Tissue Inhibitor of Metalloproteinases-4 and Progelatinase A Activation: 
The Role of the C-terminal Tail in Mediating Activation

By

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B.Sc., The University of British Columbia, 1998

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We accept this thesis as conforming
to the required standard

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April 2002

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Department of Oral Biological Medical Sciences

The University of British Columbia
Vancouver, Canada

Date April 25th, 2002
ABSTRACT

The activation of gelatinase A (matrix metalloproteinase-2) occurs on the cell surface via a unique mechanism that requires a ternary complex of membrane-type 1 matrix metalloproteinase (MT1-MMP) and tissue inhibitor of metalloproteinases (TIMP)-2, which acts as a cell surface receptor for progelatinase A. TIMP-2 binds to both the MT1-MMP and the hemopexin C-domain of progelatinase A thus linking progelatinase A to the cell surface where a second MT1-MMP can begin the activation process by cleaving the propeptide of progelatinase A. Recently, our lab has shown that TIMP-4, which shares a high degree of homology with TIMP-2, can bind and inhibit MT1-MMP and independently bind the hemopexin C-domain of progelatinase A in a manner similar to TIMP-2 but is unable to support the activation of progelatinase A.

TIMPs consist of two domains: the N-terminal inhibitory domain and the C-terminal domain. Additionally, TIMPs-2, -3, and -4 contain nine residues at the C-terminus known as the C-terminal tail. TIMPs-2 and -4 are hypothesized to bind to progelatinase A through their non-inhibitory C-domains. To investigate the differences in the interactions of TIMP-2 and TIMP-4 with progelatinase A, the C-terminal domains of TIMPs-2 and -4 were each cloned as a C-terminal fusion partner to horse heart myoglobin. The anionic C-terminal tail of TIMP-2 is hypothesized to bind a cationic site on the hemopexin C-domain of progelatinase A during activation. Therefore, to investigate the aspects of TIMP-4 that renders it deficient in the activation of progelatinase A, a set of mutations were introduced into the C-terminal tail of TIMP-2 and -4 and the activities of 8 fusion proteins
were investigated. Myoglobin-C-TIMP-2 (MbcT2) bound progelatinase A with reduced affinity relative to full length TIMP-2, whereas deletion of the tail decreased the affinity further. In contrast, deletion of the C-terminal tail of C-TIMP-4 did not alter the binding of Mb-C-TIMP-4 (MbcT4) to progelatinase A and both proteins bound with only slightly less affinity than full length TIMP-4. The similarity of the binding affinities of TIMP-4 and MbcT4 to progelatinase A indicates that the N-terminal domain and C-terminal tail of TIMP-4 is unlikely to contribute to its interaction with progelatinase A. Mutations that removed the negative charges in the C-terminal tail of MbcT2 (E192V/D193Q) also resulted in a reduction in binding, as did a substitution of the C-terminal tail of MbcT2 with that of TIMP-4. Conversely, addition of negative charges to the C-terminal tail of MbcT4 (V193E/Q194D) resulted in an increased affinity for progelatinase A, as did a substitution of its tail with that of TIMP-2. The overall analysis of these fusion protein constructs show that the C-terminal tail of TIMP-2 is integral to its ability to support the activation of progelatinase A, specifically the negatively charged residues Glu192 and Asp193. The data also indicate that the reason TIMP-4 cannot support the activation of progelatinase A is because of the lack of these negatively charged residues in the TIMP-4 C-terminal tail.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM</td>
<td>A disintegrin and a metalloproteinase</td>
</tr>
<tr>
<td>ADAM-TS</td>
<td>A disintegrin and metalloprotease with thrombospondin type I modules</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein 1</td>
</tr>
<tr>
<td>APMA</td>
<td>4-aminophenylmercuric acetate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA-MMP</td>
<td>Cysteine array matrix metalloproteinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy terminal</td>
</tr>
<tr>
<td>C-TIMP</td>
<td>The carboxy terminal domain of a tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbance assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Erythroid potentiating activity</td>
</tr>
<tr>
<td>FAB</td>
<td>Fluorimetry assay buffer</td>
</tr>
<tr>
<td>hhMb</td>
<td>Horse heart myoglobin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>K_d</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>MbcTIMP</td>
<td>Fusion protein consisting of a myoglobin at the amino terminus of the molecule and the carboxy terminal domain of a tissue inhibitor of metalloproteinases at the carboxy terminus</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMPI</td>
<td>Matrix metalloproteinase inhibitors (synthetic)</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane type matrix metalloproteinase</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCPE</td>
<td>Procollagen C-terminal proteinase enhancer protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB Id</td>
<td>Protein data bank identification number</td>
</tr>
<tr>
<td>PEA3</td>
<td>Polyoma enhancer activator</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal cell-derived factor</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SV-40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
</tbody>
</table>
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CHAPTER 1 – INTRODUCTION

1.1 General overview

Many of the normal physiological processes undertaken by cells in multi-cellular organisms involve controlled remodelling of the extracellular matrix (ECM). Connective tissue remodelling is essential for normal development and growth. Processes such as wound healing, angiogenesis, and ovulation cannot occur without controlled remodelling of the ECM (Parks, 1999; Edwards et al., 1996; Nguyen et al., 2001; Smith et al., 1999). In addition to a structural role, the ECM also provides cells with biological information and signals about their environment. The ECM can sequester certain signalling molecules within its lattice, which are released upon breakdown. Exposure of cryptic sites through cleavage of ECM molecules during remodelling can also provide signals that are important for processes such as cellular growth, differentiation, migration, and apoptosis and can be a factor in the progression of disease (Boudreau and Jones, 1999; Aumailley and Gayraud, 1998; Streuli, 1999). The ECM is composed of a number of proteins and proteoglycans of which collagen is the most abundant. Collagen consists of three individual α-chains that have a right-handed triple helical conformation and exists in numerous forms that can be subdivided into fibril-forming collagens (types I, II, III, V, XI), and non-fibril-forming collagens such as the network-forming collagens (types IV and VII) (Aumailley and Gayraud, 1998).
ECM homeostasis is achieved through the collective efforts of a number of proteinase and inhibitors families including a family of enzymes known as matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). A delicate balance between MMPs and TIMPs aids in controlling the breakdown of the components of connective tissue. Errant expression of MMPs and/or TIMPs resulting in an imbalance can lead to diseases such as periodontitis, arthritis, and atherosclerosis (Birkedal-Hansen et al., 1993; Birkedal-Hansen, 1993; Yamanishi and Firestein, 2001; Newby et al., 1994; McDonnell et al., 1999). MMPs and TIMPs also play a role in neoplasia, especially in tumour angiogenesis and metastasis (Kleiner and Stetler-Stevenson, 1999). Indeed, many MMPs are prognostic indicators in various cancers (Murray et al., 1996; Yamamoto et al., 2001; Gohji et al., 1996; Curran and Murray, 2000); (Egeblad and Werb, 2002). Synthetic MMP inhibitors (MMPIs) are currently undergoing clinical trials for the treatment of several cancers because of the importance of MMPs in the progression of cancer. However, it is difficult to judge their effectiveness in early phase clinical trials because MMPIs are anti-proliferative and anti-angiogenic but not cytotoxic (Hoekstra et al., 2001; Egeblad and Werb, 2002). MMPIs are also under investigation for the treatment of arthritis and heart disease (Elliott and Cawston, 2001; Creemers et al., 2001).

Because of the vast and varied roles of MMPs and TIMPs in normal physiological processes and in disease, it is important to study how MMPs and TIMPs interact
and bind to each other. This knowledge may be useful in controlling tissue remodelling and may lead to the discovery of strategies to combat diseases that involve the uncontrolled degeneration of the ECM such as rheumatoid arthritis, cancer and a number of cardiovascular diseases. MMP-2 or gelatinase A is an enzyme that is implicated in many diseases including cancer because of its ability to degrade type IV collagen, the basement membrane collagen, although recently, there has been evidence that its actions on the basement membrane causes the exposure of cryptic sites and release of growth factors and cytokines, which is in turn responsible for its effects on tumour progression (Giannelli and Antonaci, 2002). Indicative of the role of gelatinase A in neoplasia is the fact that gelatinase A deficient mice have reduced tumour angiogenesis and progression (Itoh et al., 1998). Because of the importance of gelatinase A in disease processes, it is important understand how its inactive precursor, progelatinase A, is activated on the surface of cells. It is hoped that knowledge of the activation mechanisms of this enzyme will lead to a better understanding of its role in the progression of cancer so that therapeutic agents can be designed to combat this disease. The role of MMPs and TIMPs in many processes are currently the subject of intense research but of particular of interest is the fact that TIMP-2, besides its ability to regulate MMP activity through inhibition, has also been shown to participate in the activation of progelatinase A, thus regulating the activation of MMPs (Strongin et al., 1995). It was recently shown that another TIMP, TIMP-4 is not able to promote the activation of progelatinase A although it can interact with progelatinase A in a manner similar to TIMP-2 (Bigg et al., 2001;
Toth et al., 2000; Hernandez-Barrantes et al., 2001). This study investigates the dichotomous relationship between progelatinase A and TIMPs-2 and -4 and why TIMP-4 cannot support the activation of progelatinase A.

1.2 Matrix Metalloproteinases

Matrix metalloproteinases, also known as matrixins, are a family of endopeptidases that are collectively capable of degrading most components of the ECM. The MMPs utilize a zinc ion for catalysis and have a requirement for structural zinc and calcium ions. MMPs are members of a superfamily of metalloproteinases, the metzincins, that are characterized by their reliance on zinc for catalysis and a conserved methionine which constitutes the “Methionine turn” which forms the end of the lower edge of the active site (Bode et al., 1999). MMPs have a conserved zinc-binding motif with the sequence HExxHxxGxxH in which the underlined histidine residues coordinate the catalytic zinc ion (Overall, 2001).

1.2.1 Structure of MMPs

There are 22 human MMPs known to date. The MMPs are structurally related and have conserved regions of homology and can be subdivided into different categories including the collagenases, stomelysins, gelatinases, matrilysins, and membrane-type MMPs (MT-MMPs) (Table 1). With the exception of the matrilysins and MMP-23 (cysteine array (CA)-MMP), MMPs are organized into...
Table 1: Members of the Matrix Metalloproteinase Family

<table>
<thead>
<tr>
<th>MMP Designation</th>
<th>Common Name</th>
<th>Sub-Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Collagenase-1</td>
<td>Collagenase</td>
</tr>
<tr>
<td></td>
<td>Interstitial collagenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibroblast collagenase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tissue collagenase</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Gelatinase A</td>
<td>Gelatinase</td>
</tr>
<tr>
<td></td>
<td>72-kDa gelatinase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72-kDa type IV collagenase</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Stromelysin-1</td>
<td>Stromelysin</td>
</tr>
<tr>
<td></td>
<td>Proteoglycanase</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Matrilysin-1</td>
<td>Matrilysin</td>
</tr>
<tr>
<td></td>
<td>PUMP-1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Neutrophil collagenase</td>
<td>Collagenase</td>
</tr>
<tr>
<td></td>
<td>Collagenase-2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Gelatinase B</td>
<td>Gelatinase</td>
</tr>
<tr>
<td></td>
<td>92-kDa gelatinase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>92kDa type IV collagenase</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Stromelysin-2</td>
<td>Stromelysin</td>
</tr>
<tr>
<td>11</td>
<td>Stromelysin-3</td>
<td>Stromelysin</td>
</tr>
<tr>
<td>12</td>
<td>Metalloelastase</td>
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</tr>
<tr>
<td></td>
<td>Macrophage elastase</td>
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</tr>
<tr>
<td>13</td>
<td>Collagenase-3</td>
<td>Collagenase</td>
</tr>
<tr>
<td>14</td>
<td>Membrane-Type (MT)1-MMP</td>
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<td>15</td>
<td>MT2-MMP</td>
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<td>16</td>
<td>MT3-MMP</td>
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<td>17</td>
<td>MT4-MMP</td>
<td>Membrane-Type</td>
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<tr>
<td>18</td>
<td>Collagenase-4</td>
<td>Collagenase</td>
</tr>
<tr>
<td></td>
<td>xCol4 (Xenopus, no known</td>
<td></td>
</tr>
<tr>
<td></td>
<td>human homologue)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Formerly MMP-18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RAS1-1</td>
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<tr>
<td>20</td>
<td>Enamelysin</td>
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<td>21</td>
<td>XMMP (Xenopus)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human homologue unpublished</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>CMMMP (Chicken)</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Cysteine Array (CA)-MMP</td>
<td></td>
</tr>
<tr>
<td>23a</td>
<td>Formerly MMP-21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIFR-1</td>
<td></td>
</tr>
<tr>
<td>23b</td>
<td>Formerly MMP-22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIFR-2</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Femalysin</td>
<td>Membrane-Type</td>
</tr>
<tr>
<td>25</td>
<td>MT5-MMP</td>
<td>Membrane-Type</td>
</tr>
<tr>
<td>26</td>
<td>Leukolysin</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Matrilysin-2</td>
<td>Matrilysin</td>
</tr>
<tr>
<td></td>
<td>Endometase</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Human homologue of MMP-22</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Epilysin</td>
<td></td>
</tr>
</tbody>
</table>
three basic domains: the propeptide domain, which is responsible for maintaining enzyme latency; the catalytic domain; and the carboxy (C)-terminal hemopexin domain, which contains exosites for substrate specificity (Figure 1). MMPs also contain an N-terminal signal peptide. Some MMPs also have additional domains such as the C-terminal transmembrane domain and cytoplasmic tail of some MT-MMPs (Sato et al., 1994). Other MT-MMPs are known to be glycosylphosphatidyl inositol anchored to the cell membrane (Itoh et al., 1999; Kojima et al., 2000). Many of the MMPs have domains that share homology to matrix proteins. These include the fibronectin-like domains of gelatinase A and B and the \( \alpha_2(V) \) collagen chain of gelatinase B (contained within the O-linked oligosaccharide domain) (Figure 1) (Overall, 2001; Opdenakker et al., 2001).

1.2.2 Regulation of MMPs

Highly controlled regulation of MMP activity is essential due to the potentially devastating effects of MMPs. There are three main mechanisms of regulation for MMPs: transcriptional, post-transcriptional, and post-translational. Together, these mechanisms tightly and differentially control the levels and activity of MMPs in tissues so that they can participate in normal physiological processes without causing harm. A breakdown in the regulatory mechanisms of MMPs can lead to serious disease and is thought to contribute greatly to tumour angiogenesis and metastasis (Egeblad and Werb, 2002).
Matrilysin 1 and 2

Collagenase-1, -2, and -3, Stromelysin-1, and -2, Metalloelastase, Enamelysin, and MMP-19

Stromelysin-3

Membrane-Type-1, -2, -3, and -5 MMP

Membrane-Type-4 and -6 MMP

Gelatinase A

Gelatinase B

MMP-21 (X MMP)

Cysteine-Array-MMP

Figure 1: Schematic representation of the different MMPs and their domain structure.
Firstly, the expression of MMPs is regulated transcriptionally. Members of the MMP family are differentially and tissue specifically regulated. While some MMPs genes are inducible, others, such as MMP-2, are not readily inducible and are more constitutively expressed at a basal level. The readily inducible MMPs (MMP-1, -3, -7, -9, -10, -12, and -13) are under the influence of two main regulatory elements found within their promoter regions, the activating protein-1 (AP-1) site and the polyoma enhancer activator (PEA3) site (Curran and Murray, 2000; Sternlicht and Werb, 2001). The AP-1 element located at approximately -70 base pairs upstream of the transcriptional start site, is important for both basal transcription of the inducible MMP genes and for repression of transcription. Basal transcription and transactivation of MMPs require that AP-1 interact with other cis-acting elements such as PEA3 (Benbow and Brinckerhoff, 1997). Interleukin-1 (IL-1), tumour necrosis factor α (TNF-α), interferon-β, 12-O-tetradecanoylphorbol 13-acetate, glucocorticoids, retinoids, thyroid hormones, progesterone, androgens, and transforming growth factor β (TGF-β) all act in some way on the AP-1 site to modulate the expression of inducible MMPs (Curran and Murray, 2000).

MMPs can also be post-transcriptionally regulated via increased mRNA stability. This is the case for gelatinase A. Upon addition of TGF-β to human gingival fibroblasts, the abundance and stability of gelatinase A mRNA was markedly increased (Overall et al., 1991). Another post-transcriptional regulation mechanism is zymogen activation. MMPs are synthesized as inactive zymogens
that contain an ~80 amino acid amino (N)-terminal prodomain which is responsible for maintaining enzyme latency (Figure 1). The prodomains of all MMPs except CA-MMP (Pei, 1999) contain a cysteine switch motif PRCG(N/V)D where the cysteine residue (underlined) coordinates the active site zinc, which excludes a water molecule necessary for catalysis, thus preserving enzyme latency (Overall, 2001). Most MMPs are secreted by cells in a latent, proenzyme/zymogen form, which is later converted to the active form by the cleavage of the prodomain. The exceptions are MMP-11 (stromelysin-3), the MT-MMPs and CA-MMP. Stromelysin-3 and the MT-MMPs are activated intracellularly by furin cleavage at the furin recognition sequence before secretion or transport to the cell surface, respectively (Pei and Weiss, 1995; Sato et al., 1996). CA-MMP is N-terminally tethered to the cell surface as a zymogen by an N-terminal signal anchor and is released as a fully active enzyme following proteolytic cleavage at a proprotein convertase motif that is located between the pro and catalytic domains (see Figure 1) (Pei et al., 2000). The activation of MMPs in vitro can also be accomplished by the addition of organomercurials such as aminophenylmercuric acetate (APMA) (Stricklin et al., 1983). Most MMPs can be activated by other proteases such as serine proteinases (i.e. plasmin or trypsin) (Okada and Nakanishi, 1989; Nagase et al., 1990; Murphy et al., 1992a; Lee et al., 1996). However, gelatinase A is different in that it lacks serine protease cleavage sites in the prodomain (Okada et al., 1990). Thrombin has been shown to induce activation of progelatinase A in microvascular endothelial cells (Nguyen et al., 1999). This pathway is hypothesized to be
independent of the thrombin receptor and is thought to involve thrombin forming a complex with thrombomodulin. The complex then converts protein C to activated protein C, which then activates progelatinase A (Nguyen et al., 2001). MMPs can also activate other MMPs, as is the case with MMP-3 (stromelysin-1), which can activate MMP-1, -8, and -13 (collagenase-1, -2, and -3) (Murphy et al., 1987; Knauper et al., 1993; Knauper et al., 1996a). Gelatinase A and collagenase-3 can activate MMP-9 (gelatinase B) (Fridman et al., 1995; Knauper et al., 1997). MT1-MMP is also an important activator of MMPs and is capable of activating procollagenase-3 (Knauper et al., 1996b). However, the most intriguing mechanism of MMP activation involving MT1-MMP is the activation of progelatinase A (Sato et al., 1994). Activation of progelatinase is lacking in MT1-MMP deficient mice indicating that MT1-MMP is the primary activator of progelatinase A in vivo (Zhou et al., 2000). This mechanism of activation occurs at the cell surface and requires a ternary complex of MT1-MMP and one of the inhibitors of MMPs, TIMP-2, to tether progelatinase A to the cell surface in order for another TIMP-2 free MT1-MMP molecule to cleave progelatinase A. The activation of progelatinase A by MT1-MMP and these progelatinase A/TIMP-2 interactions will be discussed in detail in section 1.3.4 of this thesis.

The final mechanism of MMP regulation occurs post-translationally via MMP inhibitors. The major physiological inhibitors of MMPs are TIMPs. Currently, there are four known members of the TIMP family, numbered 1-4. TIMPs noncovalently inhibit MMPs stoichiometrically at a 1:1 molar ratio. The
expression of TIMPs is also tightly regulated to maintain the local balance between MMPs and TIMPs to control ECM breakdown and accumulation. Their function and importance will also be discussed in greater detail in section 1.3 of this thesis. Besides the TIMPs, other endogenous inhibitors of MMPs include the plasma protein α2-macroglobulin (Sottrup-Jensen and Birkedal-Hansen, 1989), the noncollagenous NC1 domain of type IV collagen (Netzer et al., 1998), and a C-terminal fragment of procollagen C-terminal proteinase enhancer protein (PCPE) (Mott et al., 2000). The NC1 domain and C-terminal fragment of PCPE are protein subdomains that have structural similarity to the TIMPs. α2-macroglobulin may be the major inhibitor of MMP in tissue fluids because it is so abundant and α2-macroglobulin/MMP complexes are irreversibly cleared by scavenger receptor-mediated endocytosis (Sternlicht and Werb, 2001; Egeblad and Werb, 2002). The inhibitory interactions between the NC1 domain or the C-terminal fragment of PCPE and MMPs appear to be of lower affinity than that of TIMPs, although they can inhibit angiogenesis and tumour growth much like TIMPs (Petitclerc et al., 2000).

1.2.3 Gelatinase A

Gelatinase A (72-kDa gelatinase/type IV collagenase) is a 72 kDa proteinase that is constitutively expressed and secreted by most cell types. It contains an 80 amino acid propeptide, a 338 amino acid catalytic domain that has within it three 58-residue tandem repeats similar to the collagen-binding domain of fibronectin (the fibronectin type II-like modules), a 28 amino acid hinge region, and a 185
amino acid hemopexin C domain (Figures 1 and 2) (Collier et al., 1988; Morgunova et al., 1999). Its matrix-derived substrates include types I, III, IV, V, VII, X, and XI collagens, gelatin, fibronectin, laminin, aggrecan, elastin, and decorin (Sternlicht and Werb, 2001; Egeblad and Werb, 2002; Imai et al., 1997). In addition to its role in matrix degradation, gelatinase A can also cleave non-structural molecules such as, pro-TGF-β (Yu and Stamenkovic, 2000), pro-IL-1β (Schonbeck et al., 1998), and pro-TNF-α (Gearing et al., 1994), resulting in a variety of biological activities. Recently, it was shown by McQuibban et al. that gelatinase A cleaves chemokines including stromal cell-derived factor (SDF)-1 (McQuibban et al., 2001) and monocyte chemoattractant protein (MCP)-3 (McQuibban et al., 2000). Cleavage of SDF-1 and MCP-3 destroys their chemotatic ability and the cleaved MCP-3 has been shown to act as a receptor antagonist, preventing functional MCP-3 from binding to its receptors (McQuibban et al., 2000; McQuibban et al., 2001). This suggests that gelatinase A plays an important role in the regulation of inflammatory responses. C-terminally truncated gelatinase A has been shown to exhibit full proteolytic activity in the degradation of gelatin and type IV collagen (Fridman et al., 1992), thus the hemopexin C domain of gelatinase A does not appear to be required for its gelatinolytic activity. However, it appears to be necessary for its collagenolytic activity (Patterson et al., 2001), although it does not bind to native collagen (Wallon and Overall, 1997). The gelatinase A hemopexin C domain is also essential for interaction with its inhibitors, namely TIMPs (Murphy et al., 1992b;
Figure 2: The crystal structure of progelatinase A including the prodomain, the catalytic domain including the three fibronectin like modules, the hinge region and the hemopexin C domain as indicated. Note the cysteine of the cysteine switch shown in space-fill, coordinating the active site Zn2+. Part of the hinge region remains unresolved due to the molecular motion of that region. PBD Id: 1CK7 (Morgunova et al., 1999).
Ward et al., 1994). Thus the hemopexin C domain is important for the activation of progelatinase A by a ternary complex involving TIMP-2 and MT1-MMP (Strongin et al., 1995).

The ability of invasive tumour cells to degrade connective tissue stroma and basement membrane is pivotal to metastasis (Liotta and Stetler-Stevenson, 1991; Stetler-Stevenson et al., 1993) and the ability of endothelial cells to degrade the basement membrane and migrate through the ECM is essential for angiogenesis (Nguyen et al., 2001). Since type IV collagen is the major basement membrane structural protein, its degradation is a key event in tumour spread and growth. Thus, understanding and studying the structure and function of the proteases involved in metastasis and angiogenesis and their inhibitors, and their regulation is essential for understanding and controlling cancer. Due to the ability of gelatinase A to degrade type IV collagen, it has been implicated in the breakdown of the basement membrane. Cell lines transfected with gelatinase A have been shown to have increased invasiveness (Kawamata et al., 1995) and metastatic tumours often have high levels of gelatinase A expression either in the tumour itself or more commonly in the surrounding stroma (Stetler-Stevenson and Yu, 2001; Egeblad and Werb, 2002). Interestingly, gelatinase A knockout mice show reduced tumour growth and metastasis (Itoh et al., 1998). A tumour cannot grow larger than $1\text{mm}^3$ before it requires its own blood supply for nutrients and waste removal, thus gelatinase A has also been implicated in tumour angiogenesis (Nguyen et al., 2001). The role of gelatinase A in
angiogenesis was established by Kato et al., who showed that gelatinase A deficient mice show reduced corneal angiogenesis (Kato et al., 2001). The hemopexin C domain of gelatinase A can block angiogenesis by displacing active gelatinase A from the integrin $\alpha_\text{v}\beta_3$, providing further evidence of the role of gelatinase A in angiogenesis (Brooks et al., 1998; Bello et al., 2001).

1.3 Tissue Inhibitor of Metalloproteinases

The tissue inhibitors of metalloproteinases are a family of naturally occurring protein inhibitors that are responsible for modulating the activity of MMPs. Currently, there are four known mammalian TIMPs. They inhibit MMPs by forming a non-covalent, stoichiometric complex. The four TIMPs are highly homologous proteins both between the different members of the family and interspecies. Human TIMPs share approximately 40-50% identity and 50-70% similarity (Figure 3) (Douglas et al., 1997). TIMPs also have conserved gene structures and are located within introns of synapsin genes, indicating that the different TIMPs are likely due to gene duplications (Brew et al., 2000).

1.3.1 Structure of TIMPs

TIMPs are organized into two distinct, independently folding domains: the N-terminal domain and the C-terminal domain (Figure 4). TIMPs also contain a short leader sequence consisting of 23-29 amino acids, which is cleaved off to produce the mature protein. TIMPs share a high degree of similarity in their tertiary structure. Each TIMP contains 6 loops held together by 12 conserved
Figure 3: Sequence alignment of human TIMPs. Sequences of TIMP-1 (Accession number P01033), TIMP-2 (P16035), TIMP-3 (P35625), and TIMP-4 (Q99727) were obtained from the National Center for Biotechnological Information protein data bank and aligned using Megalign from DNASTar. Residues that match the consensus are shown as ".". When all residues match the consensus, the residue of the consensus is shown, otherwise "." is shown. Note the 9 extra residues present in TIMPs-2, -3, and -4, C-terminal tail (underlined).
Figure 4: Crystal Structure of TIMP-2. The N-terminal and C-terminal domains are as indicated. The N-terminus is marked by residue Cys1. The C-terminal tail is not visible due to its disordered nature in the crystal. The approximate location of the C-terminal tail is indicated by the arrow. PDB Id: 1BR9 (Tuuttila et al., 1998).
cysteine residues that form disulphide bonds (Williamson et al., 1990). The disulphides are essential for the function of TIMPs as reduction and alkylation of the inhibitors abrogate their inhibitory activity (Cawston et al., 1981). Each domain of the TIMPs contains 3 loops with the N-terminal domain consisting of loops 1-3 and the C-terminal domain consisting of loops 4-6 (Williamson et al., 1990). The N-terminal domain of the TIMPs is highly conserved, especially the first 22 amino acids, and this domain is responsible for the inhibitory effect of TIMPs (Willenbrock and Murphy, 1994). The C-terminal regions of the TIMPs are much more diverse and may have a role in the binding efficiencies and specificities of the TIMPs to different MMPs as well as the other effects of TIMPs. The divergence of the C-terminal domain of TIMPs in conjunction with the differences in their expression has led to the hypothesis that TIMPs have differing biological roles (Douglas et al., 1997).

1.3.2 Mechanism of inhibition

The mechanism of the inhibition of MMPs by TIMPs involves the first five residues of the N-terminus of TIMPs binding to MMPs in a substrate-like manner. The first residue, Cys1, coordinates to the active site zinc of MMPs while the hydroxyl group of the side chain of Ser2/Thr2 hydrogen bonds to the catalytic Glu of MMPs, thereby displacing a water molecule that is involved in catalysis (Williamson et al., 1994; Gomis-Ruth et al., 1997; Fernandez-Catalan et al., 1998; Stratmann et al., 2001a; Butler et al., 1999b). The C-terminal domain of TIMPs is not required for inhibition to occur, as C-terminally truncated TIMP
mutants (N-TIMPs) can efficiently inhibit MMPs (DeClerck et al., 1993; Willenbrock and Murphy, 1994). However, the C-terminal domain may provide extra contact residues because the rate of inhibition by full length TIMPs is greater than that of the N-TIMPs (Willenbrock and Murphy, 1994; Stratmann et al., 2001a; Stratmann et al., 2001b). It is likely that the C-terminal domain of the TIMPs contacts the hemopexin C domain of MMPs in the inhibitory complex as C-terminally truncated MMPs and matrilysin, which have no hemopexin C domain, bind TIMPs with greatly reduced affinity (Bigg et al., 1994; Baragi et al., 1994).

### 1.3.3 TIMP-1

TIMP-1 is a 184 amino acid glycoprotein that was discovered in the mid 1970's as a very stable protein that inhibited collagenase activity (Bauer et al., 1975) and was subsequently partially characterized by Stricklin and Welgus in 1983 (Stricklin and Welgus, 1983). Its molecular weight ranges from 28.5-30 kDa depending on its glycosylation state. TIMP-1 contains 2 separate N-glycosylation sites; one in loop 1 and another in loop 2. It has been shown that the inhibitory activity of TIMP-1 is unaffected by the loss of its carbohydrate moieties as mutation of the glycosylation sites have no effect on its ability to inhibit MMPs (Caterina et al., 1998). TIMP-1 was not fully cloned until 1985, when Docherty et al. discovered that it was identical to erythroid potentiating activity (EPA) cloned by Gasson et al. earlier in 1985 (Docherty et al., 1985; Gasson et al., 1985). Therefore from the time it was first cloned, it was known that TIMP-1 was a
multifunctional molecule that could both inhibit MMPs and act as EPA. The EPA activity of TIMP-1 was later shown to be separate from that of its MMP inhibitory activity, as destruction of its inhibitory activity through mutation did not affect its erythroid potentiating activity (Chesler et al., 1995). TIMP-1 has been shown to have growth factor effects (Hayakawa et al., 1992) and anti-apoptotic effects that are independent of its inhibitory ability (Guedez et al., 1998).

TIMP-1 is a soluble protein secreted by many cell types, whose expression is regulated by a variety of factors such as phorbol esters, growth factors, and cytokines. TIMP-1 is expressed in most tissues although its levels are highest in the uterus, decidua, and ovary (Waterhouse et al., 1993; Young et al., 2002), which is in agreement with its involvement in reproductive processes that require matrix remodelling, such as ovulation (Inderdeo et al., 1996; Einspanier et al., 1999; Tsafriri and Reich, 1999). TIMP-1 has also been shown to reduce tumour cell growth, metastasis, and angiogenesis (Khokha, 1994; Johnson et al., 1994).

With the exception of the MT-MMPs (Will et al., 1996; Butler et al., 1997), TIMP-1 inhibits all MMPs with high affinity, as well as a member of the ADAM family (a disintegrin and a metalloproteinase) of metzincins, ADAM-10 (Amour et al., 2000). Besides being able to form an inhibitory complex with MMPs, TIMP-1 has been found complexed with progelatinase B (Wilhelm et al., 1989). Binding of TIMP-1 to progelatinase B via its C-terminal non-inhibitory domain at a site other than the catalytic site of progelatinase B is suggested since: TIMP-1 cannot bind to progelatinase B in its usual inhibitory fashion because of the interference
of the pro-domain and as this proenzyme-TIMP complex inhibits other MMPs (Kolkenbrock et al., 1995); The complex inhibits the dimerization of gelatinase B and its ability to form a complex with MMP-1 (Goldberg et al., 1992); It also protects progelatinase B from activation by stromelysin-1 (Ogata et al., 1995).

1.3.4 TIMP-2
TIMP-2 is a 197 amino acid 21 kDa protein (Figure 4) that was discovered as a metalloproteinase inhibitor complexed to progelatinase A and was first characterised by Stetler-Stevenson et al. who, through amino acid sequencing of overlapping peptides, recognized its homology to TIMP-1 (DeClerck et al., 1989; Goldberg et al., 1989; Stetler-Stevenson et al., 1989). TIMP-2 is a soluble protein that is widely expressed in most tissues at relatively high levels (Young et al., 2002). Unlike TIMP-1, TIMP-2 is more constitutively expressed (Overall, 1994). TIMP-2 can inhibit all known MMPs with high affinity including the MT-MMPs (Will et al., 1996; Butler et al., 1997; Vincenti, 2001). TIMP-2 is a multifunctional molecule as it also possesses growth-stimulating activity in a manner similar to TIMP-1 and has been isolated as a factor promoting cell survival and an autocrine factor that stimulates simian virus (SV) 40 transformed human fibroblasts and osteosarcoma cells (Matsumoto et al., 1993; Nemeth and Goolsby, 1993; Woessner, 1996). TIMP-2 has also been shown to inhibit the proliferation of human microvascular endothelial cells (Murphy et al., 1993). In addition, TIMP-2 can suppress apoptosis in cells and inhibit tumour progression, metastasis, and angiogenesis (Guedez et al., 1998; Gomez et al., 1997).
From the time TIMP-2 was discovered, its ability to bind progelatinase A was known. Goldberg et al. showed that progelatinase A purified from HRAS-transformed human bronchial epithelial cells, SV-40 transformed lung fibroblasts and skin fibroblasts was noncovalently complexed to TIMP-2 in a stoichiometric manner (Goldberg et al., 1989). Goldberg et al. also showed that the TIMP-2/progelatinase A complex can by activated by organomercurials and inhibited by additional TIMPs (Goldberg et al., 1989). This indicated that the binding of TIMP-2 to progelatinase A does not involve the catalytic domain of progelatinase A, much like the TIMP-1/progelatinase B complex. TIMP-2 does not associate with C-terminally truncated progelatinase A (Murphy et al., 1992b) and therefore the TIMP binding site is likely to be located on the hemopexin C domain of progelatinase A. Olson et al. showed through kinetic analyses that the binding of TIMP-2 to both pro and active gelatinase A was biphasic with a higher and lower affinity site and that the gelatinase A hemopexin C domain contains the higher affinity binding site (Olson et al., 1997).

As the active site in the proform is unavailable for binding and as these TIMP/proMMP complexes retained MMP inhibitory activity (Kolkenbrock et al., 1991), it was hypothesised that TIMPs can bind proMMPs with their C-terminal domain. TIMP-2, unlike TIMP-1, contains 9 extra residues at the C-terminus known as the C-terminal tail. The C-terminal tail of TIMP-2 has a number of negatively charged residues which are hypothesised to contribute to the binding of TIMP-2 to the positively charged hemopexin C domain of gelatinase A. The
binding site of TIMP-2 on progelatinase A is, as expected, on the hemopexin C domain and has been mapped by Overall et al. It is located on the peripheral rim of the gelatinase A hemopexin C domain at the junction between hemopexin modules III and IV and involves a number of cationic residues (Figure 5) (Overall et al., 1999). Deletion of the anionic C-terminal tail of TIMP-2 was shown to slow its rate of inhibition and it was demonstrated that ionic interactions were involved (Willenbrock and Murphy, 1994). The C-domain to hemopexin C domain interaction between TIMP-2 and progelatinase A is hypothesised to be important in the activation of progelatinase A (Butler et al., 1998). With the discovery of the cell surface activator of progelatinase A, MT1-MMP (Sato et al., 1994), it was soon hypothesised that the role of TIMP-2 in the TIMP-2/progelatinase A complex may be to aid in the activation of the zymogen. Activation of progelatinase A by TIMP-2 is a seemingly paradoxical function for this inhibitor. Cell surface activation was first shown by Overall et al. who reported that concanavalin A (con A) added to human fibroblasts induced the activation of progelatinase A (Overall and Sodek, 1990). The progelatinase A activation effect of con A is hypothesized to induce upregulation and clustering of MT1-MMPs at the cell surface (Fishman et al., 1996; Gervasi et al., 1996). The activation of progelatinase A at the cell surface is hypothesized to occur via a ternary complex mechanism (Figure 6). TIMP-2 plays a significant role in the ternary complex in that it forms part of a progelatinase A receptor on the cell surface by binding and inhibiting an MT1-MMP molecule with its inhibitory domain. This receptor acts to
Figure 5: Crystal structure of the gelatinase A hemopexin C-domain in stereo. Shown above are cationic residues hypothesised to participate in the binding of TIMP-2 to progelatinase A. The residues are located on blades III and IV of the hemopexin C-domain. The four blades of the four-bladed beta propellor structure of the gelatinase A hemopexin C domain are numbered as indicated. Modified from PDB Id 1CK7 (Morgunova et al., 1999).
Figure 6: Diagrammatic representation of the ternary complex mechanism of the cell surface activation of progelatinase A. Activation of progelatinase A requires TIMP-2 binding the hemopexin C domain of progelatinase A through its non-inhibitory C-terminal domain and the association of TIMP-2 with activator proteinases, the membrane type (MT)1-MMPs, to form a trimolecular complex. TIMP-2, together with MT1-MMP, forms a receptor for progelatinase A that brings progelatinase A to the cell surface where it is cleaved by another free MT1-MMP molecule.
recruit progelatinase A to the cell surface through the interaction of the C-domain of TIMP-2 to the hemopexin C domain of progelatinase A, thus forming a tri-molecular complex (Butler et al., 1998). Another free molecule of MT1-MMP can then initiate the activation process by cleaving the prodomain of gelatinase A between residues Asn37 and Leu38 (Will et al., 1996). This enables the autocalytic processing of the remainder of the prodomain between residues Asn80 and Tyr81 to result in active enzyme (Figure 6) (Will et al., 1996). In order for progelatinase A activation to occur, TIMP-2 concentrations must be optimal. If there is too little TIMP-2, activation is not supported. If there is too much TIMP-2, activation is inhibited because TIMP-2 inhibits all the available MT1-MMP (Butler et al., 1997). MT2 and MT3-MMP can also initiate the activation of progelatinase A at the cell surface although their mechanisms of action are unknown (Butler et al., 1997; Takino et al., 1995). The activation mechanism of progelatinase A by MT2-MMP does not require TIMP-2 or TIMP-4, although TIMP-3 involvement has not been ruled out (Morrison et al., 2001).

1.3.5 TIMP-3

TIMP-3 is a 188 amino acid glycoprotein that is unique among the TIMPs in that it is a relatively insoluble and ECM associated. TIMP-3 was first discovered as a matrix bound molecule produced by cells undergoing transformation (Blenis and Hawkes, 1984). The matrix-binding component of TIMP-3 was first attributed to its C-terminal domain (Langton et al., 1998), however, it is now thought to be the due to the interaction of the N-terminal domain with the sulphated
glycosaminoglycans of the ECM (Yu et al., 2000). TIMP-3 also has a 9 amino acid C-terminal tail like TIMP-2, although its tail is less anionic and it contains one N-glycosylation site (Wilde et al., 1994). As its glycosylation site is located within the C-terminal domain, removal of the carbohydrate moiety of TIMP-3 does not affect inhibition. It has been further shown that both the glycosylated and unglycosylated forms of TIMP-3 can localize to the ECM, indicating that glycosylation is not required for the ECM binding properties of TIMP-3 (Langton et al., 1998). TIMP-3 has also been shown to interact with the hemopexin C domain of gelatinases. However, unlike the other TIMPs, TIMP-3 can interact with the hemopexin C domain of both gelatinases A and B (Butler et al., 1999a) although the significance of these interactions are unknown.

TIMP-3 also inhibits all known MMPs with high affinity (Butler et al., 1999a; Vincenti, 2001). However, TIMP-3, like TIMP-1, also inhibits ADAMs such as TNF-α converting enzyme (TACE or ADAM-17) (Amour et al., 1998) and ADAM-10 (Parks, 1999), which implies that the structure of the catalytic domain of ADAMs is similar to that of the MMPs. Interestingly, TIMP-3 also inhibits members of another branch of the ADAM family, a disintegrin and metalloprotease with thrombospondin type I modules (ADAM-TS), specifically ADAM-TS4 (aggrecanase-1), and ADAM-TS5 (aggrecanase-2) (Hashimoto et al., 2001; Kashiwagi et al., 2001). The aggrecanases are enzymes that have been shown to cleave aggrecan at sites that result in fragments that are seen in
various forms of arthritis and thus a reduction in the expression of TIMP-3 may be involved in the progression of arthritis.

TIMP-3 is found in various tissues with thymus, heart, kidney and ovary having the highest levels (Young et al., 2002). TIMP-3 is also expressed in the Bruch's membrane of the eye where mutations in TIMP-3 are known to cause Sorsby's fundus dystrophy (Weber et al., 1994). Like TIMPs-1 and -2, TIMP-3 has also been shown to inhibit tumour growth, metastasis and angiogenesis although there have been reports of TIMP-3 enhancing tumorigenicity and invasiveness as well (Anand-Apte et al., 1996; Baker et al., 1999; Anand-Apte et al., 1997; Sun et al., 1996). However, unlike TIMPs-1 and -2, TIMP-3 has the ability to cause apoptosis in cells (Ahonen et al., 1998; Baker et al., 1998). Although synthetic MMP inhibitors cannot cause apoptosis at physiologically relevant concentrations, this function of TIMP-3 is attributed to its inhibitory activity as mutation of its N-terminal cysteine residue abrogates the effect (Bond et al., 2000). It is thought that the apoptotic effect of TIMP-3 is due to its ability to stabilize TNF-α receptors on cell surfaces (Smith et al., 1997). TIMP-3 also stimulates detachment of transformed cells from the ECM and appears to accelerate morphological changes associated with cell transformation (Yang and Hawkes, 1992). In addition, mutations in TIMP-3 are responsible for an autosomal dominant retinal degenerative disease called Sorsby's fundus dystrophy, which leads to blindness (Weber et al., 1994). Mutations in TIMP-3 that are responsible for Sorsby's fundus dystrophy usually result in an unpaired...
cysteine in the C-terminal domain, giving rise to TIMP-3 dimers that can inhibit MMPs and bind to matrix components but have impaired turnover and therefore accumulate in the eye causing degeneration (Langton et al., 2000; Sternlicht and Werb, 2001). Because of the role of TIMP-3 in a genetic disease, it was suggested that the other TIMPs could have roles in genetic diseases as well, specifically, TIMP-4 and dilated cardiomyopathy as TIMP-4 is highly expressed in the heart, although no known genetic defects in the TIMP-4 gene have yet been revealed in affected individuals (Olson et al., 1998).

1.3.6 TIMP-4

In 1996, the newest member of the TIMP family, TIMP-4, was described. TIMP-4 was the first TIMP cloned by analysis of expressed sequence tags. A partial clone was first identified from a human brain library and the full-length cDNA was later isolated from a human heart cDNA library (Greene et al., 1996). TIMP-4 is a 195-aa, 22 kDa protein. Like TIMP-2, human TIMP-4 is not glycosylated, although rabbit TIMP-4 is (Riley et al., 1999a; Devlieger et al., 2000; Douglas et al., 1997). TIMP-4 has the ability to inhibit all known MMPs, much like the other TIMPs (Vincenti, 2001). However, it is a much more potent inhibitor of gelatinase A and matrilysin-1 than of the other MMPs (Liu et al., 1997). TIMP-4 shares a high level of sequence similarity with TIMP-2 and TIMP-3. TIMP-4 is only ~37% identical and ~57% similar to TIMP-1 but it is ~50% identical and ~69% similar to TIMP-2 and ~50% identical and ~68% similar to TIMP-3 (Douglas et al., 1997; Greene et al., 1996). Because of the high homology between TIMP-2 and TIMP-
4, it has been suggested that they inhibit MMPs by similar mechanisms. Studies show that TIMP-4 is very similar to TIMP-2 in its MMP inhibitory profile. TIMP-4, like TIMP-2 is a much more potent inhibitor of gelatinase A than gelatinase B (Liu et al., 1997). Although TIMP-2 and TIMP-4 both bind gelatinase A strongly, TIMP-4 has been shown to be a less potent inhibitor of gelatinase A than TIMP-2 (Bigg et al., 2001). However, TIMP-2, like gelatinase A, is expressed in a wide range of tissues, which is not the case with TIMP-4 (Goldberg et al., 1989).

TIMP-4 transcripts are expressed at high levels in the heart, blood vessels, platelets, and brain, at lesser levels in the thymus and testis, and at extremely low or non-existent levels in all other tissues (Greene et al., 1996; Frolichsthal-Schoeller et al., 1999; Vaalamo et al., 1999; Young et al., 2002). In the adult mouse, TIMP-4 is expressed in the brain, heart, ovary and skeletal muscle (Leco et al., 1997). Because of its limited expression, it has been suggested that TIMP-4 is a tissue specific inhibitor of MMPs and may function as part of an acute response to tissue remodeling (Greene et al., 1996; Leco et al., 1997). However, in rat, TIMP-4 appears to have a wider expression profile which includes retina, smooth and skeletal muscle, skin, pancreas, heart, brain, lung, kidney, and testis (Wu and Moses, 1998).

The aberrant expression of TIMP-4 is thought to be a factor in a number of diseased states. Although TIMP-4 is not normally expressed in the lung, it has been found in interstitial macrophages, plasma cells, and alveolar epithelial cells
of the lungs of patients with idiopathic pulmonary fibrosis (Selman et al., 2000). TIMP-4, like the other TIMPs, has been found to inhibit tumour angiogenesis and growth. It has also been found to initiate apoptosis of transformed cardiac fibroblasts and inhibit the migration and invasion of cardiac fibroblasts in a Boyden chamber assay (Tummalapalli et al., 2001). Also, the addition of recombinant human TIMP-4 and overexpression of TIMP-4 has been shown to alter the invasive potential of human breast cancer cells using a Matrigel assay (Liu et al., 1997; Wang et al., 1997). Cells transfected with TIMP-4 and orthotopically injected into nude mice exhibited significant inhibition of tumour growth by 4-10 fold in primary tumour volumes and a significant decrease was seen in axillary lymph node and lung metastases as compared to non-transfected cells (Wang et al., 1997). TIMP-4 expression has not been detected in most breast cancer cell lines. This low to nonexistent level of expression could be due to down-regulation of the gene during malignant progression (Wang et al., 1997). However, TIMP-4 appears to be expressed in the stromal cells. It has been suggested that stromal expression of TIMP-4 by fibroblasts surrounding breast carcinomas could be a host response to attempt to balance the local tissue degradation due to tumour cell invasion. Availability of excess TIMP-4 relative to MMP could create a microenvironment in the tumoral-stromal interface where subsequent tumour cell invasion can be inhibited by TIMP-4 (Leco et al., 1997). TIMP-4 also appears to be expressed mainly in the stroma in non-small cell lung carcinoma (Thomas et al., 2000). In clear cell renal cell carcinomas, a downregulation of TIMP-4 expression is attributable to the chromosomal loss of
3p, which contains the loci for TIMP-4 (Hagemann et al., 2001). Although TIMP-4 can inhibit tumour growth in vitro, there are conflicting views of its actions in vivo. Delivery of the TIMP-4 gene inhibits Wilm's tumour growth yet has been shown to stimulate mammary tumorigenesis (Celiker et al., 2001; Jiang et al., 2001).

The high level of expression of TIMP-4 in the brain suggests that it has a potential role in this organ. TIMP-4 is expressed in both human embryonic central nervous system stem cells and mature and differentiated central nervous system cells (Frolichsthal-Schoeller et al., 1999). In addition, TIMP-4 transcripts have been found to increase during the development of the rat brain and are found in high levels in the embryonic dorsal root ganglia, indicating a role for TIMP-4 in the developing central nervous system. In the adult rat brain, TIMP-4 mRNA is found primarily in the cerebellum; particularly in the cerebellar Purkinje cells (Fager and Jaworski, 2000). TIMP-4 also appears to play a role in the progression of gliomas. TIMP-4 levels are elevated in low and middle grade gliomas and downregulated in high grade gliomas with respect to the lower grade gliomas indicating a negative correlation with glioma malignancy (Groft et al., 2001).

A depression of TIMP-4 levels is also thought to have a role in the wound healing of ligaments, obesity, and the degradation of ECM at the periprosthetic interface of artificial hips (Reno et al., 1998; Maquoi et al., 2002; Sasaki et al., 2001). In
addition, TIMP-4 is expressed during certain reproductive processes: TIMP-4 is found in human milk with the maximum at 7 days and is thought to be involved in the protective actions of milk (Cheung et al., 2001); TIMP-4 has also been found in the sertoli cells and gonocytes of human fetal testis and the granulosa and theca cells of the follicle in equine ovaries (Robinson et al., 2001; Riley et al., 2001); During the second trimester of pregnancy, low amounts of TIMP-4 protein are present in extraembryonic coelomic fluid and in amniotic fluid (Riley et al., 1999a); In addition, it has been shown by the use of cDNA arrays that TIMP-4 levels are increased in pre-term labour without chorioamnionitis (Marvin et al., 2002). A decrease in the secretion of TIMPs, including TIMP-4, from the chorion laeve of the fetal membranes and the deciduas parietalis into the amniotic fluid is apparent during active labour. This suggests that decreased levels of TIMP-4 play a role in the detachment of fetal tissues from the uterus during childbirth, allowing proteases to function (Riley et al., 1999b).

Of particular interest is the fact that the highest level of TIMP-4 expression is found in the heart where human cancer metastasis rarely occurs (Greene et al., 1996). The expression of TIMP-4 in the heart is thought to be regulated by cytokines as TNF-α and IL-1β, which transiently increase TIMP-4 protein levels (Li et al., 1999). Dollery et al. found that vascular injury produced elevated TIMP-4 levels in rats and that TIMP-4 reduces the invasion of rat vascular smooth muscle cells in vitro (Dollery et al., 1999). TIMP-4 levels have also been shown to be decreased by over 50% in the left ventricle at 1 and 8 weeks following
myocardial infarction in rats (Peterson et al., 2000). However, because of its high levels of expression in the normal heart, it has been speculated that absence of this expression plays a role in idiopathic dilated cardiomyopathy (Douglas et al., 1997; Gunja-Smith et al., 1996; Olson et al., 1998). The amount of TIMP-4 is significantly reduced in idiopathic dilated cardiomyopathy (Li et al., 1998). The TIMP-4 gene was mapped to chromosome 3p25, a region that contains a locus for dilated cardiomyopathy, providing further evidence for the role of TIMP-4 in heart failure (Olson et al., 1998). TIMP-4 levels are also decreased in rats with spontaneously hypertensive heart failure (Li et al., 2000) and spontaneously hypertensive heart failure rats treated with the drug ramipril had increased TIMP-4 levels and decreased the mortality rate (Seeland et al., 2002), again indicating a role for TIMP-4 in heart failure. TIMP-4 is also suspected to play a role in the pathology of atherosclerosis (Douglas et al., 1997).

1.4 Aims of research, hypothesis, and experimental approach

Understanding of the TIMP family is constantly increasing due to their apparent importance in the prevention of tumour angiogenesis and metastasis. Although much is known about TIMP-2 and its role in the remodelling of tissues, TIMP-4, due to its relatively recent discovery, is less well understood. Because of the high homology between TIMP-4 and TIMP-2, much of what is true for TIMP-2 was assumed to also apply to TIMP-4. Indeed, there appears to be some functional redundancy in the actions of TIMP-2 and TIMP-4. In addition to its
strong inhibitory effect on gelatinase A, it was suggested that TIMP-4 could also play a role in the activation of progelatinase A like TIMP-2 (Bigg et al., 1997). TIMP-4 was reported to bind to the gelatinase A hemopexin C domain although it has a lower binding affinity ($K_d$ of $1.7 \times 10^{-7}$) than TIMP-2 ($K_d$ of $6.6 \times 10^{-8}$) (Bigg et al., 1997). TIMP-4, like TIMPs-2 and -3 has a C-terminal tail and TIMP-4 was shown to interact with progelatinase A at or near to the TIMP-2 binding site based on the competitive inhibitory effect that TIMP-2 has on TIMP-4 in the binding of the C-domain of progelatinase A (Bigg et al., 1997). It was suggested that TIMP-4 could either compete with TIMP-2 to prevent activation or it could participate in activation itself. However, this has since been refuted by further work from Bigg et al. who showed that although TIMP-4 can bind progelatinase A (Bigg et al., 1997) and inhibit MT1-MMP (Bigg et al., 2001), it cannot form a trimolecular complex and promote the activation of progelatinase A (Bigg et al., 2001; Toth et al., 2000; Hernandez-Barrantes et al., 2001). It was also shown that TIMP-4 cannot compete TIMP-2 from the gelatinase A hemopexin C domain but TIMP-2 can compete off TIMP-4 that is bound to the gelatinase A hemopexin domain (Bigg et al., 2001). However, TIMP-4 can inhibit progelatinase A activation at higher concentrations (Bigg et al., 2001; Toth et al., 2000). This inhibition of progelatinase A activation is assumed to be due to the ability of TIMP-4 to inhibit MT1-MMP, similar to the effect of excess TIMP-2 on the cell surface activation of progelatinase A.
Thus the aim of this thesis is to identify the aspects of TIMP-4 that precludes its participation in the activation of progelatinase A as opposed to TIMP-2, which can support the activation of progelatinase A. Since the anionic C-terminal tail of TIMP-2 is hypothesized to play a role in its association with the cationic hemopexin C domain of gelatinase A, it is interesting to note that although TIMP-4 has a C-terminal tail like that of TIMP-2 and -3, its tail is less anionic than that of TIMP-2 (net negative charge of 1 as opposed to 4). Therefore my hypothesis was that TIMP-4 cannot support activation because of the less negatively charged nature of its C-terminal tail and that the negatively charged C-terminal tail of TIMP-2 allows it to bind to the hemopexin C domain of gelatinase A in a manner that supports activation.

In an effort to better understand the role that TIMP-4 plays in the regulation of gelatinase A and to elucidate the molecular determinants of gelatinase A activation, the C-terminal domain of TIMP-4 (C-TIMP-4) was cloned, expressed, and purified. The C-terminal domain of TIMP-2 (C-TIMP-2) was previously cloned in our lab. Because of the small size of the C-TIMPs (~8kDa), they were cloned into a vector containing horse heart myoglobin (hhMb) cDNA and expressed as a fusion protein (MbcTIMP) on the C-terminal side of hhMb separated by a glycine linker (Figure 7). Since the hhMb contains a heme group, it has a distinctive red colour, which makes it relatively easy to track both spectrophotometrically at 409 nm (Soret band) and visually. This also allows for
Figure 7: Schematic representation of the MbcTIMP recombinant proteins. The hatched section represents hhMb. The white sections represent portions of TIMP-2. The black sections represent portions of the proteins that came from TIMP-4. The amino acid sequences shown represent the C-terminal tail and the tail mutations performed. (Not to scale)
the accurate quantification of the concentration of the MbcTIMPs as the soret absorbance is concentration dependent.

It was hypothesized that the negative charges in the C-terminal tail of TIMP-2 allow it to dock to the hemopexin C domain of progelatinase A, providing stronger and faster interactions than TIMP-4. The amino acid sequences of the C-terminal tails of TIMP-2 (QEFLDIEDP) and TIMP-4 (KEFVDIVQP) only differ by four residues (underlined). Thus 6 mutations involving the C-terminal tails of TIMPs-2 and -4 were generated to determine the binding contribution of the differing residues. To determine the contribution of the C-terminal tail to the binding of the gelatinase A hemopexin C domain, mutations were made to remove the C-terminal tails of both TIMPs-2 and -4 resulting in the mutants MbcT2AT and MbcT4AT respectively. To determine the contribution to binding of the negative residues Glu192 and Asp193 within the C-terminal tail of TIMP-2, mutations were also made to remove these negative charges, making it more like the TIMP-4 tail (MbcT2Δq'). In addition, mutations were introduced into the C-terminal tail of MbcT4 to add in the charged residues contained within the tail of TIMP-2 (MbcT4+q'). The C-terminal tails of MbcT2 and MbcT4 were also substituted with one another (MbcT2→T4 and MbcT4→T2) to determine if the chimeras would show altered binding to gelatinase A hemopexin C domain or progelatinase A (Figure 6). It was hypothesised that these recombinant proteins would be useful tools in determining why TIMP-4 cannot participate in the activation of progelatinase A by MT1-MMP but TIMP-2 can. This thesis presents
experiments which reveal that the fusion C-TIMP proteins can bind progelatinase A. I also present data demonstrating the differences between C-TIMP-2 and C-TIMP-4 in the binding of progelatinase A in the context of progelatinase A activation through the use of various binding assays.
CHAPTER 2 – MATERIALS AND METHODS

2.1 Cloning of pGYMC-MbcTIMP-4

Primers for polymerase chain reaction amplification (PCR) were designed to correspond to the 5' and 3' sequences of the C-terminal domain of hTIMP-4. The 5' primer, 5'cTIMP4 (5'-CGGGGGGCTAGCGGCTGCCAAATCACC-3') and 3' primer, 3'cTIMP4 (5'-GTCAAGCTTCTAGGGCTGAACGATGTC-3') contain Nhe I and Hind III restriction sites, respectively, for directional cloning (underlined). The C-terminal coding region of hTIMP-4 (from Gly128 to Pro195) was amplified from the full length cDNA located on pBS II SK-hTIMP-4 (obtained from Dr. Y. E. Shi, Department of Pediatrics, Long Island Jewish Medical Center, The Long Campus for the Albert Einstein College of Medicine, New Hyde Park, New York) (Greene et al., 1996). The following were the conditions under which the PCR was performed: 50 pmol of primer 5'TIMP4, 50 pmol of 3'TIMP4, 1 ng of pBS II SK-hTIMP-4, 43.5 μl of Reaction Mix (20 μl of 10x Mg-free PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 9.0 at 25 °C, 1.0% Triton X-100), 200 μM of dNTP mix (25% (v/v) each of dATP, dCTP, dGTP, dTTP), nanopure dH₂O to 43.5 μl), 12 μl of 25 mM MgCl₂, and 2 units of Taq polymerase in a 200 μl volume followed by the addition of 200 μl of mineral oil. The PCR was performed on a Cetus DNA amplification machine (Perkin-Elmer) and the reaction times were as follows: a 5 min denaturation period at 94 °C followed by 30 cycles of 94 °C for 40 s, 58 °C for 90 s, 72 °C for 60 s followed by a 10 min incubation period at 72 °C. C-TIMP-4 PCR product was recovered on a 1% agarose gel and purified by QIAEX II
Agarose Gel Extraction Kit. The gel-purified C-TIMP-4 was ligated into the expression vector pGYMC-MbMCS (Pelman and Overall unpublished) using the Nhe I and Hind III sites. The ligation reaction was performed using a 1:3 vector to insert ratio and 1 unit of T4 ligase (Gibco-BRL) at 16 °C for 16 h and 3 μl of the resulting ligation reaction was transformed into sub-cloning efficiency *Escherichia coli* (E. Coli) DH5α (Gibco-BRL) by heat shock and plated onto LB-agar plates containing 100 μg/ml ampicillin. The plates were incubated at 37 °C for 16 h. Colonies were selected for growth in liquid media + 100 μg/ml ampicillin and plasmid DNA was isolated using the QIAprep Spin Miniprep kit from Qiagen. Potential clones were screened by restriction enzyme digestion and the resulting positives were sent to the Nucleic Acids-Protein Service (NAPS) Unit at the University of British Columbia for automated sequencing (500 ng pGYMC-MbcTIMP-4 and 3.2 pmol primers M13R or M13F-17 per reaction). The sequencing reaction was analyzed on an Applied Biosystems (ABI) Prism 377 using ABI’s AmpliTaq FS DyeDeoxy™ Terminator Cycle Sequencing chemistry.

### 2.2 Creation of the MbcTIMP mutant constructs

Six additional pGYMC-MbcTIMP constructs were created using the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene. The mutagenesis reactions were performed according to the protocol provided in the kit and the resulting clones were sequenced using the Perkin Elmer Applied Biosystems Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the purified reaction products were analyzed by automated sequencing at the NAPS
sequencing unit. The sequencing data was analyzed using Sequencher 4.1.2 from Gene Codes Corporation, which confirmed the fidelity and accuracy of the constructs.

2.2.1 Mutagenesis of pGYMC-MbcTIMP-2

Primers were designed to create three constructs based on the C-TIMP-2 construct that was previously cloned in our laboratory, pGYMC-MbcTIMP-2 (King and Overall unpublished). To create the mutant lacking a C-terminal tail (MbcT2ΔT), the following primers were designed to introduce a stop codon nine residues before the end of MbcTIMP-2 at position 186 (Gln186Stop) based on the numbering of full-length TIMP-2 starting from Cys1 (mutated base(s) in bold and italics): CT2ST1 5' - GCGCGGCAGCCCCCCAAGTAGGATTTTCTCGACATC - 3'
CT2ST2 5' - GATGTCGAGAAACTCCTdCTTGGGGGGCGCCGCCGCGC - 3'.

Primers were also designed to create the mutant lacking key charged residues located near the end of the anionically charged tail of MbcTIMP-2 (Glu192Val/Asp193Gln), MbcT2Δq:
5'2T4#1 5' - GGAGTTTCTCGACATCGTGAGCCATAACTGCAGGCATG - 3'
3'2T4#1 5' - CATGCCTGCAGTTATGGCTGC4CGATGTCGAGAAACTCC - 3'.

To mutate the MbcTIMP-2 tail to resemble the tail of TIMP-4, primers with two additional mutations (Gln186Lys/Leu189Val) were designed and introduced to the MbcT2Δq' template to make MbcT2→T4:
5'2T4#2 5' - CGCCCCCCCAAGAAGGAGTCTCGACATCGTG - 3'
3'2T4#2 5' - CACGATGTCGACAACCTCCTTCTTGGGGGGCG - 3'.
2.2.2 Mutagenesis of pGYMC-MbcTIMP-4

In addition to the three mutants based on the pGYMC-MbcTIMP-2 construct, primers were designed to create an additional three constructs based on pGYMC-MbcTIMP-4. To create the mutant lacking a C-terminal tail (MbcT4ΔT), the following primers were designed to introduce a stop codon nine residues before the end of MbcTIMP-4 at position 187 (Lys187Stop) based on the numbering of full-length TIMP-4 starting from Cys1 (mutated base(s) in bold and italics):

CT4ST1 5’ - GCCACCTGCCTCTCAGGTAGGAGTTTGGTTGACATC - 3’
CT4ST2 5’ - GATGTCAACAAACTCCTACCTGAGGAGGCAGGTG - 3’.

Primers were also designed to create the mutant with the negatively charged residues in the C-terminal tail of MbcTIMP-4 (Val193Glu/Gln194Asp), MbcT4+q’ that mimics the charged residues located in the C-terminal tail of TIMP-2:

5’4T2#1 5’ - GAAGGAGTTTGGACATCGAGGACCCCTAGAAGCTTGGCACTG - 3’
3’4T2#1 5’ - CAGTGCCAAGCTCTAGGGGTCTCGATGTCACAAAACCTCCTC - 3’.

To mutate the MbcTIMP-4 C-terminal tail to resemble the C-Terminal tail of TIMP-2, primers with two additional mutations (Lys187Gln/Val190Leu) were designed and introduced to the MbcT4+q’ template to make MbcT4→T2:

5’4T2#2 5’ - CCTGCCTCTCAGGCAGAGTTTTGTGACATCGAGG - 3’
3’4T2#2 5’ - CCTCGATGTCAGGAAACCTCCTGCTGAGGAGGCAGG - 3’.
2.3 Screening of *E. coli* cell lines for protein expression

pGYMC-MbcTIMP-4 was transformed into the following *E. coli* cell lines in order to determine the most suitable cell line for expression: BL21, BL21 Gold, BL21 + pTrix, BL21 + pGroesl, BL21 + pMagic, DH5α, DH5αF', JM101, Le392F', R1360, RZ1032, TB1, and XLI (blue). 5 mL cultures of Superbroth (1% (w/v) trypticase peptone, 0.8% (w/v) yeast extract, 0.5% NaCl, 0.001% (v/v) glycerol, pH 7.4) +100 μg/ml ampicillin were inoculated with each transformed cell line. 5 ml control cultures of superbroth without ampicillin were inoculated with each cell line (non-transformed). The cultures were grown for 16 h at 37 °C. Each of the resulting bacterial cultures were split into two tubes and the bacterial cell pellet was collected by centrifugation at 13,000 x g for 2 min in microfuge tubes. The cell pellets for all tubes were resuspended in 250 μl of 1 x NET (100 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8) and sonicated on ice. 250 μl of 4 x SDS-PAGE sample buffer and 500 μl of ddH₂O were added to one of the two tubes of lysed cells from each culture. The other tubes of lysed cells were collected by centrifugation at 13,000 x g for 2 min and the supernatant (soluble protein fraction) transfered into a new microfuge tube containing in 250 μl of ddH₂O and 250 μl of 4 x SDS-PAGE sample buffer. The remaining protein pellets (insoluble inclusion body fractions) were resuspended in 750 μl of ddH₂O and 250 μl of 4 x SDS-PAGE sample buffer. The samples were analysed on 15% SDS-PAGE gels.
2.4 Stability testing of MbcTIMP-4

To determine suitable storage and working buffer conditions for MbcTIMP proteins, stability testing was performed according to a previously published protocol for optimization of solvent conditions for NMR spectroscopy of proteins (Lepre and Moore, 1998). Partially purified MbcTIMP-4 was used to screen buffer conditions. The following buffer conditions were tested: 100 mM Sodium Phosphate at pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0; 100 mM Tris at pH 7.0, 7.5, 8.0, 8.5, and 9.0; 100 mM HEPES at pH 6.5, 7.0, 7.5, 8.0, and 8.5; 100 mM Sodium Carbonate at pH 9.0, 9.5, 10.0, and 10.5; and Phosphate buffered saline (PBS – 137 mM NaCl, 2.68 mM KCl, 4.29 mM Na₂HPO₄•7H₂O, 1.47 mM KH₂PO₄, pH 7.4).

2.5 Expression, refolding and purification of recombinant proteins

All MbcTIMP recombinant proteins were expressed in the E. coli cell line BL21 Gold (see figure 8 for flow diagram of purification scheme). Transformed BL21 Gold cells were grown in 5 ml seed cultures of superbroth + 100 µg/ml ampicillin for 16 h at 37 °C. Four 2 L flasks containing 700 ml of superbroth + 100 µg/ml ampicillin were inoculated with 700 µl of seed culture. The cultures were grown for 24 h at 37 °C and the bacterial cells collected by centrifugation (6000 x g for 15 min) in a Sorvall RC-5B refrigerated superspeed centrifuge. The cell pellet was washed once with 300 ml of 1 x NET and then resuspended in 250 ml of lysis buffer (20 mM Tris, 0.15 M NaCl, 5 µg/ml DNase, 0.1 mg/ml Lysozyme, 10 mM Benzamidine, 2 mM MgCl₂ pH 8). The cells were lysed for 16 h at 4 °C and
2.8 L overnight culture (E. coli BL21 Gold, grown 24 hrs).

Media (Discarded) → P₁ (Cell Pellet)

Resuspend in Lysis Buffer for 16 h at 4 °C. Sonicate. → 15,000 x g for 15 min.

Soluble protein → P₂ (Inclusion Bodies)

Resuspend in Solubilization Buffer for 16 h at 4 °C. → 19,000 x g for 1 h.

Add 5 mg of heme/100 mL of solubilized protein → Soluble Protein → P₃

Refold protein (dialyze into 0.1 M Bicarbonate buffer pH 10)

Dialyze into 10 mM Tris pH 8 → Benzamidine Sepharose → Unbound → DEAE Sepharose

Wash with equilibration buffer → Unbound → Nickel Chelate Sepharose

Wash with eq buffer + 1 M NaCl, W₁

Wash with 10 mM, Tris pH 7, W₂

Wash with 10 mM, Tris pH 7, W₃

Elute 0-500 mM Imidazole gradient in 10 mM Tris, pH 7

Dialyze into 10 mM Tris 1 pH unit below pl → CM Sepharose

Wash with 10 mM Tris, pH 7, W₁

Dialyze into 50 mM Tris, 0.15 M NaCl, pH 8 → pool → Elute 0-500 mM NaCl in 10 mM Tris

Quantify protein

Aliquot → Flash freeze protein

Figure 8: Flow diagram of the purification scheme for MbcTIMPs.
then sonicated on ice to ensure complete cell lysis and to shear the remaining DNA. The inclusion bodies were harvested by centrifugation for 1 h at 19,000 x g. The inclusion body pellet was solubilized in 125 ml of solubilization buffer (20 mM Tris, 0.15 M NaCl, 6 M Guanidine hydrochloride, pH 8) for 16 h at 4 °C. The solubilized protein was then separated from the insoluble protein by centrifugation (19,000 x g for 1 h) and the supernatant was decanted off and the volume measured. 5 mg/100 ml of Hemin (Sigma H-5533) was added to the solubilized protein for the reconstitution of myoglobin during refolding and the volume of the solubilized protein was made up to 250 ml with refolding buffer (55 mM Na$_2$CO$_3$, 45 mM NaHCO$_3$, 0.02% (w/v) NaN$_3$, pH 10) before dialysis into refolding buffer and then 10 mM Tris at the pl of the myoglobin fusion protein. The refolded protein was applied to Benzamidine Sepharose resin to remove serine proteases and the unbound fraction was then applied to 30 ml of equilibrated DEAE Sepharose. The NaCl concentration in the unbound fraction from the DEAE column was slowly raised to 0.5 M and then applied to a 30 ml Ni-Sepharose column, washed with 10 bed volumes of equilibration buffer (W1), 10 bed volumes of equilibration + 1 M NaCl (W2), and 10 bed volumes 10 mM Tris pH 7 (W3), before elution by a 0-500 mM imidazole gradient in 10 mM Tris pH 7. Elution fractions were pooled and dialysed into 10 mM Tris, 1 pH unit below the pl of the myoglobin fusion protein in no salt conditions and then applied to a 10 mL CM-Sepharose column to remove the lower molecular weight contaminating proteins. The column was washed with 10 bed volumes of 10 mM Tris at the equilibration pH (W4) and the recombinant protein was eluted using a
0-500 mM NaCl gradient in 10 mM Tris and dialysed into Tris storage buffer (50 mM Tris, 0.15 M NaCl, pH 8) for storage at -80 °C until use. If further purification was required, elution fractions from the CM-Sepharose column were pooled and dialysed into 10 mM Tris, 1 pH unit above the pi of the myoglobin fusion protein in no salt conditions and then applied to a DEAE-Sepharose column. The column was washed with 10 bed volumes of 10 mM Tris at the equilibration pH and the purified protein was eluted using a 0-500 mM NaCl gradient and then dialysed into Tris storage buffer.

2.6 Analysis of the MbcTIMP recombinant proteins

2.6.1 SDS-PAGE analysis

0.1 µg of each MbcTIMP recombinant protein, TIMP-2 and -4 standards, and a recombinant myoglobin standard were electrophoresed on 15% polyacrylamide separating gels using the Laemmli denaturing SDS discontinuous gel electrophoresis method (Laemmli, 1970). Protein bands were visualized by silver staining.

2.6.2 Western blotting analysis

Proteins were prepared and electrophoresed as above and the gels soaked in Western blot semi-dry transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% methanol) for 10 min prior to transferring onto Immobilon-P membranes (Millipore) activated according to the manufacturer's protocol. The transfer was performed on a Pharmacia Biotech Multiphor II apparatus at 100 mA per gel for 1
h. The membranes were then blocked with 5% skim milk powder in 50 mM Tris, 0.15 M NaCl, pH 8 for 16 h at 4 °C. Membranes were washed with 50 mM Tris, 0.15 M NaCl, + 0.05% (v/v) Tween between blocking, application of primary and secondary antibodies, and development. Peptide antibodies previously available in our laboratory and raised in rabbits to the C-terminal tail of TIMP-2 and the C-terminal tail of TIMP-4 (GGLRKEFVDIVQ-NH$_2$ where the underlined residues differ from the natural sequence) as described by Overall et al., (Overall et al., 2000), and an antibody raised to recombinant hhMb were used to detect the proteins of interest. Secondary antibody was purchased from Bio-Rad (EIA Grade Affinity Purified Goat Anti-Rabbit IgG (H+L)-HRP Conjugate). Development of membranes was performed using ECL™ Western blotting detection reagents from Amersham Pharmacia Biotech according to their instructions.

2.6.3 Mass spectrometry analysis

Electrospray ionization mass spectrometry was performed on the MbcTIMP recombinant proteins and hhMb to further assess the fidelity of the expressed and purified proteins. 10 μg of each recombinant protein in storage buffer was analysed on a SCIEX API 300 (Perkin-Elmer) in single quadrupole mode. All samples were found to contain both N-terminal processed (minus methionine) and unprocessed forms of the expected proteins.
2.6.4 Soret peak analysis

To determine if the MbcTIMPs were fully reconstituted by the addition of heme during refolding, soret analysis was performed on a MbcTIMP ± the addition of 1 molar equivalent excess heme. Analysis was performed on a Varian Cary spectrophotometer. The spectrophotometric absorbance reading at 409 nm was used to determine the concentration of the MbcTIMPs. In order to determine the concentration of the recombinant MbcTIMPs, first spectrophotometric readings were taken of known amounts of serially diluted hhMb at 409 nm and a linear standard curve was constructed. This curve was then used to calculate the approximate number of moles within each sample of MbcTIMP.

2.7 Microwell solid phase binding assay

In order to test the binding interactions between the C-TIMP fusion proteins and the gelatinase A hemopexin C domain, a solid phase assay, enzyme-linked immunosorbent assay (ELISA) was used. 0.5 µg of TIMP-2 (cloned, expressed and purified from Chinese hamster ovary cells by Dr. C. J. Morrison) (Overall et al., 2000; Bigg et al., 2001), TIMP-4 (cloned, expressed and purified from baby hamster kidney cells by Dr. H. F. Bigg) (Bigg et al., 2001), MbcTIMP, or hhMb in 100 µl of Voller's buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 0.02% (w/v) NaN₃, pH 9.6) was immobilized onto 96-well plates for 16 h at 4 °C. The wells were washed four times with ~300 µl of Tris buffered saline (TBS – 20 mM Tris, 0.15 M NaCl, pH 7.4) + 0.05% (v/v) Tween using a Dynex Technologies Ultrawash Plus 96 well plate washer. To block the remaining binding sites on the wells, 200 µl of
blocking buffer (2.5% BSA (w/v) in TBS) was added to each well and incubated at 25 °C for 1 h. After blocking, the wells were washed as above prior to adding serial dilutions of gelatinase A hemopexin C domain (previously cloned in our laboratory by U. M. Wallon. Expressed and purified by A. E. King) (Wallon and Overall, 1997), which was incubated for 1 h at 25 °C. The excess gelatinase A hemopexin C domain was washed off as above and replaced with an affinity-purified antibody raised to a 6 histidine tag (anti-His$_6$ antibody) located at the N-terminus of the recombinant gelatinase A hemopexin C domain. After incubation for 1 h at 25 °C, the anti-His$_6$ antibody was washed off as above. Diluted secondary antibody (1/1000 in TBS + 0.25% (w/v) BSA + 0.05% (v/v) Tween), purchased from Bio-Rad (EIA Grade Affinity Purified Goat Anti-Rabbit IgG (H+L)-AP Conjugate), was then added and incubated for 1 h at 25 °C. The secondary antibody was then washed off as above and detection by alkaline phosphatase levels was quantified by p-nitrophenyl phosphate substrate (Sigma) in an ELISA plate reader (Molecular Devices) at 405 nm.

2.8 Affinity columns

To test for an interaction between gelatinase A and the MbcTIMPs, various mini-columns such as gelatin Sepharose and gelatinase A hemopexin C domain coupled affi-gel were attempted.
2.8.1 Gelatin Sepharose mini columns

100 µl of gelatin Sepharose resin (Pharmacia) was equilibrated with equilibration buffer (50 mM Tris, 0.15 M NaCl, 0.05% (v/v) Brij-35, pH 8) and then blocked with 2.5% (w/v) BSA. The resin was then washed with 2 x 1 ml of equilibration buffer. 2 µg of progelatinase A (cloned, expressed and purified from TIMP-2 -/- cells by Dr. G. S. Butler) (Bigg et al., 2001) was incubated for 1 h at 25 °C with 2 µg TIMP-2, -4, MbcT2, MbcT4, MbcT2ΔT, MbcT4ΔT, or hhMb prior to addition to the resin. The resin was washed with equilibration buffer and subsequently eluted with equilibration buffer + 10% DMSO and then 4 x SDS-PAGE sample buffer. The resulting fractions were analysed by SDS-PAGE.

2.8.2 Affi-gel Coupled mini columns

Gelatinase A hemopexin C domain (expressed and purified by I. Mak) (Wallon and Overall, 1997), MT1-MMP hemopexin C domain (previously cloned, expressed and purified in our laboratory by E. Tam) (Overall et al., 2000), BSA and a PBS control were coupled to Affigel-10 (Bio-Rad) according to the protocol provided by the manufacturer (~0.685 mg to 3ml of resin). The resins were blocked with ethanolamine as described in the protocol and then equilibrated with equilibration buffer + 0.02% (w/v) sodium azide. Resin was further blocked with 2.5% BSA and washed with equilibration buffer. 0.65 nmoles of TIMP-2, MbcT2, MbcT4, or hhMb was loaded onto 100 µl of resin, twice, and allowed to incubate for 1 h at 25 °C. The resin was then washed with 1 ml of equilibration buffer, 1 ml of equilibration buffer + 1 M NaCl, and equilibration buffer + 10% DMSO and
then eluted with equilibration buffer + 0.1 M EDTA and 4 x SDS-PAGE sample buffer. The column fractions were analysed by SDS-PAGE.

2.9 Velocity sedimentation

A 5-20% (w/w) sucrose gradient in 50 mM Tris, 0.15 M NaCl, 0.05% Brij-35, pH 8 was formed by layering 0.5 ml of 5%, 10%, and 15% sucrose solutions, on top of 0.5 ml of 20% sucrose solution in a Beckman Ultra-Clear centrifuge tube and letting stand for 1 h at room temperature. The gradients were then placed on ice for a further 30 min before layering 100 μl of sample. Samples consisted of 8.5 μg of the catalytically inactive mutant progelatinase A E375A (pE375A) (Crabbe et al., 1994) expressed in TIMP-2 -/- cells and purified by Dr. G. S. Butler (McQuibban et al., 2001) was incubated with 4.7 μg TIMP-2, 4.7 μg TIMP-4, 5 μg MbcT2, or 4.92 μg MbcT4 for 7 h at 4 °C, each protein on its own or pE375A from Chinese hamster ovary cells that is secreted pre-complexed with TIMP-2 (expressed and purified by Dr. G. S. Butler) (Bigg et al., 2001). The appropriate marker proteins (1 μg each hhMb, carbonic anhydrase, ovalbumin, BSA, phosphorylase B with carbonic anhydrase being omitted for samples containing C-TIMP and BSA being omitted for samples containing progelatinase A due to similarity in size) were added to the sample prior to layering on the gradient. The tubes were then spun at 50,000 rpm for 16 h at 4 °C in a Beckman Ultracentrifuge. Fractions were collected and analysed by SDS-PAGE.
2.10 Glutaraldehyde cross-linking

2.10.1 Chemical cross-linking

TIMP-2, TIMP-4, MbcT2, and MbcT4 were incubated with or without gelatinase A hemopexin C domain (expressed and purified by myself) (Wallon and Overall, 1997) at 6:1, 4:1, 3:1, and 2:1 molar ratios of TIMP:gelatinase A hemopexin C domain for 1 h at room temperature. Glutaraldehyde was then added to 0.5% (v/v) for 30 min at room temperature to cross-link interacting proteins. The reactions were stopped by addition of an equal volume of 2 x SDS-PAGE sample buffer. The interactions were analysed by SDS-PAGE and Western blotting (for TIMP-4 only) using the anti-TIMP-4 tail antibodies. MbcT2, MbcT4, MbcT2ΔT, MbcT4ΔT, and hhMb were also incubated with or without progelatinase A at 2.5:1 molar ratios of MbcTIMP or hhMb:progelatinase A and cross-linked as above. The reactions were stopped with equal volumes of 2 x non-reducing SDS-PAGE sample buffer and analysed by SDS-PAGE and zymography.

2.10.2 Analysis by zymography

Samples were run at 20 mA/gel on 10% SDS-PAGE gels containing 0.025% gelatin. The gels were then incubated twice in 2.5% (v/v) Triton X-100 for 15 min each and then rinsed 3 x with dH₂O before incubation in zymography assay buffer (20 mM Tris, 0.15 M NaCl, 5 mM CaCl₂, pH 8) for 5 h at 37 °C. The gels were then stained with Coomassie R-250.
2.11 Enzyme capture assay

In order to test the binding interactions between the C-TIMP fusion proteins and full-length progelatinase A, an enzyme capture approach was used. 0.5 μg of TIMP-2, TIMP-4, MbcTIMP, or hhMb in 100 μl of Voller's buffer was immobilized onto 96-well fluorimetry plates for 16 h at 4 °C. The wells were washed 3 x with 200 μl of TBS. To block the remaining binding sites on the wells, 200 μl of blocking buffer (1% BSA (w/v) in TBS) was added to each well and incubated at 25 °C for 1 h. After blocking, the wells were washed as above prior to the addition of serial dilutions or progelatinase A or 250 μg or 125 μg of progelatinase A in fluorimetry assay buffer (FAB – 100 mM Tris, 10 mM CaCl₂, 100 mM NaCl, 0.05% (v/v) Brij-35, pH 7.5), which was incubated for 2 h at 25 °C. The excess progelatinase A was washed off as above and the progelatinase A was activated by adding 180 μl of 2 mM 4-aminophenylmercuric acetate (APMA) and incubating for 1 h at 25 °C. 20 μl of fluorescence substrate QF-24 ((7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH₂) (Knight et al., 1992) was added to each well and the reaction was monitored on a Molecular Devices Fmax fluorescence plate reader for 4 h at 37 °C. The progelatinase A that was bound to the plate was also eluted off with 4 x non-reducing sample buffer and analyzed by zymography as above.
CHAPTER 3 – RESULTS

3.1 Recombinant protein expression and characterization

C-TIMP-4 was cloned as a fusion protein C-terminal to hhMb for protein interaction studies with progelatinase A. C-TIMP-2 was previously cloned in our laboratory. Six mutants were created based on the MbcT2 and MbcT4 constructs (Figure 7). These mutants, like MbcT2 and MbcT4, were sequenced and found to be correct and containing the desired mutation(s). Mini-expression showed that the *E. Coli* strain BL21 was suitable for expression and that the MbcTIMPs are expressed and accumulated in insoluble inclusion bodies (Figure 9). Initial purification attempts revealed that the MbcTIMPs did not contain heme upon refolding and required reconstitution and that the MbcTIMPs were unstable and tended to precipitate in phosphate buffers and at lower pH. Stability testing was performed to determine suitable buffer conditions for the purification and storage of MbcTIMPs. MbcTIMPs were determined to be stable in Tris and carbonate-based buffers at neutral to high pH (data not shown). Typically, purification of the recombinant MbcTIMPs involved harvesting and lysing the MbcTIMP expressing BL21 cells, recovery of the insoluble inclusion bodies, solubilization of the inclusion bodies, reconstitution of heme during refolding and a series of chromatography columns. Figure 10 is an example of a purification scheme, in this case for MbcT4+q" (see figure 7 for schematic of this mutant). To remove proteases that may degrade the MbcTIMPs, benzamidine Sepharose was used. DEAE Sepharose, an anion exchange resin, was used to remove
Figure 9: Mini expression of MbcT4 in BL21 revealed that the protein is expressed and accumulated in insoluble inclusion bodies. Transformation of pGYMC-MbcTIMP-4 vector into E. Coli BL21 cells resulted in an extra band at an apparent molecular weight of approximately 26kDa as indicated by the arrows, which is in agreement with the predicted molecular weight of MbcT4. The BL21 cells were harvested, lysed, and centrifuged to separate the soluble (supernatant) and insoluble (pellet) protein fractions. Western blot analysis with the α-C-TIMP-4 antibody indicates that this protein is likely MbcT4.
Figure 10: Purification of the mutant MbcT4+q−. MWt denotes molecular weight, B1 denotes before Benzamidine and DEAE Sepharose columns, B2 denotes before Ni²⁺ chelate column, W1-W4 are as indicated in section 2.5 of chapter 2 (also see figure 8 for the detailed purification scheme). Pooled fractions are as indicated. Panel A: SDS-PAGE analysis of the DEAE Sepharose column fractions. The U1 fraction was loaded onto the nickel chelate column shown in panel B. The pooled fractions from the nickel chelate column were dialysed and further purified on a CM Sepharose column as shown in panel C.
many contaminating proteins (Figure 10 A). Ni\(^{2+}\) chelate Sepharose was used to exploit the metal chelate binding properties of the heme (Figure 10 B), and the cation exchange, CM Sepharose column was used for further purification and was useful in removing the lower molecular weight contaminating proteins (Figure 10 C). The majority of the MbcTIMPs were purified to homogeneity after the CM Sepharose column. The recombinant proteins could be tracked visually due to their red colour, indicating that reconstitution was successful (Figure 11 A). Heme reconstitution studies showed that the fusion proteins were fully reconstituted and no additional heme was required (Figure 11 B). The concentrations of the recombinant proteins were calculated from a standard curve based on a serial dilution of a known amount of hhMb. The extinction coefficient for the soret absorbance of hhMb was calculated to be 149,415 M\(^{-1}\)cm\(^{-1}\) (see table 2 for protein concentrations and yields). The fidelity of gene expression and identity of the proteins was confirmed by electrospray ionisation mass spectrometry. As shown in table 3, all MbcTIMPs tested contained both N-terminal methionine processed and non-N-terminal methionine processed forms. The wild-type and mutant recombinant C-TIMPs were purified to homogeneity as shown in figure 12. To confirm the presence of the mutations in the expressed proteins, Western blot analysis was performed as shown in panels B and C of figure 12. The anti-hhMb antibody recognized all Mb-fusion proteins. The anti-C-TIMP-2 tail (α-cT2 tail) antibody recognized TIMP-2, MbcT2, MbcT4+q\(^{-}\), and MbcT4→T2 while the anti-C-TIMP-4 tail (α-cT4 tail) antibody recognized only
Figure 11: Spectrophotometric properties of the Mb-fusion proteins. Panel A: MbcT4+q', when reconstituted with heme, can be visually tracked during purification due to red colouring of the protein. Panel B: Spectrophotometric analysis is performed on 1.32 nmoles of reconstituted, heme containing MbcT2ΔT after purification (thin line – before additional heme). Shown above is the spectrum from 200-600nm with the soret peak absorbance at 409nm. The peak soret absorbance does not change significantly upon the addition of an additional 1 molar equivalent of heme to the already reconstituted MbcT2ΔT with a larger increase in the absorbance around 380nm indicating the presence of excess heme (thick line – after additional heme). This indicates that the reconstitution during refolding process was successful and that no further reconstitution of the MbcTIMPs is necessary.
Table 2: Protein yields from purification process

<table>
<thead>
<tr>
<th>Protein</th>
<th>Absorbance at 280 nm&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Absorbance at 409 nm&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Highest concentration pool (mg/ml)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Approximate overall yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MbcT2</td>
<td>1.43</td>
<td>4.92</td>
<td>0.841</td>
<td>6</td>
</tr>
<tr>
<td>MbcT4</td>
<td>1.44</td>
<td>2.87</td>
<td>0.492</td>
<td>3</td>
</tr>
<tr>
<td>MbcT2ΔT</td>
<td>0.3</td>
<td>0.99</td>
<td>0.162</td>
<td>1</td>
</tr>
<tr>
<td>MbcT4ΔT</td>
<td>6.0</td>
<td>17.1</td>
<td>2.82</td>
<td>17</td>
</tr>
<tr>
<td>MbcT2Δq&lt;sup&gt;-&lt;/sup&gt;</td>
<td>1.05</td>
<td>3.66</td>
<td>0.625</td>
<td>8</td>
</tr>
<tr>
<td>MbcT4+q&lt;sup&gt;-&lt;/sup&gt;</td>
<td>9.74</td>
<td>29.6</td>
<td>5.09</td>
<td>93</td>
</tr>
<tr>
<td>MbcT2→T4</td>
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<tr>
<td>MbcT4→T2</td>
<td>1.53</td>
<td>4.01</td>
<td>0.689</td>
<td>12</td>
</tr>
</tbody>
</table>

1 Absorbance of the highest concentration pool after multiplying with the dilution factor.

2 Calculated from the soret absorbance (409 nm) using the extinction coefficient 149,415 M<sup>-1</sup> cm<sup>1</sup>.
Table 3: Molecular mass determination of horse heart myoglobin and myoglobin C-TIMP fusion proteins by electrospray ionization mass spectrometry

<table>
<thead>
<tr>
<th>Protein</th>
<th>Measured mass + N-terminal methionine</th>
<th>Δ N-terminal methionine</th>
<th>Δ Mass from predicted + N-terminal methionine</th>
<th>Δ N-terminal methionine</th>
</tr>
</thead>
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<tr>
<td>hhMb</td>
<td>17086</td>
<td>16954</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>MbcT2</td>
<td>25534</td>
<td>25400</td>
<td>+5</td>
<td>+3</td>
</tr>
<tr>
<td>MbcT4</td>
<td>25658</td>
<td>25526</td>
<td>+4</td>
<td>+4</td>
</tr>
<tr>
<td>MbcT2ΔT</td>
<td>24448</td>
<td>24312</td>
<td>+7</td>
<td>+2</td>
</tr>
<tr>
<td>MbcT4ΔT</td>
<td>24598</td>
<td>24472</td>
<td>+1</td>
<td>+6</td>
</tr>
<tr>
<td>MbcT2Δq−</td>
<td>25520</td>
<td>25388</td>
<td>+8</td>
<td>+8</td>
</tr>
<tr>
<td>MbcT4+q−</td>
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<td>+3</td>
</tr>
<tr>
<td>MbcT2→T4</td>
<td>25502</td>
<td>25372</td>
<td>+4</td>
<td>+6</td>
</tr>
<tr>
<td>MbcT4→T2</td>
<td>25688</td>
<td>25556</td>
<td>+3</td>
<td>+3</td>
</tr>
</tbody>
</table>

1 All the proteins measured registered peaks for ± N-terminal methionine although to varying degrees. For hhMb, MbcT4ΔT, MbcT2Δq−, MbcT4+q−, and MbcT4→T2, the + Met peak is greater than the Δ Met peak. The Δ Met peak is substantially larger than the + Met peak for MbcT2, MbcT2ΔT, and MbcT2→T4. The + Met and Δ Met peaks are approximately the same size for MbcT4.

2 Predicted mass was determined from the amino acid sequence minus 2 Da per disulfide bond.
Figure 12: SDS-PAGE and Western blotting analysis of MbcTIMP recombinant proteins as compared to full length TIMPs-2 and -4 and hhMb. Samples were analysed ± reduction with 65 mM DTT. Panel A: Wild-type recombinant C-TIMPs were purified to homogeneity. Shown are the masses of MbcT2 and MbcT4 from mass spectrometry. The full-length TIMPs have an apparent molecular weight of approximately 22 kDa and hhMb has an apparent molecular weight of 18 kDa. Continued on next page.
Figure 12: SDS-PAGE and Western blotting analysis of MbcTIMP recombinant proteins as compared to full length TIMPs-2 and -4 and hhMb. Panel B: Wild-type recombinant MbcT2 as compared to mutants based on the C-TIMP-2 construct. Panel C: Wild-type recombinant MbcT4 as compared to mutants based on the C-TIMP-4 construct.
TIMP-4, MbcT4, and MbcT2→T4. The lack of recognition for MbcT2Δq' by the α-cT2 tail antibody and the ability of the α-cT2 tail antibody to recognise MbcT4+q' indicates that the α-cT2 tail antibody relies on the negatively charged residues Glu192 and Asp193 for specificity. The lack of recognition for MbcT4+q' by the α-cT4 tail antibody indicates that the epitope for the antibody includes the residues Val193 and Gln194. However, because the α-cT4 tail antibody also does not recognise MbcT2Δq', this indicates that the epitope for the α-cT4 tail antibody includes more of the C-terminal tail of TIMP-4. There was no difference in apparent molecular weight or antibody recognition ± reduction with the fusion proteins. The lack of dimers or multimers in the absence of DTT (Figure 12 A) shows that the majority of the protein is in the monomeric form, indicating that the proteins are correctly folded.

3.2 Microwell Solid Phase Binding Assay

To determine whether the MbcTIMPs can bind gelatinase A hemopexin C domain, enzyme-linked immunosorbent assays (ELISAs) were performed. All MbcTIMPs tested could bind gelatinase A hemopexin C domain, however, the binding affinities varied with coating protein and were inconsistent although TIMP-2 consistently bound to gelatinase A hemopexin C domain with much greater affinity than any other protein tested (data not shown). As well, MbcT4 and MbcT4ΔT were shown to have similar binding affinities to gelatinase A hemopexin C domain (Figure 13).
Figure 13: Comparison of the binding of MbcT4 and MbcT4ΔT to gelatinase A hemopexin C domain. Both MbcT4 and MbcT4ΔT bind with similar affinities ($K_d$ approximately $4 \times 10^{-6}$) to gelatinase A hemopexin C domain.
3.3 Affinity Columns

Another method of testing for protein interactions is through the use of mini-affinity columns. Two types of columns were used: gelatin Sepharose and affigel. Gelatin Sepharose exploits the fact that progelatinase A can bind gelatin through its collagen binding domain. When TIMP-2 is pre-incubated with progelatinase A, a small amount of TIMP-2 co-eluted with progelatinase A using 10% DMSO (data not shown). However, TIMP-4, MbcT4, and MbcT2 did not co-elute with progelatinase A (data not shown). TIMP-2 could bind gelatinase A hemopexin C domain coupled affi-gel resin and not the blocked control resin while hhMb could not bind either (data not shown). However, MbcT2 and MbcT4 bound both the hemopexin C domain coupled resin and the control resin (data not shown). Addition of detergent, blocking proteins, or coupling of a different protein to the control resin did not abrogate the interaction between the MbcTIMPs and the control resin, thus no useful data was obtained from this method.

3.4 Velocity Sedimentation

Velocity sedimentation was employed to determine if the wild-type C-TIMP fusion proteins, MbcT2 and MbcT4 could interact with progelatinase A. The predicted masses of the complexes are approximately 97kDa. Pre-complexed TIMP-2 and a catalytically inactive mutant of progelatinase A, E375A (pE375A) (Crabbe et al., 1994) were found to co-elute (Figure 14 A). Figure 14 B shows the SDS-PAGE analysis of the elution fractions from velocity sedimentation of TIMP-2 free pE375
Figure 14: Determination of protein interactions using velocity sedimentation. 8.5 μg of progelatinase A E375A (pE375A) was incubated with 4.7 μg TIMP-2 or TIMP-4 or 5 μg of MbcT2 or MbcT4 for 7 hours. Samples plus markers were then layered on a 5-20% sucrose gradient and centrifuged for 16 hours. Densitometry analysis was performed on the silver stained SDS-PAGE analyses (representative gel shown in panel B) and plotted (panels A, C-F). The arrows indicate the peak fraction of the protein marker indicated. The TIMP or MbcTIMP standard represents the elution profile of the TIMP or MbcTIMP when not complexed with pE375A. Continued on the following page.
Figure 14: Determination of protein interactions using velocity sedimentation. Panel A: Pre-complexed pE375A and TIMP-2 co-elute. Panel B: SDS-PAGE analysis of the elution fractions of pE375A incubated with TIMP-2 (densitometry presented in panel C). In panel C, and D, TIMP-2-free pE375A was complexed with TIMP-2 and TIMP-4 respectively. Panel E and F show the results of pE375A incubation with MbcT2 and MbcT4 respectively.
complexed with exogenous TIMP-2 (see figure 14 C for the densitometry analysis of this gel). TIMP-2 or TIMP-4 incubated with TIMP-2 free pE375A eluted in earlier fractions as compared to the TIMP-2 or TIMP-4 standards as shown in figure 14 C and D respectively. When MbcT2 or MbcT4 were incubated with pE375A, there was a shift in the peak fraction as compared to the MbcT2 or MbcT4 standards indicating a binding interaction between the MbcTIMPs and pE375A (Figure 14 E and F respectively).

3.5 Chemical Cross-linking

To confirm an interaction between the MbcTIMPs and progelatinase A, glutaraldehyde cross-linking was performed. In figure 15 A, different concentrations of gelatinase A hemopexin C domain were incubated with TIMP-2, a known interactor, to determine protein concentrations that would give detectable levels of the TIMP-2/gelatinase A hemopexin C domain complex. TIMP-4 was also shown to interact with gelatinase A hemopexin C domain in figure 15 B. An increasing level of gelatinase A hemopexin C domain gave rise to an increase in the TIMP-4/gelatinase A hemopexin C domain complex as seen in figure 15 B that was identified by Western blotting using the α-C-TIMP-4 tail antibody. The interaction between MbcT2 and progelatinase A was confirmed by the presence of the MbcT2/progelatinase A complex band in both a silver stained gel and zymogram as indicated in figure 15 C. A band representing the MbcT4/progelatinase A complex was also present when MbcT4 was cross-linked
Figure 15: Determination of protein interactions using glutaraldehyde cross-linking. Proteins were incubated together for 1 h prior to the addition of glutaraldehyde (GA) to 0.5%. Reactions were incubated for a further 30 min before stopping with equal volume 2 x SDS-PAGE sample buffer. For gelatinase A (MMP-2) hemopexin C domain (HexCD) and progelatinase A (proMMP-2), (+) denotes ¼ molar equivalent, (++) denotes ½ molar equivalent, (+++) denotes 1 molar equivalent versus TIMP, MbcTIMP, or hhMb. Continued on the following page.
Figure 15: Determination of protein interactions using glutaraldehyde cross-linking.
Panel A: Three different concentrations of HexCD were incubated with TIMP-2 to determine the appropriate concentration to use in this experiment. Panel B: Two different concentrations of TIMP-4 were incubated with HexCD. The TIMP-4/HexCD complex and the TIMP-4 homodimer were visualized by Western blotting using the α-C-TIMP-4 tail antibody. Panels C-F: MbcTIMPs were incubated at ¼ molar equivalent to proMMP-2 A. Position of the MMP-2/MbcTIMP complex on the silver stain (upper) and the zymogram (lower) are indicated. Panel G: Addition of glutaraldehyde to pro-incubated proMMP-2 and hhMb does not give rise to proMMP-2/hhMb complexes.
to progelatinase A as indicated in figure 15 D. Figure 15 E and F show that the tail-less mutants MbcT2ΔT and MbcT4ΔT bound progelatinase A respectively. In contrast, hhMb did not interact with progelatinase A and therefore no hhMb/progelatinase A complex bands are seen in either the silver stain or through zymography (Figure 15 G).

3.6 Enzyme Capture Assay

To quantitate the binding interaction between progelatinase A and the MbcTIMPs, an enzyme capture experiment was performed. The results of the assay were visualized by two methods: fluorimetry and zymography.

3.6.1 Visualization by Fluorimetry Assay

Progelatinase A captured on a fluorimetry plate by binding to coated TIMPs/MbcTIMPs or control proteins was APMA activated and subjected to a fluorimetry assay. Bound progelatinase A was quantified by measuring the rate of cleavage of a quenched fluorescent peptide substrate (Figure 16) (Knight et al., 1992). The results are normalized to the amount of enzyme captured by TIMP-2. Figure 16 demonstrates that TIMP-2 can capture higher amounts of progelatinase A than any other proteins tested. The amount of enzyme captured by MbcT2 was reduced by the loss or mutation of the C-terminal tail. MbcT4 did not capture as much enzyme as MbcT2 and loss of its C-terminal tail did not affect its ability to capture progelatinase A. The introduction of charges present
Figure 16: Visualization of enzyme capture assay by fluorimetry. Progelatinase A captured by the coating protein on a fluorimetry plate was APMA activated and detected by cleavage of a fluorogenic substrate. The values are expressed in fold of the amount of progelatinase A captured by TIMP-2.
in the C-terminal tail of TIMP-2 in MbcT4+q' and MbcT4→T2 increased ability to capture progelatinase A when compared to MbcT4.

3.6.2 Visualization by Zymography

Progelatinase A bound to TIMP-2, TIMP-4, the MbcTIMPs, or hhMb coated on a fluorimetry plate was visualized using zymography, a sensitive method for the detection of gelatinase A bands by degradation of gelatin contained within a polyacrylamide gel by enzyme present in the protein band. TIMP-2 captured more progelatinase A than TIMP-4 as shown in figure 17 A. In contrast, hhMb could not capture any progelatinase A (Figure 17 A). MbcT2 captured less progelatinase A than TIMP-2 as shown in figure 17 B. MbcT4 and MbcT4ΔT captured similar but low amounts of progelatinase A. Again, figure 17 B shows that the loss of the negative charges or mutation of the C-terminal tail of MbcT2 reduced the amount of progelatinase A captured. Introduction of the negative charges in the C-terminal tail of TIMP-2 in MbcT4+q' and MbcT4→T2 captured amounts of progelatinase A similar to MbcT2.
Figure 17: Visualization of enzyme capture assay by zymography. Serial dilutions of progelatinase A were overlaid on the coating protein on a fluorimetry plate. After activation and fluorimetry assay, the proteins were eluted with 4 x non-reducing SDS-PAGE sample buffer and analysed by zymography. In panel A, progelatinase A eluted from wells coated with full length TIMPs-2 and -4 and hhMb are analysed as positive and negative controls. Panel B shows the progelatinase A captured when the MbcTIMPs are the coating proteins.
CHAPTER 4 – DISCUSSION

4.1 Perspective

Because of the importance of type IV collagen degradation in tumour metastasis and angiogenesis, gelatinase A, a type IV collagenase, has been the subject of much research. Especially of interest is the unique ability of progelatinase A to be activated at the cell surface. This cell surface activation is paradoxical as it involves an inhibitor of MMPs, TIMP-2, effective over a narrow concentration range. Since TIMP-4 is highly homologous to TIMP-2 and can bind to both progelatinase A and MT1-MMP, it was proposed that TIMP-4 would also support the activation of progelatinase A (Bigg et al., 1997). However in 2001, Bigg et al. demonstrated that TIMP-4, unlike TIMP-2, cannot participate in the activation of progelatinase A, but can inhibit activation by MT1-MMP at higher concentrations (Bigg et al., 2001). It was also demonstrated that TIMP-4 could inhibit progelatinase A activation by MT2-MMP (Morrison et al., 2001). This has led to the work in this thesis, which addresses the issue of why TIMP-2 can participate in the activation of progelatinase A, but TIMP-4 cannot. TIMP-2 is hypothesized to bind progelatinase A hemopexin C domain through its C-terminal domain (Overall et al., 2000), utilizing an anionic C-terminal tail to aid in binding to the cationic residues of the hemopexin modules III and IV (Overall et al., 1999). As TIMP-4 does not possess these negatively charged residues in its C-terminal tail, we hypothesised that these negative charges may play an important role in promoting the interaction between TIMP-2 with progelatinase A. By cloning the C-terminal domain of TIMPs-2 and -4 and generating C-terminal tail mutants we
investigated the essential components that enable TIMP-2 to play a role in the activation of progelatinase A, which are absent in TIMP-4.

4.2 Recombinant protein expression, purification and characterization

In order to dissect the protein interactions that occur in the activation of progelatinase A, the C-domains of TIMPs-2 and -4 were cloned as fusion proteins C-terminal to horse heart myoglobin (hhMb) and expressed in E. coli. It has never been directly shown that the C-terminal domains of TIMPs interact with progelatinase A through its hemopexin C domain, as C-TIMPs have not previously been expressed independently. It was assumed that since the complexed TIMP-2 was inhibitory, the N-terminal domain didn't interact and therefore the C-terminal domain was interacting with progelatinase A (Kolkenbrock et al., 1991). It was demonstrated by Butler et al., in 1998 that the catalytic domain of progelatinase A was unable to bind to full-length TIMP-2 (Butler et al., 1998), indicating that the hemopexin C domain is required for the TIMP-2/progelatinase A interaction to occur. Yeast-two-hybrid analysis later confirmed that the gelatinase A hemopexin C domain is sufficient to interact with full-length TIMP-2 (Overall et al., 2000; McQuibban et al., 2000). The cell line BL21 DE3 Gold was used to express the fusion proteins because it was found to have good expression levels. We predicted that since hhMb is soluble when expressed in E. coli (Tang et al., 1994) and easily purified by metal chelate chromatography that the recombinant fusion proteins would be soluble and easy to purify. However, this was not the case as the myoglobin C-TIMPs
(MbcTIMPs) when expressed, were located in inclusion bodies (Figure 9). This posed a significant problem, as upon denaturation, solubilization and refolding, the heme contained within the hhMb was lost. This made the proteins difficult to purify and decreased their stability.

Besides the problems encountered with the loss of heme, it was soon discovered that the MbcTIMPs were unstable in phosphate-based buffers and at low pH. Exposure to low pH resulted in precipitation of the recombinant proteins as did freeze thawing in phosphate buffers. I therefore scanned for suitable storage and purification buffers. This was accomplished by the protocol established by Lepre and Moore for optimisation of solvent conditions for NMR spectroscopy of proteins (Lepre and Moore, 1998). It was discovered that the MbcTIMPs are relatively stable in tris-based buffers and very stable in carbonate-based buffers. Unreconstituted MbcTIMPs were less stable than MbcTIMPs containing heme and tended to precipitate during dialysis.

In addition, the benefits of visually tracking the recombinant protein and using spectrophotometry to quantify the fusion proteins was lost as the heme is responsible for the spectrophotometric absorbance of hhMb at 409 nm. However, heme can be reconstituted into the fusion protein during refolding by addition to the solubilized protein solution. Heme reconstitution is normally accomplished by adding a 1:1 molar ratio of heme to myoglobin (Paulson et al., 1979). However, as the concentration of MbcTIMP in the protein preparation
was unknown, an excess amount of heme was typically added. Reconstituted MbcTIMP binds metal chelate columns and can be visually tracked (Figure 11 A). The red colour of the protein solution indicates that the reconstitution process was successful. However, the excess heme made purification difficult because heme is known to bind to the surface of other proteins and therefore purification of the MbcTIMPs required several steps (Figure 10). Benzamidine Sepharose resin was first added to the refolded protein solution to remove serine proteases that degrade MbcTIMPs. Because the pl of the MbcTIMPs is higher than many proteins, these proteins would be negatively charged at the pl of the MbcTIMPs and therefore would bind DEAE Sepharose while the MbcTIMPs would not and therefore many contaminating proteins were removed (Figure 10 A). The unbound fraction from the DEAE Sepharose, anion-exchange column was then applied to a Ni\(^{2+}\) chelate column to exploit the metal chelate binding properties of myoglobin (Figure 10 B). The pooled fractions from the Ni\(^{2+}\) chelate column were dialysed to one pH unit below the pl of the recombinant proteins and then applied to a CM-Sepharose, cation-exchange column. This would allow the MbcTIMPs to bind to the resin and was effective in removing the lower molecular weight contaminating proteins (Figure 10 C).

The MbcTIMPs were also subjected to soret analysis to determine if the heme reconstitution was complete. The addition of additional heme to the purified proteins did not significantly alter the soret absorbance while increasing the absorbance at ~380nm where unincorporated heme absorbs (Figure 11 B),
indicating that the reconstitution was complete. Soret analysis also provided an excellent way to determine the concentration of the MbcTIMPs. As the absorbance of heme containing hhMb at 409 nm is concentration dependent, a standard curve was constructed using known amounts of hhMb. This allowed for the calculation of the molar concentration of the MbcTIMPs based on their soret absorbance, using the molar extinction coefficient of 149,415 M⁻¹cm⁻¹. The approximate overall yields of protein purification are presented in table 2. In addition to soret analysis, the purified recombinant proteins were subjected to electrospray ionization mass spectrometry to confirm the fidelity of gene expression, N-terminal methionine processing, and identity of the MbcTIMP proteins (table 3). The results of the mass spectrometry showed that all the MbcTIMPs and hhMb had both N-terminally processed (-met) and non N-terminally processed (+met) species and that the masses were within the limits of experimental error.

The recombinant proteins were also visualized by SDS-PAGE followed by silver staining and Western blotting with the α-Mb, α-cT2 tail and α-cT4 tail antibodies, which were previously available in our laboratory (Figure 12). The α-cT2 tail and α-cT4 tail antibodies were previously described (Bigg et al., 2001). The apparent molecular weight of MbcT2 and MbcT4 does not change upon reduction with DTT, unlike the full-length TIMPs (Figure 12 A). This could be because the full-length TIMPs have 6 disulphides whereas the MbcTIMPs only have three disulphides, making a shift in apparent molecular weight less visible. The
absence of visible dimeric/multimeric bands in the fractions run without reduction indicate that the majority of the protein does not contain intermolecular disulphides. Silver staining showed that the proteins were purified to homogeneity and Western blotting with the anti-Mb antibody confirmed the presence of the hhMb fusion tag. As expected, MbcT2 and MbcT4 are recognized by the α-cT2 tail and α-cT4 tail antibodies, which are raised to the C-terminal tails of TIMPs-2 and -4 respectively and the tail-less mutants (MbcT2ΔT and MbcT4ΔT) are not recognized by either α-C-TIMP tail antibody, thus confirming the loss of their C-terminal tails (Figure 12 B and C). It is interesting to note that both MbcT4 tail mutants (MbcT4+q⁻ and MbcT4→T2) are recognized by the α-cT2 tail antibody and not the α-cT4 tail antibody (Figure 12 C), which confirmed the success of the mutations and indicated that the negatively charged residues are the major determinants of specificity for the α-cT2 tail antibody. However, in the case of the MbcT2 tail mutants, MbcT2→T4 is recognized by the α-cT4 tail antibody whereas MbcT2Δq⁺ is recognized by neither tail antibody (Figure 12 B). This could be because the epitope for the α-cT2 tail antibody encompasses the charged residues that are removed in the MbcT2 mutants and added in the MbcT4 mutants while the epitope for the α-cT4 tail antibody encompasses a larger portion of the tail. The α-cT2 tail antibody does not recognize full length TIMP-2 as well as MbcT2 and this low level of detection of TIMP-2 has previously been noted by others in our laboratory.
4.3 Protein interaction studies

The purpose of this thesis was to study the protein-protein interactions between progelatinase A or the gelatinase A hemopexin C domain and the different MbcTIMPs compared with full length TIMPs. Protein-protein interactions can be detected by many different methods including enzyme-linked immunosorbent assay (ELISA), affinity columns, and glutaraldehyde cross-linking, each of which has their own advantages and disadvantages. In addition to these methods, detection of interactions was performed by velocity sedimentation and enzyme capture assays. It was hoped that these experiments would provide insight into the ability of TIMP-2 to participate in the activation of progelatinase A.

4.3.1 Microwell solid phase binding assay

An ELISA is a solid phase assay where a secondary protein bound to protein coated onto the wells on an ELISA plate is detected by antibodies. This proved to be an unreliable method of detecting interactions between the proteins of interest. Although the MbcTIMPs were able to bind to gelatinase A hemopexin C domain, the binding affinities obtained were different if TIMPs or MbcTIMPs were coated on the plate versus if gelatinase A hemopexin C domain was coated on the plate. The presence of Brij 35 in the protein preparations also appeared to affect the results. There is also a large amount of error associated with this kind of assay as it involves the accurate pipetting of a primary protein, serial dilution of a secondary protein, and the accurate pipetting of primary antibody, secondary antibody, and substrate over a 96-well plate using a multichannel pipette. Plus, it
also depends on the affinity of the primary antibody for the second protein and only the second protein. In the end, the only useful information gained from these experiments was that TIMP-2 binds with greater saturation and affinity to the gelatinase A hemopexin C domain than TIMP-4 or any of the MbcTIMPs (data not shown) and that MbcT4 and MbcT4ΔT bind with similar affinities (K	extsubscript{d} approximately 4 x 10^{-6}) to gelatinase A hemopexin C domain (Figure 13).

4.3.2 Affinity columns
Through the use of gelatin Sepharose mini columns, we previously showed that TIMP-4 can bind to progelatinase A (Bigg et al., 1997). This experiment was repeated and I could show binding of TIMP-2 to progelatinase A, but I could not reliably reproduce the result with TIMP-4. I was also unable to show that any of the MbcTIMPs tested bound to progelatinase A. In order to test the interaction between the hemopexin C domain and the MbcTIMPs, affinity columns using hemopexin C domain coupled to affi-gel resin were used. These columns showed that TIMP-2 could bind to the gelatinase A hemopexin C domain as opposed to PBS control resin while hhMb could not bind either of the resins. However, MbcT2 and MbcT4 bound to both the gelatinase A hemopexin C domain-coupled resin and the PBS control resin (data not shown). In an effort to reduce the non-specific interactions between the MbcTIMPs and the affi-gel resin, resin coupled to BSA and MT1-MMP hemopexin C domain were used as controls with no impact. In addition to these measures, the mini-columns were also blocked again with BSA prior to loading the MbcTIMPs onto the columns.
Unfortunately, the MbcTIMPs appeared to bind to the resin itself and this interaction was not reduced by the use of detergents or 1 M NaCl and the MbcTIMPs could only be eluted from the resin with 4 x SDS-PAGE sample buffer. The ability of these recombinant proteins to adhere directly to resin could be because the packing interface of the C-TIMP, which is normally closely interacting with the N-terminal domain of the TIMPs, is exposed in the fusion protein. It is likely that the residues along this interface are more hydrophobic contributing to its non-specific binding to resin.

4.3.3 Velocity sedimentation

Another method of detecting protein-protein interactions is by velocity sedimentation. This method is useful for detecting interactions between proteins that have a large difference in molecular weight. Therefore to assess the protein interactions, full-length progelatinase A was used. Moreover, the hemopexin C domain, which is 29kDa, is too similar in size to the TIMPs and MbcTIMPs and therefore complexes between them would be difficult to resolve. Since the sucrose is unlikely to disrupt protein-protein interactions, the progelatinase A and TIMP or C-TIMP should remain complexed during the centrifugation and elute in the same fractions. As elution is accomplished from the bottom of the tube, the higher molecular weight proteins or protein complexes would elute in earlier fractions. A non-catalytically competent progelatinase A mutant, E375A (pE375A) made in Chinese hamster ovary cells (by Dr. G. S. Butler) is secreted as a complex with TIMP-2. This complex was used to test the ability of velocity
sedimentation to isolate complexed pE375A and TIMPs. pE375A was used in these experiments because progelatinase A autoactivates and autodegrades over time. When analysed by SDS-PAGE and densitometry, the pre-complexed pE375A and TIMP-2 co-elute (Figure 14 A), which indicated the suitability of this method for detection of pE375A-TIMP/C-TIMP complexes. When TIMP-2 and pE375A (expressed in TIMP-2 -/- cells) were incubated together to form a complex, there was a shift in the fraction number in which TIMP-2 eluted as compared to TIMP-2 alone (Figure 14 B and C). As an excess amount of TIMP-2 was added, not all TIMP-2 was complexed with progelatinase, resulting in the two distinct TIMP-2 peaks shown in figure 14 C. When progelatinase A and TIMP-4 were incubated together, there was also a shift in the peak elution fraction of TIMP-4 to coincide with the peak elution fractions of pE375A (Figure 14 D). However, the shift is more subtle, which likely reflects the lower affinity of TIMP-4 to pE375A compared with TIMP-2. When tested, both MbcT2 and MbcT4 peaked at an earlier fraction number when incubated with pE375A than when incubated on their own (Figure 14 E and F). This suggests that both MbcT2 and MbcT4 can bind pE375A like TIMP-2 and TIMP-4. The second, higher molecular weight peak found in the elution profiles of uncomplexed TIMP-2, TIMP-4, and MbcTIMP-4 could be because of protein aggregation resulting in homodimers. Unfortunately, the resolution of this experiment is relatively poor as the dimension of the tubes used was 2cm long with a diameter of 0.5cm. This meant that subtle differences in binding and weak binding interactions could not be detected and therefore the remaining recombinant proteins were not tested.
The resolution issue could be resolved by using a narrower but longer tube to perform this experiment but a centrifuge with a suitable rotor was not available. However, this experiment provided evidence that the recombinant C-TIMPs can bind to progelatinase A.

4.3.4 Chemical cross-linking

The velocity sedimentation showed that MbcT2 and MbcT4, like TIMPs-2 and -4, can bind progelatinase A, so to confirm an interaction between the MbcTIMPs and progelatinase A or gelatinase A hemopexin C domain, glutaraldehyde cross-linking was employed. This method is only able to determine whether or not proteins interact and is unable to quantitate the interaction in solution. However, it is a useful tool for demonstrating protein interactions. To determine whether the method of glutaraldehyde cross-linking could show an interaction between TIMPs and gelatinase A and to determine the concentration of proteins to use, this method was first tested on TIMPs-2 and -4 and gelatinase A hemopexin C domain. Although glutaraldehyde cross-linking produced some TIMP homodimers, as an increasing amount of gelatinase A hemopexin C domain was added, the homodimers species lessened and the TIMP/gelatinase A hemopexin C domain complex became dominant (Figure 15 A and B). This indicates that the gelatinase A hemopexin C domain can bind to TIMP-2 and -4 and therefore the same conditions could be used to determine if the recombinant C-TIMPs could also bind gelatinase A hemopexin C domain. Progelatinase A was used in cross-linking experiments with the MbcTIMPs because the sizes of the
recombinant proteins and gelatinase A hemopexin C domain are very similar and therefore homodimers would be indistinguishable from heterodimers. Moreover, progelatinase A interactions with C-TIMPs would be more natural. Using progelatinase A had the added advantage of being able to detect the complexes by the highly sensitive technique of zymography. In figure 15 C and D, MbcT2 and MbcT4 are once again shown to interact with progelatinase A by the presence of an extra band in the silver stain and the shift in progelatinase A activity in the zymogram as indicated by the arrows. The addition of glutaraldehyde to the reactions appears to have an effect on effectiveness of the gelatinase A to cleave the gelatin embedded in the gel. This could be due to intramolecular cross-linking within the gelatinase A thereby inactivating a small proportion of the enzyme present in the samples. The tail-less mutants (MbcT2ΔT and MbcT4ΔT) were also shown to interact with progelatinase A (Figure 15 E and F) whereas hhMb did not interact (Figure 15 G). Because removal of the C-terminal tails of MbcT2 and MbcT4 does not stop the proteins from interacting with progelatinase A, it was assumed that the remaining mutants would interact with progelatinase A. This is because the tails of the remaining mutants contain only a few amino acid substitutions and are likely to interact with progelatinase A if the tail-less mutants can and therefore MbcT2Δq', MbcT4+q', MbcT2→T4, and MbcT4→T2 were not tested using this method.
4.3.5 Enzyme Capture Assay

The glutaraldehyde cross-linking experiment demonstrated that the MbcTIMPs, with or without their C-terminal tails, could interact with progelatinase A. In order to quantify these interactions, an enzyme capture method was employed. Enzyme capture is a solid phase assay much like ELISA. TIMP-2, TIMP-4, the MbcTIMPs, hhMb, and ovalbumin were coated on a high protein adsorption fluorimetry plate. Progelatinase A was added to the wells, incubated, and then the wells were washed. The progelatinase A that was bound to the coating protein was then APMA-activated and a fluorimetry assay was performed to detect gelatinase A activity. As the amount of enzyme captured by the coating protein increases, the rate of substrate cleavage increases accordingly. Therefore, the rate of cleavage of a quenched fluorescent peptide substrate was used to determine the relative amount of enzyme “captured” by the coating protein. Figure 16 shows the rates of gelatinase A captured by TIMP-2, TIMP-4, and the MbcTIMPs, while hhMb and ovalbumin served as controls. The rates are expressed relative to the rate of progelatinase A captured by TIMP-2. However, it must be noted that the activity captured by TIMP-2 and TIMP-4 most probably does not represent the total amount of enzyme captured by these proteins, as some activity would be blocked due to the inhibitory activity of the TIMPs, as the enzyme is activated by APMA, it is possible for full-length TIMPs to bind the catalytic domain through an inhibitory interaction. This inhibitory interaction between the full length TIMPs and activated gelatinase A becomes more apparent as the concentration of progelatinase incubated on the coating protein
is lowered. Despite this, the assay shows that TIMP-2 binds progelatinase A more avidly than TIMP-4, which is consistent with our previous data (Bigg et al., 1997). It also shows that MbcT2 binds to progelatinase A with a higher affinity than MbcT4, although much lower than TIMP-2. The lower activity seen in MbcT2 wells than in TIMP-2 wells could be because of N-terminal domain contributions to binding of progelatinase A. This would be consistent with data from our lab that shows that N-TIMP-2 can bind gelatinase A hemopexin C domain in low salt conditions, which could indicate a small role for the N-terminal domain of TIMP-2 in progelatinase A binding (Wallon and Overall, unpublished data). Another possibility is that the normally close interaction between the N- and C-terminal domains of TIMPs stabilizes the TIMP C-terminal domain. The lack of the N-terminal domain in the MbcTIMPs could destabilize C-TIMP leading to a weaker interaction between it and gelatinase A hemopexin C domain. It is also possible that the hhMb fusion domain sterically hinders the interaction between C-TIMP-2 and progelatinase A. The ability of MbcT4 to bind progelatinase A is only slightly lower than that of TIMP-4, unlike the difference between TIMP-2 and MbcT2. This is an indication that perhaps the majority of the difference between the ability of TIMP-2 and MbcT2 to bind progelatinase A is because of N-terminal contributions or C-TIMP destabilization as steric hinderance would likely affect the binding of MbcT4 to progelatinase A as well. As expected, MbcT2∆T bound less progelatinase A than MbcT2 indicating a role for the C-terminal tail of TIMP-2. When the charges in the C-terminal tail of TIMP-2 were removed as in the case of MbcT2∆Q, binding of progelatinase A
was also reduced. This suggests that the negatively charged residues in the C-terminal tail play a dominant role in the interaction between C-TIMP-2 and gelatinase A hemopexin C domain. However, removal of the C-terminal tail of C-TIMP-4 does not appear to significantly affect its ability to bind to progelatinase A. This indicates that the remainder of the C-domain of TIMP-4 is able to interact with the gelatinase A hemopexin C domain but this interaction is not enhanced by the presence of the C-terminal tail of TIMP-4. Significantly, addition of the charges found in the tail of TIMP-2 to C-TIMP-4 increases the amount of progelatinase A captured when compared to MbcT4, providing further evidence that lack of these charges are involved in binding to the hemopexin C domain of gelatinase and that lack of these charges in the TIMP-4 C-terminal tail contribute to its inability to participate in activation. Also, substitution of the C-terminal tail of C-TIMP-4 with that of TIMP-2 increases the rate of progelatinase A captured as compared to MbcT4 which again suggests that the differences between the C-terminal tails of TIMPs-2 and -4 prevent TIMP-4 from supporting the activation of progelatinase A. As expected of the negative controls, hhMb and ovalbumin captured the lowest activity of all proteins tested.

Enzyme capture can also be visualized through zymography as the proteins in the wells can be eluted with 4x SDS-PAGE sample buffer and electrophoresed on zymograms. Again, this assay shows that TIMP-2 binds progelatinase A to a greater extent than TIMP-4 while the hhMb-coated wells do not have a significant amount of gelatinase A activity (Figure 17 A). Like the results in the fluorimetry
assay, MbcT2 captured more progelatinase A activity than MbcT4 but less than TIMP-2 (Figure 17 A and B). MbcT2ΔT captures less activity than MbcT2 whereas MbcT4ΔT appears to have captured an amount of progelatinase A similar to MbcT4 (Figure 17 B). The activity in the MbcT2Δq' zymogram was less than that of MbcT2 again indicating that the negative charges Glu192 and Asp193, that were removed in MbcT2Δq' play a role in the binding of TIMP-2 to progelatinase A. MbcT2→T4 was not significantly different from MbcT2Δq'. Interestingly, when the corresponding residues in the C-terminal tail of TIMP-4 (Val193 and Gln194) are mutated to the negatively charged residues found in the C-terminal tail of TIMP-2, as is the case with MbcT4+q', an increase in the amount of gelatinase A captured, is seen (Figure 17 B) which is consistent with the fluorimetry data. The activity seen in wells coated with MbcT4→T2 is also increased as compared with MbcT4 and this result is also consistent with the result from the fluorimetry assay and is likely due to the higher affinity of the C-terminal tail of TIMP-2 to progelatinase A.

From the location of the TIMP-2 binding site on the gelatinase A hemopexin C domain mapped by Overall et al. in 1999 and the data presented above, it appears that the anionic C-terminal tail of TIMP-2 is involved in the TIMP-2/progelatinase A interaction. It is likely that the negative character of the C-terminal tail leads to an interaction between it and the clusters of cationic residues that form the TIMP-2 binding site. The negative residues Glu192 and Asp193 appear to be especially important in the interaction between C-TIMP-2
and progelatinase A as removal of these charges reduces its ability to bind progelatinase A. As the negative charges within the C-terminal tail of TIMP-2 are localised and within close proximity to one another, cationic clusters located on or near the rim of blade III such as Lys547/Lys549/Lys550/Lys558/Arg561 and Lys566/Lys567/Lys568, (Figure 18) could serve to attract and bind the anionic TIMP-2 tail, which stabilizes the interaction between TIMP-2 and the gelatinase A hemopexin C domain. The C-domain of TIMP-2 could then associate with the cationic charges of blade IV. In addition, hydrophobic interactions are likely to be involved in the packing interface around the charged residues. The work in this thesis shows a clear role for the negative residues Glu192 and Asp 193 in the anionic tail of TIMP-2 which indicates that the lack of these charges in the C-terminal tail of TIMP-4 is partially responsible for its inability to participate in the activation of progelatinase A.
Figure 18: Hypothetical binding site for the C-terminal tail of TIMP-2 on the hemopexin C domain of gelatinase A. The two clusters of cationic charges hypothesized to bind to the anionic tail of TIMP-2 are coloured in dark grey (Lys547/Lys549/Lys550/Lys558/Arg561) and black (Lys566/Lys567/Lys568). Modified from PDB Id 1CK7 (Morgunova et al., 1999).
CHAPTER 5 – CONCLUSION

The data from the velocity sedimentation and the glutaraldehyde cross-linking experiments showed that the MbcTIMPs can interact with progelatinase A in a manner similar to full length TIMP-2 and TIMP-4. To further dissect the interaction between the C-TIMPs and progelatinase A and to quantify the contribution to binding of the C-terminal tail, an enzyme capture was performed using the MbcTIMPs as “bait”. From the use of different tail mutants in the enzyme capture assay, the contribution to binding of C-terminal tails of TIMPs-2 and -4 was characterized. The data from both the fluorimetry and the zymography analyses of the enzyme capture assay indicate a role for the C-terminal tail of TIMP-2 in its binding to progelatinase A, thereby supporting the hypothesis that the C-terminal tail of TIMP-2 is involved its binding to progelatinase A. In addition, it was shown by removal of the charged residues Glu192 and Asp193 that these negative charges contribute to the binding of C-TIMP-2 to progelatinase A and that lack of these negative charges in the C-terminal tail of TIMP-4 decrease its affinity for progelatinase A as compared to TIMP-2. This lowered affinity of TIMP-4 for progelatinase A is likely responsible for its inability to form a trimolecular complex and support activation. In addition, mutation of the C-terminal tail of TIMP-4 to that of TIMP-2 increased the affinity of C-TIMP-4 to progelatinase A and this is likely to be due to the addition of the charged residues in the C-terminal tail as addition of these charges also increase the affinity of C-TIMP-4 for progelatinase A. This study has increased our understanding of the inability of the TIMP-4/MT1-MMP complex to act as a cell
surface receptor of progelatinase A. It has also shed light on the role of TIMP-2 in the activation of progelatinase A through elucidation of the molecular determinants involved in the participation of TIMP-2. The nature of the less negatively charged C-terminal tail of TIMP-4 does not allow it to bind to progelatinase A with as strong an affinity as TIMP-2. This weaker interaction could account for the inability of TIMP-4 to support progelatinase A activation. The C-terminal tail of TIMP-4 also contains a positively charged lysine residue at position 187, which could inhibit its binding to the positively charged hemopexin C domain. It is also possible that TIMP-4 lacks key N-terminal interactions with the gelatinase A hemopexin C domain that contribute to the TIMP-2/progelatinase A interaction. In addition, TIMP-3, like TIMP-4 can bind progelatinase A although it has not been shown to participate in the MT1-MMP mediated activation of progelatinase A (Butler et al., 1999a). This could be because the C-terminal tail of TIMP-3 has a net charge of 0, including a positively charged lysine residue at the same position as the one in the C-terminal tail of TIMP-4. TIMP-1, the only member of the TIMP family without a C-terminal tail is also the only member of the TIMP family unable to bind to progelatinase A. However, TIMP-1 does form a complex with progelatinase B although the progelatinase B/TIMP-1 interaction does not appear to mediate the activation of progelatinase B, which is activated through other pathways. It is likely that TIMP-1 binds to progelatinase B at a site similar to the TIMP-2 binding site on progelatinase A as TIMP-1 and gelatinase B are homologous to TIMP-2 and gelatinase A respectively. The interactions between C-TIMPs and progelatinase
A could be further investigated through cell based activation studies and fluorimetry based competition assays for additional characterization the role of the C-terminal tail, and specifically Glu 192 and Asp 193, in the activation complex.
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