MATRIX METALLOPROTEINASE SUBSTRATE RECOGNITION,
CHARACTERIZATION AND PROTEOMIC DISCOVERY

by

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ABSTRACT

The matrix metalloproteinase (MMP) family of secreted and cell membrane proteases are known to cleave all extracellular matrix proteins and a large number of bioactive molecules. However, the complete role of MMPs *in vivo* is not fully known. Thus, it is pertinent to identify all substrates of MMPs and to also characterize their interactions with substrates in order to comprehend the molecular mechanisms that lead to proteolysis. Membrane type I-MMP (MT1-MMP, MMP-14) and MMP-2 (gelatinase A) are cooperative dynamic components of a cell surface proteolysis apparatus involved in regulating the cellular signaling environment and pericellular collagen homeostasis. Here, we have investigated the structural elements of MT1-MMP and MMP-2 involved in the recognition of type I collagen, in order to dissect the enigmatic mechanism of native (triple helical) collagen cleavage by MMPs. Both MT1-MMP and MMP-2 consist of a catalytic domain tethered to a C domain by a linker peptide. MMP-2 contains an additional insert of three fibronectin type II modules within the catalytic domain. We demonstrate that recombinant proteins representing the C domain of MT1-MMP and the fibronectin domain of MMP-2 act in a dominant negative manner to block MT1-MMP and MMP-2 cleavage of type I collagen. These domains alone are also capable of perturbing the native structure of collagen. Together, these results reveal an important divergence in the mode of collagen cleavage between these two enzymes. To probe the *in vivo* role of MT1-MMP, we developed a rapid proteomic screen for identifying new MT1-MMP substrates in cells using isotope coded affinity tag (ICAT) method of protein quantification. The abundance levels of several extracellular proteins were altered upon overexpression of MT1-MMP, which we took as evidence of proteolytic modification. These potential substrates were confirmed biochemically confirmed and here we report that interleukin (IL)-8, secretory leukocyte protease inhibitor (SLPI), death receptor-6, and connective tissue growth factor (CTGF) are previously undescribed substrates of MT1-MMP. Moreover, the utility and quantitative nature of ICAT as a proteomic screen for protease substrate discovery should make it adaptable for studying the proteolytic function of other protease classes in complex and dynamic biological contexts.
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<td>2D-GE</td>
<td>two dimensional gel electrophoresis</td>
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<tr>
<td>ADAM</td>
<td>a disintegrin and metalloproteinases</td>
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<td>APMA</td>
<td>4-aminophenylmercuric acetate</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>carboxyl-terminal</td>
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<tr>
<td>CBD</td>
<td>collagen binding domain</td>
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<td>CD</td>
<td>circular dichroism</td>
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<td>ConA</td>
<td>concanavalin A</td>
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<td>CTGF</td>
<td>connective tissue growth factor</td>
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<tr>
<td>DMEM</td>
<td>dulbecco's modified essential medium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EGF</td>
<td>epidermal-like growth factor</td>
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<td>ESI</td>
<td>electrospray ionization</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphitidylinositol</td>
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<tr>
<td>GRO</td>
<td>growth related oncogene</td>
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<td>ICAT</td>
<td>isotope coded affinity tag</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
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<td>LCD</td>
<td>linker hemopexin C domain</td>
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<td>LPS</td>
<td>lipopolysaccharides</td>
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<td>MALDI</td>
<td>matrix-assisted laser desorption ionization</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>MCP</td>
<td>monocyte chemoattractant protein</td>
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<td>MD-LC</td>
<td>multi-dimensional liquid chromatography</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>MMPi</td>
<td>MMP inhibitors</td>
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<td>MS</td>
<td>mass spectrometry</td>
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<td>MS/MS</td>
<td>tandem mass spectrometry</td>
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<tr>
<td>MT</td>
<td>membrane type</td>
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<tr>
<td>N</td>
<td>amino-terminal</td>
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<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>pl</td>
<td>isoeletric point</td>
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<td>r</td>
<td>recombinant</td>
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<tr>
<td>s</td>
<td>soluble</td>
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<td>SDF</td>
<td>stromal derived factor</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SLPI</td>
<td>secretory leukocyte protease inhibitor</td>
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<td>TACE</td>
<td>TNF alpha converting enzyme</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TOF</td>
<td>time of flight</td>
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<tr>
<td>TS</td>
<td>thrombospondin</td>
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<tr>
<td>uPA</td>
<td>urokinase plasminogen activator</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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In looking back at what has been the journey of my Ph.D., it has been a somewhat uneven and unexpected road. For me research science has been very intriguing and gratifying, yet also an incredible test of patience and willpower. I could not have navigated this road without the help of so many individuals.

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I've had a wonderful time during my Ph.D. and I am extremely proud of many of my accomplishments. However, one event stands out in my mind and that was the day I met my future wife to be, Clara. And one of my greatest accomplishments was when I convinced her to marry me. She has been there every step of the way, never once questioning my methods or my decisions. Her love and encouragement has kept me afloat through many failures and I couldn't have imagined finishing this degree without her by my side. All this while trying to finish her own graduate and medical degree. She infuses me with her energy, which makes me strive to perform my very best.

I would like to end by dedicating this thesis to my loving grandfather. Though I have only seen him a handful of times in my life, his enduring love for me and my academic career has been priceless. He has gone through an amazing transformation from a man who resisted the idea of my father and his siblings attending university to being one of my greatest supporters. For that I will always reflect on him when I look back at my time here at UBC.

With that I'm ready to close this chapter of my life! I wish all my colleagues in science all the best and to a future full of publications. If I have missed anybody's name here then I am truly sorry, I can't be perfect.

Eric M. Tam
CHAPTER 1: INTRODUCTION

I. THE MATRIX METALLOPROTEINASE FAMILY

Forty-two years ago, Jerome Gross and Charles Lapiere reported an "activity" in tadpoles during the process of tail resorption (1). This activity, characterized as the unique ability to degrade collagen at neutral pH, became ascribed to an enzyme now known as collagenase. Thus, this was the beginning of a new exciting field in matrix biology and the first discovery of a new enzyme family now known as the matrix metalloproteinases (MMPs). The first human collagenase was subsequently isolated by John Jeffrey and coworkers in 1970 (2), and since then the MMP family has grown to 23 members in humans. This large family of secreted and membrane-bound, zinc-dependent endopeptidases were found to degrade not only collagen but all components of the extracellular matrix and were involved in many aspects of normal physiology and pathology due to their role in extracellular matrix remodeling and turnover (3). Yet this view of MMPs as specialized matrix degrading enzymes has been evolving as of late. We and others have shown that MMPs are capable of performing highly specific and limited cleavages of a number of bioactive molecules to modulate many aspects of cell behavior (reviewed in (4,5)). The use of genetically modified mice has contributed greatly in uncovering new functions for MMPs in vivo (6). As a result, MMP biology which was once thought to be primarily concerned with matrix degradation, has now grown extremely complex and encompasses many aspects of biology such as cell proliferation, differentiation, migration, apoptosis, angiogenesis, bone morphogenesis and the immune response (5,7). Given this complexity, there is a need to advance the study of MMPs from in vitro experimentation into biological settings representing in vivo conditions, which will require the technology of proteomics (8). In this introductory chapter, I will review the following topics of MMP biology: enzyme structure and regulation, substrate recognition, extracellular and non-extracellular substrates, gene knockout mice, and the application of proteomics for the study of MMPs.

II. THE STRUCTURE OF MMPS

The 23 members of the MMP family are designated by their common names based on their substrate specificity as well as a sequential numeric nomenclature, and are grouped accordingly to their modular domain organization (Fig. 1.1). The archetypal MMPs consist of the subgroups, collagenases, stromelysins, gelatinases, and matrilysins, as well as MMP-12, -19, -20, and -27. The convertase activatable MMPs consist of the membrane-associated MMPs, MMP-11, -21, -23 and -28. Most MMPs display the general structure of a signal peptide followed by a propeptide and a catalytic domain, which is joined by a linker peptide to the C-terminal hemopexin-like domain (Fig. 1.1).

A. Propeptide

The crystal structure of proMMP-2, -3 and -9 revealed that the MMP propeptide consists of three perpendicular α-helices followed by a segment–composed of an invariant PRCGVPD motif–which enters the active site cleft (9-11). The unpaired cysteine (Cys) residue in this motif coordinates the catalytic zinc found in the active site, in what is referred as the "cysteine-switch", thereby maintaining enzyme latency.
The propeptide is removed from the catalytic domain by a series of proteolytic cleavages to reveal a mature catalytically competent enzyme.

**Archetypal MMPs**
- Collagenases
  - Collagenase-1 (MMP-1)
  - Collagenase-2 (MMP-8)
  - Collagenase-3 (MMP-13)
- Stromelysins
  - Stromelysin-1 (MMP-3)
  - Stromelysin-2 (MMP-10)
- **Other MMPs**
  - Metalloelastase (MMP-12)
  - Enamelysin (MMP-20)
  - MMP-27 (MMP-22, C-MMP)

**Convertase-activatable MMPs**
- Membrane Associated
  - MT1-MMP (MMP-14)
  - MT2-MMP (MMP-15)
  - MT3-MMP (MMP-16)
  - MT5-MMP (MMP-24)
- **Other MMPs**
  - Stromelysin-3 (MMP-11)
  - MMP-21 (X-MMP)
  - Epilysin (MMP-28)

**Gelatinases**
- Gelatinase-A (MMP-2)
- Gelatinase-B (MMP-8)

**Matrilysins**
- Matrilysin (MMP-7)
- Matrilysin-2 (MMP-26)

**FIG. 1.1 Schematic representation of the human MMP family.** The 24-members of the human matrix metalloproteinase (MMP) family are classified based on their domain organization. Archetypal MMPs possess a signal peptide, a propeptide, a catalytic domain that contains the active site zinc (Zn), a linker peptide, and the C-terminal hemopexin-like domain. Gelatinases are distinguished by the presence of a triple repeat of fibronectin modules within the catalytic domain while matrilysins lack the entire hemopexin domain. Convertase-activatable MMPs possess a proprotein convertase cleavage site within the propeptide. This group includes the membrane associated MMPs, which membrane tethered by either a type I or type II transmembrane domain or a glycosylphosphatidylinositol (GPI) anchor. MMP-23 is unique in that it possesses a cysteine-rich and an immunoglobulin (Ig)-like domain in place of the hemopexin domain.
B. Catalytic Domain

The crystal and solution structure of the catalytic domain has been determined for a number of MMPs (see (14) for References). The polypeptide folds of all the catalytic domains are essentially superimposable and consist of a 5-stranded β-sheet, 3 α-helices and interconnecting loops (Fig. 1.2A). The top wall of the active site cleft is formed by strand IV and helix B (Fig. 1.2A). The latter contains the first two histidines and the glutamate of the zinc-binding consensus sequence \( \text{HEXXHXXGXXH} \) found in all MMPs (15). The last histidine is found on the extended loop region following helix B, which forms the base of the active site (Fig. 1.2A). These three histidines and a fixed water molecule, which is simultaneously hydrogen-bonded by the glutamate, coordinate the catalytic zinc (16). Upon binding of a protein substrate into the active site cleft, the carbonyl group of the scissile bond displaces the zinc-coordinated water molecule, which in turn, attacks the carbonyl carbon to initiate peptide bond hydrolysis. Substrate residues on either side of the scissile bond are of the following designation: Pn-P2-P1-scissile bond-P1'-P2'-Pn' (17). Analogously, the corresponding protease sites which recognize these substrate residues are designated S2, S1, S1', S2' and so forth. The extended loop region following helix B also contains a conserved methionine as part of a "Met turn" structure (15) and the S1' pocket, a major determining factor in MMP substrate specificity (16). The S1' subsite accommodates what is to become the new N-terminus (P1' residue) and its size and shape varies considerably among MMPs.

C. Linker Peptide

Extending from helix C of the catalytic domain is a linker peptide (16) that joins into the C-terminal hemopexin-like domain (Fig. 1.2B). This peptide is rich in proline residues and varies in length for the various MMPs—the shortest being 16 residues (MMP-1, 8, 13) and the longest being 54 residues (MMP-9). In both MMP-9 (18) and MT1-MMP (19), this region has been shown to be O-linked glycosylated, which may have implications for domain organization and substrate specificity. The linker peptide is also susceptible to hydrolysis by trypsin (20) or autolytic cleavage (21,22), which leads to the release of the catalytic domain from the hemopexin domain.

D. Hemopexin C Domain

With the exception of the matrilysin subgroup (MMP-7 and MMP-26) and MMP-23 (Fig. 1.1), all MMPs are expressed with a C-terminal domain that displays sequence similarity to hemopexin, a plasma heme binding and transporting protein (23). The crystal structures of MMP-1, -2, -9, and -13 (see (14) for References) reveal that the like the catalytic domain, the hemopexin C domains are very similar in structure (16) and are organized into a 4 bladed β-propeller structure with each blade composed of 4 anti-parallel β-sheets and a single α-helix (Fig. 1.2B). A single disulfide bond between blades IV and I stabilizes the structure. Though the hemopexin C domain possesses no catalytic activity, it is involved in substrate recognition conferring specificity, and is involved in the binding of the tissue inhibitors of metalloproteinases (TIMPs) (24). The hemopexin C domain of MMP-9 homodimerizes through non-covalent hydrophobic contacts made between blade IV of two molecules (25). This phenomenon has also been reported for MT1-MMP (26), which however conflicts with observations by our lab that a
recombinant protein representing the hemopexin C domain of MT1-MMP does not homodimerize (27). In MT2-MMP, a RGD motif is found in the hemopexin C domain, which may mediate integrin binding (28).

**FIG. 1.2** Three-dimensional structures of MMPs. *A.* The catalytic domain of MMP-2 (red). The active site histidines (green) are shown coordinating the active site zinc (cyan). The catalytic glutamate (yellow) is also shown, while the β-sheets (I–V) and α-helices (A–C) are labeled. *B.* The hemopexin domain of MMP-2 (yellow). The four blades of the β-propeller (I–IV) and the linker peptide are labeled. *C.* The full-length structure of MMP-2. The catalytic domain (red), the fibronectin type II modules (green) and the hemopexin C domain (yellow) are shown.
E. Fibronectin Type II Modules
In the gelatinase subgroup (MMP-2 and -9) (Fig. 1.1), three fibronectin-type II modules are found inserted between the strand V and helix B of the catalytic site (Fig. 1.2C). Each module consists of two anti-parallel $\beta$-sheets connected by an $\alpha$-helix and several disulfide bonds (10,29-31). The two $\beta$-sheets of each module form a hydrophobic pocket, which is figured to be involved in substrate binding (30,31). Interestingly, the propeptide of proMMP-2 was found to associate with the hydrophobic pocket of the third fibronectin module, in a manner that is predicted to be analogous to substrate binding (10).

F. Membrane Tethering Domains
Membrane-type MMPs (MT-MMPs) comprise the largest subgroup within the MMP family (Fig. 1.1). This subfamily of 6 members are tethered to the cell membrane by either a type I transmembrane region followed by a short cytoplasmic tail (MT1-, MT2-, MT3-, and MT5-MMP) (28,32-34) or a glycosylphosphatidylinositol (GPI) anchor (MT4- and MT6-MMP) (35,36), both of which are found following the C-terminal hemopexin-like domain. Sequence analysis reveals that there are putative tyrosine, threonine, and serine phosphorylation sites within the cytoplasmic tail suggesting that type I transmembrane MT-MMPs may have a role in cell signaling (37). The cytoplasmic tail of MT1-MMP has also been shown to be important for membrane localization (38), internalization (39), oligomerization (40), intracellular trafficking (41,42) and binding of intracellular partners (43).

G. Additional Motifs and Domains
While archetypal proMMPs are secreted and activated extracellularly, convertase-activatable MMPs, which include all the MT-MMPs, MMP-11, MMP-23 and MMP-28 (Fig. 1.1), are distinguished by the presence of a proprotein convertase sequence RR(K/R)R at the C-terminus of the propeptide and can be activated intracellularly by furin and other proprotein convertases (44-48). MMP-23, a recently discovered type II transmembrane MMP (49), is synthesized as a membrane-bound precursor due to an N-terminal signal anchor and a proprotein convertase sequence within its propeptide domain (50). It is hypothesized that proprotein convertase cleavage regulates both activation and secretion of MMP-23 (50). MMP-23 is also unique among MMPs as the linker region and the hemopexin-like domain is replaced by a cysteine-array and immunoglobulin-like domain (Fig. 1.1) (51).

H. Exosites: Important Features in MMPs
Although peptide bond hydrolysis occurs within the catalytic cleft, MMPs possess substrate-binding motifs located on domains or modules outside the active site. Termed exosites, these functional units can influence the activity of MMPs in several ways (52). By providing the additional contact areas for specific substrates, exosites can broaden the substrate profile of an MMP as well as modulate substrate selectivity. These additional binding sites may also regulate enzyme localization in cells and tissues. The influence of exosites on substrate cleavage is best exemplified in the study of MMP cleavage of collagen (52). Collagen is an unique substrate in that it is composed of three polypeptide chains interwound to form a triple helix which renders the protein resistant to cleavage by most proteases with the exception of
members of the MMP family. For these enzymes, MMP-1, -8, and -13, the hemopexin C domain was absolutely required for triple helical collagen cleavage (22,53-55). In the absence of the hemopexin C domain, the catalytic domain is only capable of cleaving single chain substrates such as denatured collagen (gelatin) and peptide substrates. The linker peptide in MMP-8 has also been shown to play a role in collagenolysis (56,57), although there is no evidence that it binds collagen. MMP-2, a potent enzyme in degrading gelatin, is also a weak collagenase (58,59). However, in contrast to other MMPs, the hemopexin C domain of MMP-2 does not bind collagen (60,61). Instead, the fibronectin type II modules bind both denatured and native type I collagen with a preference for gelatin (62). The $S_3'$ subsite of MMP-1 (63,64) and MMP-8 (65) are also critical for triple helical collagen cleavage. The Glu$^{190}$-Tyr$^{191}$ bond in MMP-1 (63) and the corresponding residues, Asn$^{188}$-Tyr$^{189}$, in MMP-8 (65) exist in a cis conformation as shown by x-ray crystallography and is predicted to play a role in collagen. Interestingly, in MMP-2 and MMP-9 this cis bond has been replaced with the insertion of the fibronectin type II modules.

III. REGULATION OF MMP ACTIVITY

The proteolytic activity of MMPs is strictly localized and regulated in normal physiology by a coordinated network of mechanisms. It is when this homeostatic balance is perturbed that pathological conditions can then arise. The three main levels of MMP regulation are transcription, proenzyme activation and inhibition. Other mechanisms such as cell secretion and internalization, shedding and autodegradation, tissue localization, and oligomerization can also play a role in fine-tuning MMP proteolytic activity.

A. Transcriptional Regulation

MMP gene expression as a whole is very complex due to the large number of MMPs and the numerous roles they play in biology. Expression is regulated temporally and spatially, and varies for different cell types and tissues—healthy or diseased—and can be rapidly induced in response to stimuli such as injury, inflammation, or tumor cell invasion (66). Currently, most of our knowledge in the regulation mechanisms of MMPs has been through the study of cancer (67). Several cytokines and growth factors such as interleukin (IL)-1, tumor necrosis factor (TNF-$\alpha$), interferon (IFN), epidermal-like growth factor (EGF), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) are potent inducers of MMP expression in many cell types, while transforming growth factor (TGF)-$\beta$ has been shown to be both an inducer and suppressor (68). Other known suppressors of MMP expression include glucocorticoids, progesterone and retinoic acids (69). The extracellular matrix is also a regulator of MMP gene transcription. Type I collagen stimulates the expression of MMP-1 (70) and MT1-MMP (71,72), while fragments of laminin-5 (73) and fibronectin (74) have been shown to upregulate MMP-1 and -13 transcription, respectively. Binding of these soluble or extracellular matrix ligands to their respective receptors leads to intracellular signaling through pathways such as the p38 mitogen activated protein (MAP) kinase pathway (75,76), and the induction of transcription factors. Many MMP promoters contain activated protein-1 (AP-1) sites, which are recognized by the AP-1 binding proteins, c-fos and c-jun (77). Other transcription factors known to bind to MMP promoters include the ETS protein family, NF-$\kappa$B, and p53 (5).
B. Enzyme Inhibition

The major endogenous inhibitors of MMPs in tissues are the TIMPs, a family of four inhibitors: TIMP-1, -2, -3 and -4, that bind tightly to MMPs in a noncovalent 1:1 stoichiometry (78). TIMPs play a critical role in the homeostasis of MMP activity in vivo—imbalance in TIMP expression and MMP activity are associated with diseases such as cancer (79). A variety of agents such as cytokines, growth factors, hormones, retinoids, lipopolysaccharides (LPS) and phorbol esters are known to stimulate TIMP gene transcription (80). TIMP-2 is ubiquitously expressed in most human tissues and to a lesser extent TIMP-1 and -3, while the expression of TIMP-4 is restricted to the brain and heart (81).

The general structure of the TIMPs (21-30 kDa) consist of an N-terminal inhibitory domain composed of a 5-stranded β-barrel, a central helical region, and C-terminal domain composed of 2 anti-parallel and 2 parallel β-strands and an α-helix (82) (Fig. 1.3A). Together, the two domains form an elongated wedge that fits into the active site cleft of MMPs (83,84). The first four residues of the inhibitor extend from the main body of the protein, stabilized by two disulfide bonds, and interface with the substrate binding grooves (P1-P1'-P2'-P3') of the MMP active site in a manner analogous to the binding of a peptide substrate (Fig. 1.3A). The α-amino and carbonyl groups of the conserved Cys1 residue coordinate the active site zinc in a bidentate fashion, thus displacing the zinc-bound water molecule that is required for catalysis.

The four TIMP members inhibit most MMPs, with the exception of TIMP-1, which is a poor inhibitor for the type I transmembrane MT-MMPs (MT1-, MT2-, MT3- and MT5-MMP) (78). TIMP-3 is unique among the TIMPs as it is also a potent inhibitor of ADAMs (a disintegrin and metalloproteinases) including ADAM-10 (85), ADAM-12 (86), ADAM-17 or TNF-α converting enzyme (TACE) (87), and members of the ADAM-thrombospondin (TS) family, ADAMTS-4 and ADAMTS-5(88). In addition to MMP inhibition, TIMP-1 and TIMP-2 have been shown to possess other biological activities. Both TIMP-1 and TIMP-2 have erythroid potentiating (89,90) and growth promoting activity (91,92), which has been shown to be independent of MMP inhibition (92,93). These activities appear to be mediated by cell receptor binding (92,94) to stimulate the activity of kinases such as cAMP-dependent protein kinase (95) and the mitogen activated protein kinase (96,97). While the N-domain is responsible for inhibition, the C-domain of TIMPs is important for several non-inhibitory interactions. The C-domains of TIMP-2 (98) and TIMP-4 (99) binds the hemopexin C domain of MMP-2 to form a non-inhibitory complex (Fig. 1.3B). This interaction between TIMP-2/MMP-2 is critical for activation of proMMP-2 by MT1-MMP on the cell surface (100). The significance of the TIMP-4/MMP-2 interaction is not known, as proMMP-2 is not activated in the presence of TIMP-4 (101). The C-domain of TIMP-1 also binds the MMP-9 hemopexin domain and could play a role in proMMP-9 activation (102), while the TIMP-3 C-domain binds sulfated glycosaminoglycans and is important for the localization of the inhibitor to the extracellular matrix (103).
FIG. 1.3 Three-dimensional structures of MMP/TIMP complexes. A. TIMP-2 (blue) is shown in an inhibitory complex with the catalytic domain of MT1-MMP (red). The disulfide bonds of TIMP-2 are shown in yellow. Groups coordinating the active site zinc (cyan) are shown in green and include the active site histidines of MT1-MMP and the amino and carbonyl groups of Cys¹ from TIMP-2. B. Non-inhibitory binding of TIMP-2 to MMP-2. TIMP-2 binds the hemopexin C domain of MMP-2 leaving the enzyme catalytically competent.

While TIMPs are the major inhibitors of MMPs in tissue, the abundant serum protein, α-macroglobulin, is most likely the major inhibitor of MMPs in fluid phase (104,105). RECK, a GPI-linker glycoprotein, inhibits MMP-2, -9, and -14 (106,107) and may play a significant role in regulating MMP activity in vivo as Reck -/- mice embryos display severe defects in type I and IV collagen remodeling (107). The tissue factor pathway inhibitor (TFPI)-2, has also been shown to inhibit MMP-1 and -13 (108). The C-domain of procollagen C-terminal proteinase enhancer (PCPE) is similar to the inhibitory domain of TIMPs and inhibits MMP-2 (109). Another protein displaying similarity to the TIMPs and has MMP inhibitory activity is the non-collagenous (NC)1 domain of type IV collagen (110,111). Endostatin, a proteolytic fragment of type XVII collagen, inhibits both MMP-2 and MMP-14 (112), while MMP-2 has also shown to be inhibited by a soluble form of β-amyloid precursor protein (APP) (113).
**i. Synthetic Inhibitors**

The development of small molecule MMP inhibitors (MMPIs) by the pharmaceutical industry for treatment of diseases, most prominently cancer, began 30 years ago. Batimastat (British Biotech), a peptide mimetic hydroxamic acid inhibitor based on the MMP cleavage sequence in collagen, became the first MMPI to reach human clinical trials for cancer (114). Batimastat displayed broad-spectrum inhibitory activity against MMPs, however due to problems with oral bioavailability, was replaced by marimastat, another broad-spectrum hydroxamate inhibitor. Based on the crystal structure of the MMP active site, second-generation MMPIs, prinomastat (Agouron) and tanomastat (Bayer), were designed as “deep pocket” inhibitors and were shown to be potent inhibitors of MMP-2 and -9 but not MMP-1 (115). Despite the initial promise of these MMPIs for cancer treatment, the success of marimastat, prinomastat and tanomastat in clinical trials has been limited (116). One major problem is that patients undergoing treatment develop musculoskeletal pain and inflammation, thus limiting the effective tolerable dose. These side effects have been associated with the inhibition of the ADAMs (a disintegrin and metalloproteinases). A new thiol-based broad spectrum MMPI, BMS-275291 (Bristol-Myers Squibb)—which has reduced activity against the ADAMs—has not shown musculoskeletal side effects (117). BMS-275291 is currently in phase III clinical trials for prostate cancer (118).

Recently, new classes of MMPIs have been in development. Doxycycline, a member of the tetracycline family of antibiotics, inhibits both the activity and production of MMPs (119). Another tetracycline-analog, metastat (Collagenex), is now in phase II clinical trials for Karposi’s sarcoma (118). Bisphosphonates, used for treating osteolytic activity in bone metastases, are also effective inhibitors of MMP activity at many levels (120). New MMPIs that are selective for certain MMPs have also been developed. Biphenyl compounds containing 1 or 2 thiol groups were shown to be selective inhibitors of MMP-2 and -9 (121, 122). Koivunen et al. (123) demonstrated that the disulfide containing cyclic peptide, CTTHWGFTLC, was selective for MMP-2 and -9, but not MMP-8, -13 and -14, and improved the survival of tumor bearing mice. Epigallocatechin-3-gallate (EGCG), a flavanol found in green tea is an inhibitor of MMP-2, -9 and -14 (124). Interestingly, the mechanism of ECGC inhibition does not appear to involve zinc chelation.

**C. Proenzyme Activation**

MMPs are expressed as latent zymogens—maintained by the cysteine switch mechanism—and must be proteolytically activated in order to be catalytically competent (12). *In vitro*, chemical agents such as 4-aminophenylmercuric acetate (APMA), HgCl₂, N-ethylmaleimide, SDS, chaotropic reagents and reactive oxygens initiate proteolytic activation by disrupting the interaction between the unpaired cysteine and the catalytic zinc to allow a water molecule to coordinate with the zinc (13). The enzyme, now catalytically competent, undergoes autoactivation where the propeptide is successively truncated by two sequential cleavages—intramolecular and intermolecular—to yield an intermediate and a mature, fully active enzyme, respectively. *In vivo*, the two cleavages required for the removal of the MMP propeptide can be accomplished by other MMPs such as MMP-3 (125, 126), MMP-7 (127) or the MT-MMPs (37), or by
extracellular serine proteases such as plasmin, urokinase plasminogen activator (uPA) or thrombin (128-131). The basic recognition sequence in convertase-activatable MMPs is located immediately upstream of the N-terminus of the mature enzyme. Pei and Weiss (44) first demonstrated that proMMP-11 is activated intracellularly by a single cleavage by furin. MT3-MMP (45) is also activated by furin, while MT1-MMP activation is regulated by both furin-dependent and independent pathways (46,47). There is also evidence that furin can activate MT1-MMP on the cell surface (132). Furin-activated MT5-MMP is shed into the medium (133) due to a second cryptic furin recognition site within its stem region upstream from the transmembrane domain (48).

D. The Complex Mechanism of ProMMP-2 Activation
The complex mechanism of proMMP-2 activation has been extensively studied (100,134-138). MMP-2 is secreted as a latent enzyme but unlike other MMPs is primarily activated at the plasma membrane, a process that requires the presence of TIMP-2 (134,135). It was subsequently discovered that MT1-MMP, a new member to the MMP family and the first transmembrane MMP described, was a cell surface activator of proMMP-2 (32). The current model of proMMP-2 activation by MT1-MMP is shown in Fig. 1.4. Secreted TIMP-2 associates with MT1-MMP through inhibition of the MT1-MMP catalytic domain (Fig. 1.4A). The C-domain of TIMP-2 is available to interact with the proMMP-2 C-domain and serves as a receptor for recruiting the latent gelatinase to the cell surface through the formation of the MT1-MMP/TIMP-2/proMMP-2 ternary complex (Fig. 1.4B). The MMP-2 propeptide is then cleaved at the Asn37-Leu38 bond by an adjacent, uninhibited molecule of active MT1-MMP (Fig. 1.4C). The final cleavage at Asn80-Tyr81 occurs intermolecularly and is dependent on the concentration of active MMP-2 present. The mechanism of release of proMMP-2 is not clear, though MT1-MMP is susceptible to autocatalytic degradation that results in the release of the catalytic domain from the hemopexin C domain (139,140) (Fig. 1.4D). Overall, the levels of TIMP-2 levels are critical in this mechanism as there must be a fine balance between uninhibited and TIMP-2-complexed MT1-MMP molecules for proMMP-2 activation to proceed efficiently (100). In addition, colocalization of inhibited and uninhibited MT1-MMP molecules is also critical. Homophilic interactions between the hemopexin C domain of MT1-MMP are thought to play a role in MT1-MMP clustering on the cell surface (26), a process that is also promoted by the lectin, concanavalin A (27).

Several labs have shown that native collagen induces cell surface expression of MT1-MMP and proMMP-2 activation (72,141,142), which is thought to occur through ligation of β1-integrins (142-144) and MAPK signaling pathways (145). This may represent a feedback loop as MT1-MMP and MMP-2 can both cleave collagen, while MMP-2 degrades gelatin. Concomitant with increased proMMP-2 activation and MT1-MMP upregulation is the autolytic shedding of the MT1-MMP catalytic domain and the formation of a 44-kDa product on the cell membrane (142,146) consisting of the linker peptide, the hemopexin C domain, and the transmembrane/cytoplasmic domain of MT1-MMP (139).
FIG. 1.4 Cell Surface Activation of proMMP-2.  A. MT1-MMP is activated intracellularly and is expressed on the cell surface while the latent form of MMP-2 and TIMP-2 are secreted. TIMP-2 inhibits active MT1-MMP on the cell surface, a process that is highly dependent on TIMP-2 and MT1-MMP concentrations.  B. The TIMP-2/MT1-MMP complex acts as a receptor for proMMP-2 and recruits the gelatinase to the cell surface.  C. Colocalizing, uninhibited MT1-MMP molecules cleave proMMP-2 bound to the TIMP-2/MT1-MMP receptor at Asn$^{37}$-Leu$^{38}$ to generate the MMP-2 activation intermediate. MT1-MMP is also susceptible to autolytic cleavage at sites within the linker peptide and the catalytic domain.  D. Fully active MMP-2 is generated through autolytic intermolecular cleavage (not shown) by MMP-2 activation intermediates. MT1-MMP autodegradation generates the 44-kDa form, which lacks the catalytic domain and may serve as a release mechanism for the active MMP-2/TIMP-2 complex.

Other MT-MMPs, MT2- (28), MT3- (33), MT5- (133), and MT6- (147) with the exception of MT4-MMP (148) can also cleave the propeptide of MMP-2. The catalytic domain of MT1-MMP (84) contains a 10-residue loop the deviates from catalytic domain structure of other MMPs, which has been shown to be required for proMMP-2 cleavage (149). Recently MT3-MMP has also been shown to possess this loop.
In cells, activation of proMMP-2 by MT2-MMP does not require TIMP-2 (151), whereas MT3-MMP activation is potentiated by both TIMP-2 and TIMP-3 (152). The catalytic domain of MT3-MMP also formed a ternary complex with proMMP-2 in the presence of TIMP-2 and TIMP-3 suggesting that the mechanism of activation may proceed in a manner similar to the MT1-MMP pathway. TIMP-4, which also binds the proMMP-2, cannot form trimolecular complexes with either MT1-MMP or MT3-MMP and proMMP-2 (101,152). Interestingly, analysis of various tissues from MT1-MMP and TIMP-2-deficient mice revealed significant reductions in active MMP-2, which suggests that the TIMP-2/MT1-MMP complex represents the major pathway of proMMP-2 activation in vivo (153,154).

IV. SUBSTRATES OF MMPs

MMPs have been traditionally thought of as catabolic enzymes responsible for the bulk degradation and turnover of the extracellular matrix. It is now becoming recognized that MMPs also perform highly specific and limited substrate cleavages to expose cryptic sites within the matrix, generate bioactive matrix fragments, and to release signaling molecules from the matrix, all of which have profound effects on cell behavior (155). Moreover, MMPs directly process a large number of signaling molecules including growth factors, cytokines, chemokines, cell surface receptors and adhesion molecules (7). With such broad substrate specificity, MMP activity encompasses almost every aspect of biology.

A. Extracellular Matrix Substrates

i. Collagen

The ability to cleave and degrade collagen at neutral pH is the most recognized feature of the MMP family. Collagen is the major constituent of the extracellular matrix and provides the scaffold upon which the structure and shape of cells, tissues and organisms is maintained and for the attachment of various proteins, macromolecules and cells (156). Native collagen is composed of three polypeptides designated as α-chains interwound to form a triple helix, a conformation which requires that glycine (Gly) be present at every third amino acid residue (Gly-X-Y motif). Proline (Pro) and hydroxyproline (Hyp) are often found in the X- and Y- positions following the Gly. Of the 25 types of collagen in mammals to date, type I is the most abundant. Together with type II, III, V, and XI, it forms the class of fibril-forming collagens. Fibrils are supermolecular bundles of triple helical collagen stacked upon one another and stabilized by cross-linking, and provides the structural integrity needed for tissues such as tendons, ligaments and bone. MMPs 1, 8, 13, and 14 cleave native type I, II and III collagens at the Gly1775-Ile/Leu1776 position to generate 3/4 N-terminal and 1/4 C-terminal fragments (157). It is not clear how MMPs distinguish this site given the number of repeating glycine residues, although it has been observed that the region of the scissile bond is low in Pro and Hyp which is known to lead to a reduction in the triple helical content (158-160). Native collagen in its triple helical form represents a unique problem for collagenases. Crystal structures have shown that the MMP active site cleft (5 Å) is not large enough to accommodate the collagen triple helix (15 Å) (16). Therefore, there must be localized unwinding of the triple helix at the cleavage site in order for individual cleavage of each α-chain. As mentioned before, the active site cleft and the hemopexin C domain represent the major binding sites for the collagen helix, although the exact location
of exosite on the hemopexin C domain has yet to be determined. Based on these observations, we have
previously proposed several models in which two-point binding of the collagen triple helix by the MMP
catalytic domain and the hemopexin C domain may induce various modes of stress at the cleavage site in
order to destabilize the helix for individual α-chain cleavage (52).

MMP-2 and -9 also cleave fibrillar collagens, type V and XI, at a single position to yield a 3/5 C-terminal
fragment (161,162), while MMP-3 cleaves type XI collagen in the N-terminal telopeptide region (163).
The basement membrane collagen, type IV, is cleaved by MMP-2, -3, -7 and -9, although the gelatinases
were found to be more efficient (164,165). Collagens VII, VIII, X and XI are also cleaved by various
MMPs (5). MMP cleavage of native collagen destabilizes the triple helix allowing collagen to become
susceptible to denaturation (166,167) and further cleavage. Gelatin is susceptible to proteolysis by many
MMPs, however, MMP-2 and -9 are the most efficient (157). Collagen degradation is not just a catabolic
event as cleaved collagen fragments have been shown to possess biological activity. MMP cleavage of
native collagen to generate gelatin can reveal cryptic RGD sites that have been shown to promote the
survival and growth of melanoma cells (168). A cryptic site in type IV collagen is exposed upon MMP-9
cleavage and is proangiogenic (169). MMP-9 also cleaves type IV collagen in the NC-1 domain of the
α3-chain to release the fragment tumstatin, an inhibitor of angiogenesis (170,171). Cleavage of the NC-1
domain of the α1-chain in type XVII collagen by several MMPs including MMP-9 (172), releases the anti-
angiogenic molecule, endostatin, an inhibitor of endothelial cell proliferation (173) and MMP activity (112).

ii. Non-collagenous Proteins
Proteoglycans and glycoproteins make up the bulk of the non-collagenous material in the extracellular
matrix and are substrates of many MMPs. Although the cartilage proteoglycan aggrecan is degraded
primarily in vivo by ADAMTS-4 and -5, a number of MMPs including 1, 3, 7, 9, 13, and 14 have been
shown to hydrolyze the Asn³⁴¹-Phe³⁴² bond in the aggrecan IGD domain (174). The large chondroitin
sulfate proteoglycan, versican, is cleaved by MMP-2, -7 and -9 (175,176) while fibromodulin is processed
by MMP-13 to release the N-terminal tyrosine sulfate-rich region (177). Membrane-anchored betaglycan
and syndecan-1 are shed by MT1- and MT3-MMP (178), and by MT1-MMP and MMP-7 (179,180),
respectively. Syndecan-1 cleavage by MMP-7 has been shown to stimulate neutrophil influx through the
release of the chemokine, KC (180). In contrast, both decorin and brevican are degraded by MMPs
(181,182). The multi-functional glycoprotein, fibronectin, is degraded by most MMPs with the exception of
MMP-8 and -9 (5). However, limited proteolysis of the fibrin and cell binding sites of fibronectin by MMP-
1, -2 and -7 exposes cryptic sites able to stimulate cell differentiation and migration (183). In addition,
proteolysis by MMP-2 within the heparin-binding site reveals a site with anti-adhesive activity (184).
Laminin-5, a heterotrimeric glycoprotein consisting of α3, β3, γ2-chains found in basement membranes, is
cleaved by MMP-2, -3, -13, -14 and -20 at the N-terminus of the γ2-chain to reveal a cryptic site that
promotes cell motility (185-187). Fibrinogen and cross-linked fibrin are both processed by MMP-3, -7, -9
and -14 (188,189) while elastin is cleaved by macrophage derived MMPs including 2, 7, 9 and 12
(164,190). Matricellular proteins, osteonectin and thrombospondin-5 are cleaved by MMP-3 and MMP-9,
respectively (191,192) and MMP-2 and -9 both cleave latent transforming growth factor-β binding protein (LTBP)-1, a potential mechanism for the release of TGF-β from the extracellular matrix (193). Several insulin-like growth factor binding proteins (IGFBPs) have been shown to be substrates of MMPs. IGFBP-1 is cleaved by MMP-2, -3, -7 and -11 (194) and MMP-3 cleavage of insulin-like growth factor (IGF)-I·IGFBP-1 complex restores the mitogenic activity of IGF-I (194). IGFBP-3 is degraded by MMP-1, -2, -3, -7, -9 and -19 (195-198), while IGFBP-5 is degraded by MMP-1 and -2 (199).

B. Non-matrix Substrates

i. Cytokines and Growth Factors

MMPs can process cytokines and growth factors, many of which are expressed as membrane bound precursors that require proteolytic cleavage for release and activation. MMP-3 releases mature heparin binding-EGF from the cell surface by cleaving the precursor in the juxtamembrane domain (200). MMP activity also regulates the release of mature TNF family member proteins, proTNF-α, TNF-related activation-induced cytokine (TRANCE) and Fas ligand (201-203). Although ADAM-17 (TACE) has been identified as the primary sheddase of TNF-α in many cell types (204,205), particularly under LPS stimulation (206), MMP-mediated cleavage may represent an independent mechanism for TNF-α release in the absence of LPS (207). Latent TGF-α is a potential MMP substrate as APMA stimulated TGF-α shedding from the cell surface in the absence of ADAM-17 (208). Latent TGF-β is secreted but is localized to the cell surface and undergoes proteolytic activation by MMP-9 and -14 cleavage of the N-terminal propeptide (209,210). MMPs can both activate the IL-1β precursor (33 kDa) and degrade the mature form of cytokine (18 kDa) (211,212). Soluble interferon IFN-β is also degraded by MMP-9 (213) and upon cleavage by MMP-1, -3, -7 and -13, connective tissue growth factor (CTGF) loses the ability to bind VEGF, which reduces its angiogenic activity (214).

ii. Chemokines

Chemokines were recently identified as substrates of MMPs (215-219). Specific cleavage within the first 4-9 residues of the chemokine N-terminus modulates the binding of these molecules with cell surface receptors and subsequent chemotaxis response. MMP-2 cleavage of CC chemokine, monocyte chemoattractant protein (MCP)-3, reduces inflammation as the truncated form occupies chemokine receptors but does not induce signaling (215). Cleaved MCP-3 was also generated by MMP-1, -3, -13 and -14. Other CC chemokines, MCP-1, 2 and 4, were also cleaved by MMPs to generate receptor antagonists (218). In contrast, N-terminal cleavage of CXC chemokines, IL-8 and ENA-78, by MMP-9 generates a more active form of these molecules (216,219). In the case of ENA-78, this initial processing is transient as the cleaved form is rapidly degraded by MMP-9 with further incubation (219). Another CXC chemokine, granulocyte chemoattractant protein (GCP)-2, is also cleaved by MMP-8 and -9 at the N-terminus, although this had no affect on the biological activity of the molecule (219). MMP cleaved stromal derived factor (SDF)-1α loses affinity for its receptor CXC receptor-4 (217), but becomes highly cytotoxic to neuronal cells through binding of an unidentified G-protein coupled receptor (220).
iii. Transmembrane Receptors and Adhesion Proteins

MMPs also regulate cell behavior in response to soluble factors by processing cell membrane receptors. MMP-2 and MMP-9 cleavage of fibroblast growth factor receptor (FGFR)1 and IL-2 receptor-α, respectively, abrogates FGF and IL-2 binding (221,222). Several MT-MMPs cleave the ectodomain of low density lipoprotein related protein (LRP), which inactivates the ligand binding function of this receptor (223). MT1-MMP also cleaves gC1qR and tissue transglutaminase (224,225). Several MMPs, most notably MMP-12, can cleave the urokinase plasminogen activator receptor (uPAR) at Thr^86-Tyr^87 to release domain 1, which abolishes uPA and vitronectin binding (226-228). Through the cleavage of cell-cell or cell-matrix adhesion molecules MMPs have been shown to modulate cell migration and apoptosis. MMP-3 and MMP-7 cleavage of E-cadherin to release a soluble 80-kDa soluble fragment induces type I collagen invasion (229) and contributes to the development of an invasive phenotype (230). The presence of TIMP-1 reduced N-cadherin fragmentation in hepatic stellate cells implicating MMPs in N-cadherin degradation (231). MMP-1 cleaves L-selectin, a regulator of leukocyte migration (232) and MMP-9 sheds ICAM-1, which may be important in tumor cell resistance to NK cell-mediated cytotoxicity (233). MMPs have also been shown to process integrins: MT1-MMP can act as an integrin convertase for the pro-αv subunit (234) and MMP-7 cleaves β4 integrin at two susceptible sites, one of which appears to be inactivating (235). In vascular smooth muscle cells, transfection with MT1-MMP and MT3-MMP results in the cleavage of focal adhesion kinase (FAK) (120 kDa) to generate a 90-kDa fragment (236). MT1-MMP also cleaves CD44 to stimulate cell motility (237), and β-amyloid precursor (APP) is cleaved by MT1-MMP at a site upstream from the α-secretase site (238) to release a soluble form of APP that lacks the inhibitory domain against MMP-2 (113).

iv. Proteases and Inhibitors

The proteolytic pathways of MMPs, ADAMs, and several serine proteases appear to be interconnected. In addition to activating MMP-2, MT4-MMP was recently shown to activate ADAMTS-4, by removing the spacer domain in the C-terminal region (239). ADAM-28 is activated through the removal of its N-terminal propeptide domain by MMP-7 (240). MMP-12, -13, and -14 degrade Factor XII (241); however, MMP-3 hydrolyzes the Glu^143-Leu^144 bond within uPA to release the uPAR binding and kringle domain while sparing the uPA enzymatic activity (242). The precursor of plasmin, plasminogen, is processed by MMP-2, -3, -7, -9 and -12 (243-246) to generate angiotatin, an inhibitor of angiogenesis (247). Several serine proteinase inhibitors are inactivated by MMP cleavage. Members of the serpin family of inhibitors including α1-proteinase inhibitor, α1-antitrypsin, antithrombin III, α1-antichymotrypsin are inactivated by MMP proteolysis of the reactive site loop (248-250). Other serpins that are inactivated by MMPs include α1-antitrypsin, α2-antiplasmin, and plasminogen activator inhibitor (PAI)-1 (251-253). Several MMPs including MMP-1, -7, -8, -9 and -12 cleave the kunitz-type inhibitor, tissue factor proteinase inhibitor (TFPI), which results in the loss of anticoagulant activity (254,255).
V. THE STUDY OF MMPS IN VIVO

In the post-genome era, the most important issue in MMP biology is to understand the biological role of all MMPs in vivo. This knowledge would be critical for the development and administration of future anti-MMP therapies. An area that has significantly advanced our knowledge of MMP activity in vivo has been the use of genetically modified mice (6,256).

A. MMP Knockout Mice

Although most mice with targeted disruptions of MMP genes, with the exception of MMP-14, are viable, fertile and appear healthy, the use of innovative experimental challenges has revealed many specific defects due to the lack of MMP activity. Mmp-2 -/- mice grew more slowly than wild-type mice but showed no gross anatomical abnormalities (257). However, these mice developed severe arthritis compared to wild-type mice when challenged with arthritogenic antibodies suggesting that MMP-2 plays a protective role in inflammatory joint disease (258). Mice lacking MMP-3 showed delayed wound healing response (259) and accelerated adipocyte differentiation during mammary gland involution (260). The disruption of the Mmp-7 gene in the Min (multiple intestinal neoplasia) mouse resulted in a decrease in intestinal tumorigenesis (261). Mmp-7 deficient mice also displayed a reduction in Fas-mediated apoptosis of epithelial cells during prostate involution (203) as well as reductions in procryptdin activation leading to a loss in antimicrobial activity (262). Mmp-8 null mice develop normally, were fertile and displayed no observable differences from wild-type mice in several tissues that were examined (263). However, male Mmp-8 -/- displayed a higher incidence of susceptibility of skin tumors than female Mmp-8 -/- mice, as a result of a delayed influx of neutrophils followed by a prolonged accumulation of inflammatory cells. Mmp9 -/- mice showed abnormal bone development as a result in defects in the vascularization and ossification (264) and delayed osteoclast recruitment (265) in long bones. The loss of MMP-9 in HPV16 transgenic mice resulted in a decrease in carcinoma incidence due to the lack of MMP-9 activity in inflammatory cells (266). Mmp-11 null mice were fertile and were indistinguishable to their wild-type counterparts (267). However, in a chemical induced carcinoma model, lower incidences of tumors were observed in several tissues of Mmp-11 -/- mice compared to wild-type mice. Mice deficient in MMP-12 were resistant to cigarette smoke induced emphysema (268). Of all the MMP gene knockouts to date, Mmp-14 -/- mice exhibit the most severe alterations (153,269). MT1-MMP deficient mice developed severe growth impairments as early as 5 days and most animals died within 50-90 days (269). The lack of MT1-MMP resulted in many defects in skeletal development such as severe osteopenia and skeletal dysplasia, abnormal cranial morphogenesis, defects in growth plate and endochondrial ossification (153,269). In addition cells lacking MT1-MMP impaired ability to degrade type I collagen (269) and activate proMMP-2 (153). Angiogenesis was also defective in these mice as observed using a corneal angiogenesis model (153).

B. Proteomics and Degradomics

Another area that will be critical for studying MMP activity in vivo is proteomics. Proteomics promises to uncover new biological roles for MMPs by providing the technology to study MMPs on a proteome-wide
scale in complex biological settings. The terms degradome, which refers to the entire repertoire of proteases, substrates and inhibitors in a given proteome, and degradomics, the application of genomic and proteomic techniques to study the degradome, were coined in an effort to address new emerging concepts and techniques for the study of MMPs and proteases in general (8).

Proteomic techniques, such as two-dimensional gel electrophoresis (2D-GE) and multi-dimensional liquid chromatography (MD-LC), allow entire proteomes to be fractionated before analysis by mass spectrometry (MS), the primary analytical technique for protein detection and identification (270). Tandem MS (MS/MS) is the standard technique for peptide sequencing in a high-throughput manner (271). Two-dimensional gel electrophoresis separates proteins according to their charge using isoelectric focusing in the first dimension, and by their electrophoretic mobility in the second dimension, generating a constellation of protein spots on a 2D gel (272). Protein identification requires excision of protein spots followed by in-gel digestion with trypsin to generate peptides, which are then sequenced by MS/MS. Limitations of 2D-gels include poor separation of hydrophobic proteins and proteins with extreme p/s, and a low detection limit for proteins in low abundance (273). In MD-LC, proteins are first digested into peptides and then separated using a biphasic liquid chromatography column consisting of strong cation exchange resin as the first phase and reversed-phase resin as the second phase before entering the mass spectrometer (274,275). Although MD-LC can detect proteins over a wide range of hydrophobicities, p/l and abundance, both of these techniques are well suited for the study of MMPs in complex samples such as cells, tissue and fluids and can be used to compliment all existing studies of MMP knockout mice.

C. MMP Substrate Identification

The most important aspect in determining the biological role of MMPs is substrate identification. Identification of all MMP substrates, potential and actual, is a technical challenge given the number of MMPs in humans and the fact that each enzyme displays activity against a multitude of substrates. To date, the majority of MMP substrates have been identified by hypothesis-driven approaches based on intuitive analysis of MMP and substrate expression, localization, and activity in cells tissues or disease models (5,7). Screening methods is another approach for identifying MMP substrates. We have previously used the hemopexin C domain of MMP-2 as a bait protein in a yeast two-hybrid screen to identify potential binding partners (215). The MMP-2 hemopexin C domain contained an exosite for the chemokine, MCP-3, which was identified as a substrate of MMP-2. Screens using synthetic peptide libraries (276,277) and phage display peptide libraries (278-281) have determined the preferred substrate specificity sequence for different MMPs. Analysis of genetically modified mice can also lead to identification of new substrates. In MMP-7 deficient mice, the failure to process pro-cryptdins results in the accumulation of these antimicrobial peptides in the small intestine (262). These mice consequently have decreased antimicrobial activity compared to wild-type mice. Alpha 1-proteinase inhibitor (α1-PI), was identified as substrate of MMP-9 by analyzing antibody induced-blister formation in Mmp9 -/- mice.
MMP-9 null mice are also deficient in myelin reformation due to their failure to process the inhibitory proteoglycan, NG2 (283).

While these approaches have been successful in MMP substrate identification, many of them such as yeast two-hybrid and the generation and analysis of MMP knockout mice, have the disadvantage of being time consuming and laborious. The use of peptide libraries has also yielded few biological substrates (278-280). This may be due to the fact that peptide libraries only offer information regarding the primary structure of a substrate and does not take into account the three-dimensional conformation of proteins or the contribution of MMP exosites in substrate recognition and cleavage. And although the use of MMP knockout mice is unequivocal in assigning substrates to MMPs, analysis can be problematic when there are redundant and compensatory enzymes as is the case with many MMP family members. In lieu of these limitations, we are interested in adapting and developing new proteomic techniques for high-throughput screening of MMP substrates on a proteome-wide scale. The advantages of using proteomics for identifying MMP substrate are evident. The prospective of identifying MMP substrates can now be greatly increased because analysis is performed on complex biological samples. Using 2D-PAGE or MD-LC separation coupled with MS identification, we can rapidly screen for candidate MMP substrates within a proteome for changes in abundance, mass or other physical characteristics, which can be used as evidence of proteolytic activity in vivo. MMP activity can now be assessed in the context of a natural milieu of inhibitors, substrates and other proteases, as opposed to studying MMP cleavage in vitro or MMP-substrate associations within a yeast nucleus.

i. Quantitative Proteomics

In dynamic biological systems, proteins levels are constantly changing as a result of alterations in gene transcription and translation, protein transport and secretion, and post-translational modifications—often in response to biological stimuli. Using quantitative proteomics, a branch of proteomics that is concerned with the quantification of proteins, a comparative analysis of protein abundance can be performed for a catalog of proteins from two systems, stimulated (experimental) and unstimulated (control). Upon experimental manipulation of individual or global MMP activity, changes in protein abundance can provide clues to potential substrates that may have undergone MMP-mediated proteolytic modifications such as protein shedding or release, degradation leading to clearance or neo-protein generation.

Although, 2D-gel analysis has been routinely used for comparative analysis, there are inherent problems with comparative spot matching between two gels due to the lack of reproducibility in this technique (284). This would also undoubtedly make assigning cleaved protein to parent proteins difficult. To date there has been only one report using 2D-gels for analyzing proteolytic activity in vivo (285). In comparison, protein quantification using mass spectrometry avoids this problem by combining the two samples for MS analysis (286). Prior to this, proteins from experimental and control are coded using stable isotopes in order to distinguish them during MS analysis. Relative quantification of identical proteins from the two different sources can be achieved based on the stable isotope dilution theory—the relative signal intensity.
between two chemically identical but isotopically different analytes in a mass spectrometer is a representation of their relative abundance (287). Although first used as method for internal standardization, stable isotope coding has emerged as the primary technique for protein quantification. There are now several strategies for the labeling of peptides with stable isotopes using metabolic labeling and targeted labeling of the N- or C-terminus or specific residues (288).

**ii. Isotope Coded Affinity Tag (ICAT) Method**

The most characterized method of stable isotope labeling is the isotope coded affinity tag (ICAT) method (289-292) (Fig. 1.5). Central to this method is the ICAT reagent, which consists of a biotin group for affinity purification, an isotope-containing linker, and a thiol-reactive group for labeling the Cys residues of proteins. The reagent exists in two isotopic forms, heavy and light, coded by the presence or absence of 8 deuterium atoms (d8 vs. d0) in the linker of the reagent. For comparative analysis, equivalent amounts of proteins from two different samples are labeled with either the heavy or light ICAT reagent (Fig. 1.5). Subsequently, the differentially labeled samples are combined and trypsin-digested into peptides. Non-protein material, excess ICAT reagent and trypsin are removed from the peptides using a strong cation-exchange column. ICAT-labeled peptides are purified from non-labeled peptides using an avidin column. The mixture of heavy and light labeled peptides is then fractionated by MD-LC. Identical peptides from the two different samples, heavy and light, elute simultaneously and are ionized using an electrospray ionization (ESI) source. The mass of the peptide ions are then analyzed by the mass spectrometer (Fig. 1.5, Full Scan Mode) and a relative abundance ratio between the heavy and light labeled peptides is determined by comparing the signal intensities of the two peptides. The most intense peptide ions are further selected for collision-induced dissociation (CID) to generate a fragment spectra, which is queried against a human protein database for sequence determination (Fig. 1.5, MS/MS Mode). The sequence of the tryptic peptide is then used to identify the parent protein.

**VI. THEME AND HYPOTHESES**

The broad focus of the work presented in this thesis has been the study of MMP substrates. Type I collagen was one of the first substrates described for MMPs, yet the mechanism of how native collagen is cleaved by MMPs is still not known. At the time this work was started, the role of the MT1-MMP hemopexin C domain in type I collagen cleavage was not clear. In Chapter 2, we hypothesized that this domain was critical for the cleavage of collagen and also for the regulation of pericellular collagenolysis due the presence of the MT1-MMP hemopexin C domain on the cell surface as part of the 44-kDa autocatalytic product of MT1-MMP. To test this hypothesis, we generated a recombinant protein representing the linker peptide and the hemopexin C domain of MT1-MMP and demonstrated that it acted as a dominant negative mutant in inhibiting MT1-MMP mediated type I collagen cleavage in vitro and in cell culture. These findings were published in the Journal of Biological Chemistry (293). There are conflicting reports by our lab and others to the role of the hemopexin C domain and the fibronectin type II modules of MMP-2 in hypothesized that these residues were also important for native collagen binding.
FIG. 1.5 Schematic of the ICAT Method.
and cleavage. In Chapter 3, we demonstrate that a recombinant protein representing the fibronectin type II modules of MMP-2 (MMP-2 CBD) acts as a dominant negative and blocks collagen cleavage by full-length MMP-2. A mutant MMP-2 CBD protein that contains the following mutations Trp$^{316}$Ala and Trp$^{374}$Ala did not. These findings were recently accepted for publication in the Journal of Biological Chemistry (294). In Chapter 4, we have screened for novel substrates of MT1-MMP using the proteomic technique of ICAT•MD-LC•MS/MS. We hypothesized that upon overexpression of MT1-MMP, protease-mediated shedding and cleavage events will result in alterations in protein abundance in the conditioned media, making it amendable to quantitative proteomic analysis. In our screen, several proteins were identified and confirmed biochemically as previously uncharacterized substrates of MT1-MMP. These findings have been recently published in the Proceedings of the National Academy of Sciences (295).

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CHAPTER 2

Collagen Binding Properties of the MT1-MMP Hemopexin C Domain:
The Ectodomain of the 44-kda Autocatalytic Product of MT1-MMP Inhibits Cell Invasion by Disrupting Native Type I Collagen Cleavage*

I. INTRODUCTION

Type I collagen is the most abundant protein of the extracellular matrix and is an important structural component in blood vessels, skin, tendons, ligaments, and bone (1). Accordingly, the synthesis and degradation of type I collagen is tightly regulated. Disruptions in this homeostasis can lead to diseases such as pulmonary fibrosis, scleroderma, arthritis, and osteoporosis, which if untreated, can result in loss of tissue function and integrity. In a number of cancer cells the capacity to degrade type I collagen and invade through type I collagen matrices often correlates with metastatic potential (2)—a characteristic that is as important for the local dissemination of tumor cells as type IV collagen degradation and basement membrane penetration is for metastasis (3). Despite the importance of maintaining correct collagen homeostasis in tissues, the proteases responsible for type I collagen degradation in vivo remain unclear.

An intracellular pathway may play an important role in collagen degradation (4) that, in bone, utilizes the cysteine protease cathepsin K at low pH (5). Extracellularly, fibrillar type I collagen may be degraded at neutral pH by several matrix metalloproteinases (MMPs), a 24-member family of zinc-dependent endopeptidases in man (2). The major collagenolytic MMPs are the secreted collagensases, MMP1, 8, and 13 (6), and the cell surface membrane type (MT)1-MMP (7,8). MT1-MMP also activates collagenase-2 (MMP13) (9) and is the primary activator of MMP2 (10), a gelatinase that exhibits weak native type I collagenolytic activity (11-13).

MMPs share a common overall structure consisting of a propeptide, catalytic domain, linker (also called a hinge), and a hemopexin C domain (14). While the majority of MMPs are secreted as latent zymogens, MT-MMPs, the largest subgroup of MMPs, are membrane anchored by the presence of a type I transmembrane sequence and cytoplasmic tail (MT1-, MT2-, MT3-, and MT5-MMP) or by glycosylphosphatidylinositol linkage (MT4- and MT6-MMP) (14). MT1-MMP is activated intracellularly by proprotein convertase-dependent or -independent pathways (15,16) and is expressed as an active protease on the surface of many normal and pathological cell types (10,17). The importance of MT1-MMP is indicated by its requirement for the invasion of endothelial and cancer cells through type I collagen matrices (18-20). Moreover, mice deficient in MT1-MMP developed severe aberrations in type I collagen-abundant tissues, such as bone and skin, and the mice exhibited arthritis and scleroderma (21,22). In man, homoallelic loss-of-function mutations in the MMP2 gene result in excessive bone resorption and arthritis (23). This condition resembles the phenotype of the MT1-MMP knockout mouse.

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supporting the close functional connection of MMP2 and MT1-MMP in regulating pericellular collagen homeostasis in the mouse and man.

Native type I collagen consists of two \( \alpha_1(l) \) chains and one \( \alpha_2(l) \) chain interwound in a right-handed triple helix that is resistant to cleavage by most proteinases at neutral pH with the exception of the MMP collagenases (14). Because the active site of collagenolytic MMPs can only accommodate a single \( \alpha \)-chain, cleavage of the three \( \alpha \)-chains occurs sequentially at the single collagenase-susceptible site, Gly\(^{775}\)-Ile/Leu\(^{776}\), to generate 3/4- and 1/4-collagen fragments. To achieve this, the collagen helix must be initially unwound by a triple helicase mechanism in order to expose the scissile bonds. This critical step requires the presence of collagen binding exosites (14), in addition to elements within the active site (24-26). In MMP1, 8, and 13, the hemopexin C domain supports binding to collagen and is required for native collagen cleavage (27-32). Deletion or mutation of the MMP-8 linker also reduces collagenolysis (32,33). Furthermore, synthetic peptide analogs of the MMP-1 linker bound collagen and inhibited collagen cleavage (34). Interestingly, the 35-amino acid residue linker of MT1-MMP is twice the length of other collagenase linkers (18 residues), however, the significance of this and its role in collagen cleavage have yet to be examined.

The regulation of MT1-MMP activity, MMP2 activation and pericellular type I collagen levels is complex. In a variety of cells, stimulation by fibrillar type I collagen has been shown to increase the cell surface expression of MT1-MMP and induce the cellular activation of pro-MMP2 (35-41). This response is in part dependent on \( \beta_1 \) integrin clustering and signaling (39,41,42) and is potentially self regulating as type I collagen is susceptible to MT1-MMP and MMP2 proteolysis (43). Concentration of MT1-MMP by overexpression (44,45) or clustering interactions favors MMP2 activation (46-48) and collagenolysis (49). Concomitant with increased MT1-MMP expression and MMP2 activation is the autocatalytic processing of MT1-MMP at Gly\(^{284}\)-Gly\(^{285}\) to shed the catalytic domain from the hemopexin C domain, which is retained on the cell membrane (39,45,50). Hence, the ectodomain of the residual 44-kDa MT1-MMP fragment (Gly\(^{285}\)-Val\(^{362}\)) on the cell surface consists of the linker, hemopexin C domain, and stalk segment only (see Fig. 2.1A) and so is catalytically inactive. The significance of the 44-kDa MT1-MMP \textit{in vivo} is not clear. In addition to being present at increased levels following cell binding to type I collagen, the 44-kDa MT1-MMP has also been detected on the surface of tumor cells (39,50). During MMP2 activation, TIMP-2-free MT1-MMP must be in close proximity to a trimeric complex of MT1-MMP/TIMP-2/pro-MMP2 in order to activate the bound pro-MMP2 (51). The mechanisms of MT1-MMP oligomerization are not clear. Recombinant hemopexin C domain of MT1-MMP did not form oligomers in solution nor modulate MMP2 activation when added to cells (46). Recent reports using transmembrane MT1-MMP chimera and deletion mutants have suggested that the hemopexin C domain can mediate homophilic complex formation of cellular MT1-MMP for efficient MMP2 activation (47,48). Expression of a transmembrane tethered MT1-MMP hemopexin C domain lacking the linker, termed PEX (Thr\(^{312}\)-Val\(^{362}\)), in HT1080 cells inhibited MT1-MMP oligomerization, the cellular activation of pro-MMP2 and Matrigel invasion (47), a function previously attributed to MMP2 proteolytic activity against type IV collagen (52).
Considering that MT1-MMP is a collagenase, we hypothesized that exosites on the hemopexin C domain would bind to type I collagen and be essential for collagenolytic activity. Thus, the autolytically generated 44-kDa MT1-MMP ectodomain would be predicted to modulate pericellular collagenolysis on the membrane through dominant-negative interactions. Since native type I collagen stimulates MMP2 activation, we also hypothesized that collagen binding by the hemopexin C domain of MT1-MMP would modulate MMP2 activation with 44-kDa MT1-MMP opposing these effects in vivo. Experiments reported here demonstrate that collagen binding by the MT1-MMP hemopexin C domain is essential for collagenolytic activity and enhancement of MMP2 activation by MT1-MMP. Inhibition of this interaction either in vitro or on the cell surface inhibits collagen degradation. Together, these studies suggest a novel feedback mechanism through which generation of the 44-kDa MT1-MMP autolysis product regulates pericellular collagenolytic activity and subsequent invasive potential.

II. EXPERIMENTAL PROCEDURES

Materials—Rat-tail type I collagen was prepared as previously described (53). Vitrogen® was purchased from Cohesion (Palo Alto, CA). Biotin-labeled type I collagen was prepared as previously described (54). Human placental type I collagen was purchased from Sigma (Saint Louis, MS). The triple helical nature of collagen was confirmed by the absence of trypsin sensitivity at an enzyme/substrate ratio of 1:10 over 3 h. The general hydroxamate inhibitor BB2116 was provided by British Biotech Pharmaceuticals (Oxford, UK). Hydroxamate inhibitor GM6001 and AB8102 (blocking antibody raised against the human MT1-MMP catalytic domain) were purchased from Chemicon (Temecula, CA). The polyclonal antibody RP1MMP-14 (raised against the MT1-MMP linker) was purchased from Triple Point Biologics (Portland, OR). The affinity-purified polyclonal antibodies, αMT1-CD and αHis₆ were described previously (46).

Synthetic Peptides and Recombinant Proteins—The following MT1-MMP linker peptide analogs were synthesized: MT1-L18 (R³⁰²PSVPDKPNPTYGPNIC³¹⁵) (University of Victoria, Canada) and MT1-L35 (G²⁸⁵ESGFPTKMPQPRRTPSVPDKPKNP TYGPNIC³¹⁵) (Tufts University, MA), and verified by mass spectrometry. Recombinant domains of human MT1-MMP and MMP2 were expressed in E. coli as N-terminal His-tagged proteins. The MT1-MMP hemopexin C domain (CD) with or without the linker (L) (MT1-LCD, Gly²⁸⁵-Cys⁵⁰⁸ and MT1-CD, Gly³¹⁵-Cys⁵⁰⁸) (see Fig. 2.1A) and the MMP2 hemopexin C domain with the linker (MMP2-LCD, Gly⁴⁴⁶-Cys⁶⁶⁰) were prepared as previously described (46). Recombinant human MMP2-CBD (Val²²⁰-Gln³⁹³) (collagen binding domain consisting of three fibronectin type II modules) was prepared previously (53). Any bacterial endotoxins in purified recombinant protein preparations were removed by polymyxin B agarose columns (Sigma). The fidelity of purified recombinant proteins was confirmed by electrospray ionization mass spectrometry (46) and N-terminal Edman sequencing of protein bands cut from the membrane of Western blots. Human soluble (s) MT1-MMP, truncated C-terminal to the hemopexin C domain (sMT1-MMP), was kindly provided by British Biotech Pharmaceuticals (Oxford, UK). Recombinant human MMP2, TIMP-1 and TIMP-2 were expressed in a mammalian cell system and purified as previously described (55) or kindly provided by Dr. H. Nagase (Imperial College School of Medicine, UK).
**Electrophoretic Techniques**—Samples in reducing (65 mM dithiothreitol) or non-reducing sample buffer (125 mM Tris-HCl, pH 6.8, 2.0 % SDS, 2.0 M urea, 0.05 % bromophenol blue) were separated on 15% SDS-PAGE gels and analyzed by either silver nitrate staining or by Western blotting using αMT1-CD and αHis6 antibodies. Enhanced chemi-luminescence (ECL) detection was performed according to manufacturer's instructions (Amersham Pharmacia Biotech). For zymographic analysis, samples were separated under non-reducing conditions on 10% SDS-PAGE gels co-polymerized with 0.5 mg/ml gelatin. Gels were washed for 30 min with 2.5 % Triton X-100, rinsed with deionized water, and incubated with assay buffer (100 mM Tris, pH 8.0, 30 mM CaCl2, 0.05 % Brij, 0.025% NaNO3) at 37 °C for 4 h before staining with Coomasie Brilliant Blue G250.

**Gel-filtration chromatography**—Purified MT1-LCD (0.5 mg) was subjected to gel-filtration chromatography on a Superdex 75 column equilibrated with PBS (10 mM Na2HPO4, 1.8 mM NaH2PO4, 2.7 mM KCl, 140 mM NaCl, pH 7.4) and run on an AKTA Purifier (Amersham Pharmacia Biotech). Protein elution was monitored at 215 nm. Molecular weight standards used were BSA (67 kDa), ovalbumin (43 kDa), chymotrypsin A (25 kDa) and ribonuclease A (13.7 kDa).

**Solid-Phase Binding Assays**—Native and heat-denatured type I collagen (rat tail) (5 μg/ml) were diluted in 15 mM Na2CO3, 35 mM NaHCO3, 0.02% NaN3, pH 9.6 (100 μl) and coated onto 96-microwell plates (Falcon) overnight at 4 °C as described previously (53,56). Wells coated with myoglobin served as a control for non-specific binding. The coated wells were blocked with 1% BSA to which serially diluted recombinant proteins in PBS (100 μl total volume) were added and incubated for 1 h at room temperature. After extensive washes, bound proteins were quantitated using affinity-purified polyclonal antibodies followed by incubation with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody. Substrate, p-nitrophenyl phosphate disodium (Sigma), was added to the wells and color development was monitored at 405 nm in a Thermomax plate reader (Molecular Devices).

**Ligand Blot Assays**—Proteins (5 μg) in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl were filtered onto an Immobilon-P® membrane (Millipore) by vacuum. Membranes were blocked with 1% BSA in PBS and incubated with biotin-labeled native type I collagen in PBS/Tween 20 for 1 h. Bound collagen was visualized using horseradish peroxidase (HRP)-conjugated streptavidin and ECL detection.

**Enzyme Assays**—Biotin-labeled type I collagen (0.025 pmol) was incubated with either sMT1-MMP or MMP2 in assay buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM CaCl2, 3.8 mM NaNO3, 0.05 % Brij) for 18 h at 28 °C. MMPs were activated with 2 mM 4-aminophenylmercuric acetate. Recombinant proteins and BB2116 in assay buffer were added to the reactions where indicated. Following digestion, samples were separated by 7.5 % SDS-PAGE and analyzed by Western blotting using streptavidin-HRP and ECL detection. α1(I) and α2(I) chains were quantitated by scanning densitometry and the percentage of native collagen cleavage was calculated as previously described (57). Cleavage of the quenched fluorescent substrate, Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH2, was performed as described...
previously ((55)). MT1-LCD and MT1-CD in assay buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM CaCl$_2$, 0.05 % Brij) were added to the reaction where indicated.

**Transmembrane MT1-MMP and MT1-MMP Hemopexin C Domain Constructs**—The mammalian expression vector pCR3.1-Uni (Invitrogen) carrying human MT1-MMP cDNA was the generous gift of Dr. D. Pei (University of Minnesota, MN). To express cell (c) surface transmembrane MT1-MMP deletion mutants, cMT1-CD (Δ112-315) and cMT1-LCD (Δ112-284) two-step overlapping PCR was used with T7 and reverse primer as external primers and 5'-CGAAGGAAGCGCCCCAACATCTGTGACGGGAAC-3' and 5'-ACAGATGTTGGGGCGCTTCTCGAACATTGCC-3' (cMT1-CD, Δ112-315), or 5'-CGAAGGAAGCGCGGTGAGTCAGGGTTCCCCACC-3' and 5'-CCCTGACTCACCGGCCTTCTGCAACATTGCC-3' (cMT1-LCD, Δ112-284) as internal primer pairs. The catalytically inactive MT1-MMP (Glu240Ala) mutant construct was generated using 5'-GGTGGCTGTGCACGCGCTGGGCCATGCC-3' and 5'-GGCATGGCCCAGCGCGTGCACAGCCACC-3' (Glu240Ala) primers. Full-length constructs were synthesized by PCR with T7 and reverse primers, digested with Hind III and EcoR I, ligated back to pCR3.1-Uni vector and fully sequenced.

**Cell Culture and Stable Transfection**—Early passage human gingival fibroblasts were kindly provided by Dr. D. Brunette (University of British Columbia, Canada) and maintained in Dulbecco's modified essential medium (DMEM) containing 10% newborn calf serum (Life Technologies). MDA-MB-231 breast carcinoma cells were kindly provided by Dr. V.G. Jordan (Northwestern University, IL) and cultured in DMEM (Cellgro) supplemented with 10% fetal bovine serum (U.S. Bio-technologies Inc). MDA-MB-231 cells were transfected with MT1-MMP cDNA constructs using FuGENE 6 (Roche) according to manufacturer's instructions. Stable cell lines were clonal-selected and maintained in medium containing 1 mg/ml G418 (Mediatech Inc). For each line, 5 clones were pooled and used in the experiments.

**Transwell Invasion and Migration Assay**—MDA-MB-231 cell invasion and migration assays through type I collagen (human placental) were performed as described previously (41). Endotoxin-free recombinant proteins, linker peptide analogs and antibodies in PBS were added to the cell media with BSA or IgG as controls. GM6001 was added to the cells in DMSO.

**Collagen Gels**—To prepare collagen gels, 8 volumes of Vitrogen was neutralized with 1 volume of 10x concentrated PBS and 1 volume of 0.1 M NaOH. Fibroblasts were detached with PBS containing 0.54 mM EDTA and 1.1 mM glucose, and resuspended in a neutralized Vitrogen solution (2.0 mg/ml) containing 11.3% DMEM and 2.5% new born calf serum. The cell/collagen solution (75 μl) was then transferred into 96-well tissue culture plates and incubated at 37 °C for 1 h to allow for collagen polymerization. Cells were supplemented with DMEM containing 2.5% new born calf serum for 18 h. Collagen gels were then rinsed with DMEM and cells were cultured under serum-free conditions with or without MT1-LCD (endotoxin-free, in PBS) for the duration of the experiment. Cell conditioned media was replaced every 24 h and analyzed by gelatin zymography after 72 h.
Latex Beads—Native and denatured type I collagen (100 μg/ml) were incubated with latex beads (1%) (Sigma) for 1 h at room temperature to allow for adsorption. The beads were then washed with PBS and blocked with 1% BSA for 1 h. Beads not absorbed with collagen served as a control. Blocked beads were rinsed with PBS and resuspended in DMEM at a concentration of 0.2% (v/v). Fibroblasts cultured in 96-well tissue culture plates were rinsed and incubated in serum-free medium for 1 h prior to incubation with the latex beads in DMEM (100 μl). Endotoxin-free MT1-LCD in PBS was added the latex bead preparations where indicated. Cells were cultured for 24 h after which the conditioned cell media was analyzed by gelatin zymography.

III. RESULTS

Recombinant Protein Expression—To characterize the hemopexin C domain of MT1-MMP and the ectodomain of 44-kDa MT1-MMP, two forms of the MT1-MMP hemopexin C domain were cloned and expressed in E. coli. MT1-LCD (Gly^{285}-Cys^{508}) corresponds to the N-terminus of 44-kDa MT1-MMP and includes both the linker and the hemopexin C domain (Fig. 2.1A/v). MT1-CD (Gly^{315}-Cys^{508}) consists of the hemopexin C domain only (Fig. 2.1A/iv). Yields of purified protein were typically ~20 mg from 3 L of liquid culture. The identities of the purified proteins were confirmed by Western blotting with αMT1-CD antibody (Fig. 2.1B) and αHis_{6} (data not shown). Non-reducing SDS-PAGE analysis demonstrated the absence of dimeric intermolecular disulfide cross-linked aggregates. Reducing SDS-PAGE and electrospray mass spectrometry determination of the purified protein masses were consistent with the predicted masses. As shown in Fig. 2.1B, both MT1-LCD (27,894 Da) and MT1-CD (24,612 Da) were within 1-2 Da of the predicted mass after accounting for the removal of the N-terminal methionine and hydrogen atoms after disulfide bond formation. Edman sequencing confirmed N-terminal methionine processing and the presence of the N-terminal His_{6} tag (Fig. 2.1B). MT1-LCD did not form non-covalent multimeric complexes under native conditions as shown by the elution of a single peak at 28 kDa corresponding to the monomeric form of MT1-LCD upon gel filtration chromatography (Fig 2.1C).

Collagen Binding Properties of the MT1-MMP Hemopexin C Domain—We first assessed the collagen binding properties of the MT1-MMP hemopexin C domain by performing solid-phase binding assays with type I collagen, the preferred collagen substrate of MT1-MMP. As shown in Fig 2.2A, binding of MT1-CD and MT1-LCD to native collagen was similar indicating that the linker had little apparent affect on collagen binding affinity. Unlike MMP2-CBD, both MT1-CD and MT1-LCD did not bind denatured collagen (Fig. 2.2B), confirming specificity. As a control, MMP2-LCD did not bind native or denatured type I collagen as shown previously (56). Binding of native type I collagen to MT1-CD and MT1-LCD was confirmed by ligand blot analysis with MMP2-LCD and BSA serving as negative controls (Fig. 2.2C).
FIG. 2.1 MT1-MMP hemopexin C domain constructs and linker peptide analogs. A, the domain structures of (i) pro-MT1-MMP and (ii) 44-kDa MT1-MMP are shown in a linear diagram. The signal sequence (SS), propeptide domain (PRO), stalk segment (ST), transmembrane sequence (TM), and cytoplasmic tail (CYT) of MT1-MMP are indicated. Schematic representations of (iii) MT1-MMP linker peptide analogs, MT1-L18 and MT1-L35, and (iv) hemopexin C domain constructs, MT1-CD and MT1-LCD, are shown with N and C terminal residues indicated. B, purified MT1-CD and MT1-LCD (0.1 μg) were electrophoresed on 15 % SDS-PAGE gels under reducing (+DTT) and non-reducing (-DTT) conditions. Gels were analyzed by either silver staining or by Western blotting using αMT1-CD antibody. Protein masses measured by electrospray ionization mass spectrometry (Mass), predicted masses, and N-terminal Edman sequence analysis are indicated. C, MT1-LCD was subjected to FPLC gel filtration chromatography on a Superdex 75 column and elution was monitored at 215 nm. The elution volumes of the void volume ($V_v$) and column volume ($V_t$) and molecular weight standards are indicated.
Fig. 2.2 Type I collagen binding properties of MT1-MMP hemopexin C domain constructs. Serial dilutions of MT1-CD and MT1-LCD were incubated in 96-microwell plates coated with native (A) or heat-denatured (65 °C for 1 h) (B) type I collagen (0.5 μg/well) as described under solid phase assays in “Experimental Procedures”. MMP2-CBD and MMP2-LCD were included as positive and negative controls, respectively. Bound recombinant domains were detected using α-His<sub>6</sub> antibody. C, ligand blot assay. Immobilized MT1-CD and MT1-LCD, and control proteins, BSA and MMP2-LCD, (5 μg each) were incubated with biotin-labeled type I collagen (0.1 ng/ml) in PBS as described in “Experimental Procedures”. Bound collagen was detected using streptavidin-HRP and ECL detection.

Collagen/MT1-MMP Hemopexin C Domain Interactions during Collagen-Induced MMP2 Activation—Physical clustering of MT1-MMP was previously shown to facilitate the pro-MMP2 activation reaction by increasing the proximity of catalytically active MT1-MMP to the trimeric activation complex (51). Owing to the collagen binding properties of the MT1-MMP hemopexin C domain, we postulated that type I collagen may function as an in vivo mechanism to directly bind and concentrate cell surface MT1-MMP to facilitate the cellular activation of pro-MMP2. To test this, human gingival fibroblasts were cultured in three-dimensional type I collagen gels for 72 h to stimulate the activation of pro-MMP2. Soluble MT1-LCD was added to the cultures to compete with endogenous MT1-MMP for collagen binding. As shown in Fig. 2.3A, activation of pro-MMP2 in the cell cultures was reduced with increasing concentrations of MT1-LCD. Control cells cultured on plastic did not activate pro-MMP2. To confirm this response, latex beads coated with type I collagen were found to stimulate pro-MMP2 activation in fibroblasts cultured on plastic (Fig. 2.3B). Consistent with our observations of cells in collagen gels, induction of pro-MMP2 activation by native collagen-adsorbed beads was reduced by the presence of MT1-LCD to the levels seen with BSA-adsorbed beads (Fig. 2.3B). The requirement for fibrillar collagen was confirmed as gelatin-adsorbed beads did not stimulate pro-MMP2 activation. In the absence of latex beads, the addition of soluble native collagen to fibroblasts cultured on plastic produced inconsistent and variable levels of activation (data not shown). Together, these results demonstrate that native type I fibrillar collagen interactions with
the MT1-MMP hemopexin C domain in fibroblasts may concentrate cell surface MT1-MMP to stimulate
the cellular activation of pro-MMP2.

Effect of Exogenous MT1-MMP Hemopexin C Domain on Collagenolysis by sMT1-MMP and MMP2—
Studies of collagenases have shown that the hemopexin C domain is required to support binding to and
cleavage of collagen (27-29,31,32,58). To examine the role of the hemopexin C domain in MT1-MMP
collagenolysis, recombinant hemopexin C domain constructs were incubated with sMT1-MMP and biotin-
labeled type I collagen. Reactions were performed at 28 °C to maintain collagen triple helicity, as
confirmed by the lack of collagen cleavage in the presence of trypsin even at a 1:10 enzyme/substrate
mole ratio (data not shown). sMT1-MMP cleaved native type I collagen (Fig. 2.4A) and was inhibited by
TIMP-2 and BB2116 (data not shown). As seen in Fig. 2.4A (left panel), the sMT1-MMP cleavage of
native type I collagen was inhibited by the presence of MT1-LCD in a concentration-dependent manner.
In contrast, neither MT1-CD (Fig. 2.4A, right panel) nor the control protein, MMP2-LCD, had any effect on
cleavage. The percentage of α-chain cleavage for each reaction was quantitated by scanning
densitometry and graphically plotted against the amount of MT1-LCD or MT1-CD added (Fig. 2.4B). The
presence of hemopexin C domain proteins at the end of each reaction was confirmed by Western blot
analysis (Fig. 2.4C). As a control, MT1-CD and MT1-LCD did not affect sMT1-MMP activity against the
quenched fluorescent peptide, Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH₂ (Table 2.1) demonstrating that
inhibition by MT1-LCD is specific for triple helical substrates and that peptide bond cleavage by MT1-
MMP does not require the hemopexin C domain. Owing to the unique association between MT1-MMP and MMP2 in vivo, we assessed whether the MT1-MMP hemopexin C domain may affect MMP2 collagenolysis. Similar to that observed for MT1-MMP, MT1-LCD, but not MT1-CD, disrupted MMP2 cleavage of native type I collagen (Fig. 2.5).

**FIG. 2.4** Recombinant MT1-MMP hemopexin C domain reduces MT1-MMP collagenolysis. A, biotin-labeled type I collagen was incubated in the absence (C) or presence of sMT1-MMP (1 pmol) for 18 h at 28 °C. Recombinant proteins, MT1-LCD, MT1-CD and MMP2-LCD were added at the indicated mole equivalents relative to sMT1-MMP. Reactions were separated by SDS-PAGE (7.5 %), followed by Western blotting using streptavidin-HRP. B, percentage of α-chain cleavage was determined by densitometric analysis as described in "Experimental Procedures" and plotted against the amount of soluble recombinant hemopexin C domain added. C, MT1-LCD and MT1-CD were detected in reaction samples by SDS-PAGE (15 %) and Western blotting using the αMT1-CD antibody.

**Table 2.1 Quenched fluorescent peptide cleavage by sMT1-MMP.** Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH₂ (0.1 nmol) was incubated in the absence (Buffer) or presence of sMT1-MMP (Control) (0.035 pmol) for 1 h at 37 °C. MT1-LCD and MT1-CD were added to the reaction at 100-fold mole excess to sMT1-MMP.

<table>
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<tr>
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<th>Buffer</th>
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<th>MT1-LCD</th>
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<td>Rate of cleavage (RFU x 10⁻³·s⁻¹)</td>
<td>0.05</td>
<td>6.7</td>
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Collagen Binding Properties of MT1-MMP Linker Peptide Analogs and the Effect on Collagenolysis—Although both MT1-MMP hemopexin C domain constructs share similar binding properties, only MT1-LCD disrupted collagenolysis. Since this result indicated an important role for the linker in native collagen cleavage, we generated two synthetic peptide analogs to further study the effect of the MT1-MMP linker on MT1-MMP collagenolysis. From clustal alignments, we synthesized the peptide analog MT1-L18 (Arg^{302}-Cys^{319}) which corresponds to an 18-amino acid residue region of similarity possessed by the collagenolytic MMPs, MMP1, 2, 8, and 13 (Fig. 2.6A). MT1-L35 (Gly^{285}-Cys^{319}) encompasses the entire MT1-MMP linker and includes the unique 17-amino acid residue region that is N-terminal to the homologous 18-amino acid residue region (Fig. 2.6A). As shown in Fig. 2.6B, neither MT1-L18 nor MT1-L35 showed affinity for native (Fig. 2.6Bi) or denatured type I collagen (Fig. 2.6Bii), indicating that the MT1-MMP linker alone does not contribute to collagen binding or that the collagen binding site spans the junction of the linker and hemopexin C domain. Similarly, both peptide analogs did not disrupt native type I collagen cleavage by sMT1-MMP, even at 1000-fold molar excess (Fig. 2.6C). To determine if either linker peptide sequence could confer regulatory activity on the MT1-CD polypeptide, MT1-L18 or MT1-L35 were added to the reaction mixture containing MT1-CD. As shown in Fig. 2.6C, no inhibition of collagenolysis was observed. In a second set of experiments, MT1-LCD inhibited collagen cleavage as previously observed (Fig. 2.4), regardless of whether MT1-L18 or MT1-L35 was added. Since the presence of the linker sequence and the hemopexin C domain together as separate polypeptides is not sufficient for disrupting cleavage, these data suggest that the ability of the MT1-LCD to inhibit collagenolysis is context and/or conformation specific.

Cellular Invasion of Type I Collagen is Inhibited by a 44-kDa MT1-MMP Ectodomain Fragment — Active MT1-MMP is efficiently processed to a 44-kDa ectodomain fragment containing the MT1-LCD sequence (Gly^{285}-Cys^{506}) that is retained on the cell membrane (39,45,50). Since the soluble MT1-LCD inhibits...
Characterization of MT1-MMP peptide linker analogs. A, sequence alignment of collagenolytic MMP linkers using Megalign (DNASTAR Inc.) (Clustal method). Conserved residues are denoted with (*). Sequences of the peptide analogs of MT1-MMP are indicated. B, 96-well plate was coated with either native (i) or denatured (ii) type I collagen (rat tail) (0.5 μg/well). Serial dilutions of MT1-LCD, MT1-L18, and MT1-L35 were added and bound protein/peptide was detected using RP1MMP-14 antibody, which recognizes the linker. C, biotin-labeled type I collagen was incubated in the absence (C) or presence of sMT1-MMP (1 pmol) for 18 h at 28 °C. Mole excesses of MT1-MMP hemopexin C domain constructs (CD and LCD) (100-fold) and linker peptide analogs (L18 and L35) (1000-fold) were added to the reaction where indicated. Reactions were separated by SDS-PAGE (7.5 %), followed by Western blotting using streptavidin-HRP.

Native collagen cleavage by sMT1-MMP, we hypothesized that 44-kDa MT1-MMP may also function in a similar manner at the cell surface to modulate the collagenolytic activity of transmembrane MT1-MMP. To test this hypothesis, we used MDA-MB-231 breast carcinoma cells, which express endogenous MT1-MMP in the absence of detectable levels of MMP2. Invasion of 3-dimensional collagen gels overlaid onto a porous polycarbonate filter requires collagenolytic activity (41). In control experiments, MDA-MB-231 cellular invasive activity was inhibited by the hydroxamate inhibitor GM6001, indicating a requirement for metalloproteinase activity (Fig. 2.7A). TIMP-2 significantly reduced invasion (p<0.05) whereas TIMP-1 had no effect (59), confirming the dependence for MT-MMPs in MDA-MB-231 cell invasion. A blocking antibody against the MT1-MMP active site (Fig. 2.7A, anti-MT1) also reduced invasion compared to IgG controls (p<0.05), identifying MT1-MMP as the critical protease in this process. Indeed, overexpression of MT1-MMP on MDA-MB-231 cells increased collagen invasion approximately 2.5-fold compared to vector transfectants (p<0.05, Fig. 2.7A black bars). Furthermore, expression of the inactive mutant, MT1-MMP (Glu240Ala), on MDA-MB-231 cells resulted in inhibition of invasion to below control values and suggesting that this species may function as a dominant negative mutant.
FIG. 2.7 Invasion of MDA-MB-231 cells is MT1-MMP-dependent and is inhibited by recombinant MT1-MMP hemopexin C domain. A and B, invasion of type I collagen. Cells (2.5 x 10⁵) were seeded onto Transwell filters (8 μm pore) coated with a type I collagen gel (20 μg) and allowed to invade for 24 h as described under “Experimental Procedures”. Non-invading cells were removed from the upper chamber with a cotton swab. Filters were then stained and cells, adherent to the underside of the filter, were enumerated using an ocular micrometer. The average of triplicate experiments were normalized to corresponding controls (designated 100%) and are presented with standard deviation being shown (* p < 0.05). A, parental MDA-MB-231 cells (white) were allowed to invade in the presence of Me₂SO (DMSO), GM6001 (10 μM), BSA (10 nM), TIMP-1 (10 nM), TIMP-2 (10 nM), purified rabbit IgG (IgG, 10 μg/ml), or AB8102 antibody (anti-MT1, 10 μg/ml). MDA-MB-231 cells expressing MT1-MMP or MT1-MMP(E240A) (black) were also analyzed. Results are expressed as % of control invasion (versus BSA and Vector, as appropriate). B, MDA-MB-231 cells expressing MT1-MMP (2.5 x 10⁵) were incubated with DMSO, GM6001 (10 μM) and MT1-LCD, MT1-CD, and BSA (4 μM and 30 μM) and allowed to invade for 24 h. Results are expressed as % of control invasion (versus BSA). I acknowledge Yi Wu from the laboratory of Sharon Stack (North Western University, Chicago, IL) for performing the stable cell transfections and the collagen invasion assays with the recombinant protein that I provided.

To determine whether the MT1-LCD could inhibit cell-associated collagenolytic activity, cells were incubated with either BSA, MT1-LCD, or MT1-L35. At low concentrations (4 μM), the invasion of MT1-MMP transfected (Fig. 2.7B) or parental (data not shown) MDA-MB-231 cells was unaffected. MT1-L35 did not affect invasion at any concentration tested (data not shown). However, since MT1-LCD binds native collagen, the effective concentration of free protein available to the cells may be reduced by binding to the collagen filters. Therefore, the highest concentration possible with these protein preparations (30 μM) was used to ensure saturation of binding sites within the collagen-coated filters and availability of free protein at the cell surface to interact with MT1-MMP. Under these conditions, collagen invasion was significantly reduced (p<0.05, Fig. 2.7B) demonstrating inhibition of cell-associated MT1-MMP collagenolytic activity and confirming the in vitro analysis of MT1-LCD inhibiting collagen cleavage.

Because autolysis of transmembrane MT1-MMP leads to the accumulation of a cell surface 44-kDa MT1-MMP ectodomain fragment containing the linker and hemopexin C domain but lacking the active site, the effect of this cell-associated product on cellular MT1-MMP-mediated collagenolysis was assessed. For this experiment, transmembrane cellular constructs of MT1-LCD (Gly²⁸⁵-Val⁵⁸², designated cMT1-LCD) and MT1-CD (Pro³¹⁶-Val⁵⁸², designated cMT1-CD) (Fig. 2.8A) were expressed in MDA-MB-231 cells and
type I collagen invasion assessed relative to vector-transfected controls. Intracellular furin processing of these constructs at R\textsuperscript{111} generates the 44-kDa MT1-MMP and the linker-deleted form thereof. Expression of cMT1-LCD significantly reduced invasion to similar levels seen with soluble MT1-LCD when compared to cells expressing cMT1-CD (p<0.05, Fig 2.8). This confirms the in vitro biochemical results and demonstrates the importance of the MT1-MMP linker-hemopexin C domain in native collagen cleavage by cells. In control experiments, migration of MDA-MB-231 cells toward type I collagen, a process

\[\text{FIG. 2.8 Recombinant 44-kDa MT1-MMP inhibits MDA-MB-231 cell invasion.} \]

A, linear diagram of MT1-MMP and deletion mutants, cMT1-LCD (Δ112-284) and cMT1-CD (Δ112-315). The signal sequence (SS), propeptide domain (PRO), stalk segment (ST), transmembrane sequence (TM), and cytoplasmic tail (CYT) are indicated. Invasion of type I collagen (B) and migration (C) of MDA-MB-231 cells expressing MT1-MMP deletion mutants, cMT1-LCD and cMT1-CD, were assessed. B, cells (2.5 x 10\textsuperscript{5}) were seeded onto Transwell filters (8 μm pore) coated with a type I collagen gel (20 μg) and allowed to invade for 24 h as described under "Experimental Procedures". C, cells (2.5 x 10\textsuperscript{5}) were seeded onto Transwell filters coated with a thin layer of collagen on the underside and incubated for 1.5 h to permit migration. In both assays, non-invading or non-migrating cells were removed from the upper chamber with a cotton swab. Filters were then stained and cells, adherent to the underside of the filter, were enumerated using an ocular micrometer. The average of triplicate experiments were normalized to the vector control (designated 100%) and are presented with standard deviation as shown (* p < 0.05). I acknowledge Yi Wu for performing the stable cell transfections and the collagen invasion assays.
independent of collagenase activity (41,60) was unaffected by expression of cMT1-LCD or cMT1-CD (Fig. 2.8C). As these data clearly demonstrate the ability of cMT1-LCD to modulate type I collagen cleavage by transmembrane MT1-MMP, our results suggest that a function of the endogenous MT1-MMP autolysis product, 44-kDa MT1-MMP, is to regulate pericellular collagenolytic activity.

IV. DISCUSSION
As an integral membrane protein, MT1-MMP appears suited for coordinating the homeostatic catabolism of pericellular type I collagen under the guide of the cell (61-64). MT1-MMP mediates collagen degradation directly by cleaving native collagen and, indirectly, by activating MMP-13 (9), and the gelatinase and weak collagenase, MMP2 (11-13). Spatially and temporally, these two distinct activities of MT1-MMP regulate collagenolytic and gelatinolytic activities on the cell surface. Since MT1-MMP is a critical initiator and effector in the pericellular collagenolytic cascade, the regulation of its biological activity is very important in physiological and pathological collagen remodeling. The studies reported here have revealed the importance of the MT1-MMP hemopexin C domain and linker in the mechanism collagen cleavage, and demonstrated the role of collagen binding to MT1-MMP in stimulating MMP2 activation by cells. Moreover, these actions may be modulated in a dominant negative manner by the 44-kDa remnant form of MT1-MMP on the cell surface, revealing a novel regulatory function in proteolysis for an autolytic fragment of a protease.

The structure of collagen presents a challenge for proteolytic cleavage as indicated by the low $k_{cat}/K_m$ values for collagenases (65). Despite several studies from a number of laboratories, the triple helicase mechanism remains enigmatic (14). Our use of recombinant domains and polypeptides to probe the exosite requirements of MT1-MMP for collagenolysis revealed similar domain requirements for triple helicase activity as the secreted collagenases. The binding of the MT1-MMP hemopexin C domain, with or without the linker, to native collagen is consistent with previous reports for the collagenolytic MMPs (27,30-32). The hemopexin C domain of MMP2, in contrast, does bind native collagen stably (56). Interestingly, the MT1-MMP hemopexin C domain does not bind denatured collagen. This suggests that following cleavage, subsequent denaturation of the collagen would result in the release of MT1-MMP from the cleaved substrate facilitating turnover.

Inhibition of sMT1-MMP collagen cleavage using MT1-MMP hemopexin C domain constructs required the presence of the linker indicating that collagen binding, by the hemopexin C domain alone, is not sufficient to disrupt collagenolysis. This requirement was also observed in MMP2 collagenolysis as MT1-LCD, but not MT1-CD, blocked MMP2 cleavage of native collagen. Protein engineering studies of MMP-1 and MMP-8 have previously shown a role for the linker in triple helicase activity (33,34,66); however, our studies have revealed some unique features of the MT1-MMP linker. Souza et al proposed that the MMP-1 collagenase linker, owing to its proline content, intercalates with the collagen triple helix, thereby displacing individual $\alpha$-chains for cleavage (67). We found that MT1-MMP linker peptide analogs of either the full-length 35-amino acid residue linker or the 18-amino acid residue region corresponding to that
found in the secreted collagenases, did not bind native or denatured type I collagen. These results indicate that the MT1-MMP linker may not bind or intercalate with the collagen triple helix as proposed for the MMP-1 linker. Potentially, the full collagen binding exosite of the MT1-MMP hemopexin C domain that recognizes the 3/4-1/4-collagen site may span the linker/hemopexin C domain junction, thereby accounting for the lack of collagen binding by the linker analogs alone. The MT1-MMP linker, when connected to the hemopexin C domain, may act as a specificity determinant directing binding of the protease to the 3/4-1/4-collagen cleavage site. Thus, competition from MT1-LCD, but not MT1-CD, may block MT1-MMP from binding collagen here and so inhibit cleavage. Topographically, the MT1-MMP, and other collagenase linkers, may also correctly configure the catalytic domain relative to the hemopexin C domain for collagenolytic competence. Indeed the MT1-MMP linker has predicted rigidity due to the presence of 9 proline residues, with the X-ray crystallographic structure of the MMP-1 linker (68) also indicating that the collagenase linker is not flexible. Hence, MT1-LCD binding of collagen may sterically disrupt the collagenolytic configuration of sMT1-MMP at the 3/4-1/4 collagen cleavage site, thereby inhibiting cleavage.

The importance of MT1-MMP in collagen homeostasis is supported by the finding that fibrillar type I collagen induces cell surface expression of MT1-MMP and subsequent MMP2 activation through transcriptional and non-transcriptional pathways (35-40,69). Induction of MT1-MMP transcription is dependent on $\beta_1$ integrin receptors and actin cytoskeleton rearrangement (37,42). Clustering of $\beta_1$ integrins by collagen ligation or antibody crosslinking induces de novo expression of MT1-MMP and subsequent MMP2 activation (36,39,41). Interestingly, our data reveals that collagen may also assemble MT1-MMP on the cell surface via binding to the hemopexin C domain, thereby increasing the local concentration of MT1-MMP for collagenolysis and efficient MMP2 activation. In view of the demonstrated absence of oligomer formation by the MT1-LCD used here, we interpret the reduction in collagen-induced MMP2 activation by MT1-LCD to be the result of competitive binding for collagen between the exogenous MT1-LCD and cell surface MT1-MMP, rather than competitively disrupting any MT1-MMP/MT1-MMP binding interactions. Indeed this interaction between MT1-MMP and collagen may represent a biological mechanism similar to that observed with ConA, which clusters MT1-MMP on the cell surface during MMP2 activation (46). As previously shown, ConA increases the matrix degradative phenotype of the cell through transcriptional and post-transcriptional regulation of MMP and TIMP genes that was reflected by extensive endogenous collagen degradation in the conditioned media (49). Cleavage of $\beta_1$ integrin-ligated collagen also releases bound pro-MMP2, which can now enter the activation pathway, which otherwise is recalcitrant to activation (70). Hence, pericellular collagen has multiple effects in binding and regulating the activities of collagenolytic MMPs, representing an unusual relationship between a protease and cognate substrate that appears to contribute to the homeostatic maintenance of collagen levels.

MT1-MMP activity on the cell surface is further regulated by endocytosis (71,72), TIMP binding (55,73) and trimolecular complex formation (55,74), as well as the autolytic shedding of the catalytic domain to yield 44-kDa MT1-MMP (45,50,75). Currently, the role of 44-kDa MT1-MMP in vivo is not clear. It has
been reported recently that the hemopexin C domain and the cytoplasmic tail of MT1-MMP mediate homophilic interactions that increase MMP2 activation (47,48). Using HT1080 cells Itoh et al (47) reported that expression of MT1-MMP PEX (Thr\textsuperscript{313}-Val\textsuperscript{582}), a truncated form of 44-kDa MT1-MMP that lacks most of the linker and hence is similar to cMT1-CD used here, reduced MMP2 activation and subsequent Matrigel invasion, presumably by disrupting the formation of oligomeric MT1-MMP complexes—PEX is unfortunately a confusing designation for the MT1-MMP hemopexin C domain as PEX was already the name of a cell surface zinc metallopeptidase belonging to the neprilysin family (76-78). As reported here and previously (46), we have found no evidence for oligomerization using MT1-LCD or MT1-CD, emphasizing the importance of cell membrane context or the stalk segment, transmembrane sequence and cytoplasmic tail in these proposed complexes. Unlike the effects of MT1-LCD in disrupting the collagen-induced activation of MMP2 shown here, the inability of soluble MT1-LCD or MT1-CD to competitively block ConA-induced MMP2 activation in cells cultured on plastic reported previously (46) indicates the importance of cellular context for these effects and highlights the difference in collagen-mediated activation of MMP2, which is blocked by MT1-LCD, from activation induced by MT1-MMP overexpression or ConA, which is not.

In our previous studies of chemokine cleavage by MMP2, we found that MCP-3 and SDF-1α binding to the hemopexin C domain markedly improved the catalytic efficiency of cleavage (79,80). Notably, the addition of recombinant MMP2 hemopexin C domain to mixtures of chemokine and active MMP2 in enzyme assays could totally block substrate cleavage (80). Therefore, the presence of the entire 35-amino acid residue linker and hemopexin C domain in the 44-kDa MT1-MMP ectodomain suggested to us that this autolytic product has the potential to antagonize the proteolytic activity of MT1-MMP in a dominant-negative manner by interacting with native collagen. Our data demonstrates that expression of cMT1-LCD (Gly\textsuperscript{285}-Val\textsuperscript{582}), representing the 44-kDa MT1-MMP in its entirety (45) on MDA-MB-231 cells inhibits MT1-MMP-mediated type I collagen cleavage and cell invasion. The inhibitory effect of cMT1-LCD expression on cell invasion was confirmed by the addition of soluble MT1-LCD to MT1-MMP-transfected cells. Since MDA-MB-231 cells do not express MMP2, the effect of cMT1-LCD expression and MT1-LCD on cell invasion is distinct from that reported previously (47) and discussed above. Nonetheless, the capacity of MT1-LCD to also block MMP2 native collagen cleavage may amplify the downregulation of collagenolysis \textit{in vivo} by blocking MMP2 in addition to MT1-MMP. Invasion was also inhibited with the expression of the dominant negative mutant, MT1-MMP (Glu\textsuperscript{240}Ala), further supporting the role of MT1-MMP in collagen invasion and of the hemopexin C domain in collagen binding and triple helical collagen cleavage. Consistent with our biochemical analysis, neither the expression of cMT1-CD nor the addition of soluble MT1-CD affected cell invasion to a significant degree, confirming the importance of the MT1-MMP linker in context with the hemopexin C domain in collagenolysis. In view of these effects, it is also likely that 44-kDa MT1-MMP may reduce MMP2 activation by reducing MT1-MMP clustering mediated by pericellular collagen. However, this could not be directly tested since MDA-MB-231 cells do not express MMP2. Together, these results clearly reveal the 44-kDa MT1-MMP as a novel inhibitor of pericellular type I collagen cleavage by MT1-MMP and MMP2 activities. Our studies also
indicate the feasibility of designing new MMP inhibitors that target the substrate rather than the protease. This new class of inhibitors may exert highly selective substrate specific protease inhibition while sparing the cleavage of other substrates in the protease degradome. Similarly, targeting the protease exosite rather than the active site may also represent new avenues of substrate-specific inhibition to achieve levels of specificity not possible with active site inhibitors (81).

FIG. 2.9 Potential role of the 44-kDa MT1-MMP in pericellular collagen degradation. A, collagenolytic cell profile. Upon collagen-induced engagement of β1 integrins and intracellular signaling, expression of MT1-MMP is upregulated on the cell surface. Increased MT1-MMP expression promotes the cleavage of native collagen (1) and the release of collagen-bound pro-MMP2, which now enters into the activation pathway (2). The conversion from collagenolysis to gelatinolysis commences with the formation of the trimolecular complex (3), which reduces MT1-MMP collagenolytic activity, and the activation of pro-MMP2, which is enhanced by the collagen-mediated assembly of MT1-MMP (4). Collagen binding by TIMP2-inhibited MT1-MMP in the trimolecular complex may also block collagen cleavage by uninhibited MT1-MMP. B, gelatinolytic cell profile. Following pro-MMP2 activation and MT1-MMP autolysis, the 44-kDa MT1-MMP accumulates on the cell surface, binds native collagen, and suppresses collagen degradation by inhibiting MT1-MMP (5) and MMP2 (6) collagenolysis, but not MMP2 gelatinolysis (7). Stimulation of cell surface MT1-MMP expression is reduced due to the absence of native collagen and β1 integrin engagement.
The degradation of pericellular type I collagen is revealed to be a dynamic-self regulated process. We have previously proposed models regarding the regulation of pericellular type I collagen levels upon \( \beta_1 \) integrin stimulation of MT1-MMP and MMP2 activity (14,39,70). Our investigation into the role of the 44-kDa MT1-MMP ectodomain adds a new dimension to this homeostatic process. As modeled in Fig. 2.9, fibrillar type I collagen induces a \( \beta_1 \) integrin-dependent increase in MT1-MMP expression on the cell surface, thus favoring an initial collagenolytic phase. Our data shows that the collagen binding properties of the MT1-MMP hemopexin C domain are necessary for native collagen cleavage (Fig. 2.9A). As suggested previously (70), the release of collagen-bound pro-MMP2 from the cell surface following collagen cleavage by MT1-MMP allows pro-MMP2 reservoirs to be optimally activated temporally and spatially in relation to its substrate. Collagen binding by the MT1-MMP hemopexin C domain also potentiates MMP2 activation, most likely by concentrating MT1-MMP/TIMP-2/pro-MMP2 complexes with TIMP-free MT1-MMP (Fig. 2.9A). Furthermore, in the MMP2 activation process, MT1-MMP collagenolytic activities are suppressed by TIMP-2 binding to form the trimolecular pro-MMP2 complex, and by MT1-MMP autolysis—converting the proteolytic signature of the cell from collagenolytic to gelatinolytic. Following MT1-MMP autolytic shedding, our data shows that the 44-kDa MT1-MMP continues to bind collagen, further reducing pericellular collagenolysis by MT1-MMP and MMP-2 (Fig. 2.9B). Overall, these intimately related and complex events allow for a conversion of proteolytic activity to take place on the cell surface. This shift from a collagenolytic to a gelatinolytic profile may be important for maintaining pericellular collagen levels. Thus collagen is a unique substrate: By binding the proteases responsible for its cleavage, these interactions recruit and regulate collagenolytic and gelatinolytic activities in a homeostatic manner. Hence, the studies reported here reveal several new aspects in the biology of MT1-MMP as a consequence of native type I collagen binding by the hemopexin C domain. This also provides a novel explanation for the generation of MT1-MMP clusters on the cell surface and adds a new layer of control to the complex regulation of focal proteolysis by MT1-MMP and MMP2.

V. REFERENCES


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CHAPTER 3

Characterization of the Distinct Collagen Binding and Cleavage Mechanisms of Matrix Metalloproteinase 2 and 14 (Gelatinase A and MT1-MMP): The Differential Role of MMP Hemopexin C Domains and the MMP-2 Fibronectin Type II Modules in Collagen Triple Helicase Activities*

I. INTRODUCTION
The extracellular matrix provides all tissues and organs with form, mechanical strength, and physical properties. In addition, the extracellular matrix provides self-reporting dynamic feedback to the resident cells upon alterations in tissue integrity, infiltration of nonresident pathological or host defense cells, and pathological processes. This occurs in response to degraded matrix components revealing cryptic motifs, to released or activated signaling molecules otherwise sequestered by the matrix, or to liberated neoproteins generated by proteolytic processing. Of the fibrillar collagens that provide most of the mechanical strength to mineralized and nonmineralized tissues, type I collagen is the most abundant and important (1). Type I collagen is also a reservoir for and regulator of cytokine release (2) and also for organizing and promoting the activation of cell membrane proteases important for collagen cleavage (3-5). Hence, connective tissue remodeling and collagen metabolism in particular, is an important aspect of normal tissue and organ homeostasis. Indeed, in many pathologies the normal balance of connective tissue synthesis and degradation is perturbed leading to altered tissue and organ function due either to loss of collagen or to the destructive effects of fibrosis (6-8). Thus, it is critical to elucidate the molecular basis of collagen homeostasis and catabolism as fundamental aspects in the understanding of human disease. In turn, this knowledge is needed to devise new therapeutic strategies to modulate collagen degradation and the effects of connective tissue destruction in many diseases.

Monomeric type I collagen, which consists of two α1(I) chains and one α2(I) chain interwound into a triple helix, is axially polymerized to form supermolecular microfibrils and higher order structural fibrils (1). Collagen crosslinkage (9) further leads to the formation of mechanically strong and proteolytically resistant collagen fibers arranged in different arrays according to the nature and demands of nonmineralized and mineralized tissues. At neutral pH, non-mineralized type I collagen is believed to be cleaved extracellularly by collagenases of the matrix metalloproteinase (MMP) family (10). The secreted soluble collagenases MMP-1 or interstitial collagenase, MMP-8 or neutrophil collagenase, and MMP-13 or collagenase-3 slowly cleave collagen at the Gly775-Ile/Leu776 bond (11), which yields 3/4 and 1/4 fragments that are thermally unstable at body temperature (12,13). MMP-2 is a potent gelatinase that is believed to be important in the final clearance of degraded and denatured collagen (14,15) with a controversial role in cleaving native type IV collagen (16-18). However, MMP-2 also has weak native type

I collagenase activity in rodents (19,20), man (5,21,22), and on triple helical synthetic peptides (23,24). The membrane type (MT) MMPs also exhibit collagenase activities (5,25) with MT1-MMP being considered a critical enzyme of native collagen metabolism (26-28).

The mechanism by which triple helical collagen is cleaved by MMPs has been extensively studied using engineered proteases, type I collagen, and triple helical peptide analogues. Despite this, it is still far from being understood (29,30). As the width of the MMP active site cleft (5 Å) (31) cannot accommodate all three α-chains (15 Å diameter) (32), the collagen triple helix must first unwind prior to cleavage in a process termed triple helicase activity. The MMP catalytic domain in isolation cannot cleave triple helical collagen and requires exosites—substrate-binding sites outside of the active site cleft—for native collagen cleavage (29). All collagenolytic MMPs contain a hemopexin carboxy-terminal (C) domain, which is linked to the catalytic domain by a flexible linker peptide (10,29). In the collagenases MMP-1, -8, -13 and -14, the hemopexin C domain binds collagen and is absolutely required for the cleavage of triple helical collagen (5,33-37). However, soluble collagen has been reported to be cleaved by the isolated MMP-8 catalytic domain at 37 °C (38). As demonstrated for MMP-8 and MMP-14, the linker peptide also plays a role in collagenolysis (5,39,40). Interestingly, the MMP-2 hemopexin C domain, does not bind native or denatured type I collagen (41,42). Instead this functionality is accomplished by the fibronectin type II modules within the MMP-2 catalytic domain which are arranged as a compact collagen binding domain (CBD) (43,44). Although this domain potentiates gelatin cleavage (18,22), its role in collagen triple helicase activity is still unclear (29).

In our recent study we reported the critical role of the hemopexin C domain and linker of MT1-MMP in collagen recognition, cleavage and in blocking MT1-MMP and MMP-2 collagenolysis when present as a recombinant domain or natural autolysis product on the cell surface (5). In the present report, using circular dichroism spectroscopy we demonstrate for the first time that the MT1-MMP linker hemopexin C domain and MMP-2 CBD fibronectin type II modules perturb the secondary structure of native type I collagen upon binding. Although this specific interaction cooperatively increased the rate of collagen cleavage by the collagenases MMP-1 and MMP-8 it was not sufficient to allow for collagen cleavage by the noncollagenolytic proteases MMP-7 and trypsin. However, despite the lack of collagen binding by the MMP-2 hemopexin C domain it was still absolutely required together with the fibronectin type II modules for native type I collagen cleavage.

II. EXPERIMENTAL PROCEDURES

Proteins—Extraction and purification of rat tail tendon type I collagen; the preparation of biotin-labeled bovine type I skin collagen; and the preparation, purification and characterization of recombinant human MMP hemopexin C domains expressed with the linker (L): MT1-LCD (Gly285-Cys508) and MMP-2 LCD (Gly446-Cys669); or without the linker, MT1-CD (Gly315-Cys508), were previously described (5,42). Recombinant human MMP-2 collagen binding domain (CBD) proteins consisting of all three fibronectin type II modules, CBD (Val220-Gln393) (44), or as a N-terminal module 1 deletion, CBD23 (Ala278-Gln393),
and the CBD23 mutant, W316A/W374A, are described elsewhere\(^1\). All recombinant domains are numbered from the initiating Met\(^1\) of the full-length enzyme. Human soluble (s) MT1-MMP lacking the stem, transmembrane and cytoplasmic tail (5), MMP-8\(^2\), and MMP-2 (45) have also been described elsewhere. Human MMP-1 was expressed and purified using the pGWIGH vector kindly provided by British Biotech Pharmaceuticals (Oxford, UK).

**Expression of C-terminally Truncated MMP-2**—The following primers: 5' (GCTCTATGGGGCCTGACCTGACATTGACCTTG GC) and 3' (GCCAAGGTCAATGTCAGGTCAGGCCCCATAGAGC) (target bases are underlined) were used to introduce a STOP codon in proMMP-2 (template pGLA/pGWIGH) to create a truncated form, N-MMP-2, which lacks the hemopexin C domain (\(\Delta419-631\)). The entire DNA sequence was confirmed by sequencing and stable CHO (Chinese Hamster Ovary) cell clones were selected using mycophenolic acid as previously described (46) and characterized by Western blotting with rabbit polyclonal antibody αCBD123 (raised against the collagen-binding domain) (44). Roller bottle cultures (850 cm\(^2\), Becton Dickinson) were incubated in 50-100 ml serum-free medium (CHO-S-SFMII, Gibco BRL) and harvested every 1-2 days for up to 10 days. N-MMP-2 was purified as follows: filtered conditioned medium was passed through 10-15 ml of gelatin-Sepharose 4B equilibrated with column buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM CaCl\(_2\), 0.025 % sodium azide, 0.04 % Brij 35). After washing with column buffer including 1 M NaCl, bound proteins were eluted with 10% dimethyl sulfoxide (DMSO). Peak fractions were pooled, dialyzed into column buffer and passed through a 4 ml column of heparin-Sepharose CL-6B to remove full-length wild-type MMP-2. The flow-through was loaded onto a small gelatin-Sepharose 4B column (2 ml) and washed and eluted as before. Peak fractions were dialyzed in storage buffer (50 mM Tris-HCl pH 7.8, 10 mM CaCl\(_2\), 0.1 M NaCl).

**Circular Dichroism**—Collagen and recombinant protein domains were incubated either alone or in combination for 2 h at 28 °C. Samples were transferred to a 1 mm path-length quartz cell and ellipticity (mdeg) was measured from 197 nm to 250 nm using a J810 (Jasco) spectropolarimeter at 28 °C. The CD spectra of native collagen alone were also determined at various temperatures maintained by a computer controlled Neslab water bath thermal jacket during thermal denaturation. Ellipticity values were averaged between triplicates and plotted against wavelength. CD spectra were measured for proteins alone: native collagen, MT1-LCD, MT1-CD, MMP-2 CBD, MMP-2 LCD, and as mixtures of native collagen with the individual recombinant protein domains. Calculated CD spectrums were produced by summing the ellipticity values for collagen and the recombinant domains alone (47) and compared with the measured CD spectra of the corresponding collagen/recombinant domain mixtures to assess the extent of perturbations in collagen secondary structure.

**Enzyme Assays**—Biotin-labeled type I collagen (0.025 pmol) was incubated with MMPs in assay buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM CaCl\(_2\), 3.8 mM NaN\(_3\), 0.05 % Brij) for 18 h at 28 °C (5). MMPs were activated with 2 mM APMA for 1 h at 37 °C prior to the addition of substrate. Recombinant protein domains were added for the duration of the assay at various mole ratios of protein:enzyme as
indicated. Collagenase assays were terminated with the addition of SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 2.0 % SDS, 2.0 M urea, 0.05 % bromophenol blue) and then analyzed by 7.5 % SDS-PAGE and Western blotting using either streptavidin-HRP and ECL detection (Amersham Pharmacia Biotech) or infrared dye labeled-streptavidin (Molecular Probes) and detection using the Odyssey Infrared Imaging System (LI-COR) at 700 nm.

Quenched fluorescent substrates Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH₂ (48) and fluorescein-labeled gelatin (DQ gelatin, Molecular Probes) were incubated with APMA-activated MMP-2 in assay buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM CaCl₂, 0.05 % Brij) for 1-2 h at 37 °C. Recombinant MMP domains were added for the duration of the experiment as indicated. Fluorescence was measured using the POLARstar Optima (BMG Labtechnologies) using excitation and emission filters of 320 nm and 405 nm for Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH₂, and 485 nm and 520 nm for DQ gelatin. Substrate cleavage was monitored and reported as arbitrary fluorescence units (units sec⁻¹ nM⁻¹).

Metabolically labeled (¹⁴C)-human type I gelatin (19) was incubated with MMP-2 for 3 h in assay buffer in the presence of MMP-2 LCD, 5 % DMSO, or increasing amounts of CBD. Digestion was terminated by precipitation with 10 % trichloroacetic acid /1 % tannic acid at 0 °C. The radioactivity of degraded gelatin peptides in the supernatant was counted by scintillation spectroscopy with quench correction.

III. RESULTS
We have previously shown that the hemopexin C domain of MT1-MMP binds and is required for native collagen cleavage (5). We hypothesized that upon native collagen binding the MT1-MMP hemopexin C domain prepares the collagen for cleavage by localized disruption of the triple helix in the immediate vicinity of the Gly⁷⁷⁵-Ile/Leu⁷⁷⁶ cleavage site. To test this, saturating amounts of recombinant MT1-MMP hemopexin C domain with or without the linker (Fig. 3.1A, insert) were incubated with native type I collagen and changes in protein secondary structure were measured using CD spectroscopy. As shown in Fig 3.1A, left panel, a deviation in the spectra was observed in the 199-205 nm region for the collagen/MT1-LCD sample where the measured minimum (203 nm, -45.1 mdeg) was shallower and occurred at a longer wavelength than that of the calculated minimum (202 nm, -48.7 mdeg). Similar spectral changes also occurred upon heat denaturation of native collagen (Fig. 3.1B). These structural changes were not seen in the collagen/MT1-CD sample (Fig. 3.1A, right panel) and so are consistent with the inability of MT1-CD compared with MT1-LCD to block MT1-MMP cleavage of collagen (5). Thus these experiments indicate that there are different modes or sites of collagen binding for recombinant MT1-MMP hemopexin C domain with or without the linker.

Given the importance of the hemopexin C domain in MT1-MMP collagenolysis (5), collagen helix perturbation induced by the linker and hemopexin C domain components of full length MT1-MMP is likely to be an essential component of the triple helicase mechanism. Although the deviation between the measured and calculated spectra predicts a change in protein secondary structure of the collagen/MT1-
FIG. 3.1 Structural perturbation of native collagen upon binding of recombinant MT1-MMP hemopexin C domain. 

A. The measured (—) and calculated (—) CD spectra of native type I collagen (0.02 nmol) in the presence of MT1-LCD (2 nmol, left panel) or MT1-CD (2 nmol, right panel) at 28 °C are shown with ellipticity expressed in millidegrees (mdeg). Calculated spectra of collagen in combination with either MT1-LCD or MT1-CD were generated as described in "Experimental Procedures". 

B. Thermal denaturation of native collagen (5.2 nmol) alone. Spectra were measured at 25, 30, 35 and 40 °C. 

C. Biotin-labeled collagen (0.025 nmol) was incubated in the absence (C) or presence of collagenases, MMP-1 (0.08 pmol) and MMP-8 (0.03 pmol), for 18 h at 28 °C. MT1-LCD was added at the indicated molar equivalents (1-10) relative to enzyme. 

D. Biotin-labeled collagen (0.025 nmol) was incubated with sMT1-MMP (1 pmol) for 18 h at 28 °C in the presence of buffer, MT1-LCD or MT1-CD at 10 molar equivalents relative to enzyme. Trypsin was incubated with biotin-labeled collagen at the indicated enzyme:substrate ratios for 3 h at 28 °C. Samples were separated by SDS-PAGE (7.5%) followed by Western blotting and enhanced chemiluminescence using streptavidin-HRP. Full length type I collagen α(1)-chains (α1, α2), intramolecularly crosslinked α-chains termed β-chains (β), and their 3/4-length cleavage fragments (βA, α1A, α2A) are indicated.

LCD complex, it does not distinguish in which protein the change occurred. However, the similarity with the change in CD spectra of native collagen upon mild heat denaturation (Fig. 3.1B) suggests, but does not conclusively show, that the structural alteration originated from or led to perturbations in the structure of collagen. Therefore, as a further indication that the changes occurred in the collagen, we examined its susceptibility to cleavage by two secreted collagenases in the presence of MT1-LCD (Fig. 3.1C). The absence of collagen degradation by trypsin alone, even at a ratio of 1:10 (enzyme to substrate), confirmed the native structure of the type I collagen in these assays (Fig. 3.1D). Whereas MT1-MMP activity was blocked by the addition of MT1-LCD (Fig. 3.1D) as we recently reported (5), the addition of MT1-LCD unexpectedly enhanced collagen cleavage by MMP-1 and MMP-8. This occurred even at low
(0.1) mole ratios (Fig. 3.1C) in a concentration-dependent manner and was particularly apparent when MMP-1 and MMP-8 were incubated at enzyme/substrate ratios that produced minimal cleavage as shown. Hence, the apparent localized disruption of the collagen triple helix by recombinant MT1-LCD rendered it more amendable to cleavage by MMP-1 and -8 in trans in a cooperative manner. In the full length MT1-MMP, a similar action of the linker and hemopexin C domain in cis is likely the mechanism of triple helicase activity of MT1-MMP. However, when in trans the MT1-LCD blocks MT1-MMP collagen cleavage indicating that the MMP-1 and MMP-8 hemopexin C domains utilize a different binding site which does not compete with MT1-MMP hemopexin C domain binding.

**FIG. 3.2** Recombinant MMP-2 CBD blocks MMP-2 cleavage of gelatin and collagen. **A,** DQ gelatin (1 μg) was incubated at 37 °C with APMA-activated MMP-2 (2.6 pmol) in the absence (Control) or presence of recombinant domains (446 pmol): MMP-2 CBD, MMP-2 LCD, MT1-LCD, and MT1-CD. The rates of cleavage after 2 h incubation are shown. **B,** (14C)-gelatin (4 ng) was incubated with APMA-activated MMP-2 in the presence of MMP-2 CBD, MMP-2 LCD and DMSO (5%). **C,** Biotin-labeled collagen (0.025 nmol) was incubated in the absence (C) or presence of 0.84 pmol MMP-2 (0) for 18 h at 28 °C. MMP-2 CBD and MMP-2 LCD were added at the indicated molar equivalents (1-100) relative to MMP-2. After assay, the reaction products were analyzed by SDS-PAGE electrophoresis and visualized by Western blotting and enhanced chemiluminescence using streptavidin-HRP. Full-length α(1)chains (α1, α2), β-chains (β), and 3/4-fragments (βA, α1A, α2A) of the type I collagen are indicated. **D,** The quenched fluorescent septapeptide Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH2 (0.1 nmol) was incubated at 37 °C with MMP-2 (0.4 pmol) in the absence (Control) or presence of MMP-2 CBD (40 nmol). The cleavage rate after 1 h is shown.
The molecular requirements and domain interactions for MMP-2 cleavage of collagen are not clear. Using recombinant proteins of the MMP-2 fibronectin type II modules (MMP-2 CBD) and the hemopexin C domain (MMP-2 LCD) as competitive inhibitors, we examined the contribution of each in the cleavage of native and denatured type I collagen. As shown in Fig. 3.2A, addition of MMP-2 CBD reduced the rate of cleavage of internally quenched fluorescein-labeled gelatin by MMP-2 approximately ~3-fold compared to the buffer control. These data were confirmed using (¹⁴C)-metabolically labeled human type I gelatin as substrate (Fig. 3.2B). In contrast, the addition of MMP-2 LCD, MT1-LCD and MT1-CD had no effect on gelatin cleavage (Fig. 3.2A and B). These present results are consistent with the binding properties of these domains we previously reported: MMP-2 CBD binds gelatin and native type I collagen with $K_d$ in the sub μM range (44), but neither the MMP-2 LCD (42) nor the MT1-MMP hemopexin LCD or CD (5) domains bind gelatin. Interestingly 5% DMSO, which is used during biochemical purification to elute MMP-2 bound to gelatin Sepharose by the CBD, only reduced gelatinolysis by ~30% so revealing the intrinsic propensity of the MMP-2 catalytic domain for gelatin cleavage.

To determine the role of the MMP-2 CBD and hemopexin C domain on native type I collagen cleavage, these domains were incubated with collagen and MMP-2 for 18 h. By monitoring the generation of the $\alpha_1^A$ and $\alpha_2^A$ fragments we found that recombinant MMP-2 CBD inhibited cleavage of native type I collagen in a concentration-dependent manner whereas the MMP-2 LCD, which does not bind collagen (42), had no effect on cleavage (Fig. 3.2C). Consistent with this, the cleavage of the collagen $\beta$-components was similarly affected. In comparison to macromolecular substrates, MMP-2 cleavage of the peptide substrate Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH$_2$ was unaffected by the presence of CBD (Fig. 3.2D) or MMP-2 LCD (data not shown) in the reactions. This indicates that the exosites on the CBD only potentiate cleavage of macromolecular substrates that can both bind to the CBD and also contact the catalytic centre at more N-terminal sites in the bound substrate. Hence, by competitive inhibition experiments the importance of the collagen binding properties of the MMP-2 exosite domains was demonstrable: the fibronectin-type II modules both bind and block gelatin and collagen cleavage whereas the MMP-2 LCD neither binds nor blocks cleavage of gelatin and collagen. Indeed, the CBD can quantitatively elute proMMP-2 from collagen and gelatin affinity columns (44). Notably, the collagen binding properties and role of the MMP-2 LCD differs from the MT1-LCD, which binds and blocks native type I collagen cleavage by both MT1-MMP and MMP-2 (5).

Given the similar activities of competitive amounts of exogenous MMP-2 CBD and MT1-LCD proteins in blocking collagen cleavage in trans of the cognate parent proteases, we next investigated whether the fibronectin type II modules of MMP-2 could also perturb the collagen triple helix like MT1-LCD. The CD spectra of native type I collagen/MMP-2 CBD complexes was compared with the calculated spectra generated and analyzed as before (Fig. 3.3A). A large deviation was observed between the two spectra in the region of 197-227 nm for collagen/MMP-2 CBD (Fig. 3.3A left panel), where the measured minimum at 198 nm was significantly greater than the corresponding minimum of the calculated spectrum.
This general flattening of the absorption curve is similar to that observed for the CD spectra of collagen upon heat denaturation shown here (Fig. 3.1B) and as previously reported (1). The deviation observed for the collagen/MMP-2 CBD sample is considerably more significant than that seen for MT1-LCD, which may reflect either a different mode of binding or the 2-fold increase in native type I collagen affinity of the MMP-2 CBD (44) compared with MT1-LCD (5). In comparison, there was little or no difference between the measured and calculated CD spectra of collagen/MMP-2 LCD (Fig. 3.3A right panel) consistent with the absence of any collagen binding properties of the isolated domain (42).

FIG. 3.3 Structural perturbation of native collagen upon binding of recombinant MMP-2 domains. A, The measured (—) and calculated (—) CD spectra of native type I collagen (0.02 nmol) in the presence of MMP-2 CBD (2 nmol, left panel) or MMP-2 LCD (2 nmol, right panel) at 28 °C are shown. Calculated CD spectra of collagen in combination with recombinant MMP-2 domains were generated as described in "Experimental Procedures". B, Biotin-labeled collagen was incubated in the absence (C) or presence of MMP-1 (0.05 pmol) and MMP-8 (0.09 pmol) with MMP-2 CBD added at the indicated molar equivalents (1-100) relative to enzyme. After incubation for 18 h at 28 °C samples were separated by SDS-PAGE (7.5 %) and the collagen chains were visualized by in-gel infrared imaging at 700 nm after the gels were incubated with streptavidin-Alexa680. Full-length α(I)-chains (α1, α2), β-chains (β), and 3/4-fragments (β*, α1, α2) of the type I collagen chains are indicated. I acknowledge Todd Moore for his work in the cloning, expression and purification of the W316A/W373A protein.

To probe the biological significance of the secondary structure changes in the collagen/MMP-2 CBD complexes, MMP-1 and MMP-8 were added and incubated for 18 h at 28 °C. As shown in Fig. 3.3B, MMP-2 CBD binding of collagen enhanced cleavage by both collagenases in trans in a concentration-dependent manner. Hence, this suggests that the structural perturbations that occur in the collagen triple helix upon binding the CBD, but not the MMP-2 LCD, is an important component of the triple helicase mechanism of MMP-2. Notably, neither MMP-7 nor trypsin exhibited native collagen cleavage properties in the presence of the MMP-2 CBD or the MT1-LCD (not shown). Since both these enzymes cleave gelatin efficiently, this indicates that upon binding of the exosite domains, the structure of collagen is only perturbed by local denaturation and not completely unwound. In addition, collagen cleavage appears to
be only be enhanced in a synergistic manner by proteases that display intrinsic collagen binding and
collagenolytic activity. As neither MMP-7 nor trypsin bind collagen, the competitive displacement of the
collagen-bound exosite domains by these enzymes is therefore unlikely to occur and so this would restrict
access of these proteases to the individual \( \alpha \)-chains. Since the CBD had the opposite effect and
competitively blocked MMP-2 collagenolysis in trans this also suggests that MMP-1 and MMP-8 bind
collagen at a different, but likely nearby, site or sites to that recognized by the CBD.

![Diagram](A.png)

**FIG. 3.4** N-MMP-2 cleavage of gelatin requires the fibronectin type II modules. A, Biotin-labeled
collagen (0.025 nmol) was incubated in the absence (C) or presence of 0.84 pmol N-MMP-2 (0) for 18 h
at 28 °C. MMP-2 CBD and MMP-2 LCD were added at the indicated molar equivalents (1-100) relative to
N-MMP-2. Collagen was analyzed by SDS-PAGE electrophoresis and visualized by Western blotting and
enhanced chemiluminescence using streptavidin-HRP. Intact \( \alpha_1 \) and \( \alpha_2 \) chains of the type I collagen are
indicated. B, DQ gelatin (1 \( \mu \)g) was incubated with APMA-activated N-MMP-2 (2.8 pmol) in the absence
(Control) or presence of MMP-2 CBD (446 pmol) for 2 h at 37 °C. I acknowledge Georgina Butler for
construction and production of N-MMP-2 enzyme.

A hemopexin C domain deleted form of MMP-2 (N-MMP-2) has been previously shown to retain
gelatinolytic and type IV collagenolytic activities (49) but the deletion did result in the loss of type I
collagenase activity (22). Given the effect of the MMP-2 CBD on the structure of collagen and in
enhancing collagenolysis by MMP-1 and MMP-8, we investigated whether exogenous MMP-2 CBD could
restore the collagenolytic activity of N-MMP-2 in trans. We generated N-MMP-2 and also found that it did
not cleave native type I collagen (Fig. 3.4A) confirming that the MMP-2 hemopexin C domain has a role in
triple helical collagen cleavage. However, unlike MMP-1 and MMP-8, the addition of exogenous MMP-2
CBD did not rescue MMP-2 collagenolytic activity, nor did the addition of exogenous MMP-2 LCD. To
further explore the mechanics of gelatin degradation we found that the cleavage rate of internally
quenched fluorescent-labeled gelatin by N-MMP-2 (Fig. 3.4B) was comparable with full length MMP-2
(Fig. 3.2A), confirming previous reports (41-44) that the hemopexin C domain is not required for
gelatinolysis. Also like full length MMP-2, exogenous MMP-2 CBD effectively reduced gelatin
degradation 5-fold (Fig. 3.4B) confirming the importance of the fibronectin type II modules in gelatin
binding and cleavage. Hence, these results demonstrate that in MMP-2 the linker/hemopexin C domain plays a role in collagenolysis despite a lack of affinity for collagen as an isolated domain.

FIG. 3.5 The additive effect of fibronectin type II modules in collagen and gelatin cleavage by MMP-2.  

A, Recombinant proteins (1 μg) CBD123, CBD23, and the CBD23 mutant W316A/W374A were analyzed by SDS-PAGE (15%) and stained with Coomasie R250 (0.1%).  

B, DQ gelatin (1 μg) was incubated with APMA-activated MMP-2 (2.6 pmol) in the absence (Control) or presence of MMP-2 recombinant proteins (446 pmol): CBD123, CBD23, and W316A/W374A, for 2 h at 37 °C.  

C and D, CBD23, and W316A/W374A were added with MMP-2 (2.6 pmol) at the indicated molar equivalents and incubated with biotin-labeled collagen for 18 h at 28 °C. Samples were separated by SDS-PAGE (7.5 %) and the collagen was visualized by infrared in gel imaging at 700 nm after the gels were incubated with streptavidin-Alexa680. Full-length type I collagen α(1)-chains (α1, α2), β-chains (β), and 3/4-fragments (β^A, α1^A, α2^A) are indicated. I acknowledge Todd Moore for construction and production of the W316A/W373A protein.

To directly show the importance of the fibronectin type II modules in MMP-2 gelatinolysis and collagenolysis, we generated a MMP-2 CBD mutant that does not bind collagen. In other work we have demonstrated that substitution of Trp^{316} and Trp^{374}—found at the base of the hydrophobic pit on the surface of the 2nd and 3rd fibronectin type II modules of MMP-2—with alanine abrogated gelatin and collagen binding in each of these modules. Recombinant CBD23 (Ala^{278}-Gln^{393}), which contains the 2nd and 3rd fibronectin type II modules only, and the non-collagen binding CBD23 mutant, W316A/W374A, were compared with recombinant MMP-2 CBD as a control (which for clarity in describing these experiments is referred to as CBD123). The recombinant proteins, W316A/W374A, CBD23, and CBD123...
CBD123 inhibited MMP-2 gelatin degradation ~3-fold (Fig. 3.5B). Using CBD23 the importance of all three fibronectin modules was observed. The absence of the 1st fibronectin type II module resulted in a ~33% reduction in gelatinolysis compared with MMP-2 controls incubated in the absence of exogenous CBD. As hypothesized, no inhibition was observed with the gelatin-binding null mutant CBD23, W316A/W374A (Fig. 3.5B). Fifty percent inhibition of MMP-2 cleavage of native type I collagen by CBD23 occurred at a molar equivalent of 8.5:1 domain to enzyme (Fig. 3.5C). In contrast, 50% inhibition of MMP-2 collagenolysis by W316A/W374A was only observed at very high mole ratios (85:1) (Fig. 3.5D). Hence, the fibronectin modules in the CBD cooperate to enhance collagen degradation by MMP-2.

IV. DISCUSSION

In view of the pivotal importance of collagen in the structure and function of the body, the control of its degradation is a critical aspect in the homeostasis of most tissues and organs. However, the role of the collagenolytic MMPs in cleavage of collagen matrices in vivo is unclear (50) due in part to the inappropriateness or availability of suitable mouse models. MMP-1 shows an extremely restricted expression pattern of two isoforms in mice that until recently were not believed to even exist in rodents (51). The MMP-13 knockout and transgenic mice have yet to be reported, whereas the MMP-8 knockout was only recently generated and found to have very little abnormalities in collagen degradation at sites of tissue challenge (52). Mmp2 knockout mice, though smaller at birth than wild-type littermates, develop normally without any apparent deficits in collagen turnover (53). However, in humans the Mmp2 gene mutations, R101H and Y244X, in three consanguineous families leads to a deficiency in MMP-2 expression (54) with afflicted individuals displaying severe growth restrictions and many skeletal defects (55,56). This indicates that in humans, MMP-2 is critical for the balance of bone matrix protein synthesis and degradation, in which type I collagen is the major structural element. The MT1-MMP knockout mouse also shows the critical importance of this proteinase for collagen metabolism in the skeleton and cartilage where severe developmental defects are manifested (26,27). These similar phenotypes are consistent with the spatial and functional association of MMP-2 and MT1-MMP on the cell surface in forming the MMP-2/MT1-MMP proteolytic axis and for their synergistic involvement in the cleavage of collagen in the pericellular matrix (5,29) as well as of cell surface receptors and cytokines involved in regulating connective tissue metabolism (57).

MT1-MMP is a potent endogenous activator of proMMP-2 that forms a ternary activation complex on the cell membrane with proMMP-2 linked via a TIMP-2 bridge (45,46,58-60)—binding outside the catalytic domain (61,62) on the lower outer rim of the hemopexin C domain (63,64). Collagen is an interesting and potentially unique pericellular matrix protein that initiates its own clearance. At high concentrations pericellular collagen interacts with cell receptors to induce MT1-MMP gene expression (4). Subsequent association between collagen and the MT1-MMP hemopexin C domain forms MT1-MMP multimers that leads to the activation of proMMP-2 (5). MMP-2 then completes the degradation of partially cleaved collagen and gelatin generated by MT1-MMP activity. Although membrane clustering of MT-MMPs
increases the efficiency of MMP-2 activation this also results in MT1-MMP autolysis to a cell surface bound 44-kDa form comprised of the linker/hemopexin C domain (65,66). We recently found that this isolated cell surface form of the MT1-MMP hemopexin C domain retains collagen binding properties and thereby functions in a dominant negative manner to suppress collagenolysis by both MT1-MMP and MMP-2, and to suppress MMP-2 activation (5). Hence, collagen may regulate its own levels in the pericellular environment by a unique dynamic feedback mechanism involving the regulation of the expression, activation and activity of the proteinases responsible for its degradation.

Despite these spatially and functionally intimately related activities of MT1-MMP and MMP-2 on the cell surface the mechanisms of collagen binding and cleavage occur quite differently in these two proteases. Whereas MT1-MMP utilizes the hemopexin C domain to bind native collagen and this is an absolute requirement for collagenolysis, MMP-2 binds collagen by the fibronectin type II modules. These modules form an alternate collagen binding domain to the hemopexin C domain, which in previous work (41,42) was shown not to bind native type I collagen. Hence, it was earlier concluded that the mechanisms of triple helicase activity of MMP-2 must be fundamentally different from the collagenases (29,42). Our present work supports this hypothesis and provides a mechanistic explanation for enzymatic cooperativity in collagenolysis. In MMP-2, a very different interaction with native collagen occurs from MT1-MMP that is driven by the MMP-2 CBD and not the hemopexin C domain. Using recombinant MMP-2 CBD in dominant negative experiments to reduce cleavage of both native and denatured collagen by MMP-2, we demonstrated the pivotal role for the fibronectin type II modules in MMP-2 collagenolysis. Mechanistically, we found by CD spectroscopy that recombinant MMP-2 CBD alone can induce a perturbation in the secondary structure of native collagen. This perturbation was identified to be a destabilization of the helix as the collagen became more susceptible to MMP-1 and MMP-8 cleavage. However, our data revealing the critical importance of the fibronectin type II modules in MMP-2 collagenolysis differ from those reported by Patterson et al. (22). Using a deletion approach, these authors reported that a MMP-2 mutant lacking the fibronectin type II modules (ΔCBD) could still cleave native type I collagen and so concluded that the fibronectin type II modules are not involved in this process. We have previously described native 3/4-collagen cleavage as the signature activity of MMP-5 (19) before identifying this protease as MMP-2 (19,20). The MMP-2 ΔCBD mutant also did not further process the native 3/4-collagen fragments into smaller fragments (22). However, it is unclear how MMP-2 would bind collagen in the absence of the fibronectin type II modules given that the CBD can quantitatively elute MMP-2 from collagen and gelatin (44). Moreover, it is unknown what the effects of such a large deletion are on the structural and functional properties of the enzyme toward macromolecular substrates despite MMP-2 ΔCBD showing very similar synthetic peptide cleavage kinetics to that of full length MMP-2. Patterson et al. (22) therefore proposed that the fibronectin type II modules are only required for gelatinolysis. Although our data is not in agreement with their overall findings, our competition experiments do support the role of the CBD in gelatin degradation as N-MMP-2 degraded gelatin with similar efficiency to MMP-2 and the addition of recombinant CBD inhibited gelatin degradation by both full length and N-MMP-2.
The fibronectin type II modules of MMP-2 have been structurally studied by NMR as a separate domain or individual modules (67-69) as well as in the context of the full-length proMMP-2 (70). These structures reveal that the three fibronectin type II modules form a compact domain, which we termed the collagen binding domain in view of its native collagen binding properties that distinguish it from gelatin binding domains only (44). It is situated adjacent to the active site cleft extending N-terminal from the S3' subsite, a region identified as being important for collagen cleavage in MMP-1 (71) and MMP-8. The three individual modules are joined by flexible linkers and face outward and can bind more than one collagen α-chain simultaneously (44). Each fibronectin type II module consists of two sets of double-stranded anti-parallel β-sheets perpendicularly arranged, connected by two loops and a short α-helix, stabilized by two disulfide bonds (67-70). The two β-sheets are arranged to form a hydrophobic surface, lined with aromatic residues. Mutational analysis (72) and NMR (67-69) have identified several aromatic residues that may be involved in gelatin binding. By mutagenesis we have also identified the nearby Phe295 and Trp316 in the 2nd fibronectin module and the homologous Phe53 in the 3rd fibronectin module to be critically important in gelatin binding. A substitution to alanine at these sites was found to eliminate the gelatin binding properties of each module. Using a CBD23 construct with the tryptophan mutations W316A/W374A that no longer binds gelatin nor blocks MMP-2 gelatinolytic activity in competition experiments, we specifically confirmed the importance of the collagen binding properties of the CBD in collagen and gelatin cleavage by MMP-2.

Despite the importance of the CBD, an enigmatic role for the MMP-2 hemopexin C domain remains since its deletion in the mutant N-MMP-2 eliminates native type I collagen cleavage as previously found (22). The addition of recombinant MMP-2 LCD to N-MMP-2 did not restore collagenolytic activity indicating that a contiguous tertiary structure is required for triple helical collagen cleavage. In this respect, the interaction of the MMP-2 LCD with collagen also differs from that of the MT1-LCD, which enhanced collagen cleavage by MMP-1 and MMP-8, and the hemopexin C domain of MMP-1 (71). In the latter report, these authors found that following the loss of collagenolytic activity upon removal of the MMP-1 hemopexin C domain a minor amount of collagenolytic activity of N-MMP-1 was restored with the addition of the MMP-1 hemopexin C domain in trans.

In isolation the MMP-2 hemopexin C domain does not bind native collagens (42). Therefore, this indicates that an interaction with collagen must occur in the context of the full-length enzyme. However, the mode of cooperativity between the MMP-2 catalytic domain and the hemopexin C domain is unclear, but may involve the formation of a collagen-binding groove between the catalytic and hemopexin C domains. In consideration of the 3D structure of MMP-2 (70) and the site of TIMP-2 interaction on the lower surface of the hemopexin C domain at the junction of blades III and IV (63), the most likely site for collagen binding resides on the upper surface of the hemopexin C domain that is immediately juxtaposed with the lower edge of the catalytic domain at the junction of hemopexin blades I and II as previously proposed (29). Near this location the linker peptide could also be potentially involved in collagen
interaction by analogy with its role in other collagenolytic MMPs (73) including MT1-MMP (5). However, the linker peptide itself does not confer the ability to bind collagen alone or in combination with the hemopexin C domain as it is present in the MMP-2 LCD used here and previously (42). Another mechanism of cooperativity in the full-length enzyme may involve the stabilization of the conformation of the lower subdomain of the catalytic domain, which may be required for triple helical collagen cleavage.

FIG. 3.6 Model of different collagen binding sites and triple helicase mechanisms of MT1-MMP, MMP-2, -1 and -8. A, Schematic of sMT1-MMP, MMP-2, MMP-1, MMP-8 and type I collagen. Enzyme domains and the collagenase cleavage site (775-776) are indicated, but not drawn to scale. B, Models for native collagen binding and unwinding by MMPs. MT1-MMP, MMP-1 and -8 utilize the hemopexin C domain to bind collagen in the vicinity of the cleavage site and to induce localized helix unwinding, whereas MMP-2 utilizes the CBD for this function. MMP-1 and -8 are also proposed to bind at a different site than MT1-MMP and MMP-2. For clarity, MT1-MMP and MMP-2 have been arbitrarily shown to bind C-terminal to the cleavage site while MMP-1 and -8 bind N-terminal, but it is equally as likely that these enzymes bind on the opposite sides. C, Binding and localized unwinding in the vicinity of the collagenase cleavage site by recombinant MT1-LCD and MMP-2 CBD. By competitive inhibition this interaction blocks collagen cleavage by MT1-MMP and MMP-2, respectively, but promotes enhanced cleavage by MMP-1 and -8, which are proposed to bind at a different site.
We have also revealed the importance of the linker peptide in collagen binding and structural perturbation of native collagen by MT1-MMP. Although the MT1-CD binds collagen, it does not block MT1-MMP collagen cleavage when added in competition experiments reported here and previously (5) nor did it disrupt the secondary structure of collagen as revealed by CD spectroscopy. In contrast, the MT1-LCD protein binds collagen, disrupts its secondary structure and competitively blocks MT1-MMP activity. We also recently found that the linker peptide alone neither binds collagen nor modulates collagenolysis by MT1-MMP (5). Together, this reveals that linker/hemopexin C domain cooperativity only occurs when in contiguous structure. Other work has also revealed the importance of the linker in collagenolysis (39,40) leading to earlier suggestions that the MMP-1 and MMP-8 collagenase linker intercalates with the individual α-chains of the collagen triple helix which allows for their sequential cleavage (29,32,74). Our data supports the hypothesis that the linker, with the hemopexin C domain, perturbs the secondary structure of collagen enabling cleavage of the individual α-chains to occur. Thus, this appears to be the mechanism of collagen triple helicase activity in MT1-MMP. Recombinant MT1-LCD, by binding to the same site that the linker and hemopexin C domain of MT1-MMP does when in context with the full length MT1-MMP, may mask the cleavage site or compete with MT1-MMP for binding like the CBD does with MMP-2 (Fig. 3.6A and C). However, since MMP-1 and MMP-8 showed enhanced cleavage in the presence of MT1-LCD, this also indicates that these proteases may bind collagen at a different site (Fig. 3.6B) but in the same localized region perturbed by the MT1-LCD domain interaction, thus facilitating collagen cleavage by these enzymes (Fig. 3.6C). Nonetheless, complete local denaturation of the collagen does not occur since trypsin and MMP-7, which are potent gelatinases, were unable to access individual α-chains in the perturbed collagen structure or displace the exosite domains and initiate cleavage of the native collagen. Thus, these results highlight the importance of the linker in this process since the MT1-CD did not induce structural perturbations in the collagen nor affect collagenolytic behavior of MMP-1, MMP-8, or MT1-MMP.

Notwithstanding our present data and the considerable amount of work from several labs investigating the mechanisms of collagen triple helicase, the mechanism of collagenase triple helicase activity remains elusive. Previously, we have proposed several potential mechanisms by which three-point binding of the collagen helix by the catalytic domain, the linker peptide and the hemopexin C domain drives collagen unwinding by the simultaneous binding at all three sites that can only occur upon perturbations being generated in the collagen structure (29) (Fig. 3.6B). That is, collagenolytic MMPs can bind native collagen at two sites but this alone does not generate helicase activity. In order to simultaneously fulfill the binding propensity of a third site the collagen has to bend or unravel in order to make this contact. In contrast, MMP-2 has evolved a different mechanism of collagen triple helicase activity. Due to the spatial orientation of each fibronectin type II module in the MMP-2 fibronectin domain (70), we hypothesize that the three modules bind and interdigitate within the collagen triple helix to splay the α-chains aside in preparation for cleavage (Fig. 3.6B). This activity would be driven by the binding preference of the fibronectin type II modules for denatured collagen versus native collagen (44) and so provides an alternative mechanism for the molecular tectonics of collagenolysis. However, fully unraveling the
mystery of collagen triple helicase activity most likely will only be forthcoming with the development of dynamic imaging techniques or other sophisticated approaches, without which only partial glimpses of the mechanism appear possible.

V. FOOTNOTES

3. Dean, R., Butler, G., an Overall, C.M. Manuscript in preparation

VI. REFERENCES


CHAPTER 4

Membrane Protease Proteomics: Isotope Coded Affinity Tag MS Identification of Undescribed Membrane Type-1-Matrix Metalloproteinase Substrates*

I. INTRODUCTION

In all living organisms, proteases exert high order post-translational control over a diverse range of cellular functions. Altered protease expression and substrate proteolysis are pivotal elements in the pathogenesis of many diseases (1) with 53 hereditary genetic diseases of proteolysis recognized (2). Indeed, proteases represent ~10% of current drug targets (3). Elucidating the substrate repertoire of a protease is critical to understanding its biological role, but given the large number of proteases (>553) present in the human genome (2), this is a daunting task. Innovative approaches using combinatorial or positional scanning libraries of fluorogenic (4) and inhibitory peptides (5), and oriented (6) and phage display (7) peptide libraries can determine consensus protease cleavage sequences. However, bioinformatic identification of proteins containing these sequences followed by biochemical and in vivo validation of proteolytic susceptibility has led to the identification of relatively few biologically relevant new substrates. This is not surprising since the majority of substrates in vivo are proteins and not peptides. Moreover, substrates must colocalize with proteases, spatially and temporally. While useful, these techniques are inherently limited in their power as they do not consider the influence of protein conformation, post-translational modification, protease exosites, and substrate availability in vivo (8). Serial analysis of protein libraries and "exosite scanning" by yeast two hybrid screens, although time consuming, have proven effective in identifying new protein substrates (9). Nonetheless, since proteases do not operate alone but more commonly in amplification cascades or regulatory circuits in the presence of a multitude of interacting proteins, substrates, and cleavage products, and often in distinct compartments, biologically relevant protease substrates may differ from theoretical activities inferred from in vitro experiments. In the face of such biochemical complexity this emphasizes the need to identify protease-cleaved substrates, and not just enzyme activities, in cells, tissues and whole organisms (10,11). Thus, rapid techniques of cleaved substrate discovery on a system-wide basis are needed to directly identify new substrates in complex biological settings and to quantitate differences in substrate processing as disease biomarkers and as surrogate markers of antiproteolytic drug treatment.

Proteomics offers the potential to identify protease substrates in complex biological samples in a system-wide approach that has been termed "degradomics" (12). However, apart from isolated reports (13), proteomic approaches for protease substrate identification have not been widely developed. In part, this may be due to the difficulty in relating cleavage products with parent protein spots on 2D gels or to mass

spectrometry (MS) spectral peaks. Difficulties in quantification also hinder comparative analyses. Recently, techniques have been developed that incorporate stable isotopes into proteins by metabolic or post-translational labeling (reviewed in (14)), thereby allowing MS quantification of the relative amounts of protein present in two samples (15). In isotope coded affinity tag (ICAT) labeling (16) proteins in two samples that are to be compared are reduced and labeled by reductive alkylation of cysteines using biotin-tagged reagents that are chemically identical but differ in isotopic composition and mass. The two isotopically distinct labeled protein samples are then combined and thereafter treated identically enabling quantitative comparisons to be made. Tryptic peptides are prepared and the biotin-tagged cysteine-containing peptides only are avidin column purified for mass determination. Spectral peak analysis of the isotopically resolved peptides from the two sources enables quantitation of the relative amounts of protein levels to be made. Differentially expressed proteins are then identified by tandem MS (MS/MS) sequencing in an iterative approach. ICAT has been recently refined by labeling with $^{13}$C$_9$ to avoid the slight alteration in hydrophobicity occurring in deuterium-labeled peptides and by use of cleavable linkers to remove the biotin moiety after elution.

Matrix metalloproteinase (MMP) 14, or membrane type (MT)1-MMP, is one of 23 members of an important protease family historically associated with the cleavage of extracellular matrix proteins (17). MT1-MMP is constitutively activated by furin processing, it is essential for normal growth and development (18), and is upregulated in cancer and arthritis (19). MT1-MMP degrades fibrillar collagen and fibronectin (20,21) and activates MMP-2 (gelatinase A) (22), which is also heavily implicated in cancer metastasis. However, like most proteases, the breadth of potential in vivo substrates and biological role of MT1-MMP is largely unknown. This is important to address since of all MMP gene knockouts, only the MT1-MMP-/- mouse exhibits strong developmental abnormalities (18).

Here we have developed a functional proteomic screen to identify new protease substrates in cell cultures. We hypothesized that the levels of individual proteins in conditioned culture medium would be differentially altered following cell transfection with MT1-MMP and thus would be amenable to ICAT and MS/MS identification. Following proteolysis, secreted proteins may be reduced in amount if degraded or processed and subsequently cleared following reductions in stability, and that other proteins may increase in quantity if proteolytically shed from the cell surface or released from the pericellular matrix. We report that ICAT is applicable to the proteomic analysis of proteolytic activity in complex cellular systems with the identification of multiple new signaling molecules as novel substrates of MT1-MMP.

II. EXPERIMENTAL PROCEDURES

Cell Culture. Human MDA-MB-231 breast carcinoma cells stably transfected with FLAG-tagged human MT1-MMP, a catalytically inactive MT1-MMP mutant (E240A) in which the active site Glu is replaced, or pCR3.1 vector alone under G418 selection were as described (21). MT1-MMP expression was confirmed by Western blotting and flow cytometry. MT1-MMP activity was confirmed by zymographic analysis of
MMP-2 activation when the transfected cells were incubated with human proMMP-2 expressed from Timp2-/- cells (23).

For protein analysis, confluent transfected MDA-MB-231 cells grown in DMEM, 10% FBS and G418 (1 mg/ml), were washed with PBS and incubated in DMEM alone to eliminate serum contamination. After 48 h the medium was replaced with DMEM and ascorbic acid (50 μg/ml). The conditioned medium was harvested (48h), protease inhibitors (6.7 μM EDTA, 1 mM PMSF, 10 μM leupeptin, 1 μM pepstatin A) were immediately added, and the conditioned medium clarified by centrifugation (30 min, 1000 x g) and filtration (0.22 μm). Amicon stirred cells and Centripreps™ (3-kDa cutoff, Millipore) were used to concentrate proteins 100-200x.

ICAT Labeling, Chromatography and MS/MS. Conditioned medium proteins (100 μg) from MT1-MMP-transfectants were labeled with isotopically heavy (13C)9-cleavable ICAT™ reagent (Applied Biosystems) (2h 37°C) for comparison with proteins from vector control or the E240A inactive MT1-MMP mutant-transfectant conditioned medium, which were labeled with the light (13C)0-ICAT reagent. Sample pairs were combined (MT1-MMP/vector or MT1-MMP/E240A), trypsin digested (18h 37°C), and fractionated on a cation-exchange column (4x15 mm) by elution with 350 mM KCl, pH 3.0 to remove the trypsin and free label. Labeled peptides were purified using an avidin-affinity column (4x15 mm) and eluted with 20% acetonitrile, 0.4% TFA. Dried samples were incubated in 95% TFA (2h 37°C) to cleave the biotin tag from the ICAT-labeled peptides. Samples were fractionated by multi-dimensional liquid chromatography (MD-LC), first on a 500 μm ID x 15 mm BioX-SCX column (LC Packings) and eluted as 50 μl fractions (25, 50, 75, 100, 150, 200, 250, 500, 1000, 2000 mM ammonium acetate, pH 4.0). Eluted peptides were concentrated and desalted on a 300 μm ID x 1 mm PepMap nano-trapping column prior to loading onto a 75 μm ID x 15 cm PepMap nano-separation column. Peptides were then fractionated using an acetonitrile gradient (0-64%, 35min) before entering the nano-spray ionization source (New Objective Inc). MS analysis of the separated peptides was performed using a QStar Pulsar™ I mass spectrometer (MD-Sciex, Genome BC Proteomics Centre). MS/MS fragmentation (2s, 65-1800 m/z) was performed on 3 of the most intense ions as determined from a 1 s survey scan (300-1500 m/z). ICAT ratios of isotopically heavy (13C)9- to light (13C)0-labeled tryptic peptides were determined using ProlCAT software and averaged for multiple peptides derived from a single parent protein. Using MASCOT, proteins were identified from peptide sequences queried against the NCBI nonredundant protein database with human sequence filtering. Labeling and analysis was performed 3 times.

Protease and Substrates. Soluble (s) human MT1-MMP lacking the transmembrane and cytoplasmic tail was expressed and purified (21). Human interleukin (IL)-8, growth related oncogene (GRO)-α and GRO-γ were chemically synthesized and purified (24). Human secretory leukocyte protease inhibitor (SLPI) (ICN) and human death receptor-6 fused via a Factor Xa site to the Fc region of IgG (R&D) were purchased. Human connective tissue growth factor (CTGF) was a kind gift from Dr D. Brigstock (Columbus, OH).
Human proTNFα fused to GST and a hydroxamate MMP inhibitor, BB2116, were provided by British Biotech. Human tissue inhibitor of metalloproteinases (TIMP)-1 and -2 were expressed and purified (25). Monoclonal antibodies against human fibronectin (MAB1891, Chemicon) and death receptor-6 (MAB1441, R&D) were purchased.

**Assays.** Recombinant and synthetic proteins were incubated with sMT1-MMP in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂, 3.8 mM NaN₃, 0.05% Brij, pH 7.4 for 18h at 37°C. Reaction products were separated by Tris-glycine or Tris-tricine SDS-PAGE and stained by Coomassie R-250 or Western blotted. The mass and sequence of the cleavage products were determined by MALDI-TOF MS and N-terminal Edman sequencing, respectively.

### III. RESULTS

**MT1-MMP Expression.** FLAG-tagged MT1-MMP and the 44-kDa form were detected on transfected cells (Fig. 4.1A,B), which activated exogenous proMMP-2 (Fig. 4.1C), confirming the presence of active MT1-MMP on the cell surface. Vector transfectants expressed only very low levels of endogenous MT1-MMP (Fig. 4.1S). No secreted active MMPs were detected using a general MMP quenched-fluorescent substrate (25) (not shown).

**ICAT and MD-LC MS/MS Analysis.** In comparing the relative abundance ratios of ICAT-labeled peptides from MT1-MMP transfectants with those from the vector or E240A MT1-MMP controls, we hypothesized that those proteins with an average labeled-peptide ICAT ratio <1.0 (MT1-MMP/control) had undergone MT1-MMP-mediated degradation or processing that triggered clearance; proteins with ratios >1.0 were hypothesized to have been shed from the cell membrane or pericellular matrix by MT1-MMP activity. Although ICAT is sensitive and highly reproducible (Abersold, 2003 #73), to reduce the probability of false positives we only analyzed proteins with ICAT ratios <0.5 or >1.5, which typically numbered from 100-150 identified proteins. Without inline MD-LC fractionation, the number of proteins so identified was reduced to less than 100. Intracellular proteins were not further considered. A selective list of the biologically interesting proteins that may be potential MT1-MMP substrates was generated from the two data sets (Table 4.1) based on deduced roles in cancer metastasis and embryonic development. These included protease inhibitors (SLPI and skin derived anti-leukoproteinase), chemokines (IL-8, GRO-α, GRO-γ, macrophage migration inhibitory factor), cytokines (TNFα, CTGF), cell receptors (death receptor-6, neuropilin-1), latent transforming growth factor binding protein-4S and complement component-3. Extracellular matrix proteins (fibronectin, EGF-containing fibulin-like extracellular matrix protein-1) and shed MT1-MMP and proMMP-1 (collagenase-1) were also elevated in the MT1-MMP cell conditioned medium.

**Chemokine Processing.** To test the hypothesis that ICAT can identify potential protease substrates we biochemically analyzed a number of the proteins from Table I for cleavage by sMT1-MMP using recombinant or synthetic pure protein as substrate. Since chemokines are a recently recognized class of
FIG. 4.1 Characterization of Cells Expressing MT1-MMP and Inactive Mutant E240A MT1-MMP. (A) Cell lysates from MT1-MMP, E240A and vector-transfected MDA-MB-231 cells were analyzed by SDS-PAGE (10%) and Western blotting. Full length MT1-MMP (55 kDa) and the 44-kDa form are indicated. (B) Cells labeled with either α-FLAG (Sigma) or α-human MT1-MMP antibody (AB815, Chemicon) (dark line) or no primary antibody (light line) were analyzed by fluorescent flow cytometry on a FACScan. (C) Media from MT1-MMP and vector-transfected cells, with or without exogenous proMMP-2 (72 kDa) added, were analyzed by gelatin zymography. Fully activated (62 kDa) and activation intermediate (68 kDa) MMP-2 are indicated. I acknowledge Yi Wu for performing the Western blot analysis and Charlotte Morrison for performing the flow cytometry and gelatin zymography analysis.
Table 4.1 Identification by ICAT and MS/MS Analysis of Cellular Proteins in the Conditioned Medium of MDA-MB-231 Cultures Displaying Altered Abundance due to MT1-MMP Activity.

<table>
<thead>
<tr>
<th>Protein*</th>
<th>MT1-MMP/control mean ratio</th>
<th>Peptide sequences§</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLPI</td>
<td>4.95</td>
<td>CCMGMCGBK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YKKPECQSDWQCPGK</td>
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<td></td>
<td></td>
<td>CVNHYGGYLCLPK</td>
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<tr>
<td>EFEMP-1</td>
<td>3.90</td>
<td>VCSSCPVGTFTTR</td>
</tr>
<tr>
<td>DR-6</td>
<td>3.79§</td>
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<td></td>
<td></td>
<td>GEWTCIAYSQLR</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>ProMMP-1</td>
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<td></td>
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<tr>
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<td></td>
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</tr>
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</tr>
<tr>
<td></td>
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</tr>
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</table>

*The following protein names are abbreviated: SLPI, secretory leukocyte protease inhibitor; EFEMP-1, EGF-containing fibulin-like extracellular matrix protein-1; DR-6, death receptor-6; SKALP, skin derived anti-leukoproteinase; CTGF, connective tissue growth factor; LTBP-4S, latent transforming growth factor binding protein-4S; TNFα, tumor necrosis factor-alpha; TIMP-1, tissue inhibitor of metalloproteinase-1; MIF, macrophage migration inhibitory factor; GROα, growth related oncogene-alpha; IL-8, interleukin-8; GROγ, growth related oncogene-gamma; C3, complement component 3.

†Control protein was from vector transfected cells unless stated otherwise.
### FIG. 4.2 MT1-MMP Processing of IL-8 and SLPI.

(A-C) CXC chemokines or SLPI were incubated with sMT1-MMP for 18h at 37°C. Samples were then separated on 15% Tris-tricine gels under non-reducing conditions. (A) MT1-MMP cleavage of IL-8 and sequence of the cleavage site. (B) MT1-MMP did not cleave GRO-α or GRO-γ as analyzed by SDS-PAGE. MALDI-TOF MS revealed identical masses for both chemokines in the presence or absence of MT1-MMP. (C) SLPI was detected by Western blotting using α-SLPI antibody. SLPI cleavage was blocked by TIMP-2 added with sMT1-MMP or after preincubation (not shown). N-terminal sequences are shown. I acknowledge Charlotte Morrison for performing the cleavage assay of SLPI.

MMP substrates (9,26-28) it was of interest that the chemokines IL-8, GRO-α and GRO-γ showed decreased levels upon MT1-MMP expression. N-terminal sequencing confirmed the proteolytic removal of the first 5 residues of IL-8 by sMT1-MMP in vitro to generate a truncated form beginning with the sequence SAKELR (Fig. 4.2A). However, SDS-PAGE and MALDI-TOF showed no change in mass for GRO-α, and GRO-γ following incubation with sMT1-MMP (Fig. 4.2B). Complement component-3, which...
displayed a similar ICAT ratio to GRO-γ (Table 4.1), was also not susceptible to MT1-MMP proteolysis in vitro (not shown) revealing that MT1-MMP expression can decrease the levels of these proteins by indirect means.

FIG. 4.3 MT1-MMP Shedding of Fibronectin and Death Receptor-6. (A) MT1-MMP and vector transfected MDA-MB-231 cells were incubated in serum-free DMEM with or without inhibitors (TIMP-1 & 2, 100nM). Shed fibronectin was then purified from the 48-h media using a gelatin-Sepharose column and eluted with 8 M urea in PBS before analysis by SDS-PAGE (5%) and Western blotting with α-fibronectin monoclonal antibody. (B) Fibronectin was incubated with sMT1-MMP (+,++) in vitro for 18h at 37°C. (C) DR6/Fc fusion protein was treated with increasing amounts of sMT1-MMP and separated by SDS-PAGE (10%). Cleavage products are indicated by arrows. (D) DR6/Fc was cleaved with Factor Xa (5h, 37°C) prior to digestion with sMT1-MMP (18h, 37°C). Samples were then electrophoresed and Western blotted using α-DR6 antibody. Factor Xa (1) and MT1-MMP (2) cleaved-DR6/Fc products are shown.

**Sheddase Activity.** Levels of the secretory leukocyte protease inhibitor, SLPI, were greatly increased upon MT1-MMP expression (Table 4.1). Biochemical assays revealed that sMT1-MMP cleaved SLPI in a TIMP-2-inhibitable manner to generate a single 12.3-kDa product with an N-terminus of SGKSFKA (Fig.
4.2C). This sequence is identical to the intact inhibitor and so cleavage occurred near the C-terminus, which may release the cleaved SLPI from association with the cell layer proteins.

Medium levels of fibronectin, a known substrate of sMT1-MMP (20), were also significantly elevated upon MT1-MMP expression (Table 4.1), implying that MT1-MMP may shed fibronectin from the cell surface. This was confirmed by gelatin-Sepharose affinity purification and quantitation of fibronectin from the conditioned medium of the transfectants (Fig. 4.3A, B). Fibronectin shedding was inhibited by adding TIMP-2 to the cultures, but not TIMP-1. Since TIMP-1 spares MT1-MMP in its inhibitory profile, this confirms the specific requirement for MT1-MMP activity in the proteolytic release of fibronectin from the cell surface. Shed immunoreactive protein, detected using an antibody that recognizes the 15th type III module of fibronectin, migrated with the expected molecular weight (220 kDa +DTT) of the full length protein (Fig. 4.3A); cleaved fibronectin fragments were never observed in the conditioned medium; and the ICAT-labeled peptides $^{67}$TYLGNALVCTCYGGSR$^{84}$ and $^{2199}$LCCQLGFGSGHFR$^{2214}$ (Table 4.1), which

![MT1-MMP Processing of ProTNFa and CTGF](image-url)

**FIG. 4.4** MT1-MMP Processing of ProTNFa and CTGF. (A) ProTNFa/GST fusion protein was incubated with sMT1-MMP (18 h 37°C) with or without BB2116. Samples were separated by SDS-PAGE (5-15%). MT1-MMP cleavage sites within the TNFa pro-region were determined by N-terminal sequencing as schematically shown. Mature TNFa commences at VRSSSRT. (B) CTGF was treated with sMT1-MMP (+,++) for 18 h at 37°C and analyzed by SDS-PAGE (15%) and MALDI-TOF. MT1-MMP cleavage sites were located by N-terminal sequencing to be in the CTGF linker. The IGF binding protein module (IGFBP), von Willebrand factor type 1C module (vWFC-1), thrombospondin 1 module (TSP-1) and the cysteine-knot containing C-terminal module (CYS) of CTGF are indicated. Insufficient protein was present to sequence the 18.8-kDa fragment. No sequence was attainable for intact CTGF, indicative of a modified N-terminus. I acknowledge Chris Overall for performing cleavage assay of pro-TN Fa/GST.
are proximal to the N and C-termini of the molecule, displayed similar abundance ratios to the other fibronectin peptides. Since the solubilized form of fibronectin is intact, overexpressed MT1-MMP did not appear to degrade fibronectin in a cellular context, despite its activity on this protein in biochemical assays (Fig. 4.3B). Although processing of fibronectin cannot be discounted as a shedding mechanism this indicates that cleavage of a fibronectin-binding cell surface molecule by MT1-MMP is the mechanism for release.

Death receptor-6 is a member of the TNF receptor family and consists of a 350-residue ectodomain and a cytoplasmic death domain (29). Using a death receptor-6 fusion protein, DR6/Fc, as a substrate for in vitro assays with sMT1-MMP we found loss of intact protein and generation of two immunoreactive fragments at 55 and 50 kDa (Fig. 4.3C). To verify that cleavage occurred within the ectodomain and not the Fc region, DR6/Fc was first treated with Factor Xa to cleave a susceptible site between the ectodomain and the Fc region. Upon subsequent incubation with sMT1-MMP in vitro, a 25-kDa product was generated from the 40-kDa ectodomain confirming susceptibility of DR6 to MT1-MMP proteolysis (Fig. 4.3D).

Cytokine Substrates. The inflammatory cytokine, TNFα, had a MT1-MMP/vector ICAT ratio of 1.29 that indicated shedding to the medium. Although this was below our arbitrary 1.5 ICAT ratio cutoff, proTNFα can be shed by MMP-7 (30). Therefore, the susceptibility of proTNFα to MT1-MMP processing was investigated using a proTNFα/GST fusion protein as substrate. This was cleaved by sMT1-MMP to generate a product corresponding to the correct size of mature TNFα (~16 kDa) (Fig. 4.4A). N-terminal sequencing revealed that in addition to cleavage at the Ala76-Val77 bond, which generates the fully active TNFα, 3 other bonds in close proximity in the pro-region of TNFα were also processed by sMT1-MMP in vitro (Fig. 4.4A).

Lastly, we analysed recombinant CTGF, a member of the CCN (CTGF/cysteine-rich 61/nephroblastoma overexpressed) family of extracellular matrix-associated signaling molecules (31) for cleavage by sMT1-MMP. Three distinct lower molecular weight fragments were generated (Fig. 4.4B). Sequencing of the 17.5 and 16.2-kDa sMT1-MMP cleavage products revealed an identical N-terminus, YRLEDT, corresponding to cleavage at the Ala181-Tyr182 bond (Fig. 4.4B).

IV. DISCUSSION

High throughput protein quantitation and identification by ICAT and MD-LC MS/MS is a powerful technique that we have adapted for the proteomic investigation of protease function and substrate discovery in a dynamic cellular milieu. In MDA-MB-231 cells, we specifically identified a number of proteins that were biochemically confirmed as novel substrates of MT1-MMP thereby validating the utility of ICAT MD-LC MS/MS for substrate discovery in cell based systems. The majority of substrates discovered to date have been from serial approaches using available proteins or hypothesis-driven intuition using time-consuming biochemical and genetic approaches or peptide libraries and
bioinformatics (12). The alternative of using proteomic techniques to screen for novel protease substrates is advantageous because of its coverage on a system-wide scale, speed, and resolution of even complex biological samples. However, to date there have been few proteomic studies except for using 2D-PAGE and stable isotope dilution (13). In comparison to gel-based analyses, liquid chromatography mass spectrometric approaches provide more extensive and rapid proteome coverage, the abundance of isotopically-labeled peptides can be quantitated, and cell membrane proteins are amenable for MS analysis (reviewed in (14)). Nonetheless, protein abundance sets detection limits and until improvements are made in sample preparation and chromatography prior to mass spectrometry it is not yet possible to localize cleaved substrates or to analyze whole tissues by ICAT because of the added sample complexity and variability. Moreover, differences in protein abundance as measured by ICAT must be interpreted with caution—some cleaved substrates may not change levels and the levels of some uncleaved proteins may alter just by expression of the transfected gene—an important caveat for interpreting any study using protease-transfected cells. Thus, ICAT and MD-LC MS/MS should be considered as a screen for potential protease substrates that must be confirmed biochemically and in vivo when possible, but offers general applicability to other protease classes for functional proteomic investigation of protease function in complex cell samples.

The pool of potential substrates available to MT1-MMP expressed in MDA-MB231 cells will contain overlapping candidates with normal mammary epithelium as well as unique targets. The majority of proteins identified as potential MT1-MMP substrates were not extracellular matrix molecules. Rather, the levels of a variety of signaling proteins were specifically modulated upon expression of MT1-MMP. Of these, IL-8, GRO-α and GRO-γ are important for the regulation of leukocyte migration during wound healing (32,33). MT1-MMP is also known to participate in wound healing and is expressed by monocytes. IL-8, GRO-α and GRO-γ belong to the subgroup of CXC chemokine family members that contain the ELR motif near the N-terminus, which is essential for neutrophil chemotactic activity (34). MT1-MMP processing of IL-8 removes the first 5 residues, which is more active than the untruncated form. In contrast to MMP-9, which processes a different site in IL-8 and also GROα (28), MT1-MMP does not cleave GRO-α or GRO-γ demonstrating selective susceptibility of CXC chemokines to MMP cleavage. Therefore, the reduction in the levels of these chemokines appears to be an indirect upstream response to the expression of MT1-MMP, but biologically may be as important a consequence of MT1-MMP activity.

A number of protease inhibitors, including the plasma serine protease inhibitor SLPI, are also essential for normal wound repair. SLPI suppresses monocyte MMP production and is resistant to neutrophil collagenase (35). Here, ICAT identified SLPI as a potential MT1-MMP substrate which we biochemically confirmed was processed near its C-terminus. Since the inhibitory site of SLPI lies within the C domain (36) this reveals a novel intersection between the serine and metallo proteases in which the activity of a serine protease inhibitor is predicted to be abrogated upon MT1-MMP proteolysis.
Expression of recombinant proteases may occur at levels greater than that seen in some normal tissues thereby favoring proteolysis of less preferred or low abundant substrates. Although sMT1-MMP can degrade fibronectin in vitro, we found that in cell culture MT1-MMP activity only led to the shedding of intact fibronectin from the MDA-MB-231 cell surface—suggesting that the levels of the recombinant MT1-MMP expressed were not artificially extreme or generated cleavage artifacts. This result not only highlights the importance of biochemically confirming proteomic data, but equally important is the biological validation of biochemically determined proteolytic activity with cell-based and in vivo studies at the genetic or proteomic levels. Although the mode of fibronectin release is not clear, it has been reported that MT1-MMP degrades transglutaminase, a fibronectin co-receptor, thereby reducing both the adhesion and migration of cells on fibronectin (37).

We also found that MT1-MMP cleaved the ectodomain of death receptor-6, a transmembrane receptor that regulates apoptosis through its cytoplasmic death domain and activation of the TRADD/FADD/Caspase-8 pathway (29). Although the purpose of our study was to develop a novel proteomic approach for protease substrate discovery and not to fully explore here the biological ramifications of the cleavage of newly identified substrates, it is reasonable to hypothesize that MT1-MMP proteolysis of the ectodomain may regulate death receptor 6 signaling and apoptosis. Ligands that bind death receptor-6 belong to the TNF family of cytokines, one of which, TRANCE, is cleaved by MT1-MMP (38). Although ADAM-17 is the enzyme thought to be primarily responsible for the generation of mature TNFα from a membrane bound precursor, particularly under LPS stimulation (39,40), MMP-7 can also process TNFα (30). Here, we extend the repertoire of MMPs that can cleave proTNFα by showing that MT1-MMP cleaves at the Ala^76^-Val^77^ bond within the pro-region of TNFα to generate mature, fully active TNFα—a potentially important mechanism in non-LPS induced inflammation.

CTGF was also identified as a potential MT1-MMP substrate by ICAT that we then showed was cleaved by sMT1-MMP in vitro at the Ala^181^-Tyr^182^ bond. This site is within the linker that connects the insulin-like growth factor binding protein domain and von Willebrand factor type-1C domain of CTGF with the thrombospondin-1 and C-terminal domains (Fig. 4.3B). Cleavage here potentially disengages matrix-binding domains from the signaling functions of CTGF leading to release from the cell layer. Hashimoto et al (41) also recently described CTGF cleavage by soluble MMPs 1, 3, 7 and 13 at Met^194^-Ile^195^ within the CTGF linker, with a minor cleavage site at Ala^181^-Tyr^182^ . CTGF is unstable in vivo being converted into low molecular weight forms (10–20 kDa) by an unidentified protease (42). Interestingly, conditioned medium from serum-stimulated mouse fibroblasts did not degrade CTGF (43) suggesting that a cell surface protease was responsible for CTGF processing. These results are consistent with our data demonstrating that the cell surface MT1-MMP can process CTGF in cell culture to generate ~17-kDa fragments.

The novel substrates identified here reveal that MT1-MMP may be an important cell signaling protease and adds to recent studies describing new MMP substrates that are redefining our outlook of the biological role of this protease family (12). Traditionally, MMPs have been viewed as enzymes of
catabolism being primarily involved in the degradation and turnover of the extracellular matrix (17). MMPs are now known to process a diverse range of extracellular proteins other than those of the matrix (44). Like other proteases MMPs, regulate and refine many aspects of protein function including activity, localization, shedding, exposure of cryptic binding sites and release of neoproteins and thereby achieve precise control over many cell processes. Thus, elucidating the substrate repertoires or degradomes of proteases is essential for understanding their in vivo roles. By establishing the physiological function of a protease, its role in pathology can be more readily ascertained. This in turn is a necessary prerequisite for drug target validation. No one technique will provide complete coverage of the protease substrate landscape, but overall MD-LC ICAT MS/MS provides a powerful new functional degradomic approach that renders the quantitative analysis of proteolysis in defined cell cultures feasible and one that should be generally applicable to elucidate the biological role of other proteases.

V. REFERENCES


38. Schlondorff J, Lum L, Blobel CP: **Biochemical and pharmacological criteria define two shedding activities for TRANCE/OPGL that are distinct from the tumor necrosis factor alpha convertase.** *J Biol Chem* 2001, 276:14665-14674.


CHAPTER 5:

Conclusions and Future Directions

The work I have presented in this thesis has focused largely on the topic of MMP substrates—an aspect that is central to our understanding of MMP biology. More specifically, I have researched how a substrate such as native collagen is recognized and cleaved by MMPs and the possible biological implications of this interaction. Further discussion of recombinant domains for the study of exosites will be given here as well as our future directions regarding our study of MT1-MMP collagen cleavage. Though I have studied MT1-MMP-mediated shedding and degradation of extracellular proteins using ICAT, there have been several improvements to 2D-GE for quantitative protein analysis. This and how ICAT can be improved for studying proteolysis will also be discussed here.

I. RECOMBINANT DOMAINS TO STUDY MMP FUNCTION

The use of recombinant domains is a large part of the work presented in the Chapters 2 and 3. By expressing the domain of a MMP as recombinant protein, one can simplify the design of experiments and the interpretation of data compared to the full-length enzyme. In addition, unequivocal assignments of binding sites and functionality can be made. However, a disadvantage is that new properties of the domain may be revealed, such as a hydrophobic surface that is buried in the full-length protein, when the recombinant form is expressed in isolation. Despite careful selection of the recombinant domain boundaries, the loss of contiguous tertiary structure may affect functionality such as case where a binding site spans a designated boundary. The alternative of using deletion or truncated enzymes is informative for loss of function studies but provides indirect evidence in regards to the properties of the deleted domain, as there may be the potential for structural perturbations in the mutant enzyme. The best approach in assessing function directly is site-directed mutagenesis as it has the least potential for inducing changes in the MMP structure. However, in the absence of a rational strategy, this approach can be laborious. Characterization of recombinant domains can help in the rational design and screening of mutations, an approach that I have used for investigating collagen binding by the MMP-2 fibronectin type II modules (Chapter 3).

Another useful feature of recombinant domains is their ability to act as dominant negative mutants in inhibiting specific enzyme function. For example, the recombinant MMP-2 hemopexin C domain blocked binding of MMP-2 to αvβ3 integrin and reduced angiogenesis (1), while the recombinant MMP-9 hemopexin C domain reduced MMP-9 gelatin degradation and tumor cell invasion (2). I demonstrated that membrane-tethered and soluble recombinant MT1-MMP hemopexin C domain reduced tumor cell invasion through type I collagen (Chapter 2) and the recombinant fibronectin type II modules of MMP-2 blocks collagen and gelatin degradation (Chapter 3). This use of dominant negative mutants potentially represents a new therapeutic approach for inhibiting MMP activity. Pfeifer et al (3) demonstrated that...
delivery of MMP-2 hemopexin C domain to endothelial cells by using lentiviral vectors suppress angiogenesis in several animal and human models.

Characterization of recombinant MMP domains can also benefit in the design of new MMP inhibitors. There is considerable interest in developing MMP inhibitors that do not target the active site as the major setback of active site MMP inhibitors has been the lack of specificity, which results in adverse side effects. Targeting MMP exosites using small molecule inhibitors such as organic molecules or peptides has great potential for improving specificity by disrupting specific MMP/substrate interactions only. Recently, Bjorkland et al. (4) recently demonstrated that a peptide, CGYGRFSPPC, binds the fibronectin type II modules of MMP-9 and inhibits gelatin degradation. To our knowledge, this is the first report of an MMP exosite inhibitor. Molecules that target non-substrate binding sites have also been shown to modulate MMP activity. In the same report, the authors found the peptide, CRVYGPYLLC, which binds the hemopexin C domain of MMP-9, blocks αvβ3 integrin binding and tumor cell invasion into Matrigel (4). Similarly, a small organic molecule that binds αvβ3 integrin blocks MMP-2 binding and inhibits angiogenesis (5). Thus, there appears to be therapeutic potential in targeting MMP exosites and non-substrate binding sites as an alternative to the active site.

II. MMP BINDING AND CLEAVAGE OF COLLAGEN

Previously, in vitro binding studies using the recombinant domains of MMP-2, MT1-MMP, TIMP-2 and TIMP-4 domains has led to the dissection of many details of MMP-2 activation pathway (6-12). Using the MT1-MMP recombinant hemopexin C domain as dominant negative mutant, I have further explored this pathway in the context of pericellular collagen (Chapter 2). In addition, an updated model for the degradation of native type I collagen at the pericellular interface by a proteolytic axis involving the cooperative activity of MT1-MMP and MMP-2 (Fig. 9, Chapter 2) was proposed. Critical to this model is collagen recognition and cleavage by MT1-MMP and proMMP-2 activation. To test the validity of this model I propose to generate a mutant of MT1-MMP that is deficient in collagen binding using site-directed mutagenesis approach. Based on our evidence that the hemopexin C domain and the linker peptide of MT1-MMP are involved in collagen binding and cleavage (Chapter 2), I hypothesize that amino acid substitutions in these two domains alone or in combination would disrupt collagen binding to produce a non-collagenolytic enzyme. In the absence of a crystal structure of a MMP with a collagen substrate, molecular modeling can aid in the design of our mutagenesis (reviewed in (13)). While the structure of the MT1-MMP catalytic domain structure has been solved in complex with TIMP-2 (14), the hemopexin C domain of MT1-MMP has yet to be determined. However, since all MMP hemopexin C domain structures described to date are very similar (15), known structures of isolated hemopexin C domains and full-length structures of MMP-1 (16) and -2 (17) can be used as templates to model the full-length MT1-MMP molecule, which can then be used to dock with known structures of triple-helical peptide substrates as demonstrated previously for MMP-1 (18) and MMP-8 (19) in order to predict critical residues involved in collagen binding. One would predict that introduction of this collagen null-binding MT1-MMP mutant into our system would result in decreased MMP-2 activation due to the lack of proMMP-2 release from MT1-
MMP cleaved native collagen and reduced MT1-MMP clustering on the cell surface as predicted in the model (Fig. 9, Chapter 2). In Chapter 3, I demonstrated that unlike wild-type MMP-2 CBD23, a mutant protein in which residues Trp316 and Trp374 have been replaced with alanine, does not block MMP-2 degradation of native and denatured collagen. To unequivocally determine if these residues are critical for collagen and gelatin cleavage, the following single, double and triple mutations: W258A, W258A/W374A and W258A/W316A/W374A, were made in the cDNA of full-length MMP-2. The hypothesis is that these three mutants will display various degrees of deficiencies in substrate binding and cleavage compared to wild-type MMP-2. These mutant proteins will also allow us to systematically determine the contribution of each of the three fibronectin modules to MMP-2 function.

III. PROTEOMICS FOR MMP SUBSTRATE IDENTIFICATION

In understanding the biological roles of MMPs in vivo, one of the most significant challenges is the identification of all MMP substrates, potential and actual. Previous reports (20,21) have successfully shown that comparative or quantitative proteomics can be used to assess proteolytic activity in vivo and for substrate identification. Two-dimensional gel electrophoresis is a valid method for studying proteolysis (20). Compared to ICAT, which provides at best indirect evidence of post-translational modifications, 2D-gels are suited for analyzing the proteolytic cleavage of proteins directly due to its separation principles (22). Comparative analysis using 2D-GE in the past required that the two different protein samples were resolved on separate gels, which were then stained and scanned digitally for comprehensive analysis of spot intensity and patterns using software (23). However, there are many difficulties associated with this method as a result of irreproducibilities in protein staining and electrophoresis (24). This has been improved recently with the development of difference gel electrophoresis (DIGE) (25). In this technique, protein samples to be compared are differentially labeled with spectrally distinct fluorescent dyes and then separated on the same gel thus avoiding many technical issues with spot matching. Once resolved, the gel is scanned at different wavelengths in order to visualize the two samples.

The advantage of ICAT•MD-LC•MS/MS over 2D-gel analysis, is that proteome coverage is more extensive due to the enormous resolving power of MD-LC, which does not discriminate against the hydrophobicity, pi, or molecular weight of a protein. However, proteins lacking cysteines are invisible to the method while proteins low in cysteine residues are not well represented. Despite these limitations I was able to demonstrate that ICAT was amendable for studying protease activity in cells (Chapter 4). Though indirect, relative protein quantitation using MS provided a method to screen for proteolytic modifications upon MMP overexpression. From the screen, several proteins were biochemically confirmed as MMP substrates. Nonetheless, conventional ICAT is generally not thought to be well suited for direct analysis of post-translational modifications such as proteolysis as it is unable to distinguish among various protein isoforms (parent and cleaved substrates).

To address this issue, new stable isotope reagents and protein targeting strategies are being developed that may allow for direct mass spectrometric identification and quantification of cleaved substrates. One
possible approach is to label all unmodified protein N-termini which would include the neo N-termini of cleaved proteins using a primary amine specific ICAT reagent. The identity of cleaved proteins as well as the cleavage site can be determined by MS/MS analysis, while the ICAT analysis provides quantitative information about the cleavage. However, there are several technical challenges to this method. Most reagents that will derivatize the N-terminus will also modify the amino groups of lysine residues (26,27). Because trypsin does not cleave at modified lysine residues, peptides generated from a tryptic digest may be relatively large and unsuitable for MS analysis. An alternative may be to use other enzymes such as glu-C or chymotrypsin to digest the proteome following N-terminal labeling. There is also an issue of sample complexity and the confidence in protein identification given that there will be only one labeled peptide per protein. However, perhaps by performing extensive fractionation using MD-LC, the number of peptides entering the MS can be decreased thus improving mass measurements for subsequent database searching.

Another option for identifying cleaved substrates is labeling at the C-terminus. This is usually accomplished enzymatically through the tryptic digestion of proteins in the presence of $\text{H}_2^{18}\text{O}$. By digesting control and experimental samples in $\text{H}_2^{16}\text{O}$ and $\text{H}_2^{18}\text{O}$, respectively, quantitative analysis of proteins can be made (28). Trypsin (29), chymotrypsin (30), lys-C (28) and glu-C (31) all introduce two $^{18}\text{O}$ (4 amu) per peptide. This is because these enzymes continually form ester intermediates with peptide until both $^{16}\text{O}$ on the carboxyl group have been replaced. It would be interesting to determine if MMPs, which do not form acyl intermediates, would incorporate two $^{18}\text{O}$ as well. The major advantage for using this method for MMP substrate identification is that only proteins that are cleaved by the MMP are labeled with $^{18}\text{O}$. However, in this regard, digestion can only be performed in vitro under strict conditions in order to reduce background proteolysis, which can lead to false positives. Whether or not these conditions are attainable is not known. Thus, this approach maybe better suited for studying overall MMP activity of a system versus the activity of a specific family member. Nonetheless, this method has the potential to directly identify substrates and sites of proteolysis, and to quantify amount of cleaved substrates in complex samples, and may complement N-terminal labeling.

In summary, my investigation into the subtleties of how MT1-MMP and MMP-2 recognize and cleave native collagen could set paradigms for collagen cleavage by collagenases or gelatinases. The study of MMP exosites could also aid in the development of new MMP inhibitors to target exosites. These inhibitors are predicted to show greater specificity than active site inhibitors and may possibly lead to fewer side effects. Proteomics has great potential to lead the MMP field in new directions. Already MMPs are becoming increasingly recognized as signaling proteases in addition to their role in matrix degradation. Techniques such as ICAT will continue to shed new light and expand our knowledge of MMPs and their biological roles. Thus, despite over 30 years of research, MMP biology continues to become larger and more complex than we previously imagined.
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