[Frontiers in Bioscience 13, 528-539, January 1, 2008]

MT-SP1 proteolysis and regulation of cell-microenvironment interactions

Molly R Darragh[1], Ami S Bhatt[2], Charles S Craik[3]

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

TABLE OF CONTENTS

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 matriptase, TADG-15, epithin, PRSS14, SNC19, prostamin, 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 Arg^{614} 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 P_1 position, but shows very little discrimination in the extended binding pockets. In contrast, MT-SP1, which has the same selectivity as trypsin at P_1, 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 (P_4-P_3-P_2-P_1—P_1') 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 matriptase/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,

Table 1. Summary of reported putative MT-SP1 substrates

Putative Substrate	Type of Protein	Cleavage Sequence	Physiological Implications	References
uPA[1]	Serine protease	PRFK--I	Migration, invasion, adhesion, growth	28, 39, 41, 51, 52
HGF/SF[2]	Growth factor	KQLR--V	Invasion, differentiation, growth, angiogenesis, adhesion	51, 61, 62
PAR-2[3]	G-protein coupled receptor	SKGR--S	Adhesion, proliferation, mitogenesis	28, 41, 67
Prostasin	Serine protease	PQPR--I	Cell differentiation, epidermal development	57, 58
Trask	Transmembrane glycoprotein	KQSR--K	Adhesion, morphology	74
MSP-1[4]	Growth factor	SKLR--V	Morphology, adhesion, motility, replication, nitric oxide production, phagocytosis	41, 63, 63
IGFBP-rP1[5]	Adhesion factor	RKGK--A	Adhesion, growth	42, 69
VEGFR-2[6]	Growth factor receptor	RRVR--K	Angiogenesis, survival, migration, proliferation	

Cleavage sequences are reported in the format P_4-P_1', with the scissile bond indicated by double dashes. The preferred cleavage sequence of MT-SP1 is (P_4-P_3-P_2-P_1—P_1') 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: [1]urokinase-type plasminogen activator , [2]hepatocyte growth factor/scatter factor, [3] protease-activated receptor 2, [4]macrophage stimulating protein 1, [5]insulin-like growth factor binding protein-related protein, [6]vascular 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 observed, 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 (P_4-P_1) 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 peritoneal 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 (P_4-P_1) 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^{2+} 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 (P_4-P_1) 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

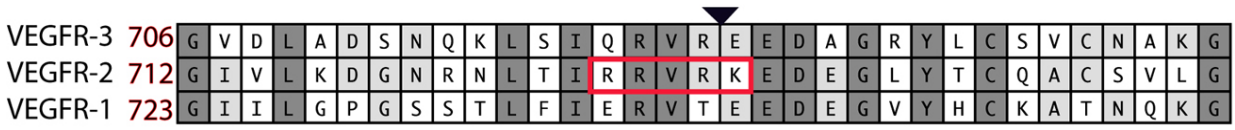

Figure 1. Amino acid alignment of the stalk region of the three vascular endothelial growth factor receptors. The MT-SP1 cleavage site in VEGFR-2 is indicated by a black triangle, with residues P_4-P_1' highlighted in red. Shading indicates percent identity within the three sequences, with darker shading 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.

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 IFGBP-rP1 in solution and demonstrating that it was able to process the protein at the same site (42). Cleavage of IGFBP-rP1 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 IFGBP-rP1 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/Flk-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.

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_4-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_4-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.

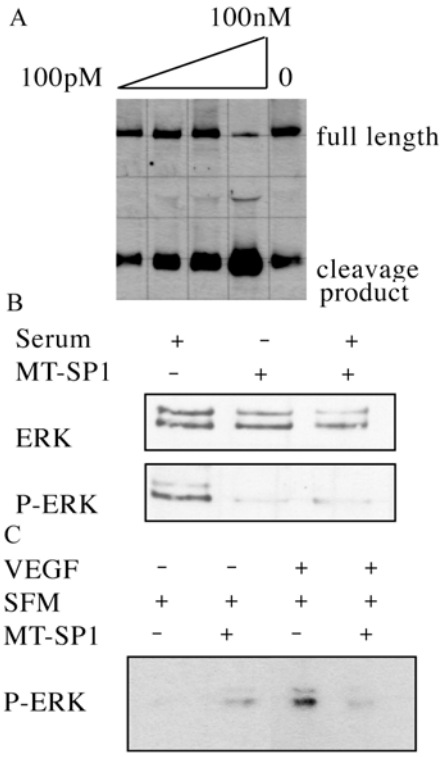

Figure 2. MT-SP1 mediated ectodomain shedding of VEGFR-2 results in receptor inactivation. A. MT-SP1 cleaves VEGFR-2 in a dose-dependent manner. Recombinant extracellular domain of VEGFR-2 fused to the human Fc region was incubated with varying concentrations of MT-SP1 for 1.5 hours. Samples were separated 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 37C. 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 VEGFR-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 are 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

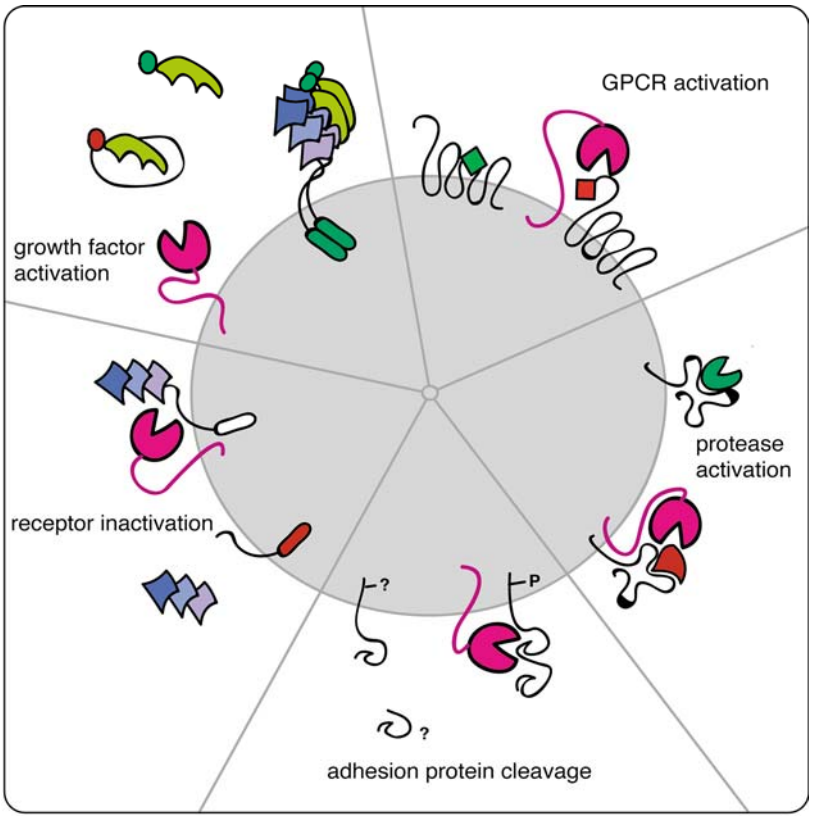

Figure 3. Summary of the putative roles of MT-SP1. MT-SP1 (in pink) is shown to be capable of playing a number of different proteolytic roles at the cell surface. Growth factor activation refers to cleavage of both MSP-1 and HGF/SF; GPCR activation represents the activation of PAR2. MT-SP1 is also capable of activating proteases uPA, prostasin 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.

tumor growth. 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

1. L. Goretzki, M. Schmitt, K. Mann, J. Calvete, N. Chucholowski, M. Kramer, W.A. Gunzler, F. Janicke and H. Graeff: Effective activation of the proenzyme form of the urokinase-type plasminogen activator (pro-upa) by the cysteine protease cathepsin l. *FEBS Lett* 297 (1-2), 112-8 (1992)
2. A.J. Rivett: Proteasomes: Multicatalytic proteinase complexes. *Biochem J* 291 (Pt 1) 1-10 (1993)
3. F. Uhlmann, F. Lottspeich and K. Nasmyth: Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit scc1. *Nature* 400 (6739), 37-42 (1999)
4. T. Dragovich, C.M. Rudin and C.B. Thompson: Signal transduction pathways that regulate cell survival and cell death. *Oncogene* 17 (25), 3207-13 (1998)
5. S.N. Levine: Enzyme amplifier kinetics. *Science* 152 (3722), 651-653 (1996)
6. R.G. Macfarlane: An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* 202 498-9 (1964)
7. Z. Werb: Ecm and cell surface proteolysis: Regulating cellular ecology. *Cell* 91 (4), 439-42 (1997)
8. M.G. Kim, C. Chen, M.S. Lyu, E.G. Cho, D. Park, C. Kozak and R.H. Schwartz: Cloning and chromosomal mapping of a gene isolated from thymic stromal cells encoding a new mouse type ii membrane serine protease, epithin, containing four ldl receptor modules and two cub domains. *Immunogenetics* 49 (5), 420-8 (1999)
9. K. List, R. Szabo, A. Molinolo, B.S. Nielsen and T.H. Bugge: Delineation of matriptase protein expression by enzymatic gene trapping suggests diverging roles in barrier function, hair formation, and squamous cell carcinogenesis. *Am J Pathol* 168 (5), 1513-25 (2006)
10. M.D. Oberst, B. Singh, M. Ozdemirli, R.B. Dickson, M.D. Johnson and C.Y. Lin: Characterization of matriptase expression in normal human tissues. *J Histochem Cytochem* 51 (8), 1017-25 (2003)
11. C.M. Benaud, M. Oberst, R.B. Dickson and C.Y. Lin: Deregulated activation of matriptase in breast cancer cells. *Clin Exp Metastasis* 19 (7), 639-49 (2002)
12. M.D. Oberst, M.D. Johnson, R.B. Dickson, C.Y. Lin, B. Singh, M. Stewart, A. Williams, A. al-Nafussi, J.F. Smyth, H. Gabra and G.C. Sellar: Expression of the serine protease matriptase and its inhibitor hai-1 in epithelial ovarian cancer: Correlation with clinical outcome and tumor clinicopathological parameters. *Clin Cancer Res* 8 (4), 1101-7 (2002)
13. R. Szabo, A. Molinolo, K. List and T.H. Bugge: Matriptase inhibition by hepatocyte growth factor activator inhibitor-1 is essential for placental development. *Oncogene* 26 (11), 1546-56 (2007)
14. M.D. Oberst, C.A. Williams, R.B. Dickson, M.D. Johnson and C.Y. Lin: The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor. *J Biol Chem* 278 (29), 26773-9 (2003)
15. K. List, C.C. Haudenschild, R. Szabo, W. Chen, S.M. Wahl, W. Swaim, L.H. Engelholm, N. Behrendt and T.H. Bugge: Matriptase/mt-sp1 is required for postnatal survival, epidermal barrier function, hair follicle development, and thymic homeostasis. *Oncogene* 21 (23), 3765-79 (2002)
16. K. Uhland: Matriptase and its putative role in cancer. *Cell Mol Life Sci* 63 (24), 2968-78 (2006)
17. A. Paoloni-Giacobino, H. Chen, M.C. Peitsch, C. Rossier and S.E. Antonarakis: Cloning of the tmprss2 gene, which encodes a novel serine protease with transmembrane, ldlra, and srcr domains and maps to 21q22.3. *Genomics* 44 (3), 309-20 (1997)
18. T. Takeuchi, M.A. Shuman and C.S. Craik: Reverse biochemistry: Use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. *Proc Natl Acad Sci U S A* 96 (20), 11054-61 (1999)
19. Y.E. Shi, J. Torri, L. Yieh, A. Wellstein, M.E. Lippman and R.B. Dickson: Identification and characterization of a novel matrix-degrading protease from hormone-dependent human breast cancer cells. *Cancer Res* 53 (6), 1409-15 (1993)
20. C.Y. Lin, J. Anders, M. Johnson, Q.A. Sang and R.B. Dickson: Molecular cloning of cdna for matriptase, a matrix-degrading serine protease with trypsin-like activity. *J Biol Chem* 274 (26), 18231-6 (1999)
21. C.Y. Lin, J.K. Wang, J. Torri, L. Dou, Q.A. Sang and R.B. Dickson: Characterization of a novel, membrane-bound, 80-kda matrix-degrading protease from human breast cancer cells. Monoclonal antibody production, isolation, and localization. *J Biol Chem* 272 (14), 9147-52 (1997)
22. C.Y. Lin, J. Anders, M. Johnson and R.B. Dickson: Purification and characterization of a complex containing matriptase and a kunitz-type serine protease inhibitor from human milk. *J Biol Chem* 274 (26), 18237-42 (1999)
23. S. Netzel-Arnett, J.D. Hooper, R. Szabo, E.L. Madison, J.P. Quigley, T.H. Bugge and T.M. Antalis: Membrane anchored serine proteases: A rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer. *Cancer Metastasis Rev* 22 (2-3), 237-58 (2003)
24. C. Kim, Y. Cho, C.H. Kang, M.G. Kim, H. Lee, E.G. Cho and D. Park: Filamin is essential for shedding of the transmembrane serine protease, epithin. *EMBO Rep* 6 (11), 1045-51 (2005)
25. M.S. Lee, K. Kiyomiya, C. Benaud, R.B. Dickson and C.Y. Lin: Simultaneous activation and hepatocyte growth factor activator inhibitor 1-mediated inhibition of matriptase induced at activation foci in human mammary epithelial cells. *Am J Physiol Cell Physiol* 288 (4), C932-41 (2005)
26. M.S. Lee, I.C. Tseng, Y. Wang, K.I. Kiyomiya, M.D. Johnson, R.B. Dickson and C.Y. Lin: Autoactivation of matriptase in vitro: Requirement for biomembrane and ldl receptor domain. *Am J Physiol Cell Physiol* (2007)
27. C. Benaud, R.B. Dickson and C.Y. Lin: Regulation of the activity of matriptase on epithelial cell surfaces by a blood-derived factor. *Eur J Biochem* 268 (5), 1439-47 (2001)
28. T. Takeuchi, J.L. Harris, W. Huang, K.W. Yan, S.R. Coughlin and C.S. Craik: Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J Biol Chem* 275 (34), 26333-42 (2000)

29. W. Ge, H. Hu, K. Ding, L. Sun and S. Zheng: Protein interaction analysis of st14 domains and their point and deletion mutants. *J Biol Chem* 281 (11), 7406-12 (2006)
30. C.D. Hoang, J. D'Cunha, M.G. Kratzke, C.E. Casmey, S.P. Frizelle, M.A. Maddaus and R.A. Kratzke: Gene expression profiling identifies matriptase overexpression in malignant mesothelioma. *Chest* 125 (5), 1843-52 (2004)
31. B. Jarzab, M. Wiench, K. Fujarewicz, K. Simek, M. Jarzab, M. Oczko-Wojciechowska, J. Wloch, A. Czarniecka, E. Chmielik, D. Lange, A. Pawlaczek, S. Szpak, E. Gubala and A. Swierniak: Gene expression profile of papillary thyroid cancer: Sources of variability and diagnostic implications. *Cancer Res* 65 (4), 1587-97 (2005)
32. J.S. Jin, D.S. Hsieh, S.H. Loh, A. Chen, C.W. Yao and C.Y. Yen: Increasing expression of serine protease matriptase in ovarian tumors: Tissue microarray analysis of immunostaining score with clinicopathological parameters. *Mod Pathol* 19 (3), 447-52 (2006)
33. J.W. Lee, S. Yong Song, J.J. Choi, S.J. Lee, B.G. Kim, C.S. Park, J.H. Lee, C.Y. Lin, R.B. Dickson and D.S. Bae: Increased expression of matriptase is associated with histopathologic grades of cervical neoplasia. *Hum Pathol* 36 (6), 626-33 (2005)
34. A.C. Riddick, C.J. Shukla, C.J. Pennington, R. Bass, R.K. Nuttall, A. Hogan, K.K. Sethia, V. Ellis, A.T. Collins, N.J. Maitland, R.Y. Ball and D.R. Edwards: Identification of degradome components associated with prostate cancer progression by expression analysis of human prostatic tissues. *Br J Cancer* 92 (12), 2171-80 (2005)
35. M. Saleem, V.M. Adhami, W. Zhong, B.J. Longley, C.Y. Lin, R.B. Dickson, S. Reagan-Shaw, D.F. Jarrard and H. Mukhtar: A novel biomarker for staging human prostate adenocarcinoma: Overexpression of matriptase with concomitant loss of its inhibitor, hepatocyte growth factor activator inhibitor-1. *Cancer Epidemiol Biomarkers Prev* 15 (2), 217-27 (2006)
36. A.D. Santin, S. Cane, S. Bellone, E. Bignotti, M. Palmieri, L.E. De Las Casas, S. Anfossi, J.J. Roman, T. O'Brien and S. Pecorelli: The novel serine protease tumor-associated differentially expressed gene-15 (matriptase/mt-sp1) is highly overexpressed in cervical carcinoma. *Cancer* 98 (9), 1898-904 (2003)
37. A.D. Santin, F. Zhan, S. Bellone, M. Palmieri, S. Cane, E. Bignotti, S. Anfossi, M. Gokden, D. Dunn, J.J. Roman, T.J. O'Brien, E. Tian, M.J. Cannon, J. Shaughnessy, Jr. and S. Pecorelli: Gene expression profiles in primary ovarian serous papillary tumors and normal ovarian epithelium: Identification of candidate molecular markers for ovarian cancer diagnosis and therapy. *Int J Cancer* 112 (1), 14-25 (2004)
38. A.V. Galkin, L. Mullen, W.D. Fox, J. Brown, D. Duncan, O. Moreno, E.L. Madison and D.B. Agus: Cvs-3983, a selective matriptase inhibitor, suppresses the growth of androgen independent prostate tumor xenografts. *Prostate* 61 (3), 228-35 (2004)
39. M. Suzuki, H. Kobayashi, N. Kanayama, Y. Saga, C.Y. Lin, R.B. Dickson and T. Terao: Inhibition of tumor invasion by genomic down-regulation of matriptase through suppression of activation of receptor-bound pro-urokinase. *J Biol Chem* 279 (15), 14899-908 (2004)
40. K. List, R. Szabo, A. Molinolo, V. Sriuranpong, V. Redeye, T. Murdock, B. Burke, B.S. Nielsen, J.S. Gutkind and T.H. Bugge: Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. *Genes Dev* 19 (16), 1934-50 (2005)
41. A.S. Bhatt, A. Welm, C.J. Farady, K.W. Vasquez, K. Wilson and C.S. Craik: Coordinate expression and functional profiling identify an extracellular proteolytic signaling pathway. *Proc Natl Acad Sci U S A* 104 (14), (2007)
42. S. Ahmed, X. Jin, M. Yagi, C. Yasuda, Y. Sato, S. Higashi, C.Y. Lin, R.B. Dickson and K. Miyazaki: Identification of membrane-bound serine proteinase matriptase as processing enzyme of insulin-like growth factor binding protein-related protein-1 (igfbp-rp1/angiomodulin/mac25). *Febs J* 273 (3), 615-27 (2006)
43. C. Leyvraz, R.P. Charles, I. Rubera, M. Guitard, S. Rotman, B. Breiden, K. Sandhoff and E. Hummler: The epidermal barrier function is dependent on the serine protease cap1/prss8. *J Cell Biol* 170 (3), 487-96 (2005)
44. K. List, R. Szabo, P.W. Wertz, J. Segre, C.C. Haudenschild, S.Y. Kim and T.H. Bugge: Loss of proteolytically processed filaggrin caused by epidermal deletion of matriptase/mt-sp1. *J Cell Biol* 163 (4), 901-10 (2003)
45. H.B. Fraser, A.E. Hirsh, D.P. Wall and M.B. Eisen: Coevolution of gene expression among interacting proteins. *Proc Natl Acad Sci U S A* 101 (24), 9033-8 (2004)
46. R. Miki, K. Kadota, H. Bono, Y. Mizuno, Y. Tomaru, P. Carninci, M. Itoh, K. Shibata, J. Kawai, H. Konno, S. Watanabe, K. Sato, Y. Tokusumi, N. Kikuchi, Y. Ishii, Y. Hamaguchi, I. Nishizuka, H. Goto, H. Nitanda, S. Satomi, A. Yoshiki, M. Kusakabe, J.L. DeRisi, M.B. Eisen, V.R. Iyer, P.O. Brown, M. Muramatsu, H. Shimada, Y. Okazaki and Y. Hayashizaki: Delineating developmental and metabolic pathways in vivo by expression profiling using the riken set of 18,816 full-length enriched mouse cdna arrays. *Proc Natl Acad Sci U S A* 98 (5), 2199-204 (2001)
47. P.F. Choong and A.P. Nadesapillai: Urokinase plasminogen activator system: A multifunctional role in tumor progression and metastasis. *Clin Orthop Relat Res* (415 Suppl), S46-58 (2003)
48. E. Hahn-Dantona, N. Ramos-DeSimone, J. Sipley, H. Nagase, D.L. French and J.P. Quigley: Activation of prommp-9 by a plasmin/mmp-3 cascade in a tumor cell model. Regulation by tissue inhibitors of metalloproteinases. *Ann N Y Acad Sci* 878 372-87 (1999)
49. G. Murphy, H. Stanton, S. Cowell, G. Butler, V. Knauper, S. Atkinson and J. Gavrilovic: Mechanisms for pro matrix metalloproteinase activation. *Apmis* 107 (1), 38-44 (1999)
50. Z. Werb, M.J. Banda and P.A. Jones: Degradation of connective tissue matrices by macrophages. I. Proteolysis of elastin, glycoproteins, and collagen by proteinases isolated from macrophages. *J Exp Med* 152 (5), 1340-57 (1980)
51. S.L. Lee, R.B. Dickson and C.Y. Lin: Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. *J Biol Chem* 275 (47), 36720-5 (2000)

52. L.M. Kilpatrick, R.L. Harris, K.A. Owen, R. Bass, C. Ghorayeb, A. Bar-Or and V. Ellis: Initiation of plasminogen activation on the surface of monocytes expressing the type ii transmembrane serine protease matriptase. *Blood* 108 (8), 2616-23 (2006)
53. P.A. Andreasen, R. Egelund and H.H. Petersen: The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell Mol Life Sci* 57 (1), 25-40 (2000)
54. M.J. Duffy: Urokinase-type plasminogen activator: A potent marker of metastatic potential in human cancers. *Biochem Soc Trans* 30 (2), 207-10 (2002)
55. J.L. Fisher, C.L. Field, H. Zhou, T.L. Harris, M.A. Henderson and P.F. Choong: Urokinase plasminogen activator system gene expression is increased in human breast carcinoma and its bone metastases--a comparison of normal breast tissue, non-invasive and invasive carcinoma and osseous metastases. *Breast Cancer Res Treat* 61 (1), 1-12 (2000)
56. J.L. Fisher, P.S. Mackie, M.L. Howard, H. Zhou and P.F. Choong: The expression of the urokinase plasminogen activator system in metastatic murine osteosarcoma: An in vivo mouse model. *Clin Cancer Res* 7 (6), 1654-60 (2001)
57. S. Netzel-Arnett, B.M. Currie, R. Szabo, C.Y. Lin, L.M. Chen, K.X. Chai, T.M. Antalis, T.H. Bugge and K. List: Evidence for a matriptase-prostasin proteolytic cascade regulating terminal epidermal differentiation. *J Biol Chem* 281 (44), 32941-5 (2006)
58. A. Shipway, H. Danahay, J.A. Williams, D.C. Tully, B.J. Backes and J.L. Harris: Biochemical characterization of prostasin, a channel activating protease. *Biochem Biophys Res Commun* 324 (2), 953-63 (2004)
59. M. Stoker, E. Gherardi, M. Perryman and J. Gray: Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* 327 (6119), 239-42 (1987)
60. D. Naka, T. Ishii, Y. Yoshiyama, K. Miyazawa, H. Hara, T. Hishida and N. Kidamura: Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. *J Biol Chem* 267 (28), 20114-9 (1992)
61. J.Y. Kang, M. Dolled-Filhart, I.T. Ocal, B. Singh, C.Y. Lin, R.B. Dickson, D.L. Rimm and R.L. Camp: Tissue microarray analysis of hepatocyte growth factor/met pathway components reveals a role for met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer. *Cancer Res* 63 (5), 1101-5 (2003)
62. C. Parr, G. Watkins, R.E. Mansel and W.G. Jiang: The hepatocyte growth factor regulatory factors in human breast cancer. *Clin Cancer Res* 10 (1 Pt 1), 202-11 (2004)
63. A. Skeel, T. Yoshimura, S.D. Showalter, S. Tanaka, E. Appella and E.J. Leonard: Macrophage stimulating protein: Purification, partial amino acid sequence, and cellular activity. *J Exp Med* 173 (5), 1227-34 (1991)
64. A.L. Welm, J.B. Sneddon, C. Taylor, D.S.A. Nuyten, M.J. van de Vijver, B.H. Hasegawa and J.M. Bishop: The macrophage stimulating protein pathway promotes metastasis in a mouse model for breast cancer and predicts poor prognosis in humans. *Proc Natl Acad Sci U S A* In Press. (2007)
65. S.R. Macfarlane, M.J. Seatter, T. Kanke, G.D. Hunter and R. Plevin: Proteinase-activated receptors. *Pharmacol Rev* 53 (2), 245-82 (2001)
66. S.R. Coughlin: Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost* 3 (8), 1800-14 (2005)
67. I. Seitz, S. Hess, H. Schulz, R. Eckl, G. Busch, H.P. Montens, R. Brandl, S. Seidl, A. Schomig and I. Ott: Membrane-type serine protease-1/matriptase induces interleukin-6 and -8 in endothelial cells by activation of protease-activated receptor-2. Potential implications in atherosclerosis. *Arterioscler Thromb Vasc Biol* (2007)
68. Y. Adachi, F. Itoh, H. Yamamoto, Y. Arimura, Y. Kikkawa-Okabe, K. Miyazaki, D.P. Carbone and K. Imai: Expression of angiomodulin (tumor-derived adhesion factor/mac25) in invading tumor cells correlates with poor prognosis in human colorectal cancer. *Int J Cancer* 95 (4), 216-22 (2001)
69. S. Ahmed, K. Yamamoto, Y. Sato, T. Ogawa, A. Herrmann, S. Higashi and K. Miyazaki: Proteolytic processing of igfbp-related protein-1 (taf/angiomodulin/mac25) modulates its biological activity. *Biochem Biophys Res Commun* 310 (2), 612-8 (2003)
70. K. Akaogi, Y. Okabe, J. Sato, Y. Nagashima, H. Yasumitsu, K. Sugahara and K. Miyazaki: Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. *Proc Natl Acad Sci U S A* 93 (16), 8384-9 (1996)
71. K. Akaogi, Y. Okabe, K. Funahashi, Y. Yoshitake, K. Nishikawa, H. Yasumitsu, M. Umeda and K. Miyazaki: Cell adhesion activity of a 30-kda major secreted protein from human bladder carcinoma cells. *Biochem Biophys Res Commun* 198 (3), 1046-53 (1994)
72. M. Murphy, M.J. Pykett, P. Harnish, K.D. Zang and D.L. George: Identification and characterization of genes differentially expressed in meningiomas. *Cell Growth Differ* 4 (9), 715-22 (1993)
73. T. Yamauchi, F. Umeda, M. Masakado, M. Isaji, S. Mizushima and H. Nawata: Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. *Biochem J* 303 (Pt 2) 591-8 (1994)
74. A.S. Bhatt, H. Erdjument-Bromage, P. Tempst, C.S. Craik and M.M. Moasser: Adhesion signaling by a novel mitotic substrate of src kinases. *Oncogene* 24 (34), 5333-43 (2005)
75. J.D. Hooper, A. Zijlstra, R.T. Aimes, H. Liang, G.F. Claassen, D. Tarin, J.E. Testa and J.P. Quigley: Subtractive immunization using highly metastatic human tumor cells identifies sima135/cdcp1, a 135 kda cell surface phosphorylated glycoprotein antigen. *Oncogene* 22 (12), 1783-94 (2003)
76. M. Scherl-Mostageer, W. Sommergruber, R. Abseher, R. Hauptmann, P. Ambros and N. Schweifer: Identification of a novel gene, cdcp1, overexpressed in human colorectal cancer. *Oncogene* 20 (32), 4402-8 (2001)
77. N. Ferrara, H.P. Gerber and J. LeCouter: The biology of vegf and its receptors. *Nat Med* 9 (6), 669-76 (2003)
78. A.K. Olsson, A. Dimberg, J. Kreuger and L. Claesson-Welsh: Vegf receptor signalling - in control of vascular function. *Nat Rev Mol Cell Biol* 7 (5), 359-71 (2006)
79. J.M. Ebos, G. Bocci, S. Man, P.E. Thorpe, D.J. Hicklin, D. Zhou, X. Jia and R.S. Kerbel: A naturally occurring soluble form of vascular endothelial growth factor receptor

2 detected in mouse and human plasma. *Mol Cancer Res* 2 (6), 315-26 (2004)
80. R.L. Kendall and K.A. Thomas: Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci U S A* 90 (22), 10705-9 (1993)
81. S. Rousseau, F. Houle, J. Landry and J. Huot: P38 map kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene* 15 (18), 2169-77 (1997)
82. B.P. Schneider and G.W. Sledge, Jr.: Drug insight: Vegf as a therapeutic target for breast cancer. *Nat Clin Pract Oncol* 4 (3), 181-9 (2007)
83. S.R. Coughlin: Thrombin signalling and protease-activated receptors. *Nature* 407 (6801), 258-64 (2000)
84. B. Barleon, P. Reusch, F. Totzke, C. Herzog, C. Keck, G. Martiny-Baron and D. Marme: Soluble vegfr-1 secreted by endothelial cells and monocytes is present in human serum and plasma from healthy donors. *Angiogenesis* 4 (2), 143-54 (2001)
85. C. Hornig, B. Barleon, S. Ahmad, P. Vuorela, A. Ahmed and H.A. Weich: Release and complex formation of soluble vegfr-1 from endothelial cells and biological fluids. *Lab Invest* 80 (4), 443-54 (2000)
86. G.H. Fong, J. Rossant, M. Gertsenstein and M.L. Breitman: Role of the flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376 (6535), 66-70 (1995)
87. J.B. Kearney, C.A. Ambler, K.A. Monaco, N. Johnson, R.G. Rapoport and V.L. Bautch: Vascular endothelial growth factor receptor flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division. *Blood* 99 (7), 2397-407 (2002)
88. F. Shalaby, J. Rossant, T.P. Yamaguchi, M. Gertsenstein, X.F. Wu, M.L. Breitman and A.C. Schuh: Failure of blood-island formation and vasculogenesis in flk-1-deficient mice. *Nature* 376 (6535), 62-6 (1995)
89. G. Brunner, U. Vettel, S. Jobstmann, M.D. Kramer and V. Schirrmacher: A t-cell-related proteinase expressed by t-lymphoma cells activates their endogenous pro-urokinase. *Blood* 79 (8), 2099-106 (1992)
90. K. List, O.N. Jensen, T.H. Bugge, L.R. Lund, M. Ploug, K. Dano and N. Behrendt: Plasminogen-independent initiation of the pro-urokinase activation cascade in vivo. Activation of pro-urokinase by glandular kallikrein (mgk-6) in plasminogen-deficient mice. *Biochemistry* 39 (3), 508-15 (2000)
91. A. Ichinose, K. Fujikawa and T. Suyama: The activation of pro-urokinase by plasma kallikrein and its inactivation by thrombin. *J Biol Chem* 261 (8), 3486-9 (1986)
92. H. Kobayashi, M. Schmitt, L. Goretzki, N. Chucholowski, J. Calvete, M. Kramer, W.A. Gunzler, F. Janicke and H. Graeff: Cathepsin b efficiently activates the soluble and the tumor cell receptor-bound form of the proenzyme urokinase-type plasminogen activator (pro-upa). *J Biol Chem* 266 (8), 5147-52 (1991)
93. P. Moran, W. Li, B. Fan, R. Vij, C. Eigenbrot and D. Kirchhofer: Pro-urokinase-type plasminogen activator is a substrate for hepsin. *J Biol Chem* 281 (41), 30439-46 (2006)
94. M.S. Stack and D.A. Johnson: Human mast cell tryptase activates single-chain urinary-type plasminogen activator (pro-urokinase). *J Biol Chem* 269 (13), 9416-9 (1994)
95. E. Yoshida, S. Ohmura, M. Sugiki, M. Maruyama and H. Mihara: Prostate-specific antigen activates single-chain urokinase-type plasminogen activator. *Int J Cancer* 63 (6), 863-5 (1995)
96. G.P. Gupta, D.X. Nguyen, A.C. Chiang, P.D. Bos, J.Y. Kim, C. Nadal, R.R. Gomis, K. Manova-Todorova and J. Massague: Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature* 446 (7137), 765-70 (2007)
97. J. Sun, J. Pons and C.S. Craik: Potent and selective inhibition of membrane-type serine protease 1 by human single-chain antibodies. *Biochemistry* 42 (4), 892-900 (2003)

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

http://www.bioscience.org/current/vol13.htm