longer treatment may be required to elicit an effect. The 12 hour treatments

with either a single or double dose of ODNs (Figure 3.3a and 3.3b, respectively), both AS1 and AS2 (final concentrations of 0.7 μ M and 5 μ M, respectively) showed inhibition of ChIMP-3 synthesis. The single dose of AS1 and AS2 inhibited ChIMP-3 synthesis 41% and 42%, respectively (3.3a, lanes 1 and 5) and the double dose produced 50% and 75% inhibition, respectively (3.3b, lanes 1 and 5) when compared to their respective no ODN control (3.3a and 3.3b, lane 3). In comparison to the AS1 and AS2, the corresponding



Figures 3.3a and 3.3b. Analysis of antisense ODN inhibition of ChIMP-3 synthesis in transforming CEF by SDS-PAGE. [35 S]methionine-labeled ECM were analyzed on a 15% polyacrylamide-SDS Laemmli gel and autoradiographed, as described under "Materials and Methods". Panel a: ECM from CEF treated with one dose of ODNs: AS1 (lane 1), R1 (lane 2), no ODN control (lane 3), R2 (lane 4), and AS2 (lane 5). Panel b: ECM from CEF treated with two doses of ODNs, identical lane assignments as in Panel a. Lane 6 contains [14 C]-labeled protein molecular weight standards (GIBCO BRL).

random ODNs, R1 and R2 (both at a final concentration of 5 μ M), did not significantly inhibit ChIMP-3 synthesis (3.3a and 3.3b, lanes 2 and 4), indicating that the effects of AS1 and AS2 were specific. In fact, the level of ChIMP-3 appears to be greater in R1 and R2 treated cells (3.3a, lanes 2 and 4) than the no ODN control (3.3a, lane 3) and it may appear that R1 and R2 may be stimulating synthesis of proteins in the cells. However, these levels appear to be similar to the no ODN control in the parallel set (3.3b, lane 3) and hence may be due to experimental variations. Similarly, the levels of ChIMP-3 in R1 and R2 (3.3b, lanes 2 and 4) appear to be lower than the control (lane 3), but appear to be similar to the no ODN control of the parallel set (3.3a, lane 3).

In Figures 3.3a and 3.3b (lanes 1 and 5), it is noteworthy that, in general, there is a lower amount of radiolabel incorporated in ECM proteins of AS1 and AS2 treated cells. This, most likely, was not due to toxicity of the ODNs to the cells since phase-contrast microscopic examination of the cells had shown no morphological signs of toxicity. It is possible that the decrease in radiolabel incorporation could be due to a decrease in cell proliferation as a result of inhibition of ChIMP-3 biosynthesis. This would be in agreement with the observations of Yang and Hawkes (1992), who showed that growth-retarded cells, maintained under the low serum conditions used in these experiments, were stimulated to divide when cultured on ChIMP-3-coated surfaces.

In this experiment, although the treatments were performed in triplicates, the samples had to be pooled for analysis due to low incorporation of the radiolabel. Hence, subsequent experiments were performed in triplicates in 60-mm dishes (instead of 35 mm) with a larger cell number (1×10^6 instead of 4×10^5) to increase the total amount of radioactivity of samples and allow analysis of replicates. However, the antisense effect in these

subsequent experiments was marginal at similar ODN concentrations of 5 μ M. An experiment with higher ODN concentrations of AS2 (15 μ M and 50 μ M), in addition to 5 μ M, performed to determine the effect of ODN concentration on ChIMP-3 synthesis, also did not show significant inhibition of ChIMP-3 synthesis.

Analysis of the CM from this experiment showed an unexpected interaction. The CM were analyzed by protease-substrate gels to test the specificity of the antisense ODNs in inhibiting ChIMP-3 synthesis without affecting other related TIMPs, such as ChIMP-2 (chicken TIMP-2) present in the CM of CEF. As shown in Figure 3.4, treatment with 5, 15 and 50 μ M AS2 and R2 revealed that the ChIMP-2 levels were almost constant and were not significantly different from the controls. However, the ODN-treated CM showed the presence of two additional inhibitors ~24 and ~27 kDa. The mobilities of these inhibitors were identical to those of authentic ChIMP-3 and ChIMP-a, as determined on a separate gel. These inhibitors are usually found exclusively in the ECM. The amounts of ChIMP-3 and ChIMP-a in the CM was proportional to the concentration of the ODN added, but was independent of the sequence of the ODNs since both AS2 and R2 produced similar effects (Figure 3.4). It can be concluded from these data that either: (1) ODNs stimulate cells to synthesize additional ChIMP-3 and ChIMP-a which are released into the media and not bound to the ECM, or (2) the ODNs interfere in some way with the binding of ChIMP-3 and ChIMP-a to the ECM and cause their release. Also, as shown in Figure 3.5, the autoradiogram of the protease-substrate gels revealed radiographic bands that were exactly coincident with the inhibitor bands and their radiographic intensities corresponded directly to intensities of the inhibitor bands stained with Coomassie Blue. Collectively, these results indicated that interactions were

occurring between the ODNs and *de novo* synthesized ChIMP-3 and ChIMP-a, at least in part, resulting in a change in location of these inhibitors from their exclusive ECM location to the CM. However, the magnitude of such interactions may not be large, since the amount of ChIMP-3 present in the ECMs of ODN-treated cells was not significantly different from the untreated controls.

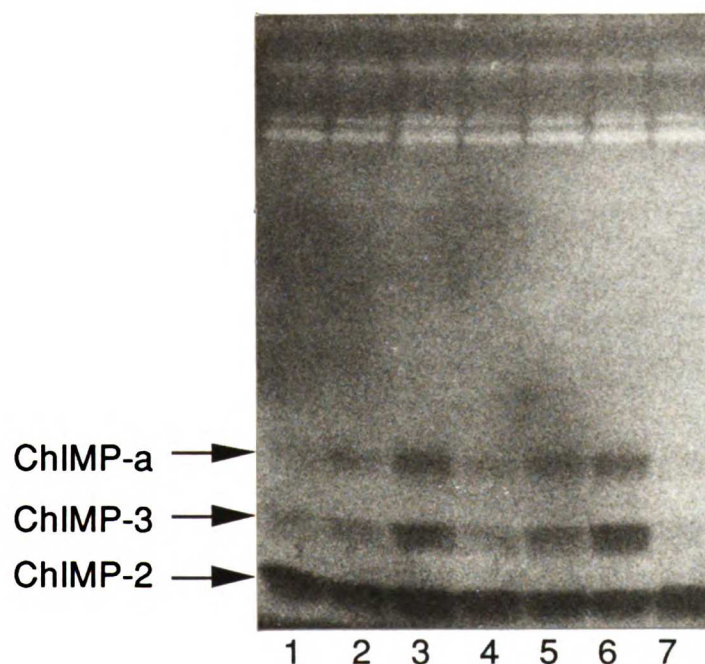


Figure 3.4. Protease-substrate gel analysis of conditioned media from CEF treated with ODNs, as described under "Materials and Methods". Lanes 1, 2 and 3 contain samples from cells treated with 5, 15 and 50 μ M AS2 ODN, respectively. Lanes 4, 5 and 6 contain samples from cells treated with similar concentrations of R2 ODN. Lane 7 contains no ODN treatment control. Note the relatively constant amount of ChIMP-2 present in all treatments, including the control, but the amounts of ChIMP-3 and ChIMP-a present in the CM clearly appear to be proportional to the ODN concentration used. In contrast, note the absence of ChIMP-3 and ChIMP-a in the control (lane 7).

The protease-substrate gels were repeated after taking additional measures, such as centrifugation of the CM samples at 10,000 $\times$ g for 30 minutes to remove any cellular debris or disrupted ECM that could potentially contribute to artifacts. This was in light of observations made by Staskus *et al.*, (1991), where minute amounts of ChIMP-3 were occasionally observed in CM, but were readily sedimented at 10,000 $\times$ g. Results with the centrifuged CM samples were similar to those presented in Figures 3.4 and 3.5.

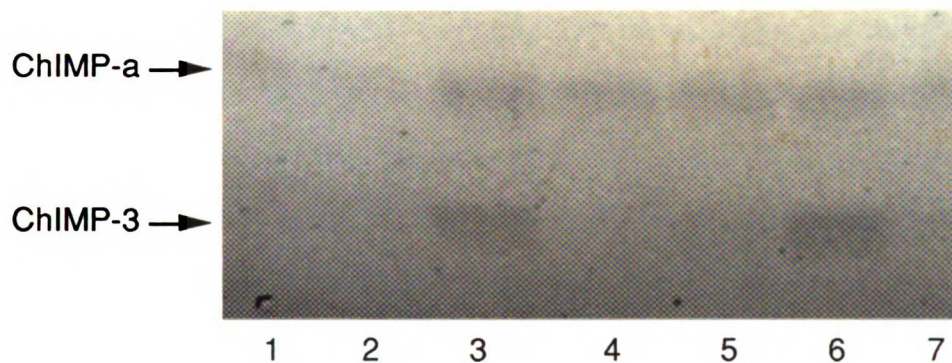


Figure 3.5. Autoradiogram of protease-substrate gel analysis of conditioned media from CEF treated with ODNs. The gel presented in Figure 3.4 was dried and subjected to autoradiography (40 days at -70°C). Due to faint signals, this autoradiogram was scanned electronically and contrast enhanced by a computer program (Image 1.42, NIH) to improve clarity. The bands labeled ChIMP-3 and ChIMP-a are coincident with the inhibitory bands shown in Figure 3.4. Note the correspondingly higher signals in samples treated with higher ODN concentrations, especially for ChIMP-3. ChIMP-a signals appear to be coincident with an unknown protein (lane 7) that shows no inhibitory activity (Figure 3.4, lane 7). Hence, the concentration effect for ChIMP-a is not as clearly discernible as for ChIMP-3.

The change in location of the inhibitors produced by ODNs could possibly be due to electrostatic interactions between the negatively charged ODNs and the inhibitors, at least one of which, ChIMP-3, is known to be a basic protein with a pI of >9.0 (the pI of ChIMP-a is not known). Supporting data for this is presented in detail in Chapter 4. Briefly, a similar experiment with DNA clearly show a change on location of ChIMP-3 and ChIMP-a into the CM with a concomitant decrease in these inhibitors in the ECM. These observations precluded the continuation of experiments with unmodified ODNs to determine the role of ChIMP-3 during transformation by functional assays such as cell detachment and proliferation, since the properties of ChIMP-3, that is now present in a "soluble" form in the CM, are not yet known. It is conceivable that the properties of ChIMP-3 in a "soluble" form may be very different from its properties as an ECM-bound protein. A precedent for this is illustrated by J1/tenascin, that has been studied both in a substrate-bound form and a soluble form. J1/tenascin stimulates neurite outgrowth in a substrate-bound state, but inhibits such growth when present in a soluble form (Lochter *et al.*, 1991).

3.4 Summary

In summary, results of experiments involving antisense ODN-mediated inhibition of ChIMP-3 were variable. At least one experiment showed good inhibition of ChIMP-3 synthesis, while in others, where some experimental parameters were changed, the effects were marginal. However, no single factor could be unequivocally identified as being responsible for the lack of significant ODN effect. However, the data presented in this chapter

clearly suggests that the ODNs produced a change in location of the ECM-specific inhibitors, ChIMP-3 and ChIMP-a. The mechanism of their release into the CM in a sequence independent but a concentration dependent manner is most likely by electrostatic interactions between the proteins and the nucleic acids. This type of interactions may have far reaching consequences in the ability of antisense ODNs to be specific in their effect, especially in therapeutic applications. The negative charge on unmodified ODNs clearly precludes their use in antisense inhibition of ChIMP-3 for determining its role in oncogenic transformation. One approach to circumvent this problem would be to use modified neutral ODNs, such as methylphosphonates. Clearly, interactions between these ODNs and ChIMP-3 would not be anticipated.

Chapter 4

Interactions of Charged Polymers with TIMPs

4.1 Introduction

Interactions between specific molecules mediate important and complex events in a cell or organism. Common examples of such interactions would include DNA-enzyme binding, RNA-ribozyme interaction, enzyme-substrate interaction, receptor-ligand binding, etc. These interactions involve different or similar classes of molecules such as nucleic acids, proteins, carbohydrates, lipids, etc.. While some of these interactions may be highly specific involving predominantly the 3-dimensional structures of the molecules involved, or the primary sequence, others are dependent on gross characteristics such as charge, charge density and hydrophobicity. Typically, interactions such as protein-DNA binding can be demonstrated directly by experiments such as DNA footprinting, gel retardation and filter binding.

In antisense ODN-mediated inhibition studies (Chapter 3), ChIMP-3 and ChIMP-a, two ECM-specific proteins, were detected in the conditioned media of cultured CEF when these cells were treated with ODNs. This change in location appeared to be sequence independent, since both the antisense ODNs and random control ODNs were capable of eliciting the effect. Furthermore, the amount of ChIMP-3 present in the CM appeared to be directly proportional to the concentration of ODN used. It was hypothesized that the change in location of the inhibitors was due to electrostatic interactions between the negatively charged unmodified ODNs and basic residues of these proteins. To test this hypothesis, several charged and uncharged polymers were used to determine their effect on the extracellular

localization of these inhibitors. It was anticipated that these studies would also provide insight into the molecular interactions that bind these inhibitors so strongly to the matrix.

4.2 Materials and Methods

4.2.1 Treatment of Cultured CEF with Polymers: CEF infected with tsRSV-LA24 were cultured and ECM prepared as described earlier, with the exception of seeding the cells at the tertiary stage in 35-mm culture dishes or 6-well cluster plates (Falcon Labware) in serum-free 2.0.0+ media (Media 199, 2% tryptose phosphate broth, 1 g/L glucose). Cells were allowed to attach and recover over a period of 12-24 hours at 41 °C before treatment. Sterile solutions of polymers, listed in Table 4.1, were made in 2.0.0+ and added to the cells in a 10 to 100 µl volume, unless indicated otherwise. In some experiments, at the time of addition of polymers, the medium was changed (see specific figure legends). Concentrations of these compounds ranged between 0.01 µg/ml and 1000 µg/ml. After 10 hours of treatment at the transformation-permissive temperature of 35 °C the cells were examined by phase-contrast microscopy before the CM was collected and the ECM was prepared. Both CM and ECM samples were analyzed by protease-substrate gels, as described earlier.

4.2.2 Treatment of Sterile ECM with Polymers: Certain polymers, such as poly(lysine), were toxic to the cultured cells and therefore the experimental design was modified to allow preparation of the sterile ECM so that the effect of the various polymers on the binding of ChIMP-3 and ChIMP-a within this matrix could be determined. A disadvantage of using isolated ECM instead of

Table 4.1. List of Polymers Used

Polymers	Molecular Weight	Concentration ^a	
		µg/ml	µM
Poly(acrylic acid)	2,000	0.01 - 1000	0.05 - 500
	5,000	0.01 - 1000	0.002 - 200
	90,000	0.01 - 1000	0.0001 - 10
	450,000	0.01 - 1000	0.000022 - 2.2
	1,000,000	0.25 - 1000	0.00025 - 1
Poly-D-Glutamic acid	2,000 - 15,000	0.01 - 1000	0.0007 - 70
	15,000 - 50,000	0.01 - 1000	0.0002 - 20
	50,000 - 100,000	0.01 - 1000	0.0001 - 10
Poly-L-Glutamic acid	15,000 - 50,000	1 - 100	0.02 - 2
Poly-L-Aspartic acid	5,000 - 15,000	0.01 - 1000	0.0007 - 70
	15,000 - 50,000	0.01 - 1000	0.0002 - 20
Dextran Sulfate	5,000	0.01 - 1000	0.002 - 200
	50,000	0.01 - 1000	0.0002 - 20
	500,000	0.01 - 1000	0.00002 - 2
Heparin Sulfate	6,000 - 25,000	0.01 - 1000	0.00065 - 65
DNA (Salmon sperm) (Calf Thymus)	Sheared - variable	2 - 1000	
	Sheared - variable	2 - 1000	
Hyaluronic Acid	20,000 - 5,000,000	0.1 - 100	0.00002 - 0.02
Hyaluronic Acid (fractionated digest)	10,500 - 11,500	0.0005 - 0.05	0.45 - 45
Poly-L-Lysine	15,000 - 30,000	10 - 1000	0.33 - 33
Poly-L-Arginine	15,000 - 50,000	1 - 100	0.02 - 2
Poly-L-Alanine	10,000 - 25,000	1 - 100	0.04 - 4
Poly-L-Histidine	15,000 - 50,000	1 - 100	0.02 - 2
Chondroitin Sulfate A	5,000 - 50,000	10 - 1000	0.36 - 36
Chondroitin Sulfate B	5,000 - 50,000	10 - 1000	0.36 - 36
Chondroitin Sulfate C	5,000 - 50,000	10 - 1000	0.36 - 36

^a Based on average molecular weight

a cell monolayer would be that potential interactions of these inhibitors with cell surface molecules would be eliminated. Sterile ECM was prepared essentially as described earlier, with the exception of using sterile solutions and aseptic procedures. These compounds were incubated at 35 °C for 24-48 hours with aseptically prepared CEF ECM (prepared on 35-mm culture dishes). The incubation solutions (or "pseudo-CM") were collected and ECM was solubilized in Laemmli sample buffer (without reducing agent), as described earlier. The samples were then analyzed by protease-substrate gels.

4.2.3 Interaction of ChIMP-3 and ChIMP-a with Natural and Synthetic Polymers:

a. Binding to DNA: A gel shift assay was used to determine if the observed change in location of ChIMP-3 or ChIMP-a from the ECM to the CM was mediated by binding to the polymers used. DNA was used as a model polymer, primarily due to the relative ease in radiolabeling DNA and availability of established electrophoretic methods, such as a gel-shift assay, to determine binding. PCR was used to generate a ~500-bp ³⁵S- and 139-bp ³²P-labeled DNA fragments using either ChIMP-3 cDNA or Hm1 muscuranic receptor cDNA as templates, respectively. Primers 3 and 4 (described under section 2.2.1) were used for amplifying the 500-bp fragment, whereas two 35-bp primers were used amplify the 139-bp fragment. After initial denaturation at 94 °C for 5 minutes, each of the 30 PCR cycles consisted of denaturation at 98 °C for 1 second, annealing at 50 °C for 1 minute and extension at 72 °C for 4 minutes. The amplified DNA was used without purification. CEF cultured in 35-mm dishes were incubated with the DNA at final concentration of approximately 1 µg/ml in serum-free 2.0.0+ medium, as described earlier.

CM samples were assayed by protease-substrate gels. The gel-shift assay was performed using a 10% polyacrylamide gel in a low-ionic strength buffer system (1 mM EDTA, 3.3 mM sodium acetate, 6.7 mM Tris (pH 7.5)), according to Strauss and Varshavsky (1984). The untreated radiolabeled DNA fragments were used as controls in the assay. Following electrophoresis, the gel was dried and autoradiographed.

b. Binding of ChIMP-3 and ChIMP-a to Heparin and Poly(lysine):

Experiments were also performed to determine specific binding of ChIMP-3 and ChIMP-a to heparin and poly(lysine). Aseptically prepared ECM from CEF in 100-mm dishes was incubated at 35 °C for 20 hours with 10 ml of a 25% v/v suspension of each of the following: immobilized heparin (Sigma; or Affi-Gel heparin, BioRad, heparin content ≥ 0.6 mg/ml), immobilized poly(lysine) (Sigma), unconjugated agarose beads (BioGel A, BioRad) or agarose powder (GIBCO BRL) as controls. Both heparin and poly(lysine) were immobilized on agarose beads by covalent linkages. All suspensions of agarose and agarose beads were made in serum-free 2.0.0+ media containing 100 units/ml penicillin and 100 μ g/ml streptomycin. Following incubation, the suspensions were collected, the dishes rinsed thoroughly with distilled water to remove residual agarose and the ECM solubilized, as described earlier. A sample (100 μ l) of each suspension was diluted with 900 μ l of CMF-PBS and centrifuged for 15 minutes at 10,000 $\times$ g. The pellet was then rinsed twice with 1 ml of CMF-PBS and the bound proteins were solubilized in Laemmli sample buffer (50 μ l) without a reducing agent. In a third set of experiments to confirm binding of ChIMP-3 and ChIMP-a to heparin, Affi-Gel heparin beads were incubated with CEF during culture at 35 °C for 10 hours. The medium containing the beads was collected. The beads were rinsed and

the bound proteins solubilized as described above. Poly(lysine)-agarose beads were not used during culture due to the potential toxicity of poly(lysine). All CM, ECM and bound-protein samples generated in the experiments described above were analyzed by protease-substrate gels.

4.3 Results and Discussion

Almost all polymers listed in Table 4.1 were used on a weight basis instead of a molar basis because most of them were available as a heterogeneous mix of various molecular weights (the range or the average molecular weight of which were indicated). Molar concentrations presented in Table 4.1 are based on the average molecular weight of the polymer. Several compounds were used in a concentration range as wide as 6 orders of magnitude i.e. 0.01 $\mu\text{g/ml}$ to 1000 $\mu\text{g/ml}$. This not only permitted evaluating effects over wide concentration ranges, but also allowed the molar ranges of the different polymers to overlap and allow comparisons amongst them.

The data presented in Figure 4.1 indicate that the addition of increasing amounts of DNA (salmon sperm) to cultures of transforming CEF causes a gradual appearance of ChIMP-3 and ChIMP-a activities in the CM (upper panel, lanes 1 - 4). A comparison of lane 1 (2 μg DNA/ml medium) with lane 4 (1000 μg DNA/ml medium) clearly shows that whereas at the lower concentration only two activities are apparent (a 70-kDa gelatinase and ChIMP-2 inhibitor) at the higher concentrations two additional inhibitor activities, whose mobilities are identical to those of ChIMP-3 and ChIMP-a in the adjacent lane 5, are also evident. Furthermore, it is clear that in all CM samples (lanes 1 - 4) the 70-kDa gelatinase and ChIMP-2 activities are

identical. However, the activities of the ChIMP-3 and ChIMP-a inhibitors increase with increasing concentration of DNA added to the cultures.

These data confirm those reported in Chapter 3 (Figure 3.4 and 3.5) which first demonstrated the appearance of ChIMP-3 and ChIMP-a in the CM after exposure of cells to small ODNs. In the previous experiment it was not possible to determine if this effect was due to increased synthesis of these inhibitors, which may remain in the medium instead of binding to the ECM (perhaps because their binding sites are saturated), or to the release of previously bound inhibitors from the ECM. The experiments reported in Figure 4.1 (lower panel) clearly support the latter possibility. Analysis of the metalloproteinase inhibitor activities in ECM samples from DNA-treated cultures indicates that there is a decrease in activities of ChIMP-3 and ChIMP-a in the ECM concomitant with an increase in their activities in the CM. It appears that the total inhibitor activity remains constant as a function of DNA concentration and that the decrease in the ECM is inversely related to the increase in the CM. This effect is dependent on the concentration of DNA added to the cells.

Other polyanionic polymers such as poly(acrylic acid), dextran sulfate and heparin sulfate elicited the same effect from transforming CEF (Figure 4.2 - 4.4). Similar data were also obtained from anionic poly(amino acids), such as poly(aspartic acid) and poly(glutamic acid) and glycosaminoglycans such as chondroitin sulfate A, B and C (data not shown). Only one polyanion tested, hyaluronic acid, failed to stimulate the release of ChIMP-3 and ChIMP-a from the ECM (Figure 4.5, lanes 1 - 3). The mere presence of carboxyl groups in hyaluronic acid instead sulfate or phosphate groups does not explain its inability to change the location of the inhibitors since poly(acrylic acid) was effective. However, the carboxyl groups in hyaluronic acid may be sterically

hindered due to the presence of bulky groups in their vicinity, and also due to the large hydrodynamic volume of hyaluronic acid in comparison to poly(acrylic acid) which is composed of smaller rigid groups.

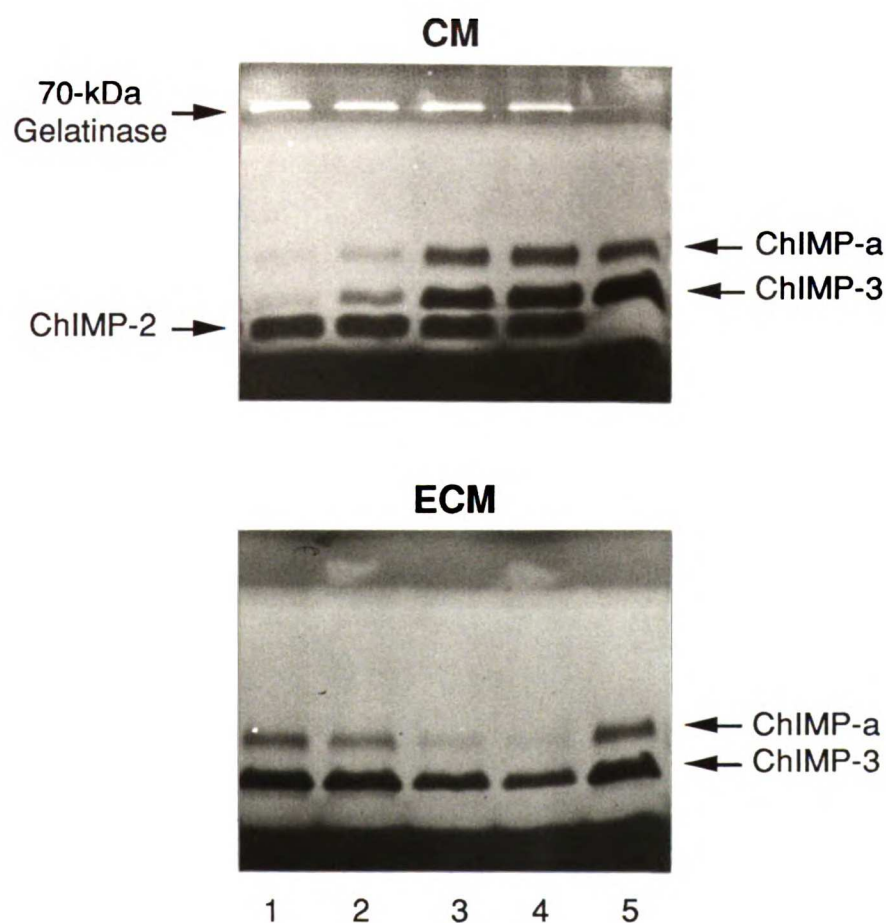


Figure 4.1. Protease-substrate gel analysis of CM (upper panel) and corresponding ECM (lower panel) from CEF treated with salmon sperm DNA. The final concentration of DNA added to the cultures was 2, 20, 200 and 1000 $\mu\text{g/ml}$ (lanes 1-4). Lane 5 in both panels is untreated ECM control. The untreated CM control (not shown) is similar to the sample in lane 1 (upper panel).

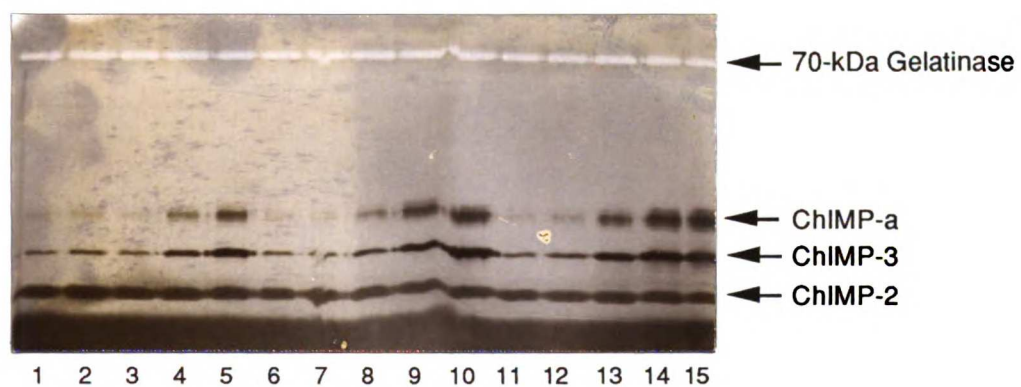


Figure 4.2. Protease-substrate gel analysis of CM from CEF treated with dextran sulfate, average MW 5,000 (lanes 1-5), dextran sulfate, average MW 15,000 (lanes 6-10) and heparin sulfate (lanes 11-15). The final concentrations of dextran sulfate or heparin sulfate added to the cultures were 0.1, 1, 10, 100 and 1000 µg/ml (left to right in each set of five treatments).

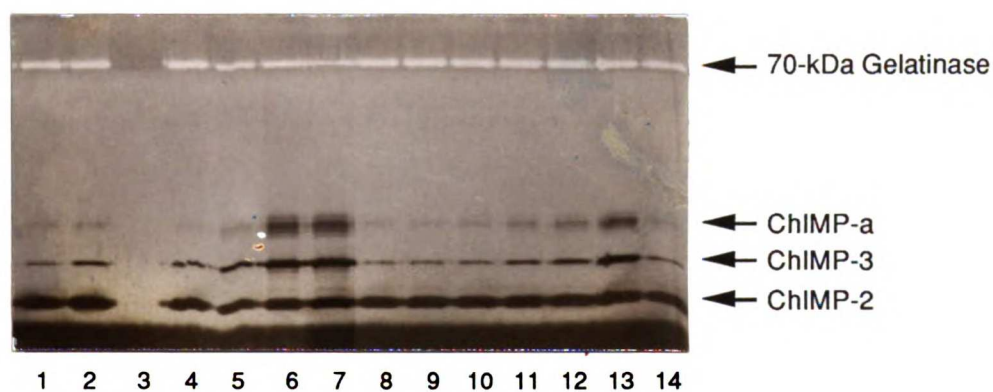


Figure 4.3. Protease-substrate gel analysis of CM from CEF treated with dextran sulfate, average MW 500,000 (lanes 1, 2, 4-7), poly(acrylic acid), average MW 2,000 (lanes 8-13). The final concentrations of dextran sulfate or poly(acrylic acid) added to the cultures were 0.01, 0.1, 1, 10, 100 and 1000 $\mu\text{g/ml}$ (left to right in each set of six treatments). Lane 14 is untreated control CM.

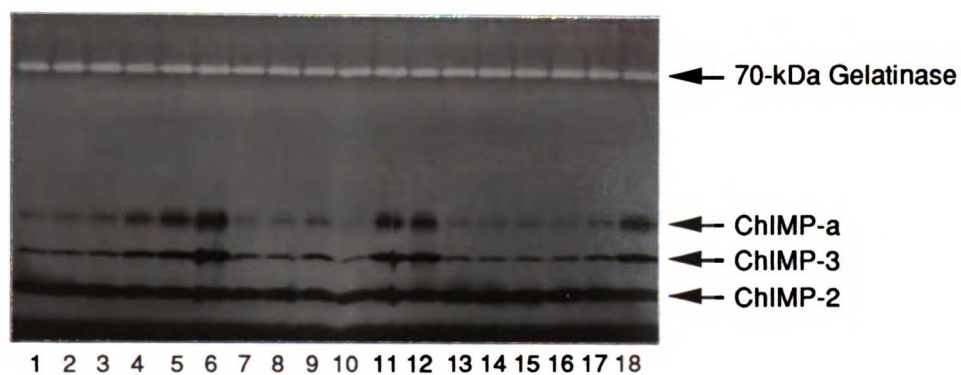


Figure 4.4. Protease-substrate gel analysis of CM from CEF treated with various molecular sizes of poly(acrylic acid): lanes 1-6 (average MW 5,000), lanes 7-12 (average MW 90,000) and lanes 13-18 (average MW 450,000). The final concentrations of poly(acrylic acid) added to the cultures were 0.01, 0.1, 1, 10, 100 and 1000 $\mu\text{g}/\text{ml}$ (left to right in each set of six treatments).

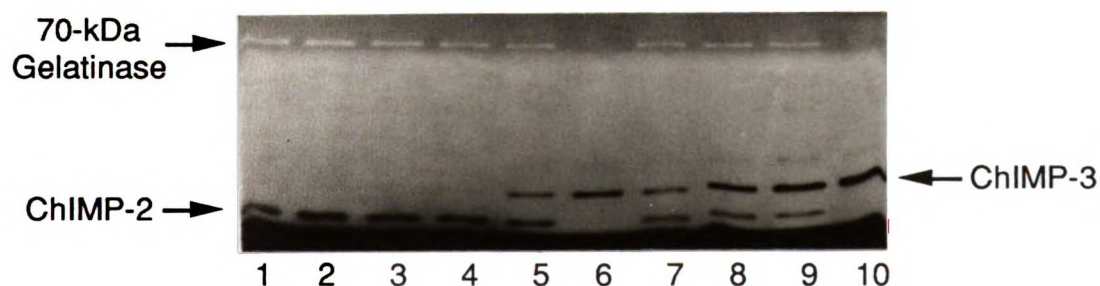


Figure 4.5. Protease-substrate gel analysis of CM from CEF treated with hyaluronic acid (lanes 1-3), poly(lysine) (lanes 4-6) and heparin sulfate (lanes 7-9). The final concentrations of agents added to the cultures were: hyaluronic acid at 0.1, 1 and 10 $\mu\text{g/ml}$, poly(lysine) at 1, 10 and 100 $\mu\text{g/ml}$ and heparin at 10, 100 and 1000 $\mu\text{g/ml}$ (left to right in all lanes). Lane 10 is untreated ECM control. Note the absence of ChIMP-2 and 70-kDa gelatinase, but the presence of ChIMP-3 (lane 6). This may be due to toxicity of poly(lysine) at 100 $\mu\text{g/ml}$ whereby *de novo* synthesis of ChIMP-2 and 70-kDa gelatinase is eliminated, but ChIMP-3 that was deposited in the ECM prior to treatment potentially interacts with poly(lysine) and moves from its normal ECM location to the CM, as discussed under "Results and Discussion".

Cells were also treated with cationic poly(amino acids), such as poly(arginine) and poly(lysine), to examine their effect on the location of ChIMP-3 and ChIMP-a. Unfortunately poly(lysine) was toxic to cells in concentrations of 100 $\mu\text{g/ml}$ and greater as determined by microscopy (cells appeared to be lysed). Also at 100 $\mu\text{g/ml}$ neither ChIMP-2 or the 70-kDa gelatinase (Figure 4.5, lane 6) were synthesized and secreted into the CM, although it appeared that poly(lysine) was not toxic to the cells at 1 or 10 $\mu\text{g/ml}$ (Figure 4.5, lanes 4 and 5). Toxicity of poly(lysine) has been observed by others at similar concentrations in cell culture (McGuire and Russell, 1990). To circumvent the problem of toxicity in cultured cells, experiments were performed in cell-free conditions using ECM that was prepared aseptically and then incubated with media containing the various polyamino acids. Surprisingly, both poly(lysine) and poly(arginine) were able not only to change the location of the inhibitors (Figure 4.6, lanes 1 - 3 and 13 - 15, respectively), but also were more effective than all of the polyanionic compounds tested (Figure 4.6, lanes 4-6, data shown for a representative polyanionic agent, poly(aspartic acid)). A marginal effect was observed with poly(histidine) (Figure 4.6, lanes 7 - 9), but uncharged poly(alanine) did not elicit any change in location (Figure 4.6, lanes 10 - 12). Protease-substrate gels were also used to evaluate the amount of inhibitors remaining behind in the ECM following treatment with the polymers. These gels indicated that as the level of inhibitors increased in the CM or "pseudo CM", there was a concomitant decrease in the inhibitors left behind in the ECM, as seen in Figure 4.7. Also, it appears that the change in location effect seems to be more dramatic for ChIMP-a than for ChIMP-3. For example as seen in Figure 4.2 (lanes 9, 10, 14 and 15), the relative amounts of ChIMP-a and ChIMP-3 appear to be equivalent in the CM of cells treated with either dextran sulfate or

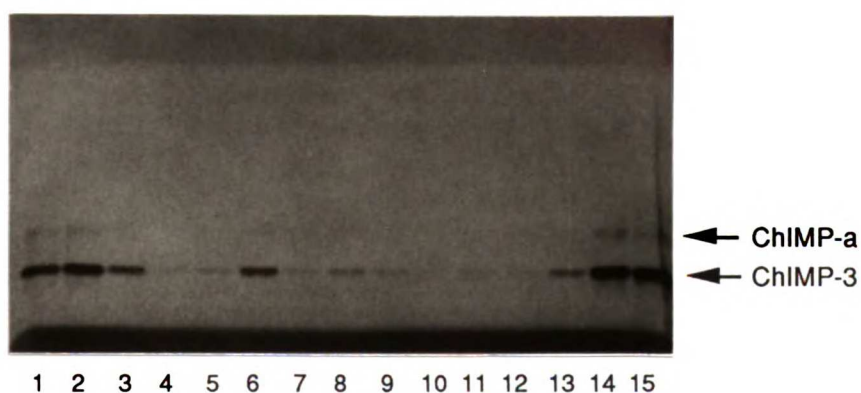


Figure 4.6. Protease-substrate gel analysis of “pseudo CM” from sterile CEF ECM incubated with media containing poly(lysine) (lanes 1-3), poly(aspartic acid) (lanes 4-6), poly(histidine) (lanes 7-9), poly(alanine) (lanes 10-12) or poly(arginine) (lanes 13-15). The final concentration of all agents in media during incubation were 1, 10 and 100 $\mu\text{g}/\text{ml}$ (left to right, except for poly(lysine) in lanes 1 - 3 where the concentrations are 10, 100 and 1 $\mu\text{g}/\text{ml}$, respectively).

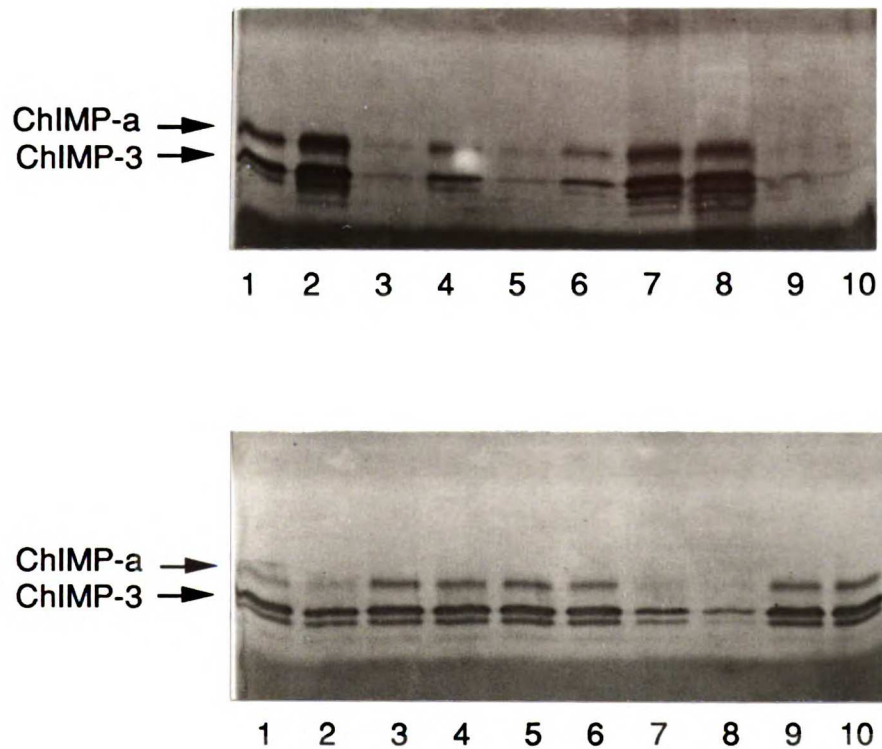


Figure 4.7. Protease-substrate gel analysis of "pseudo CM" (upper panel) and their corresponding ECM (lower panel) from sterile CEF ECM incubated with media containing heparin sulfate (lanes 1, 2), poly-D-glutamic acid, MW 50,000 (lanes 3, 4), poly(acrylic acid), MW 90,000 (lanes 5, 6) or dextran sulfate, MW 50,000 (lanes 7, 8) and media only, control (lanes 9, 10). The final concentration of all agents in media during incubation were 10 and 100 $\mu\text{g/ml}$, from left to right.

heparin sulfate, whereas ChIMP-a is most frequently observed to be the minor inhibitor in the ECM, for example as seen in Figure 4.5, lane 1.

Demonstration of binding of either ChIMP-3 or ChIMP-a to DNA by gel-shift assays was not successful. It may be possible that either no such direct interaction occurs or that the binding affinity is weak enough to be disrupted during electrophoresis. In an alternate approach to examine interactions of a model polyanionic or polycationic compound (heparin or poly(lysine), respectively) with ChIMP-3 and ChIMP-a, agarose beads covalently linked to the polymers were incubated for 20 hours with aseptically prepared ECM from transforming CEF, so that any bound material could be recovered attached to the beads. The beads were collected, rinsed extensively with buffer and the bound proteins were solubilized in Laemmli sample buffer. The solubilized proteins were analyzed by protease-substrate gels, as shown in Figure 4.8A (lanes 1 - 3). Both ChIMP-3 and ChIMP-a are clearly present in the samples obtained from the heparin-agarose beads, indicating that they were bound to heparin (lanes 2 and 3). However, there appeared to be no binding of ChIMP-3 or ChIMP-a to the poly(lysine)-agarose beads (lane 1). Also, when the supernatant fractions obtained after sedimentation of the beads were analyzed by protease-substrate gels, no inhibitors were present. At first, these observations appeared to contradict results obtained with poly(lysine) in solution, where this polymer produced a change in location of the inhibitors from their ECM-specific location into the "pseudo CM". However, it is likely that soluble poly(lysine), unlike heparin, is able to displace the inhibitors from their ECM-binding sites rather than interacting directly with the inhibitors. Conceivably this is due to the fact that poly(lysine) is more cationic than the inhibitors. In contrast, the poly(lysine)-agarose beads cannot elicit a similar response because the poly(lysine) is

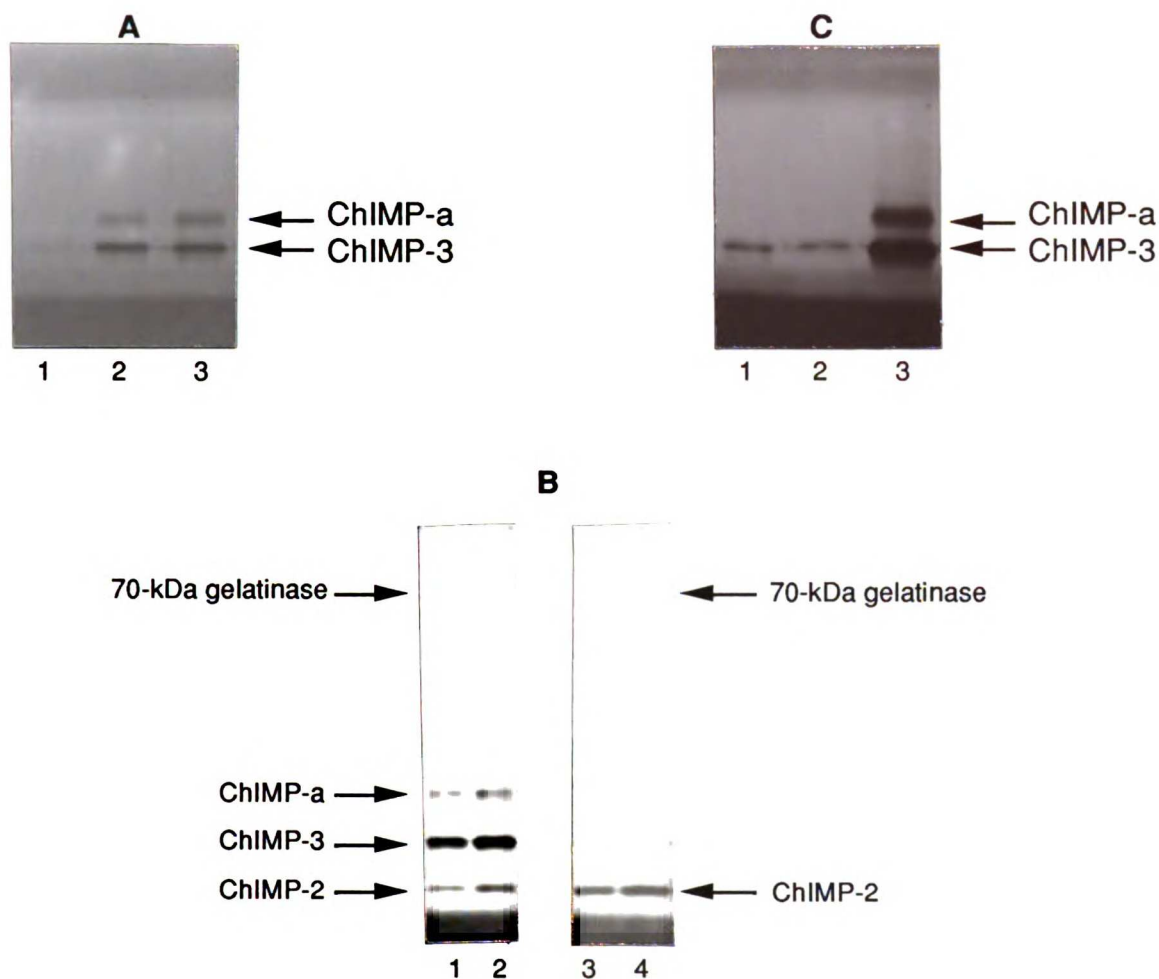


Figure 4.8. Protease-substrate gel analysis of various samples following treatment of transforming CEF or ECM from transforming CEF with heparin-agarose beads, poly(lysine)-agarose beads, unconjugated agarose beads and agarose powder. (A). Proteins solubilized from poly(lysine)-agarose beads (lane 1) and heparin-agarose beads (Sigma, lane 2 and Affi-Gel, Bio-Rad, lane 3) following incubation with CEF ECM. (B). Proteins solubilized from heparin-agarose beads (Affi-Gel, Bio-Rad) following incubation with CEF (lanes 1 and 2, two loadings of same sample). Note the presence of ChIMP-2 and the 70-kDa gelatinase in addition to ChIMP-3 and ChIMP-a, as discussed under "Results and Discussion". Lanes 3 and 4 contain the supernatant media following removal of beads by centrifugation (two loadings of same sample). Note the absence of ChIMP-3 and ChIMP-a which are bound to the immobilized heparin covalently linked to the agarose beads. (C). Lanes 1, 2 and 3 contain proteins solubilized from unconjugated agarose beads (Bio-Gel A, Bio-Rad), agarose powder (GIBCO BRL) and heparin-agarose (Bio-Rad), respectively, following incubation with CEF ECM. Note the presence of small amounts of ChIMP-3 in lanes 1 and 2 as compared to amounts in lane 3, as discussed under "Results and Discussion".

immobilized. The controls consisting of unconjugated agarose beads and agarose powder did not show any significant interaction (discussed below), indicating that the binding of ChIMP-3 and ChIMP-a to heparin was a specific interaction. Hence, it appears that both ChIMP-3 and ChIMP-a display heparin-binding properties like other proteins such as basic fibroblast growth factor (bFGF), antithrombin III (AT-III), etc. (Cardin and Weintraub, 1989). In addition to ChIMP-3 and ChIMP-a, ChIMP-2 (chicken TIMP-2) along with its companion 70-kDa gelatinase (MMP-2) is also present among heparin-agarose bound proteins (when incubations were made with cells), as seen in Figure 4.8B (lanes 1 and 2). This binding of ChIMP-2 and the chicken MMP-2 is not surprising and may have been expected because the basis of purification of TIMP-2 has been heparin affinity chromatography, where TIMP-2 is invariably isolated as a complex with MMP-2 (Goldberg *et al.*, 1989; Stetler-Stevenson *et al.*, 1989; Staskus *et al.*, 1994). The CM samples show the presence of ChIMP-2 and MMP-2 (lanes 3 and 4), suggesting that binding sites on heparin may be saturated. The controls, unconjugated agarose beads and agarose powder, showed an interaction with ChIMP-3 (Figure 4.8C, lanes 1 and 2). However, this interaction is small in comparison to the interaction of ChIMP-3 with heparin-agarose (lane 3) and may be due to the slight anionic nature of agarose resulting from the presence of sulfate groups. It is not uncommon for agarose to contain sulfates to the extent of 0.3% by weight.

The site of the interaction of these polymers with the inhibitors remains to be determined. However, since the change in location is observed with both positively and negatively charged polymers it is conceivable that the sites and/or mechanism of interactions of these compounds are different. A model for these interactions is presented in Figure 4.9. bFGF is a well characterized protein which is sequestered in the ECM much like ChIMP-3

and ChIMP-a (Vlodavsky *et al.*, 1987; Mignatti and Rifkin, 1991). Two binding sites for bFGF exist: a low affinity site where bFGF binds to either cell surface heparin or heparin sulfate proteoglycans and a high affinity bFGF receptor to which it binds following its binding to the low affinity site i.e. a dual receptor system (Folkman and Klagsbrun, 1987). In addition bFGF binds to heparin sulfate proteoglycans (HSPG) in the ECM. In this sequestered state bFGF is stabilized and protected from degradation. The basic nature of ChIMP-3 and interactions of ChIMP-3 and ChIMP-a with heparin sulfate and other negatively charged polymers suggests that these inhibitors could be bound to HSPG in the ECM in a similar fashion to bFGF. Introduction of a very large excess of exogenous polyanion would be expected to shift the equilibrium of the normal physiological inhibitor-HSPG interaction in the ECM in favor of the exogenous polyanions in the "pseudo CM" and hence bring about the change in location of the inhibitors. The ability of polycations to produce a similar effect could be attributed to displacement of the inhibitors from their ECM binding site with HSPG due to the higher net positive charge of the polycations as compared to the inhibitors.

ChIMP-3 possesses stretches of positive charges (only lysine and arginine considered as positively charged amino acids) which are uninterrupted by negative charges. These stretches are similar to those displayed by other proteins which are known to bind to heparin sulfate. Stretches with at least four uninterrupted positive charges were considered only as potential candidates for such electrostatic interactions. While 3 such stretches exist in ChIMP-3, only one such stretch exists in bovine TIMP-2 (R22-K29) as seen in Figure 4.10 (underlined). A similar stretch in the human TIMP-2 sequence, in contrast, contains 3 positively charged residues that are interrupted by an acidic residue. TIMP-2 is known to form a complex with MMP-2 which binds to

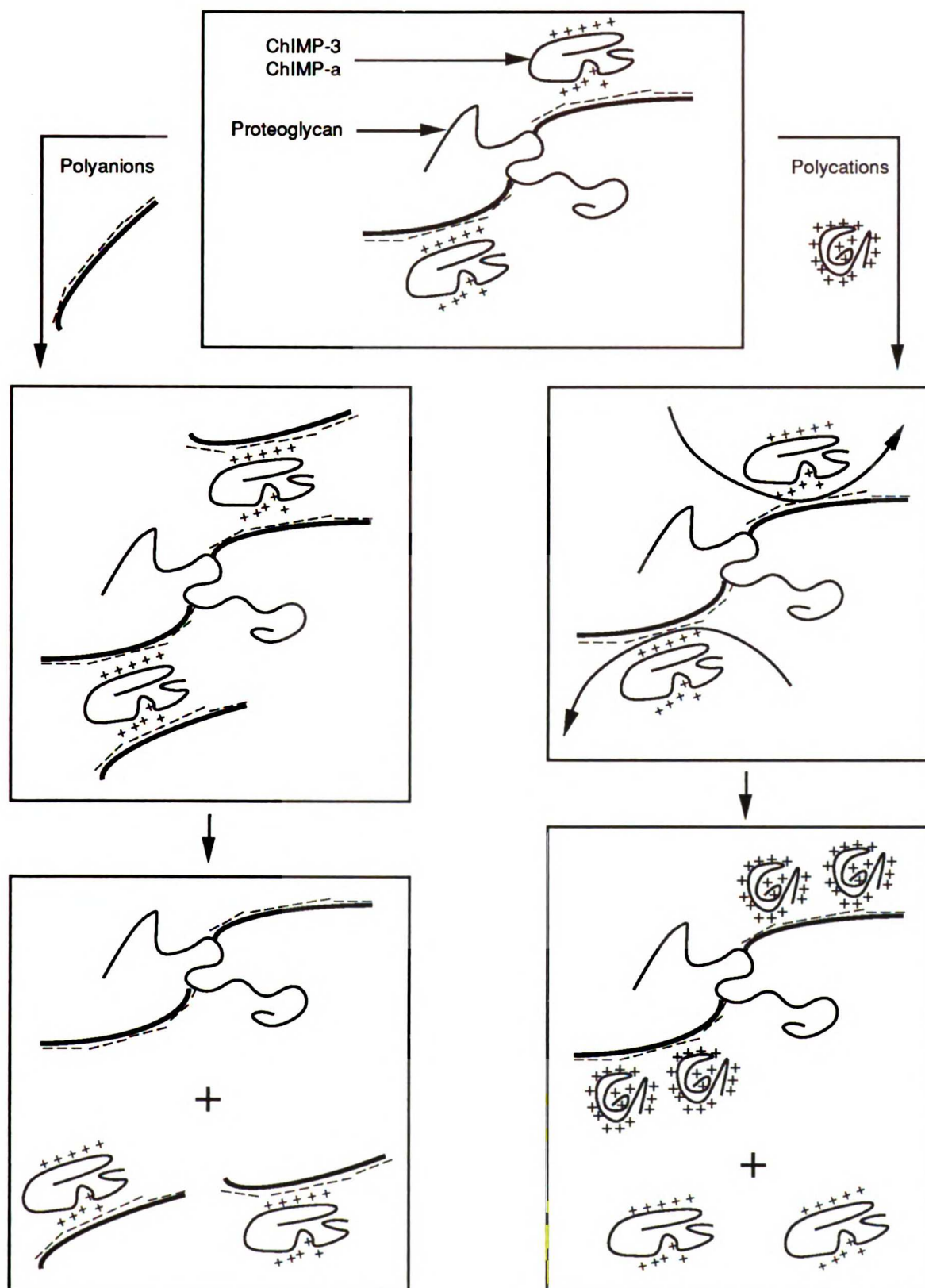


Figure 4.9. A model representing mechanisms of interactions of polymers with ChIMP-3 and ChIMP-a.

hTIMP-1 ^a	---MAPFEPLASGIL LLLWLIAPSRA[CTCVPHPQTAFCN	<u>SDLVI</u>	RAKFV	<u>QTPEVNQ</u>	---
hTIMP-2 ^b	MGAAARTLRlALG1L LLATLLRPaDA[CSCSPVHPQQAFCNADVVI	RAKA	<u>vSEkEVD</u>	SgND	
bTIMP-2 ^c	MGAAARSLPlAFClL LLGTLLRPaDA[CSCSPVHPQQAFCNADIVI	RAKA	<u>vNKkEVD</u>	SgND	
			<u>+</u>	<u>+</u>	<u>++</u>
			R22	-	K29
ChIMP-3 ^d	--MTAWLGFlAVFlC SWSLRDLVaEA[CTCVPiHPQDAFCN	<u>SDIVI</u>	RAKV	<u>vGKkLMKD</u>	g--
			<u>+</u>	<u>+</u>	<u>++</u>
			R20	-	K30
hTIMP-1 ^a	----TTLYQRyEIK- MTKMYKG	<u>FQALGDAADIRFVYTPAMES</u>	<u>SVCG</u>	YFHRSHNRSEEF	LIA
hTIMP-2 ^b	IYGNpIKRIQyEIK- qIKMFKGPE-----KDIEFiYTAPSSAVCG	<u>vSlDVG</u>	-GkKEy	LIA	
bTIMP-2 ^c	IYGNpIKRIQyEIK- qIKMFKGPD-----QDIEFiYTAPAAAVCG	<u>vSlDIG</u>	-GkKEy	LIA	
ChIMP-3 ^d	----pFGTMRYTVK- qMKMYRGE	<u>FQI</u>	---MPHVQYiYTEASE	<u>SLCG</u>	vKlEV--NkYQyLIT
		<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>
		R38	-	R48	
hTIMP-1 ^a	GK-LQDGLLHITTCs FVAPWNSLSLAQRRG FT-KTYTVGCEE	<u>CTV</u>	FPCL	SIPCKLQSG	
hTIMP-2 ^b	GKAEGDGKMHitlCD FIVPwDTlSTQKkS ln-hrYQMGC-E	<u>Cki</u>	TRCPMIP	CYISSP	
bTIMP-2 ^c	GKAEGNGNMHitlCD FIVPwDTlSATQKkS ln-hrYQMGC-E	<u>Cki</u>	TRCPMIP	CYISSP	
ChIMP-3 ^d	GR-VYEGKVYTGlCN WYEKWdRlTlSQrkG ln-hrYHLGC-G	<u>Cki</u>	RPCYYL	PCFATSK	
		<u>+</u>	<u>++</u>	<u>+</u>	<u>+</u>
		R103	-	K137	
hTIMP-1 ^a	THCLWTDQLLQGSEKGF QSR	<u>HLACL</u>	PREPGLC	TWQSLRSQIA-----	207
hTIMP-2 ^b	DeCLWMDWVTEKNI	Ng	QakFFACiKrSDGSC	AWyrgAappKQEF	LD IE
bTIMP-2 ^c	DeCLWMDWVTEKNI	Ng	QakFFACiKrSDGSC	AWyrgAappKQEF	LD IE
ChIMP-3 ^d	NeCIWTDMLS	NFGHSg	h	QakHYACiQrVEGYC	SWyrgWappDKTIIN ATdp 212

Figure 4.10. Comparison of TIMP sequences to determine stretches of at least four positively charged amino acids (underlined) that are not interrupted by acidic residues. Three such stretches are present in the ChIMP-3 sequence, while only one is present in the bovine TIMP-2 sequence. The cysteine residues are in bold.

^a Docherty *et al.*, (1985); Gasson *et al.*, (1985); Carmichael *et al.*, (1986)

^b Boone *et al.*, (1990); Stetler-Stevenson *et al.*, (1990)

^c Boone *et al.*, (1990)

^d Pavloff *et al.*, (1992)

heparin, but the mechanism of this binding is not known. It is conceivable that this stretch may be involved in the binding to heparin, since this region appears to be the common denominator for the ability of TIMP-2 and ChIMP-3 to bind heparin. Alternatively, the binding of TIMP-2:MMP-2 complex to heparin may be occurring by a different mechanism, in which case the other two stretches of positively charged residues of ChIMP-3 may be involved in the binding of ChIMP-3 to heparin and/or its ECM site. In ChIMP-3, two short stretches of 11 amino acids containing 4 and 5 positive residues in each are present close to the N-terminus (R20-K30 and R38-R48), while the longer third stretch of 35 amino acids containing 7 positive residues is closer to the C-terminus (R103-K137). In addition to the size differences between these stretches, a striking difference is the presence of 4 cysteine residues in the longer stretch (C120, C122, C127 and C132) of which C127 and C132 potentially form a disulfide bond (by analogy with the structure of TIMP-1, Williamson *et al.*, 1990). Also 2 additional cysteine residues are present in close proximity at C95 and C140. Together, these cysteine residues could conceivably impose considerable restraint and rigidity in the 3-dimensional structure of this R103-K137 region of the protein. In contrast the two shorter stretches are devoid of cysteine residues and hence may be less restrained to form secondary structures such as alpha-helices.

These two shorter regions (R20-K30 and R38-R48) showed propensity to form alpha-helices when examined by using the Chou-Fasman (CF) and Garnier-Osguthorpe-Robson (GOR) algorithms (Computer Graphics Laboratory, UCSF) which predict secondary structure patterns. When these regions are modeled as alpha-helices using the helical wheel model (Schiffer and Edmundson, 1967), the positively charged amino acids clearly form a high density charged surface while the interspaced uncharged amino acids

form a cluster away from the charged surface (Figures 4.11a and 4.11b). In contrast, the GOR algorithm predicted no propensity for the longer region, R103-K137, to form an alpha helix, while the CF algorithm predicted that only R103-K110 (unconstrained by disulfide bonding) could form an alpha helix. When only the unconstrained region (R103-R115) was modelled as a helical wheel (Figure 4.11c), the basic residues (dark circles) lie scattered around the helix. However, the constraints imposed by the presence of disulfide bonds could conceivably position basic residues present in this region (C120-K137, Figure 4.11c, shaded circles) in close proximity to basic residues in the helical portion. On the other hand, as predicted by the GOR algorithm (absence of a helical structure), this segment may be interacting with anionic polymers by clustering of the basic residues in the tertiary structure of the protein. Three dimensional folding of the protein may bring other distant but critical positively charged amino acids within close proximity of the charged surface to enable its interaction with its target molecule. Evidence of the critical nature and importance of distant amino acids is evident from studies with AT-III (Gandrille *et al.*, 1990) and von Willebrand factor (Sobel *et al.*, 1992) which have indicated that mutations in these amino acids could lead to decreased or no interaction and thereby resulting in a disease state.

Another potential site for interaction of ChIMP-3 could be hyaluronic acid. Several reports have indicated a consensus sequence for interaction of positively charged proteins with hyaluronic acid. However, experiments with both CEF in culture and sterile ECM, indicated no or very weak interaction between ChIMP-3 and exogenously added hyaluronic acid or specific size fractions of hyaluronic acid digests that cause displacement of ChIMP-3 from the matrix. Unpublished data from our laboratory indicate

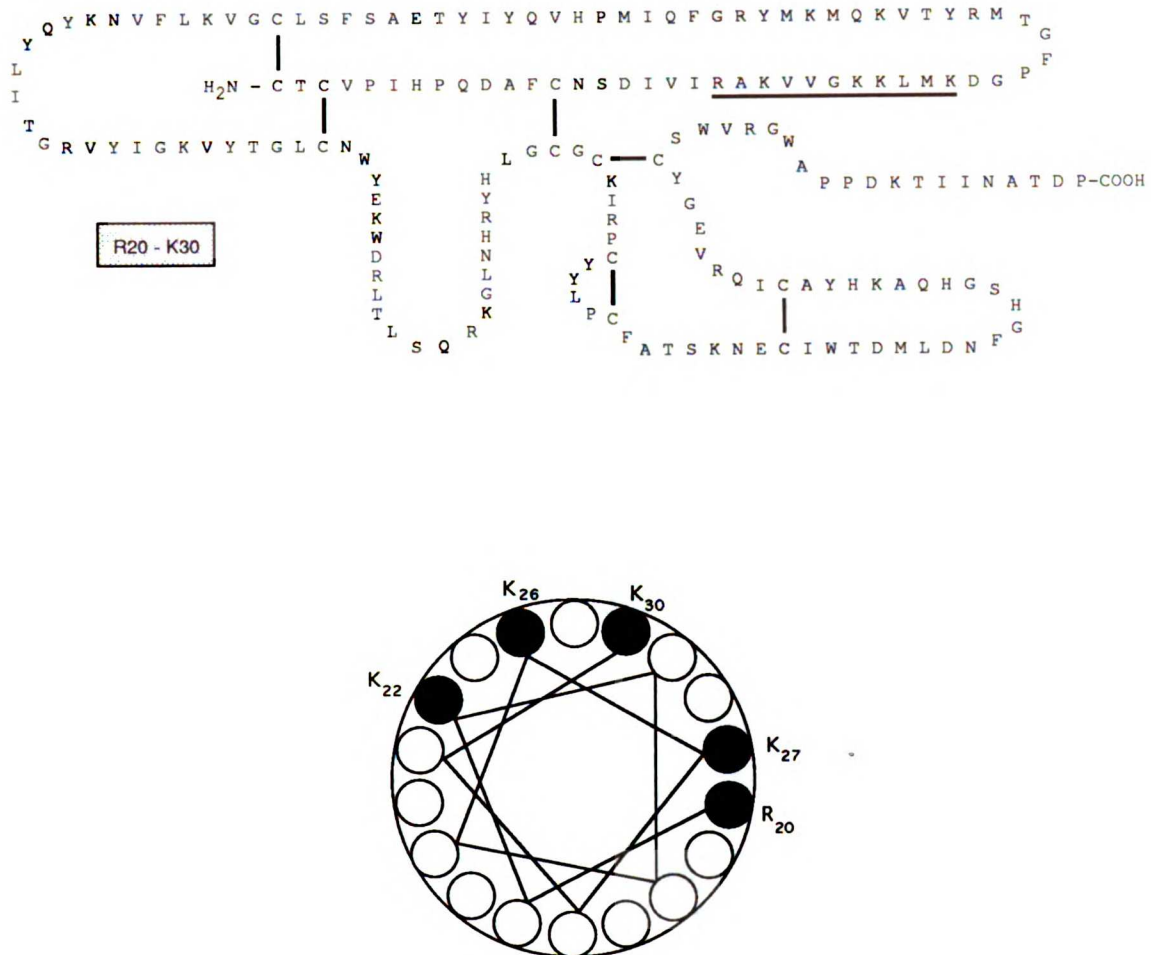


Figure 4.11a. A model of the primary structure of ChIMP-3 and a helical wheel model of a segment, R20-K30. The primary structure is based on disulfide bond assignments made by Williamson *et al.*, 1990 for TIMP-1. The helical wheel model assumes 3.6 residues/helical turn (Schiffer and Edmundson, 1967). Note the clustering of basic residues (dark circles) on one face of the helix.

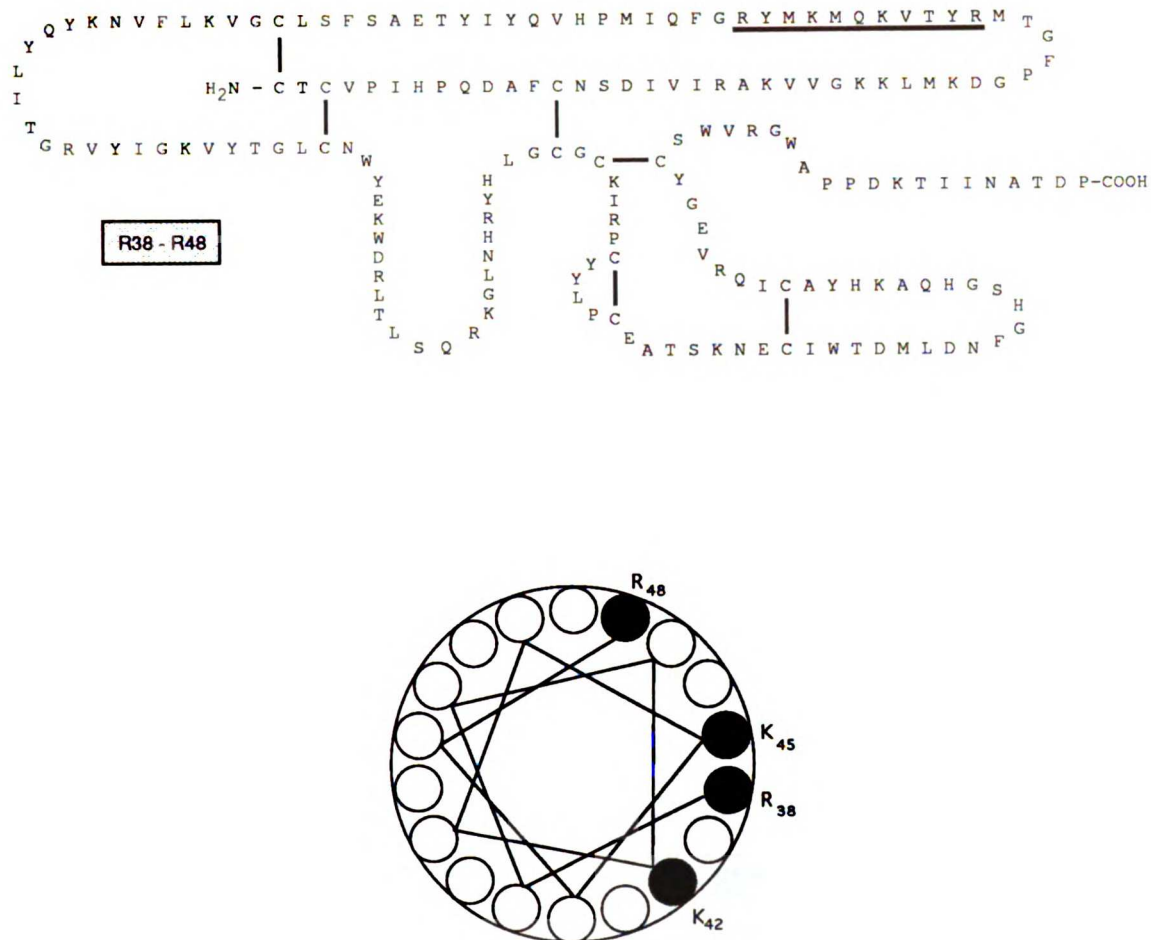


Figure 4.11b. A model of the primary structure of ChIMP-3 and a helical wheel model of a segment, R38-R48. The primary structure is based on disulfide bond assignments made by Williamson *et al.*, 1990 for TIMP-1. The helical wheel model assumes 3.6 residues/helical turn (Schiffer and Edmundson, 1967). Again, note the clustering of basic residues (dark circles) on one face of the helix.

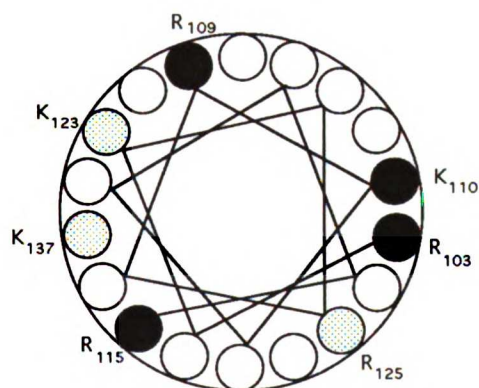
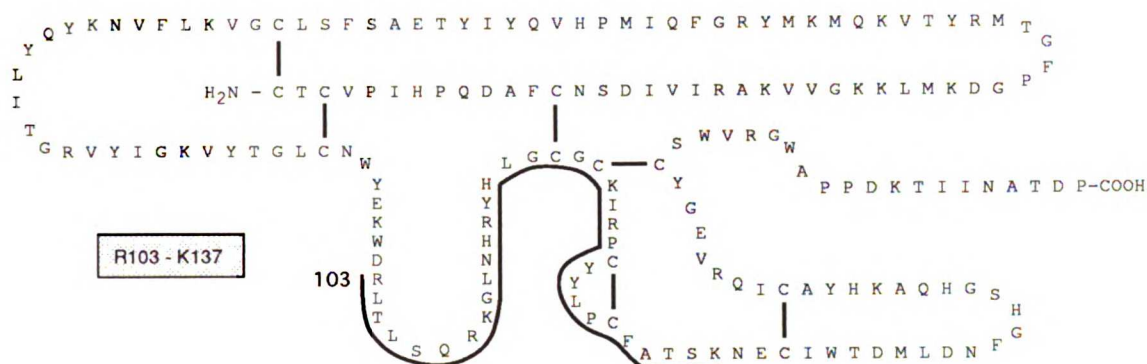


Figure 4.11c. A model of the primary structure of ChIMP-3 (segment R103-K137, underlined) and a helical wheel model of a segment, R103-R115. The primary structure is based on disulfide bond assignments made by Williamson *et al.*, 1990 for TIMP-1. The helical wheel model assumes 3.6 residues/helical turn (Schiffer and Edmundson, 1967). Only a portion of the first half (R103-R115) of the segment R103-K137 has been modeled as an alpha helix because the 3 disulfide bonds in the latter half could conceivably confer rigidity to this region. Although the basic residues modeled as alpha helix (dark circles) are scattered around the helix, in the tertiary structure they could be in close proximity to basic residues present in the remaining portion (shaded circles) of the segment, as discussed under "Results and Discussion".

that hyaluronic acid does bind to ChIMP-3. Tritiated hyaluronic acid isolated from transforming CEF binds to ChIMP-3 on a nitrocellulose membrane blot (Malczewski and Hawkes, unpublished data) and shows saturation binding to immobilized ChIMP-3 (Te-Tuan Yang and Hawkes, unpublished data). Therefore at this point, there is no clear explanation for the lack of effect of hyaluronic acid on ChIMP-3 location apart from the fact that this glycosaminoglycan is itself involved in numerous interactions with other matrix molecules and possibly the inhibitors interact with hyaluronic acid but remain bound within the matrix.

Smaller polymers appear to be more effective in eliciting a change in location of ChIMP-3 and ChIMP-a, i.e. poly(acrylic acid) with average molecular weights of 2,000, 5,000 and 90,000 were more effective than the larger 450,000 molecular weight polymer (Figure 4.3, lanes 8-13 and Figure 4.4). This difference in effect may be due to reduced accessibility of the large polymer to ChIMP-3 and ChIMP-a, that are presumably bound to component(s) of the ECM. This may partly explain the lack of effect with hyaluronic acid: it is an extremely large polymer (up to 5,000 kDa) and it has a large hydrodynamic volume (1000 times its anhydrous volume) which could limit its accessibility to the ECM components, particularly in the presence of cells. However, factors other than accessibility may be involved in the lack of effect by hyaluronic acid because fractionated lower molecular weight (~11,000 kDa) also did not elicit any effect (data not shown). AT-III requires at least a hexasaccharide heparin to achieve optimum binding, although higher molecular weight heparin also binds AT-III (Ishihara *et al.*, 1993). It may be interesting to extend these studies determine the smallest polymer which could effectively produce a change in location of ChIMP-3 and ChIMP-a.

4.4 Summary

Experiments presented in this chapter confirmed the results presented in Chapter 3. The change in location of ChIMP-3 and ChIMP-a from their exclusive ECM location into the conditioned media when treated with oligodeoxynucleotides (chapter 3) was also observed with polyanionic polymers such as salmon sperm DNA, heparin sulfate, dextran sulfate and poly(acrylic acid). Both polyanionic (with the exception of hyaluronic acid) and polycationic polymers, such as poly(lysine), caused the change in location. Interestingly, the polycationic polymers were more effective in eliciting the location change. Both ChIMP-3 and ChIMP-a clearly bound to heparin but not to poly(lysine), indicating that their mechanisms of interaction were different. The polyanions appear to competitively bind to the inhibitors and cause a change in their location, whereas the polycations probably competitively displace the inhibitors from their ECM binding site(s). At least three stretches of basic residues, uninterrupted by negatively charged amino acids, are present in the primary sequence of ChIMP-3. These stretches, either individually or collectively, may be involved in the binding of ChIMP-3 to polyanionic polymers.

Chapter 5

Characterization of Human TIMP-3

5.1 Introduction

Although chicken TIMP-3 (ChIMP-3) has been purified (Staskus *et al.*, 1991), cloned and sequenced (Pavloff *et al.*, 1992), the presence of its counterpart in the human system has not yet been reported. A major aim of this thesis was to identify human TIMP-3 and to purify sufficient quantities of the protein so that it could be characterized. Initial screening of two available cell lines indicated the presence of a matrix-specific metalloproteinase inhibitor ~24 kDa in size which is similar to the molecular size of 21.8 kDa for ChIMP-3. However, the relative amount of the putative human TIMP-3 in these cell lines was several fold lower than the amount of ChIMP-3 synthesized by transforming CEF. Two different approaches were pursued to obtain higher amounts of TIMP-3. These were screening of cell lines to find one that expressed TIMP-3 at higher levels and the stimulation of TIMP-3 synthesis by treatment of cells with certain common modulators such as phorbol myristate acetate, dexamethasone and retinol acetate.

Generally, proteins can be purified by a wide variety of techniques, which may be used either independently or in any number of combinations. These techniques include the various chromatographic methods (HPLC, gel-filtration, ion-exchange, immunoaffinity, etc.), one dimensional or two dimensional gel electrophoresis and immunoprecipitation. In recent years with the advent and rapid advances in automated microsequencing the isolation and purification of proteins to homogeneity is no longer imperative if the only aim is to obtain limited primary sequence information. The primary requirements for successful microsequencing are resolution of the

protein of interest from other closely or comigrating proteins by either 1- or 2-dimensional gel electrophoresis followed by positive identification of the protein of interest after electrophoretic transfer to a suitable membrane.

5.2 Materials and Methods

5.2.1 Cell Culture and Preparation of ECM: All cells were of human origin with the exception of chicken embryo fibroblasts (CEF), which were used as a control. Gingival fibroblasts (GF11 and 1292) were obtained as a gift from Dr. D. Richards at UCSF. The following cells were purchased from American Type Culture Collection: transformed primary embryonal kidney (293), neuroblastoma cells (SK-N-SH), normal whole embryo (FHs 173We), cervix epitheloid carcinoma (HeLa S3), colon adenocarcinoma (Caco-2), ileocecal adenocarcinoma (HCT-8) and fibrosarcoma cells (SW 684 and Hs 913T). All human cells were cultured in 100-mm culture dishes (Falcon Labware) at 37 °C in a humidified atmosphere of 5% CO₂/95% air with the exception of SW 684 cells, which were cultured without CO₂. Cells were cultured in the following media supplemented with 5% fetal bovine serum (FBS): SW 684 in Leibovitz L-15, GF11 and 1292 in MEM-a, FHs 173We and Hs 913T in DMEM with 4.5g/l glucose, 293 in a 50:50 mix of DMEM with 1g/l glucose and F12, SK-N-SH in DMEM with 4.5g/l glucose and without sodium pyruvate, HCT-8 in RPMI 1640, Caco-2 in MEM with non-essential amino acids and HeLa S3 in F12. To eliminate the contribution of serum proteins to the analysis of the ECM, confluent cultures were reseeded 1:2 to 1:3, in their corresponding media without serum, and cultured for 48 hours before harvesting ECM as previously described (Blenis and Hawkes, 1983). Basically the protocol

consisted of aspiration of the media, rinsing the cells with Ca^{2+} -, Mg^{2+} -free phosphate buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 0.1% w/v ϵ -amino caproic acid (buffer P) and then incubating the cells in buffer P containing 5 mM EGTA at 37 °C for at least 15 minutes. The cells were then carefully removed from the dish surface by repeated gentle squirting until all the cells were lifted from the dish surface. Dishes were then rinsed at least twice with buffer P and finally once with ice-cold water. ECM was then solubilized in a small volume, typically 40-60 μl , of Laemmli sample buffer without β -mercaptoethanol. Control CEF were cultured and infected with the temperature-sensitive mutant, LA24, of Rous sarcoma virus as described by Blenis and Hawkes (1983).

5.2.2 Cell Culture on Collagen-Coated Dishes in Serum-Free Media:

Culturing cells in serum-free conditions often compromises their proliferation and their attachment to the culture dish surface. To alleviate these shortcomings, 293 cells were cultured on culture dishes coated with sterile collagen (Vitrogen 100, Collagen Corp., Palo Alto, CA) and the cells were then seeded in serum-free media. Coating of dishes with collagen was achieved by diluting the stock collagen solution (3 mg/ml) to 50 $\mu\text{g}/\text{ml}$ in 12 mM acetic acid and incubating 5 ml of this diluted solution in each 100-mm dish at 37 °C overnight. Before seeding the cells, the coating solution was aspirated and the dish surface was rinsed once with serum-free media.

5.2.3 Serum-Free Cell Culture: In addition to culturing 293 cells on collagen-coated dishes, a serum-substitute was evaluated to allow cells to grow in a more conducive environment. Ultrosor® G (GibcoBRL), a low protein serum-substitute, was used at a final concentration of 1% in serum-free

media. 293 cells were slowly weaned off the routinely used 5% FBS concentration over 2 to 3 passages by using successively decreasing amounts of FBS. The cells were then routinely cultured in serum-free media with 1% Ultrosor®-G.

5.2.4 Protease-Substrate Gel Electrophoresis: Metalloproteinase inhibitory activities in ECM and conditioned media (CM) samples were assayed by electrophoresis on protease-substrate gels essentially as described earlier under section 3.1.6. Molecular weight standards for electrophoresis included phosphorylase B ($M_r = 97,400$), bovine serum albumin ($M_r = 66,200$), ovalbumin ($M_r = 45,000$), carbonic anhydrase ($M_r = 31,000$), soybean trypsin inhibitor ($M_r = 21,500$) and lysozyme ($M_r = 14,400$) and were purchased from Bio-Rad Laboratories, Richmond, CA. The proteins were denatured in 6 M guanidine HCl, reduced with β -mercaptoethanol and acetylated with iodoacetamide to block sulfhydryl groups. The standards were then dialyzed against 62.5 mM Tris-HCl (pH 6.8) and the protein aggregate was dissolved by addition of SDS to a concentration of 10 mg/ml. The reduced and alkylated standards migrated identically to the untreated standards under reducing conditions of electrophoresis, and provided discrete bands under the nonreducing conditions required for protease-substrate gels. These modified molecular weight standards were prepared by Dr. Staskus in our laboratory.

5.2.5 Treatment of FHs 173We ECM with PNGase F: A FHs 173We ECM sample solubilized in Laemmli sample buffer was diluted 5-fold with water to decrease the SDS concentration to approximately 0.4 - 0.5%, as required by the protocol (PNGase F, also referred to as N-glycosidase-F, New England Biolabs Inc., Beverly, MA). NP-40 and sodium phosphate (pH 7.5) were then added

from 10X stock solutions to provide final concentrations of 1% and 50 mM, respectively. The mixture was split in half: to one half 1000 units of PNGase F was added in a volume of 1 μ l, while the other half received 1 μ l water. The samples were incubated at 37 °C for 6 hours and analyzed by protease-substrate gels, as described earlier.

5.2.6 Preparation of Anti-ChIMP-3 Antibodies: Two polyclonal anti-ChIMP-3 antibodies were prepared in our laboratory. The first was raised against the pure protein and the second against a synthetic N-terminal peptide. ChIMP-3 from transforming chicken embryo fibroblasts was purified to homogeneity as described previously (Staskus *et al.*, 1991; Yang and Hawkes, 1992). Polyclonal antibodies were raised in a single New Zealand White rabbit following two injections of ChIMP-3 (20 μ g protein/immunization). A titer of 1:3000, as determined by ELISA using pure ChIMP-3, remained constant for three subsequent bleeds. The antibodies recognize denatured and denatured/reduced ChIMP-3 on Western blots. The anti-ChIMP-3 antiserum detects a single, strong 22-23-kDa band on blots of reduced ECM proteins from transforming CEF. The second antibody was raised against a peptide (synthesized by Dr. Te-Tuan Yang) corresponding to amino acid residues 3 through 13 of ChIMP-3: NH₂-Cys-Val-Pro-Ile-His-Pro-Gln-Asp-Ala-Phe-Cys-COOH. This peptide was synthesized by solid phase methods on polyamide-kieselguhr resin using the Fmoc (fluorenylmethylcarbonyl) group to provide α -amino protection and its sequence was confirmed by the Edman technique. It was coupled to both keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) by use of the heterobifunctional reagent, sulfo-maleimido benzoyl-N-hydroxysuccinimide, which couples free sulfhydryl groups on peptides to free amino groups on the carrier protein. The coupling was

performed under reducing conditions to ensure that the two cysteines in the peptide were reactive. The KLH- and BSA-conjugates were used to immunize New Zealand White rabbits (under contract with the Berkeley Antibody Company). Sera were analyzed by ELISA using the alternate hapten carrier conjugate, in each case, as screening antigen. The KLH-peptide was the most immunogenic and provided antisera with titers in the range of 10,000-12,000. These antibodies recognize both ChIMP-3 and ChIMP-a (another, as yet unidentified, TIMP) on Western blots of ECM proteins from transforming CEF. As the N-termini of this family of proteins are so similar, this result is not surprising. We do not know yet if these anti-peptide antibodies will recognize either "native" ChIMP-3 or ChIMP-a.

5.2.7 Western Blotting and Probing with Antibodies: CM from FHs 173We were dialyzed overnight against 0.01% w/v SDS using Spectra/Por-1 tubing (6,000-8,000 molecular weight cutoff, Spectrum Medical Industries) and then concentrated 150-fold by low-speed centrifugation, under vacuum, in a Speed-Vac (Savant Instruments). The concentrated CM and solubilized ECM from FHs 173We cells were reduced in β -mercaptoethanol (final concentration 5% v/v) and electrophoresed by SDS-PAGE on a 1.5-mm thick, 15% polyacrylamide slab gel. Electrophoretic transfer to PVDF membrane (Immobilon-P™, Millipore) was performed using 10 mM CAPS buffer, pH 11.0 containing methanol (10% v/v) (Matsudaira, 1987). Transfer was accomplished at 300 mA for 2 h. Non-specific binding sites on the membrane were blocked with 1% w/v nonfat dry milk powder (Carnation) in TBS (10 mM Tris-HCl, pH 7.5; 150 mM NaCl) at 4 °C overnight. The blot was then incubated with the primary antibodies (diluted 1:500 in TBS containing 1% w/v BSA and 0.02% w/v sodium azide) either overnight at 4 °C or at room

temperature for at least 8 h. All primary antibodies were polyclonal and prepared in rabbits using purified TIMP-1, TIMP-2, ChIMP-3 or a synthetic N-terminal peptide of ChIMP-3 (above). Antisera against TIMP-1 and TIMP-2 were obtained from Drs. Stanley Zucker and William Stetler-Stevenson, respectively. The blots were rinsed extensively in the following sequence: one rinse with TBS, one rinse with TBS containing 0.1% v/v Tween 20 and 0.5 M NaCl, two rinses with TBS with 0.1% v/v Tween 20 and two rinses with TBS. The blots were then incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories) (diluted 1:3000 in TBS containing 1% w/v BSA and 0.02% w/v sodium azide) at room temperature for 2-4 h and rinsed extensively as described above. The blots were developed using a substrate kit for alkaline phosphatase (Bio-Rad Laboratories).

5.2.8 Determination of Intra-Molecular Disulfide Bonding: Confluent FHs 173We cultures were subcultivated 1:4 in serum-free DMEM with 4.5 g/L glucose in presence of 40 μ Ci cell-labeling grade [35 S]methionine (Amersham) per 100-mm dish for 48 h and the ECM was harvested as described earlier. The labeled ECM was electrophoresed on a 12%-polyacrylamide protease-substrate slab gel either in presence or absence of 5% v/v β -mercaptoethanol (final concentration) in adjacent lanes and processed as described under section 3.1.6. After staining, the gel was soaked for 15 minutes in an autoradiographic signal enhancer solution (EnlighteningTM, DuPont), dried and exposed to X-ray film (Kodak, XAR-2) at -70 $^{\circ}$ C. ECM samples were also electrophoresed on SDS-15%-polyacrylamide prepared according to Laemmli (1970). The proteins were transferred electrophoretically to PVDF membrane and immunostained, as described earlier.

5.2.9 TPCK-Trypsin Treatment of FHs 173We ECM: ECM was prepared from ~90% confluent FHs 173We cells, as described above until the ice-cold water rinsing step. Following the water rinse, 4 ml of TPCK-trypsin (Cooper Biomedical) in 1 x TD (5 units/ml) was added and incubated for 15 minutes at 37 °C. The trypsin solution was aspirated and the dishes rinsed at least three times with buffer P and twice with ice-cold water. ECM was solubilized in Laemmli buffer without β -mercaptoethanol. Control ECM with no trypsin treatment was made simultaneously. The ECM samples were analyzed by protease-substrate gels and by Western blotting.

5.2.10 SDS-PAGE Based Purification Strategies: The purification of ChIMP-3 to homogeneity by SDS-PAGE (Staskus *et al.*, 1991) was aided by both a significant level of expression of this protein and a sufficient amount of protein present after electrophoresis to be visualized by use of the positively charged detergent cetyltrimethylammonium bromide (Staskus *et al.*, 1991; Yang and Hawkes, 1992). This is a staining procedure where certain proteins (such as ChIMP-3) appear as clear bands in an opaque background when visualized under oblique light. However a similar approach to locate the human TIMP-3 on SDS-PAGE gels was not successful, probably due to smaller amounts of the protein in the samples being analyzed. Hence other methods for locating and purifying TIMP-3 were evaluated.

Several precautions, as suggested by Hunkapiller *et al.* (1983), were taken during all purification procedures to avoid N-terminal blockage of proteins. All SDS-polyacrylamide gels used were made with fresh reagents (Bio-Rad Laboratories) and freshly made solutions. The separating gels were overlaid with the separating gel buffer within 2 hours following polymerization and were aged at room temperature for periods ranging

between 24 to 48 hours. Gels were often used within 4 hours of pouring the stacking gels. Sodium thioglycolate was used as a free radical scavenger in the electrophoresis cathode buffer at final concentrations of either 0.1 mM or 1 mM (except in protease-substrate gels). Electrophoretic transfer of proteins was essentially performed as described earlier for immunoblotting using 10 mM CAPS buffer (pH 11.0 containing 10% v/v methanol), with the exception of using ProBlott™ PVDF membrane (Applied Biosystems, Foster City CA) instead of Immobilon-P in some experiments (where indicated) and transferred for a longer time (12 hours at 240 mA instead of 2 hours at 300 mA). Proteins transferred to PVDF membranes, when stained, were incubated with Coomassie Blue R-250 (final concentration of 0.1%) in acetic acid/methanol/water (1:50:49) for 1 minute. The blot was then destained in methanol/water (50:50), rinsed extensively with water, dried and stored at -20 °C before being subjected to sequencing. All N-terminal microsequencing procedures were performed using an Applied Biosystems Model 473A protein sequencer which utilized standard chemistry and programs (courtesy of Dr. F. Masiarz, Chiron Corp., Emeryville, CA).

5.2.10.1 Modified Protease-Substrate Gels: Protease-substrate gels were considered as a means to unambiguously identify and accurately locate the inhibitors. Briefly, a narrow gel slice from a protease-substrate gel was developed and stained and used to locate TIMP-3 on the untreated remaining part of the gel. Proteins were then eluted from the excised gel slices and re-electrophoresed on a SDS-polyacrylamide gel followed by electrophoretic transfer to PVDF (details below). Routinely, protease-substrate gels are only 0.75-mm thick to facilitate quicker exchange of SDS in the gel with Triton X-100 and to allow optimal development to detect inhibitors. However, in

order to purify significant amounts of TIMP-3, thicker gels of 1.5 mm were evaluated to permit loading of sufficient amounts of ECM samples. Also, gelatin, which is used as a substrate in these protease-substrate gels at a final concentration of 1 mg/ml, is present in the eluted samples at high enough concentrations to potentially cause distortions in subsequent gel electrophoreses. Hence, efforts were directed to modify the protease-substrate gels for use with thicker 1.5 mm-gels which contained reduced amounts of gelatin. These modified gels contained gelatin at either 0.2 mg/ml or 0.05 mg/ml which correspond to one-fifth and one-twentieth of the original amount of gelatin. Also development of these gels was monitored at 2.5, 5, 8, 11 and 33 hours post-incubation (instead of overnight), since reduced amounts of gelatin represent smaller amounts of substrate for the same amount of gelatinase to degrade. Based on data obtained from initial experiments, an incubation time of 5 hours and 1.5 mm protease-substrate gels containing 0.05 mg/ml gelatin were chosen for subsequent use. Gel slices were excised from the unstained portion of the gel based on migration of the inhibitor in the stained portion of the gel. The proteins were electrophoretically eluted in a commercial elution apparatus (Elutrap™, Schleicher and Schuell) using 25 mM Tris, 192 mM glycine, 0.1% SDS as the elution buffer. The proteins were precipitated with methanol (final concentration 90% v/v) at -20 °C overnight. The proteins were collected by centrifugation (Sorvall, RC-6) at 10,000 × g at 4 °C and solubilized in Laemmli sample buffer. Samples were electrophoresed and transferred to PVDF (as described under section 5.2.7). A section of blot was immunostained (as described above) while the remainder was stained with Coomassie Blue R-250. The immunostained section was aligned with the Coomassie stained

section to locate the inhibitor. The stained band was excised and subjected to automated microsequencing.

An additional modification to this approach for purification of the human TIMP-3 was to use similar protease-substrate gels, but a different gel-buffer system because some studies have indicated advantages (mainly decreased N-terminal blockage of proteins) of using gels and electrophoretic buffers that are close to neutral pH instead of alkaline conditions of the Laemmli system (Moos *et al.*, 1988). Protease-substrate gels were cast using the near neutral pH buffer system suggested by Jovin (Jovin, 1973c; Jovin, 1973a; Jovin, 1973b). In this system the gel buffer contains bisTris, pH 6.61 while the upper cathode and lower anode electrophoresis buffers contain TES, bisTris, 0.1% SDS (pH 7.25) and bisTris (pH 5.90) respectively. Following electrophoresis, all further steps were as described earlier for protease-substrate gels cast using the Laemmli system, including elution, re-electrophoresis and transfer to PVDF for microsequencing.

5.2.10.2 Transfer of Size Fractionated ECM to PVDF Membranes: In a slightly different approach, FHs 173We ECM samples treated with 5% v/v β -mercaptoethanol were electrophoresed on a 3-mm thick preparative SDS-15% polyacrylamide slab gel prepared according to Laemmli (1970) (ECM from 12 dishes per 8.5-mm wide lane) and a wide gel band encompassing molecular sizes of ~20- to 30-kDa was excised. Proteins were eluted by incubation of crushed gel slices in 50 mM ammonium bicarbonate, 0.5% SDS and 5 mM DTT at 37 °C for ~24 hours. Proteins were concentrated using ultrafiltration devices with 10K molecular weight cutoff (Filtron) and diafiltered by 3 rinses of water to facilitate removal of SDS and salts. The retained fraction was then re-electrophoresed and transferred to PVDF. TIMP-3 was identified by

immunostaining a section of the blot, as described above, while the remainder of the blot was stained with Coomassie Blue R-250 and aligned with the immunoblot to identify TIMP-3. The protein band on the membrane was excised and subjected to N-terminal microsequencing.

5.2.10.3 Transfer of All ECM Proteins to PVDF Membranes: N-terminal sequencing of samples from the modified protease-substrate gels and from size fractionated ECM were unsuccessful. This was probably due to N-terminal blockage of the protein since protein samples were visible after staining with Coomassie. Usually, staining of proteins with Coomassie is an indication that the amount of protein is sufficient for N-terminal sequencing. In light of the low abundance of the human TIMP-3 in the ECM samples, both in terms of absolute amounts and in comparison to ChIMP-3, electrophoretic transfer of either total ECM proteins or partially purified TIMP-3 to PVDF was considered as an alternative to purification of the protein to homogeneity for the sole purpose of obtaining protein for N-terminal sequencing. Such an approach would involve fewer manipulations of the protein, which may be desirable to prevent possible N-terminal blockage of the proteins and other detrimental processes such as degradation, aggregation, adsorptive losses, etc.. Also, in the approach to transfer all ECM proteins to PVDF, ECM was prepared from FHs 173We cells that were seeded at a 2-fold higher density (1.25×10^6 cells/100-mm dish, density of confluent cultures) than routine cultures to maximize the amount of TIMP-3 present (although the relative amount of inhibitor, i.e. ChIMP-3, deposited in the ECM may decrease at higher cell densities, Blenis and Hawkes, 1983).

In the approach of direct transfer of all ECM proteins to PVDF, ECM prepared from 45 culture dishes (100 mm) of FHs 173We cells was solubilized

in Laemmli sample buffer containing β -mercaptoethanol (final concentration 5% v/v) and electrophoresed in three lanes (width 8.5 mm) through a preparative SDS-polyacrylamide gel (12% acrylamide, 3-mm thick) using the gel/buffer system described by Laemmli (1970). Following electrophoresis, proteins were transferred to PVDF membrane (ProBlott™, Applied Biosystems) in 10 mM CAPS buffer, pH 11.0 containing methanol (10% v/v). A section of the blot containing a small aliquot of ECM from FHs 173We cells was excised and probed with the polyclonal antibody to the ChIMP-3 peptide, as described above, to locate TIMP-3. The antibody-probed blot was then aligned with the Coomassie-stained blot and used to locate the TIMP-3 bands. These were excised and half of the sample was subjected to N-terminal microsequencing and the remainder reserved for amino acid composition analysis using the Pico-Tag method (Waters Associates) (Bidlemeier *et al.*, 1984).

5.3 Results and Discussion

Initial experiments with two human cell lines (embryonic kidney and neuroblastoma) indicated that the human TIMP-3 represents a relatively small percentage of ECM proteins in contrast to ChIMP-3, which is a major component of the ECM of chicken embryo fibroblasts during the early stages of transformation. As a result, it was difficult to identify the human TIMP-3 among other proteins on polyacrylamide gels stained with silver or Coomassie Blue, or autoradiograms of [³⁵S]methionine-labeled proteins separated by SDS-PAGE (data not shown). To date, the only assays that have allowed identification of the protein are protease-substrate gel electrophoresis and Western blot analysis using anti-ChIMP-3 antibodies as probes.

Preliminary experiments were directed at determining the stimulatory effects, if any, of TPA, dexamethasone and retinol acetate on TIMP-3 synthesis in human fibroblasts (1292 and Hs 913T). None of these compounds was able to stimulate TIMP-3 synthesis significantly (data not shown). Hence, a variety of cultured human cells were screened by protease-substrate gel electrophoresis to identify cells that expressed TIMP-3 at a high level. All cells were seeded and cultured in serum-free conditions for 48 hours to eliminate any possible exogenous contribution of metalloproteinase inhibitors from serum. Protease-substrate gel analysis of fetal bovine serum (FBS) revealed not only the presence of substantial amounts of TIMP-1 and TIMP-2 (molecular sizes of ~29 and ~21 kDa, respectively) as expected, but also showed small amounts of an inhibitor at a molecular weight of ~24 kDa (Figure 5.1, lanes 1, 2), which is similar to the expected size of the human TIMP-3 (Figure 5.1, lanes 3 - 5 and 8). If human cells were cultured in 100-mm dishes under routine conditions with 10 ml of media containing 5% FBS, the cell monolayer would be exposed to 500 μ l of serum. Typical analysis of the ECM from cultured cells by protease-substrate gels involves electrophoresis of one one-thousandth of the material isolated from a single 100-mm culture dish. Hence, a volume of 0.5 μ l of FBS would represent the maximum amount of inhibitors that could be present in the ECM of such a sample, if it was assumed that all of the inhibitors from the serum were sequestered and deposited into the ECM. As shown in Figure 5.1, this inhibitory activity (at ~24 kDa) in 0.5 μ l FBS (lane 2) appears as approximately 50% of the activity seen when ECM corresponding to one-thousandth of a dish is loaded from cultured human cells (lane 5); i.e. this inhibitor from the serum would be a major contaminant, if it was assumed that the entire amount present the serum was being sequestered and deposited in the ECM. Due to the close

similarity in its molecular size to chicken TIMP-3 (21.8-kDa), the 24-kDa inhibitor could possibly be bovine TIMP-3 and may be a contaminant released from tissue debris during sera collection. Hence, on the basis of this preliminary experiment it was considered crucial to eliminate the use of serum in the culture of human cells in order to rule out any potential contamination of human TIMP-3 with the bovine counterpart. In addition,

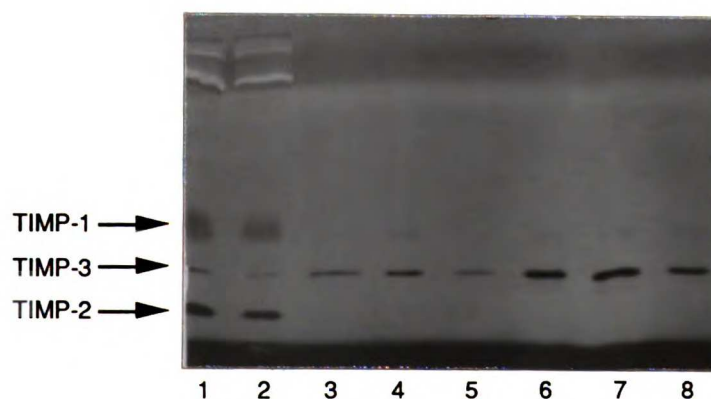


Figure 5.1. Protease-substrate gel analysis of fetal bovine serum and ECM from human and chicken cells. Lanes 1 and 2 contain 1 μ l and 0.5 μ l of fetal bovine serum. Lanes 3, 4, 5 and 8 contain ECM corresponding to one-five hundredth of a 100-mm culture dish from human FHs 173We cells cultured independently at different times. Lane 6 contains similar ECM amount from CEF, whereas samples loaded in lanes 6 and 8 were combined and loaded in lane 7. Note the human TIMP-3 in lanes 3-5 is slightly larger in size than ChIMP-3 in lane 6, although they are indistinguishable when loaded together in the same lane (lane 7).

human cells (e.g. 293) cultured on collagen-coated dishes in serum-free media or in serum-free media supplemented with serum-substitute Ultrosar-G showed improved attachment and spreading of cells in comparison to cells cultured in serum-free media. However, there appeared to be no significant difference in the amounts of inhibitors produced and deposited in the ECM (data not shown). Hence, all cells were routinely cultured in serum-free media.

Figure 5.2 shows protease-substrate gel analysis of the ECM of a representative sample of human cells screened in this study. The amount of ECM loaded per lane (lanes 4-11) for each cell type was normalized to the number of cells (4000 or 8000 cells, in odd and even numbered lanes, respectively) present on the dish at the time of isolating ECM. ECM from 40,000 cells was loaded for CEF (lane 12). As seen in Figure 5.2, both transformed and normal human cells express and deposit at least 3 metalloproteinase inhibitors in the ECM: a major inhibitor (~24-25 kDa) and two minor inhibitors (~29 and ~30 kDa). The major inhibitor is distinctly ECM-specific and is not present in the conditioned media from the same cells. TIMP-1 and TIMP-2 (molecular weights of ~29 and ~21 kDa, respectively) however are clearly present in the conditioned medium from FHs 173We cells (Figure 5.2, lanes 2 and 3). Based on the data that follow, it appears that the 24-kDa inhibitor is the human TIMP-3.

The protease-substrate gel (Figure 5.2) also indicates that the amount of metalloproteinase inhibitory activity that can be attributed to TIMP-3 produced by different human cell lines varies considerably (e.g., Figure 5.2, lanes 4 and 5 compared to lanes 8 and 9). In general, the amount of TIMP-3 activity expressed by human cells is considerably smaller than that produced by transforming chicken embryo fibroblasts. FHs 173We and 293 cell lines are

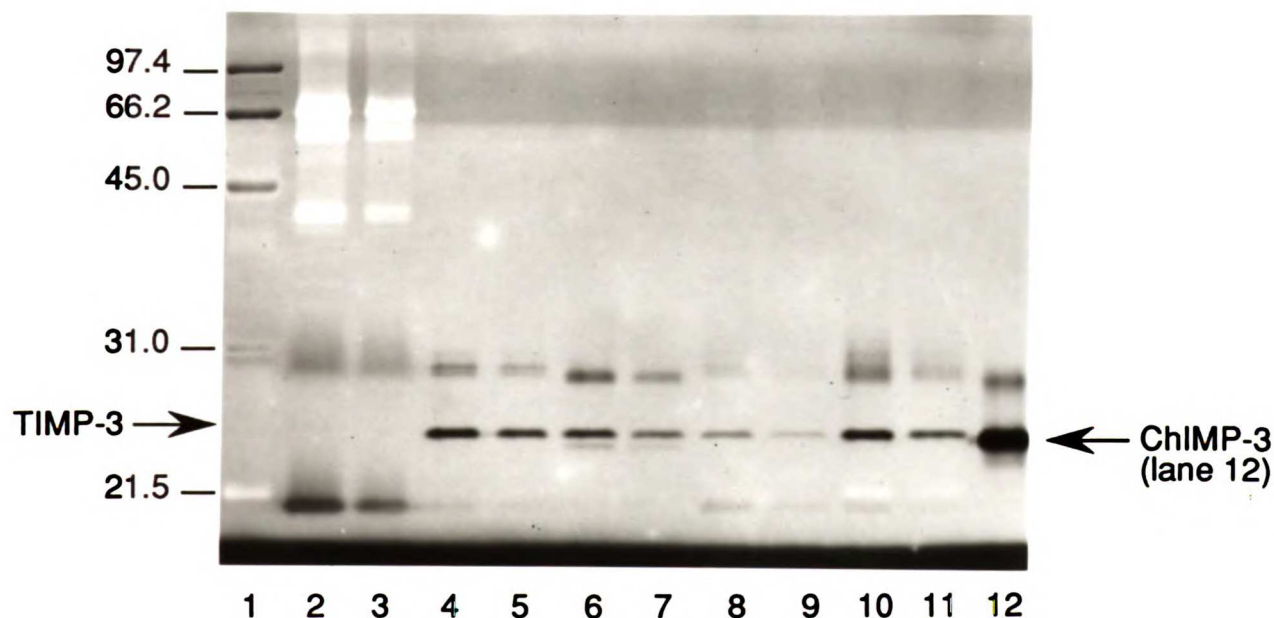


Figure 5.2. Protease-substrate gel analysis of human and chicken ECM. Lane 1 contains BioRad low range molecular weight protein standards: phosphorylase *b* ($M_r = 97,400$), bovine serum albumin ($M_r = 66,200$), ovalbumin ($M_r = 45,000$), carbonic anhydrase ($M_r = 31,000$) and soybean trypsin inhibitor ($M_r = 21,500$); lanes 2 and 3 are concentrated FHs 173We CM equivalent to 300 ml and 150 ml, respectively; lane 12 is CEF ECM. All other lanes contain two loadings of human ECM, as described under "Materials and Methods". Lanes 4 and 5 are FHs 173We; lanes 6 and 7 are Hs 913 T; lanes 8 and 9 are SK-N-SH and lanes 10 and 11 are 293 cells. The additional band immediately below the TIMP-3 band in lanes 6 and 7 is not consistently observed.

two exceptions and produce comparable amounts of TIMP-3, as determined by protease-substrate gel electrophoresis. This is substantiated by preliminary estimates for the amounts of TIMP-3 produced by LA24-RSV-infected CEF and FHs 173We cells of approximately 40 fg/cell and 54 fg/cell, respectively. The calculations were based on data from amino acid analyses. Although the values are very similar, it should be pointed out that the isolation techniques used to recover the TIMP-3 from the chicken and human cells were different. Therefore, a direct comparison of the two may not be reliable. The data for the LA24-RSV-infected CEF were generated by analysis of protein purified as described earlier by Staskus *et al.*, (1991). Another factor which influences the amount of TIMP-3 that can be recovered from the cell cultures is the surface area occupied by individual cells. The FHs 173We cells are approximately 8-fold larger than chicken embryo fibroblasts and thus the amount of TIMP-3 deposited per unit area of culture dish is correspondingly lower for the human cells than for the transforming chicken cells.

With reference to the amount of TIMP-3 produced by various cells, the embryonic origin of the FHs 173We and 293 cells may well be significant. Indeed, we have observed high levels of expression of human TIMP-3 mRNA extracted from various fetal tissues (Pavloff and Hawkes, unpublished data). Furthermore, TIMP-3 appears to be developmentally regulated in mouse embryos (Apte *et al.*, 1994a), although it is also expressed in some adult tissues (Leco *et al.*, 1994). Another cell line that produces comparable amounts of TIMP-3 is the fibrosarcoma, Hs 913T. Again, the fibroblastic origin of these cells and their tumorigenic properties may be related to this observation.

One interesting feature of mammalian TIMP-3 is that its electrophoretic mobility in protease-substrate gels is slower than that of

chicken TIMP-3, despite the fact that ChIMP-3 is expected to be slightly larger. Assuming no post-translational modifications, the deduced molecular size of ChIMP-3 is 21.8 kDa whereas both mouse (Leco *et al.*, 1994) and human (Apte *et al.*, 1994b) are 21.7 kDa. However, both mouse (from Balb 3T3 cells) and human TIMP-3 consistently migrate with identical mobilities (data not shown) and behind ChIMP-3 in the protease-substrate gels (as shown for human TIMP-3 in Figure 5.2). From these data, the apparent sizes of human and chicken TIMP-3 are 24.8 kDa and 24.1 kDa, respectively. Thus both mammalian TIMP-3 activities can be attributed to proteins whose electrophoretic mobilities are retarded in comparison to the chicken protein. The reason for this aberrant behavior is unknown, but may be due to slightly different tertiary structures, to post-translational modifications or to interactions with conditioned media components of the protease-substrate gel during electrophoresis.

The two minor inhibitors, IMP-a and IMP-b, are most clearly seen in the ECM of FHs 173We cells (Figure 5.2, lanes 4 and 5). These inhibitors are not observed as consistently as TIMP-3 and migrate as bands that are either smaller (lanes 6 and 7) or larger (lane 10), by ~1-2 kDa, than the most frequently observed size of ~29 and ~30 kDa (lane 4). This variability, which may be due to different extents of glycosylation (see below), precludes their identification as two distinct species at this time.

Several smaller metalloproteinase inhibitory activities were observed when 293 cells were cultured on collagen-coated dishes (Figure 5.3). The molecular weights of these inhibitors were approximately 13, 15, 16 and 17 kDa and appeared to be more abundant than the ~24-kD inhibitor. These smaller inhibitors were seen infrequently and seemingly were present in the ECM of cells that were cultured on collagen-coated dishes for extended time

periods, usually greater than 7-10 days. Since there is a significant decrease in the ~24-kDa inhibitor, it is conceivable that these inhibitors are its proteolytic degradation products that have retained metalloproteinase inhibitory activity. Establishing an unambiguous relationship, if any, of these inhibitors to the ~24-kDa inhibitor or other known TIMPs would require their purification and N-terminal sequencing.

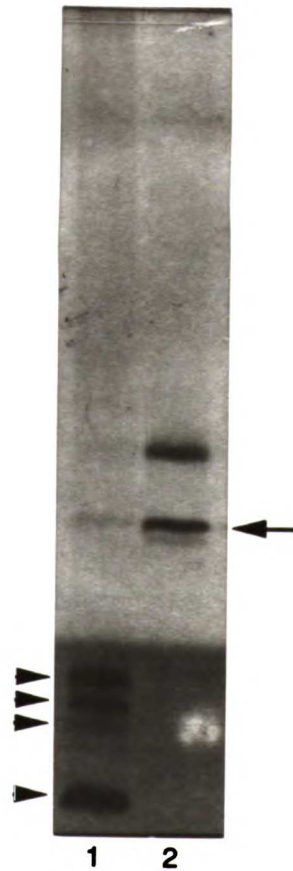


Figure 5.3. Protease-substrate gel analysis of ECM from 293 cells cultured on collagen-coated culture dishes. Lanes 1 and 2 contain ECM from 293 cells cultured either on collagen-coated dishes or on untreated dishes, respectively. Note the presence of at least 4 inhibitor bands (arrowheads) which are smaller in molecular weight than the human TIMP-3 (arrow).

In contrast to the two minor inhibitors in the ECM, TIMP-3 is not N-glycosylated. Figure 5.4 shows a protease-substrate gel analysis of ECM from human embryo cells (FHs 173We). It is clear that the relative mobility of TIMP-3 is identical whether or not it is pre-treated with PNGase F, which cleaves N-linked oligosaccharides from a protein backbone. In contrast, the mobilities of IMP-a and IMP-b (not resolved on this gel) are altered following incubation with the enzyme. No additional bands of activity are apparent on the gel after this treatment, probably because the deglycosylated proteins migrate with mobilities that are similar enough to the TIMP-3 to be masked by the broad band of its inhibitor activity.

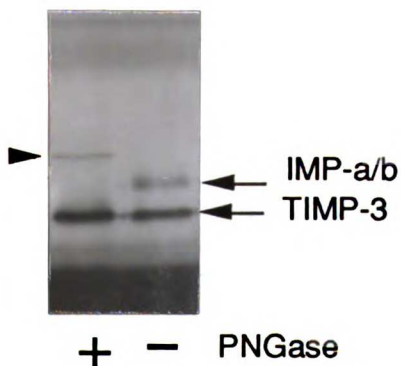


Figure 5.4. PNGase treatment of FHs 173We ECM analyzed by protease-substrate gel. ECM harvested from FHs 173We cells was treated with PNGase F (N-glycosidase-F) as described under "Materials and Methods". (+) indicates treatment with PNGase; (-) indicates no treatment. The stained band (arrowhead) is presumably PNGase (MW ~ 36 kDa) in the sample.

Evidence that this 24-kDa inhibitor is the human TIMP-3 is provided by recognition of the protein by polyclonal antibodies raised against purified ChIMP-3, but not by antibodies against human TIMP-1 or TIMP-2. Figure 5.5 shows Western blots of reduced proteins from the ECM of human embryo cells (FHs 173We). The center panel (lane 3) shows that the anti-ChIMP-3 antibody recognizes a major protein (~24 kDa) in the ECM of these cells, together with a minor band (~29 kDa). The 24-kDa band migrates with a slightly lower mobility than control ChIMP-3 in lane 4. In contrast, anti-human TIMP-1 (lane 2) and anti-human TIMP-2 (lane 5) do not recognize any proteins in the ECM samples although they recognize authentic TIMP-1 (lane 1) and TIMP-2 (lane 6) in samples of conditioned media from the whole embryo cells. However, the identity of the minor band of protein, which is also recognized by antibodies to ChIMP-3 and to its N-terminal peptide, on Western blots of ECM proteins from FHs 173 We cells is not known (Figures 5.5 and 5.6). This protein, which migrates slower than TIMP-3, could possibly be IMP-a or IMP-b, but, due to the similarity of electrophoretic mobilities of the latter two inhibitors, it is not possible to make an assignment. The fact that both IMP-a and IMP-b are glycosylated raises the question of their relationship to TIMP-1 and also to ChIMP-a. TIMP-1 is occasionally detected in ECM samples from human cells on Western blots probed with anti-TIMP-1 antibody (data not shown) and its size (28.5 kDa) is similar to that of IMP-a and IMP-b. Thus, it is possible that one of these inhibitors is TIMP-1. It is tempting to speculate that the other is glycosylated TIMP-3. The cDNA sequence for human TIMP-3 (Pavloff and Hawkes, unpublished and (Apte *et al.*, 1994b)) predicts a site for N-linked glycosylation at the C-terminus identical to that found for ChIMP-3 (Pavloff *et al.*, 1992) . Although we had originally considered the possibility that ChIMP-a was N-glycosylated ChIMP-

3, after deglycosylation it has a faster electrophoretic mobility in SDS-polyacrylamide gels than ChIMP-3 (Pavloff *et al.*, 1992). Thus, the two chicken proteins appear to differ by more than just the presence of N-linked carbohydrate. Whether or not the other minor inhibitor (IMP-a or IMP-b) from the human cells is a glycosylated TIMP-3 remains to be determined.

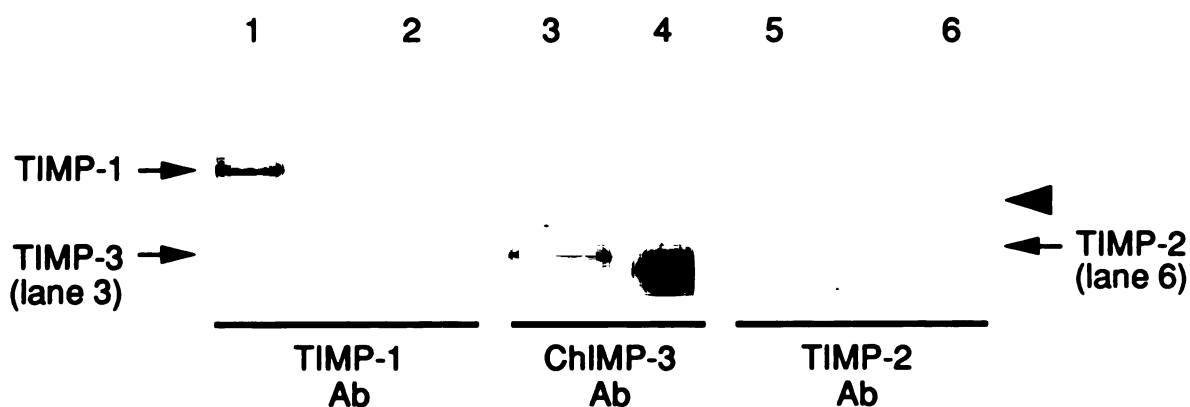


Figure 5.5. Immunostaining of human ECM with antibodies to TIMP-1, TIMP-2 and chicken TIMP-3. Lanes 1 and 6 each contain dialyzed and concentrated FHs 173We CM (equivalent to 60 ml native CM); lanes 2, 3 and 5 each contain FHs 173We ECM from approximately 2.4×10^6 cells cultured in six 100-mm tissue culture dishes and lane 4 contains CEF ECM from approximately 4×10^6 cells cultured in one 100-mm tissue culture dish. Following SDS-PAGE the proteins were transferred to PVDF membrane and probed with the various antibodies as described under "Materials and Methods".

Resistance of the human TIMP-3 to trypsin digestion was evaluated by treating freshly prepared FHs 173We ECM with TPCK-trypsin for 15 minutes before solubilizing the ECM in Laemmli sample buffer. Initially, protease-substrate gels were used to analyze the ECM because this technique is convenient and sensitive. However, the trypsin treated ECM was heavily contaminated with trypsin (presumably by binding to the culture dish and/or ECM components) which extensively degraded the gelatin in the protease-substrate gels in the region of 20-25-kDa where the human TIMP-3 migrates (data not shown). Hence, immunoblotting was used as an alternate method to evaluate resistance of the human TIMP-3 to trypsin. Immunoblotting of the ECM proteins was performed essentially as described earlier using the KLH-peptide antibody. The results are shown in Figure 5.6. The untreated control samples showed a clear TIMP-3 band (lane 5). However, the trypsin treated ECM showed no distinct band, although positive immunostaining was distinctly present in the TIMP-3 molecular size region (lane 4). It could be speculated that either tryptic fragments of higher molecular weight proteins have migrated in the MW region of TIMP-3 and have certain epitopes exposed that are being recognized by the antibody, or alternatively very small pieces of the TIMP-3 are being cleaved off, resulting in migration of these truncated proteins in the same vicinity as the uncleaved TIMP-3 and causing a smear instead of a band. Also a distinct lower MW band at ~18-kDa is immunostained (lane 4, arrowhead), which is probably a specifically recognized tryptic fragment of TIMP-3. Unequivocal demonstration of the effect of TPCK-trypsin on TIMP-3 and specific fragments thereof would require purified human TIMP-3, which then could be analyzed by immunoblotting.

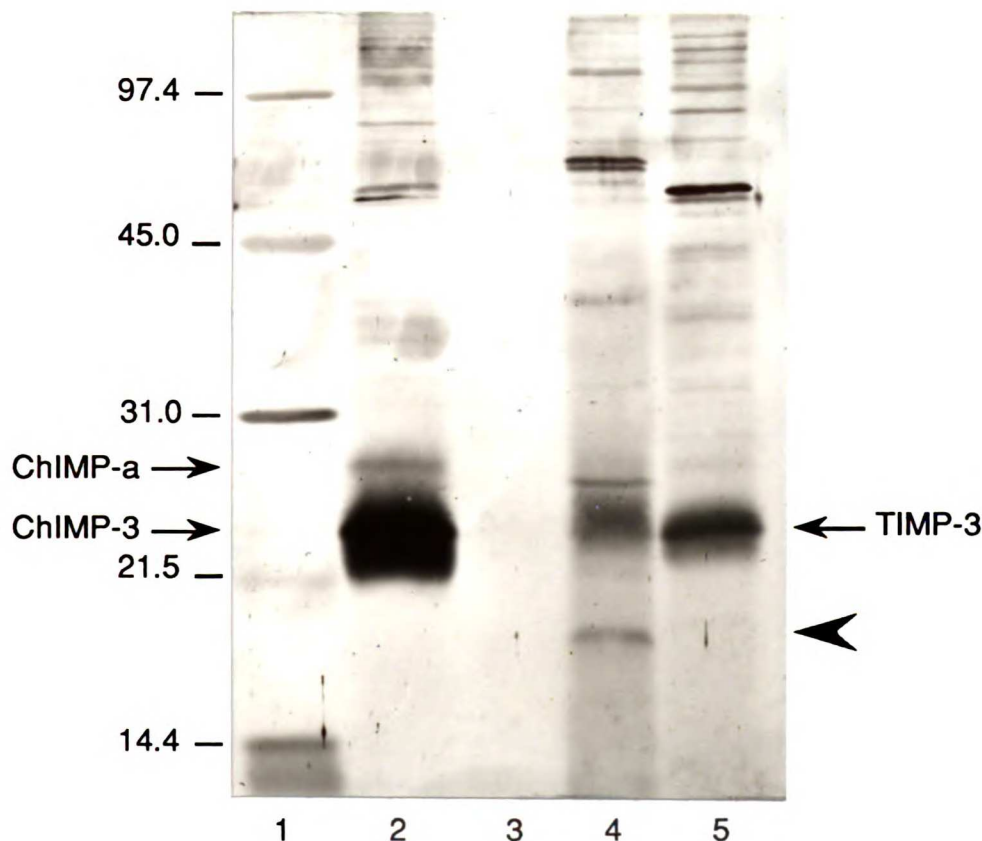


Figure 5.6. Analysis of TPCK-trypsin treated FHs 173We ECM by immunoblotting. FHs 173We ECM was treated with TPCK-trypsin (lane 4), as described under "Materials and Methods". Lane 1 contains molecular weight standards, while lane 3 is a blank lane containing sample buffer. Lanes 2 and 5 contain untreated CEF ECM and FHs 173We ECM, respectively. All samples were treated with β -mercaptoethanol (final concentration 5% v/v). Immunoblotting was performed as described under "Materials and Methods". The arrowhead indicates an immunostained band (lane 4), which is smaller than TIMP-3 and is not present in the untreated sample, as discussed under "Results and Discussion".

Several approaches were used to purify the human TIMP-3. Modifications of protease-substrate gels to thicker 1.5 mm gels containing lower amounts of gelatin (0.05 or 0.2 mg/ml instead of 1 mg/ml) were successful, as shown in Figure 5.7 for gels containing 0.2 mg/ml gelatin (similar results were obtained with 0.05 mg/ml gelatin, but are not shown). These modified gels required shorter incubation times to show metalloproteinase inhibitory activities in CEF ECM, presumably due to decreased amount of substrate that the collagenase(s) were required to degrade. Inhibitory bands are apparent after only an 2.5 hour incubation, although clear bands are visible after 5 or 8 hour incubations (Figure 5.7).

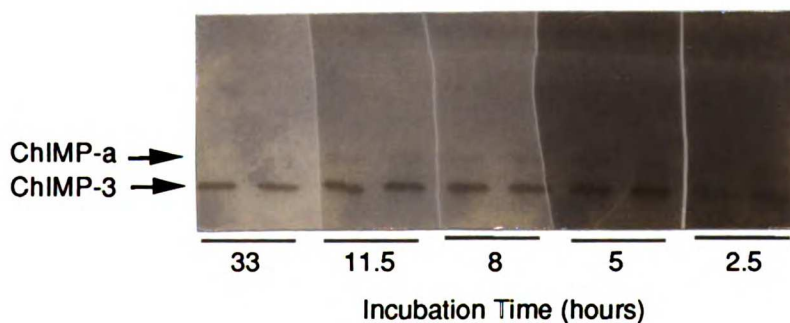


Figure 5.7. Modified Protease-Substrate Gels. Protease-substrate gels were modified to contain one-fifth and one-twentieth the amount of gelatin normally present in these gels (1 mg/ml) and to be twice as thick (1.5 mm) as normal gels. Identical amounts CEF ECM were electrophoresed in all lanes. Excised gel slices were incubated for indicated times and stained with Coomassie. The gel with one-fifth amount of gelatin is shown here. Similar results were obtained with one-twentieth amount of gelatin.

Rapid development of representative slices of 1.5-mm thick preparative-scale modified protease-substrate gels allowed identification of gel regions containing the human TIMP-3 in the retained portions of the gel. However, following elution and concentration of the protein, re-electrophoresis and transfer to PVDF membrane, no N-terminal sequence was obtained, presumably due to N-terminal blockage of the protein. Similar results were obtained with proteins eluted from broad bands (in the 20 to 30 kDa region) which were excised from standard preparative gels and re-electrophoresed, as described earlier. Again, inability to obtain any N-terminal sequence could have been due to either protein losses during the various steps or to an unacceptable degree of N-terminal blockage during protein manipulation.

Protease-substrate gels with the bisTris-TES gel system were not used for the purification of human TIMP-3 due to the poor resolution of ChIMP-3 from ChIMP-a in this system (Figure 5.8, lanes 1 and 2). The CEF ECM was used here as a model for establishing the purification procedure. The reason for the poor resolution is not known, although these inhibitors are well resolved in the Laemmli system (e.g. Figure 5.1, lane 6). However, the sensitivity of this system in detecting ChIMP-3 was far greater than with the Laemmli system. Clearly, similar amounts of CEF ECM (Figure 5.1, lane 6 and Figure 5.8, lane 2) in the Laemmli and bisTris-TES systems, respectively, display vastly disparate inhibitory activities. The major difference in the two gels systems is their pH. The Laemmli gel system is alkaline: the pH of the resolving gel is 8.8 and the pH of the electrophoresis buffer is 8.3. The corresponding pH values in the bisTris-TES system are near neutral: pH 6.6 and pH 7.25. It is conceivable that the near neutral pH conditions protect ChIMP-3 from possible deleterious effects, and hence lower inhibitory activity, of the Laemmli system. As seen in Figure 5.8 (lane 4), ChIMP-3 deposited in

the ECM by as few as 400 cells is clearly detectable. Optimization of this system could possibly provide several fold improvement in detection, and conceivably ChIMP-3 from as few as tens of cells may be detectable. In terms of amounts, this system can detect ~20 pg of protein (based on amount present per cell, discussed later in this section).

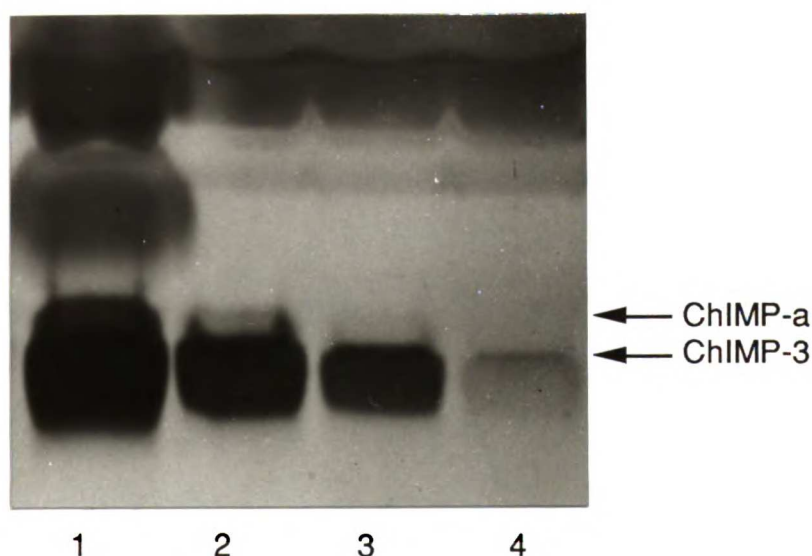


Figure 5.8. Protease-Substrate Gels in BisTris-TES Gel System. Samples corresponding to 1%, 0.2%, 0.05% and 0.01% of CEF ECM from a 100-mm culture dish (lanes 1 - 4) were electrophoresed as described under "Methods and Materials". Compare the inhibitory activity of ChIMP-3 in similar amounts of CEF ECM, as seen in normal protease-substrate gels (Laemmli gel system, Figure 5.1, lane 6) with the bisTris-TES system (this figure, lane 2).

N-terminal sequence for the human TIMP-3 (Table 5.1) was obtained from protein which was subjected to the least manipulation, i.e. where all ECM proteins were transferred to PVDF, followed by identification of TIMP-3 by Western blotting. Precautions taken to prevent N-terminal blockage of proteins, by using reagents of the highest quality, aging gels and using sodium thioglycolate as a free radical scavengers, also may have been crucial in obtaining N-terminal sequence of the human TIMP-3. However, almost 95% of the protein was still blocked, as determined by quantitation of signals obtained during N-terminal sequencing and comparing it with amino acid composition analysis of the human TIMP-3. The sequence was obtained with approximately 2 pmoles of unblocked protein.

Amino acid sequence analysis of purified human TIMP-3 indicates, for those residues determined, that its N-terminus is identical to that of ChIMP-3 (Pavloff *et al.*, 1992) and the recently cloned mouse TIMP-3 (Leco *et al.*, 1994) (Table 5.1). Of those positions where the chicken and mouse sequences differ (valine/serine at position 4 and isoleucine/serine at position 6 for ChIMP-3/mTIMP-3), no assignments could be made. Cysteines at positions 1, 3 and 13, which are highly conserved in all TIMPs, could not be determined without prior derivatization of the protein with 4-vinyl pyridine.

A comparison of all available N-terminal sequences for TIMP-1, TIMP-2 and TIMP-3, shown in Table 5.1, indicates that TIMP-2 is the most conserved, with only one variable residue; TIMP-1 is the least conserved, with five variable residues; and TIMP-3 is intermediate, with two. Based on sequence data for TIMP-3 from human, mouse and chicken, it appears that the tenth residue (Table 5.1, underlined) of an N-terminal sequence for a metalloproteinase inhibitor will predict whether or not the protein should be classified as a TIMP-1, TIMP-2 or TIMP-3. This position is invariant for each

Table 5.1

NH₂-terminal amino acid sequence of human TIMP-3

hTIMP-3	- T - - P - H P Q D A F - N - D I V
ChIMP-3 ^a	C T C V P I H P Q D A F C N S D I V
mTIMP-3 ^b	C T C S P S H P Q D A F C N S D I V
TIMP-1con ^c	C t C v P p H P Q T A F C n S D l V s a t s v
TIMP-2con ^d	C S C S P V H P Q Q A F C N v D i V i a

^a N-terminal sequence and deduced amino acid sequence (Staskus *et al.*, 1991; Pavloff *et al.*, 1992 respectively)

^b Deduced amino acid sequence (Leco *et al.*, 1994)

^c Deduced amino acid sequences from human, bovine, rat, murine and rabbit sources (Edwards *et al.*, 1986; Johnson *et al.*, 1987; Gewert *et al.*, 1987; Freudenstein *et al.*, 1990; Docherty *et al.*, 1985; Gasson *et al.*, 1985; Carmichael *et al.*, 1986; Horowitz *et al.*, 1989; Boone *et al.*, 1990; Stetler-Stevenson *et al.*, 1990)

TIMP. In TIMP-1 it is threonine, in TIMP-2 it is glutamine and in TIMP-3 it is aspartate.

Further evidence of its identity is provided by the amino acid composition of the protein, which is very similar to that of ChIMP-3 (Table 5.2). The apparent discrepancy in the high glycine content in the chicken protein (23-34 residues compared to 17 in human TIMP-3) has been attributed to contamination with this amino acid from buffers during purification (Staskus *et al.*, 1991). Indeed, the deduced amino acid composition from ChIMP-3 cDNA analysis indicates 15 glycine residues from a total of 188 amino acids in the mature protein (Pavloff *et al.*, 1992). In order to eliminate this potential problem for the human TIMP-3 the protein was isolated by a protocol which eliminated glycine-containing buffers from its final stages.

Finally, like other TIMPs, the human TIMP-3 displays evidence of intrachain disulfide bonding. Figure 5.9 shows that the reduced protein, pre-treated with β -mercaptoethanol (lanes 3 and 4) migrates slightly more slowly than the non-reduced protein (lane 1). In lane 2, where there is a gradient of reducing agent between lane 1 (without β -mercaptoethanol) and lanes 3 and 4 (with β -mercaptoethanol), the protein shows an altered pattern of mobility that is reminiscent of that observed for ChIMP-3 (Staskus *et al.*, 1991). The inhibitors are all visualized by use of an antibody directed to the N-terminus of ChIMP-3. This antibody recognizes a second chicken inhibitor (ChIMP-a) in addition to the ChIMP-3 in a sample of ECM from transforming chicken embryo fibroblasts (lane 6). Similar results were also obtained by electrophoresis of [35 S]methionine-labeled FHs 173We ECM in the presence or absence of β -mercaptoethanol through protease-substrate gels followed by autoradiography (Figure 5.10). The non-reduced samples on the protease-

Table 5.2

Amino acid composition of hTIMP-3 and its comparison with ChIMP-3

	ChIMP-3 (deduced)	hTIMP-3 (aa comp)	ChIMP-3 (aa comp)
Asx	14	15	14-15
Glx	15	19	17
Ser	8	12	14
Gly	15	17	23-24
His	7	3	3
Arg	10	9	8-9
Thr	11	11	15
Ala	8	11	9-10
Pro	9	10	8-9
Tyr	15	9	9-10
Val	11	12	11
Met	6	2	4
Cys	12	ND	ND
Ile	8	8	8-9
Leu	11	15	12
Phe	5	6	5
Lys	15	11	8-9
Trp	5	ND	ND

ND - Not determined

substrate gel permitted unambiguous identification of TIMP-3 on the autoradiogram.

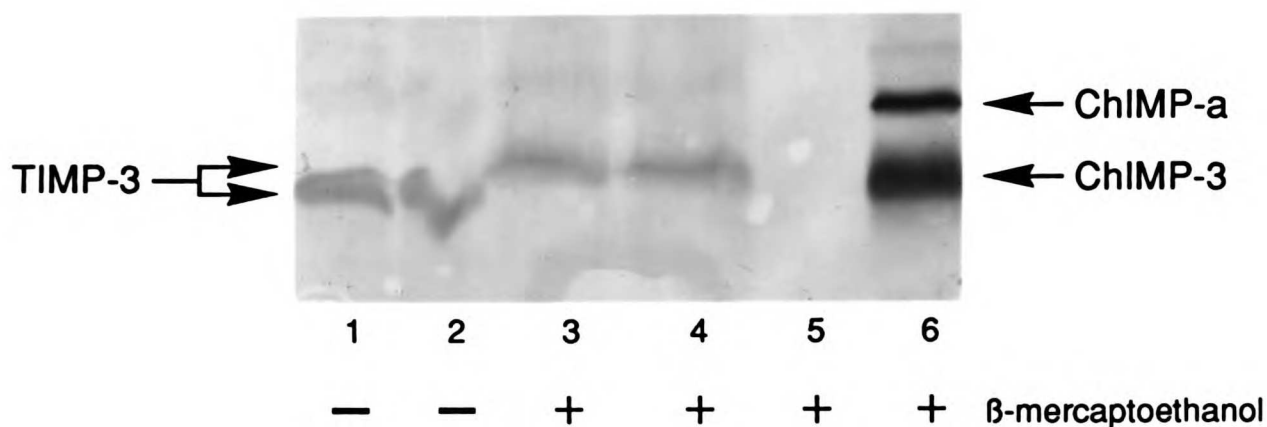


Figure 5.9. Effect of β -mercaptoethanol on the human TIMP-3 demonstrated by immunostaining. FHs 173We ECM from approximately 2.4×10^6 cells cultured in six 100-mm tissue culture dishes was loaded in each lane (lanes 1-4); CEF ECM from approximately 4×10^6 cells cultured in one 100-mm tissue culture dish was loaded in lane 6. Electrophoresis and immunostaining was performed as described under "Materials and Methods". Lane 5 contained only the sample loading Laemmli buffer. (+) indicated addition of β -mercaptoethanol (final concentration 5% v/v); (-) indicate no addition of β -mercaptoethanol.

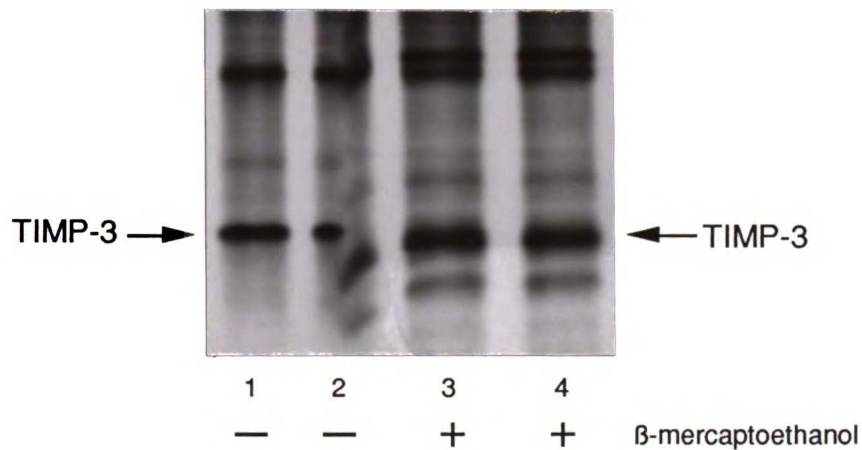


Figure 5.10. Effect of β -mercaptoethanol on the human TIMP-3 demonstrated by protease-substrate gel and autoradiography. [^{35}S]methionine-labeled FHs 173We ECM from approximately 3×10^5 cells was loaded in lane 1 and 2 and lanes 3 and 4 of a protease-substrate gel, either in the presence or absence of β -mercaptoethanol (final concentration 5% v/v), respectively. Following development and staining of the protease-substrate gel, the gel was dried and autoradiographed. The arrow indicates band on autoradiogram that corresponds to the human TIMP-3 on the protease-substrate gel. Note the characteristic change in mobility of the human TIMP-3 in lane 3 where β -mercaptoethanol has diffused from lane 2.

5.4 Summary

Data presented in this chapter provide conclusive evidence for the existence of human TIMP-3 in the ECM of cultured cells. TIMP-3 is found exclusively in the extracellular matrix and not in the conditioned media of a large number of cultured human cells. Its apparent size of 24-25 kDa, as determined by its migration on protease-substrate gels, is intermediate between TIMP-1 (28.5 kDa) and TIMP-2 (21 kDa). Like the latter two proteins, human TIMP-3 contains intrachain disulfide bonds and displays altered electrophoretic mobility in the presence of β -mercaptoethanol. The N-terminal amino acid sequence of the isolated protein is identical to that of ChIMP-3 for those residues determined and its amino acid composition is similar. The protein is not N-glycosylated, as determined by treatment with PNGase F. It also appeared to be resistant to trypsin. Finally, it is recognized by antisera raised against pure ChIMP-3 but not by anti-human TIMP-1 or anti-human TIMP-2 antibodies. The sensitivity of protease-substrate gels was improved by at least an order of magnitude using a near-neutral gel electrophoresis system. TIMP-3 present in the ECM from 400 CEF and corresponding to an amount of 20 pg was clearly detectable. In light of the multifunctional properties displayed by the TIMP family as a whole and by the chicken TIMP-3, in particular, it will be important to isolate sufficient quantities of the human protein to establish its potential role in the development of the malignant phenotype.

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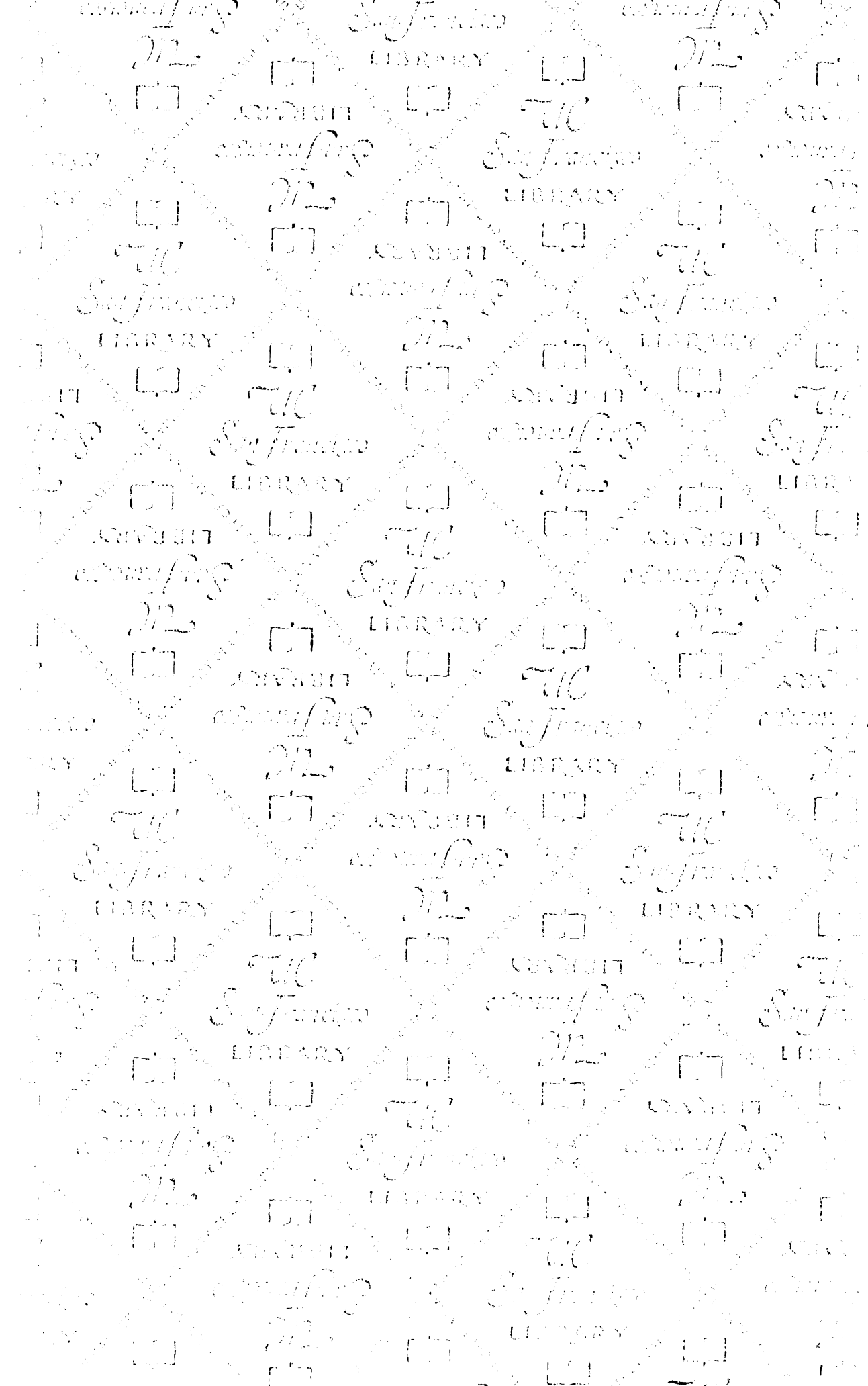
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