MT-SP1 proteolysis and regulation of cell-microenvironment interactions

Molly R Darragh1, Ami S Bhatt2, Charles S Craik3

1Graduate Program in Biophysics, University of California, San Francisco, 2Brigham and Women’s Hospital, Department of Internal Medicine, 75 Francis Street, Boston, MA 02115, 3Department of Pharmaceutical Chemistry, University of California, San Francisco

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. MT-SP1 and substrates
   3.1. Identifying putative substrates
   3.2. Protease activation
   3.3. Growth factor activation
   3.4. Receptor activation
   3.5. Additional substrates
   3.6. VEGFR-2 is inactivated by MT-SP1
4. Discussion
   4.1. MT-SP1 and cancer
   4.2. Substrate validation
5. Experimental Methods
   5.1. Recombinant VEGFR-2 cleavage reactions
   5.2. VEGFR-2 cleavage and ERK1/2 activation assay
6. Acknowledgements
7. References

1. ABSTRACT

MT-SP1 is a type II transmembrane serine protease implicated in a range of human cancers including those of the breast, cervix, ovaries, prostate, colon and gastrointestinal tract. Mouse models have shown it to be critical for proper epidermal development and postnatal survival. However, the role of this enzyme in normal and malignant biology has not yet been fully elucidated. Several groups have identified putative substrates of MT-SP1 in an effort to understand the possible biological processes in which this protease may be involved. Methods for substrate identification include comparing known protein cleavage sequences with MT-SP1 specificity data, in vitro cleavage assays, examining genetic microarrays for enzyme/substrate coexpression, immunohistochemistry for colocalization, and a variety of phenotypic observations using cell culture and mouse models. Given the inherent limitations of each individual method, substrate plausibility is best substantiated using a combination of experimental approaches. Here we review MT-SP1 substrates identified to date and the possible physiological implications of substrate cleavage in cell-microenvironment interactions. This data indicates that MT-SP1 is capable of playing roles in growth factor activation, receptor activation and inactivation, protease activation, and ectodomain shedding. We also present for the first time vascular endothelial growth factor receptor 2 (VEGFR-2) as a putative substrate for MT-SP1.

2. INTRODUCTION

Proteolysis is a critical step in many biological processes from protein degradation to viral maturation and cell cycle progression(1-3). Proteases often mediate these processes by cleaving proteins involved in signaling pathways. The high processivity of proteases makes them well suited to amplify signals within a pathway, as in angiogenesis or the blood coagulation cascade (4-6). Unlike some enzyme pairs, such as the kinase/phosphatases, proteases are not paired with protein ligases. Proteolysis is thus often a “one way” signaling event that must be regulated by protein expression, localization, activation and inhibition. This rapid, tunable, and irreversible method of protein modification allows cells to regulate a number of degradative processes and signaling pathways.

Spatial distribution and confinement of proteases ensures that the correct signals are processed in the correct environment. Proteolysis which is localized to the cell surface is of particular interest in that it can be crucial in orchestrating cell-environment interactions (7). This may include cellular responses to the environment, as when growth factors and/or receptors are modified extracellularly to initiate intracellular signaling, or it may be used to modify the environment in response to cellular signals, such as extracellular matrix (ECM) degradation.
Understanding pericellular proteolysis requires both the identification of proteases that are active at the junction between intra- and extracellular regions and the subsequent elucidation of their biological roles within an organism. Membrane-Type Serine Protease 1 (MT-SP1) is a type-two transmembrane serine protease (TTSP) which is active on the surface of a broad range of epithelial cells and thus may be important in regulating signaling across the cell membrane (8-10). MT-SP1 is coexpressed and colocalized with its cognate inhibitor hepatocyte growth factor activator inhibitor 1 (HAI-1), suggesting a system for activity modulation (11-13). Its ability to autoactivate also allows for the possibility that MT-SP1 plays an initiating role in those pathways in which it is involved (14). These characteristics, combined with its high activity and narrow specificity, make MT-SP1 a likely candidate for regulation of information trafficking across the cell surface. The possible significance of MT-SP1 as a signaling protease is also supported by evidence indicating that its activity is important both for normal development and cancer-promoting processes (15, 16). As a result, there is growing interest in MT-SP1 and its role in pericellular proteolysis.

MT-SP1 is also referred to as matrilsptase, TADG-15, epithin, PRSS14, SNC19, prostatin, and the gene designation ST14, but here we have chosen to use the name MT-SP1 to be consistent with the nomenclature of the membrane-type matrix metalloproteases (MT-MMPs) (17, 18). This protease was first described in 1993 as a novel matrix-degrading enzyme secreted by breast cancer cells (19). Later that decade, it was characterized as a multi-domain transmembrane serine protease present in both breast and prostate cancer cells (18, 20, 21). MT-SP1 was also found in complex with its cognate inhibitor, hepatocyte growth factor activator inhibitor 1 (HAI-1) as a soluble component of breast milk (22).

Like other members of the TTSP family, MT-SP1 is a mosaic protein; it consists of a short intracellular N-terminal domain followed by a transmembrane domain, a SEA domain, two CUB domains, four LDLR domains and the C-terminal trypsin-fold serine protease domain (23). The role of the small intracellular domain is unclear, though there is evidence to suggest that it might interact with the cytoskeleton to regulate localization of MT-SP1 on the cell surface (24, 25). Experimental evidence suggests that the remaining noncatalytic domains (SEA, CUB, LDLR) likely function in protein-protein interactions necessary for localization, activation, and inhibition (14, 23, 26). MT-SP1 is activated by cleavage at Arg614 with the catalytic domain remaining attached to the upstream domains via a disulfide bond (27).

The broadly degradative protease trypsin exhibits a pronounced specificity for substrates with an Arg or Lys at the P1 position, but shows very little discrimination in the extended binding pockets. In contrast, MT-SP1, which has the same selectivity as trypsin at P1, has been found to have an extended specificity profile that can be well defined. A positional-scanning synthetic combinatorial library (PS-SCL) and a substrate phage library were used to establish the preferred cleavage sequences (P2-P1-P2-P1-P1') of R/K-X-S-R—A and X-R/K-S-R—A, where X is a non-basic amino acid (28). Interestingly, this correlates well with the activation cleavage sequence of MT-SP1 itself, which is R-Q-A-R—V, and indeed MT-SP1 is able to autoactivate both in solution in the membrane-bound form (14, 18, 26).

Multiple experiments indicate that MT-SP1 is involved in processes related to normal and malignant development. Transcript quantitation and immunohistochemistry have shown that MT-SP1 is upregulated at the mRNA and protein level in a number of cancers, and in some cases can be correlated with cancer stage (for a review of matrilptase/MT-SP1 and cancer, see (Uhland 2006) (12, 16, 29-37). Small molecule and anti-sense inhibition of MT-SP1 in cell culture and xenograft mouse models suggest that MT-SP1 activity may be important in tumor growth and invasion (38, 39). Upregulation of MT-SP1 in skin is also sufficient to induce spontaneous tumor formation and carcinogen-induced squamous cell carcinoma, further suggesting oncogenic potential (40). MT-SP1 also appears to play a role in embryologic development. Null mice showed impaired development of the hair follicles and immune system and were unable to survive 48 hours past birth due to rapid dehydration through an abnormally-formed epidermal barrier (15). When the endogenous MT-SP1 inhibitor HAI-1 was deleted in mice, the placenta failed to develop properly. In a double HAI-1/MT-SP1 knock out, however, normal placenta development was restored and the mouse instead displayed only those abnormalities observed in the MT-SP1 deletion, thus suggesting that unregulated MT-SP1 activity was responsible for placental defects (13).

MT-SP1 activity and regulation appears to have important implications in both healthy and disease states; however, the precise role of MT-SP1 at the molecular level is still unclear. Assembling the most complete ‘molecular picture’ requires an understanding of the various roles a protease plays in vivo. One important step in studying proteases and their biological pathways is to identify possible substrate proteins. While enzyme/substrate relationships established in the laboratory may not always translate directly to the in vivo setting, the identification of possible pathways can help elucidate the roles that MT-SP1 is capable of playing at the cell-environment interface. Combined with data taken from organisms, this information may help support hypotheses regarding the role of the protease in vivo. Several groups have studied MT-SP1 in this fashion, and have found that it is capable of participating in a variety of signaling pathways important for both normal and malignant cellular function.

3. MT-SP1 AND SUBSTRATES

3.1. Identifying putative substrates

Currently, eight different proteins have been identified as possible MT-SP1 substrates using a combination of experimental methods. All candidates were initially selected based on the observation that they are cleaved at the cell membrane, and usually the cleavage was attributed to some unknown serine protease. Purified MT-SP1 was used to demonstrate substrate cleavage in solution. Additional experiments such as phenotypic assays,
MT-SP1 proteolysis

Table 1. Summary of reported putative MT-SP1 substrates

<table>
<thead>
<tr>
<th>Putative Substrate</th>
<th>Type of Protein</th>
<th>Cleavage Sequence</th>
<th>Physiological Implications</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>uPA</td>
<td>Serine protease</td>
<td>PRKR--I</td>
<td>Migration, invasion, adhesion, growth</td>
<td>28, 39, 41, 51, 52</td>
</tr>
<tr>
<td>HGF/SP²</td>
<td>Growth factor</td>
<td>KQLR--V</td>
<td>Invasion, differentiation, growth, angiogenesis, adhesion</td>
<td>51, 61, 62</td>
</tr>
<tr>
<td>PAR-2</td>
<td>G-protein coupled receptor</td>
<td>SKGR--S</td>
<td>Adhesion, proliferation, mitogenesis</td>
<td>28, 41, 67</td>
</tr>
<tr>
<td>Prostasin</td>
<td>Serine protease</td>
<td>PQPR--I</td>
<td>Cell differentiation, epidermal development</td>
<td>57, 58</td>
</tr>
<tr>
<td>Tnink</td>
<td>Transmembrane glycoprotein</td>
<td>KQSR--K</td>
<td>Adhesion, morphology</td>
<td>74</td>
</tr>
<tr>
<td>MSP-I</td>
<td>Growth factor</td>
<td>SKLR--V</td>
<td>Morphology, adhesion, motility, replication, nitric oxide production, phagocytosis</td>
<td>41, 63, 63</td>
</tr>
<tr>
<td>IGFBP-3p1¹</td>
<td>Adhesion factor</td>
<td>RKGK--A</td>
<td>Adhesion, growth</td>
<td>42, 69</td>
</tr>
<tr>
<td>VEGF/R²</td>
<td>Growth factor receptor</td>
<td>RRVR--K</td>
<td>Angiogenesis, survival, migration, proliferation</td>
<td></td>
</tr>
</tbody>
</table>

Cleavage sequences are reported in the format P₄-P₁', with the scissile bond indicated by double dashes. The preferred cleavage sequence of MT-SP1 is (P₄-P₃-P₂-P₁—P₁') of R/K-X-S-R—A and X-R/K-S-R—A, where X is a non-basic amino acid (28). Physiological implications include cellular processes in which the protein has been shown to be involved. Abbreviations used: ¹urokinase-type plasminogen activator, ²hepatocyte growth factor/scatter factor, ³protease-activated receptor 2, ⁴macrophage stimulating protein 1, ⁵insulin-like growth factor binding protein-related protein, ⁶vascular endothelial growth factor receptor 2

zymograms and immunoprecipitation were often used to further suggest an in vivo association with MT-SP1 (41-44). Information about residues at the endogenous cleavage site of the protein was also taken into account in evaluating it as a possible substrate of MT-SP1.

Most recently, microarray analysis of coexpression has been used to predict previously undescribed substrates (41). This approach is based on the observation that components of the same metabolic pathways may be coexpressed in a coordinated fashion to maintain proper ratios of pathway components (45, 46). Tissue and cell samples were used to calculate correlation coefficients for coexpression of MT-SP1 with described and predicted substrates, HAI-1 and common cancer markers. While coexpression may not always be possible, possibly due to different tissues of origin or inherently low expression levels of either component, tightly positive correlations can be an additional tool in evaluating enzyme/substrate relationships.

In this case, the authors were able to support previously identified relationships between MT-SP1 and certain putative substrates. They were also able to propose a new potential substrate for MT-SP1 based on coexpression data (41). This is the first use of this technology to identify a previously undescribed protein/protein interaction.

The eight putative substrates identified to date are summarized in Table 1. Cleavage of these proteins has widespread physiological implications and indicates that MT-SP1 can function as an activator of other proteases, receptors, growth factors and as a sheddase. Though the exact role of MT-SP1 in vivo is still unknown, these data suggest that it is capable of participating in a wide variety of pathways.

3.2. Protease activation

In addition to its ability to autoactivate, MT-SP1 has been shown to activate two proteases important in development and extracellular remodeling. One of the first proteins identified as being capable of being activated by MT-SP1 was urokinase-type plasminogen activator (uPA). A serine protease, uPA can be localized to the cell surface by means of its endogenous receptor, where it is activated by other serine proteases. Upon activation, uPA activates plasmin, another serine protease. These two proteases are important for a variety of processes including ECM degradation, MMP activation, and growth factor release (47-50). uPA was initially suggested as a candidate substrate for MT-SP1 based on its activation proximal to epithelial cells and its known cleavage sequence (P₄-P₁) of P-R-F-K (28, 47, 51).

Early experiments showed that MT-SP1 is indeed capable of cleaving single chain uPA (sc-uPA) and converting it into the two-chain active form in solution. Physiological relevance of this cleavage was suggested in 2004 when Suzuki et al showed that MT-SP1 is capable of activating receptor-bound sc-uPA on the surface of ovarian cancer cells, and that decreasing MT-SP1 levels using anti-sense transfection effectively decreased uPA activation without affecting its expression. Physiologically, it was also noted that the MT-SP1-deficient cells suffer a decreased ability to invade an extracellular matrix layer (39). It is known that the activated form of the uPA substrate plasminogen is able to activate uPA itself. This activity-dependent feedback loop results in slow initial activation of uPA on the cell surface in the absence of activation by an alternate protease. Kilpatrick, et al used two monocytic cell lines, one with rapid uPA activation, and another with the characteristic initial lag phase of slow uPA activation, to show that changes in levels of MT-SP1 expression correlate with plasmin-independent activation of uPAR-bound uPA (52). Active uPA is necessary not only for normal cell responses in tissue remodeling and inflammation, but it is also important for cancer invasion, metastasis and ECM degradation (53). Increased levels of uPA have also been shown to correlate with malignant phenotype and invasive potential in cancer (54-56). MT-SP1 and uPA are significantly coexpressed in both normal and some cancerous tissues (41). There is little doubt that the activation and regulation of this enzyme is important for a variety of cell responses and it appears that MT-SP1 is capable of regulating this activity.

Prostasin, a GPI-anchored serine protease, was first proposed to interact with MT-SP1 when the deletion of
prostasin in mice showed identical phenotypic deformations in the thymus, hair follicles and stratum corneum (15, 43, 44, 57). Investigation into the temporal and spatial expression of the two proteases during epidermal barrier development showed that they were colocalized and their expression was coordinated. The identification of the cleavage sequence of prostasin as P-Q-P-R—I and the observation that MT-SP1 is capable of cleaving prostasin in vitro suggest that it is MT-SP1 which is the upstream protease (57, 58). This hypothesis is supported by data showing that while wild type tissue contains both active and pro-prostasin, MT-SP1-deficient mice solely display the zymogen form of prostasin. Both the prostasin and MT-SP1 knockout mice showed a complete lack of filaggrin processing integral to terminal epidermal differentiation (43, 44, 57). This experiment makes a compelling argument that MT-SP1 and prostasin are intimately related in vivo and that MT-SP1 plays a role in the activation of prostasin.

3.3. Growth factor activation

Upon proteolytic activation, growth factors are able to bind to cell-surface receptors and activate downstream signaling pathways. MT-SP1 has been proposed as an activator of two different growth factors. Hepatocyte growth factor/scatter factor (HGF/SF) was the first of these to be identified (51). Upon binding of activated HGF/SF to its cognate tyrosine kinase receptor c-Met, epithelial cells expressing the receptor scattered and experienced an increase in local motility (59). However, HGF/SF is produced primarily by stromal fibroblasts and some tumor cells, and is activated after secretion to elicit the physiological response in epithelial cells. It was this, combined with the knowledge that HGF/SF is activated by cleavage at the sequence K-Q-L-R—V, that initially led investigators to evaluate it as a substrate for MT-SP1 (60). In vitro experiments showed that active MT-SP1 purified from human breast milk was able to cleave and activate pro-HGF/SF secreted by fibroblasts, and that this activation led to the phosphorylation of c-Met in a human lung cancer cell line (51). Subsequent genetic studies showed a tight correlation between expression of HGF/SF, MT-SP1 and c-Met in node-negative breast cancer, implying that they might be involved in the same pathway (61). HGF/SF stimulates proliferation, dissociation, migration and invasion in tumor cells and is also angiogenic (62). In normal tissue, HGF/SF plays a role in embryogenesis and tissue regeneration (59). This again indicates that activation of HGF/SF must be carefully regulated to maintain normal homeostasis, and that MT-SP1 may be responsible for at least part of this regulation.

An HGF/SF homologue, macrophage-stimulating protein 1 (MSP-1), is also cleaved by MT-SP1. This is the first substrate predicted predominantly by genomic data resulting from transcriptional profiling. In a study of 2000 human tissue and cell samples, expression of MSP-1 was found to be well correlated with that of MT-SP1 in normal tissue and a few cancers. Expression of the MSP-1 receptor, RON, is even more tightly correlated in the sampled tissues, suggesting the three might participate in a common pathway. The authors utilized primary mouse peri toneal macrophages, which express both MT-SP1 and RON, to demonstrate that proMSP-1 is activated by MT-SP1 and then elicits physiological and chemical changes in the macrophage. These effects can be blocked with MT-SP1-specific inhibitors that prevent proMSP-1 activation. MSP-1 is also cleaved by MT-SP1 in solution, and its cleavage sequence (P4-P1) S-K-L-R is in agreement with PS-SCL data (41, 63). A recent examination of co-overexpression of MT-SP1, MSP-1 and RON together in breast cancer tumors suggests that this signaling pathway may be important in tumor metastasis (64). Thus, the data so far have shown a role for MT-SP1 in the activation of MSP-1 and the subsequent regulation of cell morphology and tumor metastasis.

3.4. Receptor activation

In addition to the activation of growth factors, proteases may also be responsible for the activation or inactivation of cell-surface receptors. Protease-activated receptor 2 (PAR2) is a widely-expressed endothelial transmembrane G-protein coupled receptor that is implicated in cell proliferation, mitogenesis, adhesion, inflammation and changes in intracellular Ca++ concentration (65). PAR2 functions in a manner similar to other PARs; N-terminal cleavage allows the new N-terminus to function as a tethered ligand for the receptor itself (66). Like uPA, PAR2 was identified as a potential candidate based on its extracellular localization and cleavage sequence (P4-P1) S-K-G-R. Transcriptional microarray analysis has also shown a significant correlation between MT-SP1 and PAR2 expression both in normal and cancerous cells and tissues (41). Cleavage was verified by expressing PAR2 on the surface of Xenopus oocytes and activating them with the addition of the soluble MT-SP1 proteolytic domain (28). It was recently observed that recombinant MT-SP1 was able to induce tyrosine phosphorylation and inflammatory cytokine expression in endothelial cells via a pathway that was dependent on PAR2 expression levels. This potential epithelial-endothelial interaction is plausible; immunohistochemistry showed strong staining for MT-SP1 in blood cells adjacent to the vascular endothelium (67). MT-SP1 in this case may be used to enhance inflammatory responses in areas where the blood cells are localized. PAR2 is the first example of a cell surface receptor that can be activated on the cell surface by MT-SP1.

3.5. Additional substrates

The precise functions of two additional substrates, insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) and Trask, are not yet known, though both proteins play a role in adhesion and thus may be referred to as “adhesion factors.” IGFBP-rP1, also known as angiomodulin, mac25, and prostacyclin-stimulating factor (PSF), is expressed in a wide variety of normal tissues as well as some tumor and tumor-associated cells (68-73). Earlier studies of IGFBP-rP1 indicated that it was cleaved at R-K-G-K—A by a trypsin-like serine protease (69). In studying IGFBP-rP1 producing cancer cells, Ahmed et al noted that proteolysis was confined to
the membrane fraction of these cells, and zymogram and immunoblot analysis attributed this activity to MT-SP1. This cleavage was further confirmed by adding purified MT-SP1 to IGFBP-1P in solution and demonstrating that it was able to process the protein at the same site (42). Cleavage of IGFBP-1P1 reduced its ability to promote cell growth in the presence of insulin and insulin growth factor, but it increased cell adhesion as regulated through a syndecan-1 mediated pathway (69). While the precise function of IGFBP-1P1 is not clear, its regulation does have an effect in at least two signaling pathways.

Trask, also known as CDCP1 and SIMA 135, was first characterized as a binding partner of MT-SP1 and later as a putative substrate resulting from an initial observation that the two proteins co-immunoprecipitate in cells overexpressing Trask (74-76). Little is known about Trask except that it is a transmembrane glycoprotein phosphorylated by src kinases and that it likely plays a role in cell-cycle dependent cell adhesion. The demonstrated interaction between the two proteins, combined with the observation that cells produced two molecular weight isoforms of the protein led Bhatt et al to hypothesize that MT-SP1 was responsible for the cleavage of Trask. Indeed, the catalytic domain of MT-SP1 cleaved recombinant Trask as predicted (74). MT-SP1 activity has already been implicated in cancer, and it is interesting to note that different cancer cell lines display varying degrees of Trask cleavage, though expression levels have not been correlated to invasiveness or proliferation (16). Whether or not there is a correlation between MT-SP1 activity and Trask cleavage has not been investigated, but transcriptional profiling shows striking coordination between the expression of the two genes in normal and cancerous tissue (41, 74).

### 3.6. VEGFR-2 is inactivated by MT-SP1

Vascular endothelial growth factor receptor 2 (VEGFR-2/Fk-1/KDR), a receptor tyrosine kinase and key regulator in angiogenesis in development, has not yet been described as a putative substrate of MT-SP1 (77, 78). With the recent recovery of soluble VEGFR-2 from the plasma of both mice and humans there has been a mounting interest in defining the mechanism by which ectodomain shedding of VEGFR-2 takes place (79). Transcriptional profiling shows a modest correlation between MT-SP1 and VEGFR-2 expression, indicating that the two may participate in a common pathway (41). Here, we report that MT-SP1 is capable of cleaving VEGFR-2 and inactivating downstream signaling in cell culture.

<table>
<thead>
<tr>
<th>VEGFR-3</th>
<th>706</th>
<th>G V D L A D S N Q K L S I Q R V R E D A G R Y L C S V C N A K G</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-1</td>
<td>723</td>
<td>G I L G P G S S T L F I E R V T E E D E G V Y H C K A T N Q K G</td>
</tr>
</tbody>
</table>

**Figure 1.** Amino acid alignment of the stalk region of the three vascular endothelial growth factor receptors. The MT-SP1 cleavage site in VEGFR-2 is indicated by a black triangle, with residues P-Z-P', highlighted in red. Shading indicates percent identity within the three sequences, with darker shading indicating greater identity. VEGFR-1 does not appear to possess an MT-SP1 cleavage sequence. The Gln residue at P$_4$ and the Glu residue at P$_1'$ in VEGFR-3 probably disfavor MT-SP1 cleavage. The amino acid numbering corresponds to the receptor sequences prior to signal peptide processing.

Though shed from the cell surface, the exact cleavage sequence of VEGFR-2 not yet been defined. Identifying putative cleavage sites that agree with MT-SP1 specificity data was the initial step in evaluating it as a possible substrate. VEGFR-2 consists of seven immunoglobulin-like folded domains on the extracellular side of the membrane connected to the intracellular tyrosine kinase domain via the transmembrane and juxtamembrane domains (78). Complete ectodomain shedding of VEGFR-2 suggests that the cleavage sequence is in the juxtamembrane region between the transmembrane domain and the most C-terminal immunoglobulin domain. Soluble forms of both VEGFR-1 and VEGFR-2, but not VEGFR-3, have been observed in vivo (79, 80). In order to determine whether MT-SP1 might cleave any of the three receptors, the stalk regions of VEGFR-1, 2 and 3 were aligned using ClustalW (Figure 1). The predicted cleavage position is indicated by a black triangle. VEGFR-1 cleavage at the stalk region was deemed unlikely, as the predicted cleavage sequence does not possess a basic residue at the P$_1$ position. The predicted cleavage region in VEGFR-3 is (P$_Z$-P$_1'$) Q-R-V-R. Although this is somewhat consistent with the specificity determined by PS-SCL, the Gln at position P$_4$ is not favored. Indeed, preliminary data suggest it is not selectively cleaved at this site. The predicted cleavage sequence of VEGFR-2, R-R-V-R matches the substrate specificity profile of MT-SP1 obtained using the completely diverse PS-SCL library (28). This data justified further evaluation of VEGFR-2 as a substrate of MT-SP1.

In order to confirm the MT-SP1 cleavage site, we obtained recombinant extracellular domain of VEGFR-2 that was fused to the human Fc from a commercial source. Treatment of this protein with MT-SP1 catalytic domain resulted in two cleavage fragments. The cleavage of VEGFR-2 by MT-SP1 is dose-dependent, with nearly complete proteolysis at approximately 100 nM MT-SP1 in 1.5 hours at 37°C (Figure 2a). Cleavage fragments were separated by SDS-PAGE, blotted onto PVDF, and submitted for N-terminal sequencing. The VEGFR-2 cleavage site (P$_Z$-P$_1'$) R-R-V-R—K, suggested by sequence alignment, was confirmed by sequencing. This cleavage would result in the release of the complete extracellular domain of VEGFR-2, consistent with the naturally occurring soluble form of the receptor.
MT-SP1 proteolysis

Figure 2. MT-SP1 mediated ectodomain shedding of VEGFR-2 results in receptor inactivation. A. MT-SP1 cleaves VEGFR-2 in a dose-dependent manner. Recombinant extracellular domain of VEGFR-2 fused to the human Fc region was incubated with varying concentrations of MT-SP1 for 1.5 hours. Samples were separated by SDS-PAGE and immunoblotted with an HRP-conjugated anti-human-Fc antibody. There is a distinct increase in the intensity of the lower band, which corresponds to the cleavage product of VEGFR-2, with increasing dose of protease. Cleavage at this position results in a complete shedding of the extracellular domain of VEGFR-2. B. HUVECs were serum starved prior to treatment with MT-SP1. In the absence of protease treatment, replacement of the serum free medium with serum-containing medium results in recovery of ERK1/2 activation as measured by ERK1/2 phosphorylation (lane 1). Cells treated with MT-SP1 do not exhibit ERK1/2 activation (lane 2) even upon the re-addition of serum-containing medium as is demonstrated by the lack of phospho-ERK1/2 (lane 3). C. MT-SP1 treatment of HUVECs prevents VEGF-induced ERK1/2 phosphorylation. HUVECs were serum starved prior to addition of MT-SP1. Following incubation with MT-SP1, cells were either kept in serum free medium or treated with 50 ng/mL VEGF. ERK1/2 activation, as measured by phospho-ERK1/2 signal on immunoblot, is regained in HUVECs not treated with MT-SP1, but is absent in the MT-SP1 treated samples.

Cell culture assays were then used to test the physiological effects of MT-SP1 cleavage of VEGFR-2. Vascular endothelial growth factor (VEGF) is able to activate the kinase ERK1/2 in a VEGFR-2-dependent manner in receptor-expressing human umbilical vein endothelial cells (HUVECs) (81). This cell line was chosen for testing proteolytic receptor inactivation using MT-SP1. Phosphorylation of the downstream substrate ERK1/2 was monitored by immunoblot. VEGF and serum-induced ERK1/2 phosphorylation was blocked when HUVEC cells were pre-treated with 100 nM MT-SP1 for one hour at 37°C. Treatment of HUVEC cells with recombinant MT-SP1 was sufficient to lead to VEGFR-2 inactivation as measured by the blockade of downstream signaling in response to complete serum, and more specifically, VEGF (Figure 2b,c).

We are reporting the first demonstration of VEGFR-2 cleavage despite the previous discovery of soluble extracellular domain in serum. Though soluble VEGF-1 results from alternative splicing, this has not been reported for soluble VEGFR-2 (79). Therefore, it is plausible that a protease may be responsible for receptor shedding. VEGFR-2 has been implicated in angiogenic development and endothelial cell survival, migration, and proliferation (78, 81). MT-SP1 may be able to mediate any or all of the related pathways through VEGFR-2 inactivation. MT-SP1 is expressed in a variety of cancers, and VEGFR-2 is thought to play a central role in tumor growth in many of these cancer types. Careful regulation of angiogenesis is central to tumor growth and metastasis, and in fact VEGF is a target for the anti-cancer drug Avastin. Multiple small molecule drugs which target VEGFR-2 have also been FDA-approved and/or are currently in clinical trials (82). This pathway is critical in cancer biology and it is possible that MT-SP1-regulated receptor cleavage is important in vascular development.

4. DISCUSSION

The substrates identified thus far indicate that MT-SP1 is capable of carrying out a variety of functions in vivo (Figure 3). Not only is it able to activate growth factors necessary for signaling, but it may also regulate this signaling through the activation or inactivation of cell-surface receptors. The shedding activity that leads to VEGFR-2 inactivation can also be useful in cleaving proteins important for cell adhesion such as Trask. MT-SP1 is also able to activate at least two different proteases, indicating it might be important in a number of proteolytic cascades. Such a variety of roles for a single protease is not unheard of; thrombin, a well-studied serine protease involved in inflammation and blood coagulation, has been shown to activate PARs, process fibrinogen, release growth factors and modulate adhesion (83). MT-SP1, like thrombin, may also be an important regulator of pericellular biology through processing of some or all of the proposed substrates.

4.1. MT-SP1 and cancer

The diversity of proposed MT-SP1 substrates is interesting in light of the idea that MT-SP1 activity is upregulated in cancer. Some of the proposed catalytic actions would seem to favor tumor progression, such as the activation of uPA and the subsequent ECM degradation. On the other hand, inactivation of VEGFR-2 may oppose
Figure 3. Summary of the putative roles of MT-SP1. MT-SP1 (in pink) is shown to be capable of playing a number of different proteolytic roles at the cell surface. Growth factor activation refers to cleavage of both MSP-1 and HGF/SF; GPCR activation represents the activation of PAR2. MT-SP1 is also capable of activating proteases uPA, prostatin and stromelysin. MT-SP1 has also been shown to cleave IGFBP-rP1 and Trask, both proteins related to adhesion. The question marks indicate pathways in which the exact molecular ramifications of MT-SP1-mediated cleavage events are unclear. VEGFR-2 is the first known example of MT-SP1-mediated receptor inactivation. 

In addition to inactivating the signaling pathway, the shedding of VEGFR-2 releases a soluble form that may, like secreted VEGFR-1, function as a ‘ligand sink’ to sequester free VEGF and further inhibit pathways favoring tumor progression (84, 85). This is not entirely contradictory in that, like any other biological system, cancer likely progresses through modulations in existing pathways rather than simple on/off switches. This has been seen in adhesion regulation in cancer, wherein a decrease in adhesion is necessary for cells to move through the ECM. At the same time, some adhesion is necessary for cells to maintain contact with the ECM and locally degrade it. Therefore, both pro-adhesion and anti-adhesion signals coexist in the disease state. Similarly, angiogenesis in cancer must be carefully regulated by a number of changes in signals and receptors that, on the individual protein level, seem counterintuitive but which in concert create an environment favoring malignant development. An example can be seen in embryogenesis, where both VEGFR-1 and VEGFR-2 knockouts are lethal to the mouse embryo. When VEGFR-1 is deleted, the embryo perishes due to uncontrolled endothelial proliferation, while the VEGFR-2 knockout shows defective endothelial cell and vascular development (78, 86-88). This suggests that in this case, VEGFR-2 promotes proper endothelial development while VEGFR-1 (likely in soluble form) serves to restrain it. Obtaining the optimal balance in this environment involves both pro- and anti-angiogenic activity. Similarly, MT-SP1 may play a key role in maintaining a very specific physiological environment by cleaving both pro- and anti-cancer substrates.

4.2. Substrate validation relies on multiple experimental approaches

The intricacy of in vivo pathways makes them difficult to study reliably in the laboratory; the fact that one action is able to occur in a limited, testable setting does not ensure that it will necessarily translate to more complex environments. When studying pericellular proteolysis in particular, it is important to note that pathway components are likely to come from different cells or tissues, and cell culture studies are by nature too isolated to reflect these conditions. Enzymes can also be spatially or temporally regulated by expression, activation, and inhibition, and without this knowledge it can be difficult to predict which processes are biologically relevant. Experimental results may also be affected by reagents available to the researcher. Because of difficulties with expression of the full-length enzyme, MT-SP1 cleavage is often monitored using either the recombinant proteolytic domain or enzyme
purified from the media of immortal cells; this data may not accurately reflect proteolysis involving all upstream domains at the cell surface.

Further complicating the study of in vivo proteolysis is the idea of functional redundancy, in which one substrate may be cleaved by more than one protease. Many of the substrates presented here are known to be cleaved by alternative proteases. uPA, for example, has also been shown to be cleaved by plasmin, hepsin, plasma kallekrein, T-cell associated serine proteases, cathepsins B and L, glandular kallekrein-6, human mast cell tryptase and PSA (1, 89-95). Of course, many of these interactions may not be relevant in vivo, and those that are may depend on expression levels in different tissues as well as specific environmental conditions and/or colocalization of the enzyme and substrate pair. The functional redundancy of having many proteases cleave the same substrate may be important in finely regulating multiple pathways at once. Or, as proposed in cell-associated uPA activation, one protease may serve as an initiator but not the primary activator in a pathway (52). It is certainly possible that MT-SP1 works in concert with other proteases in some or all of its endogenous biological pathways, though this may be difficult to elucidate without information about enzyme expression and regulation in vivo.

In light of the limitations of cell culture and enzyme assays, alternative strategies should be employed to support the physiological relevance of enzyme-substrate pairs in vivo. Immunohistochemistry and transcriptional profiling of human tissue can give information about colocalization and/or coexpression to determine which interactions may be biologically plausible. These types of experiments have strengthened the arguments that PAR2, prostasin, uPA, Trask and MSP-1 could be MT-SP1 substrates in vivo (41, 57, 67). More recently, genetic profiling was followed by cell culture and in vivo experiments to suggest a functional relationship between epiregulin, COX2 and MMPs 1 and 2 in breast tumor growth and metastasis to the lungs (96).

Animal models are also extremely useful for showing physiological effects of modifying protein levels within a living organism. Though protein knockouts can be complicated by functional redundancy or limited organism survival, experiments may help demonstrate relationships between proteins, as in the case of MT-SP1 and prostasin (43, 44, 57). Animal models are also critical in determining the possible physiological roles of proteins at the organism level, such as the observation that MT-SP1 is important in epidermal development (15).

Ultimately, there is no single foolproof method for identifying substrate/enzyme pairs that are relevant in vivo. Instead, a combination of methods demonstrating physical cleavage, possible interactions in vivo and physiological consequences are necessary to fully understand proteolytic signaling.

Many different approaches have been used in the study of MT-SP1, but its role in vivo is still not clear. Though the dramatic effect of completely deleting the protein in mice indicates that it is involved in a number of pathways critical for survival, the lethality of this mutation makes it challenging to study in processes beyond early development (15). Similarly, the biochemical analysis of proteases and their putative substrates in the absence of physiological information is of questionable relevance. Further understanding of its physiological role(s) requires the use of transient and localized inhibition of the protein using specific inhibitors, as has been done in studying MT-SP1 in tumor models, or tissue-specific and/or inducible MT-SP1 knockouts (38, 97). This information, combined with our growing knowledge about possible biological pathways in which it participates, will help establish the most likely roles of MT-SP1 in vivo.

5. EXPERIMENTAL METHODS

5.1. Recombinant VEGFR-2 cleavage reactions

Recombinant human MT-SP1 catalytic domain was prepared and active site titrated as described (18). Recombinant human VEGFR-2 extracellular domain-Fc chimera was purchased (R&D Systems). Four micrograms of recombinant VEGFR-2 extracellular domain were incubated with 100nM MT-SP1 for 30 minutes at 37°C. This corresponds to a molar ratio for substrate to enzyme of 25:1. The products were then separated by SDS-PAGE on 4-20% tris-glycine gels (Invitrogen) and either silver stained or transferred onto PVDF for microsequencing or nitrocellulose for immunoblotting. Edman degradation sequencing of N-terminal VEGFR-2 cleavage product was carried out at the UC Davis Molecular Structure Facility. Immunoblotting was performed using an anti-human-Fc-HRP conjugated antibody (Bio-Rad). Blots were developed using a chemiluminescent substrate and they were exposed onto Hyperfilm (substrate and film both from Amersham Biosciences).

5.2. VEGFR-2 cleavage and ERK1/2 activation assay

HUVECs were cultured in 24 well plates to 70% confluency. At this time cells were transferred to serum-free medium for two hours. Recombinant, catalytic domain of MT-SP1 was then added for 1 hour at 37°C. Following this, fetal bovine serum, recombinant VEGF, or other growth factors were added back and cells were harvested 30 minutes post-stimulation. Cells were lysed directly into 1X Laemmli sample buffer in PBS, 6M urea, 1.5% (v/v) Triton-X100. Samples were then boiled and SDS-PAGE was performed using 4-20% tris-glycine gels (Invitrogen). Immunoblots were performed using antibodies to ERK1/2 and phospho ERK1/2 (Cell Signaling). Blots were developed using a chemiluminescent substrate and they were exposed onto Hyperfilm (Amersham Biosciences). VEGFR-2 cleavage was monitored by immunoblot using antibodies to VEGFR-2 (Cell Signaling).

6. ACKNOWLEDGEMENTS

The authors would like to thank Christopher Farady for useful discussions. We would also like to acknowledge the NIH CA072006 (CC), NIH Medical Sciences Training Grant (AB), and the NSF Graduate Research Fellowship (MD) for funding.
7. REFERENCES


expressing the type ii transmembrane serine protease plasminogen activation on the surface of monocytes

Ghorayeb, A. Bar-Or and V. Ellis: Initiation of


MT-SP1 proteolysis


**Abbreviations:** MT-SP1 (membrane type serine protease 1), HAI-1 (hepatocyte growth factor activator inhibitor 1), TTSP (type II transmembrane serine protease), MMP (matrix metalloproteinase), ECM (extracellular matrix), uPA (urokinase-type plasminogen activator), HGF/SF (hepatocyte growth factor/scatter factor), MSP-1 (macrophage stimulating protein 1), PAR (protease activated receptor), IGFBP-rP1 (insulin-like growth factor binding protein-related protein 1), VEGF (vascular endothelial growth factor) and VEGFR (vascular endothelial growth factor receptor)

**Keywords:** matriptase, substrate validation, protease signaling, VEGFR-2 cleavage, cell environment interactions, type II transmembrane serine protease

**Send correspondence to:** Dr Charles S Craik, UCSF Genentech Hall MC 2280, 600 16th St, San Francisco, CA 94158-2517, Tel: 415-476-8146, Fax: 415-502-8298, E-mail: craik@cgl.ucsf.edu