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TITLE:  Cell Surface Regulation of Matrix Metalloproteinases in Breast Cancer Cells

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Metastasis is the major cause of death in breast cancer patients and is partly caused by the action of proteolytic enzymes that degrade extracellular matrix (ECM). We have focused on the gelatinases, MMP-2 and MMP-9, two ECM-degrading enzymes that are members of the matrix metalloproteinase (MMP) family of proteases. The gelatinases are associated with the surface of breast cancer cells. MMP-2 surface binding plays a role in activation by MT1-MMP, a membrane-bound MMP that is also expressed in breast cancer. MMP-2 activation is mediated by the action of TIMP-2, a metalloproteinase inhibitor. However, the role of TIMP-2 in pro-MMP-2 activation by MT1-MMP is not clear. Although MMP-9 is identified on the cell surface, the mechanism of MMP-9 surface association and regulation remains to be investigated. The research of the Academic Award during the second year has focused on the mechanisms involved in pro-MMP-2 activation and MMP-9 surface association. We report a detailed characterization of the activation of pro-MMP-2 and the interactions of TIMP-2 and TIMP-4 with MT1-MMP during this process. We also carried phage display studies to identify MMP-9-binding proteins.
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INTRODUCTION

Background. The matrix metalloproteinase (MMP) family of endopeptidases has been shown to play a central role in the promotion of tumor invasion and angiogenesis in breast carcinomas (1, 2) by their ability to mediate the degradation of extracellular matrix (ECM) components as well as non-ECM proteins. A key aspect of proteolytic degradation in cancer cells involves the targeting of enzymes to the cell surface. Studies with various protease systems have shown that surface association of ECM-degrading proteases in cancer cells regulates enzyme activity and interactions with protease inhibitors (3, 4). To achieve their full potential in pericellular proteolysis, the members of the MMP family evolved into secreted and membrane-tethered multidomain enzymes by incorporating distinct domains that facilitate binding to ECM components and surface molecules in the case of soluble MMPs such as the gelatinases (MMP-2 and MMP-9) and unique domains that anchors the enzyme to the cell surface, in the case of the MT-MMPs.

The gelatinases (MMP-2 and MMP-9) represent a subgroup of MMPs that can degrade basement membrane collagen IV and therefore they have been implicated in metastasis in many human tumors including breast cancer (1). As soluble MMPs, the gelatinases developed unique mechanism of surface association. For example, the latent form of pro-MMP-2 binds to a surface complex of MT1-MMP with TIMP-2, which plays a role in activation of pro-MMP-2 (5). Pro-MMP-9 binds to the cell surface via the α2(IV) chain of collagen IV or via the hyaluronic receptor CD44 (3). However, their roles in MMP-9 regulation are not known. Furthermore, other surface-binding proteins may exist.

MT1-MMP is a member of the MT-MMP subfamily of MMPs that is anchored to the cell surface by the presence of a transmembrane domain (6). MT1-MMP is expressed in both breast cancer cells and in the tumor stroma (7). As membrane-tethered MMP, MT1-MMP plays a key role in pericellular proteolysis and is a known activator of pro-MMP-2 on the cell surface. The activation process involves the action of TIMP-2, which binds to active MT1-MMP forming a surface “receptor” for pro-MMP-2. The interactions of TIMP-2 with MT1-MMP and the role they play in pro-MMP-2 activation and MT1-MMP regulation are not well understood. TIMP-4, another member of the TIMP family, can form a non-covalent complex with pro-MMP-2. However its role in pro-MMP-2 activation by MT1-MMP is unknown.

Purpose. The purpose and scope of the Academic Award proposal is to focus our research efforts in understanding the cell surface regulation of the gelatinases, MMP-2 and MMP-9, and MT1-MMP with the goal to decipher the function and role of these enzymes in breast cancer progression.
Note: Since the results of the following section have been published, we respectfully request the reviewers to refer to the enclosed publications for details in experimental procedures and results.

As a continuation of our studies aimed to understand the regulatory effects of MT1-MMP inhibition in pro-MMP-2 activation, we carried out studies with synthetic MMP inhibitors (MMPI). We previously showed that pro-MMP-2 activation by MT1-MMP was a process dependent on TIMP-2 (8). Studies on the processing of MT1-MMP revealed an unexpected effect of TIMP-2. In the presence of the inhibitor, the cells accumulated the active form of MT1-MMP on the cell surface due to a direct inhibition of autocatalytic processing (8). We hypothesized that accumulation of active MT1-MMP, as a consequence of decreased enzyme turnover, would increase the number of MT1-MMP molecules available on the cell surface. In the presence of TIMP-2 this would generate additional MT1-MMP/TIMP-2 complexes and therefore enhance pro-MMP-2 activation. Hence, inhibition of autocatalytic processing would “feed” MT1-MMP molecules into the ternary complex system further enhancing pro-MMP-2 activation. This suggested the hypothesis that TIMP-2 enhancement of pro-MMP-2 activation by MT1-MMP involves both formation of ternary complex and inhibition of MT1-MMP processing. To test the importance of inhibition of MT1-MMP autocatalytic processing, we used synthetic MMPIs such as Marimastat. This inhibitor also binds MT1-MMP and induces accumulation of the active form of MT1-MMP. We showed that MMPIs could act synergistically with TIMP-2 in the promotion of pro-MMP-2 activation by MT1-MMP. In contrast, TIMP-4, an efficient MT1-MMP inhibitor, had no synergistic effect. These studies suggest that under certain conditions the pericellular activity of MT1-MMP in the presence of TIMP-2 can be modulated by synthetic and natural (TIMP-4) MMP inhibitors. This study has been published, as shown above.

We also found that the ectodomain of MT1-MMP is shed into the extracellular space, while in the membrane remains a 44-kDa inactive species. This process occurs at low TIMP-2 concentrations and is consistent with the autocatalytic nature of the MT1-MMP processing. While this process would terminate activity on the plasma membrane, the shed ectodomain contains the catalytic domain and if functional may contribute to pericellular proteolysis. The characterization of this process is ongoing.

The tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMP enzymatic activity. However, TIMP-2 can promote the activation of pro-MMP-2 by MT1-MMP. This process is mediated by the formation of a complex between MT1-MMP, TIMP-2 and pro-MMP-2. Binding of TIMP-2 to active MT1-MMP also inhibits the autocatalytic turnover of MT1-MMP on the cell surface. Thus, under certain conditions, TIMP-2 is a positive regulator of MMP activity. TIMP-4, a close homologue of TIMP-2 also binds to pro-MMP-2 and can potentially participate in pro-MMP-2 activation. We co-expressed MT1-MMP with TIMP-4 and investigated its ability to support pro-MMP-2 activation. TIMP-4, unlike TIMP-2, does not promote pro-MMP-2 activation by MT1-MMP. However, TIMP-4 binds to MT1-MMP inhibiting its autocatalytic processing. When co-expressed with TIMP-2, TIMP-4 competitively reduced pro-MMP-2 activation by MT1-MMP. A balance between TIMP-2 and TIMP-4 may be a critical factor in determining the degradative potential of cells in normal and pathological conditions.


Identification of pro-MMP-9 Interacting Proteins. Since pro-MMP-9 binds to the surface of breast cancer cells, we set up to identify novel proteins that may interact with pro-MMP-9. In our original application we proposed to utilize a biochemical approach using immobilized MMP-9 and cell extracts. Instead, we decided to try a genetic approach using phage display. We thought that this system would allow us to identify interacting proteins and obtain their gene sequence directly. To this end, we carried out studies on affinity screening of a T7 Phage displayed breast tumor cDNA library. The tumor used for making this random primed Human cDNA library (Novagen, WI) was from a 46-year old woman suffering from moderately differentiated invasive ductal carcinoma. The number of primary clones in the library was $1.6 \times 10^7$. The recombinant human pro-MMP-9 used for biopanning was the 92-kDa latent form produced in the lab using our vaccinia expression system in mammalian cells (9). Recombinant pro-MMP-9 was purified to homogeneity as described (10). Trace amounts of TIMP-1 in the recombinant pro-MMP-9 preparation were removed by Heparin-agarose affinity. During the second year (00-01), we carried out the screening assay as follows:

Protocol:

- Each well of ELISA plate was coated with 1μg of Pro MMP-9. Control wells were coated with BSA alone.
- The non-specific binding sites were blocked with 5% protease free BSA
• 3.4 x 10^9 pfu of the amplified phage display library was added in each well for affinity interaction between phage expressed proteins and Pro-MMP9. The plates were incubated for 1 hr. at 37°C to pick up high affinity clones.

• The non-binders were washed away with TBST. The stringency of washing increased with each round of screening.

• The bound phage was eluted with 0.1% SDS. Eluted phage was amplified on BLT5615 cells before each round of screening.

• The eluted bound phages were plated out at log dilutions 10^3, 10^4 and 10^5 in duplicate before amplification after each round of screening to determine the enrichment of the selected clones after each round of screening.

• After the final round of screening 170 plaques were picked up from the plates, dispersed in extraction buffer, mixed with equal volume of EDTA and heated at 65°C for 10'.

• For each of the 170 clones a PCR reaction was set up using T7 UP and DOWN primers derived from the flanking regions of the phage coat protein.

• The PCR products were purified using standard molecular biology procedures and run on 2% agarose gel.

• The PCR products were grouped in 10 sets based on their size and digested with Alu1 and run on a 2% gel to check for similar clones.

• One clone from each of the similar clone sets was sequenced using T7 UP and DOWN primers.

• From the sequences obtained, NCBI BLAST was performed using NR and SWISS PROT databases to search for homology.

Results:

Starting from a titer of 2.12 x 10^4 of the clones from wells coated with pro-MMP-9 alone almost a 100 fold enrichment of the plaques (3.76 x 10^6) was obtained after the 7th round of screening. The plaques binding non-specifically to control wells coated with BSA alone were lost completely with increased stringency of washing after the 3rd round of screening (data not shown). The selected clones were grouped in 9 sets based on their sizes ranging down from 1000 bp to 350 bps and an Alu1 digestion was performed to search for similar and different clones (Table 1). Out of 170 clones picked 59 clones were about 600 bp in size and represented the most selected clone. However, when an Alu 1 digestion was performed two patterns of digestion were observed showing that the class of clones representing 600bp actually comprised of two different sets of clones. The clones representing sizes between 350
and 375 bp turned out to be the same upon sequencing representing 19% (4+15) of the total selected clones. The next in abundance were the ~1000bp clones (18 in number) constituting 10% of the total. The remaining clones did not show a defined digestion pattern and thus were not further investigated.

Table 1: Distribution of clones after digestion with Alu1

<table>
<thead>
<tr>
<th>Clone class on the basis of size</th>
<th># of clones</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>18</td>
<td>~10%</td>
</tr>
<tr>
<td>900</td>
<td>1</td>
<td>~0.5%</td>
</tr>
<tr>
<td>800</td>
<td>5</td>
<td>~3%</td>
</tr>
<tr>
<td>620</td>
<td>30</td>
<td>~18%</td>
</tr>
<tr>
<td>607</td>
<td>29</td>
<td>~17%</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>~2%</td>
</tr>
<tr>
<td>410</td>
<td>2</td>
<td>~1%</td>
</tr>
<tr>
<td>390</td>
<td>7</td>
<td>~4%</td>
</tr>
<tr>
<td>375*</td>
<td>6</td>
<td>~4%</td>
</tr>
<tr>
<td>350*</td>
<td>26</td>
<td>~15%</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>~75%</td>
</tr>
</tbody>
</table>

* Same translated sequence 36 amino acids from the point of fusion

Blas**tp**, Blastn and tblastx were used to find significant homologies at nucleotide and protein levels. Blastp compares an amino acid query sequence against a protein sequence database. Blastn compares a nucleotide query sequence against a nucleotide sequence database. Tblastx compares six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence.

Blastp results:

The nucleotide sequence downstream of the phage coat protein and in alignment with its reading frame was translated and the sequence (only the sequence before the first stop codon was considered) of the amino acids obtained was used in blastp of NCBI NR database and the Swissprot database. The results of the blast have been summarized in Tables 2, 3, 4, 5, 6 and 7. The Bit score value ‘S’ is derived from the raw alignment score in which the statistical properties of the scoring system used have been taken into account. These scores have been normalized with respect to the scoring system; they can be used to compare alignment scores from different searches. ’E’ is the Expectation value and represents the chance occurrence of an alignment during the search. The lower the E-value, the more significant is the score. Homologies with E-values greater than 100 were restricted out from the blast search.
Table 2: blastp of the 1000bp clones

<table>
<thead>
<tr>
<th>Clone size</th>
<th># of clones</th>
<th>% of total</th>
<th># of amino acids from point of fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>~1000 bp</td>
<td>18</td>
<td>11%</td>
<td>44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>NR Database</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unnamed Protein product (AK022872) (codes for 443 aa)</td>
</tr>
<tr>
<td>Hypothetical proteinFLJ12810</td>
</tr>
<tr>
<td>Ig lambda chain precursors</td>
</tr>
<tr>
<td>S</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>23-22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Swiss Prot</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHR4 Factor H-Related Protein 4 precursor</td>
</tr>
<tr>
<td>Ig lambda chain C regions</td>
</tr>
<tr>
<td>Microtubule-associated protein 1B</td>
</tr>
<tr>
<td>Integrin β6-precursor</td>
</tr>
<tr>
<td>S</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>20</td>
</tr>
</tbody>
</table>
Table 3: blastp of a subset of 600 bp clones

<table>
<thead>
<tr>
<th>Clone size</th>
<th># of clones</th>
<th>% of total</th>
<th># of amino acids from point of fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>~600 bp</td>
<td>29</td>
<td>17.05%</td>
<td>51</td>
</tr>
</tbody>
</table>

**NR Database**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothetical Protein KIAA0559</td>
<td>25</td>
<td>7.0</td>
</tr>
<tr>
<td>Hypothetical protein FLJ13725</td>
<td>25</td>
<td>9.2</td>
</tr>
<tr>
<td>Intercellular adhesion molecule 5 precursor (ICAM)</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td>Immunoglobulin heavy chain variable region</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>Lung Cancer Antigen NY-LU-12</td>
<td>23</td>
<td>48</td>
</tr>
<tr>
<td>Melanoma Antigen MAGE A9</td>
<td>23</td>
<td>51</td>
</tr>
<tr>
<td>Chondroitin sulfate proteoglycan 2 (VERSICAN)</td>
<td>23</td>
<td>53</td>
</tr>
</tbody>
</table>

**Swiss Prot**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin IF</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>Melanoma Associated Antigen MAGE-9</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Versican Core protein Precursor</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Corticotropin releasing Factor Receptor 1 precursor (CRF-R)</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Restin (Cytoplasmic linker protein-170 alpha-2) (CLIP170)</td>
<td>21</td>
<td>39</td>
</tr>
<tr>
<td>IL-2 Receptor β-Chain</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>Integrin α-M precursor (Cell Surface Glycoprotein MAC-1)</td>
<td>20</td>
<td>63</td>
</tr>
<tr>
<td>α-subunit) (CR-3 α chain) (CD11B) Leukocyte adhesion receptor MO1 (neutrophil adherence receptor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrin α-X precursor Leukocyte adhesion glycoproteinP150, 95 α-chain (CD11c) (leu M5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrin α-D precursor (leukointegrin α-D) (CD11d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adapter Related protein Complex 2 α1 subunit (clathrin assembly protein complex 2)</td>
<td>20</td>
<td>64</td>
</tr>
<tr>
<td>MAGE-2 antigen</td>
<td>20</td>
<td>67</td>
</tr>
<tr>
<td>MAGE-12 antigen</td>
<td>20</td>
<td>85</td>
</tr>
<tr>
<td>Cell adhesion kinase (tyrosine-protein kinase CAK)</td>
<td>20</td>
<td>91</td>
</tr>
<tr>
<td>(Epithelial discoidin domain receptor 1 precursor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor I receptor precursor</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4: blastp of 350bp, 500bp and 800bp clones

<table>
<thead>
<tr>
<th>Clone sizes</th>
<th># of clones</th>
<th>% of total</th>
<th># of amino acids from point of fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>375 bp</td>
<td>6</td>
<td>3.5%</td>
<td>36</td>
</tr>
<tr>
<td>350 bp</td>
<td>26</td>
<td>15%</td>
<td>36</td>
</tr>
<tr>
<td>500 bp</td>
<td>3</td>
<td>1.76%</td>
<td>15</td>
</tr>
<tr>
<td>800 bp</td>
<td>5</td>
<td>2.94%</td>
<td>23</td>
</tr>
</tbody>
</table>

350 and 375 bp clones

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmembrane 7 superfamily member</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>Brain tumor associated protein NAG14</td>
<td>23</td>
<td>56</td>
</tr>
<tr>
<td>Hypothetical protein FLJ20772</td>
<td>23</td>
<td>74</td>
</tr>
<tr>
<td>KIAA0298 protein</td>
<td>23</td>
<td>78</td>
</tr>
<tr>
<td>VWF factor precursor (Coagulation factor VIII)</td>
<td>22</td>
<td>94</td>
</tr>
</tbody>
</table>

500 bp clone

Dipeptidyl Peptidase IV like protein                      | 20  | 82  |

800 bp clone

NR Database

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>55-C-FOS Protooncogene</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>Hypothetical protein KIAA00..</td>
<td>22</td>
<td>37</td>
</tr>
<tr>
<td>SACSIN (may function in chaperone mediated folding)</td>
<td>21</td>
<td>51</td>
</tr>
<tr>
<td>Protein Kinase CLK3</td>
<td>21</td>
<td>56</td>
</tr>
<tr>
<td>Sorting Nexin13</td>
<td>21</td>
<td>72</td>
</tr>
<tr>
<td>BRC2 (breast Cancer type2 susceptibility protein)</td>
<td>21</td>
<td>84</td>
</tr>
<tr>
<td>Brain-specific angiogenesis inhibitor 2 precursor</td>
<td>21</td>
<td>85</td>
</tr>
</tbody>
</table>

SwissProt

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sacsin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contactin precursor (glycoprotein GP135) (contains 6Ig-like</td>
<td>21</td>
<td>36</td>
</tr>
<tr>
<td>C2-type domain and 4 fibronectin typeIII-like domains)</td>
<td>20</td>
<td>74</td>
</tr>
<tr>
<td>Dipeptidyl Peptidase IV like protein-Dipeptidyl aminopeptidase related protein</td>
<td>20</td>
<td>82</td>
</tr>
</tbody>
</table>
Table 5: blastp of the 900bp clone

<table>
<thead>
<tr>
<th>Clone size</th>
<th>% of clones</th>
<th>% of total</th>
<th>% of amino acids from point of fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>900 bp</td>
<td>1</td>
<td>0.5%</td>
<td>91</td>
</tr>
</tbody>
</table>

**NR Database**

<table>
<thead>
<tr>
<th>Description</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opioid growth factor receptor</td>
<td>28</td>
<td>3.4</td>
</tr>
<tr>
<td>Suppression of tumorigenicity 5</td>
<td>27</td>
<td>7</td>
</tr>
<tr>
<td>KIAA0672 gene product</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>C-KIT proto-oncogene (Proto-oncogene tyrosine protein kinase )</td>
<td>26</td>
<td>17</td>
</tr>
<tr>
<td>Lectomedin-2 (KIAA0821 protein) (a family of heptahelical receptors with adhesion-like domains)</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>Hypothetical protein FLJ20753</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>Hypothetical and unnamed proteins</td>
<td>24</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>25-23</td>
<td>29-92</td>
</tr>
</tbody>
</table>

**Swiss Prot**

<table>
<thead>
<tr>
<th>Description</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast/Stem Cell growth factor receptor precursor (SCFR) C-KIT, CD117 antigen</td>
<td>26</td>
<td>3.2</td>
</tr>
<tr>
<td>Phospholipase C-β-3</td>
<td>24</td>
<td>7.7</td>
</tr>
<tr>
<td>Basement Membrane Specific heparan Sulfate Proteoglycan core (PERLECAN)</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>Macrophage Colony Stimulating Factor I Receptor precursor (CD115)</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Low Affinity immunoglobulin gamma Fc region receptor II B precursor (CD32)</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Alpha adducing</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Coagulation factor X precursor (Stuart Factor)</td>
<td>23</td>
<td>39</td>
</tr>
<tr>
<td>MAGE 12</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>MAGE-2</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td>IG Lambda Chain V-II region</td>
<td>22</td>
<td>49</td>
</tr>
<tr>
<td>VEGFR-2 precursor</td>
<td>21</td>
<td>70</td>
</tr>
</tbody>
</table>
### Table 6: blastp of the 400bp clones

<table>
<thead>
<tr>
<th>Clone size</th>
<th>% of clones</th>
<th>% of total</th>
<th>Number of base pairs</th>
<th>% of amino acids from point of fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>~400 bp</td>
<td>2</td>
<td>1.17%</td>
<td>414</td>
<td>64</td>
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</table>

**NR and Swiss Prot Database**

<table>
<thead>
<tr>
<th>Neuronal Pas Domain Protein</th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>38</td>
</tr>
</tbody>
</table>

### Table 7: blastp of the ~390bp clones

<table>
<thead>
<tr>
<th>Clone size</th>
<th>% of clones</th>
<th>% of total</th>
<th>Number of base pairs</th>
<th># of amino acids from point of fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>390</td>
<td>6</td>
<td>3.53%</td>
<td>369</td>
<td>53</td>
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</tbody>
</table>

**NR Database**

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solute carrier family 9 ((40-50% homology)</td>
<td>48</td>
<td>2e-06</td>
</tr>
<tr>
<td>Protein Tyrosine phosphatase</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>Myosin light chain kinase</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>Cell surface glycoprotein (CD-44)</td>
<td>23-22</td>
<td>48-62</td>
</tr>
</tbody>
</table>

**Swiss Prot**

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis specific basic protein</td>
<td>24</td>
<td>4.4</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase</td>
<td>24</td>
<td>6.1</td>
</tr>
<tr>
<td>Myosin Light chain Kinase</td>
<td>24</td>
<td>6.6</td>
</tr>
<tr>
<td>CD-44 antigen Precursor</td>
<td>22</td>
<td>22</td>
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<tr>
<td>Serum response factor (SRF)</td>
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<td></td>
</tr>
<tr>
<td>Low density Lipoprotein receptor-related protein 1 precursor</td>
<td>21</td>
<td>43</td>
</tr>
<tr>
<td>(LRP) apolipoprotein E receptor</td>
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<td></td>
</tr>
<tr>
<td>Laminin α-2 chain precursor</td>
<td>21</td>
<td>51</td>
</tr>
<tr>
<td>Clone Size (bp)</td>
<td>% of total</td>
<td># of base pairs</td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
<td>----------------</td>
</tr>
<tr>
<td>1000</td>
<td>10.59</td>
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</tr>
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<td>900</td>
<td>0.5</td>
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<tr>
<td>800</td>
<td>2.94</td>
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<tr>
<td>620</td>
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<td>607</td>
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<tr>
<td>500</td>
<td>1.76</td>
<td>504</td>
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<tr>
<td>410</td>
<td>1.17</td>
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<tr>
<td>390</td>
<td>3.54</td>
<td>369</td>
</tr>
<tr>
<td>375</td>
<td>3.5</td>
<td>354</td>
</tr>
<tr>
<td>350</td>
<td>15</td>
<td>327</td>
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</tbody>
</table>

**blastn and tblastx results:** Blast of human EST database with the translated nucleotide sequence downstream of phage coat protein in six reading frames took care of any early termination codons that may have been introduced due to frame shifts caused by sequencing errors since the entire nucleotide sequence beyond the point of fusion was used for homology search. This search showed that the most abundant clones (clones of 1000 and 600 bps) showed binding to sequences with a high score S to the α2 (I) chain of collagen I (Table 8). Interestingly, we reported that the α2(IV) chain of collagen IV bound with high affinity to pro-MMP-9 (11). Also a high score was obtained for clones showing homologies to binding with IL-5. Chance occurrence of these clones was remote as revealed by low E
values of 2e-73 and 3e-28. Clones of 350 bp size, representing 15% of the selected clones, showed homologies with dipeptidyl peptidase with a relatively modest Score of 27.2. Clones 800, 607 and 375 were artifacts since they showed homologies with the subjects representing DNA strands in reverse orientation albeit with 100% homology.

Discussion. The foregoing results have been obtained from screening of a library by specific protein-protein interactions. The search should have yielded proteins or peptides showing 100% homology and high scores. Looking at tables 2, 3, 4, 5, 6 and 7, we find that the score values range anywhere between 20-36 on an average (S=48 for 390 and is an exception). These scores are low when compared to the scores obtained at the nucleotide level. One may attribute this fundamental dichotomy in the results obtained by protein and nucleotide blasts respectively to frame shifts caused by sequencing errors. Nevertheless, these differences make it difficult to draw any meaningful conclusions. In view of the constraints inherent in the technique alternative strategies may have to be employed for identifying MMP-9 binding proteins. We have initiated studies with the yeast two-hybrid system to search for MMP-9 binding proteins

KEY RESEARCH ACCOMPLISHMENTS

- Characterized the processing of MT1-MMP and described the major enzyme forms
- Reported the synergistic and enhancing effects of TIMP-2 and synthetic MMPIs in pro-MMP-2 activation
- Showed that TIMP-4 cannot promote pro-MMP-2 activation by MT1-MMP
- Demonstrated the localization of MMP-2 and MT1-MMP in caveolae
- Carried out a search for MMP-9 binding proteins using a phage display system

REPORTABLE OUTCOMES

Published papers.

TIMP-2 acts synergistically with synthetic MMP inhibitors but not with TIMP-4 to enhance the 


Differential roles of TIMP-4 and TIMP-2 in pro-MMP-2 activation by MT1-MMP. Bioch. 

Abstracts.

with MT1-MMP cannot promote pro-MMP-2 activation. Differential roles of TIMPs in regulation of 
pericellular proteolysis.” Meeting of the International Metastasis Society, London, UK, September 
2000.

The matrix metalloproteinases (MMPs) have been associated with tumor invasion and metastasis. 
Recent evidence suggests a role for the membrane type 1 (MT1)-MMP in metastasis and angiogenesis. 
MT1-MMP is a membrane bound MMP that is known to be the physiological activator of pro-MMP-2. 
A model for the activation of pro-MMP-2 has been proposed in which the catalytic domain of MT1-
MMP binds to the N-terminal domain of TIMP-2 while TIMP-2 C-terminal domain binds to the 
hemopexin-like domain (HLD) of pro-MMP-2. Moreover, studies have shown that the amount of 
active MT1-MMP (57 kDa) on the surface is regulated by TIMP-2. Thus, TIMP-2 can act as a positive 
regulator of MMP activity. Like TIMP-2, TIMP-4 has been shown to bind to the HLD of pro-MMP-2. 
Thus, TIMP-4 can potentially participate in the activation of pro-MMP-2 by MT1-MMP. Here we co-
expressed MT1-MMP with or without TIMP-2 or TIMP-4 in TIMP-2 null cells and examine their 
ability to promote pro-MMP-2 activation and accumulation of active MT1-MMP. Whereas TIMP-2 
enhanced pro-MMP-2 activation by MT1-MMP, TIMP-4 had no effect. Interestingly, both inhibitors 
induced the accumulation of the 57-kDa active form of MT1-MMP in the cells consistent with their 
inhibitory activity. Co-expression of TIMP-4 with TIMP-2 inhibited pro-MMP-2 activation. These 
studies demonstrate that TIMP-4 is a true MT1-MMP inhibitor but is unable to participate in pro-
MMP-2 activation. We propose that a balance between TIMP-2 and TIMP-4 in tumor tissues may be a 
critical factor in determining the invasive/degradative potential of tumor cells.

Cell behavior is strictly dependent on the cell's ability to control its immediate microenvironment through surface proteolysis. The members of the MMP family play a pivotal role in pericellular proteolysis. We have been studying the surface regulation of two distinct MMPs, MT1-MMP and MMP-9, known for their role in cancer metastasis and angiogenesis. MT1-MMP activity on the surface is partly controlled by a delicate balance between trafficking and insertion of active enzyme to the plasma membrane and its turnover by autocatalytic processing. The concentration of active MT1-MMP on the cell surface is positively influenced by natural and synthetic MMP inhibitors (MMPIs), which sustain enzyme presence in the membrane and, under restricted conditions, may promote MT1-MMP-dependent activity. Under low inhibitor concentrations, active MT1-MMP is processed to an inactive membrane-tethered form of 44 kDa while the entire catalytic domain (MT1-MMP\textsubscript{cat}) is shed into the extracellular space. Shedding of MT1-MMP\textsubscript{cat} is significantly reduced by both TIMP-2 and TIMP-4 as well as by broad spectrum MMPIs consistent with being an autocatalytic event. Binding of TIMP-2 but not of TIMP-4 to MT1-MMP is also essential for pro-MMP-2 activation. However, MMPIs can enhance, synergistically, the effect of TIMP-2 on pro-MMP-2 activation. In contrast, TIMP-4 inhibits this effect. Structurally, neither the cytosolic tail nor the hinge region of MT1-MMP are necessary for pro-MMP-2 activation in the presence of TIMP-2 suggesting that these domains are not required for ternary complex formation and catalytic activity on the cell surface. Taken together these studies suggest that a delicate balance of MT-MMP, TIMPs and MMPIs can alter pericellular proteolysis and activation cascades by regulating the nature of membrane-tethered forms and the shedding of the catalytic domain.


Cell behavior is strictly dependent on the cell's ability to control its immediate microenvironment through surface proteolysis. The members of the MMP family play a pivotal role in pericellular proteolysis, which is partly determined by innate domain features in enzyme structure or by interactions with specific cell surface components. We have been studying the surface regulation of two distinct MMPs, MT1-MMP and MMP-9, known for their role in cancer metastasis and
angiogenesis. MT1-MMP activity on the surface is partly controlled by a delicate balance between trafficking and insertion of active enzyme to the plasma membrane and its turnover by autocatalytic processing. The concentration of active MT1-MMP on the cell surface is positively influenced by natural and synthetic MMP inhibitors, which sustain enzyme presence in the membrane and, under restricted conditions, may promote MT1-MMP-dependent activity. Under low inhibitor concentrations, MT1-MMP is processed to an inactive membrane-tethered form while the entire catalytic domain is shed into the extracellular space. These results suggest that MT1-MMP activity in the pericellular compartment is positively influenced by TIMPs and may involve both membrane-bound and soluble enzymes possibly with different biological functions. Accumulating evidence suggests that gelatinases (MMP-2 and MMP-9), which are secreted MMPs, can also associate with the cell surface. In the case of pro-MMP-2, surface association is mediated by an MT1-MMP/TIMP-2 complex, which promotes pro-MMP-2 activation by facilitating zymogen binding to the cell surface. Pro-MMP-9 has been identified on the surface of a variety of cells where it binds with high affinity. Several proteins have been described as potential “MMP-9 receptors.” However, their significance for MMP-9 activity and inhibition is unclear. Furthermore, in certain cases, strict criteria for ligand-receptor association have not been clearly established. The role of surface binding in pro-MMP-9 activation is unknown since a surface activation mechanism of pro-MMP-9 has yet to be identified. Such a mechanism should take into account that solution phase activation of pro-MMP-9 is kinetically very efficient. These and other issues regarding MMP-9 surface binding and the dilemmas that they present for our understanding of pericellular proteolysis will be discussed.


The metalloproteases, MMPs, are essential to normal tissue remodeling processes and have also been implicated in pathological conditions such as arthritis, cardiovascular diseases and cancer. The gelatinases, MMP-2 and MMP-9, have been shown to play a key role in angiogenesis and tumor metastasis. Synthetic MMP inhibitors with therapeutic value for these conditions are highly sought. All the currently available inhibitors take advantage of chelation of the active site zinc ion and lack specificity. For this purpose we have resorted to the design of mechanism-based inhibitors for the gelatinases. We have designed, synthesized and kinetically characterized the first mechanism-based inhibitor for MMP-2 and MMP-9. The compound, (4-phenoxyphenylsulfonyl)methylthirane, comprises a biphenyl moiety which fits in the gelatinases active site as demonstrated by energy-
minimization modeling of the enzyme-inhibitor complexes. This binding mode brings the sulfur of the thirane group into the coordination sphere of the zinc ion. Zinc coordination is expected to render the carbon of the thirane moiety electrophilic, making it susceptible of being modified by a nucleophile, possibly the carboxy group of the active site glutamic acid residue (Glu-404), leading to the formation of an ester bond and to irreversible enzyme inactivation. Kinetic characterization of enzyme inhibition by this compound using synthetic fluorogenic substrates, was consistent with the proposed mechanism for gelatinase inactivation. Slow binding inhibition was observed for both MMP-2 and MMP-9 with \( K_i \) values of 13.9 ± 0.4 and 600 ± 200 nM, respectively. In contrast the \( K_i \) values for the other MMPs tested, MMP-3, MMP-7 and MMP-1, are in the micromolar range. Analogous compounds with longer backbones for the point of attachment of the thirane moiety, or the corresponding oxirane derivatives, served as poor competitive inhibitors at best, and there were cases of no inhibition. The collective results indicate high selectivity of the aforementioned compound for the inhibition of gelatinases and should serve as a paradigm for the design of inhibitors for other closely related enzymes.

CONCLUSIONS

1. Active MT1-MMP undergoes a process of autocatalysis resulting in the generation of a membrane-tethered species of 44 kDa without the catalytic domain. TIMP-2, TIMP-4 and synthetic MMPIs inhibit this autocatalytic processing inducing accumulation of the active species on the cell surface. Due partly to this process, there is enhanced activation of pro-MMP-2 and thus TIMP-2 and MMPIs can act synergistically to promote pro-MMP-2 activation by MT1-MMP. Thus, under certain conditions synthetic MMPIs can promote MT1-MMP-dependent pericellular proteolysis. These findings are relevant for understanding the potential effects of MMPIs in cancer tissues.

2. TIMP-4 cannot promote pro-MMP-2 activation by MT1-MMP in spite of its ability to bind pro-MMP-2. TIMP-4 also competes with TIMP-2 and thus inhibits its ability to promote pro-MMP-2 activation by MT1-MMP. This suggests the possibility that in breast tumor tissues, a balance between TIMP-2 and TIMP-4 may regulate the degree of pro-MMP-2 activation.

3. We have carried out phage display studies to search for pro-MMP-9 binding proteins. These studies have provided conflicting data as explained above. However, several interesting proteins have
been identified. The reliability of this method is unclear and therefore we are not confident of the findings.

REFERENCES


APPENDICES


Tissue Inhibitor of Metalloproteinase (TIMP)-2 Acts Synergistically with Synthetic Matrix Metalloproteinase (MMP) Inhibitors but Not with TIMP-4 to Enhance the (Membrane Type 1)-MMP-dependent Activation of Pro-MMP-2*

Marta Toth†, M. Margarida Bernardo‡, David C. Gervasi‡, Paul D. Soloway§, Zhiping Wang§, Heather F. Biggs§, Christopher M. Overall§, Yves A. DeClerck§, Harald Tschesche*, Michael L. Cher†, Stephen Brown§, Shahriar Mobashery**, and Rafael Fridman***

From the †Departments of Pathology, ‡Urology and §§Chemistry, Wayne State University, Detroit, Michigan 48201, the §Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263, the ‡Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, the ‡Division of Hematology-Oncology and Department of Pediatrics, Children’s Hospital Los Angeles, University of Southern California, Los Angeles, California 90027, and the **Department of Biochemistry, Faculty of Chemistry, University of Bielefeld, W-4800 Bielefeld 1, Germany

The membrane-type I matrix metalloproteinase (MT1-MMP) has been shown to be a key enzyme in tumor angiogenesis and metastasis. MT1-MMP hydrolyzes a variety of extracellular matrix components and is a physiological activator of pro-MMP-2, another MMP involved in malignancy. Pro-MMP-2 activation by MT1-MMP involves the formation of an MT1-MMP-tissue inhibitors of metalloproteinases 2 (TIMP-2)-pro-MMP-2 complex on the cell surface that promotes the hydrolysis of pro-MMP-2 by a neighboring TIMP-2-free MT1-MMP. The MT1-MMP-TIMP-2 complex also serves to reduce the intercellular reactive oxygen species (ROS) and to suppress the activity of TIMP-2. The activation of active MT1-MMP on the cell surface. Evidence shown here in Timp-2-null cells demonstrates that pro-MMP-2 activation by MT1-MMP requires TIMP-2. In contrast, a C-terminally deleted Timp-2-ΔTIMP-2-ΔT IMP-2, unable to form ternary complex, had no effect. However, ΔTIMP-2 and certain synthetic MMP inhibitors, which inhibit MT1-MMP autolysis, can act synergistically with TIMP-2 in the promotion of pro-MMP-2 activation by MT1-MMP. In contrast, Timp-4, an efficient MT1-MMP inhibitor, had no synergistic effect. These studies suggest that under certain conditions the pericellular activity of MT1-MMP in the presence of TIMP-2 can be modulated by synthetic and natural (TIMP-4) MMP inhibitors.

Proteolytic degradation of extracellular matrix (ECM) is a fundamental aspect of cancer development and a key event in tumor-induced angiogenesis and tumor metastasis. A major group of enzymes responsible for ECM degradation in cancer tissue is the matrix metalloproteinase (MMP) family (1–4). The MMPs are zinc-dependent multidomain endopeptidases that, with few exceptions, share a basic structural organization comprising propeptide, catalytic, hinge, and C-terminal (hemopexin-like) domains (1, 5). All MMPs are produced in a latent form (pro-MMP) requiring activation for catalytic activity, a process that is usually accomplished by proteolytic removal of the propeptide domain. Once activated, all MMPs are specifically inhibited by a group of endogenous tissue inhibitors of metalloproteinases (TIMPs) that bind to the active site, inhibiting catalysis (1). Over the last 5 years, the MMP family has been expanded to include a new subfamily of membrane-anchored MMPs known as membrane-type MMPs (MT-MMPs), which to date includes six members (6–12). The MT-MMPs, with the exception of MT4-MMP, are unique because they are anchored to the plasma membrane by means of a hydrophobic stretch of approximately 20 amino acids, leaving the catalytic domain exposed to the extracellular space. This organization makes the MT-MMPs perfectly suited for regulation of pericellular proteolysis. MT1-MMP (MMP-14) was the first member of the MT-MMP family to be discovered and has been shown to be the major physiological activator of pro-MMP-2 (gelatinase A) on the cell surface (6, 12). The role of MT1-MMP in pericellular proteolysis is not restricted to pro-MMP-2 activation, since MT1-MMP is a multifunctional enzyme that can also degrade a variety of ECM components (13–16) and hence can play a direct role in ECM turnover. MT1-MMP has been recently shown to be the first member of the MMP family indispensable for normal growth and development, since mice deficient in MT1-MMP exhibit a variety of connective tissue pathologies and a short life span (17, 18). Furthermore, both MMP-2 (19) and MT1-MMP (20–26) have been associated with metastatic potential in many human cancers, angiogenesis (27), and enhanced tumor cell invasion in experimental systems (28–31). This has raised considerable interest in understanding the regulation of these MMPs because they represent an important target for development of novel drugs aimed at inhibiting tumor metastasis and angiogenesis (3, 32, 33).

Studies on the mechanism of activation of pro-MMP-2 by MT1-MMP revealed a complex role for TIMP-2 in this process. A model for the activation of pro-MMP-2 has been proposed in which the catalytic domain of MT1-MMP binds to the N-termi-

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‡ The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane type MMP; MMP, MMP inhibitor; TIMP, tissue inhibitor of metalloproteinase; PAGP, polyacrylamide gel electrophoresis; pAb, polyclonal antibody; pfu, plaque-forming units; DMEM, Dulbecco’s modified Eagle’s medium.

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nal portion of TIMP-2, leaving the negatively charged C-ter-
mini region of TIMP-2 available for the binding of the he-
mospain-like domain of pro-MMP-2 (12, 34–38). This ternary
complex has been suggested to cluster pro-MMP-2 at the cell
surface near a residual TIMP-free active MT1-MMP molecule,
which is thought to initiate activation of the bound pro-MMP-2.
Pro-MMP-2 activation would occur only at low TIMP-2 con-
centrations relative to MT1-MMP, which would permit availability
of active MT1-MMP to activate the pro-MMP-2 bound in the
ternary complex (39). Thus, under restricted conditions,
TIMP-2 is thought to promote the activation process by acting
as a molecular link between MT1-MMP and pro-MMP-2. We
have recently shown that TIMP-2, besides its role in ternary
complex formation, has direct and critical effects on MT1-MMP
processing, which influence the profile and spatial localization
of MT1-MMP forms (40). Biochemical and cellular evidence
showed that binding of TIMP-2 to active MT1-MMP (57 kDa)
inhibits autocatalytic degradation, leading to accumulation of
active TIMP-2 on the cell surface. In the absence of TIMP-2,
MT1-MMP levels in rapidly processed cells increased by a 44-kDa
massed inactive enzyme (40, 41). Thus, under controlled condi-
tions, TIMP-2 may act as a positive regulator of MT1-MMP activity
by promoting the availability of active MT1-MMP on the cell
surface and consequently may support pericellular proteolysis.
Since some of the effects of TIMP-2 on MT1-MMP activities are
related to its inhibitory activity, we wished to examine the
effects of synthetic and physiological MMP inhibitors (MMPi)
on MT1-MMP processing and its ability to promote pro-MMP-2
activation with TIMP-2. Although several types of MMPi have
been developed (3, 32, 33, 42–47), little is known about their
effects on the processing and activity of membrane-tethered
MMPs, which exhibit unique properties. Here we show for the
first time that synthetic MMPi and a C-terminally truncated
TIMP-2 but not TIMP-4, which inhibit MT1-MMP activity, act
synergistically with TIMP-2 to promote pro-MMP-2 activation
by MT1-MMP. These studies demonstrate the complexity of
MT1-MMP regulation and provide new insights into the roles
of TIMP-2, TIMP-4, and MMPi in this process.

EXPERIMENTAL PROCEDURES

Cell Culture—Nonmalignant monkey kidney epithelial BS-C-1 (CCL-
29) and human fibrosarcoma HT-1080 (CCL-121) cells were obtained
from the American Type Culture Collection (ATCC, Manassas, VA)
and cultured in Dulbecco’s modified Eagle’s medium (DMEM)
supplemented with 10% fetal bovine serum and antibiotics. HeLa S3 cells were ob-
tained from ATCC (CCL-2.2) and grown in suspension in MEM Spinner
medium (Quality Biologics, Inc., Gaithersburg, MD) supplemented with
5% horse serum. All other tissue culture reagents were purchased from
Life Technologies, Inc.

Isolation of Immortalized Timp2 Mutant Mouse Fibroblasts—Adult
skin fibroblast cells were isolated from heterozygous (+/−) Timp2 mut-
ant mice and immortalized by retroviral infection using a Ha-ras
and v-myc-producing, replication-defective retrovirus as described previ-
ously (48). A G418 selection protocol was used to select for homozgyous
Timp2−/− fibroblasts (33) from an immortalized (−/−) HI-11 cell line (49) as
described (49). Detailed methods for the isolation and selection of the
immortalized (+/−) and (−/−) isogenic cell lines will be reported elsewhere.
The homozygous and heterozygous Timp2 mutant cells were grown in
DMEM supplemented with 10% fetal bovine serum and antibiotics.

Recombinant Genes—The production of the recombinant vaccinia virus
(vTP7-3) expressing bacteriophage T7 RNA polymerase has been described by
Fuerst et al. (50). Recombinant vaccinia virus expressing either human pro-MMP-2, TIMP-2, or MT1-MMP under control of the T7 promoter were obtained by homologous recombinant as described previously (55, 40, 61)
comprising, Synthetic MMP Inhibitors, and Antibodies—
Human recombinant pro-MMP-2, TIMP-2, and TIMP-1 were expressed in HeLa S3 cells infected with the appropriate recombinant vaccinia viruses and purified to homogeneity, as described previously (52). Hu-
man recombinant TIMP-4 was expressed in baby hamster kidney cells and purified from the conditioned medium by sequential application to
Red Sepharose, phenyl-Sepharose, Q-Sepharose, and Ze3+—charged che-
lating Sepharose columns as described. A C-terminally truncated hu-
man TIMP-2 ending at Cys248 (Δ-TIMP-2) was constructed and ex-
pressed in mammalian cells as described previously (53). The recom-
binant proteins of TIMP-2 and Δ-TIMP-2 were purified by metal-act-
ive site titration with recombinant TIMP-2 as described (56). Batimastat (BB-94) and marimastat (BB-2516), two hydroxamate-based MMP inhibitors (53, 45, 47), and BB-2116, a bor-
onate-containing MMP inhibitor, were obtained from British Biotech
(Annapolis, MD). The mechanism-based MMP inhibitor SB-3CT was
synthesized and characterized as previously reported (42). Stock solu-
tions of marimastat (1 mM), batimastat (1 mM), BB-2116 (20 mM),
and SB-3CT (30 mM) were prepared in Me2SO. The rabbit anti-TIMP-2 polyclonal antibody (pAb) and the anti-TIMP-2 monoclonal antibody
CA-101 were previously described (57). The rabbit pAb 457 to TIMP-
2 and TIMP-1 (50, 58) has been previously described (55). The rabbit pAb 160 to
TIMP-1 (40, 59) and the rabbit pAb to human TIMP-4 were a gener-
ous gift from Dr. Amy Sang (Florida State University, Tallahassee, FL).

Expression of MMP-2 by Vaccinia Infection—To express MMP-2,
confuent cultures of BS-C-1 or Timp2−/− mutant cells in 6-
or 12-well plates were co-infected with 5–10 pfu/cell each of vTP7-3 and vTP7-MTI viruses for 45 min in infection medium (DMEM plus 2.5% fetal bovine serum and antibiotics) at 37 °C. As a control, the cells were infected only with the vTP7-3 virus as described (40).

Natural and Synthetic Inhibitor Treatment and Pro-MMP-2 Activ-
ation—After infection, the media were aspirated, and the cells were
washed with serum-free DMEM and replaced with fresh serum-free
DMEM supplemented with or without various doses of purified human recombiant TIMP-2. After various times at 37 °C, the media were
aspirated; the cells were rinsed with DMEM and then incubated (15–30
min, 37 °C) in fresh media supplemented with 10 ng/ml pro-MMP-2. The
media were then collected, and the cells were rinsed twice with cold
phosphate-buffered saline and solubilized in cold lysis buffer (25 mM
Tris-HCl (pH 7.5), 1% IGEPAL CA-630, 100 mM NaCl, 10 μg/ml apro-
tinin, 1 μg/ml leupeptin, 2 mM benzamidine, and 1 mM phenylmethyl-
sulfonyl fluoride). The lysate fractions were analyzed for pro-MMP-2
activation by gelatin zymography and/or immunoblot analysis for as-
essment of TIMP-1 and TIMP-2 expression. To examine the effects of synthetic MMP inhibitors, Δ-TIMP-2, Ala + TIMP-2, and TIMP-4 on TIMP-2-dependent
activation of pro-MMP-2, the TIMP-2-infected cells were incubated (16 h, 37 °C) with the appropriate MMP inhibitors (various doses)
diluted in serum-free DMEM. Then the media were aspirated, and the
media were aspirated. The cells were rinsed with DMEM. TIMP-2 (10 ng/ml) was added to the cells in serum-free DMEM for a 5–30 min incubation at 37 °C. The media were aspirated, followed by a wash with DMEM to remove un-
bound TIMP-2. The cells were then incubated (15 min, 37 °C) with serum-free DMEM supplemented with 10 ng/ml pro-MMP-2. Analysis of pro-
MMP-2 activation and of the profile of MT1-MMP forms in the cell
lysates was carried out as described below.

Gelatin Zymography and Immunoblot Analysis—Gelatin zymo-
graphy was performed using 10% Tris-glycine SDS-polyacrylamide gels
containing 0.1% gelatin. Briefly, samples of lysates or media were mixed with Laemmli sample buffer (60) without reducing agents and
without heating and then subjected to SDS-polyacrylamide gel electro-
phoresis. After electrophoresis, the gels were washed extensively (61) and then dried. The gelatinolytic bands were visualized by staining
with Coomassie blue. In addition, the gelatinolytic bands were stained
with Coomassie blue. Detection of the active enzyme was performed
using the enhanced chemiluminescence system (Pierce) according to the
manufacturer’s instructions.

Enzyme Inhibition Studies—MT1-MMP activity was monitored with
the fluorescence-quenched substrate MOCAPGLA_p(Asp)_AR-
NH2 (62). Fluorescence was measured with a Photon Technology Inter-
national (PTI) spectrofluorometer interfaced to a Pentium computer,
equipped with the RatioMaster™ and FeIX™ hardware and software,
respectively. The cuvette compartment was maintained at 22 °C. Exci-
tation and emission passes of 1 and 3 nm, respectively, were used.
Substrate hydrolysis was monitored at emission and excitation wave-lengths of 328 and 393 nm, respectively. Fluorescence measurements were taken every 4 s. Less than 10% hydrolysis of the fluorogenic substrate was monitored, as described by Knight (62). For slow binding inhibition, progress curves were obtained by adding enzyme (0.5 µM) to a mixture of fluorogenic substrate (7 µM) and varying concentrations of inhibitor in buffer R (50 mM HEPEs (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, 0.01% Brij-35, and 1–5% Me₂SO; final volume 2 ml) in acrylic cuvettes with stirring and monitoring the increase in fluorescence with time for 15–30 min. The progress curves were nonlinear least squares fitted to Equation 1 (63),

\[ F = v_p + (v_o - v_p) (1 - \exp(-kt)) /k + F_o \]  

(1) where \( v_o \) represents the initial rate, \( v_p \) is the steady state rate, \( k \) is the apparent first order rate constant characterizing the formation of the steady-state enzyme-inhibitor complex, and \( F_o \) is the initial fluorescence, using the program SCIENTIST (MicroMath Scientific Software, Salt Lake City, UT). The obtained \( k \) value, \( v_o \), and \( v_p \) were further analyzed according to Equations 2 and 3 for a one-step association mechanism.

\[ k = k_{cat} + k_{on} / [E]_0(1 + [S]/K_{m}) \]  

(2)

\[ (v_o - v_p)v_o = [1]/(K_e(1 + [S]/K_{m})) \]  

(3)

The \( K_m \) and \( k_{on} \) values for the reaction of MT1-MMP-cat with the fluorogenic substrate were determined to be 6.9 ± 0.6 µM and 0.67 ± 0.03 s⁻¹, respectively. Intercept and slope values, obtained by linear regression of the \( k_{versus} \) inhibitor concentration plot (Equation 2), yielded the association and dissociation rate constants \( k_{on} \) and \( k_{off} \) respectively, and the inhibition constant \( K_i \) (\( k_{off}/k_{on} \)). Alternatively, \( K_i \) was determined from the slope of the \( (v_o - v_p)v_o \) versus \([I]\) plot according to Equation 3. The dissociation rate constant was determined independently from the enzyme activity recovered after dilution of a pre-formed enzyme-inhibitor complex. To this end, typically 60 nM of enzyme was incubated with 80 nM of inhibitor for a sufficient time to reach equilibrium (>45 min) at 25.0 °C. The complex was diluted 400-fold into 2 ml of buffer R containing fluorogenic substrate (10 µM final concentration). Recovery of enzyme activity was monitored for ~60 min. The fluorescence \( v versus \) time traces were fitted, using the program SCIENTIST, to Equation 4,

\[ F = v_p + (v_o - v_p) (1 - \exp(-kt_{diss})) /k_{diss} + F_o \]  

(4)

where \( v_o \) represents the initial rate (very small), \( v_p \) is the rate observed when the EI complex is completely dissociated, and \( k_{diss} \) is the first order rate constant of EI dissociation. In light of the slow dissociation of the MT1-MMP-cat-TIMP-2 complex, the direct analysis of the \( k_{diss} \) parameter for the wild type TIMP-2 was not possible and was estimated based on a 10-fold difference observed between the slopes of the linear portions of the dissociation curves for the complexes of MT1-MMP-cat with TIMP-2 (steady state rate) and for wild type TIMP-2. For competitive inhibition, initial rates were obtained by adding enzyme (0.5 µM) to a mixture of fluorogenic substrate (7 µM) and varying concentrations of inhibitor in buffer R (final volume 1 ml) in quartz semimicro cuvettes and monitoring the increase in fluorescence with time for 5–10 min. The initial velocities were determined by linear regression analysis of the fluorescence \( v versus \) time traces using Felix¥. The initial rates were fitted to Equation 5 (64),

\[ v_o/v_o = (K_m + [S])/(K_m(1 + [IV]) + [S]) \]  

(5)

where \( v_o \) and \( v_o \) represent the initial velocity in the presence and absence of inhibitor, respectively, using the program SCIENTIST.

**RESULTS**

**Pro-MMP-2 Activation and MT1-MMP Processing in Timp2 Mutant Cells**—Using a vaccinia expression system, we have recently shown that immortalized monkey kidney epithelial BSC-1 cells infected to express MT1-MMP could activate pro-MMP-2 (40). Under these conditions, this process appeared to be independent of TIMP-2, since in infected BSC-1 cells expression of the endogenous inhibitor was significantly suppressed (35, 61). However, studies were inconclusive with regard to the requirement of TIMP-2 for pro-MMP-2 activation, since a residual amount of endogenous inhibitor could not be ruled out. To establish the importance of TIMP-2 in the activation of pro-MMP-2 by MT1-MMP, we used homozygous (−/−) and heterozygous (+/−) Timp2 mutant mouse fibroblasts (65) that were immortalized by retroviral infection. We tested the expression of TIMP-2 in both cell types by immunoblot analysis. As shown in Fig. 1A, TIMP-2 was only detected in the Timp2 (+/−) mutant cells, as reported with the primary fibroblast cells (65). The cells were then tested for activation of exogenous pro-MMP-2 after treatment with concanavalin A (66), and neither cell variant activated pro-MMP-2 regardless of the presence of TIMP-2 (data not shown), suggesting a low level of endogenous MT1-MMP expression. We therefore infected the Timp2 (+/−) and (−/−) mutant cells to express MT1-MMP using the recombinant vaccinia virus (vTF7-3) and the T7 RNA polymerase virus (vTF7-5) (40). As control, the cells were infected with the vTF7-5 virus alone. MT1-MMP expression (60- and 44-kDa bands) was detected only in the cells infected to express MT1-MMP (Fig. 1B, lane 2) but not in the control-infected cells (Fig. 1B, lane 1) in both cell types (only the homozygous cells are shown in Fig. 1B).

The ability of the expressed MT1-MMP to promote pro-MMP-2 activation with or without TIMP-2 in this cellular system was examined by gelatin zymography of the lysate fraction. To this end, after infection, the cells were incubated with or without exogenous TIMP-2, washed to remove unbound inhibitor, and incubated with exogenous pro-MMP-2. The latter was added, since both the (+/−) and (−/−) Timp2 mutant cells do not produce detectable pro-MMP-2 (Fig. 1C, lanes 2 and 5). As shown in Fig. 1C, the (+/−) Timp2 mutant cells expressing MT1-MMP activated pro-MMP-2 only after the addition of exogenous TIMP-2 (Fig. 1C, lane 7). In contrast, the Timp2 (+/−) mutant cells expressing MT1-MMP activated pro-MMP-2 regardless of exogenous TIMP-2 presence (Fig. 1C, lane 6), without TIMP-2; lane 7, with TIMP-2). Control-infected homozygous and heterozygous Timp2 mutant cells consistently failed to activate pro-MMP-2 regardless of exogenous TIMP-2.
Fig. 2. Effect of TIMP-2, Δ-TIMP-2, TIMP-4, and TIMP-1 on pro-MMP-2 activation in Timp2 (−/−) mutant cells. A, confluent Timp2 (−/−) mutant cells were co-infected to express MT1-MMP as described in the legend to Fig. 1 and incubated (16 h, 37 °C) with TIMP-2 (0–100 nM). After a gentle wash, the cells were incubated with 10 nM pro-MMP-2 for 15 min at 37 °C. The lysates were subjected to gelatin zymography (concomitant conditions) and reducing 10% and 15% SDS-PAGE for detection of MT1-MMP and TIMP-2, respectively, by immunoblot analysis with pAb 437 to MT1-MMP and monoclonal antibody CA-101 to TIMP-2 followed by detection with enhanced chemiluminescence. The ~50-kDa band is monomer specific. B, Timp2 (−/−) mutant cells infected to express MT1-MMP were incubated (16 h, 37 °C) with 2.5 nM TIMP-2 (lane 1), 25 nM Δ-TIMP-2 (lane 2), 10 nM TIMP-4 (lane 3), or 25 nM TIMP-1 (lane 4). After the incubation, the media were aspirated, and the cells were washed with DMEM followed by the addition of serum-free DMEM containing 10 nM pro-MMP-2. After a 15-min incubation at 37 °C, pro-MMP-2 activation was determined in the cell lysate fraction by gelatin zymography. The asterisks show the pro-MMP-2 added to the media. C, Timp2 (−/−) mutant cells infected to express MT1-MMP were incubated (16 h, 37 °C) without (lane 1) or with (lane 2) 10 nM exogenous recombinant TIMP-4. The cell lysates were subjected to reducing 10% SDS-PAGE followed by immunoblot analysis using a specific rabbit pAb to TIMP-4. Lane 3 shows 10 ng of the purified recombinant TIMP-4.

The presence (Fig. 1C, lane 3, without TIMP-2; lane 4 with TIMP-2). Taken together, these studies establish the importance of TIMP-2 for the MT1-MMP-dependent activation of pro-MMP-2 and are in agreement with recent in vitro and in vivo studies (65, 67) with Timp2 mutant fibroblasts and mice, respectively.

We have previously shown that TIMP-2 regulates the turnover of MT1-MMP on the cell surface by binding to the active form of the enzyme (40). This process induces the accumulation of active MT1-MMP (57 kDa) on the cell surface and concomitantly decreases the amount of a membrane-associated 44-kDa form of MT1-MMP (40). N-terminal sequencing data demonstrated that the 47 kDa species starts at Tyr112 and the 44 kDa species starts at Gly232; thus the latter represents an inactive enzyme form (40). To examine the relationship between pro-MMP-2 activation and MT1-MMP processing in the Timp2-null cell system, the homozygous Timp2 mutant cells expressing MT1-MMP were analyzed for pro-MMP-2 activation and MT1-MMP forms as a function of TIMP-2 concentration. As shown in Fig. 2A (zyogram), as little as 1 nM TIMP-2 induced pro-MMP-2 activation as monitored in the cell lysate fraction. The lysates were also analyzed for MT1-MMP forms and TIMP-2 by immunoblot analyses (Fig. 2A, immunoblot). These studies show that overnight exposure to TIMP-2, at doses of >10 nM, induce a detectable accumulation of the 57-kDa species concomitantly with a reduction in the inactive 44-kDa form of MT1-MMP. Without TIMP-2 and at doses of 1 nM TIMP-2, the major species detected were the 60-kDa (pro-MT1-MMP) and the 44-kDa species. A minor 63-kDa protein represents the pro-MT1-MMP with the signal peptide, and the ~50-kDa protein is a nonspecific band. TIMP-2 was also detected in the cell lysates (Fig. 2A, immunoblot α-TIMP-2) consistent with an association of the exogenous TIMP-2 with the MT1-MMP-expressing cells (38).

Importance of Ternary Complex on Pro-MMP-2 Activation by MT1-MMP in the Timp2-Null Cell System—Previous studies demonstrated that the formation of an MT1-MMP-TIMP-2-pro-MMP-2 (ternary) complex on the cell surface is required for pro-MMP-2 activation (12, 36, 39). To demonstrate the importance of this complex in the Timp2 null cellular system, we used a C-terminally truncated form of TIMP-2, Δ-TIMP-2, incapable of binding pro-MMP-2 (data not shown) and therefore unable to form the ternary complex (36, 53). In addition, we tested recombinant TIMP-4, known to bind to pro-MMP-2 (68). Due to their lower affinity for MT1-MMP, Δ-TIMP-2 (shown in Table 1) and TIMP-1 (36, 69) were added to the homogenous cells at concentrations 10-fold higher than that of wild type TIMP-2. As shown in the zymogram of Fig. 2B, 2.5 nM TIMP-2 (Fig. 2B, lane 1) efficiently promoted pro-MMP-2 activation. In contrast, Δ-TIMP-2 (Fig. 2B, lane 2; 25 nM) and TIMP-1 (Fig. 2B, lane 4; 25 nM) failed to induce pro-MMP-2 activation. Interestingly, exogenous TIMP-4 also had no effect on activation (Fig. 2B, lane 3; 1–100 nM, only 10 nM shown), consistent with the results of Bigg et al. (53). In addition, co-expression of MT1-MMP with TIMP-4 in the (−/−) Timp2 mutant cells using the vaccinia expression system had no effect on pro-MMP-2 activation. Immunoblot analysis demonstrated the cell association of the exogenous TIMP-4 with (−/−) Timp2 mutant cells expressing MT1-MMP (Fig. 2C, lane 2) suggesting the binding of TIMP-4 to MT1-MMP. Taken together, these results demonstrate that only full-length TIMP-2 can promote the MT1-MMP-dependent activation of pro-MMP-2 in a process that is dependent on ternary complex formation.

TIMP-2 and MMP Inhibitors Act Synergistically to Enhance Pro-MMP-2 Activation—Previous studies suggested that, in addition to ternary complex formation, the enhancing effect of TIMP-2 on pro-MMP-2 activation was the result of a specific inhibition of MT1-MMP autocatalytic turnover on the cell surface (40). Indeed, TIMP-2 induces the accumulation of the 57-kDa form of MT1-MMP (shown in Fig. 2A). It was hypothesized that at low inhibitor concentrations relative to MT1-MMP and continuous enzyme synthesis by the cells, this process would slow down enzyme turnover, generating a fraction of inhibitor-free active MT1-MMP and hence increase pericellular proteolysis (40). Since this effect is due to inhibition of MT1-MMP activity, we hypothesized that synthetic MMPis may mimic TIMP-2 in its ability to reduce MT1-MMP turnover. We asked whether reduction of MT1-MMP autocatalytic turnover by MMPis together with ternary complex formation by TIMP-2 would enhance pro-MMP-2 activation when compared with activation promoted by TIMP-2 alone.

To test this hypothesis, the Timp2 (−/−) null cells were incubated overnight with a variety of synthetic and natural MMPis to inhibit MT1-MMP autocatalysis and induce accumulation of the active 57-kDa species. The cells were then washed to remove excess unbound inhibitors and then exposed to TIMP-2 to generate the ternary complex. After a rinse to remove unbound TIMP-2, the cells received pro-MMP-2. We tested the effects of marismat and batimat, two hydroxamate-based inhibitors (33, 45, 47; SB-3CT, a mechanism-based inhibitor (42); and Δ-TIMP-2 (53). As shown in the zymogram of Fig. 3, administration of TIMP-2 alone for 5 min was sufficient to promote pro-MMP-2 activation, as expected (Fig. 3, lane 2). However, in the cells pretreated with a 1 μM concentration of either marismat (Fig. 3, lane 3) or batimat-

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3 M. Toth, unpublished results.

TABLE I

Association, dissociation, and inhibition constants for MT1-MMP interactions with natural and synthetic MMP inhibitors

MT1-MMP was added to a solution of MOCAPGLLA(prDnp)-AR-NH₂ (7 μM) and varying concentrations of inhibitor in buffer B at 25°C. Substrate hydrolysis was monitored at excitation and emission wavelengths of 328 and 355 nm, respectively, for up to 30 min. To determine the dissociation rate constants, a mixture of enzyme (50 nM) and inhibitor (50 nM) in buffer B was incubated for 1 h at 25°C and diluted 400-fold in a solution of fluorogenic substrate (10 μM) in the same buffer containing 1% Me₂SO. Substrate hydrolysis was monitored for up to 1 h. The kinetic parameters were evaluated as described under “Experimental Procedures.” Analogous results were obtained from at least two independent experiments.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>kₐ</th>
<th>kₑ₀</th>
<th>Kᵣ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type TIMP-2</td>
<td>(2.74 ± 0.14) × 10⁶</td>
<td>2 × 10⁻⁶</td>
<td>0.07</td>
</tr>
<tr>
<td>Δ-TIMP-2</td>
<td>(2.68 ± 0.12) × 10⁶</td>
<td>(1.95 ± 0.03) × 10⁻³</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>MARIMATSTAT</td>
<td></td>
<td></td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>BATIMATSTAT</td>
<td></td>
<td></td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>BB-2116</td>
<td>8 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SB-3CT</td>
<td>110 ± 11</td>
<td></td>
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</tbody>
</table>

a Estimated value based on a 10-fold difference between the slopes of the linear portions of the dissociation curves for the complexes of MT1-MMP with Δ-TIMP-2 (steady state rate) and wild type TIMP-2.

Fig. 3. Synthetic MMPs enhance pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2. The TIMP-2 (−) mutant cells in 12-well plates were co-infected to express MT1-MMP as described in the legend to Fig. 1. After the infection, the media were aspirated and replaced with serum-free DMEM (1 ml/well) supplemented with 1 μM marimastat (lane 3), 1 μM batimastat (lane 4), 1 μM SB-3CT (lane 5), or 100 nM Δ-TIMP-2 (lane 6) followed by a 15-h incubation at 37°C. Some wells received DMEM without inhibitors (lanes 1 and 2). The cells were washed once to remove excess inhibitors and then incubated with DMEM (1 ml/well) supplemented without (lane 1) or with (lanes 3–6) 10 nM TIMP-2 for 5 min at 37°C. The media were then aspirated and replaced with fresh DMEM containing 10 nM pro-MMP-2. After 15 min at 37°C, the cells were rinsed with phosphate-buffered saline and solubilized in lysis buffer. The lysates were analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. The asterisk shows the pro-MMP-2 added to the media. The ~50-kDa band is nonspecific. This experiment was repeated at least three times with similar results.tat (Fig. 3, lane 4) or 100 nM Δ-TIMP-2 (Fig. 3, lane 6), a significant increase in active MMP-2 was observed. SB-3CT at doses of 1 μM showed a modest effect (lane 5) when compared with the other synthetic MMPs. Analysis of the lysate fractions for MT1-MMP forms by immunoblotting revealed accumulation of the 57-kDa species after treatment with marimastat, batimastat, and Δ-TIMP-2 (Fig. 3, lanes 3, 4, and 6, respectively) but not with SB-3CT (Fig. 3, lane 5). However, Δ-TIMP-2 was less efficient in inhibiting MT1-MMP conversion to the 57-kDa form (Fig. 3, lane 6, immunoblot). In the absence of TIMP-2, no activation was detected (Fig. 3, lane 1, zymogram).

Since the relationship between inhibition of MT1-MMP autocatalytic turnover and the existence of inhibitor-free MT1-MMP is dependent on the inhibitor concentration and affinity, we used inhibitor doses ranging from 0 to 500 nM to further examine the differential effects of marimastat and SB-3CT on pro-MMP-2 activation. We also tested BB-2116, a boronate-containing MMP inhibitor. In addition, we asked whether TIMP-4 would act synergistically with TIMP-2 in pro-MMP-2 activation by MT1-MMP. TIMP-4 inhibits MT1-MMP activity but cannot form a ternary complex with MT1-MMP and pro-MMP-2. As shown in the zymogram of Fig. 4A, SB-3CT had no effect on pro-MMP-2 activation at doses of 4, 20, and 500 nM (Fig. 4A, lanes 5 and 6; only 20 and 500 nM, respectively, are shown). In contrast, as little as 20 nM marimastat (Fig. 4A, lane 7) had a noticeable effect on pro-MMP-2 activation when compared with TIMP-2 alone (Fig. 4A, lane 2). BB-2116 exhibited enhancing effects with TIMP-2 at doses of 500 nM (Fig. 4A, lane 4). Consistently, both marimastat (Fig. 4A, lanes 7 and 8) and BB-2116 (Fig. 4A, lane 4) induced accumulation of the 57-kDa form of MT1-MMP. Pretreatment of the cells with TIMP-4 (100 nM) followed by TIMP-2 addition had no enhancing effect on pro-MMP-2 activation (Fig. 4B). Interestingly, TIMP-4 induced the accumulation of 57-kDa form of MT1-MMP consistent with its inhibitory activity. These results suggest that, with the exception of TIMP-4 and SB-3CT, certain synthetic MMPs and Δ-TIMP-2, which inhibit MT1-MMP activity and consequently autocatalytic degradation, can enhance MT1-MMP-dependent pro-MMP-2 activation in the presence of TIMP-2.

The Enhancing Effect of MMPs on Pro-MMP-2 Activation by MT1-MMP Requires TIMP-2 for Ternary Complex Formation—To examine the relationship between the effects of the MMPs on pro-MMP-2 activation (inhibition of MT1-MMP autocatalysis) and ternary complex formation, the TIMP-2 (−) mutant cells were pretreated with marimastat to accumulate the 57-kDa form of MT1-MMP and then were or were not exposed to TIMP-2, Δ-TIMP-2, or TIMP-1. As expected, marimastat pretreatment and the addition of TIMP-2 (Fig. 5, lanes 2) resulted in a significant increase in pro-MMP-2 activation when compared with the activation observed with TIMP-2 alone (lane 1). In contrast, administration of either Δ-TIMP-2 (lane 3) or TIMP-1 (lane 4) after the marimastat treatment had no effect. Marimastat treatment alone had no effect on pro-MMP-2 activation (lane 5). Immunoblot analysis demonstrated the presence of the 57-kDa form of MT1-MMP after marimastat treatment, as expected (Fig. 5, immunoblot). Taken together, these results indicate that inhibition of MT1-MMP turnover alone is not sufficient to promote pro-MMP-2 activation, a process that requires ternary complex formation. However, both processes can act synergistically to enhance activation.

Effect of Δ-TIMP-2 and Synthetic MMPs on MT1-MMP Activity—The results above indicated a differential inhibition of MT1-MMP autocatalytic turnover by various synthetic MMPs and Δ-TIMP-2. In order to elucidate the inhibitor effects on MT1-MMP activity observed in the cells, the interactions of the catalytic domain of MT1-MMP (MT1-MMPcat) with natural and synthetic inhibitors were characterized in a purified system. As depicted in Table I, both TIMP-2 and Δ-TIMP-2 exhibit slow binding kinetics with similar association rate constants of
**MMP Inhibitors and MT1-MMP Activity**

**Fig. 4. Synergistic effect of TIMP-2 with synthetic MMPIs and TIMP-4 on pro-MMP activation.** A, TIMP-2 (+/-) mutant cells in 12-well plates were infected to express MT1-MMP as described in Fig. 1. After the infection, the media were aspirated and replaced with serum-free DMEM (1 ml/well) supplemented without (lanes 1 and 2) or with SB-2116 (lane 3, 20 nm; lane 4, 500 nm), SB-3CT (lane 5, 20 nm; lane 6, 500 nm) or marimastat (lane 7, 20 nm; lane 8, 500 nm). After a 16-h incubation at 37 °C, the media were aspirated, and the cells were washed with DMEM to remove excess inhibitors and then incubated with DMEM (1 ml/well) supplemented without (lane 1) or with lanes 2–6) 10 nm TIMP-2 for 5 min at 37 °C. The media were then aspirated and replaced with DMEM containing 10 nm pro-MMP-2. After 15 min at 37 °C, the cells were solubilized in lysis buffer and analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. B, TIMP-2 (+/-) mutant cells in 12-well plates expressing MT1-MMP were incubated (16 h, 37 °C) without (lane 1) or with TIMP-4 (lanes 2, 1 nm; lane 3, 10 nm; lane 4, 100 nm). After removal of the unbound TIMP-4, the cells received 10 nm TIMP-2 for a 5-min incubation at 37 °C followed by the addition of 10 nm pro-MMP-2 as described above. Analysis of pro-MMP-2 activation and MT1-MMP forms were monitored by gelatin zymography and immunoblot analysis, respectively.

**Fig. 5. TIMP-2 is required for the enhancing effect of marimastat on pro-MMP-2 activation by MT1-MMP.** TIMP-2 (+/-) mutant cells infected to express MT1-MMP in 12-well plates were treated (lanes 2–5) or not (lane 1) with 1 µM marimastat overnight at 37 °C. The medium was then aspirated and replaced with medium supplemented with either 10 nm TIMP-2 (lanes 1 and 2), Δ-TIMP-2 (lane 3), or TIMP-1 (lane 4) or medium without TIMPs (lane 5). After a 30-min incubation, the medium was aspirated, and the cells were rinsed with DMEM and incubated (30 min, 37 °C) with 10 nm pro-MMP-2. The cell lysates were analyzed for pro-MMP-2 activation and MT1-MMP forms by gelatin zymography and immunoblot analysis, respectively. The asterisk shows the pro-MMP-2 added to the medium.

(2.74 ± 0.14) × 10^10 and (2.68 ± 0.12) × 10^10 m^−1 s^−1, respectively. The latter value is in agreement with that reported by Butler et al. (36) for the interaction of the (Δ128–194) TIMP-2 mutant with the catalytic domain of MT1-MMP (2.80 ± 0.45 × 10^10 m^−1 s^−1). TIMP-2 bound with a picomolar K_i (0.07 nm) and showed significant inhibition at a concentration similar to that of the enzyme itself. Δ-TIMP-2 exhibits reduced affinity (K_i = 0.73 ± 0.03 nm) due to a 10-fold higher dissociation rate constant (k_off = 1.95 ± 0.03 × 10^−3 s^−1) relative to the value for the full-length TIMP-2. The synthetic MMP inhibitors marimastat, batimastat, SB-2116, and SB-3CT show competitive inhibition and, with the exception of SB-3CT, exhibit K_i values in the low nanomolar range. These data are in agreement with the K_i values for marimastat and batimastat reported by Yamamoto et al. (70) for a mutant MT1-MMP lacking the transmembrane domain. In addition, the K_i value for marimastat with the MT1-MMPcat compares with K_i values reported for the interaction of this inhibitor with the gelatinases (IC_50 = 3–6 nm), fibroblast collagenase (IC_50 = 5 nm), and matryalin (IC_50 = 16 nm) consistent with marimastat being a nonspecific (i.e., broad-spectrum) MMP inhibitor (33, 45, 47, 71). Interestingly, SB-3CT shows an ~10–1600-fold reduced affinity (K_i = 110 nm) for MT1-MMPcat compared with the other MMPIs, in agreement with its inability to induce accumulation of the 57-kDa species of MT1-MMP and pro-MMP-2 activation with TIMP-2.

**Synergistic Effects of MMP Inhibitors on Pro-MMP-2 Activation in a Background of Endogenous Expression of TIMP-2.**—To further examine the synergistic effects of MMPIs and TIMP-2 on pro-MMP-2 activation, we used BS-C-1 cells infected to express MT1-MMP. BS-C-1 cells produce low levels of endogenous TIMP-2, which are further suppressed but not completely eliminated upon viral infection (data not shown). Consistently, BS-C-1 cells infected to express MT1-MMP can activate pro-MMP-2 without the addition of exogenous TIMP-2 (40). Thus, we used BS-C-1 cells to examine the effects of MMPIs on pro-MMP-2 activation in a cellular system expressing a background level of endogenous TIMP-2. BS-C-1 cells infected to express MT1-MMP were incubated with increasing concentrations of TIMP-2 (0–20 nm), Δ-TIMP-2 (0–500 nm), SB-3CT (0–1 µM), or marimastat (0–10 µM) followed by the addition of pro-MMP-2. In addition, we tested the effects of Ala + TIMP-2 (0–100 nm), a mutant TIMP-2 devoid of inhibitory activity, as a negative control inhibition of MT1-MMP autocalytic turnover. As shown in Fig. 6, pro-MMP-2 activation is greatly enhanced after administration of exogenous TIMP-2 (TIMP-2 panel). Both marimastat and Δ-TIMP-2 enhance pro-MMP-2 activation when compared with the basal activation detected in BS-C-1 cells in the absence of inhibitors (due to endogenous TIMP-2). Consistently, activation under these conditions is associated with accumulation of the 57-kDa species of MT1-MMP as shown in the immunoblots of Fig. 6. Both Ala + TIMP-2 and SB-3CT have no significant effects, suggesting that inhibition of the MT1-MMP autocalytic turnover is required for the synergistic effect of the synthetic and natural MMPIs with the endogenous TIMP-2. Indeed, neither Ala + TIMP-2 nor SB-3CT induces a detectable accumulation of the 57-kDa form (Fig. 6, immunoblot). Taken together, these studies indicate that inhibition of MT1-MMP turnover (accumulation of 57-kDa form) by synthetic MMPIs can enhance the effect of the endogenous TIMP-2 in pro-MMP-2 activation in the BS-C-1 cell system.

**DISCUSSION**

The studies presented here provide conclusive evidence for the complex regulation of MT1-MMP activity by TIMP-2 and further demonstrate that some synthetic MMPIs might have the potential to promote MT1-MMP-dependent activation of pro-MMP-2. Our results clearly show that pro-MMP-2 activation requires the presence of TIMP-2, since the TIMP-2-null cells are unable to activate pro-MMP-2 even after expression of MT1-MMP. However, a short (5-min) incubation with exogenous TIMP-2 and a brief incubation (15 min) with pro-MMP-2 result in a significant conversion of pro-MMP-2 to its active form. This rapid activation of pro-MMP-2 is unprecedented in a cellular system and demonstrates the high catalytic efficiency of MT1-MMP for this substrate under optimal conditions. The dependence on TIMP-2 for activation is also evident from the results with the heterozygous Timp2 (+/-) mutant cells and the BS-C-1 cells, both of which contain endogenous TIMP-2 and are able to activate pro-MMP-2 after expression of MT1-MMP without requirement of exogenous TIMP-2. Strong et al. (12) proposed that the effect of TIMP-2 on activation is mediated by a ternary complex formed between active MT1-MMP, TIMP-2, and pro-MMP-2, where the C-terminal region of TIMP-2 binds
to pro-MMP-2 to anchor thezymogen to the cell surface. Although formation of such a complex was not directly demonstrated in the present study, the results with Δ-TIMP-2, which lacks the C-terminal domain and failed to induce pro-MMP-2 activation in the Timp2(−/−) mutant cells, indirectly support the ternary complex model of pro-MMP-2 activation.

In addition to its role in ternary complex formation, TIMP-2 also influences the processing of MT1-MMP. We have recently shown that TIMP-2 prevents the autocatalytic conversion of active MT1-MMP (57 kDa) to its inactive 44-kDa species, and as a consequence the 57-kDa species accumulates on the cell surface (40). As shown here, a similar effect is induced by synthetic MMPls (41) as well as by Δ-TIMP-2 and TIMP-4. We show for the first time that some synthetic MMPls and Δ-TIMP-2 can enhance pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2. This effect is due to the accumulation of the 57-kDa MT1-MMP species as a consequence of inhibition of MT1-MMP turnover. Our kinetic data suggest that the increase in MT1-MMP-TIMP-2 complexes may be a consequence of a displacement of the bound synthetic MMPs by TIMP-2 (KΔ for TIMP-2 is approximately 1–2 orders of magnitude lower than that for the synthetic MMPs), a process that will generate more pro-MMP-2 "receptors." However, binding of small molecule inhibitors concurrently with TIMP-2 to active MT1-MMP cannot be ruled out. Regardless of the mechanism involved, pro-MMP-2 activation would require a "catalytic" quantity of the inhibitor-free MT1-MMP to hydrolyze the Asn37-Leu48 bond of pro-MMP-2 as previously shown (69). It should be noted that the enhancing effects of the MMPls on pro-MMP-2 activation in the Timp2(−/−) null cells in the presence of TIMP-2 were evident only when the synthetic inhibitors (up to 10 μM) to avoid toxic effects) were administered to the cells and removed from the system prior to the administration of the exogenous TIMP-2. Preincubation of the cells with the MMPls was necessary to induce accumulation of the 57-kDa form of MT1-MMP, a fraction of which would be expected to be inhibitor-free, since the cells are continuously producing MT1-MMP.

We postulate that these conditions (removal of excess synthetic inhibitor prior to the addition of TIMP-2 and pro-MMP-2 and continuous replenishment of MT1-MMP by the cells) generate sufficient catalytically active MT1-MMP to generate ternary complex and to process pro-MMP-2. In contrast, simultaneous administration of TIMP-2 with various doses of marimastat inhibits pro-MMP-2 activation in a dose-dependent manner (data not shown). This inhibitory effect is likely to be due to competition between TIMP-2 and marimastat for MT1-MMP binding, resulting in decreased ternary complex formation (38, 72). However, BS-C-1 cells, which contain low levels of endogenous TIMP-2, exhibit enhanced pro-MMP-2 activation upon administration of the MMPls. In this case, enhanced activation is the result of the inhibition of MT1-MMP autocatalysis and ternary complex formation is not a limiting step. The result with the BS-C-1 cells also suggests that invasive tumor cells equipped with both MT1-MMP and TIMP-2 may be subject to similar synergistic effects of synthetic MMPls on MT1-MMP activity under the right conditions.

The relationship between inhibition of TIMP-2 autocalystase and ternary complex formation was also demonstrated in the experiments in which the Timp2(−/−) mutant cells were treated with marimastat followed by administration of Δ-TIMP-2 or TIMP-1, in which case pro-MMP-2 activation was not observed. Furthermore, the Ala + TIMP-2 mutant, devoid of inhibitory activity, failed to support pro-MMP-2 activation in the BS-C-1 cells in the presence of endogenous TIMP-2 due to its inability to inhibit MT1-MMP turnover. Likewise, pretreatment of the Timp2(−/−) cells with marimastat had no effect on pro-MMP-2 activation without subsequent addition of TIMP-2 revealing that accumulation of active MT1-MMP alone is not sufficient for pro-MMP-2 activation and requires a functional TIMP-2 to generate the ternary complex. This was also demonstrated by the results with TIMP-4, which, despite its ability to inhibit MT1-MMP activity as found by Bigg et al. (2) and to bind pro-MMP-2 (68), was unable to promote MT1-MMP-dependent activation of pro-MMP-2 or to act synergistically with TIMP-2 in this process. Recent studies from Overall's laboratory (76) also show that TIMP-4 cannot form a ternary complex in a purified system and, if administered with TIMP-2, inhibits pro-MMP-2 activation by MT1-MMP in Timp2(−/−) mutant cells. Here we have shown that TIMP-4, like other MMPls, induces accumulation of the 57-kDa form of MT1-MMP, consistent with its inhibitory activity, but fails to act synergistically with TIMP-2 in the promotion of pro-MMP-2 activation. The reason for this puzzling result is yet unknown but may be related to differences in affinity between these inhibitors for MT1-MMP and pro-MMP-2. Indeed, TIMP-4 exhibits a lower affinity for pro-MMP-2 when compared with TIMP-2. Also, a potential rapid internalization of the putative MT1-MMP-TIMP-4 complex, although yet unproven, may play a role. Further enzymatic and biochemical studies are required to understand the dynamics of TIMP-4 and TIMP-2 inhibitory activities in relation to MT1-MMP functions. Nevertheless, these studies suggest that the effects of TIMP-4 on TIMP-2 may represent a natural and unique regulatory mechanism of MMP-dependent proteolysis on the cell surface in which TIMP-4 may play a counter role to that of TIMP-2, physiologically, by binding to active MT1-MMP with high affinity. We therefore propose that the long held view of a balance between MMPls and TIMPls as a key determinant of proteolytic activity and tumor progression (75) may well include a balance of TIMP-2 and TIMP-4 as a major determining factor for MT1-MMP-dependent proteolysis in cancer tissues where both inhibitors may be present.

The results presented here demonstrate that pro-MMP-2 activation by MT1-MMP at the cell surface is the result of a
highly regulated enzymatic process that involves two independent events, which under certain conditions may work synergistically to enhance MT1-MMP-dependent activation of pro-MMP-2. It should be noted that this might not be the case in all circumstances or with different MT1-MMP substrates. For example, for pro-MMP-2, our data show that a short (5-min) exposure to TIMP-2 followed by a 15-min incubation with pro-MMP-2 was sufficient to rapidly activate pro-MMP-2 without detectable accumulation of active (57-kDa) MT1-MMP. The reason for the lack of detection of active enzyme under this conditions is unclear but may be related to the detection method (immunoblotting), rapid enzyme turnover, and/or the internalization and turnover of the MT1-MMP (57 kDa)/TIMP-2 complex as recently reported (72). Under conditions of substoichiometric TIMP-2 molar concentrations relative to MT1-MMP, the efficient binding of TIMP-2 and the catalytic efficiency of MT1-MMP for its substrate result in optimal pro-MMP-2 activation (59). Thus, while rapid bursts of TIMP-2 expression will be sufficient to generate ternary complex and consequently activate pro-MMP-2 in the absence of a significant and detectable accumulation of active MT1-MMP, chronic exposure to TIMP-2 or MMPs would maintain a steady level of MT1-MMP on the cell surface due to inhibition of autocatalysis. For other MT1-MMP substrates such as ECM components, which do not require ternary complex formation to be hydrolyzed by MT1-MMP, sustained TIMP-2 expression and/or the presence of synthetic MMPs may indirectly enhance catalytic activity, as demonstrated here using pro-MMP-2 as a target substrate.

Recent accomplishments in drug design have resulted in the generation of a variety of novel MMP inhibitors with effective anti-tumor and anti-angiogenic activities in animal models of cancer (3, 4, 33, 46, 47). These encouraging results have brought some of these compounds, such as marimastat and batimatstat, to human clinical trials. The majority of the compounds undergoing testing in humans, however, lack specificity toward the various MMP families. The hydroxamates, for instance, inhibit a wide spectrum of MMPs, including MT1-MMP as herein demonstrated, often with similar affinities (70, 71). The complex outcome of MT1-MMP inhibition on catalytic activity demonstrated here raises important issues regarding the potential consequences of inhibiting MT1-MMP. The intramolecular autocatalytic turnover of MT1-MMP on the cell surface may represent an important regulatory step aimed at controlling pericellular proteolysis, a process that is likely to be favored by lateral diffusion and clustering of MT1-MMP molecules in the cell surface (74, 75). Thus, reversible inhibition of MT1-MMP activity would play a role in preventing excessive enzyme cleavage from the cell surface and indirectly favor proteolysis. Such an effect by synthetic MMP inhibitors would depend on the spectrum of activity (K_i values) elicited by each particular inhibitor against the different members of the MMP family and on their pharmacokinetics and dosing regime. The MMPs tested here exhibit different K_i values for the catalytic domain of MT1-MMP, which correlated well with their efficacy in promoting pro-MMP-2 activation with TIMP-2. We recently described the first example of a mechanism-based inhibitor for MMPs (42). This inhibitor, SB-3CT, is highly specific for inhibition of gelatinases, enzymes that were inhibited covalently by this inhibitor. SB-3CT does not perturb the metal chelation strategy for its inhibition, in contrast to the case of the existing inhibitors. We have shown here that SB-3CT is substantially less effective in inhibition of MT1-MMP, for which it was not designed, and simply behaves as a simple linear competitive inhibitor, in contrast to the case of gelatinases (42). Again in contrast to marimastat and batimatstat, SB-3CT did not show any ability to stimulate activation of pro-MMP-2 induced by TIMP-2. Thus, the design of highly specific MMP inhibitors will minimize potential adverse effects in conditions where inhibition of the MT1-MMP-2 system is a therapeutic goal.

The complex regulation of MT1-MMP activity by TIMP-2 may provide a biochemical framework for understanding several intriguing observations in human tumors and experimental models of metastasis using synthetic MMP inhibitors. High levels of TIMP-2 expression were found in various human cancers, which positively correlated with metastasis and poor survival (21, 76–79). A recent study reported that treatment of tumorbearing mice with batimatstat significantly inhibited tumor growth but promoted tumor cell invasion into the liver of a variety of human cancer cells (80). However, the mechanism for such effect was not reported. Finally, recent tumorigenicity studies with the heterozygous and homozygous Timp2 mutant cells indicate a higher incidence of tumor formation and metastasis in the heterozygous cells, suggesting a role for TIMP-2 in promotion of tumor progression.6 Our findings disclosed in this report provide one plausible explanation for these observations, that by binding to active MT1-MMP, both natural and synthetic MMP inhibitors may produce a "pool" of active MT1-MMP available to degrade ECM components and to activate pro-MMP-2. While this may represent an undesired effect of some strategies for anti-MMP therapies in cancer that are being investigated, this effect may be beneficial in pathological conditions characterized by excessive deposition of collagen such as fibrosis and connective tissue disorders where increased MMP activity might be desired. These examples and the studies presented herein emphasize the importance of a rational approach for the design of specific MMP inhibitor, which should also be based on an understanding of the regulation of MT1-MMP and likely other members of the MT-MMP subfamily by TIMPs and MMPs at the cell surface.

REFERENCES


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Differential Roles of TIMP-4 and TIMP-2 in Pro-MMP-2 Activation by MT1-MMP

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The tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMP enzymatic activity. However, TIMP-2 can promote the activation of pro-MMP-2 by MT1-MMP. This process is mediated by the formation of a complex between MT1-MMP, TIMP-2, and pro-MMP-2. Binding of TIMP-2 to active MT1-MMP also inhibits the autolytic turnover of MT1-MMP on the cell surface. Thus, under certain conditions, TIMP-2 is a positive regulator of MMP activity. TIMP-4, a close homologue of TIMP-2 also binds to pro-MMP-2 and can potentially participate in pro-MMP-2 activation. We coexpressed MT1-MMP with TIMP-4 and investigated its ability to support pro-MMP-2 activation. TIMP-4, unlike TIMP-2, does not promote pro-MMP-2 activation by MT1-MMP. However, TIMP-4 binds to MT1-MMP inhibiting its autolytic processing. When coexpressed with TIMP-2, TIMP-4 competitively reduced pro-MMP-2 activation by MT1-MMP. A balance between TIMP-2 and TIMP-4 may be a critical factor in determining the degradative potential of cells in normal and pathological conditions.

Key Words: matrix metalloproteinases; TIMP; proteases; membrane proteins; cell surface.

The activation of the zymogenic form of MMP-2 (pro-MMP-2) has been shown to be accomplished by the membrane-tethered MT1-MMP (MMP-14) (1, 2), which hydrolyses the Asn37-Leu48 peptide bond in the prodomain of pro-MMP-2 (3). To facilitate the association of the prodomain of pro-MMP-2 with the active site of MT1-MMP, pro-MMP-2 must be positioned in close association with MT1-MMP. To achieve this, TIMP-2 acts as a molecular link between pro-MMP-2 and MT1-MMP (1). It has been shown that the NH2-terminal region of TIMP-2 binds to the active site of an active MT1-MMP on the cell surface generating a pro-MMP-2 "receptor" (4). In turn, the COOH-terminal region of TIMP-2 binds to the COOH-terminal domain of pro-MMP-2, also known as the hemopexin-like domain (HLD) (1, 4-8). This trimeric MT1-MMP/TIMP-2/pro-MMP-2 complex permits the association of pro-MMP-2 to the cell surface, which eventually facilitates its activation by a neighboring TIMP-2-free MT1-MMP. Under these conditions, TIMP-2 acts as a positive regulator of pro-MMP-2 activation (9). We have recently shown that in addition to its ability to form the pro-MMP-2 receptor, TIMP-2 can also regulate the nature of MT1-MMP forms present in the cells by its ability to inhibit MT1-MMP activity (6). This effect is due to the TIMP-2 inhibition of the autolytic turnover of MT1-MMP on the cell surface, which may represent a natural mechanism of clearance of active MT1-MMP from the cell surface once the enzyme has fulfilled its pericellular proteolytic function. As a consequence of the inhibition of autoproteolytic degradation, de novo synthesis and membrane incorporation of new MT1-MMP molecules, TIMP-2 binding to active MT1-MMP results in accumulation of active MT1-MMP on the cell surface (6). These dual effects of TIMP-2 (ternary complex formation and inhibition of MT1-MMP autolytic turnover) contribute to the overall effects of MT1-MMP on the cell surface: activation of pro-MMP-2 and direct pericellular proteolysis (10, 11).

TIMP-2 belongs to a family of four TIMP inhibitors, which presently includes TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (12). Studies on TIMP-MMP interactions have shown that TIMP-4, like TIMP-2 is also capable of forming a complex with pro-MMP-2, which is mediated by binding of the inhibitor to the HLD of the enzyme (13). Thus, functionally, TIMP-4 is similar to TIMP-2. In addition, sequence analyses revealed a 70% identity between TIMP-2 and TIMP-4 (12, 14). Based on these
observations, we asked whether TIMP-4, like TIMP-2, could also promote pro-MMP-2 activation by MT1-MMP. To answer this question, we expressed human TIMP-4 in a vaccinia expression system (15, 16) and tested its ability to promote pro-MMP-2 activation and inhibition of MT1-MMP autocatalysis in mammalian cells expressing MT1-MMP. Our studies demonstrate that unlike TIMP-2, TIMP-4 cannot promote MT1-MMP-dependent pro-MMP-2 activation but can inhibit MT1-MMP autocatalytic degradation. Furthermore, if co-expressed with TIMP-2, TIMP-4 reduces the rate of pro-MMP-2 activation induced by TIMP-2.

MATERIALS AND METHODS

Cells. Nonmalignant monkey kidney epithelial BS-C-1 (CCL-20) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Immortalized homozygous Timp2 (−/−) mutant mouse fibroblasts were isolated from Timp2 deficient mice and immortalized by retroviral infection as described (7, 17, 18) and maintained in DMEM supplemented with 10% FBS and antibiotics. All tissue culture reagents were purchased from Gibco BRL (Grand Island, NY).

Vaccinia virus and construction of expression vectors. The generation of the vaccinia virus expression vector pTF7EMVCV-1 (pTF7) containing the T7 RNA promoter and the production of vTF7-3, a recombinant vaccinia virus expressing the T7 RNA polymerase, have been described by Fuerst et al. (16). The generation of the pTF7-T2 vector expressing human TIMP-2 (15) and pTF7-MT1 expressing human MT1-MMP (6) has been described. The human full-length TIMP-4 cDNA (14), a generous gift from Dr. Y. E. Shi (Albert Einstein College of Medicine, New Hyde Park, NY 11042), was amplified by the polymerase chain reaction (PCR) using the following oligonucleotide primers: 5'-CATTCATGACATTGGAGAGCTCC-3' and 5'-CTAAGCTTTCGACCATGTCAAC-3' containing the NcoI and BamHI restriction sites, respectively. The amplified TIMP-4 fragment was isolated and cloned into pTF7 vector (18) to generate the pTF7-T4 plasmid. The DNA sequence of the TIMP-4 fragment was verified by sequencing of both strands directly from the pTF7-T4 vector using an ABI377A DNA sequencer.

Expression of recombinant proteins by infection-transfection. Monkey kidney BS-C-1 or Timp2 (−/−) mutant cells in 6-well plates were infected with 30 plaque-forming units (pfu)/cell of the vTF-3 virus in DMEM containing 2.5% FBS (15). Thirty minutes postinfection, the media were aspirated and the infected cells were co-transfected with a mixture of either pTF7-MT1 and pTF7-T4 plasmids (04 μg/ml each) or pTF7-MT1 and pTF7-T2 plasmids (04 μg/ml each) to coexpress MT1-MMP with each inhibitor using Effectene (Qiagen, Valencia, CA), as described by the manufacturer. In some experiments, MT1-MMP was coexpressed with both TIMP-2 and TIMP-4 and TIMP-2 by transfecting a mixture of the three plasmids (04 μg/ml each). As controls, the infected cells were transfected with pTF7-MT1, pTF7-T4, pTF7-T2 or the empty pTF7EMVC-1 expression vectors alone. An additional control included cells infected with vTF-3 virus but nontransfected. Four h post-transfection, the media were aspirated and replaced with 1 ml/well of OPTI-MEM (Gibco BRL).

Activation of pro-MMP-2. Eighteen hours following the infection/transfection procedure, the cells received 3 nM/well of purified recombinant pro-MMP-2, purified to homogeneity as previously described (15), followed by a 4-h incubation at 37°C. The media were collected and clarified by a brief centrifugation (13,000g, 15 min, 4°C) and the cells were solubilized in 100 μl/well of cold lysis buffer (23 mM Tris–HCl [pH 7.5], 1% IGEPAL CA-630, 100 mM NaCl, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM benzamidine, and 1 mM PMSF) and centrifuged (13,000g) for 15 min at 4°C. Samples of the lysates (10 μl) were mixed with 4× Laemmli sample buffer (19) without reducing agents and without heating and subjected to gelatin zymography, as previously described (20).

Immunoprecipitation of TIMP-2 and TIMP-4. BS-C-1 cells Infected-transfected to express TIMP-2 or TIMP-4 with or without MT1-MMP, in 6-well plates, were metabolically labeled for 4 h at 37°C with 100 μCi/ml of [35S]-methionine in 1 ml/well of DMEM with 1% dialyzed FBS. The media were collected, clarified by a brief centrifugation and an aliquot incubated (16 h, 4°C) with either 5 μg of anti-TIMP-2 CA-1 antibody, a monoclonal antibody to a rabbit polyclonal antibody to TIMP-4 (21) followed by the addition of 30 μl of Protein G-Sepharose beads for an additional 3-h incubation at 4°C. After recovering the beads by a brief centrifugation, the beads were washed (5 times) with cold 50 mM Tris–HCl pH 7.5 containing 150 mM NaCl, 0.1% NP-40, and 1% glycerol and resuspended in 15 μl Laemmli sample buffer with β-mercaptoethanol followed by boiling (5 min). The immunoprecipitates were resolved by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Detection of radiolabeled proteins was performed by autoradiography.

Immunoblot analysis of MT1-MMP forms. Infected BS-C-1 cells in 6-well plates, as described above, were lysed in cold lysis buffer. The lysates were mixed with 4× Laemmli sample buffer with β-mercaptoethanol and then resolved by 10% SDS–PAGE followed by transfer to a nitrocellulose membrane as described (20). Detection of MT1-MMP and TIMP-4 were carried out using a polyclonal antibody to human MT1-MMP (pAb 437) (22) and a polyclonal antibody to human TIMP-4 (21), respectively. Detection of the antigen – antibody complex was performed using the SuperSignal Enhanced Chemiluminescence (ECL) system (Pierce, Rockford, IL), according to the manufacturer’s instructions.

RESULTS AND DISCUSSION

Our previous studies demonstrated that coexpression of human MT1-MMP with TIMP-2 in a vaccinia virus expression system resulted in enhanced activation of pro-MMP-2 (6). Activation of pro-MMP-2 by MT1-MMP was TIMP-2-dependent since no activation was observed in cells devoid of TIMP-2 (Timp2 mutant cells) (7). Furthermore, TIMP-2 presence induced the accumulation of the 57-kDa form of MT1-MMP, which represents the active enzyme starting at Y112. Concomitantly with the accumulation of the 57-kDa form there was a reduction of the 44–40-kDa autocatalytic product of MT1-MMP in the cell lysates (7). To examine the effects of TIMP-4 on pro-MMP-2 activation, the full-length human TIMP-4 cDNA was cloned into the pTF7EMVC-1 vaccinia expression vector (16) and expressed in BS-C-1 cells with or without MT1-MMP using the infection-transfection procedure. For comparison, the cells were infected-transfected to coexpress TIMP-2 and MT1-MMP. Analysis of TIMP-4 and TIMP-2 expression was monitored by immunoprecipitation of the inhibitors from the media of [35S]-labeled cells. As shown in Fig. 1, both TIMP-2 (Fig. 1, lane 2) and TIMP-4 (Fig. 1, lane 4) were detected in the media when expressed without MT1-MMP. In contrast, co-expression of the inhibitors with MT1-MMP

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resulted in a significant reduction of TIMP-2 (Fig. 1, lane 3) and TIMP-4 (Fig. 1, lane 5) from the media. We have previously shown that co-expression of TIMP-2 with MT1-MMP results in the targeting of the inhibitor to the cell surface. This differential distribution is due to the binding of TIMP-2 to active MT1-MMP resulting in a specific down-regulation of TIMP-2 protein from the media. Thus, the lack of detection of TIMP-4 in the supernatant of cells coexpressing MT1-MMP and TIMP-4 suggests that TIMP-4, like TIMP-2, associates with active MT1-MMP. Indeed, when coexpressed with MT1-MMP, TIMP-4 is found mostly in the cell lysate (shown in Fig. 3B, lane 3).

We next examined the ability of TIMP-4 to promote pro-MMP-2 activation by MT1-MMP. To this end, the BS-C-1 and the Timp2 (−/−) mutant cells were infected-transfected to express MT1-MMP with or without TIMP-2 or TIMP-4. Then, the cells received exogenous pro-MMP-2. Activation was monitored by gelatin zymography of cell lysates. As shown in Fig. 2, BS-C-1 (Fig. 2A) or Timp2 (−/−) mutant (Fig. 2B) cells coexpressing MT1-MMP and TIMP-2 (BS-C-1, Fig. 2A, lane 3; Timp2 (−/−), Fig. 2B, lane 4) converted pro-MMP-2 (refer as P) into the active MMP-2 (refer as A) form of 62-kDa. In contrast, coexpression of MT1-MMP with TIMP-4 in either BS-C-1 (Fig. 2A, lane 4) or Timp2 (−/−) mutant (Fig. 2B, lane 5) cells failed to promote pro-MMP-2 activation. BS-C-1 cells infected-transfected to express MT1-MMP alone (Fig. 2A, lane 2) exhibited a modest degree of active MMP-2 when compared to the activation observed when MT1-MMP was coexpressed with TIMP-2 (Fig. 2A, lane 3). This basal activation is due to the presence of some residual endogenous TIMP-2, which is present in the BS-C-1 cells, as reported previously (7). Indeed, in the Timp2 (−/−) mutant cells, which are devoid of TIMP-2, no evidence of active MMP-2 was observed in cells expressing MT1-MMP alone (Fig. 2B, lane 3) consistent with the absolute requirement of TIMP-2 for the MT1-MMP-dependent activation of pro-MMP-2. As expected, no activation was observed in either cell type infected with the vTF7-3 virus and transfected with the empty vector (BS-C-1, Fig. 2A, lane 1; Timp2 (−/−), Fig. 2B, lane 2). These studies establish the inability of TIMP-4 to promote pro-MMP-2 activation by MT1-MMP and demonstrate that this effect can only be mediated by TIMP-2 in spite of the ability of both inhibitors to form a non-covalent complex with pro-MMP-2 through its hemopexin-like domain (13, 15). The localization of TIMP-4 in the cell lysate when coexpressed with MT1-MMP suggested that TIMP-4 could bind to active MT1-MMP. Therefore, we wished to examine whether coexpression of TIMP-2 and TIMP-4 with MT1-MMP would affect the ability of TIMP-2 to enhance pro-MMP-2 activation. As shown in Fig. 2A, BS-C-1 cells coexpressing MT1-MMP, TIMP-2, and TIMP-4 (Fig. 2A, lane 5) exhibited a significant reduction in active MMP-2 when compared to the cells expressing MT1-MMP with TIMP-2 (Fig. 2A, lane 3). This result suggests that TIMP-4 competes for the binding of TIMP-2 to MT1-MMP and therefore prevents its ability to support pro-MMP-2 activation.

FIG. 1. Expression of TIMP-2 and TIMP-4 with and without MT1-MMP. BS-C-1 cells were infected-transfected to express TIMP-2 (lanes 2 and 3) or TIMP-4 (lanes 4 and 5) alone (lanes 2 and 4) or with MT1-MMP (lanes 3 and 5) as described under Materials and Methods. As control, cells were infected and then transfected with the empty vector. The next day, the media were collected and immunoprecipitated with antibodies to either TIMP-2 (lanes 2 and 3) or TIMP-4 (lanes 4 and 5) and Protein G-Sepharose beads. The medium of control-transfected cells (lane 1) was immunoprecipitated with both antibodies. The immunoprecipitates were resolved by reducing 15% SDS-PAGE followed by autoradiography. 14C-labeled molecular weight standards were used as reference.

FIG. 2. Effect of TIMP-4 on pro-MMP-2 activation by MT1-MMP. (A) BS-C-1 cells were infected with 30 pfu/cell of vTF7-3 and then transfected with 0.4 μg/well of each of plasmid DNA (lane 1, pTF7EMCV-1 empty vector; lane 2, pTF7-MT1; lane 3, pTF7-MT1 + pTF7-T2; lane 4, pTF7-MT1 + pTF7-T4; and lane 5, pTF7-MT1 + pTF7-T4 + pTF7-T2). Some cells were only infected with 30 pfu/cell of vTF7-3 (lane 1). At 18 h post-infection/transfection, the cells were incubated with 3 nM of purified recombinant pro-MMP-2 for an additional 4 h at 37°C followed by solubilization of the cells with lysis buffer. The lysates were then subjected to gelatin zymography. (B) Timp2 (−/−) mutant cells were infected-transfected to express MT1-MMP alone (lane 3) or with either TIMP-2 (lane 4) or TIMP-4 (lane 5) as described in A. As control, some mutant cells were infected with vTF7-3 virus only or subsequently transfected with empty vector (lane 2). Activation of pro-MMP-2 was carried out as described in A. P, I, and A refer to pro-MMP-2 (72 kDa), intermediate form (64 kDa), and active MMP-2 (62 kDa).
Early studies showed a restricted pattern of expression of TIMP-4 in human and mouse tissues with preponderant expression in heart tissue (14, 23). However, recent studies indicate a broader tissue expression of TIMP-4 in normal tissues (24–26) and enhanced expression in pathologic conditions (27–31). In various instances, the expression of TIMP-4 has been shown to overlap with that of TIMP-2 (27–29, 31). TIMP-4 has also been shown to inhibit tumor growth and metastasis of a human breast cancer cell line (32). However, its expression in human cancer tissues has not been examined in detail. Although the precise role of each TIMP in normal and pathologic conditions remains to be further evaluated, the ability of TIMP-4 to inhibit the effect of TIMP-2 on pro-MMP-2 activation suggests that a balance of these inhibitors may alter the net activity of MMP-2 with TIMP-2, under certain conditions, promoting MMP-2- and MT1-MMP-dependent proteolysis and TIMP-4 acting as a general MMP inhibitor.

Presently, no information exists in regards to the molecular interactions of TIMP-4 with MT1-MMP. The crystal structures of TIMP-2 and TIMP-1 show that TIMP-2 has an insertion at Asp\(^{30}\)-Arg\(^{42}\) that extends the beta-sheets to interact with the catalytic domain of MT1-MMP. In addition, Tyr\(^{36}\) on TIMP-2 has hydrophobic interactions with the catalytic domain of MT1-MMP, fitting into a hydrophobic pocket above the active site groove near the S2/S3 site (5). These features, which are absent in TIMP-1, may partly explain the preferred binding of TIMP-2 to MT1-MMP (3, 5, 6). Energy-minimized computer modeling of the TIMP-4 structure based on the x-ray crystal structure of TIMP-2 (5) shows that TIMP-4 (12, 14), as opposed to TIMP-1, possesses a similar insertion at Val\(^{29}\)-Met\(^{41}\) that could potentially enhance interactions with the catalytic domain of MT1-MMP (Drs. Mobashery and Kotra, Dept. of Chemistry, Wayne State University, Personal communication) and inhibit MT1-MMP activity. We have previously shown that TIMP-2 induces accumulation of the 57-kDa active form of MT1-MMP (6). This effect is a direct consequence of the inhibitory action of TIMP-2 on MT1-MMP activity, which inhibits the autocatalytic turnover of active MT1-MMP into the inactive forms of 44–40 kDa. The 44-kDa form possesses an N-terminus starting at C\(^{28}\) and its formation involves the deletion of the entire catalytic domain of MT1-MMP (6). Accumulation of active MT1-MMP and reduction of the 44-kDa form are also observed with synthetic MMP inhibitors consistent with the autocatalytic nature of this process (6). Thus, both TIMP-2 and synthetic MMP inhibitors regulate the nature of the MT1-MMP forms present in the cells. Here, we examined the lysates of BS-C-1 cells expressing MT1-MMP alone or with TIMP-4 for the profile of MT1-MMP forms by immunoblot analysis. As shown in Fig. 3, in the absence of TIMP-4, MT1-MMP is detected in its 60-, 57-, and 44–40-kDa forms (Fig. 3, lane 2) as previously reported (6). The 60-kDa protein represents pro-MT1-MMP starting at S\(^{24}\) (6). The 62-, 55-, and 37–35 kDa bands are non-specific, as they are detected in infected cells transfected with empty vector (Fig. 3, lane 1). In the presence of TIMP-4 (Fig. 3, lane 3), there is a significant increase in the 57-kDa species of MT1-MMP and a reduction in the 44-40-kDa forms. Figure 3B shows the presence of TIMP-4 in the same lysates as detected with a specific anti-TIMP-4 antibody (lane 3) (21). Similar results were obtained with the Timp2\(-/-\) mutant cells (data not shown). From these results we conclude that, like TIMP-2, TIMP-4 inhibits the autocatalytic turnover of MT1-MMP and thus, TIMP-4 is likely to form a complex with the active form of MT1-MMP. This is further supported by the lack of detection of TIMP-4 in the supernatant of cells co-expressing MT1-MMP with the inhibitor as discussed above. Although not directly proven here, these results suggest that the lack of pro-MMP-2 activation in the presence of TIMP-4 is due to the inability of this inhibitor to generate a ternary complex with MT1-MMP and pro-MMP-2. Thus, while TIMP-4 binds to active MT1-MMP, the TIMP-4/MT1-MMP complex cannot act as a receptor for pro-MMP-2 and therefore activation does not ensue. The structural constrains that impede the formation of the ternary complex awaits elucidation of the crystal structure of the TIMP-4/MT1-MMP complex. In conclusion, the results of this study demonstrate a differential role for members of the TIMP family in the inhibition of MT1-MMP activity and in MT1-MMP-dependent pro-MMP-2 activation.
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REFERENCES