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Functional Analysis of

Tissue Inhibitor of Metalloproteinases (TIMP)-3

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Functional analysis of Tissue Inhibitor of Metalloproteinases (TIMP)-3" submitted by P. Paul Beaudry in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Degradation of the extracellular matrix is an essential component of a host of normal physiological processes such as wound healing and embryo implantation, as well as abnormal processes such as invasion of cancer cells and rheumatoid arthritis. The main group of enzymes responsible for this degradation are the matrix metalloproteinases (MMPs), which are secreted as latent enzymes and are subsequently activated. Tight regulation of the MMPs occurs at several levels, including expression, activation, and through interaction with another family of proteins, the tissue inhibitors of metalloproteinases (TIMPs), multifunctional proteins which inhibit the catalyic activity of the MMPs, but are also potent effectors of cell growth and development. There are three members of the TIMP family. The newest member, TIMP-3, possesses characteristics which distinguish it from TIMPs-1 and -2, and suggest that it has a distinct role in physiology. In particular it is tightly associated with the extracellular matrix (ECM) while TIMPs-1 and -2 are freely diffusible, and it is expressed in a distinct array of tissues in both the developing and adult mouse.

The objectives of these experiments are to characterize the relative ability of TIMP-3 to inhibit two of the MMPs, gelatinases A and B, determine the role of specific domains of the TIMP-3 protein in MMP inhibition and ECM association, and to determine the effects of TIMP-3 on the growth of a range of cell types.

Using TIMPs and gelatinases produced from stably transfected cell lines in reverse zymography, a form of proteinase-substrate gel electrophoresis, it was observed that TIMP-3 can inhibit gelatinase B more effectively than TIMPs -1 or -2. Domain functions were studied by construction of chimaeric TIMP-1/TIMP-3 proteins using PCR. This revealed that the TIMP-3 C-terminus confers at least partial trafficking to the matrix, while the signal peptide and highly conserved TIMP-3 3' untranslated region (UTR) have no effect on localization. Finally, growth assays demonstrated that exogenous TIMP-3 appears to have the ability to inhibit or stimulate the growth of some cell types *in vitro,* while cycling cells overexpressing TIMP-3 were found to have an increased proportion of cells in G1 phase compared to control transfected cells.

ACKNOWLEDGEMENTS

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For my grandfather,

Gordon D. Hayward.

TABLE OF CONTENTS

Approval page ii

Abstract iii

Acknowledgements v

Dedication vi

Table of Contents vii

List of Tables x

List of figures ix

List of Abbreviations xiii

CHAPTER 1: INTRODUCTION 1
1.1 THE EXTRACELLULAR MATRIX 2
1.2 THE MATRIX METALLOPROTEINASES. 3
1.2.1 Activation of MMPs 11
1.3 THE TISSUE INHIBITORS OF MATRIX METALLOPROTEINASES (TIMPs). 12
1.3.1 Formation of TIMP-progelatinase complexes 18
1.3.2 TIMP Effects on cell growth 21
1.3.3 TIMPs as regulators of development. 23
1.4 MMPs and TIMPs IN PATHOLOGY 23
1.5 HYPOTHESIS AND OBJECTIVES. 27

CHAPTER 2: MATERIALS AND METHODS 28
2.1 RECOMBINANT DNA TECHNIQUES 29
2.1.1 Restriction Enzyme Digestion 29
2.1.2 Generation of Blunt Ended DNA Molecules 29
2.1.3 Ligation of DNA Molecules 30
2.1.4 Chimaeric cDNA Construction Using Polymerase Chain Reaction (PCR). 30
2.2 PREPARATION OF RADIOLABELLED DNA PROBES AND FILTER HYBRIDIZATION 31

2.3 PLASMID VECTORS 38
2.3.1 Preparation and Transformation of *E. coli* Bacteria with Plasmid DNA 38
2.3.2 Identification of Recombinant Plasmids . . . 39
2.3.3 Plasmid Preparations 39
2.3.4 Sequence Analysis of Plasmid DNA 40
2.4 ELECTROPHORESIS OF DNA 40
2.4.1 Electrophoresis of DNA on Agarose Gels . 40
2.4.2 Purification of DNA Fractionated on Agarose Gels 41
2.4.3 Electrophoresis of DNA on Polyacrylamide Gels 41
2.5 ELECTROPHORESIS OF RNA 42
2.5.1 Isolation of RNA from Cultured Cells 42
2.5.2 Electrophoresis of RNA on Agarose Gels . 42
2.5.3 Transfer of RNA from Agarose Gels to Solid Support - Northern Transfer 42

2.6 CELL CULTURE TECHNIQUES 43
2.6.1 Maintenance of Cell Lines 43
2.6.2 Transient Transfection of COS-1 Cells . . . 44
2.6.3 Construction of Stably Transfected BHK Cell Lines 45
2.6.4 Cell Growth Assays 45
2.6.5 Flow Cytometry Analysis of Cell Cycle . . . 46

2.7 ISOLATION AND GEL ELECTROPHORESIS OF PROTEIN . 47
2.7.1 Isolation of Protein from Cells in Culture . . 47
2.7.2 Electrophoresis of Protein on Polyacrylamide Gels 47
2.7.3 Zymography 48
2.7.4 Reverse Zymography 48
2.7.5 Transfer of Proteins to Solid Support and Probing With Biotinylated Hyaluronic Acid (BHA) 49

CHAPTER 3: RESULTS 51
3.1 Expression of Recombinant TIMPs In COS Cells and Assay by Reverse Zymography. 52
3.2 Construction of BHK Cell Lines Stably Overexpressing Gelatinase B and TIMPs. 60
3.3 Differential Reverse Zymography 71

3.4 Analysis of TIMP-3 domain function. 86
3.4.1 Role of the Signal Peptide in TIMP-3 Processing, Trafficking, and Activity. . . 92
3.4.2 Role of the TIMP-3 C-terminal Domain in Processing, Trafficking, and Activity. . . 94
3.4.3 Expression and Analysis of Truncated TIMPs Lacking a C-terminal domain. 101
3.5 Identification of glycosaminoglycans associating with TIMP-3. 103
3.6 TIMP-3 Effects on Cell Growth. 108

Chapter 4. DISCUSSION 122
4.2 Publications during this thesis 138

References 139

LIST OF TABLES

Table 1. Characteristics of the Matrix Metalloproteinases (MMPs) 8

Table 2. Primers and templates used in PCR. 35

Table 3. Primer sequences. 36

LIST OF FIGURES

Figure 1. Domain Structure of the Matrix Metalloproteinases (MMPs). . . . 6

Figure 2. Domain Structure of Tissue Inhibitors of Matrix Metalloproteinases (TIMPs). 13

Figure 3. Strategy for generation of chimaeric proteins. 32

Figure 4. Reverse zymography of TIMPs. 53

Figure 5. Amino acid sequence comparison of mouse TIMPs-1 and -3. . 56

Figure 6. Reverse zymography of deglycosylated TIMPs. 58

Figure 7. Schematic of pNUT Expression Vector Construction. 62

Figure 8. Reverse zymography and silver stain analysis of conditioned media and matrix from stably transfected BHK cell lines. 68

Figure 9. Reverse zymography incorporating chicken gelatinase A or hamster gelatinase B. 73

Figure 10. Differential reverse zymography using proteins expressed in COS-1 and BHK cell lines. 75

Figure 11. Laser densitometric analysis of the time course of reverse zymography using gelatinases A and B. 78

Figure 12. Determination of gelatinolytic activity used in differential reverse zymography. 80

Figure 13. Reverse zymography of different amounts of TIMP-1, TIMP-2, and TIMP-3. 84

Figure 14. cDNA domain breakdown of TIMP-3 protein and schematic of the PCR protocol used to generate TIMP-1:TIMP-3 chimaeric cDNAs and PCR product cloning strategy. 87

Figure 15. Reverse zymography and silver stain analysis of TIMP-3 signal peptide domain function. 90

Figure 16. Reverse zymography and silver stain analysis of TIMP-1:TIMP-3 C-terminal domain exchange and truncated TIMPs. 95

Figure 17. Northern analysis of TIMP expression in transiently transfected COS-1 cells. 99

Figure 18. Identification of glycosaminoglycans (GAGs) associating with TIMP-3. 105

Figure 19. Growth rate comparison of BHK cells overexpressing TIMP-3.109

Figure 20. Analysis of matrix maintained by BHK cells under low serum conditions. 111

Figure 21. Growth comparison of cell lines plated on TIMP-3 enriched matrix. 114

Figure 22. Cell cycle analysis of transfected BHK cells. 120

List of Abbreviations

Units of Measure

bp	- base pair
Ci	- Curie
cm	- centimetre
cpm	- counts per minute
°C	- degrees centigrade
g	- gram
kb	- kilobase
kbp	- kilobase pair
kDa	- kilodalton
μCi	- microCurie
μg	- microgram
μl	- microlitre
ml	- millilitre
mm	- millimetre
mM	- millimolar
M	- molar
ng	- nanogram
pg	- picogram
xg	- times gravitational force

Chemical Compounds/Buffers/Solutions

DTT - dithiothreitol

EDTA - ethylenediamine-tetraacetatic acid

EtBr - ethidium bromide

PBS - phosphate buffered saline

SDS - sodium dodecyl sulphate

SSC - standard saline citrate

Tris - Tris(hydroxymethyl)aminomethane

TAE - Tris-acetate EDTA buffer

TBE - Tris-borate EDTA buffer

TE - Tris EDTA buffer

TSFM - Totally serum-free medium

Nucleic Acids

ATP - adenosine 5'-triphosphate

dATP - 2'-deoxyadenosine 5'-triphosphate

dCTP - 2'-deoxycytidine 5'-triphosphate

dNTP - 2'-deoxynucleoside 5'-triphosphate

DNA - deoxyribonucleic acid

cDNA - complementary deoxyribonucleic acid

RNA - ribonucleic acid

mRNA - messenger ribonucleic acid

tRNA - transfer ribonucleic acid

General

BSA	- bovine serum albumin
ChIMP	- chicken inhibitor of metalloproteinases
CM	- conditioned media
DMEM-F12	-Dulbecco's modified Eagle's medium-Ham's F12 (1:1)
ECM	- extracellular matrix
E. coli	- *Escherichia coli*
EGF	- epidermal growth factor
FCS	- fetal calf serum
HRP	- horseradish peroxidase
LB	- Luria-Bertani bacterial medium
MMP	- matrix metalloproteinase
pBS	- Bluescript plasmid
PCR	- polymerase chain reaction
PMA	- phorbol 12-myristate 13-acetate
RT-PCR	- reverse transcription-polymerase chain reaction
SDS-PAGE	- SDS-polyacrylamide gel electrophoresis
TGFβ	- transforming growth factor β
TIMP	- tissue inhibitor of metalloproteinases

CHAPTER 1: INTRODUCTION

1.1 THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is an intricate network of macromolecules which surrounds cells. It consists of a variety of versatile polysaccharides and proteins that are secreted locally by cells and assembled into an organized meshwork (Alberts *et al.*, 1989). Variations in the relative amounts of the individual ECM components give rise to a great diversity of forms which can accommodate the specific needs of individual tissue types. ECM gives teeth hardness, tendons strength and corneas transparency. Until recent years it was thought that the ECM served mainly as a scaffold for cells, defining tissue architecture and stabilizing tissues. It is now clear that the ECM plays a much more dynamic role in physiology by regulating the development, migration, growth, shape, and metabolism of cells in contact with it (Alberts *et al.*, 1989).

The ECM is a dynamic structure which is being continually replaced by a balance of synthesis and degradation of its components. Temporary alteration of this balance is required for a number of physiological processes. Matrix degradation is a part of the remodelling of specialized matrices such as bone and cartilage, which are resorbed or expanded in response to hormonal stimuli (Alexander and Werb, 1991). In physiological processes which involve tissue architecture changes that result in removal of a whole tissue or organ, such as uterine and mammary involution, enzymes are required to break down basement membranes as well as interstitial ECM so that the components can be absorbed by scavenging cell types (Alexander and Werb, 1991). A period

of aggressive matrix breakdown is involved in the implantation of the mouse embryo into the uterine wall (Harvey *et al.*, 1995). ECM degradation is also required for the invasive migration of cells, which is necessary for responses to foreign antigens by cells of the immune system such as macrophages and neutrophils. During these processes, the balance of matrix turnover has shifted toward ECM breakdown which must, however, be tightly regulated in order to prevent excessive tissue destruction. This regulation can be imposed by increasing synthesis of new matrix to restore the balance, or it can be imposed by controlling the amount of active matrix degrading enzymes present in a given physiological situation. The level of latent enzyme synthesis, activation of latent enzyme, or specific inhibition of enzyme catalytic activity are different ways that degradative enzymes can be controlled.

Deregulation of matrix turnover underlies a range of diseases. Upsetting the balance to favor degradation is involved in development of rheumatoid- and osteoarthritis; as well, a more localized deregulation is a key step in the progression of cancer cells to a malignant state, enabling them to spread to other tissue sites (Mignatti and Rifkin, 1993). Conversely, upsetting the balance to favor the deposition of matrix results in the development of fibrotic tissue states.

1.2 THE MATRIX METALLOPROTEINASES.

Three distinct groups of proteases have been shown to play a role in the degradation of the ECM: 1) the matrix metalloproteinases (MMPs) , 2) the

serine proteinases, and 3) the cysteine proteinases. The most important of these is the MMPs, a family of zinc dependent enzymes which are secreted in a proenzyme form and are subsequently activated by mechanisms that are still poorly understood. The catalytic activity of the MMPs is inhibited through protein-protein interaction with another family of secreted proteins, the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs). MMPs have emerged as central players in matrix degradation because of their association with processes involving ECM breakdown as well as their broad, overlapping substrate specificities which enables them to collectively degrade all the proteinaceous components of the ECM (Birkedal-Hansen *et al.*, 1993). MMPs have been implicated in physiological events based on ECM breakdown which, as mentioned earlier, range from normal to pathological. Characterizing the MMPs and the mechanisms regulating their activity will be vital to our understanding these processes, in turn enabling us to treat conditions which are based on incorrect regulation of matrix homeostasis.

The MMP gene family consists of at least 14 members which can be placed into three main categories on the basis of substrate specificity, namely the collagenases, the gelatinases, and the stromelysins (Table 1). The enzymes share a number of common structural features (Figure 1): The hydrophobic signal peptide is followed by a propeptide domain which constitutes the NH_2-terminal domain of the secreted proenzyme, a catalytic domain containing the catalytic machinery as well as the zinc binding site, a

proline rich hinge region which leads into a hemopexin- or vitronectin-like COOH terminal domain that appears to play a role in substrate specificity (Birkedal-Hansen *et al.*, 1993). In addition to these structural elements, the gelatinases contain three tandem copies of a 58-amino acid residue fibronectin type II-like module, located immediately NH2-terminal of the zinc binding site (Figure 1) (Wilhelm *et al.,* 1989). It has recently been demonstrated that this domain binds specific extracellular matrix proteins with high affinity, such as denatured type I collagen, elastin, and denatured types IV and V collagen, but does not bind native types IV and V collagens nor fibronectin, which are all substrates for gelatinase B (Steffensen *et al.*, 1995). These results suggest that the fibronectin-like domain of the gelatinases may play a role in localization of the enzyme to the ECM. Variation of the core MMP structure is also seen in the newest members of the MMP family, membrane-type (MT)-MMPs, which have a transmembrane domain in addition to the structural elements characteristic of the MMPs (Sato *et al.*, 1994).

As pointed out, the MMPs are secreted as zymogens. Conversion to a catalytically active state involves cleavage of the NH2-terminal propeptide. The propeptide that is cleaved contains a highly conserved amino acid sequence around the site of an unpaired cysteine residue (PRCGVPD), which is thought to interact with the zinc atom of the active site and thus mediate the latency of the pro-enzyme (Fridman *et al.*, 1992). This interaction has been verified for at least one of the MMPs, stromelysin (Kleiner and

Figure 1. Domain Structure of the Matrix Metalloproteinases (MMPs). The 14 members of the MMP family of proteinases can be regarded as derivatives of the five-domain modular structure characteristic of the collagenases and the stromelysins. The presence of three tandem 58 amino acid fibronectin II-like domains distinguishes the gelatinases, while the transmembrane domain is unique to membrane type (MT)-MMPs. (After Birkedal-Hansen *et al*, 1993).

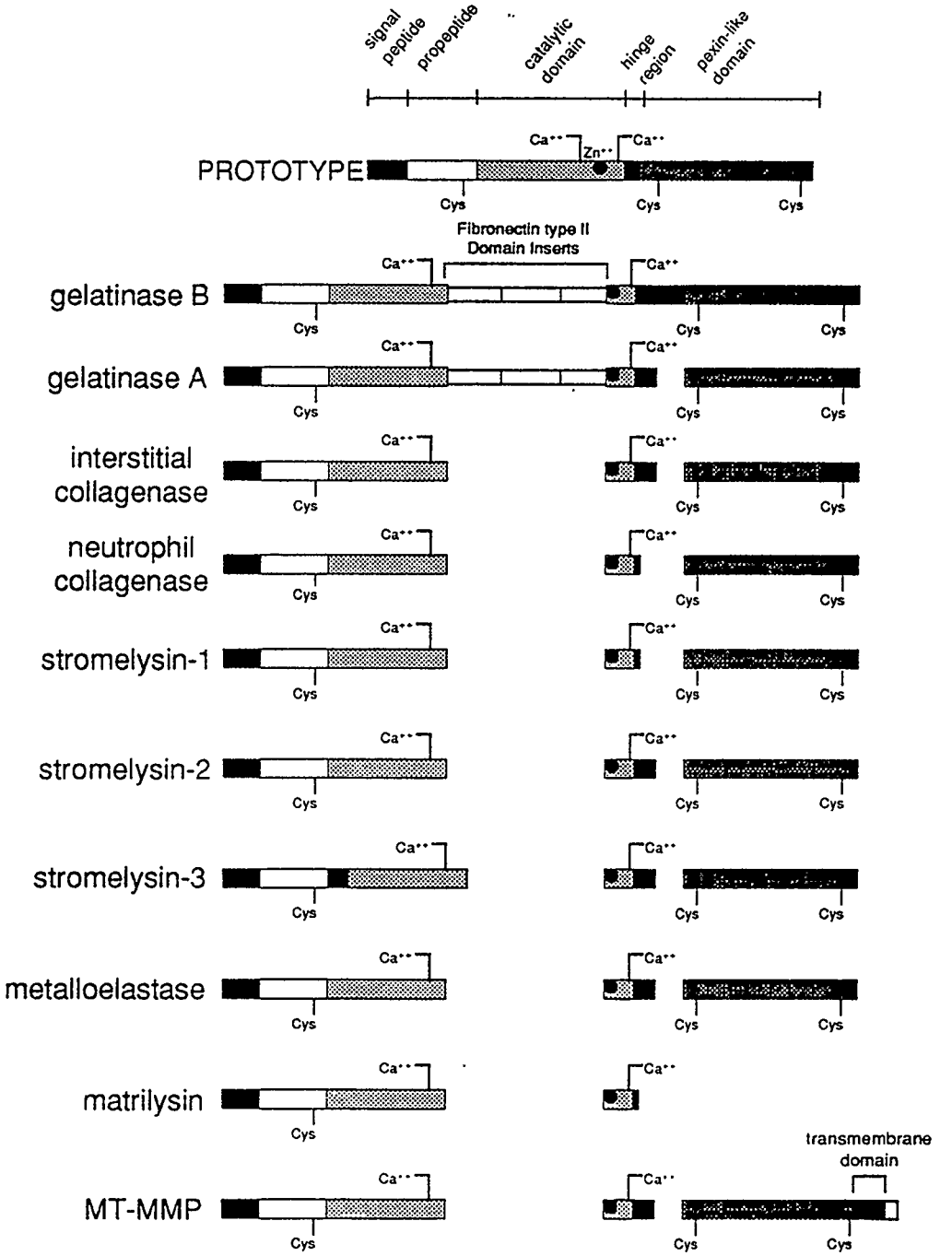

signal peptide
propeptide
catalytic domain
hinge region
pexin-like domain
PROTOTYPE
Ca++
Zn++
Cys
Fibronectin type II Domain Inserts
gelatinase B
gelatinase A
interstitial collagenase
neutrophil collagenase
stromelysin-1
stromelysin-2
stromelysin-3
metalloelastase
matrilysin
MT-MMP
transmembrane domain

Table 1. Characteristics of the Matrix Metalloproteinases (MMPs)

NAMES	DEDUCED MASS (kDa)	ECM SUBSTRATES
Collagenases		
Interstitial Collagenase MMP-1 Fibroblast Collagenase	54.1	collagens I, II, III, VII, X
Neutrophil Collagenase MMP-5 PMN collagenase	53.4	collagens I, II, and III
Gelatinases		
Gelatinase A MMP-2 72 kDa gelatinase IV collagenase	73.9	gelatins, collagen IV?, V, elastin
Gelatinase B MMP-9 92 kDa gelatinase 92 kDa IV collagenase	78.4	gelatins, collagen IV?, V, elastin
Stromelysin		
Stromelysin-1 MMP-3 transin proteoglycanase procollagenase activator	54.0	proteoglycans, fibronectin, laminin, gelatins, collagens III, IV, V, IX
Stromelysin-2 MMP-10 transin-2	54.1	proteoglycans, fibronectin, laminin, gelatins, collagens III, IV, V, and IX
Matrilysin MMP-7 PUMP-1 uterine metalloproteinase	29.7	proteoglycans, collagen IV?, fibronectin, gelatins, elastin
Others		
Stromelysin-3	54.6	?
Metalloelastase	53.9	elastin, fibronectin
Membrane-Type(MT) MMP #1	66.0	gelatinase A
MT-MMPs #2-4	?	?

Stetler-Stevenson, 1993). Also, it has been shown that mutation of the cysteine residue to a serine will result in autoactivation of fibroblast collagenase (MMP-1), and that 14 amino acid synthetic peptides based on the highly conserved N-terminal sequence containing the unpaired cysteine (TMRKPRCGNPDVAN) will inhibit the activated form of gelatinase A *in vitro* (Melchiori *et al.*, 1992). Disruption of the coordination between the cysteine residue and the zinc atom by organomercurials such as *para*-aminophenylmercuric acetate (APMA) or proteolysis, leads to the autocatalytic cleavage of the approximately 80 amino acid N-terminal profragment. As well, some members of the MMP family can be further processed to lower molecular mass active species by autocatalytic cleavage of the C-terminal domain (Fridman *et al.*, 1992).

Another highly conserved region among the MMPs resides within the N-terminal catalytic domain, where there is a short conserved region (HEXGH) that provides two zinc coordinating histidine residues and a glutamic acid which is considered part of the catalytic site (Denhardt *et al.*, 1993).

The role of the C-terminal domains of the MMPs has also been studied. In general it has been shown that C-terminal truncations do not affect proteolytic activity, but there are changes in substrate specificity (Murphy *et al.*, 1992a; Murphy *et al.*, 1992b). The type IV collagenases, gelatinase A (72kDa, MMP-2) and gelatinase B (92kDa, MMP-9) are distinguished from other members of the MMP family by the presence of three contiguous copies of the fibronectin

type II homology unit within the C-terminal domain (Figure 1). It has been shown that these fibronectin-like domains, which are similar to the collagen binding domains of fibronectin, are required for the proenzyme to bind gelatin (Strongin *et al.*, 1993), but this binding does not appear to be a rate limiting step in gelatin hydrolysis. As a result, the physiological significance of the gelatin binding ability of the gelatinases remains unclear. In addition to the fibronectin like domains, the gelatinase B C-terminal domain contains an additional 54 amino acid long proline-rich collagen V-like domain of unknown function (Wilhelm *et al.*, 1989). A model for the mechanism of fibrillar collagen breakdown is postulated by the observation that interstitial collagenase and gelatinase B can form stable complexes, so that during ECM breakdown collagen fibrils are attacked first by interstitial collagenase, which cleaves at a specific site giving 3/4 and 1/4 collagen fragments that are unstable at body temperature, leading to thermal denaturation to form gelatin, which can then be degraded by the gelatinase component of the complex (Goldberg *et al.*, 1992).

Another function of the C-terminal domains of gelatinases A and B is indicated by the observation that deletions of this domain have been shown to affect the interaction of the enzymes with specific inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMPs, see below)(Murphy *et al.*, 1992a).

1.2.1 ACTIVATION OF MMPs

The physiological mechanisms underlying MMP activation have proven elusive. This gap in our understanding may represent the most important obstacle to learning how cells utilize MMPs to degrade the ECM (Birkedal-Hansen *et al.*, 1993). It has been shown that exogenous proteinases such as trypsin, plasmin, chymotrypsin, neutrophil elastase, and plasma kallikrein, can attack a short basic sequence exposed on the surface of the collagenase molecule (Nagase *et al.*, 1990). This initial cleavage then allows a second, autocatalytic cleavage 5 to 8 residues from the unpaired cysteine in the inhibitory domain. It has been suggested that generalized MMP activation may be mediated by plasmin and trypsin. Evidence has not been sufficient to verify this model, as not all MMPs are amenable to activation by these enzymes, and their activity toward MMPs has been looked at using *in vitro* assays, so it remains to be verified that these proteinases are responsible for MMP activation *in vivo*. (Birkedal-Hansen *et al.*, 1993). Indeed, the model is emerging that specific activation pathways may be required of individual MMPs. Another model for MMP activation that has emerged is proteolysis by other MMP family members. It has been observed that stromelysins-1 and -2 (MMP-3, MMP-10) can activate interstitial collagenase (MMP-1) and gelatinase B (MMP-9, 92kDa gelatinase) (Murphy *et al.*, 1987). However, the mechanism underlying the activation of stromelysin which would be required to initiate the cascade is unknown. More recently, the newest member of the MMP family,

membrane type (MT)-MMP has been shown to function as an activator of gelatinase A (MMP-2, 72kDa gelatinase) but not gelatinase B (Sato *et al.*, 1994).

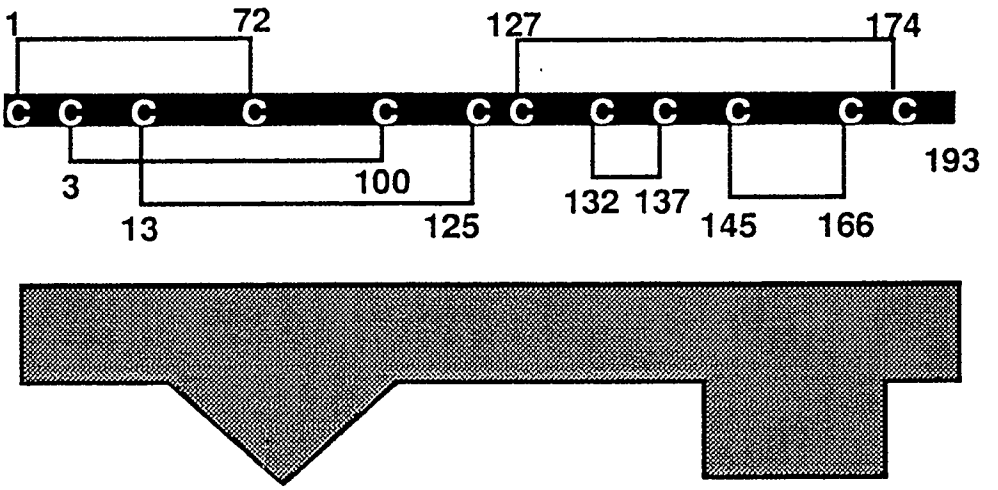
1.3 THE TISSUE INHIBITORS OF MATRIX METALLOPROTEINASES (TIMPs).

Proper maintenance of the ECM requires precise regulation of the activity of MMPs. This regulation may occur at the level of gene expression, with specific factors inducing or inhibiting expression of MMP genes as a given physiological situation requires. Another level in which the proteolytic activity of the MMPs is regulated is through tight binding with another family of proteins, the tissue inhibitors of metalloproteinases (TIMPs). To date three TIMPs have been discovered. TIMP-1 and TIMP-2 have been more thoroughly characterized than the most recently discovered TIMP-3. The TIMPs have a number of common as well as distinguishing characteristics which suggest that they may have distinct physiological roles. Also, in addition to their roles as MMP inhibitors, the TIMPs have been shown to possess cytokine properties, making them multifunctional molecules.

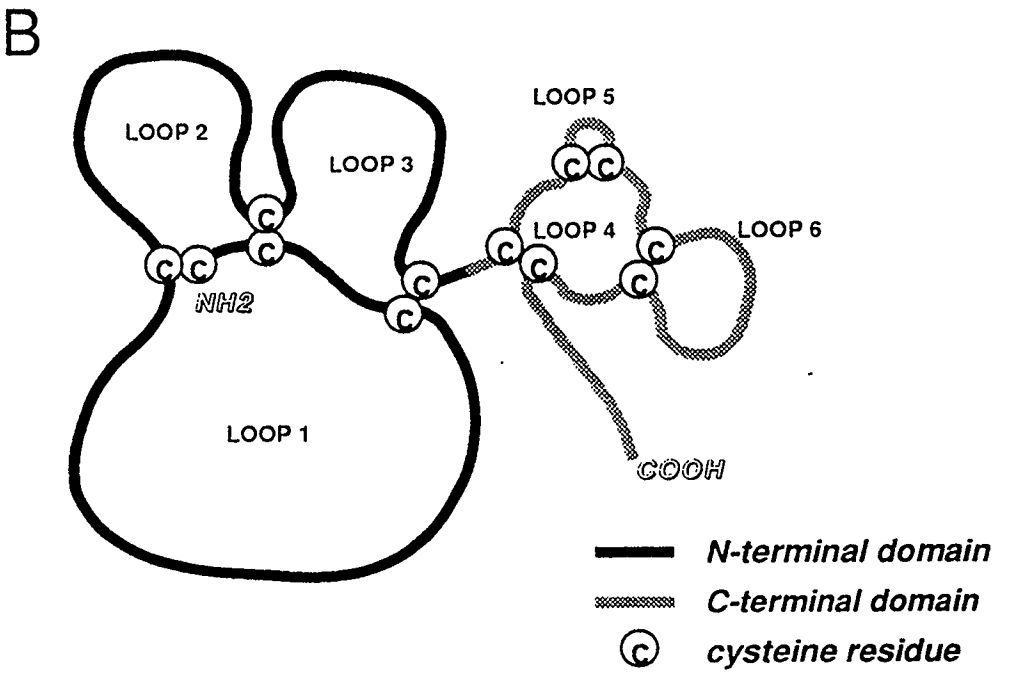
The most important conserved feature among the TIMPs is the precise spatial arrangement of 12 cysteine residues which form intrachain disulphide bonds that fold the protein into a two domain, six-looped structure (Figure 2) (Williamson *et al.*, 1990). This modular arrangement suggests functional specificity for the N- and C-terminal domains of the TIMPs, which has been verified for TIMP-1 and -2. It has been demonstrated that truncated versions

Figure 2. Domain Structure of Tissue Inhibitors of Matrix Metalloproteinases (TIMPs). The disulphide bonding pattern of the TIMPs (a) is dictated by the 12 cysteine (C) residues conserved among all of the TIMP family members. The relative positions of each cysteine and their disulphide bond assignments are shown. The pattern of disulphide bonds gives rise to a functionally modular structure which distinguishes the N- and C-terminal domains of the mature TIMP protein, further subdivided into six loops (b). (After Kleiner and Stettler-Stevenson, 1993; and Alexander and Werb, 1991).

A


1
72
127
174
193
3
100
13
125
132
137
145
166
BINDS TO AVAILABLE ACTIVE SITE OF ENZYME
BINDS TO CARBOXY TERMINUS OF PROENZYME

B


LOOP 2
LOOP 3
LOOP 5
LOOP 4
LOOP 6
NH2
LOOP 1
COOH
N-terminal domain
C-terminal domain
cysteine residue

of TIMP-1 containing only the N-terminal loops were able to form complexes with and inhibit active forms of the MMPs, suggesting that the N-terminus contains the structural features required for inhibition of catalytic activity of the enzymes (Murphy *et al.*, 1991). Similarly, the N-terminal domain of TIMP-2 has also been shown to be sufficient for inhibition of interstitial collagenase (DeClerck *et al.*, 1993). The C-terminus of TIMP-1 and -2 has been shown to be involved in the formation of specific TIMP-1:progelatinase B TIMP-2:progelatinase A complexes (see below) (Wilhelm *et al.*, 1989; Stetler-Stevenson *et al.*, 1989).

In spite of the marked similarities among the TIMPs, there are significant differences in their localization , relative MMP inhibitory capacity, structure, and inducibility which suggests a distinct role for each TIMP in physiology. TIMP-1 and -2 are freely diffusible while TIMP-3 has been characterized as tightly matrix associated (Blenis and Hawkes, 1983, 1984; Staskus *et al.*, 1991). The mechanism and functional significance of this is not understood. In general, TIMPs have been shown to have a broad spectrum of inhibitory activity toward members of the MMP family (DeClerck *et al.*, 1989; Stetler-Stevenson *et al.*, 1989; Goldberg *et al.*, 1989; Apte *et al.*, 1995). However, some evidence gathered thus far has suggested that there may, in some cases, be differential inhibition of MMPs by TIMPs. Enzyme activity assays based on measuring liberation of acid soluble gelatin peptides from radiolabelled gelatin have been used to characterize the relative inhibitory ability of TIMP-1 and -2

against gelatinase A and B (Howard *et al.*, 1991). From this it was shown that TIMP- 2 is a better inhibitor of both the autoactivated 72kDa form of gelatinase A (2 fold better inhibition than TIMP-1) as well as the 42.5 kDa activated fragment (10 fold), which lacks the C-terminal domain. As well, TIMP-2 was seen to be a better inhibitor of activated gelatinase B (7 fold). However, in similar assays using another MMP, interstitial collagenase, TIMP-1 was shown to be the more effective inhibitor (2-fold). TIMP-3 has been shown to be an effective inhibitor of chicken gelatinase A (Pavloff *et al.*, 1992), but its inhibitory ability relative to TIMP-1 and TIMP-2 for any of the MMPs is not known.

Comparison of the amino acid sequence of the three murine TIMPs reveals that TIMP-1 and -2 are 39% identical while TIMP-3 shares 27% and 40% identity with TIMP-1 and -2 respectively (DeClerck *et al.*, 1993; Leco *et al.*, 1994). All the TIMPs have typical signal peptide sequences, though it should be noted that the sequences found in mouse, human and chicken TIMP-3 have fewer leucine and isoleucine residues and contain conserved tryptophan and cysteine residues (Leco *et al.*, 1994). Structural differences between the TIMPs include the presence of N-linked glycosylation sites (Asn-X-Ser/Thr) on TIMP-1 and -3, but not on TIMP-2. The cDNA sequences for TIMP-1 and TIMP-2 code for proteins of 21 kDa, but TIMP-1 is glycosylated at two sites and as a result migrates on SDS-PAGE gels as two main glycosylation variants of 24 kDa (singly glycosylated) and 28 kDa (doubly glycosylated) (Mignatti and

Rifkin, 1993; Leco *et al.*, 1994; Murphy *et al.*, 1991). Also, TIMP-3, the cDNA of which codes for a 24 kDa protein, contains one N-linked glycosylation site located at the extreme C-terminus of the protein (Leco *et al.*, 1994). This results in a minority of the protein being detected at 27kDa with the majority migrating as unglycosylated protein at 24 kDa unless treated with an N-linked glycosidase, at which time only a 24 kDa band is detected (Hawkes and Beaudry, unpublished results). Since both glycosylated and unglycosylated forms of the TIMPs have been shown to inhibit MMPs, the functional significance of glycosylation remains unclear.

There are also marked differences in the transcriptional regulation of the TIMPs. TIMPs -1 and -3 have been shown to be highly inducible in mouse C3H 10T1/2 fibroblasts by phorbol ester (PMA), epidermal growth factor (EGF), and transforming growth factor-β1 (TGF-β1), but the on/off transcription kinetics are faster for TIMP-3 than TIMP-1 (Leco *et al.*, 1994). TIMP-2 expression has been shown to be relatively uninducible by a range of factors in mouse and human fibroblasts (Leco *et al.*,1992; Edwards *et al.*, 1995). Expression of TIMP-3, but not that of TIMP-1 or TIMP-2, was shown to be cell cycle-regulated, reaching a maximum in mid G1 in exponentially growing diploid human fibroblast cell line, WI-38 (Wick *et al.*, 1994).

Further evidence that the TIMPs have distinct roles is provided by experiments demonstrating that *in vivo* expression patterns of the three TIMP genes are distinct (Nomura *et al.*, 1989; Waterhouse *et al.*,1993; Leco *et al.*,

1994). High levels of TIMP-1 mRNA were detected in tissues undergoing osteogenesis in the mouse embryo, while in the female adult mouse, the highest level of TIMP-1 expression was seen in the uterus and corpus luteum of the ovary (Nomura *et al.*, 1989). Low levels of TIMP-1 expression are seen in murine maternal decidua, embryonic kidney, lung, and amnion (Nomura *et al.*, 1989). TIMP-2 has been shown to be highly expressed in placenta just prior to birth concomitant with increased gelatinase A expression (Waterhouse *et al.*, 1993). TIMP-3 has been shown to be expressed in adult tissues lacking expression of other TIMPs, with high levels of TIMP-3 mRNA detected in kidney, lung, and brain but only low levels in bone, a site of high TIMP-1 transcription (Leco *et al.*, 1994). In the developing mouse embryo, TIMP-3 is abundantly expressed in the surface epithelia of organs such as the developing bronchial tree, kidney, colon and esophagus, organs with extensive tubular structure (Apte *et al.*, 1994). Also, *in situ* analysis of TIMP-3 expression in implanting mouse embryos and adjacent uterine stroma shows high levels of TIMP-3 expression by maternal cells proximal to the trophoblast, where gelatinase B expression is elevated (Harvey *et al.*, 1995).

1.3.1 Formation of TIMP-progelatinase complexes

Another significant feature of TIMP-1 and TIMP-2 is their ability to form mutually exclusive complexes with the latent forms of gelatinase B and A respectively. Using gelatin affinity chromatography to purify the gelatinases it was observed that TIMP-2 copurified with progelatinase A (Stetler-Stevenson

et al., 1989) and TIMP-1 with progelatinase B (Wilhelm *et al.*, 1989). A number of experiments have shown that the C-terminal domains of both TIMP-2 and progelatinase-A are essential to formation of the TIMP-2- proenzyme complex, and that the regions involved are distinct from both the active site of the enzyme as well as the inhibitory domain of TIMP-2 (Kleiner and Stetler-Stevenson, 1993; Fridman *et al.*, 1992; Willenbrock *et al.*, 1993). It has also been shown that the C-terminal domain of gelatinase-A greatly increases the sensitivity of the enzyme to inhibition by TIMP-2, and that activated TIMP-2 - gelatinase A complexes retain 10% of the gelatinolytic activity of free activated enzyme (Fridman *et al.*, 1992). Activation of gelatinase A by MT-MMP mentioned earlier has been shown to be blocked when the proenzyme is complexed with TIMP-2 (Sato *et al.*, 1994). This relationship has been characterized in greater detail by the observation that TIMP-2 is able to abolish the enhanced level of cell-surface associated gelatinase A that normally occurs following concanavalin A stimulation of primary fibroblasts (Ward *et al.*, 1995). Further, it has been demonstrated that removal of TIMP-2 from gelatinase A results in autoactivation and rapid degradation of the enzyme (Howard *et al.*, 1991). Data assembled thus far has led to development of a model of TIMP-2-gelatinase A interaction featuring formation of a complex with increased gelatinase A stability and tightly regulated gelatinolytic activity, in that the complexed proenzyme resists activation by MT-MMP and, if activated, the complexed enzyme is only moderately catalytic but with an increased half-life

compared to free enzyme (Kleiner and Stetteler-Stevenson, 1993).

Similarly to the TIMP-2 - progelatinase A complex, characterization of the domains involved in the formation of the TIMP-1 - gelatinase B complex has revealed that the C-terminal domains of the TIMP-1 and the proenzyme are essential to the formation of the complex (Goldberg *et al.*, 1992; Murphy et al 1991). Also similar is that the presence of TIMP-1 in the complex prevents the activation of progelatinase B by the physiologically relevant activator, stromelysin-1 (Goldberg *et al.*, 1992). In addition to these similarities TIMP-1 - gelatinase B interactions have been shown to involve some unique variations. For example, when the enzyme is present in molar excess over TIMP-1, it can form a homodimer that is unable to complex with TIMP-1, and can thus be readily activated by stromelysin (Goldberg *et al.*, 1992). Also, domains involved in the homodimerization are separate from those involved in formation of the TIMP-1 - proenzyme complex. Under conditions of excess interstitial collagenase relative to gelatinase B and TIMP-1, gelatinase B forms a stable complex with interstitial collagenase, and as discussed earlier, it is postulated that this complex is physiologically relevant due to the teamwork required between these two MMPs in the breakdown of collagen. It has been shown that TIMP-1 and interstitial collagenase are mutually exclusive in binding directly to gelatinase B, and that TIMP-1 has the higher affinity for the possibly shared or overlapping binding site (Goldberg *et al.*, 1992).

The existence of a TIMP-3 - proenzyme complex is not yet determined, but

conservation of overall domain structure among TIMPs suggests that it will also enter into specific complexes with the MMPs, and that this complex or complexes may be dependent upon C-terminal functions.

1.3.2 TIMP EFFECTS ON CELL GROWTH

In addition to their role in inhibiting the MMPs, all members of the TIMP family have been characterized as having growth effects on a number of cell lines *in vitro*. These growth effects are unique for each TIMP, providing additional support for the existence of a separate role for individual TIMPs in physiology.

The amino acid sequence of TIMP-1 deduced from its cDNA is identical to that of erythroid potentiating activity (EPA), which stimulates the growth of erythroid precursors as well as an erythroleukemia cell line, K-562 (Hayakawa *et al.*, 1992). Further support for the TIMP-1 role as a mitogen comes from its characterization as a potent growth factor for a number of cell types, allowing some cell types to grow in serum-free medium at a rate comparable to medium containing fetal calf serum (Hayakawa *et al.*, 1992). In these experiments, purified human or recombinant TIMP-1 was added to cells growing in serum-free media at a concentration of 100ng/ml and shown to restore the growth of human gingival fibroblasts and Raji lymphoma cells. TIMP-2 was not able to stimulate the growth of these cell types at the concentration used for TIMP-1, but was later shown to be potently mitogenic at a much lower concentration of 10 ng/ml. Furthermore, both TIMP-1 and -2 retain significant mitogenicity

following reductive alkylation which eliminated MMP inhibitory ability (Hayakawa *et al.*, 1994), showing that the growth effects of TIMP-1 and -2 are distinct from their MMP inhibitory function. TIMP-2 has also been shown to stimulate the growth of erythroid precursors, and this effect was blocked by antibodies designed to inhibit EPA (TIMP-1), suggesting that the epitope responsible for the effect is common to both TIMP-1 and TIMP-2 (Stetler-Stevenson *et al.*, 1992). TIMP-2 has also been characterized as an autocrine growth factor produced by SV40 transformed human fibroblasts (Nemeth and Goolsby, 1993), expanding the growth stimulatory repertoire of TIMP-2.

In addition to being growth factors, TIMP-1 and TIMP-2 have also been shown to have growth inhibiting properties. Overexpression of TIMP-1 by the malignant cell line B16-F10 has been shown to reduce the tumourigenicity as well as the metastatic potential of these cells upon injection into a mouse (Khokha, 1994). TIMP-1 overexpressing cells did not display increased invasiveness as measured by *in vivo* videomicroscopy (Koop *et al.*, 1994) but foci generated grew more slowly suggesting that the reduced tumourigenicity induced by more TIMP-1 expression may not be due to increased MMP inhibition alone and the effect of TIMP-1 on the growth rate of cells may also play a role.

A number of lines of evidence have shown TIMP-2 to have growth inhibitory properties. It was initially shown that TIMP-2 is an inhibitor of neovascularization in cartilage (Moses *et al.*, 1990) and later demonstrated that

it is an inhibitor of microvascular endothelial cell proliferation *in vitro* (Murphy *et al.*, 1993).

The growth effects of TIMP-3 have not been as well characterized as TIMP-1 and TIMP-2. However, it has been observed that chicken TIMP-3 can stimulate the growth of chicken fibroblasts in serum free conditions (Yang and Hawkes, 1992). The human TIMP-3 gene was cloned on the basis of its elevated expression in mid G1 in cycling WI-38 fibroblasts (Wick *et al.*, 1994), suggesting that TIMP-3 may be involved in transit through the cell cycle. These findings suggest that TIMP-3, like TIMP-1 and -2, has a repertoire of effects on the growth of mammalian cell types.

1.3.3 TIMPs as regulators of development.

In addition to the activities outlined above, at least one of the TIMPs has been shown to act as a regulator of cell development. A 70 kDa complex that acts as a potent stimulator of steroid production by specific gonadal cells was shown to be a complex of TIMP-1 and the proenzyme form of cathepsin-L, a cysteine proteinase (Boujrad *et al.*, 1995). The stimulating activity was shown to be due to the TIMP-1 component of the complex alone, but was greatly enhanced by the presence of procathepsin-L for unknown reasons (Boujrad *et al.*, 1995)

1.4 MMPs and TIMPs in pathology.

As pointed out earlier, the TIMPs and the MMPs have been linked to a number of pathological states associated with deregulation of ECM turnover.

In the progression of a cancer from benign to the more deadly malignant phenotype, one of the characteristics tumour cells must acquire is the ability to degrade the surrounding ECM and travel through the interstitial space, eventually entering a blood vessel. The observation that gelatinases cleave soluble type IV collagen, a major component of basement membranes, has raised the question of whether these enzymes play a role in tumour cell invasion as well as intra- and extravasation (Birkedal-Hansen *et al.* 1993). A number of studies have borne out this idea. *In vitro* studies of human and murine tumour cell lines has demonstrated overproduction of a number of MMPs (Lyons *et al.*, 1991; Matrisian *et al.*, 1991; Sato *et al.*, 1992; Templeton *et al.*, 1990). The role of MMPs in invasion is also characterized by Northern and *in situ* hybridization studies of human tumour tissue which show elevation of interstitial collagenase (MMP-1) expression proximal to the tumour, but not in normal tissue adjacent to the neoplasm (Hewitt *et al.*, 1991; Urbanski *et al.*, 1992). It should be noted here that stromal cells and malignant cells cooperate to endow tumours with increased invasive potential, regardless of which cell type produces the enzyme or its precursor (Vassali and Pepper, 1994). The newest member of the MMP family, MT-MMP was initially localized to the surface of invasive lung carcinoma cells (Sato *et al.*, 1994).

Functional studies of the role of MMPs in invasive behavior have demonstrated that addition of excess peptide containing the MMP inhibitory prosegment sequence can inhibit invasive activity at the cellular level (Melchiori

et al., 1992). The first direct evidence implicating an MMP in metastasis was obtained in experiments demonstrating that nonmetastatic rat embryo cells are induced to tumourigenicity by transfection with a gelatinase B expression vector (Bernhard *et al.*, 1994). The identification of a relationship between metastasis of malignant cells and MMP activity has led to the development of synthetic MMP inhibitors which, administered systemically, have been shown to decrease the metastatic potential of tumours in the mouse (Brown, 1994; Hodgson, 1995). It is believed that these inhibitors also block tumour growth by preventing angiogenesis, since it has been shown that MMPs are required for the formation of new blood vessels (Mignatti *et al.*, 1989). Some of these inhibitors are currently in clinical trials to determine their efficacy in preventing the metastasis of human cancers.

In addition to their role in the progression of cancer, the MMPs have also been shown to play a role in the development of several degenerative diseases. The continuous or intermittent joint destruction seen in rheumatoid arthritis has been studied and shown to involve an increase in collagenase and stromelysin activity within the synovial fluid of the joint (Hayakawa *et al.*, 1991; Case *et al.*, 1989). In peridontal disease, another degenerative disorder, collagenase and gelatinase B have been shown to be elevated locally with activity proportional to disease severity (Overall *et al.*, 1991). As well, MMPs have been associated with pulmonary emphysema (D'Armiento *et al.*, 1992), atherosclerosis (Henney *et al.*, 1991), and liver fibrosis (Milani *et al.*, 1994).

The activity of MMPs is regulated at the protein level by the TIMPs. Thus it is the local balance of active enzyme and TIMP which will determine if matrix degradation will occur (Stetler-Stevenson *et al.*, 1993). The importance of TIMPs in regulating the catalytic activity of the MMPs suggests that they, too, will play a role in the progression of diseases based upon deregulation of matrix turnover. A number of studies have demonstrated an inverse relationship between level of TIMP expression and metastatic potential of tumour cells. TIMP-1 has been shown to inhibit *in vivo* metastasis in animal models (Khokha *et al.*, 1992a; 1992b; Alvarez *et al.*, 1990; Schultz *et al.*, 1988). Further, downregulation of TIMP-1 expression in Swiss 3T3 cells by antisense RNA was shown to induce tumourigenic and metastatic behaviour of the cells upon injection into nude mice (Khokha *et al.*, 1989). Similar experiments using TIMP-2 have also demonstrated its ability to reduce the invasive ability and metastatic potential of transformed cell lines (DeKlerck *et al.*, 1992; DeKlerck *et al.*, 1991; Albini *et al.*, 1991). The role of TIMP-3 in the invasion of malignant cells has not yet been characterized. TIMP-3 has, however, been shown to underlie a degenerative disorder of the retina known as Sorsby's Fundus Dystrophy (SFD) (Weber *et al.*, 1994). Pedigree analysis of individuals with this disorder allowed mapping of the autosomal dominant disease locus to the same region of the genome as TIMP-3. Affected individuals from two pedigrees were shown to have mutations in the C-terminal domain of TIMP-3 predicted to disrupt the tertiary structure of the mature TIMP-3 protein,

possibly preventing it from acting as an inhibitor of MMP activity or interfering with some as yet unidentified aspect of TIMP-3 function.

1.5 HYPOTHESIS AND OBJECTIVES.

TIMP-3 has characteristics which make it a third, distinct member of the TIMP family of proteins. By virtue of these characteristics, I hypothesize that 1) TIMP-3 forms a unique and specific relationship with one of the MMPs; 2)Like TIMP-1 and -2, distinct domains of the protein are involved in separate TIMP-3 activities, including MMP inhibition, growth effects, and localization to the extracellular matrix; and 3) TIMP-3 can affect the growth of certain cell types, contributing to its role in regulation of matrix turnover.

The specific aims of my thesis are 1) Characterization of the ability of TIMP-3 to inhibit gelatinases A and B; 2) Characterization of TIMP-3 domains required for MMP inhibition and matrix association; and 3) Determination of TIMP-3 effects on *in vitro* cell growth for a range of cell types.

CHAPTER 2: MATERIALS AND METHODS

2.1 Recombinant DNA Techniques

2.1.1 Restriction Enzyme Digestion

Restriction enzyme digestion of double stranded DNA was performed using the Pharmacia One-Phor-All buffer system (Pharmacia, Baie d'Urfe, Quebec). Reactions included restriction enzyme buffer, 0.2-20 μg of DNA, and one unit of restriction enzyme (Pharmacia, Baie d'Urfe, Quebec; Gibco-BRL, Burlington, Ontario; Boehringer Mannheim, Laval, Quebec) per microgram of DNA in a final volume of 10-50 μl. Reactions were incubated at 37°C temperature for 2-18 hours. Restriction digested DNA was analyzed by agarose gel electrophoresis (described below).

2.1.2 Generation of Blunt Ended DNA Molecules

Generation of blunt ended DNA molecules (Sambrook *et al.,* 1989) was sometimes required during subcloning of restriction enzyme digested or polymerase chain reaction (PCR) synthesized DNA products. The Klenow fragment of *E. coli* DNA polymerase (Pharmacia) was used for both the 5' to 3' fill in reaction and the 3' to 5' exonuclease reaction in the presence of all four dNTPs. Reactions were terminated by extraction of the DNA once with phenol/chloroform (1:1), followed by precipitation with ethanol according to standard procedures (Sambrook *et al.*, 1989). After precipitation, the DNA was resuspended in a small volume of TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0).

2.1.3 Ligation of DNA Molecules

Ligation of DNA molecules having either compatible protruding termini or blunt termini were carried out in essentially the same manner. Insert or plasmid DNA was restriction digested and purified by agarose gel electrophoresis (see below). Content and quality of isolated DNA was monitored by agarose gel electrophoresis prior to ligation. Ligations were set up to approximate equivalent amounts of available insert and vector ends. Ligation buffer was prepared according to Maniatis *et al.*, (1982). A typical ligation reaction included 100 ng restriction digested plasmid DNA, insert DNA (amount determined for each reaction to yield an equivalent number of DNA ends available for ligating), ligation buffer, 1 mM ATP, and 1 unit T4 DNA ligase (Pharmacia) in a 20 μl final volume. Control ligations of cut plasmid without insert were performed to assess the efficiency of generation of recombinants. Ligation reactions involving compatible restriction enzyme digested ends were incubated at 15 °C for 4-18 hours. Blunt ligations were incubated a comparable amount of time at 4°C. Generally 5-10 μl of each ligation reaction was used to transform competent *E. coli* bacteria as described below.

2.1.4 Chimaeric cDNA Construction Using Polymerase Chain Reaction (PCR).

All PCR reactions were carried out using a Techne PHC-2 thermal cycler (Mandel Scientific). Reactions were all 35 cycles of 1'@ 94°C, 2'@55°C, and 1.5' @72°C. Primers used for PCR were engineered with an EcoR1 restriction

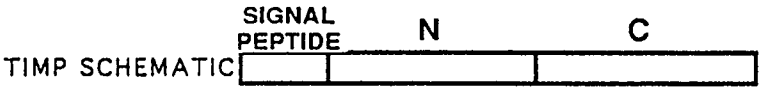
enzyme site where required for cloning of PCR product into the appropriate expression vector. Construction of mouse TIMP-3 cDNA lacking 3' untranslated region (UTR) was carried out by PCR using primer 1 (5'-GGGAATTCACAACAGCTACCATGAC-3'), which included the start codon of mouse TIMP-3 , and primer 8 (5'-GGGAATTCTGGGTTCAGGGGTCTGT-3') which included the mouse TIMP-3 stop codon . Chimaeras were generated by 2-stage PCR using junctional oligonucleotides corresponding to both domains to be joined together (Figure 3, see table 2 for description of reactions). cDNA encoding signal peptide, N- and C-terminal domains were abbreviated as S, N, and C and assigned a number to indicate the specific TIMP family member involved. The sequence of all primers used is presented in Table 3.

2.2 Preparation of Radiolabeled DNA Probes and Filter Hybridization

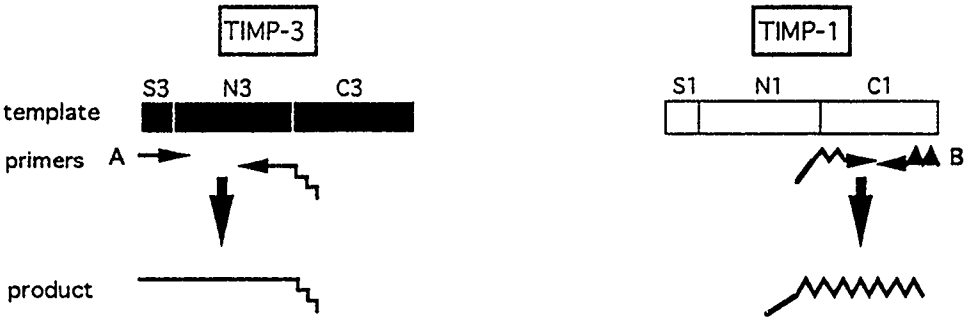
Radioactive labelling of double stranded DNA was accomplished by the nick-translation method (Sambrook *et al.*, 1989). Approximately 100 ng of DNA was labeled using 20 μCi [α-^{32}P]-dCTP (3000 Ci/mmol; Amersham, Oakville, Ontario). The reaction was incubated at 15 °C for 90 minutes, and stopped by the addition of 90 μl of 5 mM EDTA (pH 8.0)/0.1% SDS in water. The radiolabeled DNA was purified from unincorporated [α-^{32}P]-dCTP by spun column chromatography using Sephadex G-50 (Pharmacia) according to Maniatis *et al.*, (1982). The purified DNA probe was precipitated in ethanol in the presence of 100 μg yeast tRNA as carrier, and resuspended in a final

Figure 3. Strategy for generation of chimaeric proteins. A. Schematic of PCR strategy used to generate cDNA that would express chimaeric proteins. Details of PCR are presented in table 2. **B.** All PCR generated cDNA was designed with EcoR1 linkers at both 5'and 3'ends, allowing ligation of EcoR1 digested insert into the cloning site of the pXMT2 expression vector also prepared by digestion with EcoR1.

A

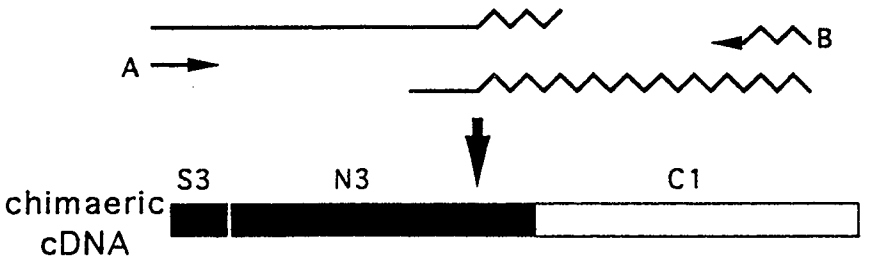


STAGE 1



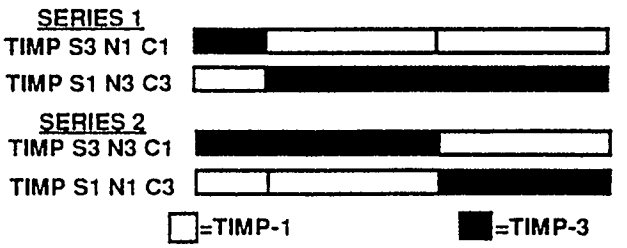
PURIFY PRODUCTS FROM EACH REACTION, MIX PRODUCTS TOGETHER AND RE-PCR WITH OLIGOS A AND B

STAGE 2



Chimaeric TIMPs to be constructed:

Control= S3 N3 C3 (wild type TIMP-3)



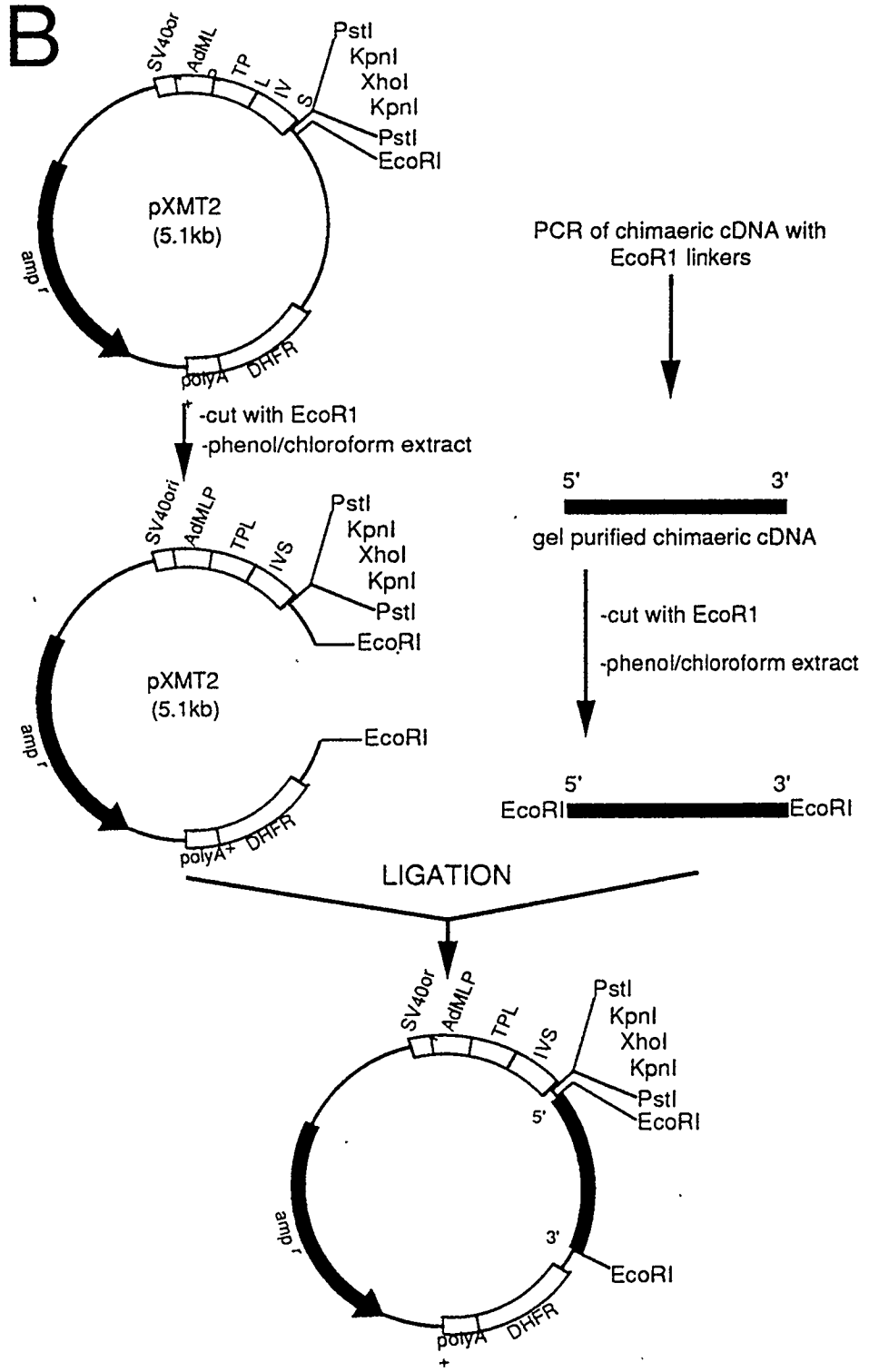

B
SV40or
AdML
TP
L
IV
S
PstI
KpnI
XhoI
KpnI
PstI
EcoRI
pXMT2
(5.1kb)
amp r
polyA
DHFR
-cut with EcoR1
-phenol/chloroform extract
SV40ori
AdMLP
TPL
IVS
PstI
KpnI
XhoI
KpnI
PstI
EcoRI
pXMT2
(5.1kb)
amp r
EcoRI
polyA+
DHFR
PCR of chimaeric cDNA with
EcoR1 linkers
5'
3'
gel purified chimaeric cDNA
-cut with EcoR1
-phenol/chloroform extract
5'
3'
EcoRI
EcoRI
LIGATION
SV40or
AdMLP
TPL
IVS
PstI
KpnI
XhoI
KpnI
PstI
EcoRI
5'
3'
EcoRI
amp r
polyA
+
DHFR

Table 2. Primers and templates used in PCR.

	PRIMERS	cDNA TEMPLATE	CHIMAERIC cDNA PRODUCT
Step 1			
rxn 1	#5, #6	TIMP-1	S1
rxn 2	#7 ,#8	TIMP-3	N3 C3
Step2			
	#5, #8	S1, N3 C3 mixed	**S1 N3 C3**
Step 1			
rxn 1	#1, #2	TIMP-3	S3
rxn 2	#3, #4	TIMP-1	N1 C1
Step2			
	#1, #4	S3, N1 C1 mixed	**S3 N1 C1**
Step1			
rxn 1	#8, #9	TIMP-3	C3
rxn 2	#5, #10	TIMP-1	S1 N1
Step 2			
	#5, #8	C3, S1 N1 mixed	**S1 N1 C3**
Step 1			
rxn 1	#4, #11	TIMP-1	C1
rxn 2	#1, #12	TIMP-3	S3 N3
Step2			
	#1, #4	C1, N3 C3 mixed	**S3 N3 C1**
	HTIMP-5' hT1ΔC-3'EcoR1	human TIMP-1	**hTIMP-1ΔC**
	#5 mT1ΔC-3'EcoR1	mouse TIMP-1	**mTIMP-1ΔC**
	TIMP-2SENSE mT2ΔC-3'EcoR1	mouse TIMP-2	**mTIMP-2ΔC**
	#1 mT3N-termE	mouse TIMP-3	**mTIMP-3ΔC**

Table 3. Primer sequences.

PRIMER #	SEQUENCE
1	5'-GGGAATTCACAACAGCTACCATGAC-3'
2	5'-GGTGGGGCACAGCTACACGCTTCCGCGCCCCAGT-3'
3	5'-ACTGGGGCGCGGAAGCGTGTAGCTGTGCCCCACC-3'
4	5'-GGGAATTCGGAAGGCTTCAGGTCAT-3'
5	5'-GGGAATTCACCAGAGATACCATGAT-3'
6 (S1)	5'-CTGGGAGAGCATGTGCAGGCCTTACTGGAAGCTA-3'
7 (S2)	5'-TAGCTTCCAGTAAGGCCTGCACATGCTCTCCCAG-3'
8	5'-GGGAATTCTGGGTTCAGGGGTCTGT-3'
9 (S3)	5'-GTGCTGGCTGTGGGGTGTGCAAGATCAAGTCCTG-3'
10 (S4)	5'-CAGGACTTGATCTTGCACACCCCACAGCCAGCAC-3'
11 (S5)	5'-ACCACCTGGGTTGCAATTGCACAGGTGTTTCCCTG-3'
12 (S6)	5'-CAGGGAAACACTGTGCAATTGCAACCCAGGTGGT-3'

volume of 100 μl TE buffer. One microlitre of the DNA probe was added to 5 ml BCS (Biodegradable Counting Scintillant; Amersham), the scintillant cocktail was counted in a Beckman LS5000CE β-counter (Beckman Instruments, Mississauga, Ontario), and specific activity was calculated (specific activity cpm/μg DNA = [cpm/μl x 100 μl] / [0.1 μg DNA labeled]). This method generally yielded probes between 10^8 and 10^9 cpm/μg DNA.

Filter hybridization of immobilized target DNA or RNA to radiolabeled probes was performed to detect specific sequences of interest. Filter preparation for various applications is described elsewhere below. Nylon filters (Amersham) were first baked at 80 °C under vacuum for 1-2 hours to fix the nucleic acid to the membrane. Filters were then placed in heat sealable plastic freezer bags and prehybridization solution consisting of 50% formamide, 5X SSC (20X SSC = 3 M NaCl, 0.3 M sodium citrate), 5X Denhardt's solution (Denhardt, 1966), 1% SDS, and 0.1 mg/ml single-stranded salmon sperm DNA (Maniatis *et al.*, 1982) was added at 4 ml per 100 cm^2 of filter. Bags were sealed with the exclusion of air bubbles and incubated with agitation for 1-2 hours at 42 °C in a hybridization oven. Just prior to addition of the radiolabeled DNA probe, the prehybridization solution was changed, probes were denatured by heating in a boiling water bath for 3 minutes, centrifuged briefly, and placed in an ice water bath. Generally, 1 x 10^6 cpm probe was added per millilitre of prehybridization buffer and the filters incubated at 42 °C for 12-18 hours.

To wash off non-specifically bound probe, the filters were removed from

the bags and placed in a plastic container with a solution of 1X SSC and 0.1% SDS, and agitated gently for 10 minutes at room temperature. A second wash in the same solution was performed at 42 °C with gentle agitation for 20 minutes. Two more washes in a solution of 0.2X SSC, and 0.1% SDS were performed at 42 °C with gentle agitation for 10 minutes. The filters were air dried on 3MM filter paper (Whatman International, Maidstone, England) at room temperature, taped to fresh 3MM filter paper, wrapped in plastic wrap, placed in an autoradiography cassette (Fisher Scientific, Ottawa, Ontario) and exposed to X-ray film (Eastman-Kodak, Rochester, New York) at -70 °C.

When necessary, the filters were stripped of bound probe by incubation in sealed plastic bags with prehybridization buffer at 75 °C for 10 minutes. Fresh prehybridization buffer was added followed by the new radiolabeled probe. Hybridization and washing were carried out as described above.

2.3 Plasmid Vectors

2.3.1 Preparation and Transformation of E. coli Bacteria with Plasmid DNA

The bacterial strain utilized for transformation purposes was *E. coli* XL1 Blue (Stratagene, La Jolla, California). Competent bacteria were prepared by the calcium chloride method described by Sambrook *et al.*, (1989). Glycerol was added to the competent bacteria to 15%, the suspension split into 200 or 400 μl aliquots, quick frozen in liquid nitrogen, and stored at -70 °C until required.

For each transformation, 100 µl of frozen competent bacteria was thawed slowly on ice. Bacteria were mixed with 5-10 µl of a ligation mixture (approximately 25 ng ligated plasmid), and stored on ice for 30-60 minutes. Cells were then heat shocked at 42 °C for 90 seconds, placed on ice for 1 minute, 0.6 ml of LB broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per litre) added, and incubated at room temperature for 30 minutes to allow expression of the ampicillin resistance marker from the plasmid. One hundred to two hundred µl of the transformed culture was spread on a 100 mm LB agar (LB broth plus 15 g agar per litre) plate containing 50 µg/ml ampicillin, allowed to dry, and incubated at 37 °C for 16-18 hours.

2.3.2 Identification of Recombinant Plasmids

Bacterial colonies containing recombinant plasmids were identified by transfer of bacterial colonies directly from an agar plate to nylon filters (Amersham; Sambrook *et al.*, 1989) and detection of inserted DNA by hybridization with a radiolabeled DNA probe. Prehybridization and hybridization of filters was performed as described above.

2.3.3 Plasmid Preparations

Small scale or mini-preparation of plasmid DNA from 2 ml bacterial cultures was performed by the alkaline lysis method (Sambrook *et al.*, 1989) to rapidly identify if any of the bacterial colonies initially detected by hybridization contained the desired recombinant plasmid. Five microlitres of each purified plasmid was subjected to digestion with the appropriate

restriction enzyme(s) and analyzed by agarose gel electrophoresis (described below).

Once bacterial cultures containing the desired recombinant plasmid were identified, large scale (300 ml) cultures were grown, plasmid prepared by the alkaline lysis method, and purified by cesium chloride density gradient centrifugation (Sambrook *et al.*, 1989).

2.3.4 Sequence Analysis of Plasmid DNA

Cesium chloride density gradient purified plasmid DNA was used for DNA sequence analysis. The dideoxynucleotide chain termination method of sequence analysis (Sanger *et al.*, 1977) was employed using the Sequenase Version 2.0 DNA sequence kit (United States Biochemical, Cleveland, Ohio) according to the protocols provided. For each plasmid to be sequenced using the Sequenase kit, 10 μCi [α-^{35}S]-dATP (>1000 Ci/mmol; Amersham) was used to radiolabel the DNA products produced in the reaction. Radiolabeled products were visualized by polyacrylamide gel electrophoresis and autoradiography as described below.

2.4 Electrophoresis of DNA

2.4.1 Electrophoresis of DNA on Agarose Gels

Fragments of DNA produced by restriction enzyme digestion or PCR were separated according to molecular size and conformation by electrophoresis on agarose gels (Sambrook, *et al.*, 1989). Prior to

electrophoresis, the DNA samples were mixed with a 6X gel loading buffer (Sambrook *et al.*, 1989) to facilitate loading onto the gels. Electrophoresis was performed as described (Sambrook *et al.*, 1989) on gels containing 0.6-2.0% agarose, 1X TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA), and 0.1 μg/ml ethidium bromide in a model H5 horizontal system for submerged gel electrophoresis (BRL, Gaithersberg, Maryland) containing 1X TAE running buffer at room temperature. Comb size and percentage of agarose was chosen to optimize separation of DNA molecules of interest. Bands of DNA were visualized with an ultraviolet light source. Photographs of the gels were taken using Polariod Type 57 film and a Polaroid 545 Land Camera (Polaroid Corporation, Cambridge, Massachusetts).

2.4.2 Purification of DNA Fractionated on Agarose Gels

When DNA fragments were to be recovered, electrophoresis was carried out as described above, and the DNA bands visualized by a hand held ultraviolet light source. The DNA fragments were electroeluted onto NA45 DEAE cellulose membranes (Schleicher and Schuell, Keene, New Hampshire) and recovered as previously described (Sambrook *et al.,* 1989). Isolated fragments were routinely checked for quality and quantity by agarose gel electrophoresis.

2.4.3 Electrophoresis of DNA on Polyacrylamide Gels

Polyacrylamide gels were prepared from a stock of 6% acrylamide / 7M urea in 1x TBE (90 mM Tris-borate pH 8.0, 2 mM EDTA) buffer in a Sequi-Gen

apparatus (Bio-Rad Laboratories, Richmond, California) according to manufacturer's instructions. After electrophoresis was complete, the apparatus was disassembled and the gel fixed in a solution of 5% acetic acid and 5% methanol for 20 minutes, blotted dry on 3MM paper, dried on a model 583 gel drier (Bio-Rad) for 2 hours, and exposed to X-ray film at room temperature for 12-18 hours.

2.5 Electrophoresis of RNA

2.5.1 Isolation of RNA from Cultured Cells

Total cellular RNA was isolated by lysing the cells directly on the culture dish by addition of a guanidine isothiocyanate buffer followed by extraction of RNA by the acid-guanidinium-phenol-chloroform (AGPC) method (Chomczynski and Sacchi 1987).

2.5.2 Electrophoresis of RNA on Agarose Gels

Samples of total RNA (10 μg) were electrophoresed on 1.1% agarose, 2.2 M formaldehyde containing gels as previously described (Maniatis *et al.*, 1982).

2.5.3 Transfer of RNA from Agarose Gels to Solid Support - Northern Transfer

Detection of mRNA species of interest after agarose gel electrophoresis was accomplished using a capillary transfer technique. Following electrophoresis the gel was rinsed in 10X SSC for 20-30 minutes, and transferred to nylon filters (Amersham) as described (Sambrook, *et al.,* 1989).

After transfer was complete (12-18 hours) the nucleic acid was fixed to the membrane by baking at 80 °C under vacuum for 2 hours and the filter probed and washed as described above.

2.6 Cell Culture Techniques

2.6.1 Maintenance of Cell Lines

COS-1 (monkey kidney fibroblast), 10T1/2 (mouse fibroblast), HT1080 (human fibrosarcoma), B16F10 (melanoma), and HS68 (human fibroblast) cell lines are all from the American Type Culture Collection (ATCC) (Rockville, Maryland, U.S.A.). BHK (baby hamster kidney) TK21- cells were a gift from Dr. Ross MacGillivray (University of British Columbia).

All cell lines were maintained in Dulbecco's modified Eagle medium-F12 medium (DMEM-F12) supplemented with 10% (5% for all BHK cell lines, or otherwise specified) fetal calf serum (FCS), 1% antibiotic and antimycotic (all from Gibco-BRL) in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. All tissue culture plastic was supplied by either Nunc (Roskilde, Denmark) or Sarstedt Canada (St. Laurent, Quebec). Cells were passaged as required by trypsin treatment and dilution (approximately 1:20) into fresh medium.

Frozen stocks of cell lines were prepared from cultures grown to approximately 80% confluence in 150 cm^2 flasks. Cells were harvested by trypsin treatment, counted on a haemocytometer, centrifuged at 500 xg, and resuspended in freezing medium (50% FCS, 45% DMEM-F12, and 5% sterile

DMSO) to yield 1 x 10^6 - 2 x 10^6 cells per millilitre. One millilitre of cell suspension was transferred to a cryovial (Nalgene, Rochester, New York), the vials positioned between two styrofoam racks, and placed at -70 °C for 12-18 hours. Frozen cells were then placed in liquid nitrogen for long term storage.

To thaw, a vial of frozen cells was removed from liquid nitrogen, placed in a 37 °C water bath for 3 minutes, the cell suspension transferred to a fresh 75 cm^2 flask containing 10 ml DMEM-F12/10% FCS, and incubated in 5% CO_2 at 37 °C. After 16-20 hours the medium was removed, replaced with 15 ml fresh DMEM-F12/10% FCS, and the cells replaced in the incubator.

2.6.2 Transient Transfection of COS-1 Cells

The monkey kidney cell line, COS-1, was used for transient expression of foreign proteins from cDNAs inserted into the pXMT2 expression vector (Sambrook *et al.,* 1989). The COS-1 cells were seeded at a density of 1 x 10^6 cells per 100 mm dish in DMEM-F12/10% FCS. The following day cells were transiently transfected with 10 μg of plasmid DNA using the calcium phosphate method of Chen and Okayama (1987) and placed at 3% CO_2. After 24 hours, the medium was replaced with fresh DMEM-F12/10%FCS, and the cells allowed to recover at 5% CO_2 for 18 hours. The cells were then washed twice with serum-free DMEM-F12 and incubated 24 hours in 10 ml serum-free DMEM-F12 prior to collection of the conditioned medium or extracellular matrix (see below).

2.6.3 Construction of Stably Transfected BHK Cell Lines

BHK TK21- cells were used for generation of stably transfected cell lines. Cells were plated at a density of 1 x 10^6 cells per 100 mm dish in DMEM-F12/5% FCS. The following day cells were transfected with 10 μg of pNUT expression vector (also a gift from Dr. MacGillivray) containing the cDNA of interest, using the calcium phosphate method of Chen and Okayama (1987) and placed at 37°C, 3% CO_2 overnight. The next day the cells were placed under fresh DMEM-F12/5% FCS and placed at 5% CO_2 overnight to recover. After 14-18 hours of recovery cells were placed under DMEM-F12/5% FCS containing 0.5 mM methotrexate. Cells were placed under fresh medium containing 0.5 mM methotrexate every 24-48 hours. After 8-12 days, surviving cells were expanded into 175 cm^2 flasks to be assayed for protein expression. Successfully transfected cells maintained in culture for extended periods were grown in media without methotrexate until every fifth or sixth passage when cells were returned to media with 0.5 mM methotrexate to maintain selective pressure.

2.6.4 Cell Growth Assays

Stably transfected BHK cells were plated out in six well tissue culture plates (Becton Dickinson, Lincoln Park, New Jersey) at low density in 5% FCS. The following day the medium was removed and the cells were rinsed 2x with serum free medium prior to being placed under the experimental serum conditions (serum free or 1%). At specified time points the cells were

harvested by trypsinizing and counted with a hemocytometer. In a second approach, stably transfected cells overexpressing TIMP-3 were plated out in six well tissue culture plates and allowed to grow to confluence. These cells were then removed using PBS containing 5mM EDTA/5mM EGTA to leave the extracellular matrix produced by the cells containing TIMP-3 relatively intact. The wells of the plate were then rinsed with deionized, sterile water to ensure complete removal of the BHK cells as possible, and the cell type to be assayed was plated onto the conditioned matrix at low density. Cells were harvested by trypsinizing at indicated time points and counted on a hemocytometer.

2.6.5 Flow Cytometry Analysis of Cell Cycle

Cells to be analyzed were washed once with serum-free medium, harvested by trypsinization and counted with a hemocytometer. 1-2x10^6 cells were taken and resuspended in 1 ml of serum-free media. 1ml of absolute ethanol was then added to the cells for a final ethanol concentration of 50%. Cells were placed at -20°C overnight and spun at 100g to pellet the next day. The cells were then washed with serum-free medium once and resuspended in 250 μl PBS containing RNAase A at a concentration of 1mg/ml, and left for 30 min. at room temperature. 1 ml of propidium iodide was added to this and left for 20 min. in the dark. The cells were then filtered through a nylon mesh screen and sorted using a Facscan cell sorter (Becton Dickinson, Lincoln Park N.J.). Cell cycle distribution was determined using CellFIT Cell Cycle Analysis Version 2.01.2 software (Becton Dickinson, Lincoln Park, N.J.).

2.7 Isolation and Gel Electrophoresis of Protein

2.7.1 Isolation of Protein from Cells in Culture

Serum-free conditioned media were isolated from cells stimulated with various agents and from cells that were transfected with expression plasmids. The medium was removed from a dish of cells 24 hours after removal of FCS and centrifuged at 1,000xg for 5 minutes to remove floating cells and cellular debris. The supernatant was decanted into a fresh tube and Tris-Cl (pH 7.5) was added to 50 mM. Conditioned media were stored at 4 °C until analyzed. When concentration of conditioned media was required, centricon-10 microconcentrators were used (Amicon Inc., Beverley MA).

Extracellular matrix was isolated by the method of Blenis and Hawkes (1983) except that the Ca^{2+}- and Mg^{2+} -free PBS contained no protease inhibitors. Extracellular matrix solubilized in 1X SDS-PAGE sample buffer (without DTT) was stored at 4 °C until analyzed.

2.7.2 Electrophoresis of Protein on Polyacrylamide Gels

Samples of conditioned media and extracellular matrix were electrophoresed on 0.1% SDS, 15% polyacrylamide gels and stained with either Coomassie or silver as described (Sambrook *et al.,* 1989). The vertical gel electrophoresis system model V16 (Gibco-BRL) or the Protean II (Bio-Rad) was used according to manufacturer's instructions for all protein

polyacrylamide gel analyses described herein. All protein electrophoresis was performed at 4 °C.

2.7.3 Zymography

Samples to be analyzed for the presence of gelatin-degrading enzymes were electrophoresed on 0.1% SDS, 10% polyacrylamide gels containing 1 mg/ml gelatin as a substrate. Powdered gelatin (Sigma, St. Louis, Missouri) was added to the water portion of the resolving gel and heated to 65 °C until dissolved. The solution was allowed to cool, the remaining ingredients added and the gel cast as described (Sambrook *et al.,* 1989). Electrophoresis was carried out at 4 °C after which the gel was washed at room temperature in a solution of 2.5% Triton X100, 50 mM Tris-Cl (pH 7.5), and 5 mM $CaCl_2$ once for 15 minutes, then a second time for 12-16 hours (overnight). The gel was then rinsed in water once and incubated at 37 °C in 50 mM Tris-Cl (pH 7.5), and 5 mM $CaCl_2$ for 18-24 hours. The gel was then stained with Coomassie blue as described (Sambrook *et al.,* 1989). The majority of the gel stains blue due to the presence of undigested gelatin; clear bands against the background blue staining indicate the presence of gelatin-degrading enzymes in the sample.

2.7.4 Reverse Zymography

Samples to be analyzed for the presence of TIMPs were electrophoresed on 0.1% SDS, 12% polyacrylamide gels containing 1 mg/ml gelatin and a source of gelatin-degrading enzyme(s). Gelatin was dissolved in the Tris-Cl component of the resolving gel as described above. Conditioned media from

BHK cells (a gift from Ross MacGillivray, University of British Columbia, Vancouver, B.C.) was added to 8.8% (vol/vol) as a source of gelatin-degrading enzyme, the water component of the gel reduced accordingly, the other components of the gel added, and the gel cast (Sambrook *et al.,* 1989). Electrophoresis was carried out at 4 °C after which the gel was washed at room temperature in a solution of 2.5% Triton X100, 50 mM Tris-Cl (pH 7.5), and 5 mM $CaCl_2$ once for 15 minutes, then again overnight. The next day, gel was rinsed in water once and incubated in 50 mM Tris-Cl (pH 7.5), and 5 mM $CaCl_2$ for 24 hours at 37°C and stained with Coomassie blue as described above. The majority of the gel does not stain as the gelatin has been degraded. Dark bands represent inhibition of gelatin degradation by components within the sample. For time course reverse zymography multiple, identical samples were run on a gel and following electrophoresis the gel was cut into strips and washed in Triton as above. During digestion at 37°C, a strip was removed every 4 hours for 24 hours and placed in Coomassie stain to fix. Amount of gelatin degradation and gelatinase inhibition was quantified using an Ultroscan XL laser densitometer (LKB Bromma, Sweden).

2.7.5 Transfer of Proteins to Solid Support and Probing With Biotinylated Hyaluronic Acid (BHA)

Proteins to be analyzed were electrophoresed as described above. Gels were then equilibrated in Western Blot transfer buffer (Sambrook, *et al.,* 1989) prior to transfer onto nitrocellulose (Bio-Rad) using a Semiphor protein transfer

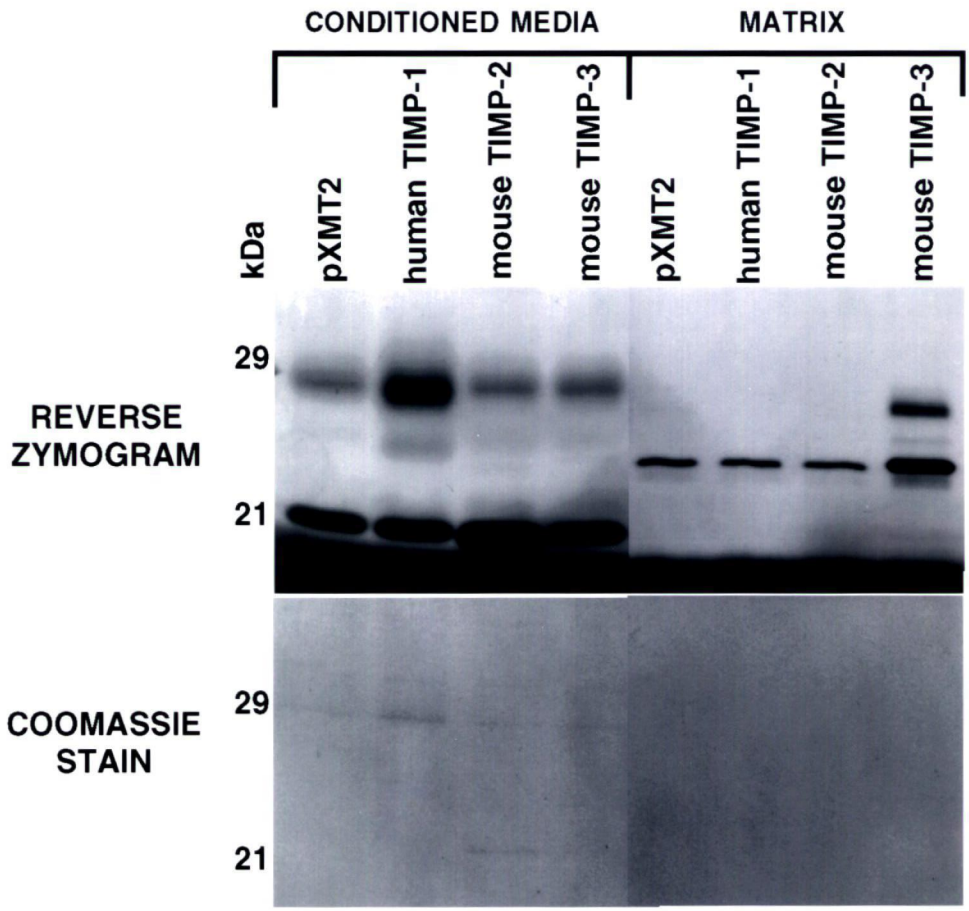
apparatus (Hoefer Scientific, San Francisco, California). Following transfer, filter was blocked in 5% skim milk powder in PBS for 1 hour at room temperature. Filter was then rinsed briefly with PBS and probed with biotinylated hyaluronic acid (BHA) (a kind gift from Dr. Eva Turley, University of Manitoba) diluted 1:250 in PBS containing 0.1% Tween-20 (VWR Scientific) for 1 hour at room temperature. Following probing with BHA the filter was washed in PBS/0.1% Tween-20 for 1 hour at room temperature then probed with streptavidin-horseradish peroxidase (HRP) conjugate (Gibco-BRL) diluted 1:1000 in PBS/0.1% Tween-20 for 1hour at room temperature. The filter was then washed and exposed to X-ray film using the ECL protocol (Amersham International, Buckinghamshire, England). Based on procedure developed by Hoare et al., 1993.

CHAPTER 3: RESULTS

3.1 Expression of Recombinant TIMPs In COS Cells and Assay by Reverse Zymography.

TIMPs are multifunctional molecules that are effective inhibitors of catalytically active MMPs. This ability to prevent MMPs from degrading their substrates allows TIMPs to be visualized by reverse zymography, a form of proteinase-substrate gel electrophoresis in which gelatin and a source of gelatinase are incorporated into a non-reducing SDS-PAGE gel. Following electrophoresis of samples which contain TIMP activity, SDS is removed from the gel, allowing proteins present to renature. This process of SDS denaturation followed by renaturation causes the incorporated progelatinase to activate and degrade the gelatin throughout the gel except in areas where TIMP activity is present and the gelatin is protected. Coomassie staining will stain any gelatin left intact and thus reveal TIMP activity. In initial experiments, I analyzed TIMP activities by transiently transfecting TIMP-1, -2, and -3 expression vectors into COS-1 cells. Conditioned media and matrix from transfected cells were harvested and analyzed for TIMP activity by reverse zymography using conditioned media from RSV transformed chicken fibroblasts (a gift from Dr. Susan Hawkes,University of California San Francisco) as a source of gelatinase A activity. Conditioned media from cells transfected with TIMP-1 contains an increased amount of TIMP activity migrating mainly at 28 kDa (Figure 4), while conditioned media from TIMP-2 transfected cells contained an increased amount of 22 kDa TIMP activity. Cells

Figure 4. Reverse zymography of TIMPs. Expression vectors directing expression of human TIMP-1, mouse TIMP-2, or mouse TIMP-3 were transiently transfected into COS-1 cells. After 18 hours in serum-free medium, the conditioned medium and matrix from each transfection were harvested and analyzed by reverse zymography for TIMP activity. In the conditioned media only, human TIMP-1 was expressed mainly as a 28 kDa gelatinase inhibitory activity (lane 2), while mouse TIMP-2 was detected as a 22 kDa activity (lane 3). Mouse TIMP-3 was detected only in the ECM as a 24 and 27 kDa activity (lane 8). Control transfected cells had detectable levels of background TIMP activity (lane 1, lane 5). SDS-PAGE followed by Coomassie staining of samples run in parallel demonstrates that the bands seen on the reverse zymogram are TIMP activities and not staining artifacts due to the presence of a large amount of protein.


CONDITIONED MEDIA
MATRIX
kDa
pXMT2
human TIMP-1
mouse TIMP-2
mouse TIMP-3
pXMT2
human TIMP-1
mouse TIMP-2
mouse TIMP-3
29
21
REVERSE ZYMOGRAM
29
21
COOMASSIE STAIN

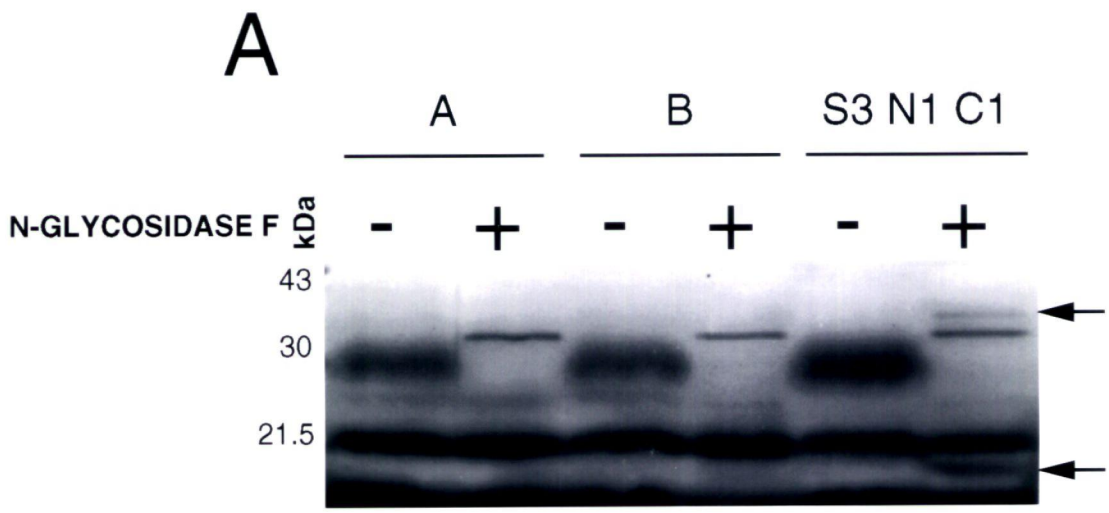
transfected with TIMP-3 had an increased amount of TIMP activity localized to the matrix and migrating mostly at 24 kDa but also detected at 27 kDa. These results demonstrate that TIMPs expressed by transfected COS-1 cells are functional and are also localized correctly, since TIMP-1 and TIMP-2 are detected in the conditioned media while TIMP-3 expressed by transient transfection is localized to the ECM. SDS-PAGE followed by Coomassie staining of samples of matrix and conditioned media demonstrates that the bands detected by reverse zymogram are due to inhibition of the gelatinase A incorporated and not the staining of a large amount of protein present in the sample (Figure 4). Reverse zymography is also highly sensitive, allowing the detection of TIMP protein at levels which are not visible by SDS-PAGE silver staining, as will be discussed in subsequent sections. Collectively, these results demonstrate that transfection of cells followed by reverse zymography of conditioned media and matrix is an effective approach to analyzing TIMPs in terms of their size and gelatinase inhibition.

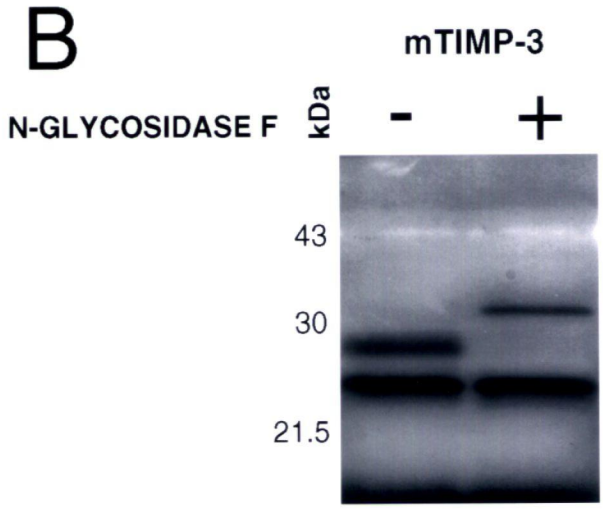
The predicted amino acid sequence of TIMP-1 indicates that there are two N-linked glycosylation sites within its N-terminal domain (Figure 5). TIMP-3 also contains an N-linked glycosylation site at the extreme C-terminus of the protein (Figure 5). To verify that glycosylation at these sites accounts for TIMP activities of less mobility than predicted by the size of the core protein, TIMPs-1 and -3 were treated with N- glycosidase F and analysed by reverse zymography (Figure 6). Deglycosylation of recombinant TIMP-1 resulted in loss

Figure 5. Amino acid sequence comparison of mouse TIMPs-1 and -3. The mature protein N-terminal of each TIMP which includes the first six cysteine residues is enclosed by the brackets. N-linked glycosylation sites are marked with an asterisk. Hyaluronic acid binding motifs in TIMP-3 are underlined. Residues conserved between mouse TIMP-1 and TIMP-3 are in italics, while residues conserved among all TIMPs are in boldface.

```
mTIMP-1 ---MMAPFASLASGI LLLLSLIASSKA [CSC APPHPQTAFCNSDLV 34
mTIMP-3 ---MTPWLG-IVVLI SCWSLGHWGAEA [CTC SPSHPQDAFCNSDIV 31

                      *
mTIMP-1 IRAKFMGSPEINE-- -----TTLYQRYKIK MTKMLKGFKAVGNA ADIRYAYTPVMESLC 94
mTIMP-3 IRAKVVGKKLVKEG- -----PFGTLVYTIK QMKMYRGFSK---M PHVQYIHTEASESLC 91

                 *
mTIMP-1 GYAHKSQNRSEEFLI TGR-LRNGNLHISAC SFLVPWRTLSPAQQR AFS-KTYSAGC]  148
mTIMP-3 GLKIEV--NKYQYLL TGR-VYEGKMYTGIC NFVERWDHITLSQRK GLN-YRYHLGC]  142


mTIMP-1 GVCT VFPCLSIPCKLESDT HCLWTDQVLVGSED- YQSRHFACLPRNPGL 185
mTIMP-3 -NCK IKSCYYLPCFVTSKN ECLWTDMLSNFGYPG YQSKHYACIRQKGGY 196

mTIMP-1 CTWRSLGAR------ ----- 205
                         *
mTIMP-3 CSWYRGWAPPDKSIS NATDP 211
```

Figure 6. Reverse zymography of deglycosylated TIMPs. Conditioned media and matrix from transiently transfected COS-1 cells were analyzed by reverse zymography before and after treatment with an N-linked glycosidase (N-glycosidase F). Deglycosylation of native TIMP-1 resulted in loss of TIMP activity at 28 kDa (A, lanes 2 and 4). Deglycosylation of S3 N1 C1 also resulted in loss of the 28 kDa TIMP activity but gave rise to two novel TIMP activities (arrows). Deglycosylation of TIMP-3 resulted in loss of the 27 kDa TIMP-3 species (B, lane 2). The band located at 30 kDa on reverse zymograms is the N-glycosidase F enzyme which is stained with Coomassie blue.


A
A
B
S3 N1 C1
N-GLYCOSIDASE F
kDa
-
+
-
+
-
+
43
30
21.5


B
mTIMP-3
N-GLYCOSIDASE F
kDa
-
+
43
30
21.5

of the 28 kDa TIMP activity, verifying that it is a glycosylated form of TIMP-1 (Figure 6a). Deglycosylation of recombinant TIMP-3 resulted in loss of the lower mobility TIMP-3 activity (Figure 6b), verifying that the 27 kDa TIMP-3 activity is the glycosylated form of the protein. The identification of both glycosylated and unglycosylated forms of TIMP-1 and TIMP-3 by reverse zymography suggests that glycosylation status does not significantly influence their MMP inhibitory ability.

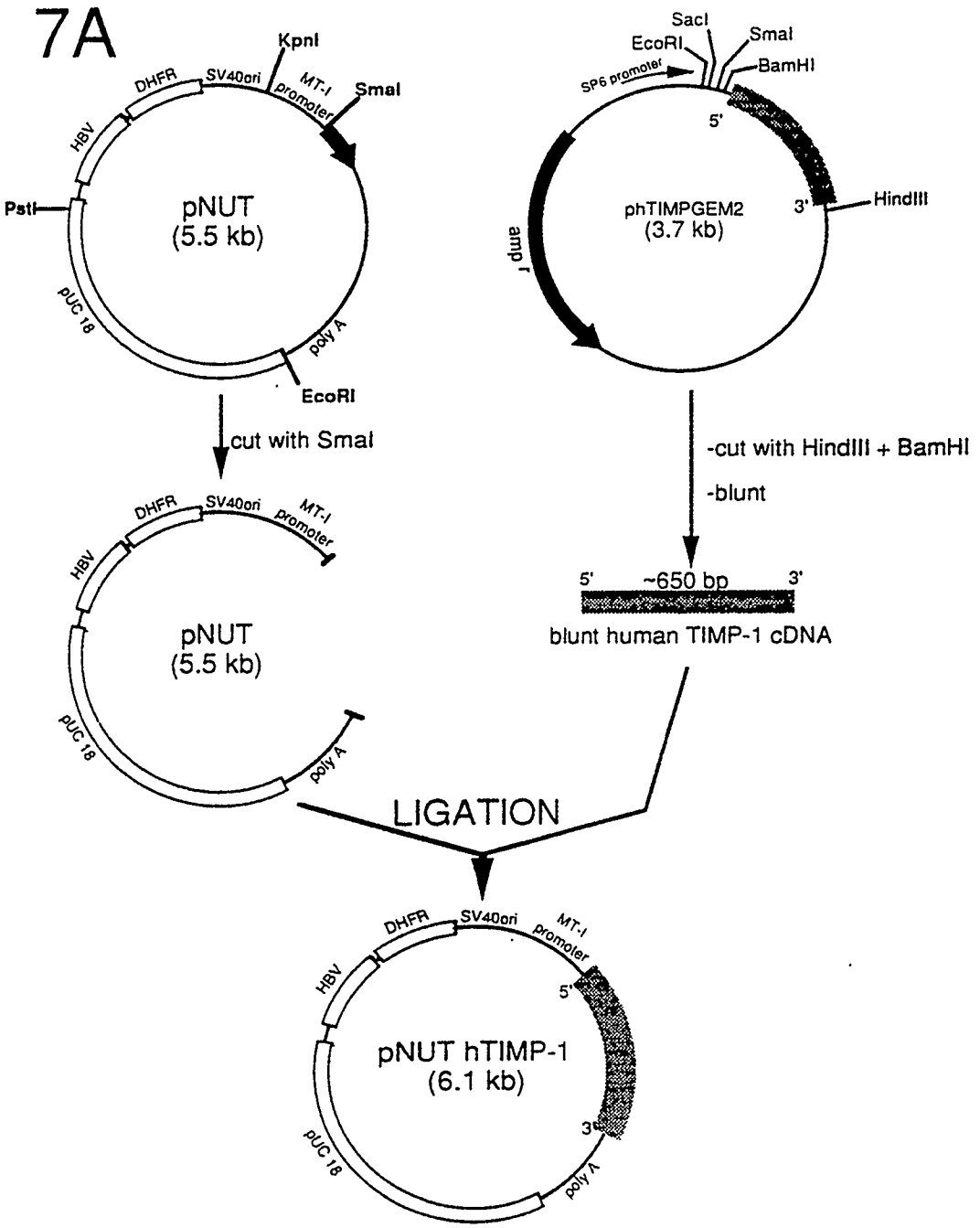
This analysis of COS-1 cell expressed TIMP proteins demonstrates that a number of characteristics of each TIMP can be represented using reverse zymography as a means of analysis. I demonstrated that TIMP-3 is primarily localized to the ECM of cells while TIMP-1 and TIMP-2 proteins are freely diffusible. Also, I demonstrated that discrete mobilities of each TIMP can be detected due to the variable glycosylation state of the protein.

3.2 Construction of BHK Cell Lines Stably Overexpressing Gelatinase B and TIMPs.

In order to facilitate analysis of the TIMP-3 protein in terms of comparison to TIMPs-1 and -2 as well as interactions with gelatinases A and B, individual cell lines stably overexpressing relevant proteins were constructed. These cell lines would serve as a steady source of recombinant proteins to be used in analysis of TIMP-3 and eliminate the need to repeat transient transfections when proteins are needed. The expression system chosen utilizes the pNUT expression vector and a BHK TK22- cell line, both

gifts from Dr. Ross MacGillivray (University of British Columbia). cDNAs encoding human TIMP-1, mouse TIMP-2, mouse TIMP-3, human gelatinase A, and mouse gelatinase B were obtained from previously constructed vectors and blunted prior to ligation into pNUT vector cut with Sma I restriction enzyme (Figure 7). Conditioned media and matrix from cells resistant to methotrexate following transfection were analyzed by gelatin zymography, to verify gelatinase expression, and reverse zymography to verify TIMP expression, while SDS-PAGE followed by silver stain was used to look at the profile of total protein production (Figure 8). Functional TIMP-1 and -2 protein was detected in the conditioned media of transfected cells, named BHK pNUT-hT1 and BHK pNUT-mT2, by both reverse zymogram and silver stain (Figure 8, lanes 3 and 4). TIMP-1 was present as two different mobility activities on the reverse zymogram (Figure 8, lane 3) inferred to be due to the presence of two N-linked glycosylation sites in the N-terminus of the mature TIMP-1 protein (Murphy *et al.*, 1991) such that monoglycosylated TIMP-1 migrates at approximately 24 kDa. Unglycosylated TIMP-1, if present, is not detectable since its molecular weight being very close to that of TIMP-2, thus the two species are not resolved by reverse zymography. TIMP-3, also detected as two distinct mobilities due to the presence of a single N-linked glycosylation site located at the extreme C-terminus of the mature protein (Leco *et al.*, 1994), was localized primarily in the matrix fraction of transfected cells (BHK pNUT-mT3) but some activity was also seen in the conditioned media (Figure 8A).

Figure 7. Schematic of pNUT Expression Vector Construction. Schematic illustration of cloning strategies used in the construction of A) pNUT-human TIMP-1, B) pNUT-mouse TIMP-2, C) pNUT-mouse TIMP-3, D) pNUT-92kDa gelatinase (human gelatinase B), E) pNUT-105kDa (mouse gelatinase B). Illustrations are not to scale. HBV- Hepatitis B virus enhancer; DHFR-Dihydrofolate reductase; SV40ori-Simian virus 40 origin of replication; MT-1 -mouse metallothionein; poly A- polyadenylation sequence.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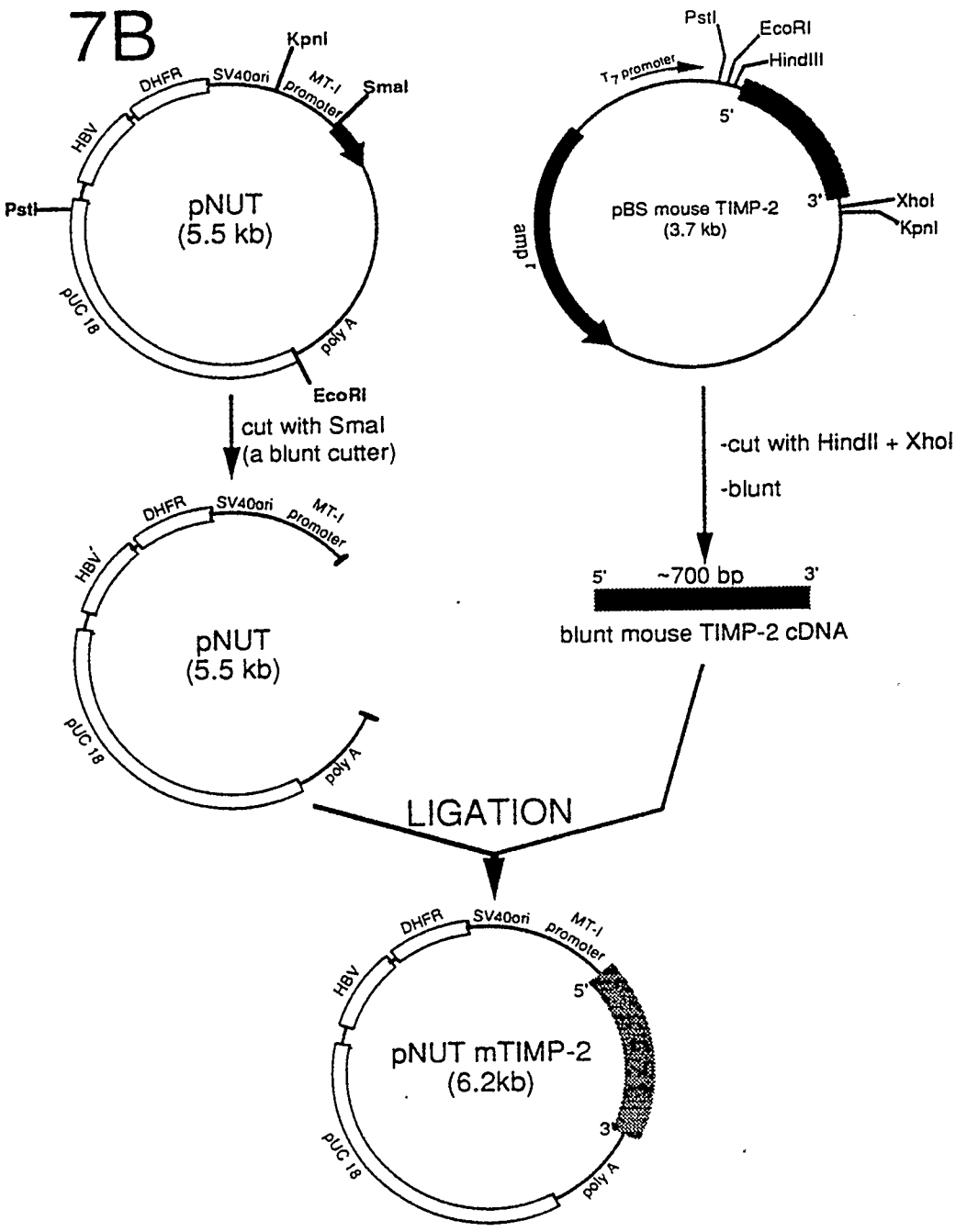
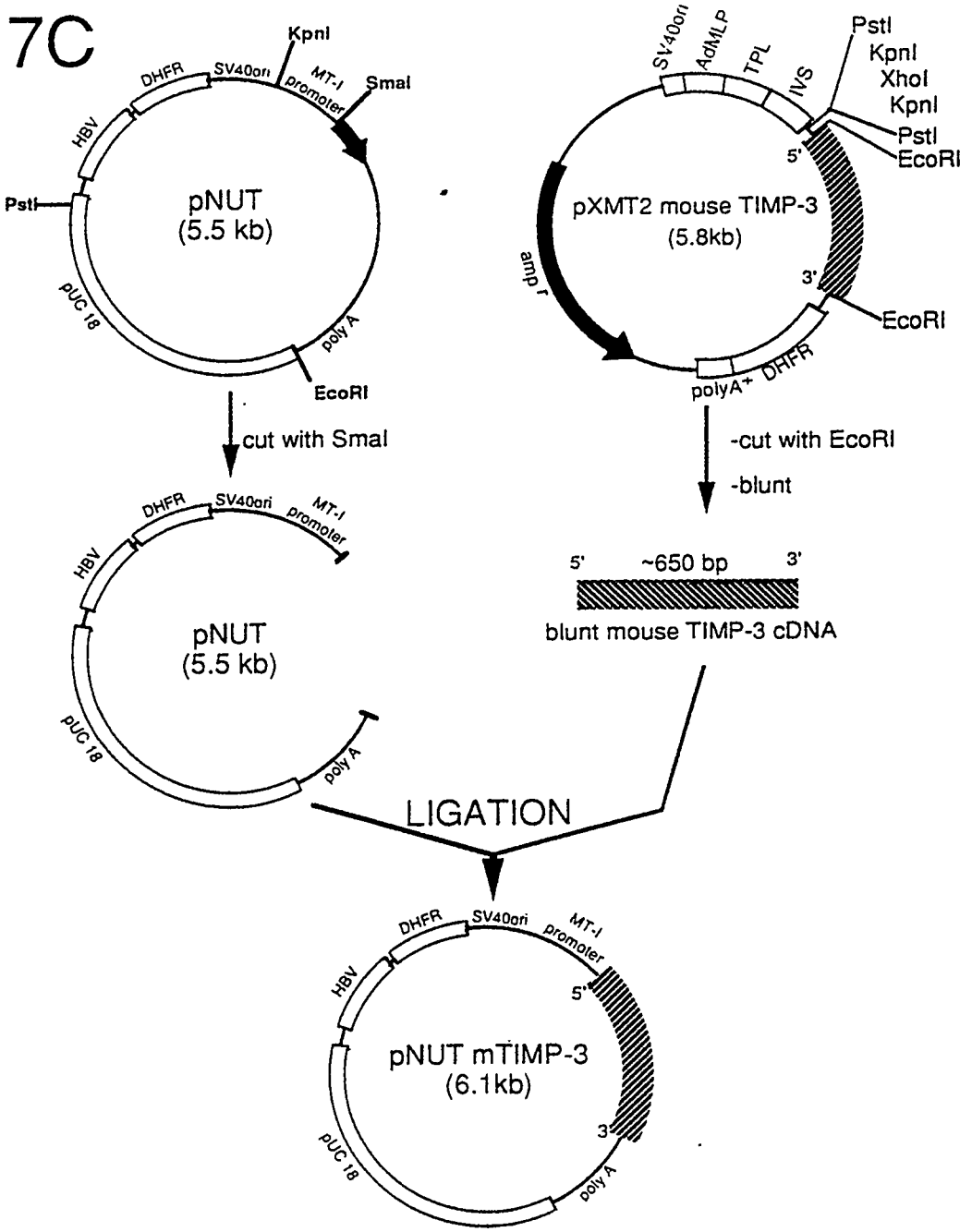
DHFR
SV40ori
MT-I promoter
SmaI
HBV
PstI
pNUT
(5.5 kb)
pUC 18
poly A
EcoRI
cut with SmaI
SacI
EcoRI
SmaI
BamHI
SP6 promoter
5'
phTIMPGEM2
(3.7 kb)
3'
HindIII
amp r
-cut with HindIII + BamHI
-blunt
5'
~650 bp
3'
blunt human TIMP-1 cDNA
LIGATION
pNUT hTIMP-1
(6.1 kb)


7B
KpnI
DHFR
SV40ori
MT-I promoter
SmaI
HBV
PstI
pNUT
(5.5 kb)
pUC 18
poly A
EcoRI
cut with SmaI
(a blunt cutter)
PstI
EcoRI
HindIII
T7 promoter
5'
pBS mouse TIMP-2
(3.7 kb)
3'
XhoI
KpnI
amp r
-cut with HindII + XhoI
-blunt
5'
~700 bp
3'
blunt mouse TIMP-2 cDNA
DHFR
SV40ori
MT-I promoter
HBV
pNUT
(5.5 kb)
pUC 18
poly A
LIGATION
DHFR
SV40ori
MT-I promoter
HBV
5'
pNUT mTIMP-2
(6.2kb)
3'
pUC 18
poly A


7C
KpnI
DHFR
SV40ori
MT-I promoter
SmaI
HBV
PstI
pNUT
(5.5 kb)
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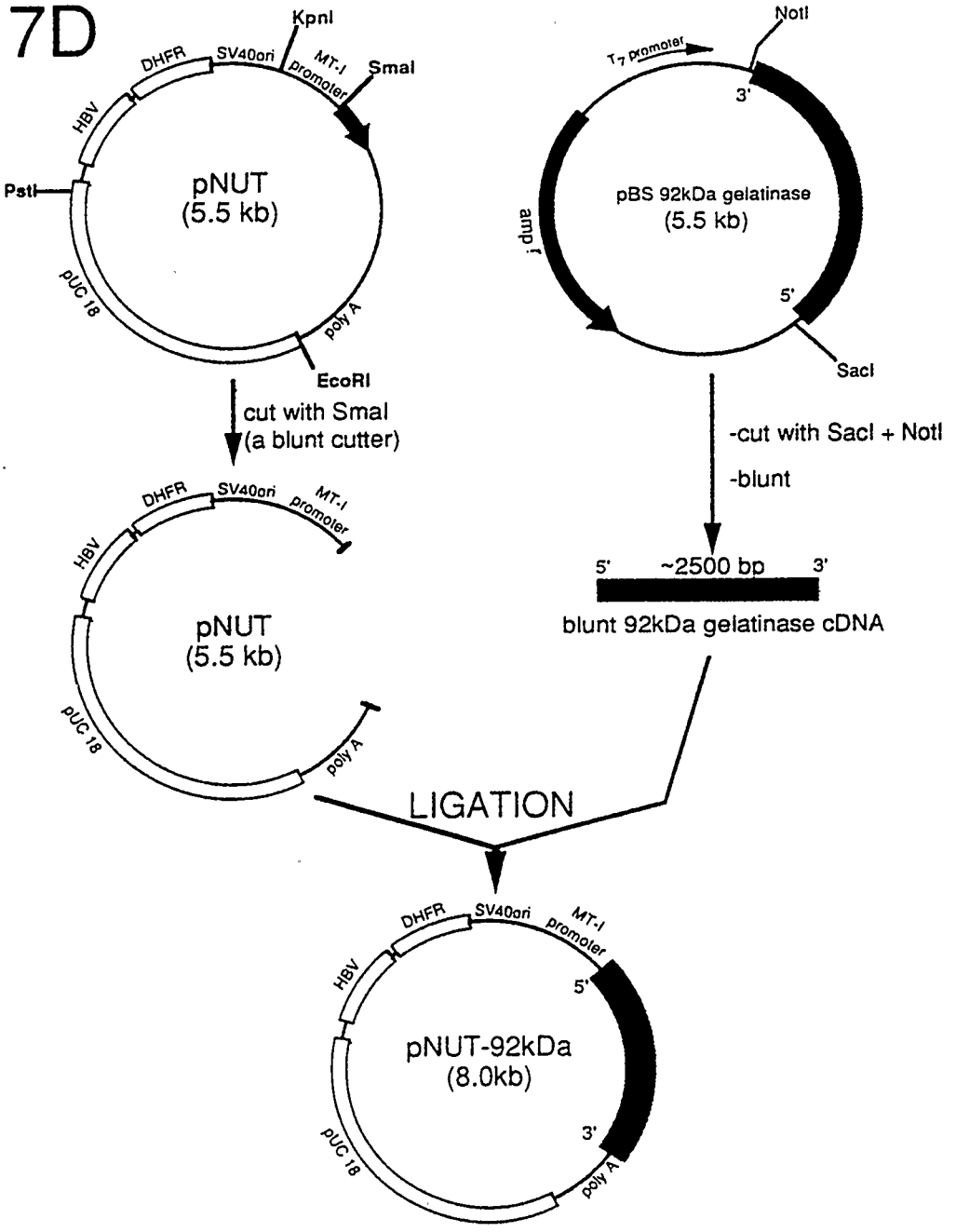
poly A
EcoRI
cut with SmaI
SV40ori
AdMLP
TPL
IVS
PstI
KpnI
XhoI
KpnI
PstI
EcoRI
5'
pXMT2 mouse TIMP-3
(5.8kb)
amp r
3'
EcoRI
polyA+
DHFR
-cut with EcoRI
-blunt
5'
~650 bp
3'
blunt mouse TIMP-3 cDNA
DHFR
SV40ori
MT-I promoter
HBV
pNUT
(5.5 kb)
pUC 18
poly A
LIGATION
DHFR
SV40ori
MT-I promoter
HBV
5'
pNUT mTIMP-3
(6.1kb)
3'
pUC 18
poly A

7D


KpnI
SmaI
DHFR
SV40ori
MT-I promoter
HBV
PstI
pNUT
(5.5 kb)
pUC 18
poly A
EcoRI
cut with SmaI
(a blunt cutter)
NotI
T7 promoter
3'
pBS 92kDa gelatinase
(5.5 kb)
amp r
5'
SacI
-cut with SacI + NotI
-blunt
5'
~2500 bp
3'
blunt 92kDa gelatinase cDNA
LIGATION
pNUT-92kDa
(8.0kb)

7E

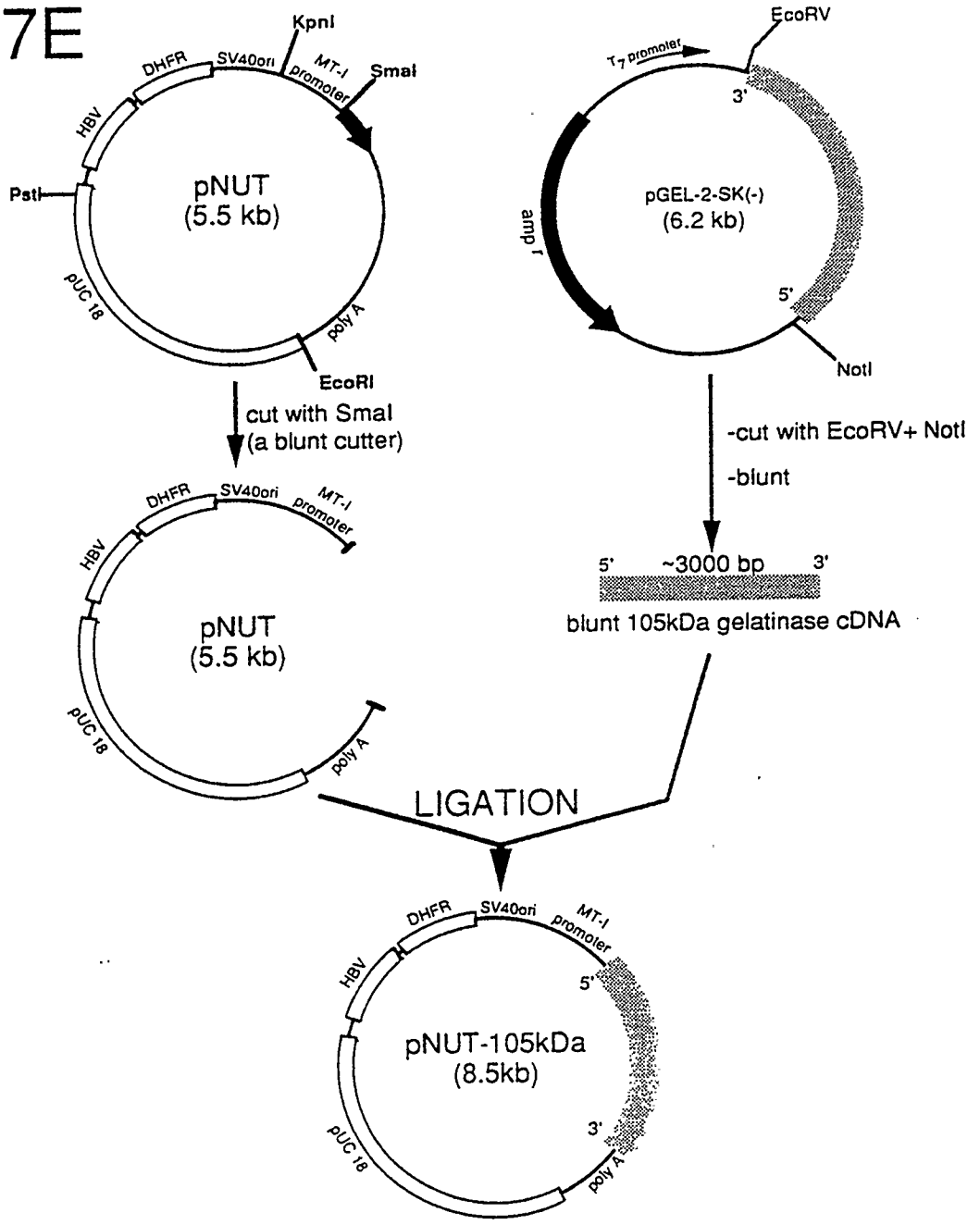
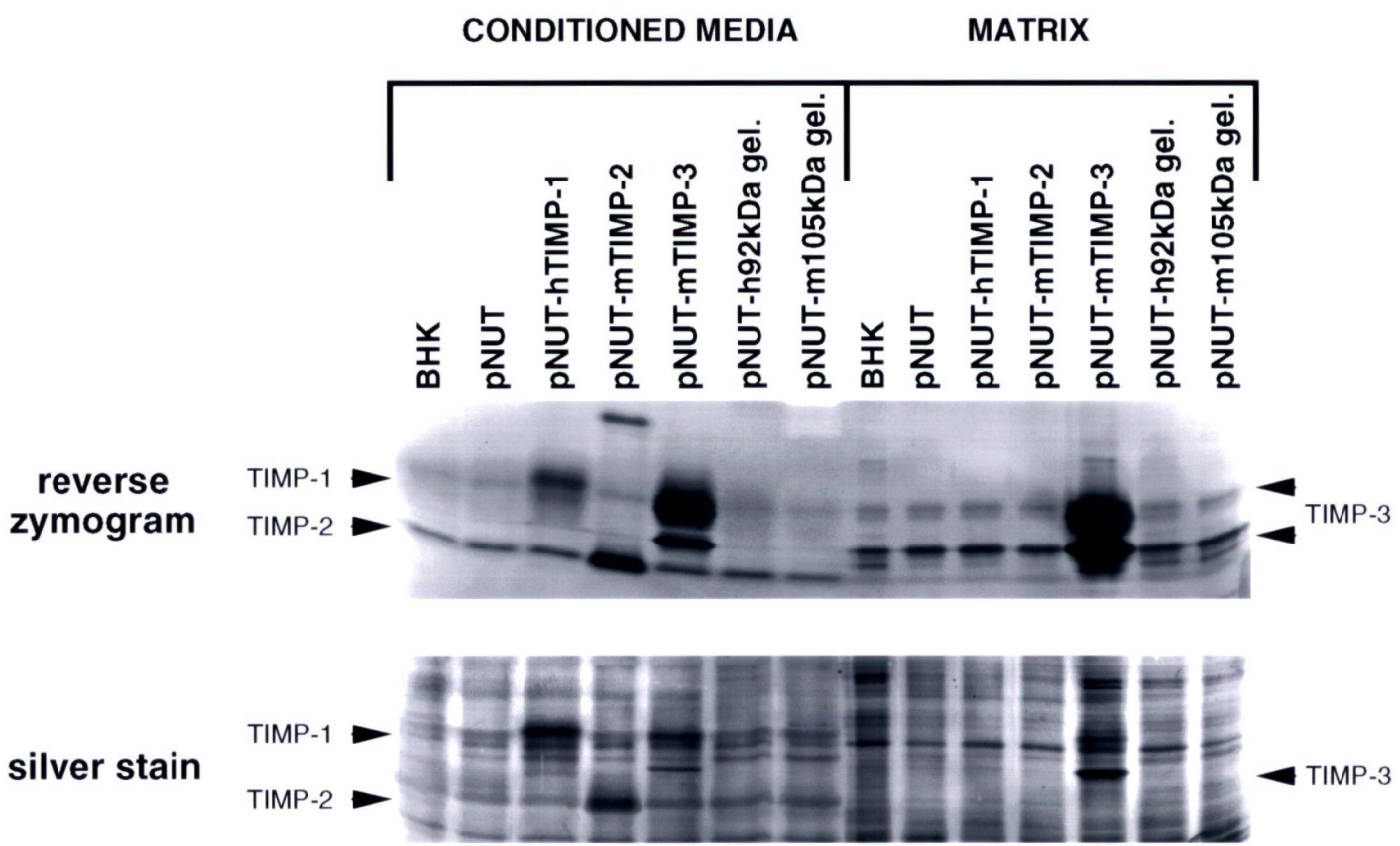
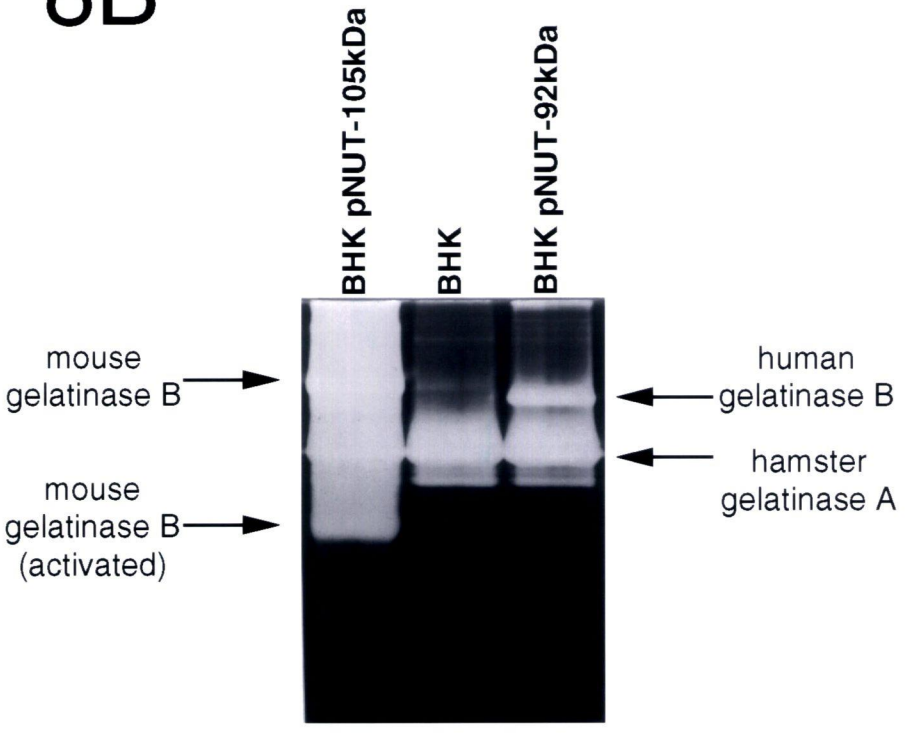

KpnI
DHFR
SV40ori
MT-I promoter
SmaI
HBV
PstI
pNUT
(5.5 kb)
pUC 18
poly A
EcoRI
cut with SmaI
(a blunt cutter)
EcoRV
T7 promoter
3'
pGEL-2-SK(-)
(6.2 kb)
amp r
5'
NotI
-cut with EcoRV+ NotI
-blunt
5'
~3000 bp
3'
blunt 105kDa gelatinase cDNA
LIGATION
pNUT-105kDa
(8.5kb)

Figure 8. Reverse zymography and silver stain analysis of conditioned media and matrix from stably transfected BHK cell lines. **A.** Following selection for transfected cells with methotrexate, cells were placed under serum-free medium for 24 hours before analysis of conditioned media and matrix by reverse zymography (A, top) and SDS-PAGE silver staining (A, bottom).


CONDITIONED MEDIA
MATRIX
BHK
pNUT
pNUT-hTIMP-1
pNUT-mTIMP-2
pNUT-mTIMP-3
pNUT-h92kDa gel.
pNUT-m105kDa gel.
BHK
pNUT
pNUT-hTIMP-1
pNUT-mTIMP-2
pNUT-mTIMP-3
pNUT-h92kDa gel.
pNUT-m105kDa gel.
reverse zymogram
TIMP-1
TIMP-2
TIMP-3
silver stain
TIMP-1
TIMP-2
TIMP-3

8B


BHK pNUT-105kDa
BHK
BHK pNUT-92kDa
mouse gelatinase B
mouse gelatinase B (activated)
human gelatinase B
hamster gelatinase A

Expression of mouse or human gelatinase B was verified by zymography (Figure 8B) of conditioned media from BHK pNUT-h92k and BHK pNUT-m105k cell lines. Untransfected BHK cells used for construction of cell lines were found to express high levels of gelatinase A as detected by zymogram (Figure 8B), and were used as a source of gelatinase A for reverse zymography. From these resultsIconclude thatIhave constructed stably transfected cell lines which individually overexpress human TIMP-1, mouse TIMP-2, mouse TIMP-3, mouse gelatinase B, and human gelatinase B. This analysis of these recombinant proteins demonstrates that the proteins are functional in terms of MMP inhibition and are also the correct size, allowing me to conclude that they are accurate representatives of *in vivo* proteins.

3.3 Differential Reverse Zymography

At the time that these studies were initiated, no information was available concerning the capacity of TIMP-3 to inhibit other members of the MMP family, and no evaluation of the relative abilities of TIMPs -1, -2, and -3 had been undertaken. A reverse zymography protocol using gelatinases A and B was developed in order to characterize the relative ability of the TIMPs to inhibit the gelatinases. Conditioned media from RSV-transformed chicken fibroblasts (a gift from Dr. Susan Hawkes) was initially used as a source of gelatinase A. Conditioned media from an SV MYC CHO cell line observed to produce increased levels of gelatinase B as indicated by zymography (Dickinson Laing, unpublished results) was used as a source of gelatinase B. Following

electrophoresis, incubation, and Coomassie staining of reverse zymograms using either gelatinase A or B as the incorporated gelatinolytic activity, it was observed that TIMP-3 could inhibit both gelatinases effectively, while TIMPs-1 and -2 were not able to inhibit gelatinase B as effectively as they inhibited gelatinase A (Figure 9).

These experiments were repeated using gelatinases produced by stably transfected BHK cells in reverse zymograms analyzing COS-1 (Figure 10A) or BHK (Figure 10B) expressed TIMPs. In reverse zymograms using mouse gelatinase B, hamster gelatinase A, and chicken gelatinase A, mouse TIMP-3 is shown to inhibit the gelatinases with comparable efficiency, while TIMP-1 and TIMP-2 are not able to inhibit gelatinase B as effectively. Time course reverse zymography of both COS-1 and BHK expressed TIMPs also clearly demonstrated a distinct superiority in the ability of TIMP-3 to inhibit gelatinase B (Figure 11). A parallel silver stained gel of the samples used for reverse zymography revealed that the amounts of the three TIMPs were equivalent, establishing that the consistently superior gelatinase B inhibitory activity of TIMP-3 was not simply due to overabundance of TIMP-3 protein (Figure 10A, 10B).

In order to ensure that these observations were not due to disparate amounts of gelatinase incorporated into each reverse zymogram, a zymogram was prepared using the same relative amounts of conditioned media to be used in reverse zymography (Figure 12A). The amount of gelatin degradation, as

Figure 9. Reverse zymography incorporating chicken gelatinase A or hamster gelatinase B. The ability of TIMPs to inhibit gelatinases A and B was assayed using reverse zymography. Conditioned media and matrix from COS-1 cells transiently transfected with TIMPs-1, -2, and -3 using the pXMT2 expression vector was electrophoresed on reverse zymograms in which conditioned media containing chicken gelatinase A (top) or hamster gelatinase B (bottom) was incorporated as a source of gelatinolytic activity. All three TIMPs are strongly represented on the reverse zymogram using gelatinase A while only TIMP-3 is strongly represented when gelatinase B is used.

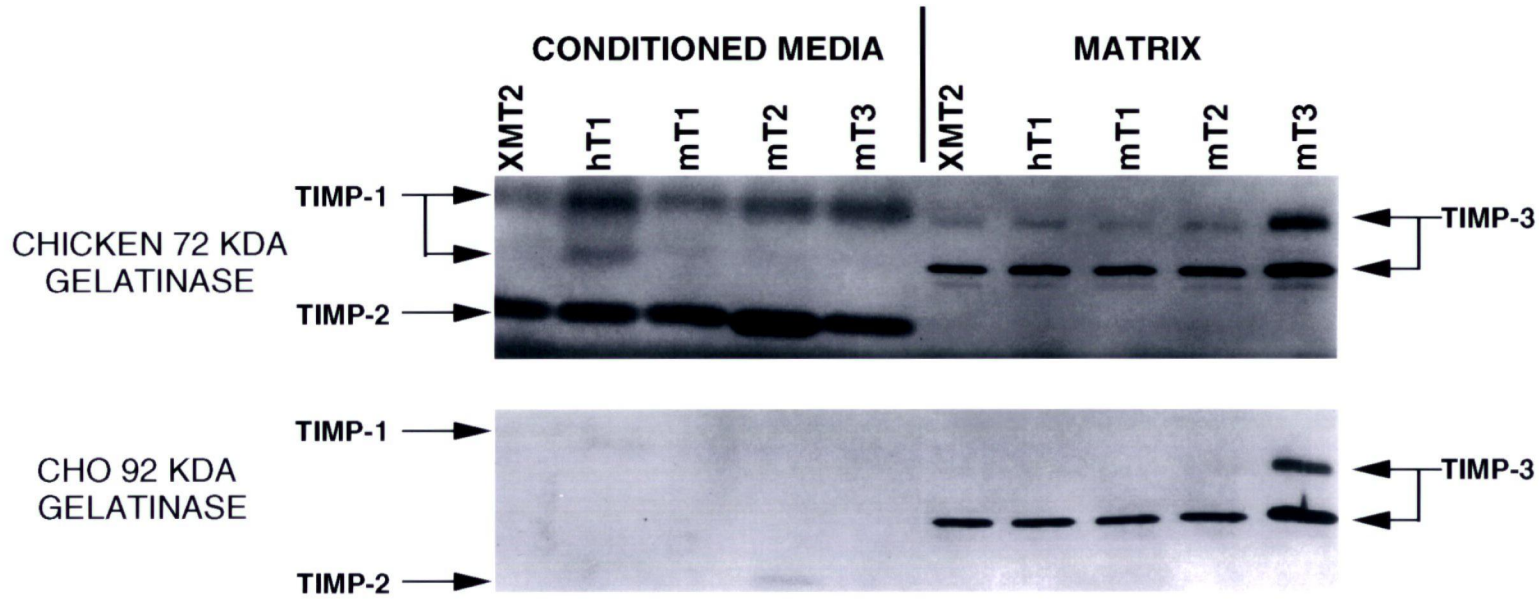
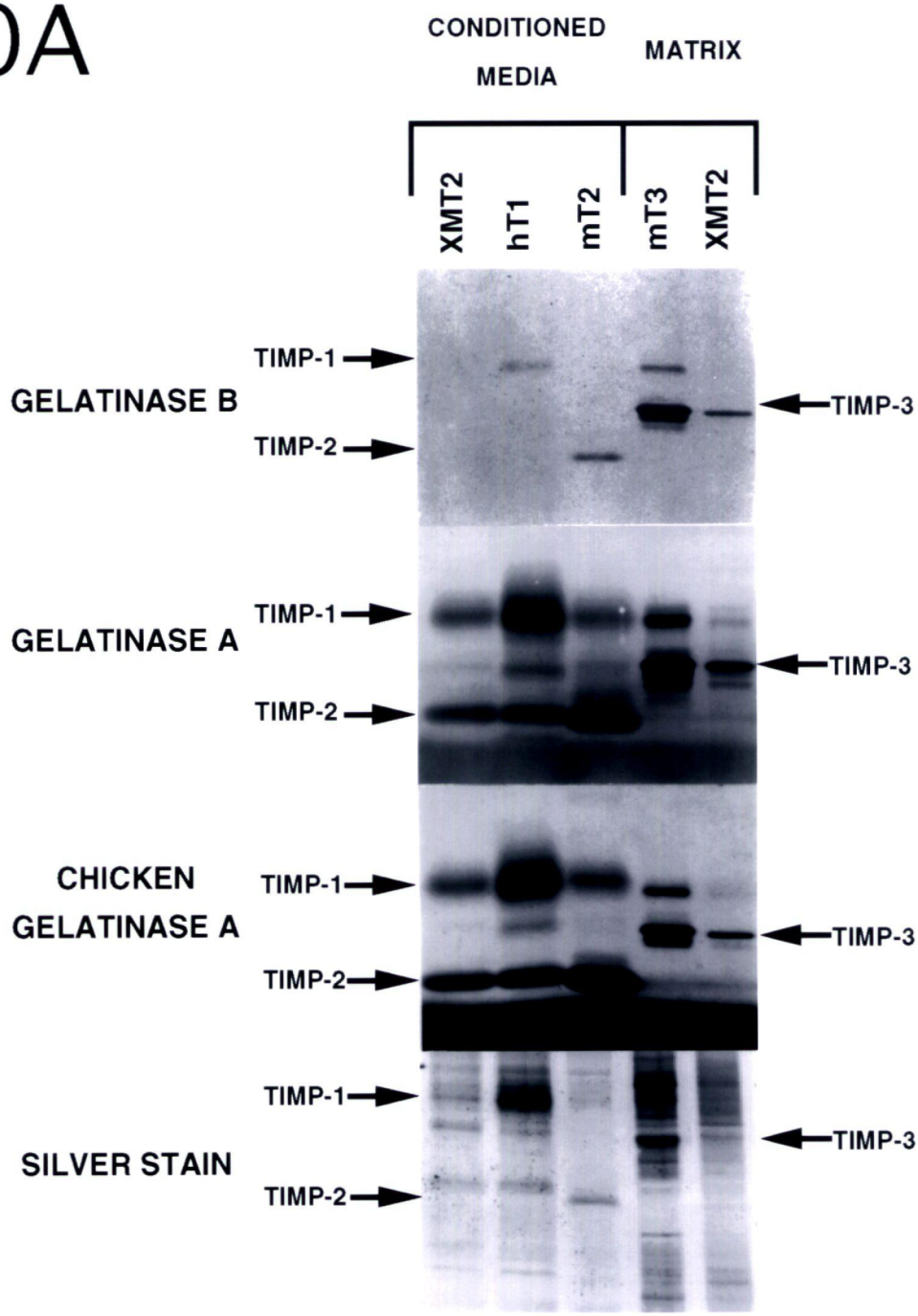

CONDITIONED MEDIA
MATRIX
XMT2
hT1
mT1
mT2
mT3
XMT2
hT1
mT1
mT2
mT3
TIMP-1
CHICKEN 72 KDA GELATINASE
TIMP-2
TIMP-3
TIMP-1
CHO 92 KDA GELATINASE
TIMP-2
TIMP-3

Figure 10. Differential reverse zymography using proteins expressed in COS-1 and BHK cell lines. Conditioned media and matrix from transiently transfected COS-1 cells (A) or stably transfected BHK cells (B) was harvested and analyzed by reverse zymography using either hamster gelatinase A, chicken gelatinase A, or mouse gelatinase B as gelatinolytic activity. TIMP-3 is observed to inhibit both gelatinases effectively while TIMPs-1 and -2 cannot block gelatinase B as well as they inhibit gelatinase A. SDS-PAGE followed by silver stain indicates relative amounts of each TIMP present. In (B), TIMP-3 in was obtained from matrix as described previously.

10A


CONDITIONED MEDIA
MATRIX
XMT2
hT1
mT2
mT3
XMT2
GELATINASE B
TIMP-1
TIMP-2
TIMP-3
GELATINASE A
TIMP-1
TIMP-2
TIMP-3
CHICKEN GELATINASE A
TIMP-1
TIMP-2
TIMP-3
SILVER STAIN
TIMP-1
TIMP-2
TIMP-3

10B

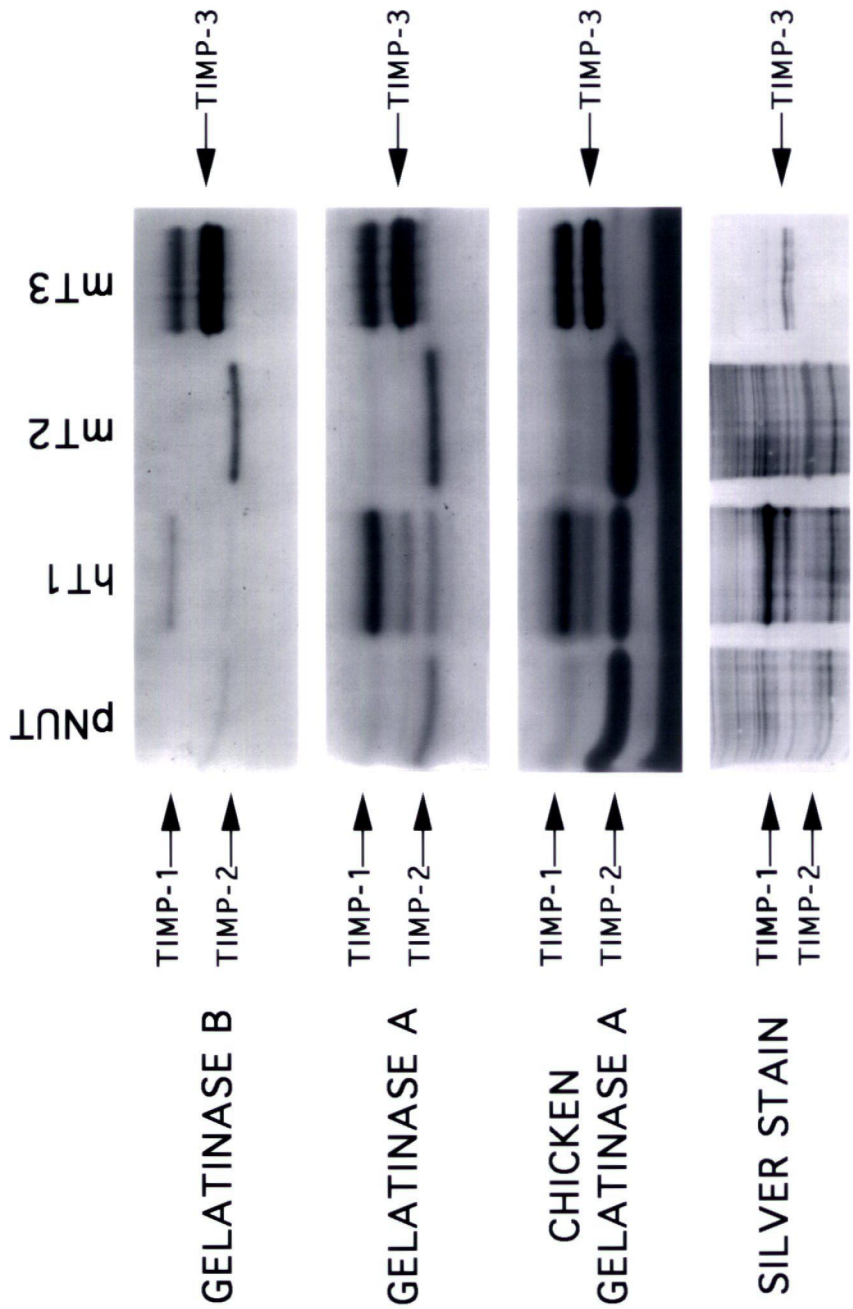

pNUT
hT1
mT2
mT3
TIMP-3
TIMP-1
TIMP-2
GELATINASE B
GELATINASE A
CHICKEN GELATINASE A
SILVER STAIN

Figure 11. Laser densitometric analysis of the time course of reverse zymography using gelatinases A and B. Reverse zymograms containing equivalent amounts of gelatinase A or B and repeat loadings of identical amounts of conditioned media or matrix containing TIMP-1, TIMP-2, or TIMP-3 were cut into strips such that each strip had the same amount of each TIMP on it. Individual strips from each type of reverse zymogram were removed from digestion conditions and fixed at four hour intervals. The amount of activity present for each TIMP at each time point was measured by laser densitometry and plotted. 100% activity is defined as the maximum amount of TIMP activity measured by the laser densitometer for each TIMP. The major 28 kDa band of TIMP-1 activity was measured as TIMP-1 activity and other species due to different N-linked glycosylation states were disregarded. Glycosylated and unglycosylated TIMP-3 were measured separately.

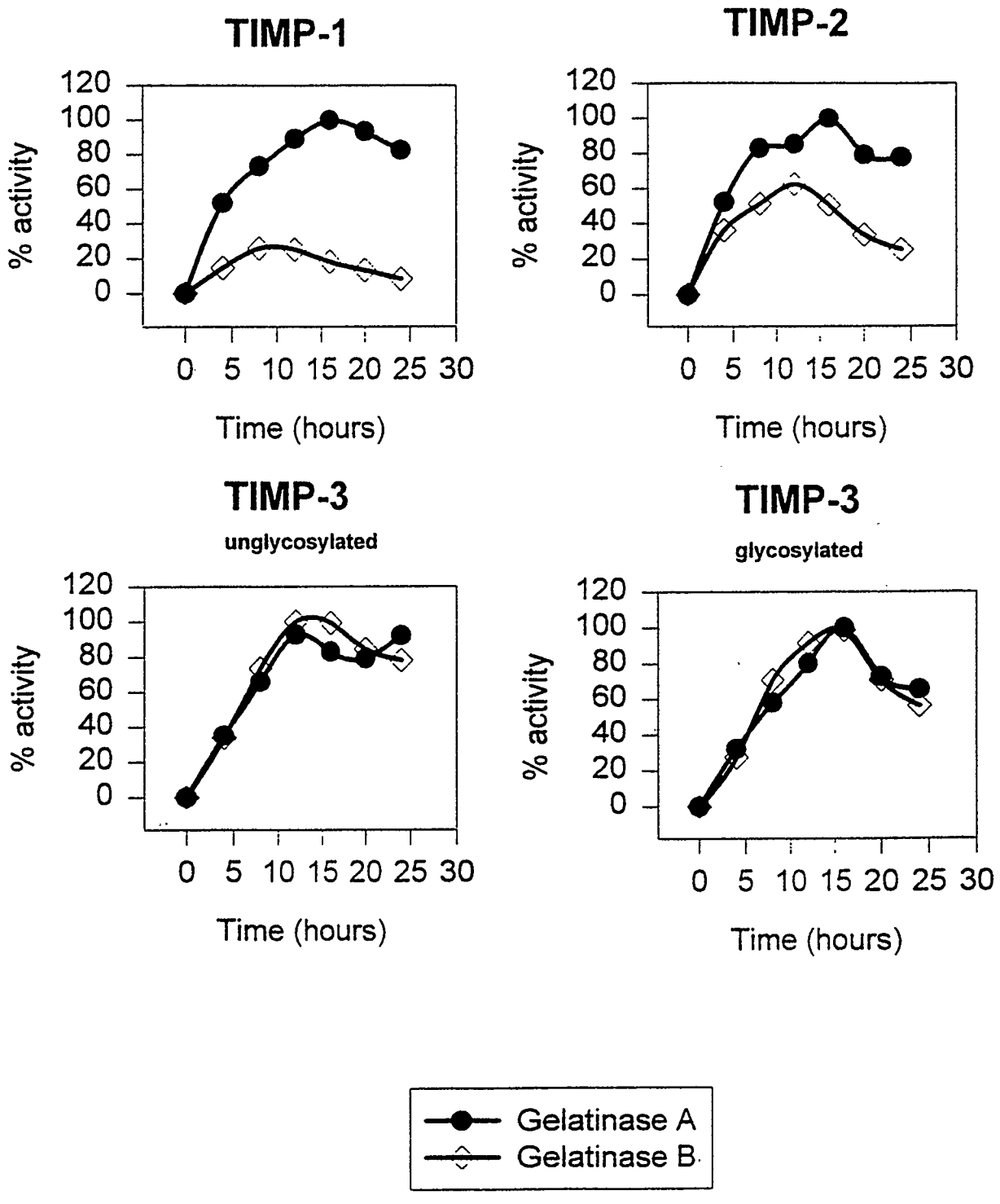
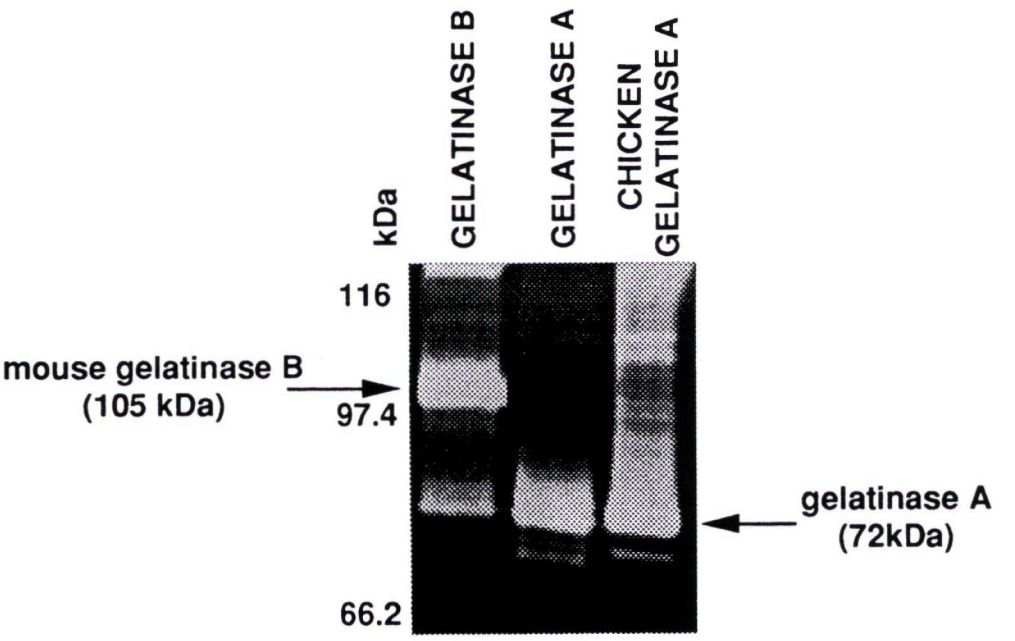
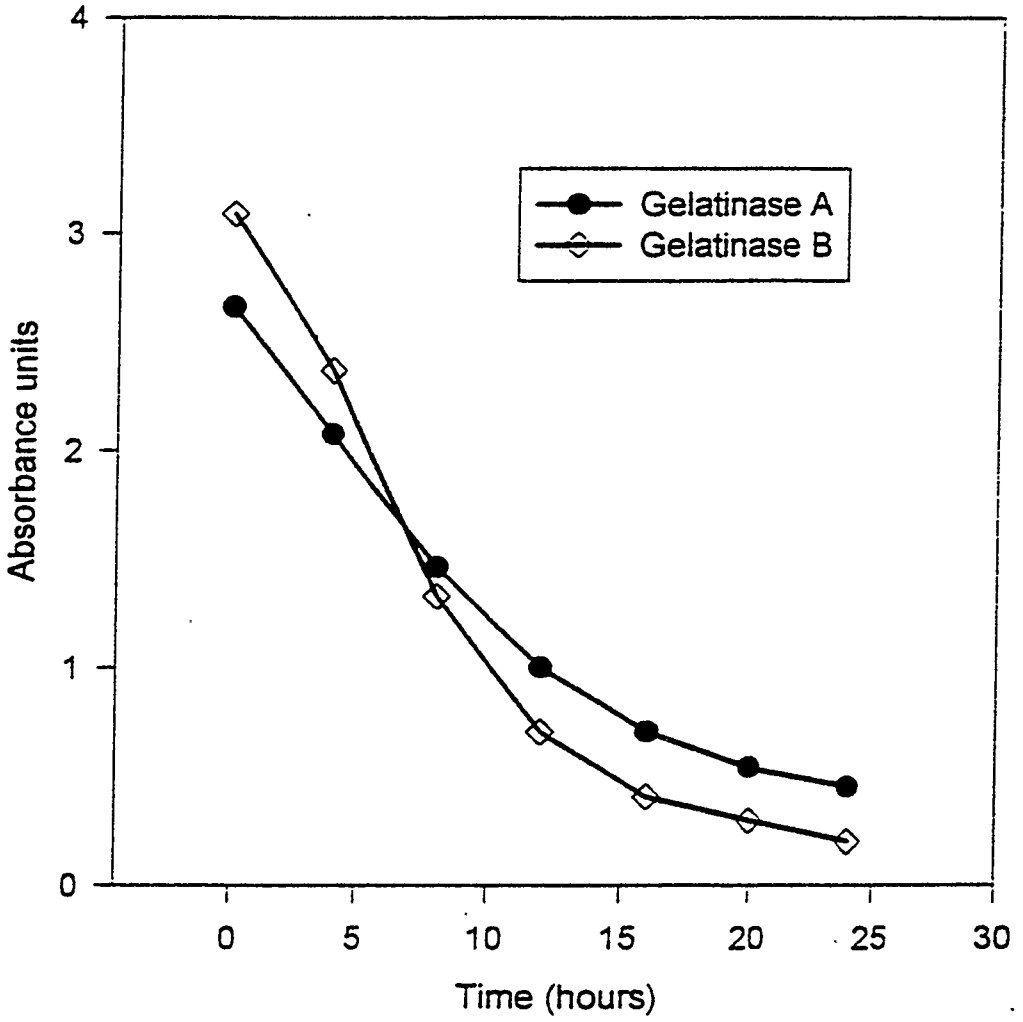

TIMP-1
% activity
120
100
80
60
40
20
0
0 5 10 15 20 25 30
Time (hours)
TIMP-2
% activity
120
100
80
60
40
20
0
0 5 10 15 20 25 30
Time (hours)
TIMP-3
unglycosylated
% activity
120
100
80
60
40
20
0
0 5 10 15 20 25 30
Time (hours)
TIMP-3
glycosylated
% activity
120
100
80
60
40
20
0
0 5 10 15 20 25 30
Time (hours)
Gelatinase A
Gelatinase B

Figure 12. Determination of gelatinolytic activity used in differential reverse zymography. Two approaches were used to demonstrated that equivalent amounts of gelatinase A and B were used in reverse zymograms that indicated differential inhibition by TIMPs (Figure 9). **A.** The same relative amounts of conditioned media incorporated into reverse zymograms were analyzed by zymography to measure amount of gelatinase A and B activity . The amount of total gelatin degradation, as indicated by estimation of the size of clear areas on the zymogram, is approximately equal by conditioned media containing gelatinase A or B. **B.** The rate of gelatin digestion within the reverse zymograms that generated the time course data in Figure 11 was measured as the baseline absorbance of each gel slice in areas not containing any TIMP activities.

12 A


GELATINASE B
GELATINASE A
CHICKEN GELATINASE A
kDa
116
97.4
66.2
mouse gelatinase B
(105 kDa)
gelatinase A
(72kDa)

12B

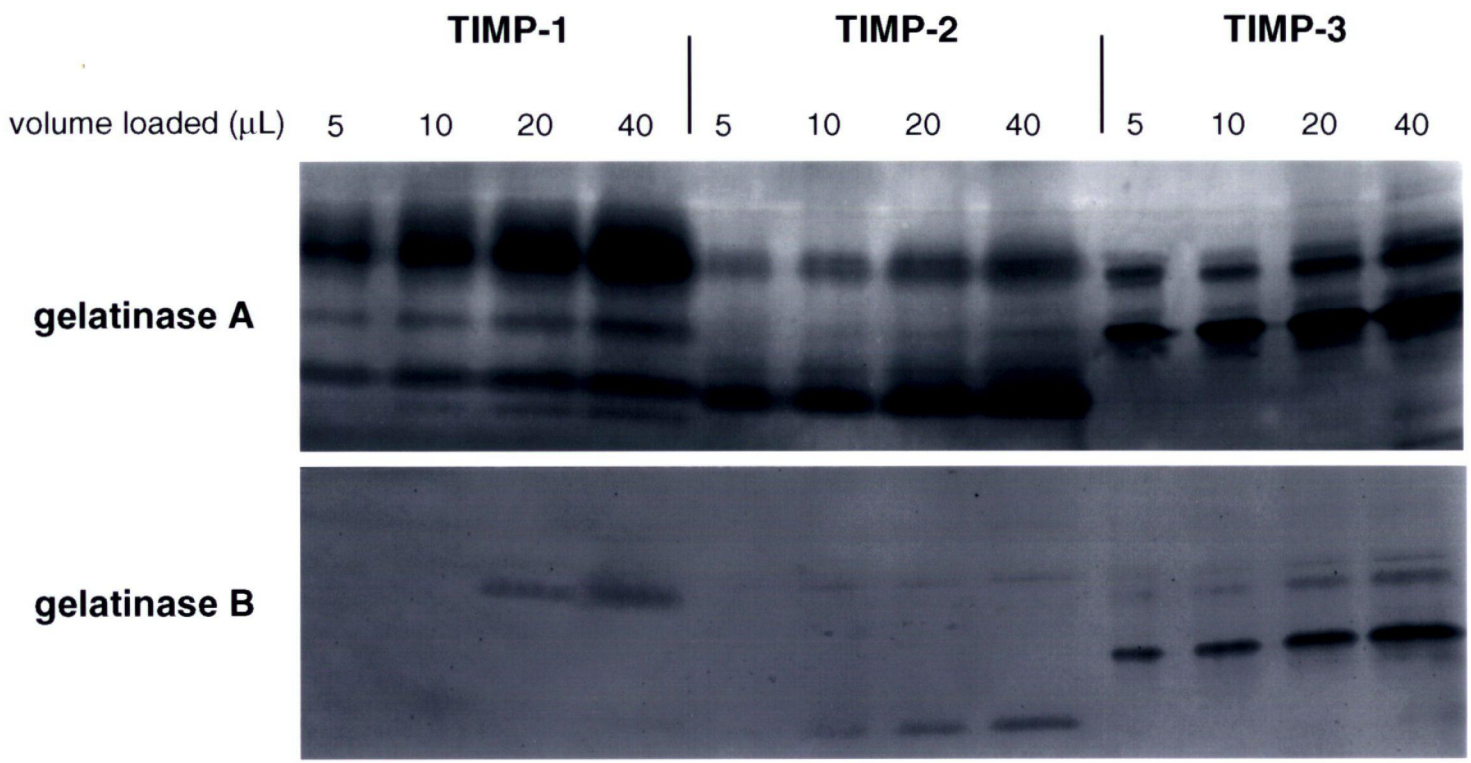

Gelatinase A
Gelatinase B
Absorbance units
Time (hours)
0
1
2
3
4
0
5
10
15
20
25
30

indicated by areas not stained, is similar for both gelatinases, suggesting that comparable amounts of gelatinase activity are present. In a second approach used to verify that equivalent amounts of each gelatinase activity were used, amount of gelatin digestion in reverse zymograms containing the same relative amounts of conditioned media that were analyzed by zymogram (Figure 10a) was determined at four hour time points over 24 hours by laser densitometry (Figure 12B). Measurement of gelatin breakdown as percent absorbance revealed similar digestion kinetics for both gelatinase A and B incorporated into a reverse zymogram.

The two dimensional nature of reverse zymography allows gelatinase activity in a given portion of the gel to be saturated by TIMP if enough TIMP is present. In order to allow us to infer differences in the relative inhibition of gelatinases by TIMPs it is necessary to ensure that the amount of TIMP does not saturate the gelatinase present, and that the TIMP would be more represented on the reverse zymogram if more were used. This was tested by carrying out reverse zymography with a range of volumes of conditioned media or matrix around the volume used for the differential reverse zymogram (Figure 13), demonstrating that the amount of each TIMP used was not saturating local gelatinase in the reverse zymogram.

In conclusion, these results demonstrate that reverse zymography analysis of the relative ability of the TIMPs to block the catalytic activity of the gelatinases suggests that TIMP-3 is intrinsically able to inhibit both gelatinase

Figure 13. Reverse zymography of different amounts of TIMP-1, TIMP-2, and TIMP-3. Increasing amounts of conditioned media (TIMP-1 and TIMP-2) and matrix (TIMP-3) from stably transfected BHK cell lines was analyzed by reverse zymography using equivalent amounts of gelatinase A or B. 20 μl of conditioned media and 5μl of matrix is equivalent to the amount used in differential reverse zymography. Stronger representation of each TIMP on both types of reverse zymogram when a larger amount was loaded indicates that the amount used for differential reverse zymography does not saturate local gelatinase activity.


TIMP-1
TIMP-2
TIMP-3
volume loaded (µL)
5
10
20
40
5
10
20
40
5
10
20
40
gelatinase A
gelatinase B

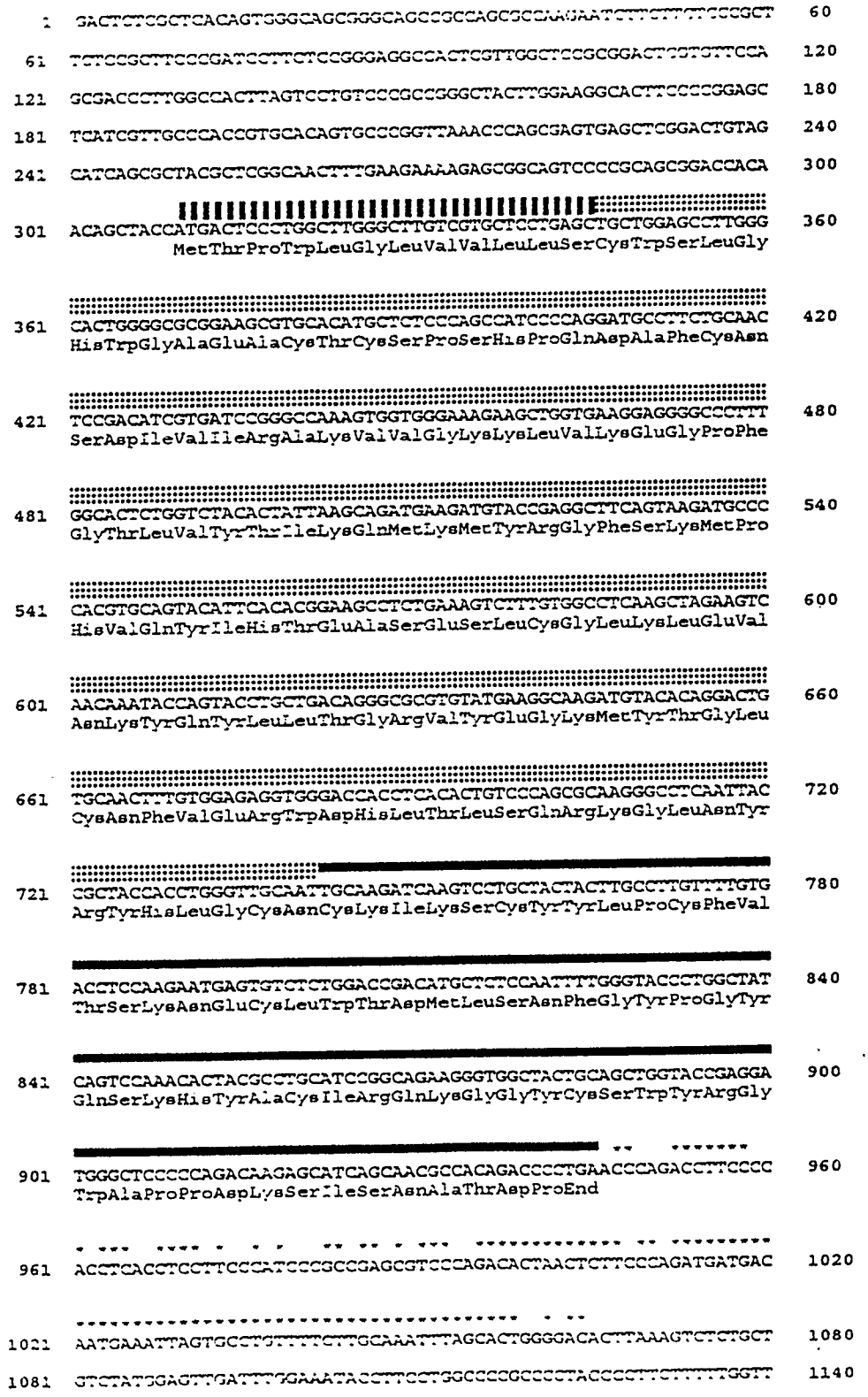
A and gelatinase B comparably, while TIMP-1 and TIMP-2 are not able to block the gelatinolytic activity of gelatinase B as effectively as they do gelatinase A. This is a novel functional characteristic of TIMP-3 which serves to further distinguish it from TIMP-1 and TIMP-2.

3.4 Analysis of TIMP-3 domain function.

I have demonstrated that TIMP-3 can be distinguished from the other TIMPs in at least two aspects of its activity: its association with the matrix and its superior ability to inhibit gelatinase B. In order to identify the regions of TIMP-3 which confer its unique properties, the distinguishing regions of TIMP-3 mRNA and protein were first studied. There are at least two such distinguishing regions whose functions are not known: one is the 3'-untranslated region (UTR) of TIMP-3, an AU rich element which is highly conserved across species and distinct from comparable regions of TIMP-1 and TIMP-2 (Figure 14); the other is the signal peptide of TIMP-3 which is distinct in its lack of leucine residues (Figure 5). The conservation of the location of twelve cysteine residues across all of the TIMP family members characterized to date suggests that the modular structure of the molecule imposed by the intrachain disulphide bridges formed by these residues will also be conserved (Figure 2). This modularity was exploited to assess the role of the TIMP-3 N- and C-terminal domains in its activity.

My initial studies expressing TIMP-3 in COS-1 cells (Figure 4) used a TIMP-3 cDNA which contained the 635 bp coding region as well as ~160 bp

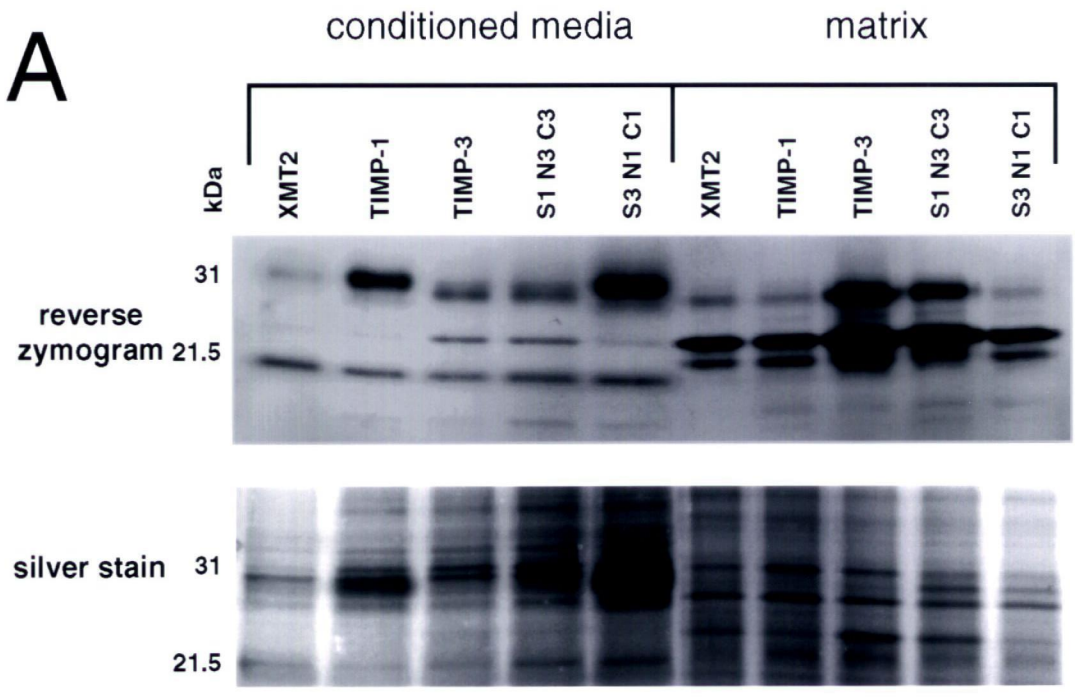
Figure 14. Domains of the TIMP-3 protein and mRNA. cDNA sequence defining the signal peptide (IIII), the N-terminal (▒▒▒), and the C-terminal (▬▬) of TIMP-3 are indicated. Regions of the 3'-UTR that are conserved between chicken and mouse TIMP-3 are also indicated (*).

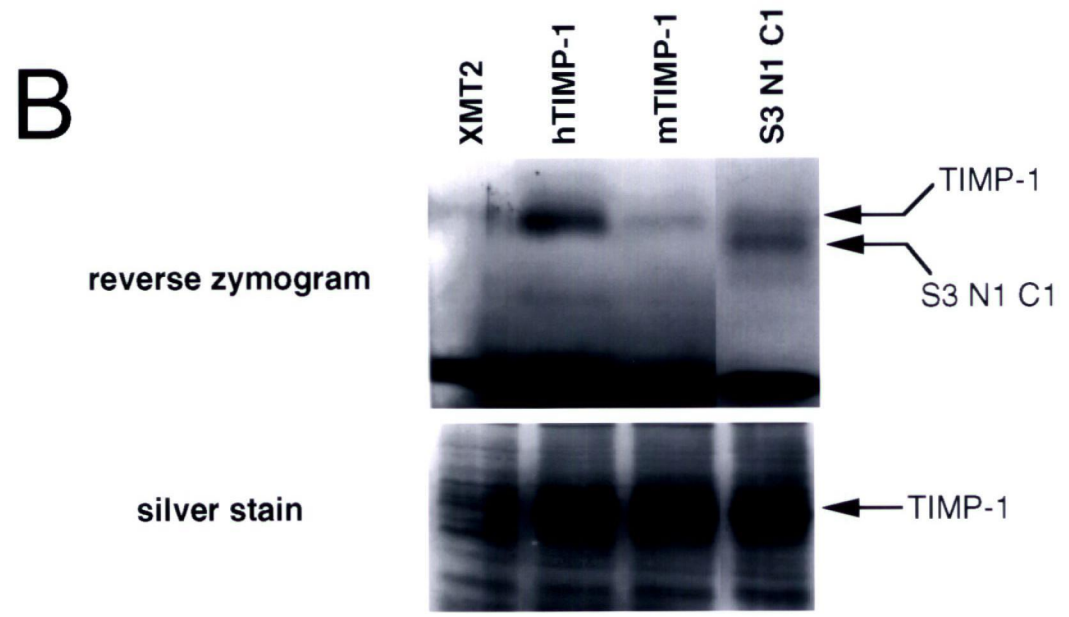

1 GACTCTCGCTCACAGTGGGCAGCGGGCAGCCGCCAGCGCCAAGAATCTTCTTCTCCCGCT 60
61 TCTCCGCTTCCCGATCGTTCTCCGGGAGGCCACTCGTTGGCTCCGCGGACTCGTGTTCCA 120
121 GCGACCCTTGGCCACTTAGTCCTGTCCCGCCGGGCTACTTGGAAGGCACTTCCCCGGAGC 180
181 TCATCGTTGCCCACCGTGCACAGTGCCCGGTTAAACCCAGCGAGTGAGCTCGGACTGTAG 240
241 CATCAGCGCTACGCTCGGCAACTTTGAAGAAAAGAGCGGCAGTCCCCGCAGCGGACCACA 300
301 ACAGCTACCATGACTCCCTGGCTTGGGCTTGTCGTGCTCCTGAGCTGCTGGAGCCTTGGG 360
MetThrProTrpLeuGlyLeuValValLeuLeuSerCysTrpSerLeuGly
361 CACTGGGGCGCGGAAGCGTGCACATGCTCTCCCAGCCATCCCCAGGATGCCTTCTGCAAC 420
HisTrpGlyAlaGluAlaCysThrCysSerProSerHisProGlnAspAlaPheCysAsn
421 TCCGACATCGTGATCCGGGCCAAAGTGGTGGGAAAGAAGCTGGTGAAGGAGGGGCCCTTT 480
SerAspIleValIleArgAlaLysValValGlyLysLysLeuValLysGluGlyProPhe
481 GGCACTCTGGTCTACACTATTAAGCAGATGAAGATGTACCGAGGCTTCAGTAAGATGCCC 540
GlyThrLeuValTyrThrIleLysGlnMetLysMetTyrArgGlyPheSerLysMetPro
541 CACGTGCAGTACATTCACACGGAAGCCTCTGAAAGTCTTTGTGGCCTCAAGCTAGAAGTC 600
HisValGlnTyrIleHisThrGluAlaSerGluSerLeuCysGlyLeuLysLeuGluVal
601 AACAAATACCAGTACCTGCTGACAGGGCGCGTGTATGAAGGCAAGATGTACACAGGACTG 660
AsnLysTyrGlnTyrLeuLeuThrGlyArgValTyrGluGlyLysMetTyrThrGlyLeu
661 TGCAACTTTGTGGAGAGGTGGGACCACCTCACACTGTCCCAGCGCAAGGGCCTCAATTAC 720
CysAsnPheValGluArgTrpAspHisLeuThrLeuSerGlnArgLysGlyLeuAsnTyr
721 CGCTACCACCTGGGTTGCAATTGCAAGATCAAGTCCTGCTACTACTTGCCTTGTTTTGTG 780
ArgTyrHisLeuGlyCysAsnCysLysIleLysSerCysTyrTyrLeuProCysPheVal
781 ACCTCCAAGAATGAGTGTCTCTGGACCGACATGCTCTCCAATTTTGGGTACCCTGGCTAT 840
ThrSerLysAsnGluCysLeuTrpThrAspMetLeuSerAsnPheGlyTyrProGlyTyr
841 CAGTCCAAACACTACGCCTGCATCCGGCAGAAGGGTGGCTACTGCAGCTGGTACCGAGGA 900
GlnSerLysHisTyrAlaCysIleArgGlnLysGlyGlyTyrCysSerTrpTyrArgGly
901 TGGGCTCCCCCAGACAAGAGCATCAGCAACGCCACAGACCCCTGAACCCAGACCTTCCCC 960
TrpAlaProProAspLysSerIleSerAsnAlaThrAspProEnd
961 ACCTCACCTCCTTCCCATCCCGCCGAGCGTCCCAGACACTAACTCTTCCCAGATGATGAC 1020
1021 AATGAAATTAGTGCCTGTTTTCTTGCAAATTTAGCACTGGGGACACTTAAAGTCTCTGCT 1080
1081 GTCTATGGAGTTGATTTGGAAATACCTTCCTGGCCCCGCCCCTACCCCTTCTTTTTGGTT 1140

of 5'-UTR and ~950 bp of the 3'-UTR, including the highly conserved AU rich element (Figure 14). To determine if either the 5' or 3'-UTR plays a role in TIMP-3 activity and localization, a cDNA containing only the 635 bp coding region was generated using PCR. The resulting cDNA was cloned into pXMT2 and transiently expressed in COS-1 cells (Figure 15A, lane 8). Recombinant protein expressed from this TIMP-3 clone was mainly localized to matrix and expressed at a level comparable to the clone with an intact 3'UTR (compare with Figure 4, lane 8). The small amount of TIMP-3 detected in the conditioned medium is due to saturation of TIMP-3 sites in the matrix because this soluble TIMP-3 was only seen when very high expression of transfected construct was achieved. This observation demonstrates that the unique 3'-UTR of TIMP-3 alone does not play a role in the localization of TIMP-3 to the ECM.

To characterize the functional regions of TIMP-3, the ability of specific TIMP-3 domains to confer aggressive gelatinase B inhibition and matrix localization on a heterologous protein, TIMP-1, was assayed. For the purposes of this analysis, three main TIMP domains were considered: the signal peptide, which, as pointed out, is unique for TIMP-3; and the N-terminal and C-terminal domains (Figure 14), which for all TIMP family members are considered to be the first six and last six conserved cysteine residues respectively, and which have been shown to be functionally modular for TIMP-1 and TIMP-2 . A series of chimaeric constructs with murine TIMP-1 were built using a two step PCR

Figure 15. Reverse zymography and silver stain analysis of TIMP-3 signal peptide domain function. A. Conditioned media and matrix from transiently transfected COS-1 cells were analyzed by reverse zymography and SDS-PAGE followed by silver stain. Expression constructs for TIMP-1 (lane 2) and TIMP-3 (lane 8) directed expression of proteins of the appropriate size. TIMP-1 was localized to conditioned media while TIMP-3 was localized mainly to the matrix. Chimaeric cDNA containing the coding region for TIMP-1 signal peptide attached to TIMP-3 N-and C-terminal domains (S1 N3 C3) directed expression of a protein that was indistinguishable from native TIMP-3 (lane 9). **B.** Chimaeric cDNA coding for TIMP-3 signal peptide with TIMP-1 N- and C-terminal domains (S3 N1 C1) directed expression of a protein that appeared identical to TIMP-1, but was detected by reverse zymography as a TIMP activity with slightly higher mobility than TIMP-1 in some transfections.


A
conditioned media
matrix
kDa
XMT2
TIMP-1
TIMP-3
S1 N3 C3
S3 N1 C1
XMT2
TIMP-1
TIMP-3
S1 N3 C3
S3 N1 C1
31
reverse zymogram
21.5
silver stain
31
21.5


B
XMT2
hTIMP-1
mTIMP-1
S3 N1 C1
reverse zymogram
TIMP-1
S3 N1 C1
silver stain
TIMP-1

strategy to generate chimaeric cDNA which was subsequently cloned into pXMT2 in order to transiently express recombinant protein in COS-1 cells (see materials and methods).

3.4.1 Role of the Signal Peptide in TIMP-3 Processing, Trafficking, and Activity.

Recombinant chimaeric proteins were constructed in which the signal peptide sequences were exchanged between mouse TIMP-1 and mouse TIMP-3. These chimaeras, designated S1 N3 C3 and S3 N1 C1, were expressed in COS-1 cells and the products were analyzed by reverse zymography and SDS-PAGE silver staining of conditioned media and matrix of transfected cells (Figure 15A). As observed previously, transfection of native TIMP-1 resulted in increased expression of TIMP-1 protein localized to the conditioned media of cells (Figure 15A, lane 2), whereas native TIMP-3 was localized primarily to the ECM (Figure 15A, lane 8), with a small portion in conditioned medium from transfected cells (Figure 15A, lane 3). Exchange of the TIMP-3 and TIMP-1 signal peptide domain did not alter the expression or localization of the mature TIMP-3 protein (Figure 15A, lanes 3, 4, 8, and 9). The TIMP-3 signal peptide had variable effects on the mature TIMP-1 protein. Some transfections with the TIMP-3 signal peptide bearing TIMP-1 resulted in the expression of mature TIMP-1 that was indistinguishable from native, recombinant TIMP-1 (Figure 15A, lanes 2 and 5), while other transfections resulted in the detection of a TIMP activity with higher mobility than native TIMP-1, while the bulk of

expressed protein was the same molecular weight as native TIMP-1 as indicated by SDS-PAGE and silver stain of recombinant proteins (Figure 15B). This suggests that the signal peptide of TIMP-3 can affect the post-translational processing of mature TIMP-1 protein such that its mobility is changed. Since reverse zymograms are non-denaturing it is not clear if this change in mobility is due to an alteration of the length of the core protein or if an alternative folding scheme is responsible.

Treatment of S3 N1 C1 recombinant protein with N-glycosidase and analysis by reverse zymography resulted in the appearance of two new TIMP bands (Figure 6), not seen following deglycosylation of native TIMP-1. This alteration in mobility of unglycosylated S3 N1 C1 protein is likely due to the same alterations which account for mobility changes seen with intact S3 N1 C1 protein (Figure 15B).

The TIMP-1 signal peptide was not seen to alter the ability of mature TIMP-3 to inhibit gelatinase B (S1 N3 C3 chimaera), nor was the TIMP-3 signal peptide seen to confer enhanced gelatinase B inhibitory capacity on the TIMP-1 mature protein (S3 N1 C1 chimaera) (results not shown).

In summary, this functional analysis of the TIMP-3 leader peptide by assaying its ability to confer changes in localization or gelatinase B inhibition on the mature TIMP-1 protein demonstrates that the signal peptide of TIMP-3 is not alone responsible for either of these characteristics of TIMP-3 which distinguish it from TIMP-1 and TIMP-2. The signal peptide of TIMP-3 does,

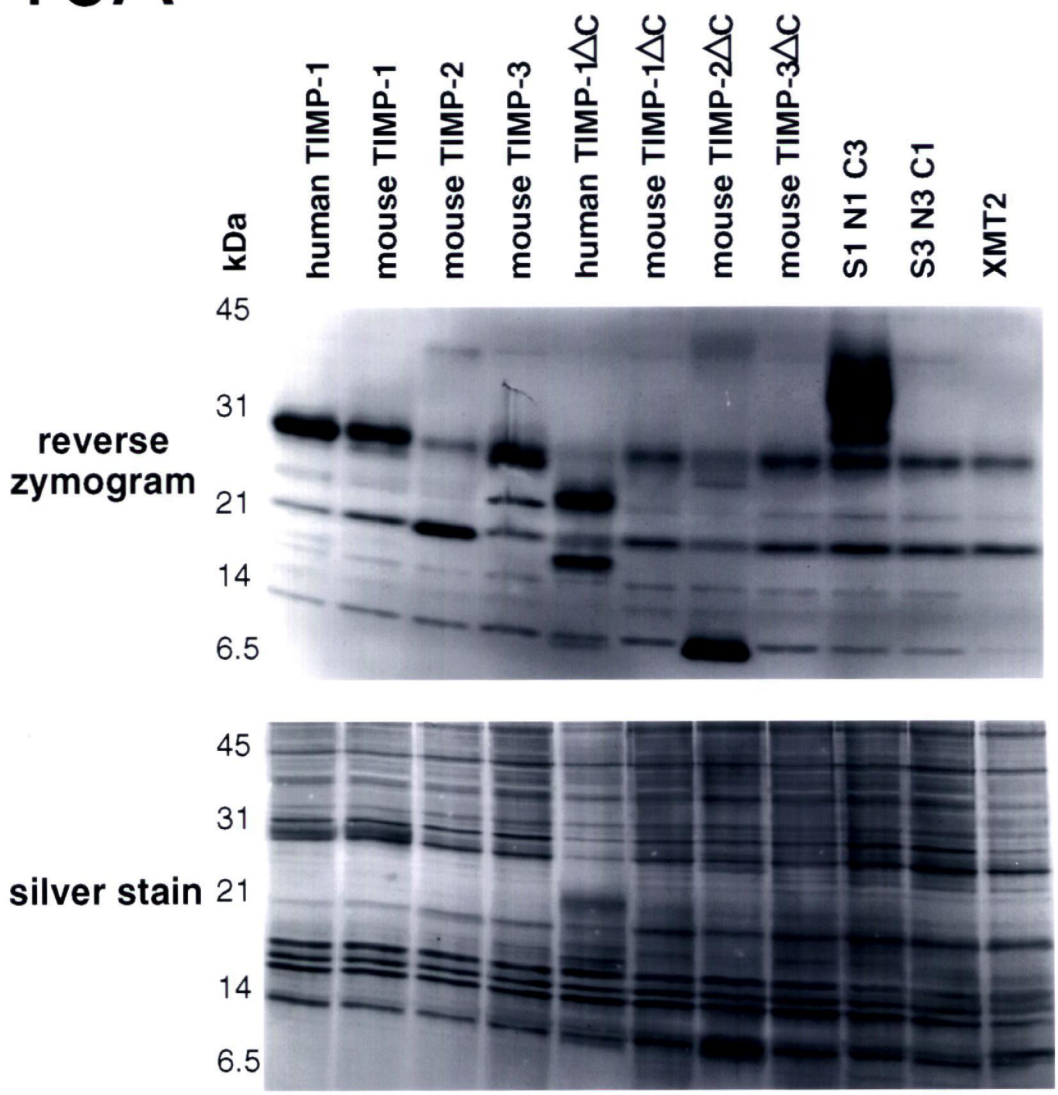
however, alter the molecular weight of mature TIMP-1, suggesting it may play a role in some aspect of post-translational processing that is unique to the TIMP-3 protein.

3.4.2 Role of the TIMP-3 C-terminal Domain in Processing, Trafficking, and Activity.

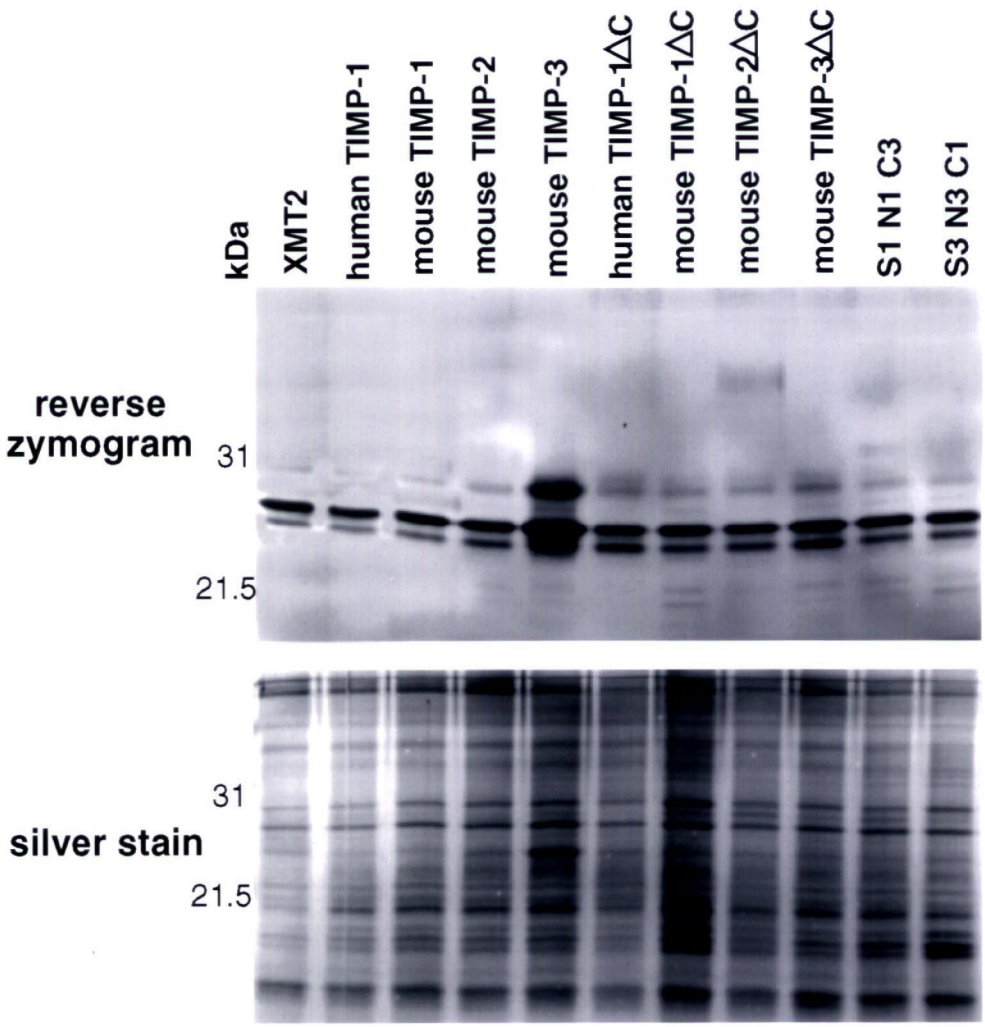
TIMPs-1 and -2 are structurally and functionally defined by their N-and C-terminal domains. To determine the role of the TIMP-3 N-and C-termini, the ability of these domains to confer TIMP-3 characteristics on a heterologous protein, TIMP-1, was determined. Chimaeric cDNA encoding an exchange of the C-terminal domain between mouse TIMP-1 and mouse TIMP-3 was expressed in COS-1 cells and analyzed by reverse zymography and SDS-PAGE silver stain of conditioned media and matrix from transfected cells (Figure 16). Conditioned media from cells transfected with a construct encoding the TIMP-3 C-terminus attached to the TIMP-1 signal peptide and N-terminus contained two species of 31 and 36 kDa detectable on reverse zymograms which were not detectable by SDS-PAGE silver staining (Figure 16a, lane 9). The mobility of the 31 kDa TIMP activity is accounted for by the predicted molecular weight of the chimaera plus two glycosylations in the N-terminal TIMP-1 domain, while the 36 kDa species is likely due to glycosylation at all three N-linked glycosylation sites present in the chimaera (two in the TIMP-1 N-terminal domain and one in the TIMP-3 C-terminal domain). Deglycosylation of this protein to verify this is currently being carried out. Matrix from cells

Figure 16. Reverse zymography and silver stain analysis of TIMP-1:TIMP-3 C-terminal domain exchange and truncated TIMPs. Conditioned media (A) and matrix (B) from transiently transfected COS-1 cells was analyzed by reverse zymography and SDS-PAGE followed by silver stain.

16A


kDa
human TIMP-1
mouse TIMP-1
mouse TIMP-2
mouse TIMP-3
human TIMP-1ΔC
mouse TIMP-1ΔC
mouse TIMP-2ΔC
mouse TIMP-3ΔC
S1 N1 C3
S3 N3 C1
XMT2
reverse zymogram
45
31
21
14
6.5
silver stain
45
31
21
14
6.5

16B


kDa
XMT2
human TIMP-1
mouse TIMP-1
mouse TIMP-2
mouse TIMP-3
human TIMP-1ΔC
mouse TIMP-1ΔC
mouse TIMP-2ΔC
mouse TIMP-3ΔC
S1 N1 C3
S3 N3 C1
reverse zymogram
31
21.5
31
silver stain
21.5

transfected with the same construct appeared the same as control transfected matrix except for a novel TIMP activity (Figure 16b, lane 10) which comigrated with the 31 kDa activity seen in the conditioned media from the same cells (Figure 16a, lane 9). This indicates that the TIMP-3 C-terminal domain can confer ECM association on a heterologous protein. Transfection with the construct encoding the TIMP-1 C-terminus attached to the TIMP-3 signal peptide and N-terminus did not result in any detectable change in TIMP activity or protein expression, compared to control transfected cells (Figure 16a, lane 10 and Figure 16b, lane 11). Sanger dideoxy chain termination sequencing of the S3 N3 C1 construct did not reveal any sequence alterations in the cDNA (results not shown), and Northern analysis of transcription of transfected cells revealed abundant S3 N3 C1 mRNA (Figure 17, lane 7), in spite of the absence of detectable protein. This suggests that the protein translated from the S3 N3 C1 chimaera is not stable enough to be exported by the cell due to interference with post translational processing imposed by the TIMP-1 C-terminal domain. The C-terminal domain of TIMP-3 was not seen to confer a change in the ability of the TIMP-1 N-terminal domain to inhibit gelatinase B (S1 N1 C3 chimaera) (results not shown). This indicates that the C-terminal of TIMP-3 is not alone able to confer the enhanced gelatinase B inhibition seen with full length TIMP-3.

Figure 17. Northern analysis of TIMP expression in transiently transfected COS-1 cells. Total cellular RNA (DNase-treated) harvested from COS-1 cells transfected with pXMT2 expression constructs or pXMT2 alone was probed with nick-translated probes for the N-terminal domain of mouse TIMP-1 (upper) or TIMP-3 (lower).

mouse TIMP-3ΔC
mouse TIMP-1ΔC
PROBE
human TIMP-1
mouse TIMP-1
mouse TIMP-2
mouse TIMP-3
mouse TIMP-1 ΔC
mouse TIMP-3 ΔC
S3 N3 C1
S1 N1 C3
S3 N1 C1
S1 N3 C3
XMT2

3.4.3 Expression and Analysis of Truncated TIMPs Lacking a C-terminal domain.

As pointed out earlier, TIMPs -1 and -2 have been characterized as functionally modular proteins in which the N-terminal domain of TIMP-1 and TIMP-2 have been shown to be sufficient for inhibition of MMPs (Murphy *et al.*, 1991; DeClerck *et al.*, 1993). To determine if the N-terminus of TIMP-3 is sufficient for inhibition of gelatinase A, cDNA encoding truncated TIMP-3 lacking a C-terminus was constructed using PCR and expressed in COS-1 cells.

Truncated human TIMP-1, mouse TIMP-1, and mouse TIMP-2 were also constructed and expressed. Reverse zymography and SDS-PAGE silver stain analysis of conditioned media and matrix from cells transfected with these constructs revealed expression of truncated human TIMP-1 and truncated mouse TIMP-2 in the soluble fraction which retained gelatinase inhibitory capacity. Three species of truncated TIMP-1 were resolved on both the reverse zymogram and the silver stain, one at 13 kDa, a second at approximately 18 kDa, and a third at approximately 25 kDa (Figure 16a, lane 5). These different mobility forms are likely due to glycosylation at the two N-linked sites present in the N-terminal of TIMP-1 (Figure 5). Alternatively, the highest molecular weight form may be a dimer of the 13 kDa species, which corresponds approximately to the predicted size of the TIMP-1 N-terminal domain without glycosylation. Truncated TIMP-2 was detected as a single species migrating at 13 kDa (Figure 16a, lane 7), which is the predicted

molecular weight of the TIMP-2 N-terminus. Mouse TIMP-1 and -3 cDNA constructs encoding a C-terminal truncation did not give rise to any detectable protein (Figure 16a, lanes 6 and 8, Figure 16b, lanes 7 and 9). Sanger-dideoxy chain termination sequencing of the cDNA did not reveal any base alterations (data not shown), and Northern analysis of transfected cells indicates that the truncated mouse TIMP-1 and -3 are expressed at the RNA level (Figure 17, lanes 5 and 6).

Similarly to the S3 N3 C1 chimaera, the TIMP-3 C-terminally truncated cDNA construct lacks a TIMP-3 C-terminal domain and does not give rise to detectable protein in spite of its transcription, suggesting that the expressed protein is not correctly processed post-translationally and cannot be exported by the cells. Since human TIMP-1 and mouse TIMP-2 C-terminal deletions were produced, TIMP-3 may be unique among TIMPs in its requirement of an intact C-terminal domain for proper protein processing and export. It is unclear why the mouse TIMP-1 C-terminal truncation cannot be detected as protein either, since it can be expressed with a TIMP-3 C-terminal (S1 N1 C3 construct, Figure 16), and its human counterpart with which it shares 74% amino acid identity can be expressed as an N-terminal region alone (Figure 16).

In analysis of the role of the TIMP-3 C-terminal domain in ECM localization and aggressive gelatinase B inhibition, the two characterized features which distinguish TIMP-3 from the other TIMP family members, I demonstrated that the C-terminal domain contains at least part of the

mechanism which underlies the association of TIMP-3 with the ECM. Also, our results assign a critical role to the C-terminal domain unique to TIMP-3 in maintaining the structure of the TIMP-3 protein. Our analysis of the other domains of TIMP-3 does not assign any role in ECM localization to the signal peptide of the TIMP-3 protein, nor to the 5'- and 3'- UTR of the mRNA. In looking for regions of TIMP-3 conferring the strongest gelatinase B inhibition of the TIMP family, it appears that neither the signal peptide nor the C-terminal domain contain the structures responsible.

3.5 Identification of glycosaminoglycans associating with TIMP-3.

TIMP-3 is distinguished from TIMPs-1 and -2 by its association with the ECM. The mechanism underlying this is not known. A nonspecific association is suggested by the basic nature of the TIMP-3 protein (pI of 9.16; Leco *et al.*, 1994). Thus the molecule would have a net positive charge at a neutral pH and may nonspecifically associate with negatively charged components of the ECM. Glycosaminoglycans (GAGs) are long, unbranched polysaccharide chains composed of repeating disaccharide units which form hydrated gels that occupy most of the extracellular space (Alberts *et al.*, 1989). One of the two sugars in a disaccharide unit is always an amino sugar that is usually sulfated. These sulfate groups together with the carboxyl groups present on most of the sugar residues make the GAGs highly negatively charged and potential TIMP-3 associating structures. Serum-free media containing purified GAGs incubated with TIMP-3 enriched cell-free matrix was used to assay the ability of the

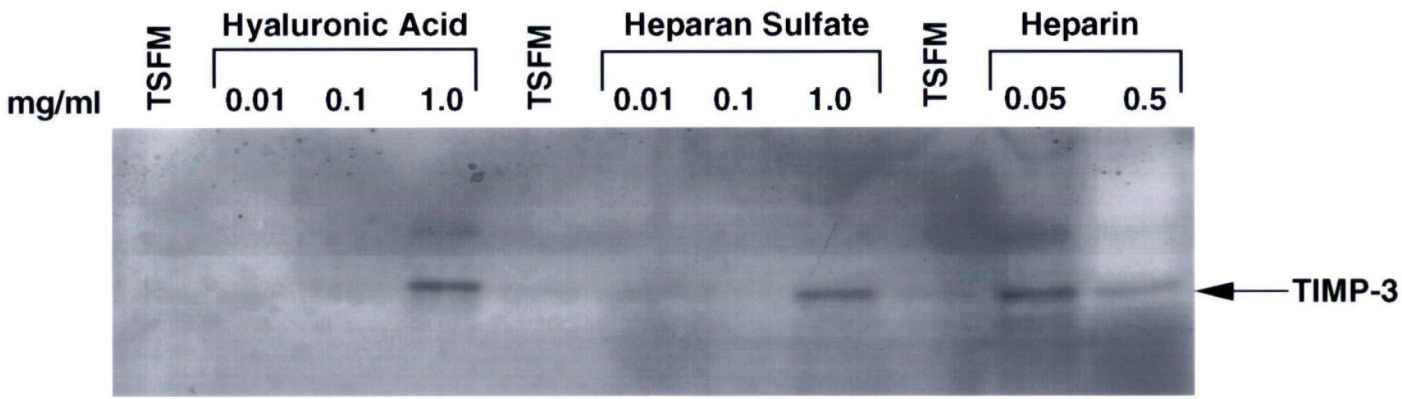
GAGs to bind TIMP-3. Reverse zymography of the serum-free medium or medium containing a known concentration of GAG revealed TIMP-3 activity that was liberated from the matrix (Figure 18A). Serum-free media containing 1mg/ml hyaluronic acid, heparin sulfate, or 0.05 mg/ml heparin was able to liberate a detectable amount of TIMP-3 from the matrix, while serum-free medium alone was not. Similar results were obtained using chondroitin sulfate A or B at a concentration of 1 mg/ml (results not shown).

These observations suggest that TIMP-3 does associate with the GAGs. Heparin, the most sulfated GAG assayed, appears to have a higher affinity for TIMP-3. This may mean that the relationship between TIMP-3 and the GAGs is based on their opposite charge, because the more sulfated residues are more negatively charged.

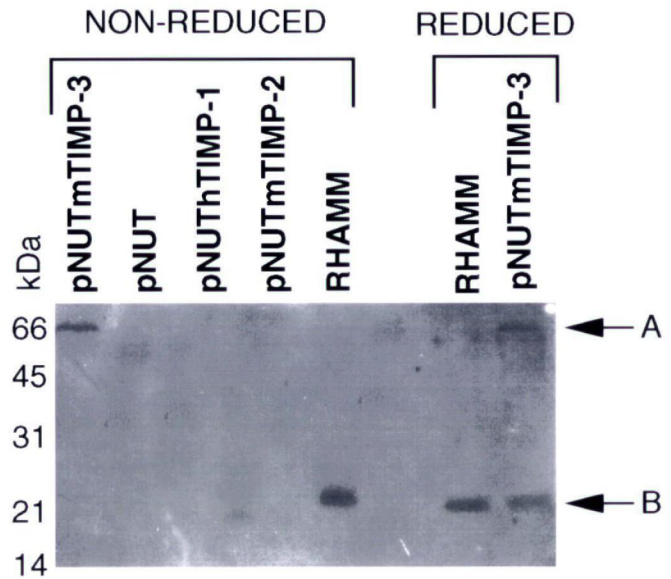
In contrast to the nonspecific GAG association with charge over the surface of the entire protein suggested by these observations, it is also possible that there are some specific domains of TIMP-3 responsible for presenting a charge configuration that recognizes specific elements of the ECM. The hyaluronic acid (HA) binding motif, BX_7B, in which two basic amino acids flank a seven amino acid stretch (Yang *et al.*, 1994), is seen in the TIMP-3 protein at 4 sites (Figure 5). In order to determine if HA does bind to TIMP-3 a procedure for detecting HA binding proteins in a transblot assay using biotinylated HA as a probe was used. Unreduced and reduced matrix from BHK pNUT TIMP-3 and control cells were electrophoresed and fixed on

Figure 18. Identification of glycosaminoglycans (GAGs) associating with TIMP-3. **A.** Reverse zymography of serum-free medium (TSFM) or serum-free medium containing purified glycosaminoglycan that was incubated on TIMP-3 enriched matrix. TSFM was not able to liberate a detectable amount of TIMP-3 activity from the matrix, while TSFM containing 1.0 mg/ml hyaluronic acid (lane 4), 1.0 mg/ml heparin sulfate (lane 8), or 0.05 mg/ml heparan (lane 10) was able to cause some TIMP-3 to be detected in the soluble fraction. A similar effect was seen with chondroitin sulfate A and B (results not shown). **B.** Samples of TIMP-3 enriched and control matrix were electrophoresed under non-reducing or reducing conditions, fixed to nitrocellulose and probed with biotinylated hyaluronic acid. 3 μg of RHAMM protein was loaded under both reducing and non-reducing conditions as a positive control.

A


mg/ml
TSFM
Hyaluronic Acid
0.01
0.1
1.0
TSFM
Heparan Sulfate
0.01
0.1
1.0
TSFM
Heparin
0.05
0.5
TIMP-3

B


NON-REDUCED
REDUCED
kDa
pNUTmTIMP-3
pNUT
pNUThTIMP-1
pNUTmTIMP-2
RHAMM
RHAMM
pNUTmTIMP-3
66
45
31
21
14
A
B

nitrocellulose prior to probing with biotinylated HA (a gift from Dr. Eva Turley, University of Manitoba). Purified recombinant Receptor for Hyaluronic Acid Mediated Motility (RHAMM; also a gift from Dr. Turley), a characterized HA binding protein, was also electrophoresed and fixed to the filter to serve as a positive control. In non-reducing conditions, biotinylated HA detected a 66 kDa protein present only in TIMP-3 enriched matrix (Figure 18B, lane 1), while RHAMM protein was detected at 22kDa (Figure 18B, lane 5). Addition of 0.1 M DTT to and boiling of TIMP-3 enriched matrix prior to transblotting led to the detection of a 22 kDa protein in addition to the 66 kDa species seen under non-reducing conditions (Figure 18B, lane 7). These observations indicate that there is a protein present only in TIMP-3 enriched matrix which may associate with hyaluronic acid. Verification of this protein as TIMP-3 will require the generation of specific antibodies. It is difficult to identify the detected proteins on the basis of the mobility of TIMP activity seen on a reverse zymogram run in parallel because of differences between reverse zymography and the Western transfer protocol, such as the presence of conditioned media and gelatin, which may affect the mobility of proteins in the gel.

This analysis of TIMP-3 interactions with the GAGs demonstrates that these molecules may be at least partially responsible for the affinity of the TIMP-3 protein for the ECM. The nature of the interaction appears to be based on the net charge of the TIMP-3 protein, which is positive at neutral pH. In addition to the charge over the surface of the entire protein being involved, our

results suggest the possibility that there are specific amino acid seqences, such as the HA binding motif, which present the appropriate charge configuration for interaction with the ECM.

3.6 TIMP-3 Effects on Cell Growth.

In addition to their function as MMP inhibitors, TIMP-1 and TIMP-2 have been characterized as growth factors for a range of cell types. In order to look for effects of endogenously expressed mouse TIMP-3 on the growth of cells, BHK pNUT-mT3 cells and controls were seeded at low density in a range of low serum conditions (serum-free and 1% FCS) and their growth rate measured by counting cell numbers at later time points. BHK cells overexpressing mouse TIMP-3 grew more slowly than control transfected cells under 1% and serum-free conditions (Figure 19). This suggests that overproduction of mouse TIMP-3 by the BHK cells can negatively affect the growth kinetics of these cells.

The amount of TIMP-3 present in the matrix of control transfected and TIMP-3 transfected cells over the course of the growth assay was determined by harvesting the matrix from cells grown in parallel and performing reverse zymography. TIMP-3 is not detectable in matrix until 4 days after plating at low density (day 2 of the growth assay) (Figure 20, lane 2) and increases to a maximum 6 days after plating (day 4 of the growth assay) (Figure 20, lane 4). Interestingly, after 9 days in low serum conditions (day 7 of the growth assay) the TIMP-3 produced by cells was detected primarily in the conditioned media, while the amount detected in the matrix is reduced (Figure 20, lane 6).

Figure 19. Growth rate comparison of BHK cells overexpressing TIMP-3. BHK cells transfected with TIMP-3 or pNUT vector alone were seeded at low density ($3X10^4$ cells/$10cm^2$) and placed under medium containing 1% FCS (upper panel) or serum-free medium (lower panel) the following day (day 0). Cells were trypsinized at the indicated time point and counted on a hemocytometer to measure growth.

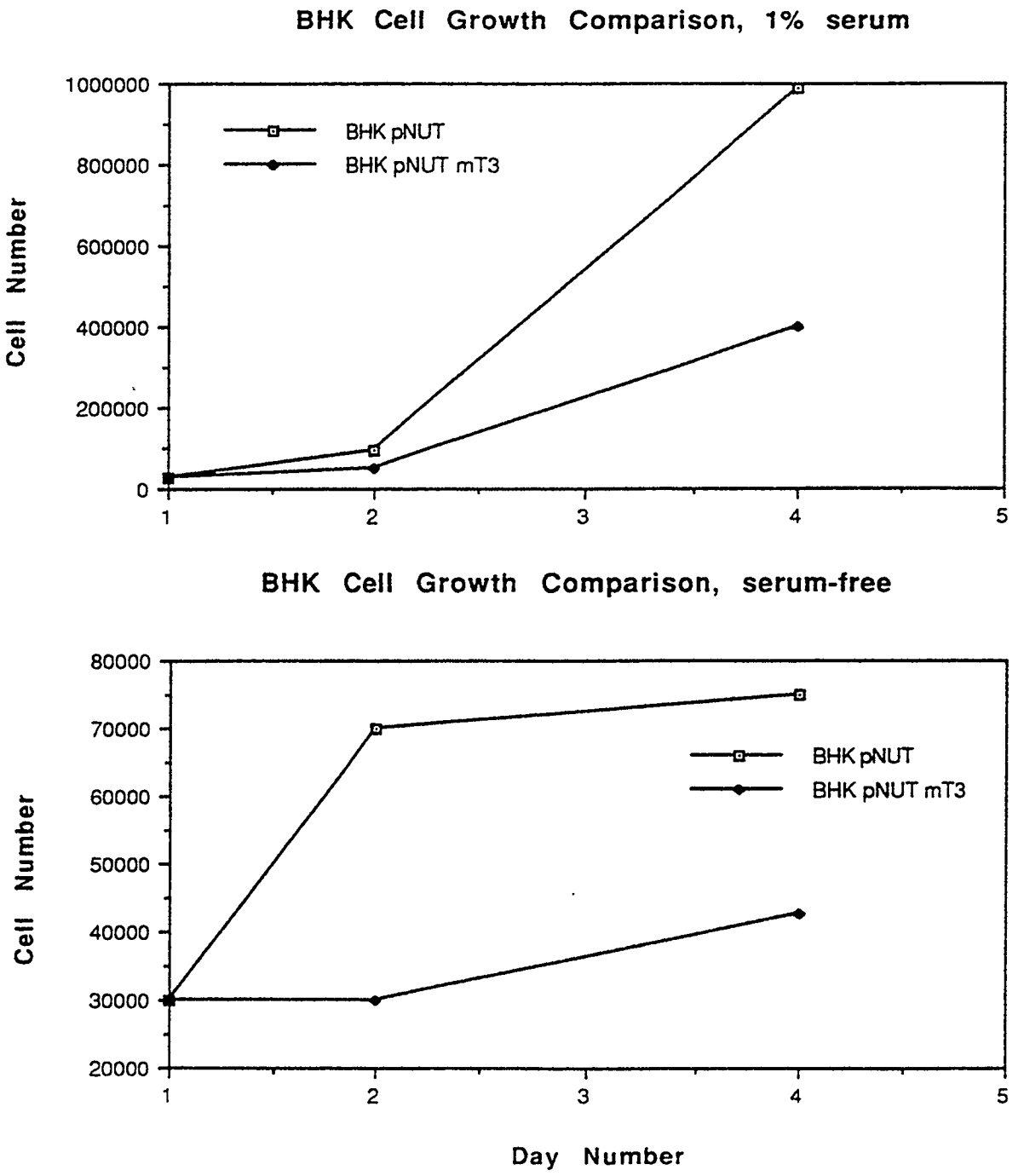
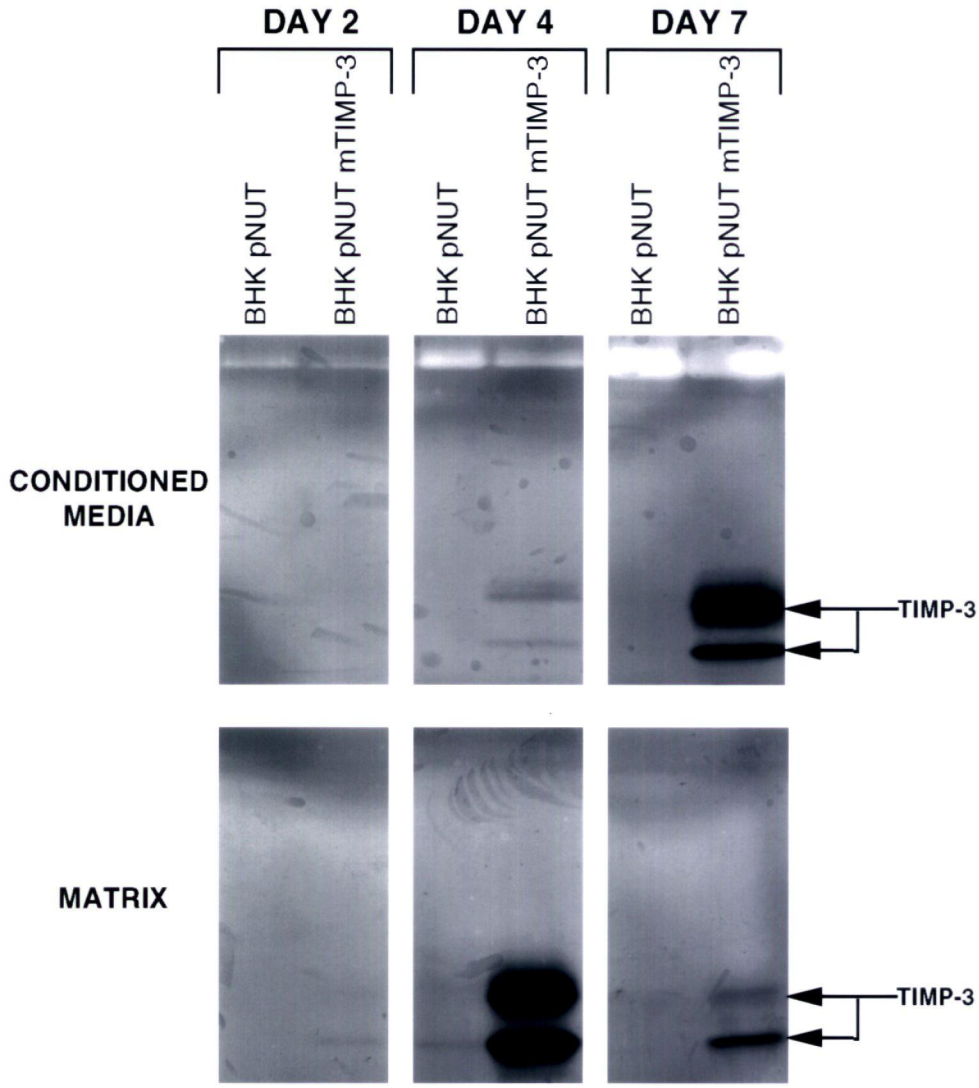

BHK Cell Growth Comparison, 1% serum
Cell Number
1000000
800000
600000
400000
200000
0
BHK pNUT
BHK pNUT mT3
1
2
3
4
5
BHK Cell Growth Comparison, serum-free
Cell Number
80000
70000
60000
50000
40000
30000
20000
BHK pNUT
BHK pNUT mT3
1
2
3
4
5
Day Number

Figure 20. Analysis of matrix maintained by BHK cells under low serum conditions. To verify the presence of TIMP-3 present in the matrix during the course of a growth assay, BHK pNUTmT3 cells and control transfected BHK cells were plated at low density and placed under low serum conditions the following day (day 0). Cells were placed under serum-free medium one day prior to harvesting conditioned media and matrix at 2, 4, and 7 days after placement in low serum.


DAY 2
DAY 4
DAY 7
BHK pNUT
BHK pNUT mTIMP-3
BHK pNUT
BHK pNUT mTIMP-3
BHK pNUT
BHK pNUT mTIMP-3
CONDITIONED MEDIA
TIMP-3
MATRIX
TIMP-3

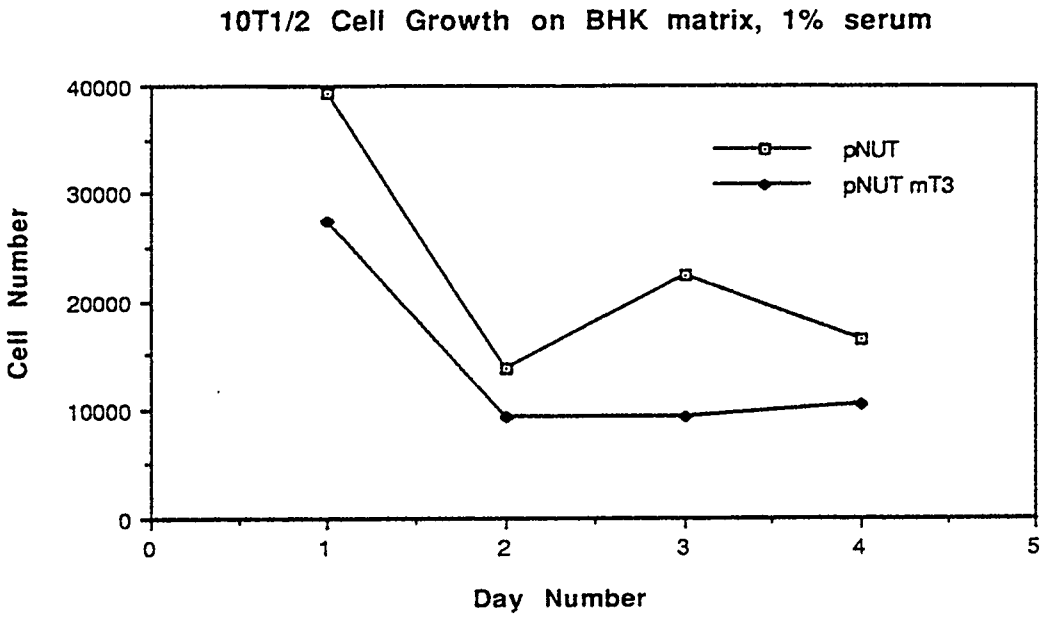
This migration of TIMP-3 into the soluble phase may negate any influence TIMP-3 would have on cell growth if TIMP-3 must be matrix associated to have such an effect, or by reducing the concentration of TIMP-3 below a threshold required for cell response.

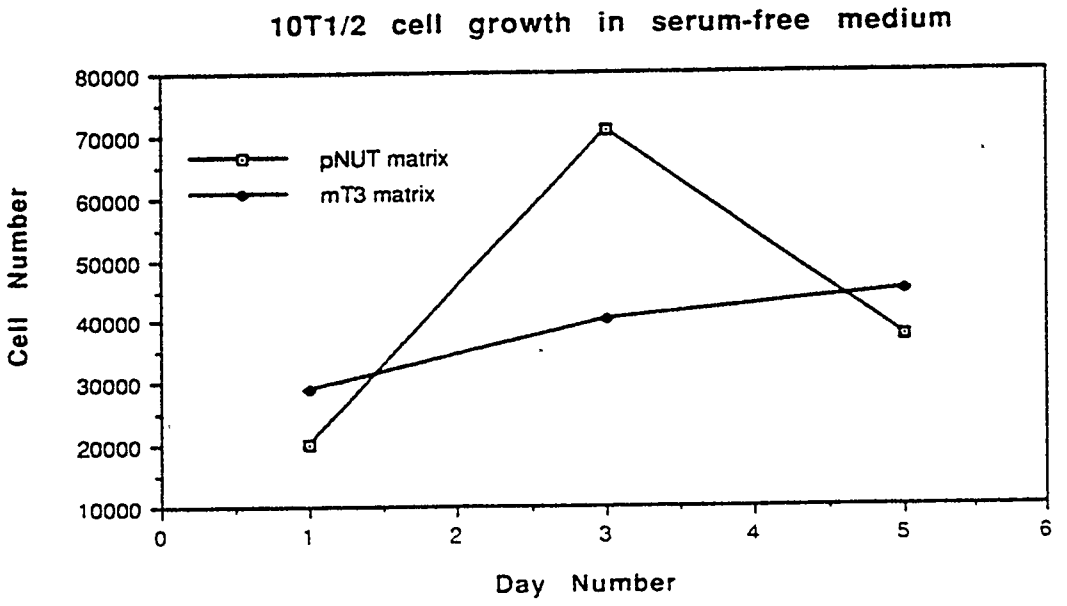
Since it is only a subset of cell types that have their growth affected by TIMPs -1 and -2, a number of different cell lines were screened for a growth response to TIMP-3. To look at the effect of exogenous TIMP-3 on the growth of a range of cell types, cells were plated onto TIMP-3 enriched matrix produced by BHK pNUT-mT3 cells and grown under a range of serum conditions. A mouse fibroblast cell line (10T1/2) (Figure 21A), and a human fibroblast cell line (HS68) (Figure 21B) were seen to have some suppression of growth when plated onto TIMP-3 enriched matrix compared to cells plated on matrix from control transfected BHK cells. Growth of a melanoma cell line (B16F10) was not affected by the presence of TIMP-3 (Figure 21C). A human fibrosarcoma cell line (HT1080) was observed to have its growth rate increased by the presence of TIMP-3 enriched matrix (Figure 21D)

The human TIMP-3 gene was cloned on the basis of its elevated expression in mid G1 in cycling WI-38 fibroblasts (Wick *et al.,* 1994). In order to determine if TIMP-3 plays a role in the passage of cells through the cell cycle, BHK pNUT-mT3 cells were grown to late log phase (~80% confluence) and sorted according to cell cycle position by flow cytometry. Determining percentage of total cells in S, G2+M, or G1 phase of the cell cycle indicates

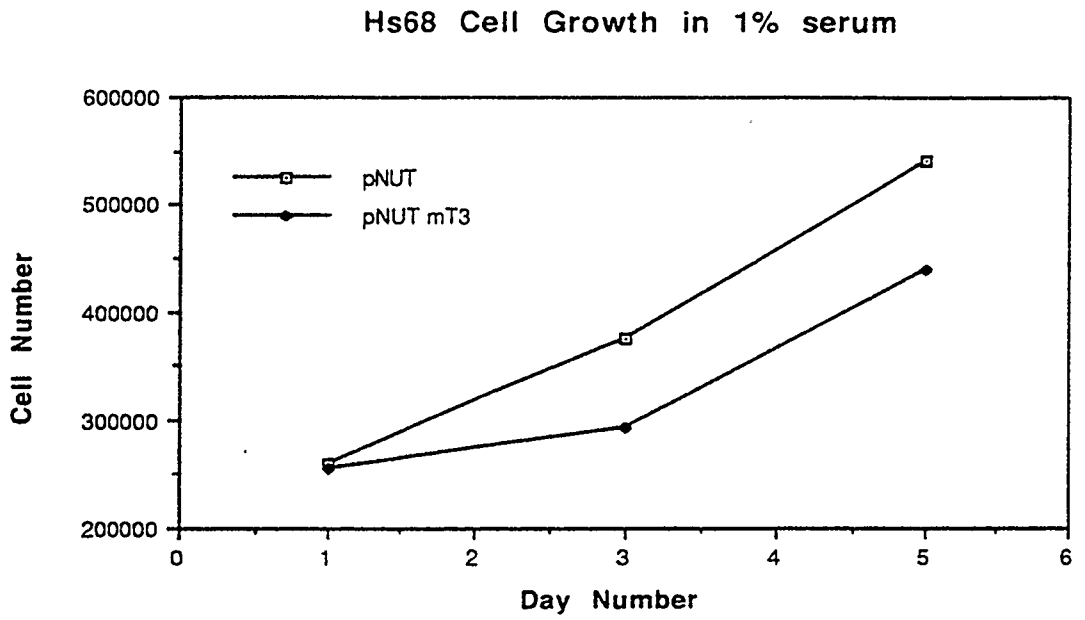
Figure 21. Growth comparison of cell lines plated on TIMP-3 enriched matrix. Cells were plated at low density (4-6X10^4/10cm^2) on matrix produced by either pNUT or pNUT-mouse TIMP-3 transfected cells and allowed to attach under normal serum conditions overnight. The next day (day0) cells were placed under low serum (1%; upper panel) or serum-free (lower panel) conditions. Cells were trypsinized and counted at the indicated time point. Cell lines tested were A, 10T1/2 mouse fibroblasts, B, HS68 human fibroblasts, C, B16F10 melanoma cells, and D, HT 1080, a human fibrosarcoma cell line.

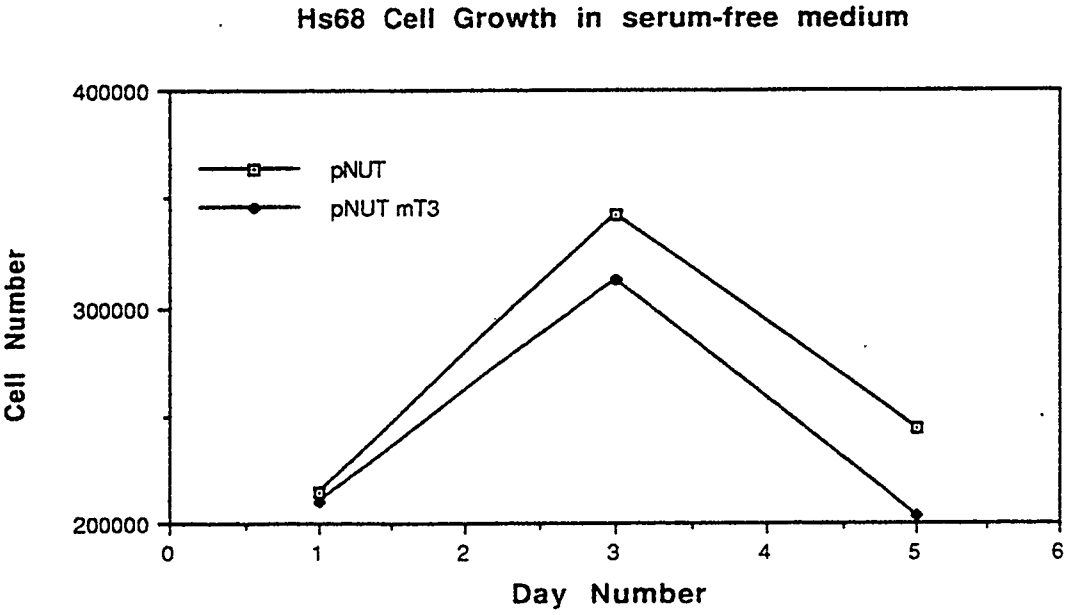
A


10T1/2 Cell Growth on BHK matrix, 1% serum
Cell Number
40000
30000
20000
10000
0
0
1
2
3
4
5
Day Number
pNUT
pNUT mT3

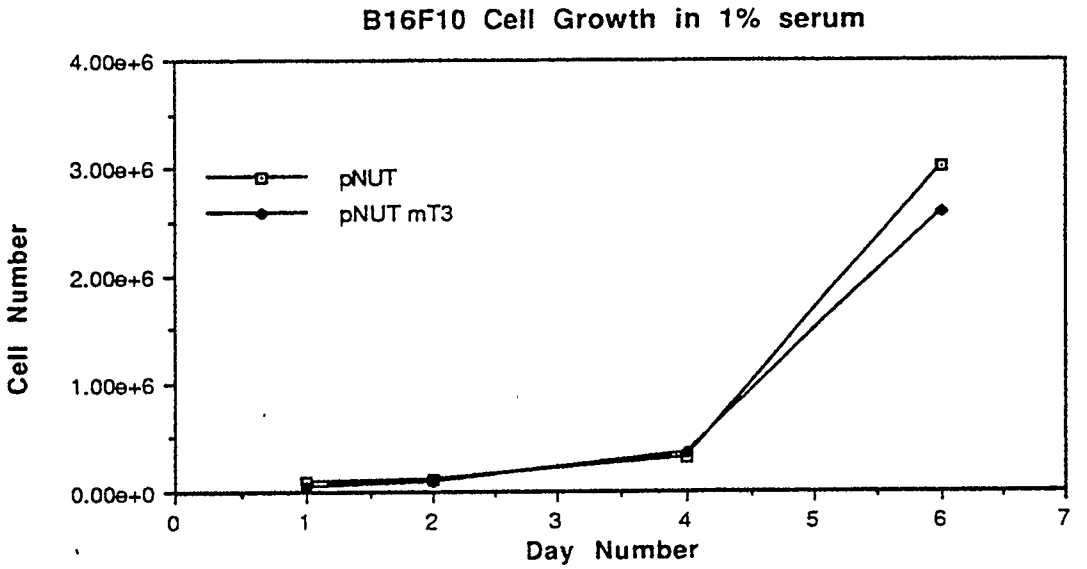

10T1/2 cell growth in serum-free medium
Cell Number
80000
70000
60000
50000
40000
30000
20000
10000
0
1
2
3
4
5
6
Day Number
pNUT matrix
mT3 matrix

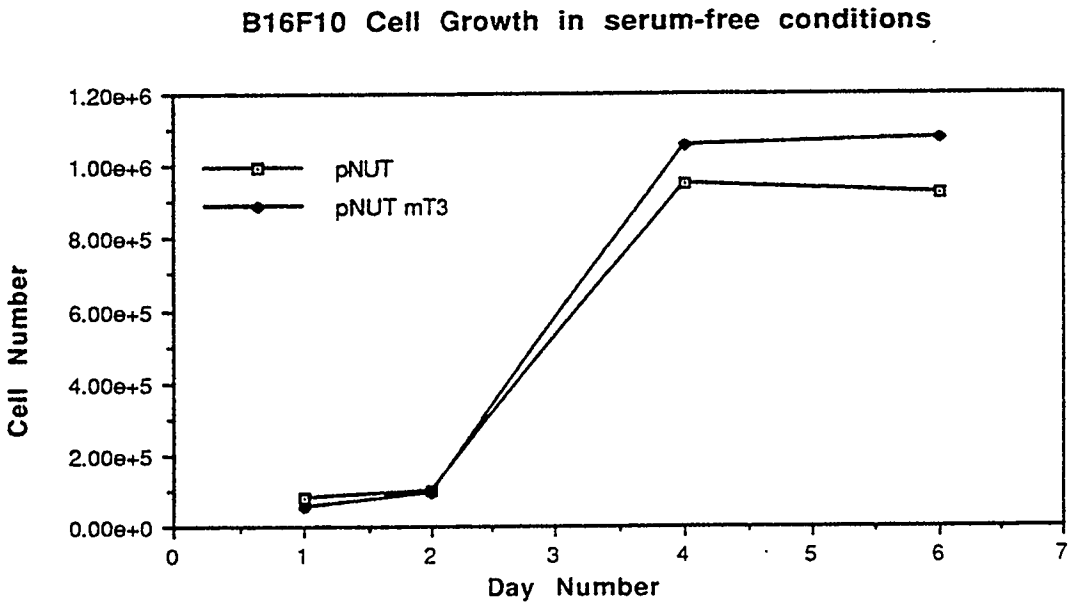
B


Hs68 Cell Growth in 1% serum
600000
500000
400000
300000
200000
Cell Number
pNUT
pNUT mT3
0
1
2
3
4
5
6
Day Number

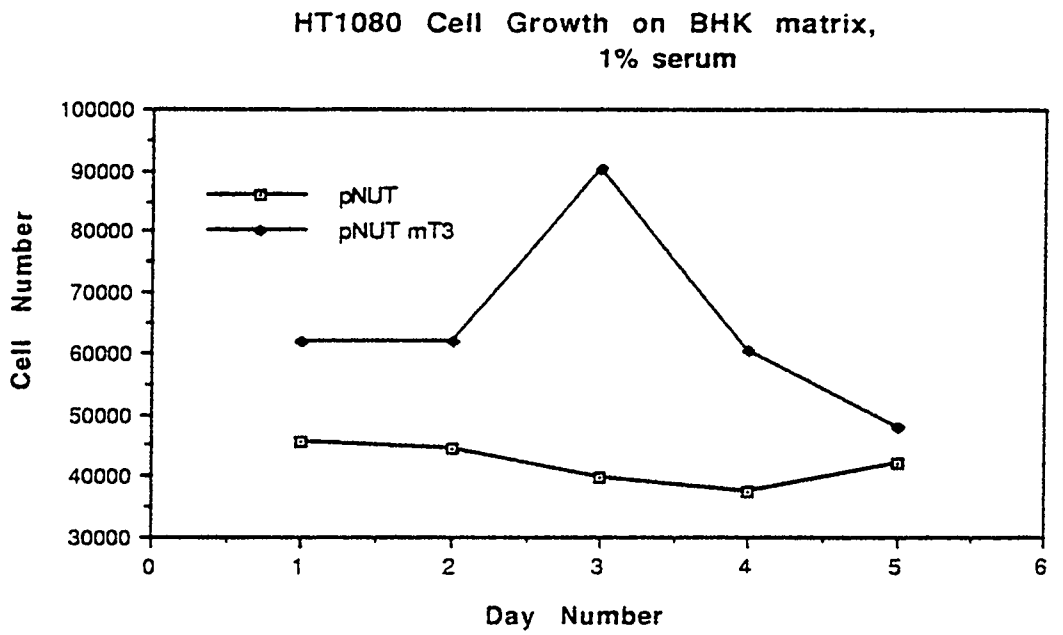

Hs68 Cell Growth in serum-free medium
400000
300000
200000
Cell Number
pNUT
pNUT mT3
0
1
2
3
4
5
6
Day Number

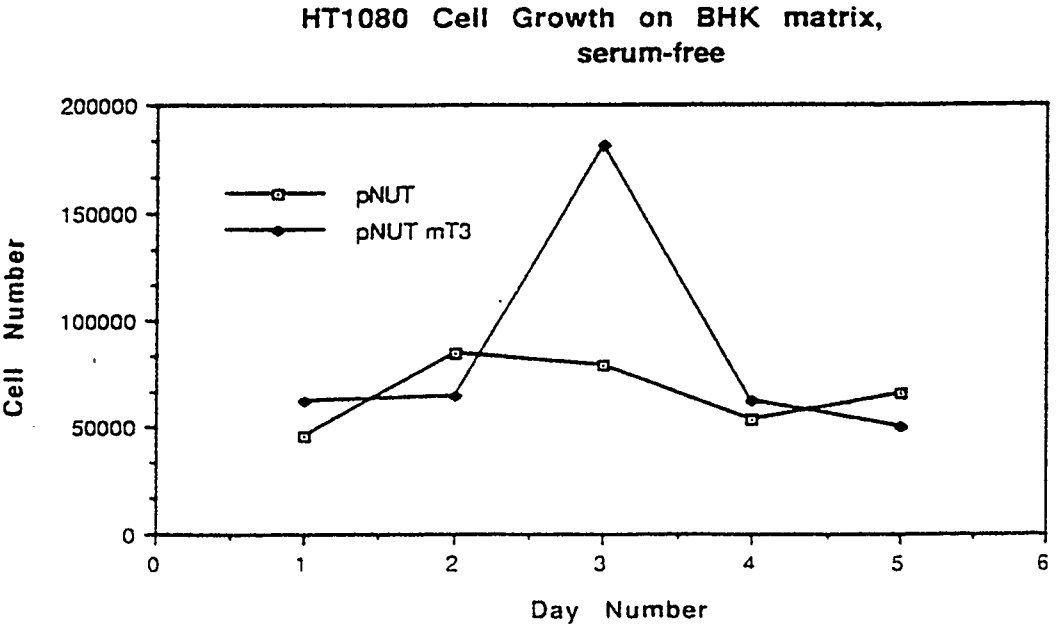
C


B16F10 Cell Growth in 1% serum
4.00e+6
3.00e+6
2.00e+6
1.00e+6
0.00e+0
Cell Number
pNUT
pNUT mT3
0
1
2
3
4
5
6
7
Day Number


B16F10 Cell Growth in serum-free conditions
1.20e+6
1.00e+6
8.00e+5
6.00e+5
4.00e+5
2.00e+5
0.00e+0
Cell Number
pNUT
pNUT mT3
0
1
2
3
4
5
6
7
Day Number

D


HT1080 Cell Growth on BHK matrix,
1% serum
Cell Number
100000
90000
80000
70000
60000
50000
40000
30000
pNUT
pNUT mT3
0
1
2
3
4
5
6
Day Number

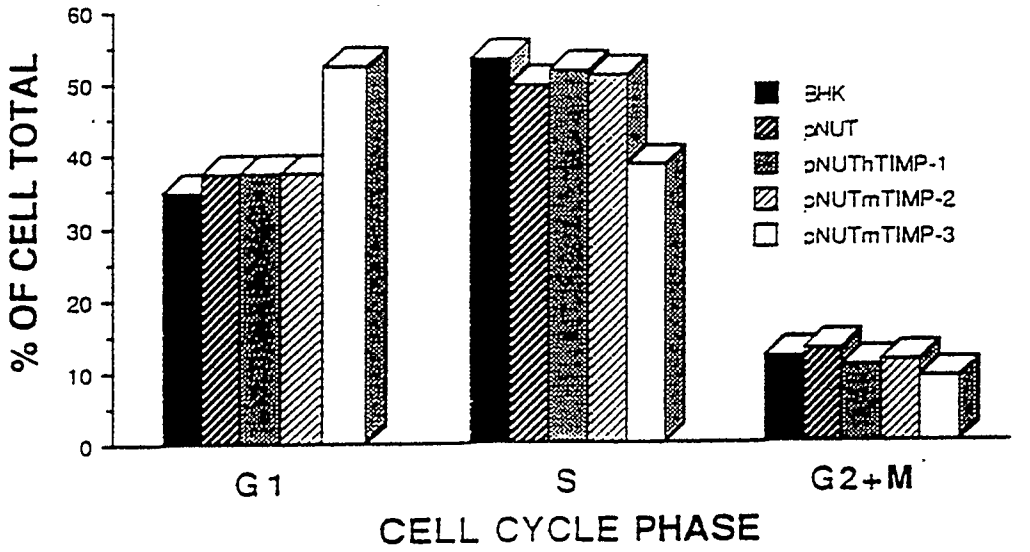

HT1080 Cell Growth on BHK matrix,
serum-free
Cell Number
200000
150000
100000
50000
0
pNUT
pNUT mT3
0
1
2
3
4
5
6
Day Number

that cells overexpressing TIMP-3 accumulate in G1 phase compared to control transfected cells and cells overexpressing TIMP-1 or TIMP-2, which are primarily localized in S-phase (Figure 22A). This G1 accumulation is not seen in cells sorted one day after plating at low density (Figure 22B), when TIMP-3 levels are undetectable (Figure 20).

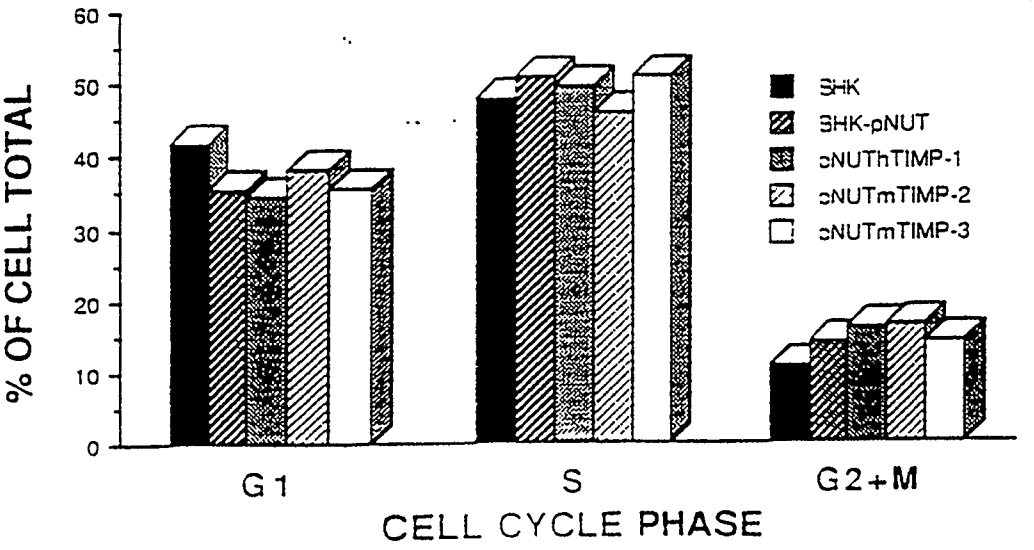
Analysis of the effest of TIMP-3 on cell growth indicates that it has some growth suppressive effect on some cell types. This effect may be interfered with by the migration of TIMP-3 out of the ECM during the course of the growth assay. Cell cycle analysis of growing cells overexpressing TIMP-3 reveals that a larger percentage of these cells are retained in G1, suggesting that TIMP-3 may have a role in progression of the cell cycle.

Figure 22. Cell cycle analysis of transfected BHK cells. Cells were sorted according to cell cycle stage by staining with propidium iodide and analysis using a flow cytometer. A. Late log phase cells showing an accumulation of TIMP-3 overexpressing cells in G1 phase. B. Cells analyzed one day after plating at low density, at which time the accumulation of TIMP-3 overexpressing cells in G1 is not seen.

A


% OF CELL TOTAL
60
50
40
30
20
10
0
G1
S
G2+M
CELL CYCLE PHASE
BHK
pNUT
pNUThTIMP-1
pNUTmTIMP-2
pNUTmTIMP-3

B


% OF CELL TOTAL
60
50
40
30
20
10
0
G1
S
G2+M
CELL CYCLE PHASE
BHK
BHK-pNUT
pNUThTIMP-1
pNUTmTIMP-2
pNUTmTIMP-3

Chapter 4. Discussion

The regulation of the synthesis and degradation of the extracellular matrix is a key aspect of many physiological processes. The Matrix Metalloproteinases (MMPs) are a family of enzymes which are likely responsible for most of the degradation of ECM and have been implicated in a number of pathological states which feature deregulation of ECM proteolysis. Tight regulation of MMP catalytic activity is required and one of the mechanisms which enforces this regulation is interaction of the MMP with the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs), a family of multifunctional proteins which, in addition to their role as MMP inhibitors, are also regulators of cell growth and development. I sought to characterize certain aspects of the mouse homolog of TIMP-3, the newest member of the TIMP family. Specifically, I looked to: determine the ability of TIMP-3 to inhibit two of the MMPs, gelatinases A and B relative to the other members of the TIMP family; identify components of the extracellular matrix that associate with TIMP-3; identify domains of the TIMP-3 protein required for inhibition of the gelatinases and association with the extracellular matrix; and determine the ability of TIMP-3 to affect the growth of cells and their progression through the cell cycle.

It is widely assumed in the MMP/TIMP field that TIMP family members are interchangeable in their capabilities as inhibitors of active MMPs (Birkedal-Hansen *et al.*, 1993; Kleiner and Stetler-Stevenson, 1993). However the relative ability of the TIMPs to inhibit the catalytic activity of the MMPs has not been studied extensively and the characterization of a stronger inhibitory

capacity of a TIMP for a specific MMP by *in vitro* analysis may lead to the identification of significant functional relationships *in vivo*. The literature in this area is sparse and conflicting: It has been reported that there is differential inhibition of gelatinases A and B by TIMP-1 and TIMP-2 (Howard *et al.*, 1991), but conversely others have reported that no significant differences exist in the ability of the TIMPs to inhibit MMPs (Apte *et al.*, 1995).

Conclusions drawn from *in vitro* studies of TIMP behavior are complicated by the artificiality of the assay conditions used to test TIMPs. The ability of the TIMPs to inhibit MMPs has previously been measured using purified proteins in soluble peptide liberation assay which has all reagents suspended in an aqueous environment. This raises the issue of the insolubility of TIMP-3, which makes comparison with TIMP-1 and 2 hazardous as it cannot normally be studied in a comparable soluble state without preventing its association with the ECM. In a recent study that used solution MMP assays, the recombinant TIMP-3 employed had been expressed in a mouse myeloma cell line (Apte *et al.*, 1995), which produces a hyperglycosylated form of the protein that cannot associate with the ECM and as a consequence may be significantly different from the ECM associated TIMP-3 used in our analysis. Reverse zymography as a means of analyzing the relative inhibitory ability of the TIMPs may address the issue of comparison between TIMPs in spite of TIMP-3 insolubility by subjecting each TIMP to the same process of SDS denaturation followed by refolding after SDS removal. It can thus be argued

that the comparative gelatinase inhibitory capacity intrinsic to each TIMP will be accurately represented on a gelatin reverse zymogram. Furthermore, it could be argued that the conditions prevailing in a gelatin reverse zymogram, namely a three dimensional network of denatured collagen substrate, gelatinase and TIMPs, may more accurately reflect an ECM microenvironment than the conditions that are achieved in a soluble gelatin degradation assay. Also, reverse zymography uses proteins from conditioned media and matrix without purification steps which can increase the opportunity for gratuitous modifications that lead to alteration of bioactivity or specificity. These qualities of reverse zymography together with its very high sensitivity and reproducibility suggest that it is an effective and accurate method for characterizing the gelatinase inhibitory aspects of TIMP function.

Our comparison of TIMP inhibition of gelatinases A and B by reverse zymography using recombinant proteins expressed by both transiently and stably transfected cell lines has provided the novel and functionally significant observation that TIMP-3 can inhibit both gelatinases equally well, while TIMP-1 and TIMP-2 cannot inhibit gelatinase B as well as they inhibit gelatinase A. The recombinant TIMPs used for this comparison are not purified so comparing gelatinase inhibitory ability between the TIMPs is limited by the absence of accurate quantitation of amount of TIMP used, but comparing the amount of TIMP protein present as indicated by SDS-PAGE and silver staining suggests that TIMP-3 is actually a better inhibitor of gelatinase B than either TIMP-1 or

TIMP-2.

The functional significance of these observations would be apparent in situations where gelatinase B is produced to facilitate acute invasive behavior, such as is seen in trophoblast cells of a mouse embryo undergoing implantation into the wall of the uterus (Harvey *et al.*, 1995). TIMP-3 appears to be the only TIMP expressed in response to this invasion, where it is upregulated in maternal uterine stroma cells immediately adjacent to the trophoblast cells producing gelatinase B (Harvey *et al.*, 1995; Reponen *et al.*, 1995). The ability of TIMP-3 to associate with the ECM may be very important in this context since it provides a spatially localized "shield" that protects the uterus against the invading embryo. It will be interesting to determine whether disturbances in this gelatinase B/TIMP-3 balance underlie the defective implantation seen in preeclampsia or choriocarcinoma, a tumour which arises due to overinvasive behavior of trophoblast cells. Gelatinase B has also been shown to be directly involved with the metastatic potential of certain cell types (Bernhard *et al.*, 1994) but the relationship of TIMP-3 to gelatinase B production during this type of cell behavior is not yet known. Also, gelatinase B appears to be upregulated in certain degenerative diseases of the kidney (Martin *et al.*, 1994). The increasing body of evidence assigning a significant role for gelatinase B in pathology makes characterizing the qualities of TIMP-3 that allow it to inhibit this enzyme better than the other TIMPs important for developing effective ways of treating such disorders.

TIMPs are highly modular proteins due to the conservation of 12 cysteine residues which form intrachain disulfide bonds that define the N- and C- terminal domains as discrete, three-looped structures (Williamson *et al.*, 1990). This structural modularity has been translated into functional modularity for at least some of the characterized activities of the TIMPs, with MMP inhibition defined as a feature of the N-terminal domain (Murphy *et al.*, 1991; DeClerck *et al.*, 1993) while the C-terminal is involved in formation of TIMP:progelatinase complexes (Stetler-Stevenson *et al.*, 1989; Wilhelm *et al.*, 1989). Isought to look at the possible functional modularity of TIMP-3 in terms of the characteristics which distinguish it from TIMPs-1 and -2: superior gelatinase B inhibition and matrix association. In our analysisIconsidered three domains, the signal peptide, the N-terminal and the C-terminal.

The mechanism underlying the unique matrix association of TIMP-3 has not been characterized. The possibility that the signal peptide may play a role in trafficking TIMP-3 to the ECM is suggested by the work of Rathjen *et al.* (1990) who demonstrated that alternate splicing of leukemia inhibitory factor (LIF) mRNA transcripts resulted in the expression of a matrix bound LIF protein which was indistinguishable from soluble LIF as a mature protein, but the ECM associated form contained a different signal peptide sequence that included a distinctive MRCRIV motif. This sequence is not seen in the signal peptide of TIMP-3. However, the TIMP-3 signal sequences in mice, humans, and chickens are distinguished by the absence of a stretch of three or four leucine

residues present in the signal peptides of TIMP-1 and TIMP-2, and the presence of conserved tryptophan and cysteine residues. By analogy with LIF it was speculated that the TIMP-3 signal peptide may play a role in trafficking TIMP-3 to the ECM. Our results suggest that the TIMP-3 signal peptide, although unique from those of TIMP-1 and TIMP-2, does not alone play a role in localizing TIMP-3 to the matrix or in providing TIMP-3 with superior gelatinase B inhibitory capabilities. The signal peptide of TIMP-3 was however observed to alter some aspect of post translational processing of the mature TIMP-1 protein, as evidenced by alteration of both glycosylated and unglycosylated TIMP-1 mobility on reverse zymograms. The characterized function of the signal peptide is recognition by the signal peptide recognition particle (SRP) which causes association of the ribosomes and nascent protein with endoplasmic reticulum (ER), resulting in trafficking of the nascent protein into the lumen of the ER where post-translational processing occurs and eventual secretion in the case of the TIMPs (Alberts *et al.*, 1989). Signal peptides are rapidly cleaved from the protein by a signal peptidase following entry into the ER and they are not considered to take part in the subsequent post-translational processing and folding which gives rise to the three dimensional mature protein. However, our data from the S3 N1 C1 chimaera suggests that there may be differences in the post-translational processing of TIMP-1 and TIMP-3 dictated by their respective signal peptide sequences. Future experiments to characterize whether the change in TIMP-1 is due to alteration

of core protein length or changes in folding will shed light on the nature of the differences in the processes TIMPs undergo prior to their secretion.

The potential role of the 3'-UTR of the TIMP-3 mRNA in protein localization was also addressed here. Although there is no specific precedent for this region of the mRNA being involved in matrix localization, it has been unexpectedly implicated in a number of cellular processes. For example it has been shown that the 3'-UTR of the β-actin mRNA is sufficient for localization of the mRNA to the lamellipodia of motile fibroblasts (Jackson, 1993). The 3'-UTR of the TIMP-3 mRNA contains a region that is highly conserved between species (Leco *et al.*, 1994) suggesting that it plays an essential role in TIMP-3 production. Our findings do not demonstrate a role for the 3'-UTR in the matrix localization of TIMP-3. The function of this region remains to be characterized.

The possibility that a discrete region of TIMP-3 was required for its matrix association was pursued further by looking for the ability of the entire N- and C-terminal domains of TIMP-3 to localize to the matrix when expressed as a chimaeric protein with TIMP-1. It was observed that the TIMP-3 C-terminal domain was able to confer matrix localization upon a heterologous protein, TIMP-1, allowing us to conclude that the C-terminal contributes to the mechanism underlying TIMP-3 localization. Given the pattern of higher and lower mobility TIMP activity seen on reverse zymograms for TIMP-1 and TIMP-3 whichIhave shown is due to glycosylation of the protein, the 36 kDa activity expressed by the S1 N1 C3 chimaera is presumably due to full glycosylation of the protein. This possibly more

glycosylated form of the chimaeric protein did not localize to the matrix, suggesting that the addition of N-linked sugars to the site on the extreme C-terminus of TIMP-3 prevents the TIMP-3 C-terminal from conferring matrix association. Both glycosylated and unglycosylated TIMP-3 are normally detected in the matrix, but the unglycosylated form is more abundant (Blenis and Hawkes, 1983, 1984; Staskus *et al.*, 1991, Leco *et al.*, 1994). It is interesting to note that in our experiments it is the glycosylated form of TIMP-3 which appears to be more abundant in the soluble phase. Further, the recombinant TIMP-3 generated from a mouse myeloma cell line (Apte *et al.*, 1995) does not associate with the ECM and is hyperglycosylated. Collectively these observations suggest that glycosylation of TIMP-3 can interfere with ECM association. One possible mechanism accounting for this effect may be suggested by the S1 N1 C3 chimaera, in which there are three glycosylation sites present, two in the TIMP-1 N-terminal and one close to the carboxy end of the TIMP-3 C-terminus, proximal to two of the four HA binding motifs in TIMP-3 (see below). If the HA binding motifs can be shown to play a key role in matrix localization, then the proximity of the glycosylation site of the TIMP-3 protein may suggest that glycosylation can affect association with the matrix possibly by steric hindrance of interaction between the HA binding domains of TIMP-3 and the ECM.

The C-terminal of TIMP-3 plays a role in its localization and thus possesses at least some of the unknown characteristics required for association with the ECM. One of these characteristics may be the highly basic nature of TIMP-3 (pI of 9.16) predicted by its deduced amino acid sequence (Leco *et al.*, 1994) which suggests

that it may interact with negatively charged residues present in the extracellular matrix. Glycosaminoglycans (GAGs) are long unbranched polysaccharide chains composed of repeating disaccharide units in which one of the two sugars is almost always an amino sugar (Alberts *et al.*, 1989). They are hydrophilic, inflexible, and highly negatively charged due to the presence of sulfate or carboxyl groups on most of their sugar residues. Collectively these attributes result in their formation of hydrated gels filling most of the extracellular space. If TIMP-3 is associating with the matrix in a nonspecific fashion, the characteristics of GAGs outlined would make them excellent candidates for such an association. Ihave demonstrated here that a number of the glycosaminoglycans can associate with TIMP-3, and perhaps some may have higher affinity for TIMP-3 than others, but the characteristics of the GAG which would impart this increased TIMP-3 affinity are unclear. Heparin was observed to pull TIMP-3 out of matrix at a lower concentration than other GAGs tested, and heparin is distinguished from other GAGs by the presence of 2.0-3.0 sulfates per dissacharide unit, while other GAGs have 0-1.8, which would possibly make it a more negatively charged GAG. These observations would support the conclusion that TIMP-3 associated with the matrix in a nonspecific, charge based-manner.

Conversely, it remains possible that TIMP-3 is involved in a specific association which is based on domains in the protein which recognize particular ECM components. Hyaluronic acid (HA) is a GAG which is widely distributed in body tissues and is seen in ECM and on cell surfaces. It has been shown to affect

cell behavior such as adhesion, motility, and growth where it is present on cell surfaces (Yang *et al.*, 1994; Hall *et al.*, 1995). A specific binding domain for hyaluronic acid has been characterized on the Receptor for Hyaluronic Acid Mediated Motility (RHAMM), a cell surface associated protein which mediates some of the effects of HA on cell behavior (Yang *et al.*, 1994). Ihave observed that HA can titrate TIMP-3 out of the matrix. The HA binding motif is characterized as two basic amino acid residues spaced seven residues apart (BX_7B), and predicted to be minimally required for HA binding activity. Four motifs are present in the predicted amino acid sequence of TIMP-3 which qualify as HA binding domains. Two of these motifs are also seen in TIMPs-1 and -2; the function of these, if there is one, is not known. In other known HA binding proteins, the HA binding domain has been shown to be required in order for the protein to bind biotinylated HA (BHA). I can conclude that there is a protein or proteins present in TIMP-3 enriched matrix, but not control matrix, which can bind to BHA. The identity of the HA binding protein(s) detected in the matrix is unclear. The 66 kDa target for BHA could represent a protein complex that contains TIMP-3. Both TIMP-1 and TIMP-2 have been shown to be involved in the formation of higher order structures with proenzyme forms of certain MMPs (Stetler-Stevenson *et al.*, 1989; Wilhelm *et al.*, 1989) and one of the cysteine proteinases (Boujrad *et al.*, 1995) with significant functional consequences. It is likely that TIMP-3 is also involved in such higher order complexes as a component of its activity, and that this complex is what is detected by BHA in the matrix. Verification that TIMP-3 is involved will require the

use of antibodies which are not yet available to us. Also, BHA is being prepared with greater specific activity for use in further experiments to characterize the relationship of HA and TIMP-3, including alteration of the putative HA-binding domains of TIMP-3 and analysis of the effect on BHA binding. These experiments will help characterize in detail the basis of the relationship between TIMP-3 and the ECM.

The *in vitro* relationship between the ECM and TIMP-3 is not stable for longer periods under low serum conditions as evidenced by the migration of TIMP-3 into the conditioned medium upon culture of TIMP-3 overexpressing BHK cells in reduced (1%) serum conditions for seven days. The basis for this dissociation is not known. Future experiments to characterize the change in conditions required for TIMP-3 to enter the soluble phase will also shed light on the mechanism of TIMP-3 association with the ECM. As pointed out, the increased proportion of TIMP-3 present in glycosylated form in the soluble phase suggests that the glycosylation state of the protein may play a role. It is interesting to note that the "soluble" TIMP-3 appears fully able to inhibit gelatinase A and gelatinase B in reverse zymography. This would suggest that TIMP-3 *in vivo* does not have a requirement for matrix association in order to block the catalytic activity of MMPs. It is not yet known if other uncharacterized aspects of TIMP-3 function are disrupted by the loss of matrix association, but detection of TIMP-3 behaving as a soluble protein raises the question of whether there is also some functional relevance *in vivo* for this change in matrix affinity. It is possible that TIMP-3 remains bound to

the ECM only for a period of time that would be required for it to serve its function, such as stopping the invading trophoblast (Harvey *et al.*, 1995) or to affect differentiation (Wick *et al.*, 1994), or some other uncharacterized activity, and then possibly as a downregulation mechanism, becomes a soluble protein until it is resorbed.

Certain TIMP constructs that were transfected did not give rise to proteins in spite of their transcription and the apparent absence of PCR induced mutations. This was seen with the S3 N3 C1 construct as well as the C-terminally truncated TIMP-3 (S3 N3) but not for the other chimaeras and deletions expressed, suggesting that the TIMP-3 C-terminal region plays a specific and vital role in protein folding. In order to verify that the protein is not actually being translated and trapped within the cell it will be necessary to perform reverse zymography on whole cell extracts of cells transfected with cDNA encoding these otherwise undetectable proteins. This may also be approached by metabolically labeling the cells to look for expressed chimaeric protein that may lack the gelatinase inhibitory capacity required for detection on reverse zymograms. A third approach may involve attempting to express the constructs in reticulocyte lysates which would presumably lack the surveillance mechanisms that are responsible for detection and degradation of incorrectly folded proteins in the intact cell. These approaches would all help to clarify the role of the TIMP-3 C-terminal domain in protein folding.

Sorsby's Fundus Dystrophy (SFD) is an autosomally dominant heritable degenerative disease of the retina which has been shown to be due to mutations

to TIMP-3 (Weber *et al.*, 1994). The mutations occurring to TIMP-3 in individuals with this disorder have been characterized as introduction of cysteine residues localized to the C-terminal domain which result in an uncharacterized gain of function. The description of function altering mutations localized to the C-terminal in SFD, together with our observation that the TIMP-3 N-terminal domain cannot be translated into a secreted protein either by itself or with a substituted TIMP-1 C-terminal domain suggests that the C-terminal of TIMP-3, in addition to being involved in ECM localization, is critical to other aspects of TIMP-3 function. Analysis of TIMP-3 cloned from individuals with SFD has started and will undoubtedly lead to identification of the role of TIMP-3 in SFD as well as the part that the C-terminal domain plays in its activities. Also, our structure-function analysis of TIMP-3 suggests that in addition to the characteristically conserved cysteines being key contributors to the structure of the protein, the TIMPs appear to have additional folding requirements which are unique to each and may reflect structural requirements for other distinguishing functions for each TIMP in addition to MMP inhibition. This is supported by the conformation stability analysis of truncated forms of human TIMPs-1 and -2, which demonstrates that the TIMP-1 N-terminus expressed by itself is 1.4-2.0 times more stable than the N-terminus of TIMP-2 (Williamson *et al.*, 1994). The C-terminally truncated forms of human TIMP-1 and mouse TIMP-2 that have been successfully expressed in our experiments will also be useful for trying to narrow down regions of these proteins that are responsible for the potent mitogenic and growth inhibitory activity that they possess.

As pointed out earlier TIMP-1 and TIMP-2 are multifunctional proteins which, in addition to their ability to inhibit MMPs, have been characterized as potent mitogens or growth inhibitors depending on the cell type (Hayakawa *et al.*, 1992; Stettler-Stevenson *et al.*, 1992; Nemeth and Goolsby, 1993). TIMP-3 appeared to be growth inhibitory for some cell types (BHK, HS68, 10T1/2), growth stimulatory for one cell type (HT 1080), while another cell type did not appear to be affected by the presence of TIMP-3 at all (B16F10). Conclusions regarding the effect of TIMP-3 on cell growth are complicated by the observation that the bulk of TIMP-3 does not remain associated with the matrix over the course of the growth assay and does, in fact, enter the soluble phase. It is possible that the effect of TIMP-3 on cell growth, if it exists, requires the TIMP-3 to be in the matrix either because it is functionally necessary or because it raises the concentration of TIMP-3 in the immediate vicinity of the cells attached to the matrix sufficiently to induce such an effect. If it can be demonstrated that the physical association of TIMP-3 with the ECM is not required for it to act as a growth effector, the detection of soluble TIMP-3 is encouraging with respect to purifying the protein for future growth assays in which it can be readily added to growing cells at a known concentration which will clarify our understanding of its effect on cell growth.

Another important experiment will be to assess the growth effects of reductively alkylated TIMP-3, which will have its MMP inhibitory ability ablated by the disruption of the protein tertiary structure. If this reduced TIMP-3 is still able to influence cell growth, I will be able to dissociate its role as an MMP inhibitor from

its role as a growth regulator.

It is possible that the growth inhibition that is imposed by TIMP-3 is based in blocking cells in G1 when overexpressed. The mechanism by which TIMP-3 imposes this block to the cell cycle is not known, nor is the physiological basis of such a block known. It has been reported that human TIMP-3 is expressed in G1 phase of the cell cycle concomitant with an arrest of cell cycle progression associated with differentiation of HL-60 cells, a myeloid cell line (Wick *et al.*, 1994). These observations raise the possibility that the expression patterns seen during development may outline the locations where TIMP-3 is directly involved in providing differentiation or growth arrest signals to cells in addition to or possibly instead of acting as an MMP inhibitor. As to the growth stimulatory effect of TIMP-3 seen, the mechanism that accounts for this is unknown, as it is also for the mitogenic activities of TIMP-1 and TIMP-2 that have been characterized. Future experiments looking at TIMP-3 expression in a range of stem cell types in relation to growth effects and differentiation will clarify this novel role for the newest member of the TIMP gene family.

4.2 PUBLICATIONS DURING THIS THESIS

Brown, C.B., **Beaudry, P.**, Dickinson Laing, T., Shoemaker, S., and Kaushansky, K. (1995). In Vitro Characterization of the Human Recombinant Soluble Granulocyte-Macrophage Colony Stimulating Factor Receptor. Blood 85:1488-1495.

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