

REVIEW ARTICLE

The cutting edge: membrane-anchored serine protease activities in the pericellular microenvironmentToni M. ANTALIS*¹, Marguerite S. BUZZA*², Kathryn M. HODGE*, John D. HOOPER† and Sarah NETZEL-ARNETT*²

*Center for Vascular and Inflammatory Diseases and Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201, U.S.A., and †Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, Queensland 4059, Australia

The serine proteases of the trypsin-like (S1) family play critical roles in many key biological processes including digestion, blood coagulation, and immunity. Members of this family contain N- or C-terminal domains that serve to tether the serine protease catalytic domain directly to the plasma membrane. These membrane-anchored serine proteases are proving to be key components of the cell machinery for activation of precursor molecules in the pericellular microenvironment, playing vital functions in the maintenance of homeostasis. Substrates activated by membrane-anchored serine proteases include peptide hormones, growth and differentiation factors, receptors, enzymes, adhesion molecules and viral coat proteins. In addition, new insights into our

understanding of the physiological functions of these proteases and their involvement in human pathology have come from animal models and patient studies. The present review discusses emerging evidence for the diversity of this fascinating group of membrane serine proteases as potent modifiers of the pericellular microenvironment through proteolytic processing of diverse substrates. We also discuss the functional consequences of the activities of these proteases on mammalian physiology and disease.

Key words: membrane serine protease, pericellular proteolysis, serine protease inhibitor, type II transmembrane serine protease (TTSP).

INTRODUCTION

The immediate context of a cell, or the cell microenvironment, is critically modulated by proteolytic activities that regulate a wide variety of developmental, physiological and disease-associated processes. Pericellular proteolysis via cell-surface-localized proteases is recognized as an important pathway by which cells interact with the cell microenvironment [1]. Many proteins in the pericellular microenvironment, including growth factors, cytokines, receptors, enzymes and cell adhesion molecules, are present as inactive precursors that require activation by endoproteolytic cleavage of peptide bonds [2]. This activation may occur through the action of proteases that are cell-surface-localized via protease receptors, but may also be mediated by proteases directly anchored on the cell surface via membrane-anchoring domains. This latter proteolytic mechanism enables spatial and directional substrate processing directly at the cell membrane and can be mediated by at least three classes of enzymes, including MT-MMPs [membrane-type MMPs (matrix metalloproteinases)], ADAMs (a disintegrin and metalloproteases) and membrane-anchored serine proteases. Biochemical, cell biological and genetic advances have revealed the importance of the last enzyme class, the membrane-anchored serine proteases, to mammalian health and survival, and have also

provided important new insights into the mechanisms regulating their roles in pericellular activation of precursor proteins.

Membrane-anchored serine proteases

Serine proteases are defined by the nucleophilic serine residue in the enzyme active site, which attacks the carbonyl moiety of the substrate peptide bond to form an acyl intermediate [3,4]. Completion of proteolysis is dependent on a catalytic triad of histidine, aspartate and serine residues, often referred to as a charge relay system. Over one third of all known proteolytic enzymes are serine proteases, and these have been grouped into families and clans based on common architectures and other functional attributes [4–6]. The serine proteases of clan PA, subfamily S1A, are a large group of enzymes that account for over 20% of the total known proteases. The most widely studied members of this group, which include trypsin, chymotrypsin and thrombin, are produced as soluble, secreted proteins or are compartmentalized within intracellular granules and released in response to stress or inflammation (Figure 1). Some members, including the plasminogen activators, may be bound to specific cell-surface receptors, enabling increased efficiency of plasminogen activation and subsequent plasmin-dependent proteolysis within the pericellular environment [7].

Abbreviations used: ANP, atrial natriuretic peptide; BNP, brain/B-type natriuretic peptide; CAP, channel-activating protease; CUB, complement C1r/C1s, Uegf, Bmp1; DESC, differentially expressed in squamous cell carcinoma; ECD, extracellular domain; EGFR, epidermal growth factor receptor; EMT, epithelial to mesenchymal transition; ENaC, epithelial Na⁺ channel; FGF-2, fibroblast growth factor-2; GPI, glycosylphosphatidylinositol; HA, haemagglutinin; HAT, human airway trypsin-like protease; HEK-293T cell, HEK (human embryonic kidney)-293 cell expressing the large T-antigen of SV40 (simian virus 40); HGF, hepatocyte growth factor; HGFA, HGF activator; IL, interleukin; IRIDA, iron-refractory iron deficiency anaemia; LDLRA, low-density-lipoprotein receptor class A-like; MDCK, Madin–Darby canine kidney; MMP, matrix metalloproteinase; MSPL, mosaic serine protease, large form; MT, membrane-type; PAI-1, plasminogen-activator inhibitor 1; PAR, protease-activated receptor; PCI, protein C inhibitor; RCL, reactive centre loop; SARS-CoV, severe acute respiratory syndrome coronavirus; SBTI, soya-bean trypsin inhibitor; SEA, sea-urchin sperm protein, enterokinase and agrin; SPD, serine protease domain; SRCR domain, scavenger receptor cysteine-rich domain; TEER, transepithelial electrical resistance; TMPRSS, transmembrane protease, serine; TTSP, type II transmembrane serine protease; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

¹ To whom correspondence should be addressed (email tantalis@som.umaryland.edu).

² These authors contributed equally to this study.

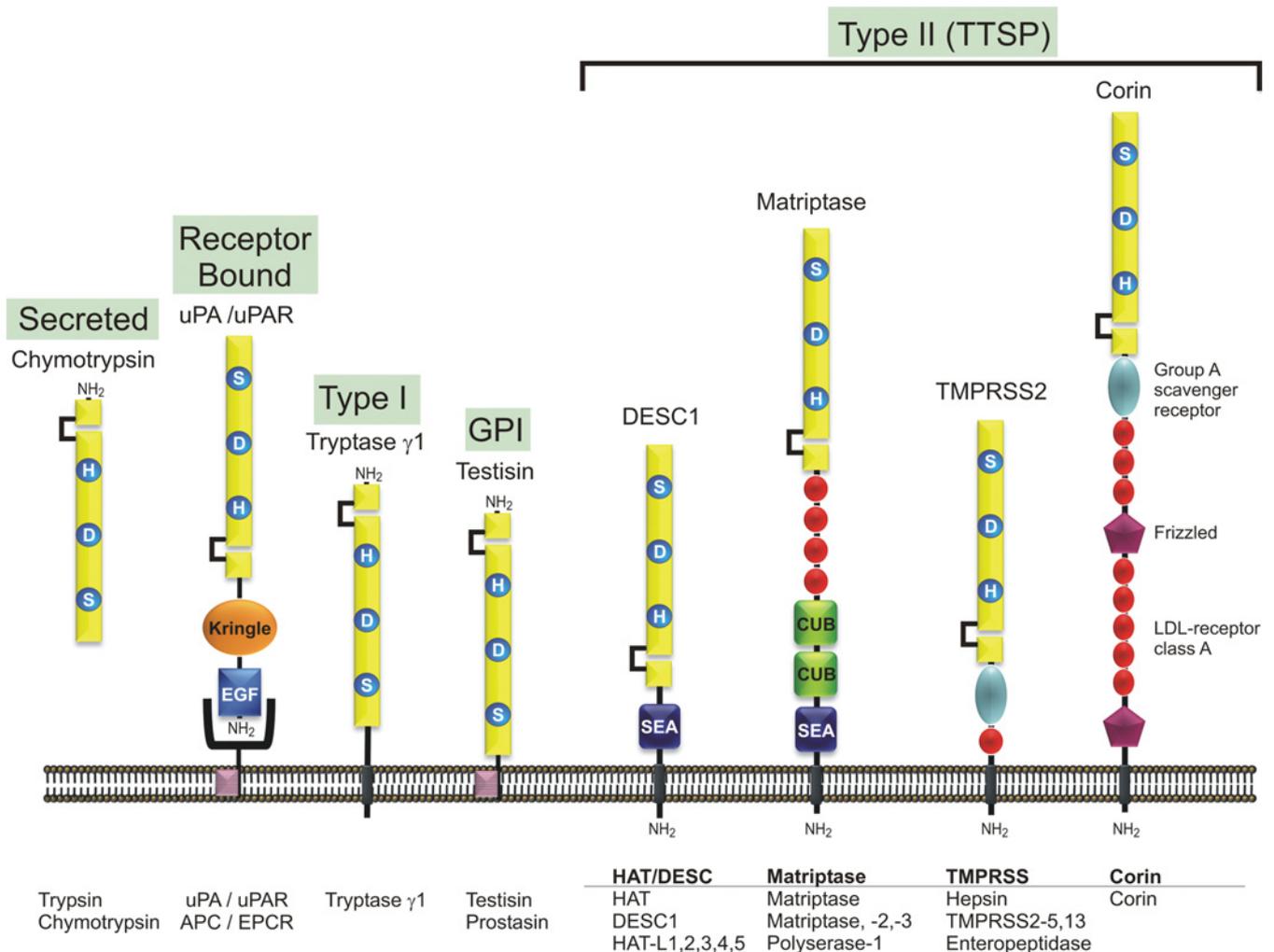


Figure 1 Membrane-anchored serine proteases are linked directly to the plasma membrane

Most of the well characterized S1A serine proteases, such as the prototype enzymes trypsin and chymotrypsin, are secreted enzymes, associated with extracellular proteolysis. Some of these enzymes [e.g. uPA and APC (activated protein C)] may participate in pericellular proteolysis by binding to specific cell-surface receptors [uPAR and EPCR (endothelial protein C receptor)]. Tryptase γ 1 is the only known type I transmembrane serine protease. The human GPI-anchored serine proteases are proastasin and testisin. The TTSPs are the largest group of pericellular serine proteases and may be divided into four subfamilies [12,21]: (i) the HAT/DESC subfamily for which the stem regions are all composed of a single SEA domain, (ii) the Hepsin/TMPRSS subfamily, each of which have a group A SRCR in their stem region, preceded by a single LDLRA domain in TMPRSS2, 3, 4, and 13 or in enteropeptidase, an array of SEA, LDLRA, CUB and MAM domains, (iii) the Matriptase subfamily, each containing a SEA domain, two CUB domains and three or four LDLRA domains in their stem region. Polyserase-1 is unique, comprising two active, and one catalytically inactive, SPDs and a stem region containing an LDLRA domain, and (iv) the Corin subfamily, consisting of a single member, corin, which possesses a complex stem region composed of two Frizzled domains, eight LDLRA domains and one SRCR domain.

Only in the last decade has it been recognized that a unique subgroup of S1A serine proteases contain N- or C-terminal domains that serve to anchor the SPDs (serine protease domains) directly at the plasma membrane [8,9]. The present review focuses on these enzymes as potent modifiers of the pericellular microenvironment through the diversity of their substrates and discusses their contributions to mammalian physiology and disease. For historical perspectives, naming, classifications into subgroups, gene structure and chromosomal localization, and tissue- and cell-specific distribution of the membrane-anchored serine proteases, the reader is referred to previous excellent reviews [8–12].

Membrane localization

The membrane-anchored serine proteases may be divided into subgroups based on structural features (Figure 1). These proteases

are tethered either by a C-terminal transmembrane domain (Type I), through a GPI (glycosylphosphatidylinositol) linkage, or via an N-terminal transmembrane domain with a cytoplasmic extension [TTSPs (type II transmembrane serine proteases)] [8,9]. Type I serine proteases and the GPI-anchored serine proteases are synthesized with a classical N-terminal signal peptide and possess a hydrophobic domain at their C-terminus. They are very similar in length, ranging from 310 to 370 amino acids. Tryptase γ 1 is the only Type I transmembrane serine protease that has been identified to date [13,14]. The C-terminal domains of proastasin and testisin are post-transcriptionally modified with GPI anchors [15–19]. TTSPs [8] lack a classical signal peptide, but instead are synthesized with an N-terminal signal anchor. This signal anchor is not removed during synthesis, but serves as a transmembrane domain that positions the protease in the plasma membrane as an integral membrane protein with a cytoplasmic N-terminus and an

extracellular C-terminus [8]. The first described TTSP was enteropeptidase, although its transmembrane molecular structure was only recognized when it was cloned [20]. The TTSPs share several structural features: an N-terminal cytoplasmic domain of variable length (20–160 amino acids), a type II transmembrane sequence, a central stem region of variable length with modular structural domains, and an extracellular C-terminal SPD. A total of 19 TTSPs have been identified and divided into four subfamilies based on phylogenetic analyses of their SPDs and the domain structure of their extracellular stem regions (Figure 1) [9,21]. These are the HAT (human airway trypsin-like protease)/DESC (differentially expressed in squamous cell carcinoma), hepsin/TMPRSS (transmembrane protease, serine), matriptase and corin subfamilies [10]. Polyserase-1 is an unusual member of the matriptase family that has a unique structure with three tandem SPDs and the ability to generate three independent serine proteases (i.e. serase-1, -2 and -3), the third of which is predicted to be enzymatically inactive due to the lack of the catalytic serine [22]. An alternatively spliced transcript encodes serase-1B, a TTSP that has a SEA (sea-urchin sperm protein, enterokinase and agrin) module in the stem region and a single protease domain with a mucin-like box at the C-terminus [23]. Differences in isoforms between humans and rodents have also been observed. For example, rodents express a secreted isoform of the human TTSP homologue HAT that lacks the transmembrane and SEA domains [24]. In contrast, pancreasin/marapsin possesses a C-terminal GPI anchor in mice, which is absent from human and chimpanzee homologues where it is only found as a secreted enzyme [25].

The membrane-anchoring domains of these enzymes contribute to their cellular trafficking and localization. Surface localization studies of the membrane-anchored serine proteases for which antibodies are available show that they are localized at plasma membranes. An exception is the type I membrane-anchored protease tryptase- γ , which is stored in the secretory granules of mast cells and does not reach the cell surface until degranulation [26]. The GPI-anchored testisin and prostaticin are found to be compartmentalized at plasma membranes within the dynamic microenvironment of specialized cholesterol-rich membrane microdomains or lipid rafts [17,18]. The cytoplasmic domains of the TTSPs, which are mosaic in nature and variable in length, are thought to contribute to the targeting of these enzymes to plasma membrane microdomains, based on the cellular sorting of other integral membrane proteins. Several of the TTSP cytoplasmic domains also contain consensus phosphorylation sites that may facilitate communication between the cell and the pericellular environment.

Membrane polarity and the EMT (epithelial to mesenchymal transition) affect the pericellular distribution of many of the membrane-anchored serine proteases. In polarized epithelial monolayers, in which the plasma membranes are separated into apical and basolateral surfaces, some of these enzymes are targeted specifically to apical and others to the basolateral regions. Matriptase is present on basolateral membranes [27,28], and specifically localizes to the laterally located adherens junctions with E-cadherin in polarized epithelial cells [28,29]. Adherens junction formation and rearrangements of the actin cytoskeleton appear to be a prerequisite for sphingosine 1-phosphate-induced matriptase localization at mammary epithelial cell–cell contacts [30]. Mutagenesis studies of the matriptase cytoplasmic domain has revealed that the juxtamembrane cytoplasmic amino acids (Lys⁴⁵, Val⁴⁷ and Arg⁵⁰) are important for matriptase targeting to the basolateral surfaces of polarized MDCK (Madin–Darby canine kidney) epithelial monolayers [31]. The matriptase cytoplasmic domain has been shown to interact with actin-associated filamin

[32], but whether this interaction is important for junctional localization in polarized epithelia has not been investigated. Hepsin also exhibits a junctional localization, co-localizing with desmosomal markers in OVCAR5 carcinoma cells, but not precisely with gap junctions, adherens junctions or tight junctions [33]. Desmosomal junctions appear to be specifically required for hepsin junctional localization, since hepsin localization at junctions does not occur in the related OVCAR5-TR cells, which lack the desmosomal junction protein desmoplakin, but retain normal adherens junctions. In contrast with these intercellular localizations, prostaticin [16,34,35], TMPRSS2 [36,37] and enteropeptidase [38,39] localize to apical membranes in polarized epithelia. The mucin-like SEA domain of enteropeptidase directs its apical targeting in MDCK epithelial monolayers [38,39]. In some cases, loss of membrane polarity, such as which occurs in tumour cells, is associated with mislocalization of the membrane serine proteases. TMPRSS2, for example, localizes to the apical surfaces of renal tubular and airway epithelial cells [36] and along the apical membrane in normal prostate epithelium [37,40,41], whereas in prostate carcinoma cells, TMPRSS2 displays a more prominent cytoplasmic localization [41].

Ectodomain shedding

Targeted release of extracellular domains from the cell surface, or ectodomain shedding, has been reported for several of the membrane-anchored serine proteases, providing a means by which these enzymes may contribute to proteolytic activities in the extracellular space. Soluble forms of prostaticin are found in human urine [42,43], and are elevated in hypertensive patients [44]. Aldosterone increases the expression and secretion of prostaticin in a kidney cortical collecting duct cell line (M-1) and increases the urinary excretion of prostaticin in rodents [45]. Prostaticin is released apically from M-1 cells by cleavage of its GPI anchor via an endogenous GPI-specific phospholipase D1 [17]. Alternatively, there is evidence that prostaticin may be shed via a tryptic-like proteolytic cleavage in its hydrophobic C-terminal domain [15,46]. The GPI-anchored testisin, on the other hand, has not been found in a soluble shed form but may be released from cell membranes by addition of exogenous bacterial PI-PLC (phosphatidylinositol-specific phospholipase C) [18].

Among the TTSPs, naturally-occurring shed forms of matriptase, matriptase-2, enteropeptidase and HAT have been detected *in vivo*. Shed matriptase was identified originally in complex with an inhibitor, HAI-I (hepatocyte growth factor activator inhibitor-1), in human milk [47], and additional shed forms have been reported in conditioned media of cultured breast epithelial lines, thymic epithelial lines and polarized intestinal epithelial cells [28,48–51]. Released enteropeptidase is found in mucosal fluid of the intestinal wall, where shedding is induced by hormones (secretin, cholecystokinin-pancreozymin) [52,53]. Soluble TMPRSS2 is a normal component of seminal fluid that accumulates within the lumen of the prostate, and in androgen-stimulated LNCaP cells, the TMPRSS2 ectodomain is released as a result of auto-activation [37,41]. Soluble forms of HAT are found in sputum from patients with chronic airway diseases [54].

The mechanisms by which the TTSPs are released are not yet fully understood and appear to vary depending on the specific protease. Porcine enteropeptidase, matriptase and matriptase-2 are shed as a result of proteolytic cleavage within their SEA domains [1,50,55], at sites that are distinct from the spontaneous processing known to occur in the SEA domain [50]. N-terminal

sequencing of matriptase isoforms isolated from milk showed proteolytic cleavage at one of two sites either in the SEA domain (Lys¹⁸⁸) or in the linker region between the SEA domain and CUB1 (complement C1r/C1s, Uegf, Bmp1 1) domain (Lys²⁰³), although the identity of the protease(s) that mediate these cleavages remain(s) to be identified. The SEA domain may be critical for the shedding of these enzymes, since a mutation that disrupts the conformation of the SEA domain inhibits shedding of matriptase-2 *in vitro* [1]. Shedding of matriptase is thought to require its proteolytic activity [56], and can occur as a result of zymogen activation and HAI-I-mediated inhibition [28,50]. Matriptase shedding may also be induced in response to PMA, causing local accumulation of the protease at the membrane, and interaction of the matriptase cytoplasmic domain with the cytoskeletal linker protein filamin, which was found to be essential for shedding [32]. PMA-induced shedding could be inhibited by the MMP inhibitor GM6001 [32], implicating the involvement of a MMP in the shedding process. In contrast, Serase-1B is shed from HEK-293T [human embryonic kidney-293 cells expressing the large T-antigen of SV40 (simian virus 40)] cells in an inactive form following cleavage within the stem domain, which was not inhibited by GM6001 [23], suggesting specific mechanisms exist depending on the protease.

MODULATION OF MEMBRANE-ANCHORED SERINE PROTEASE CATALYTIC ACTIVITY

The serine protease catalytic domain

The catalytic domains of the membrane-anchored serine proteases are highly conserved and essential to the biological and physiological functions so far ascribed to these enzymes. Despite the high sequence homology of these domains, differences in amino acids that occupy key positions confer unique substrate specificities. All of the membrane-anchored serine proteases share a serine protease tertiary domain structure with high sequence homology [9] that contains the catalytic triad of histidine, aspartate and serine residues necessary for S1 serine protease catalytic activity [8,9]. The catalytic domains are approx. 225–230 amino acids in size and are orientated in such a way that the domain is at the terminus of an extracellular region that is directly exposed to the pericellular environment. All of the membrane-anchored serine proteases are synthesized as single-chain inactive pro-enzymes or zymogens, with an N-terminal extension that acts as a propeptide, requiring proteolytic cleavage to generate the active enzyme. Activation results in a two-chain form with the pro- and catalytic domains linked by a disulfide bridge between two conserved cysteine residues [19]. Each active enzyme is defined by a binding pocket whose size, shape and charge are major determinants of substrate cleavage specificity. A specific nomenclature is used for the interaction of proteases with their substrates [57], where substrate amino acids (called P for peptide) are numbered P₁ to P_n counting outward from the N-terminal side of the peptide bond that is cleaved during hydrolysis, and where those on the C-terminal side are numbered P_{1'} to P_{n'}. Hydrolysis occurs between the P₁ and P_{1'} residues. The corresponding subsites of the enzymes are designated S_n to S_{n'}. All of the membrane serine proteases possess a conserved aspartate residue at the bottom of the S₁ substrate-binding pocket in the activated SPD which determines the preference for cleavage of substrates with a basic amino acid (arginine or lysine) in the P₁ position (Table 1).

Detailed comparative analyses of the amino acid sequences [8,9,21] combined with structural analyses of the serine protease catalytic domains of matriptase [58], hepsin [59], enteropeptidase

[60], DESC1 [61] and prostasin [62–64] have revealed insights into the unique catalytic activities of the membrane serine proteases. In most but not all cases, crystal structures were obtained using truncated catalytic domains in complex with low-molecular-mass inhibitors. These structures reveal that each catalytic domain consists of two adjacent, six-stranded β -barrel domains that are connected by three transdomain segments. The catalytic triad amino acids are located along the junction between the two barrels, whereas the active site cleft runs perpendicular to this junction. The specificity of these enzymes is determined by both the nature of the substrate-binding subsites (e.g. S₄–S_{2'}), and the differing loop regions that surround the active site. These loops can influence enzyme specificity both by 'shaping' extended binding subsites and by providing secondary exosites for substrate or inhibitor binding. For example, the loop bordering the S_{2'} pocket varies markedly in length among the membrane serine proteases, and in matriptase this loop is uniquely distorted due to a four-residue insertion, which leads to thrombin-like shielding of the prime site [58,61,65]. In contrast, DESC1 carries a one amino acid deletion in this loop compared with other TTSPs, resulting in a narrowing of the prime site [61]. The 99-loop, which limits the space for the P₂ and P₄ residues of the substrate peptide at the top of the active site cleft, exhibits pronounced sequence heterogeneity among the membrane-anchored serine proteases and contributes significantly to their respective specificities. The remarkably specific enteropeptidase is dependent on Lys⁹⁹ within its 99-loop for interaction with the P₂–P₄ aspartate residues to facilitate cleavage of its physiological substrate, trypsinogen [60]. Ion interactions may also contribute to substrate specificities. The S₁ subsite loop of prostasin directly binds the bivalent cation Ca²⁺ and exhibits a large degree of conformational variation, able to move to block or to expose the S₁ subsite [62]. This highlights a possible mechanism of regulation in pericellular spaces where ionic concentrations are variable.

In contrast with these crystal structures of truncated catalytic domains, the structure of the complete hepsin extracellular domain, including the serine protease catalytic domain and the N-terminal SRCR (scavenger receptor cysteine-rich) domain contained within the stem region was determined [59]. Interestingly, structural analyses revealed that the SRCR domain was rigidly bound to the back of the catalytic SPD [59]. Comparison of the surface charges of hepsin, DESC-1, matriptase and enteropeptidase suggest that this may be a common feature, with the stem region domains functioning as interaction partners with the exposed surfaces of the serine protease catalytic domains [61], potentially contributing to appropriate orientation of the active site cleft on the cell surface.

Extracellular domains affecting serine protease catalytic activities

There is now considerable evidence that the stem domains of the TTSPs not only contribute to surface orientation, but also modulate proteolysis by contributing to TTSP activation, the binding of substrates and interactions with other proteins. N-terminal processing within a SEA domain, present in 11 members of this family, can regulate TTSP activation [66,67]. For matriptase, and potentially other SEA domain-containing TTSPs, this domain undergoes spontaneous proteolytic processing at a conserved glycine residue [55,66], due to conformation-driven non-enzymatic hydrolysis of the peptide bond [68]. After hydrolysis, the matriptase extracellular domain remains non-covalently associated and this processing is required for matriptase zymogen activation. In addition to the contribution of the SEA domain, mutations that disrupt the domain structure

Table 1 Cleavage sequence specificities

Basic amino acids are in bold, acidic amino acids are underlined and aromatic amino acids are italicized. Substrate cleavage specificities for DESC4/HATL2, HATL4/TMPRSS11F, HATL5/TMPRSS11B and TMPRSS3 are not known. AMC, 7-amino 4-methylcoumarin; Boc, t-butoxycarbonyl; Cbz, benzyloxycarbonyl; GST, glutathione transferase; h, human; IGFBP-rP1, insulin-like growth factor-binding protein-related protein-1; m, mouse; MCA, 4-methylcoumaryl-7-amide; pNA, p-nitroanilide; pyroE, pyroglutamic acid; r, rat; TRASK, transmembrane and associated with Src kinases.

Protease	Common aliases	Gene symbol	P ₄	P ₃	P ₂	P ₁	↓	P ₁ '	P ₂ '	P ₃ '	P ₄ '	Protease source	Substrate(s)	Reference(s)		
GPI-anchored Prostasin	PRSS8, CAP1	<i>PRSS8</i>	R	K	R	K	I	S	G	K	cDNA	ENaC(SCNN1γ)	[141]			
			R/K	H/K/R	Y/W/R	R/K	X	X	X	A/S	SPD	Combinatorial peptide library. SPD	[85]			
Testisin	PRSS21, TESP, TEST1, ESP-1, tryptase 4	<i>PRSS21</i>	Boc	F	S	R	MCA					SPD	Four peptide screen	[18]		
Type I Tryptase γ1	TMT/TPSG1, PRSS31	<i>TPSG1</i>		K	W/Y/F	R	AMC					ECD	Peptide library	[217]		
Type II HAT	TMPRSS11D	<i>TMPRSS11D</i>	N	S	G	R	A	V	T	G	SPD	uPAR	[96]			
			T	Y	S	R	S	R	Y	L	SPD	uPAR	[96]			
			K	Q	T	R	G	L	F	G	cDNA	H3N2 HA protein, MDCK cells	[162]			
			Boc	F	S	R	MCA				Purified human	Peptide	[54]			
			DESC1	TMPRSS11E	<i>TMPRSS11E</i>	R	R/Q	A/R	R	V	V	G	G	ECD	18 peptide screen	[87]
						V	S	A	R	M	A	P	E	hECD, mSPD	hPAI-1 and RCL mutant	[86,87]
						F	T	F	R	S	A	R	L	mSPD	hPCI and RCL mutant	[86]
			HATL1	TMPRSS11A, DESC3	<i>TMPRSS11A</i>	S	L	L	R	S	T	S	Q	soluble protein	SARS S glycoprotein (triSpike)	[167]
						P	T	K	R	S	F	I	E			[167]
			HATL3	Neurobin, TMPRSS11C	<i>Tmprss11c</i>		V	G	R	pNA					SPD	Nine peptide screen
			R	G	H	K	V	A	G	G	cDNA	Pro-HATL3	[102]			
			N	T	Y	R	S	R	K	Y	mSPD	FGF-2 fragment	[102]			
			F	F	L	R	I	H	P	D	mSPD	FGF-2 fragment	[102]			
			C	A	N	R	Y	L	A	M	mSPD	FGF-2 fragment	[102]			
			V	R	E	K	S	D	P	H	mSPD	FGF-2 fragment	[102]			
			P	H	I	K	L	Q	L	Q	mSPD	FGF-2 fragment	[102]			
Hepsin	TMPRSS1	<i>HPN</i>	K/P	K/Q	T/N/L	R						ECD	Peptide library	[83]		
			S	Q	L	R	L	Q	G	S	ECD	Laminin-332 β3	[198]			
			K	Q	L	R	V	V	N	G	ECD	Pro-HGF	[121]			
			P	Q	G	R	I	V	G	G	cDNA, ECD	FVII	[83,91]			
			P	V	D	R	I	V	G	G	cDNA	m Pro-hepsin	[101]			
			P	R	F	K	I	I	G	G	ECD, cDNA	Pro-uPA	[93]			
			TMPRSS2	Epitheliasin	<i>TMPRSS2</i>	R	Q	S	R	I	V	G	G	cDNA	Pro-TMPRSS2	[37]
						R	Q	S	R	F	V	L	G	cDNA	HMPV F protein Vero cells	[165]
						I	Q	S	R	G	L	F	G	cDNA	1918 HA (South Carolina) 293T cells	[164]
						K	Q	T	R	G	L	F	G	cDNA	H3N2 HA protein MDCK cells	[162]
			S	K	G	R	S	L	I	G	Purified	PAR ₂	[112]			
TMPRSS4	MT-SP2, CAP2	<i>TMPRSS4</i>	Cbz	G	G	R	AMC					Purified	Peptide assay	[112]		
			I	Q	S	R	G	L	F	G	cDNA	1918 HA (South Carolina) HEK-293T cells	[164]			

continued on next page

Table 1 Continued

Protease	Common aliases	Gene symbol	P ₄	P ₃	P ₂	P ₁	↓	P ₁ '	P ₂ '	P ₃ '	P ₄ '	Protease source	Substrate(s)	Reference(s)						
TMPRSS13	MSPL	<i>TMPRSS13</i>	R	K	R	R		<u>E</u>	A	G	S	cRNA	r γ ENaC <i>Xenopus</i> oocytes	[145]						
			L	N	Y	K		T	N	S	<u>E</u>	cRNA	r α ENaC <i>Xenopus</i> oocytes	[145]						
			F	N	Y	R		T	I	<u>E</u>	<u>E</u>	cRNA	r β ENaC <i>Xenopus</i> oocytes	[145]						
			L	N	Q	R		S	I	M	<u>E</u>	cRNA	r γ ENaC <i>Xenopus</i> oocytes	[145]						
			Boc	Q	A	R		AMC				ECD	Peptide assay	[187]						
			Boc	Q/L	R/K	R		MCA					19 peptide screen	[84]						
			R/K	K	K	R		G	L	F	G	ECD	HA-based peptide	[161]						
			Boc	Q	A	R		MCA				SPD	Ten peptide screen	[220]						
			<u>D</u>	<u>D</u>	<u>D</u>	K		I	V	G	G	purified porcine, bSPD	Trypsinogen 1	[221,222]						
			<u>D/A</u>	<u>D</u>	<u>D</u>	K/R		I	V	G	G	Purified bovine, porcine	20 peptide screen	[223]						
Matriptase	MT-SP1, CAP3, TADG-15, PRSS14, ST14, SNC19, epithin (mouse)	<i>ST14</i>	R	Q	A/G/L	R		V	V	G	G	SPD	18 peptide screen	[87]						
			R/K	X	S	R		A				SPD	Combinatorial peptide library	[81]						
			X	R/K	S	R		A				SPD	Combinatorial peptide library	[81]						
			R	Q	A	R		V	V	G	G		Pro-matriptase	[69]						
			P/I	Q	A/P	R		I	T	G	G	KO, SPD	Pro-prostasin (h/m)	[99,123]						
			P	R	F	K		I	I	G	G	SPD	Pro-uPA	[81]						
			S	K	G	R		S	L	I	G	SPD	hPAR ₂ <i>Xenopus</i> oocytes	[81]						
			K	Q	L	R		V	V	N	G		Pro-HGF	[95,224,225]						
			S	K	L	R		V	V	G	G		MSP-1	[117]						
			K	Q	S	R		K	F	V	P	SPD	ECD TRASK	[226]						
Matriptase-2	TMPRSS6	<i>TMPRSS6</i>	R	K	G	K		A	G	A	A	ECD, siRNA	IGFBP-rP1	[227]						
			R	R	V	R		K	E	<u>D</u>	<u>E</u>		VEGFR2	[125]						
			Boc	Q	A/G	R		AMC				GST-SPD	Five peptide screen	[104]						
			R	R	A	R		A/V	V	G	G	ECD	18 peptide screen	[87]						
			Matriptase-3	TMPRSS7	<i>TMPRSS7</i>	A	A	P	R		pNA				mSPD	Seven peptide screen	[203]			
							F	V	R		pNA				mSPD	Seven peptide screen	[203]			
						V	S	A	R		M	A	P	<u>E</u>	mSPD	hPAI-1 and RCL mutant	[203]			
						Boc	Q	G	R		AMC				SPD	Four peptide screen	[22]			
						Polyserase-1 Serase-1B	TMPRSS9	<i>TMPRSS9</i>	Boc	Q/L	S/T/A	R		MCA				ECD	14 peptide screen	[23]
									P	R	F	K		I	I	G	G	ECD	Pro-uPA	[23]
Y	A	P							R		S	L	R	R	cDNA, ECD	Pro-ANP, HEK-293 cells	[127,128]			
R	A	P							R		S	P	K	M	ECD	Pro-BNP	[79]			
	pyroE	F							K		pNA					Five peptide screen	[228]			

of the LDLRA (low-density-lipoprotein receptor class A-like) domain and deletions of either of the CUB domains of matriptase also inhibit zymogen activation [66,69]. Also, the extracellular stem domains of corin (Frizzled domain 1 and LDLRA1–4), and unidentified domains of the enteropeptidase stem region, are important for recognition of macromolecular substrates, but are not required for cleavage of peptide substrates [70,71]. Furthermore, post-translational modifications such as N-linked glycosylation also affect biological activity of the membrane-anchored serine proteases. For example, N-linked glycosylation at sites in the CUB1 domain and serine protease catalytic domain of matriptase are required for zymogen activation [66], and glycosylation in the SPD of corin is required for both cell-surface expression and zymogen activation [72,73]. The theme emerging from these studies is that for each TTSP, its stem domain contributes uniquely to its cellular localization, activation and inhibition and that it is likely that further studies on these domains will identify important new binding partners that regulate TTSP-mediated pericellular proteolysis.

Human disease mutations affecting membrane serine protease functions

It is significant that mutations in several TTSP genes that affect protease activity have been causally linked to human diseases. For the interested reader, a comprehensive list of TTSP mutations can be found in [12]. In summary, many of these mutations result in TTSPs with missing or truncated protease domains, whereas others are point mutations predicted to influence enzyme expression or activity. Several of these point mutations have been investigated by cell transfection studies or through *in vitro* expression of the recombinant mutant proteases. These experiments have provided valuable insights into the functions of these TTSPs as well as mechanisms that regulate their proteolytic activities (summarized in Table 2). For example, missense mutations identified in the SPDs of matriptase, TMPRSS3 and TMPRSS5 result in catalytically inactive proteases, leading to human skin dysfunction in the case of matriptase and deafness in the case of TMPRSS3 and TMPRSS5. In contrast, missense mutations in the SEA or CUB1 domains of matriptase-2 cause defective zymogen activation resulting in IRIDA (iron-refractory iron deficiency anaemia) [1,74,75]. In addition, mutations in the LDLRA domains of this TTSP appear to be important for trafficking to the cell surface and subsequent zymogen activation [75]. Similarly, a 30 amino acid deletion mutation in the LDLRA1/2 domains and a point mutation in the CUB1 domain of matriptase-2 are also associated with IRIDA [76], again showing the importance of non-SPDs for TTSP function. Further highlighting this point, a point mutation within the Frizzled-2 domain of corin, which is associated with systemic hypertension, also results in impaired zymogen activation [77–79]. Several other mutations in TMPRSS3 that are linked to congenital deafness are found in the LDLRA and SRCR domains and result in proteases that fail to become activated [80]. The impact of so many disease-causing mutations in non-catalytic domains emphasizes the importance of these to the functional activities of the membrane-anchored serine proteases.

MEMBRANE SERINE PROTEASE SUBSTRATES

Substrate recognition is controlled in part by interactions between the active site of a protease and the amino acids spanning the cleavage site of its substrate. The following sections summarize

the approaches that have been used to identify the peptide substrate specificities for membrane-anchored serine proteases and experimentally identified protein cleavage sites (Table 1). Since additional factors will influence the activity and specificity of the membrane serine proteases for their substrates *in vivo*, we discuss the experimental evidence for cleavage of macromolecular substrates in various pericellular microenvironments. Although it is clear from animal models and studies of human disease that the membrane-anchored serine proteases are important components of specific physiological processes (Tables 2 and 3), much work remains to identify the relevant *in vivo* substrates for many of the membrane serine proteases.

Peptide substrate specificity

Biochemical and proteomics approaches have proved to be of considerable value as a first step for identification of substrate cleavage sites and for determining the amino acids that are preferred both on the side of and proximal to these sites. In several cases, cell-based or synthetic libraries of peptide substrates have been applied to quantitatively determine the substrate specificity of the catalytic domains of the membrane-anchored serine proteases [81–83]. Table 1 lists the known cleavage sequences of the membrane serine proteases against peptide substrates, macromolecular substrates and inhibitors. Of note, whereas the substrate specificities for hepsin, matriptase, prostasin and tryptase $\gamma 1$ have been extensively characterized using combinatorial peptide libraries, only limited or no information is available for other membrane-anchored serine proteases. The listed cleavage sequences have been determined from *in vitro* studies using purified recombinant truncated SPDs or recombinant extracellular TTSP domains including the stem region [ECD (extracellular domain)], or in some cases analysed using cell-based systems. The reader should be mindful that the pathophysiological relevance of substrate data obtained using SPDs alone may be imperfect, since truncated catalytic domains may not reveal secondary intramolecular interactions, protein and cofactor binding, or structural conformations that exist *in vivo* that can both positively and negatively affect substrate specificities.

These analyses demonstrate that all of the membrane-anchored serine proteases show a preference for cleavage of substrates with a basic amino acid (arginine or lysine) in the P₁ position, although each enzyme displays different peptide substrate specificity, and recognition of diverse macromolecular substrates. Interestingly, several membrane-anchored serine proteases, namely matriptase, prostasin, MSPL (mosaic serine protease, large form), tryptase $\gamma 1$ and hepsin, prefer multibasic residues in the P₄–P₁ positions. In fact, the multibasic peptide specificity profiles of these membrane-anchored serine proteases are similar to those of the proprotein convertases. Indeed, the furin inhibitor decanoyl-RVVKR-CMK (decanoyl-Arg-Val-Lys-Arg-chloromethylketone) is a potent inhibitor of TMPRSS13 ($K_i = 2.9$ nM) [84]. Then again, the furin-selective mutant of $\alpha 1$ -proteinase inhibitor ($\alpha 1$ AT-PDX) is not an effective inhibitor of TMPRSS13 [84] or of prostasin [85]. As a group it could be generalized that the TTSPs select against acidic residues in the P₄–P₁ positions with the exception of enteropeptidase, which possesses a highly selective preference for acidic amino acids in P₄–P₂. In the P₂ position of protein and peptide substrates, both prostasin and tryptase $\gamma 1$ have a strong preference for aromatic residues, whereas TMPRSS2 and HAT have a strong preference for small polar amino acids or glycine at this position. Other TTSPs, such as hepsin, HATL3 and TMPRSS4 can

Table 2 Disease-related human point mutations in type II proteases effect on protease function

Protease	Mutation	Location	Phenotype	Effect on function <i>in vitro</i>	Reference(s)
TMPRSS3	R216L	SPD - zymogen activation site	Non-syndromic autosomal recessive deafness	Mutant does not undergo proteolytic processing representative of auto-activation	[178]
	P404L	SPD	Non-syndromic autosomal recessive deafness	Mutant does not undergo proteolytic processing representative of auto-activation. Catalytically inactive	[80,177]
	D103G, R109W	LDLRA	Non-syndromic autosomal recessive deafness	These mutants do not undergo proteolytic processing representative of auto-activation and are unable to activate ENaC substrate compared with wild-type	[80]
	C194F W215C	SRCR SPD			
TMPRSS5	A317S	SPD	Non-syndromic deafness	SPD is catalytically inactive	[40]
Matriptase	G827R	SPD	Impaired epidermal barrier function, ichthyosis, hypertrichosis, follicular atrophoderma, corneal opacity and photophobia	Mutation is in active site binding cleft. SPD is catalytically inactive and protease is unable to auto-activate	[103,170,172]
Matriptase-2	A118D	SEA	IRIDA	Mutant protein reaches cell surface and is shed, but is unable to become activated	[1]
	D521N	LDLRA	IRIDA	LDLRA mutants cannot reach cell surface, are retained in Golgi and cannot activate. CUB mutant has reduced activation. Mutants are still able to interact with haemojuvelin and partially repressed hepcidin expression	[75]
	E552K	LDLRA	IRIDA		
	G442R	CUB-2	IRIDA		
Corin	T555I/Q568P	Frizzled-like domain-2	Decreased processing of pro-atrial natriuretic peptide resulting in hypertension and cardiac hypertrophy	Mutant reaches cell surface, but possesses impaired catalytic activity due to impaired zymogen activation	[78,79]

accommodate a variety of amino acid classes (e.g. aromatic, basic or hydrophobic) at the P₂ position.

Overall, the peptide substrate specificities of matriptase, hepsin, enteropeptidase and prostaticin determined by combinatorial peptide library screens strongly agree with the cleavage sites identified in protein substrates that have been characterized by amino acid sequencing. However, it is also interesting that in some instances, the substrate specificity predicted by active site geometry of the catalytic domain or by peptide screens does not match with the preferred cleavage sites of biological substrates or inhibitors. For example, analysis of the crystal structure of DESC1 suggests that its substrate specificity differs markedly from that of other TTSPs, requiring large hydrophobic residues in P₄/P₃, small residues in P₂, arginine or lysine in P₁ and hydrophobic residues in P_{1'} and P_{3'} [61,86]. However, kinetic analyses show that DESC1 cleaves peptide substrates with arginine in P₄, comparable with matriptase [87]. Additionally, the crystal structure of hepsin [83] predicts that large bulky side chains can be accommodated in the S₂ subsite, but a glycine residue would not provide strong interactions with the S₂ pocket. The ability of hepsin to activate pro-uPA (pro-urokinase-type plasminogen activator) and pro-HGF (pro-hepatocyte growth factor), containing phenylalanine and leucine respectively in the P₂ position, are consistent with this observation. Conversely, the hepsin substrate FVII contains glycine in the P₂ position, suggesting other interactions may be compensating for the less favourable binding into the hepsin S₂ pocket.

Zymogen activation: membrane serine proteases activating other serine proteases

The involvement of serine proteases in zymogen cascades has been long recognized [2]. Zymogen cascades involve at least two consecutive proteolytic reactions with one protease zymogen being the substrate of another previously activated protease. This strategy confers the advantage that a signal can be specifically and irreversibly amplified each time a downstream zymogen is activated, unleashing a burst of proteolytic potential [88]. A range of key biological processes rely on protease zymogen activation,

including blood coagulation, fibrinolysis, complement reaction, hormone production, metamorphosis, fertilization and digestion [2]. Although all of the membrane-anchored serine proteases are synthesized as protease zymogens, for the most part, the specific endogenous proteases that catalyse the activation of their zymogen forms *in vivo* are not known. *In vitro*, mild treatment with trypsin will convert recombinant serine protease pro-domains into active enzymes [51]; however, it is likely that specific proteases catalyse these cleavage reactions *in vivo*.

The substrate specificities of several of the membrane serine proteases are compatible with the cleavage and activation of serine protease zymogens, and a few have been demonstrated to participate in zymogen activation (Figure 2). For example, enteropeptidase produced on the brush border of the small intestine activates pancreatic trypsinogen, which in turn catalyses the conversion of other pancreatic zymogens, chymotrypsinogen, proelastase, prolipases and procarboxypeptidases, into their active forms during digestion [89]. This processing is critical for normal digestion and nutritional well-being [90]. A further example is hepsin, which intersects the blood coagulation cascade by activating Factor VII to Factor VIIa, which in turn is capable of initiating a coagulation pathway on the cell surface that leads to thrombin formation [91].

Several of the TTSPs intersect the plasminogen cascade, a key cascade critical for fibrinolysis, cell migration, extracellular matrix remodelling and MMP activation [92], through the activation of pro-uPA to its catalytically active form. Pro-uPA is relatively promiscuous with respect to its activation in contrast with most serine protease zymogens, and a wide range of both serine proteases and enzymes from other proteolytic classes can catalyse pro-uPA activation in *in vitro* assays, including the catalytic domains of hepsin, matriptase and serase-1B (Figure 3A) [23,81,93,94]. Serase-1B mediates efficient conversion of pro-uPA into active uPA, which is negatively regulated by glycosaminoglycans [23]. Active uPA processes plasminogen on the cell surface with high efficiency when bound to its cellular receptor, uPAR. Kinetic analyses demonstrate that, although matriptase is a relatively poor activator of pro-uPA in

Table 3 Physiological functions defined in murine models

Murine models are not yet available for TMPRSS3-5, TMPRSS13, HAT, HATL3-5, DESC1-4 or polyserase-1/serase-1B. GI, gastrointestinal.

Protease	Murine model	Phenotype	Function	Reference(s)
GPI-anchored	Prostasin	<i>Epidermal specific ablation: Prss8^{loxΔ}/K14-Cre mice</i>	Death within 60 h due to severe dehydration, increased epidermal permeability, defective epidermal differentiation, defective profilaggrin processing, absence of occludin	Maintenance of epidermal barrier integrity [229]
	Testisin	<i>Prss21 null mice</i>	PRSS21-deficient spermatozoa show reduced oocyte fertilization, decreased motility, angulated and curled tails, fragile necks, increased susceptibility to decapitation and failed to mount an effective swelling response upon release into hypotonic media. These defects reflect aberrant maturation during passage through the epididymis	Directs epididymal sperm cell maturation and sperm fertilizing ability [180,181]
Type II	Hepsin	<i>Hpn null mice</i> <i>Overexpression in prostate epithelium: Hpn-probasin mice</i>	Deafness, abnormal cochlear development, thyroid hormone deficiency Promotion of SV40 large T antigen-induced prostate carcinoma and metastasis, basement membrane disorganization, reduced expression of laminin-322	Unknown Tumour promotion [40,179] [230]
TMPPRS2	<i>Tmprss2 null mice</i>	No phenotype		[231]
Matriptase	<i>St14 null mice</i>	Death within 48 h due to severe dehydration, increased epidermal permeability, defective epidermal differentiation, defective hair follicle development, increased thymocyte apoptosis. Defective pro-filaggrin processing in skin. Decreased activation of prostasin in epidermis	Maintenance of epidermal barrier integrity	[99,168,169]
	<i>St14 hypomorphic mice</i>	Epidermal hyperproliferative ichthyosis, impaired desquamation, sparse hair, tooth defects. Defective pro-filaggrin processing	Maintenance of epidermal barrier integrity	[172,232]
	<i>Inducible ablation: B-actin-Cre-ER^{tm+/0};St14^{LoxP}/- mice</i>	Tamoxifen inducible ablation in adult mice. Dramatic weight loss, loss of fur, scaling of skin, oedema of intestines, dissolution of colonic tissue architecture, inflammation of orofacial surfaces. Increased intestinal and colonic crypt cell proliferation. Abnormal expression/localization of tight junction markers in small intestine epithelium. Increased intestinal and epidermal permeability	Global role in maintenance of epithelial homeostasis	[174]
	<i>GI tract ablation: Villin-Cre^{+/0};St14LoxP⁻ mice</i>	GI tract-specific ablation. Death several weeks after birth, persistent diarrhoea, low body weight, enlarged colon, increased proliferation of colonic crypt cells and loss of mucin production. Gross disruption of colonic tissue architecture, oedema and inflammation.	Maintenance of intestinal epithelial barrier	[174]
	<i>Salivary duct epithelium ablation: MMTV-Cre^{+/0};St14LoxP⁻ mice</i>	Salivary ductal epithelium specific ablation. Loss of saliva production. No obvious abnormalities	Maintenance of salivary epithelial function	[174]
	<i>Expression in murine basal keratinocytes: St14-cytokeratin 5 mice</i>	Spontaneous squamous cell carcinoma, increased susceptibility to chemical carcinogenesis	Tumour promotion	[185]
Matriptase-2	<i>Tmprss6 null mice</i>	Alopecia and a severe iron deficiency anaemia, upregulation of hepcidin expression, reduced ferroportin expression in enterocytes	Regulation of hepcidin and iron homeostasis	[157,233]
	<i>Mask mice: Tmprss6^{msk/msk} mice, chemically induced mutant phenotype</i>	Lack expression of SPD of matriptase-2 due to splicing defect. Loss of body hair and microcytic anaemia, reduced iron absorption caused by high levels of hepcidin.	Regulation of hepcidin and iron homeostasis	[153,233]
	<i>Mask mice X haemojuvelin null mice: Tmprss6^{msk/msk};Hfe2^{tm1Nca/tm1Nca}</i>	Disruption of both matriptase-2 and the membrane receptor haemojuvelin, which is known to stimulate hepcidin expression in response to elevated iron levels. Results in low hepcidin expression, high serum and liver iron, suggesting haemojuvelin is a major matriptase-2 substrate that regulates hepcidin expression.	Regulation of membrane haemojuvelin to suppress hepcidin expression	[158]
Corin	<i>Corin null mice</i>	Defective pro-atrial natriuretic peptide processing leading to hypertension, cardiac hypertrophy, increased body weight, abnormal hair pigmentation	Regulation of hypertension	[131,182]
		Abnormal hair pigmentation 'dirty blonde' phenotype	Role in specifying hair colour by suppression of the Agouti pathway	[182]

solution, it is an effective activator of uPAR-bound pro-uPA and initiates pericellular plasminogen activation on the monocyte cell surface [95]. The uPAR is also a substrate for HAT [96]. Recombinant HAT processes the full-length uPAR (D1D2D3) into a truncated (D2D3) species and promotes shedding of the

major ligand-binding D1 domain [97]. Recombinant truncated matriptase has also been shown to activate stromelysin (MMP-3) in several cell systems [98], implicating TTSPs in protease cascade networks associated with extracellular matrix degradation in tumour cell microenvironments.

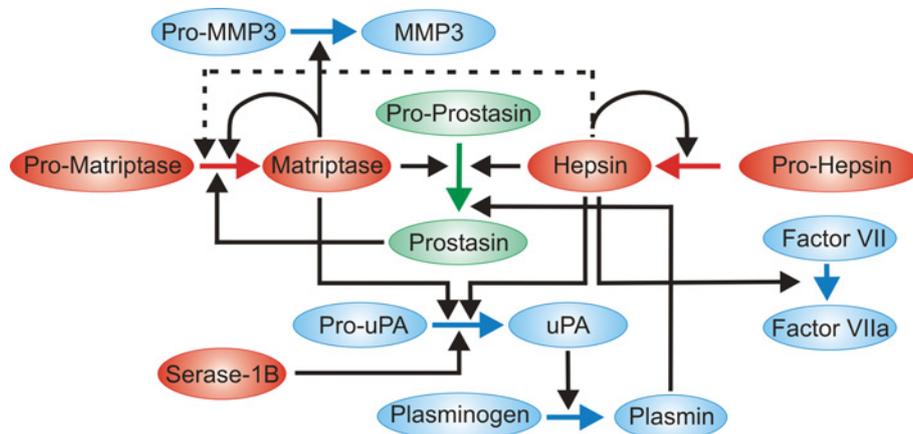


Figure 2 Membrane-anchored serine proteases participate in zymogen cascades

Pathways in which membrane-anchored serine proteases have been shown to activate, or be activated by, serine proteases *in vitro* and *in vivo*. Proteases are colour coded according to membrane localization sequences: red, TTSPs; green, GPI-anchored proteases; blue, other secreted proteases. Lines indicate activation cleavages and loops indicate auto-activation. The broken line indicates that hepsin is a weak activator of the matriptase zymogen. Membrane-anchored serine proteases intersect the coagulation cascade (Factor VII activation), fibrinolysis (pro-uPA activation) and metalloproteinase pathways (pro-MMP-3 activation). Not shown is the activation of trypsinogen by enteropeptidase.

In addition to activating other serine protease zymogens, several of the membrane-anchored serine proteases are able to activate other membrane-anchored serine proteases or to self-activate (Figure 2). The prostatic zymogen was shown to be converted into its active enzyme form by matriptase *in vitro* and *in vivo* in skin, where the matriptase/prostasin axis is a physiological activator of terminal epidermal differentiation [99]. Intriguingly, in *in vitro* assays, the zymogen form of matriptase can also be converted into the active form by the addition of soluble active prostasin or hepsin, suggesting that, in certain cellular contexts, prostasin might function both upstream and downstream of matriptase, thus constituting a positive-feedback loop similar to the amplification cascades associated with coagulation [100]. Self or autocatalytic activation of some of the purified TTSPs *in vitro* has been reported. In these cases, the specificity preference of the TTSP is compatible with the activation motif of the TTSP, and inactivation of the active site catalytic triad residues of the biochemically purified catalytic domain can be shown to prevent autocatalytic activation [10,66,101,102]. *In vitro* autoactivation has been reported for the TTSPs matriptase [66,81,103], matriptase-2 [104], hepsin [105], TMPRSS2 [37], TMPRSS3 [80], TMPRSS4 [106] and TMPRSS11C [102], although the ability to undergo an intermolecular activating cleavage requires more rigorous demonstration for several of these enzymes.

Cleavage of PARs (protease-activated receptors)

G-protein-coupled signalling receptors on the cell surface known as PARs are activated by proteolytic cleavage [107,108], and several membrane-anchored serine proteases have been investigated either as direct or indirect activators of PAR signalling. PARs are cleaved at specific amino acid sequences within the extracellular N-terminus of the receptor, exposing a new N-terminus that serves as a tethered ligand domain, initiating various intracellular signal transduction pathways [109]. The four members of the PAR family (PAR₁₋₄) may be processed by different activating or inactivating proteases. PAR₁, PAR₃ and PAR₄ are activated by thrombin, and PAR₃ has been reported to be a co-factor for the activation of PAR₄ by thrombin. PAR₂

is a target of several trypsin-like serine proteases, including trypsin, mast cell tryptase and kallikrein 4 [110,111], and is activated by the soluble catalytic domains of matriptase [81,100] and TMPRSS2 [112], resulting in protease-triggered signalling, indicative of G-protein-coupled-receptor activation (Figure 3B). Matriptase was also found to induce release of pro-inflammatory cytokines, including IL (interleukin)-6 and IL-8 in endothelial cells through activation of PAR₂ [113]. Truncated HAT has been shown to induce amphiregulin release through PAR₂-mediated ERK (extracellular-signal-regulated kinase) activation and TACE [TNF α (tumour necrosis factor α)-converting enzyme] activity in airway epithelial cells [114]. Although it has not been shown that prostasin proteolytically activates PAR₂, the expression of prostasin was found to play a modulatory role in PAR₂ signalling in prostate epithelial cells [115], and more recent studies show that soluble recombinant prostasin can activate PAR₂ indirectly through a matriptase-dependent mechanism [100]. Whether membrane-tethered serine proteases are direct physiological activators of PARs juxtapositioned on plasma membranes in the pericellular environment will be important to investigate. Indeed, recent studies suggest that this may be the case, since full-length matriptase co-expressed in a lung fibroblast cell line in the presence of HAI-1 was shown to activate PAR₂ after activation of the matriptase zymogen by soluble matriptase or prostasin [100].

Growth factor processing

Proteolytic cleavage governs many multi-component signalling events, including growth factor activation and availability. *In vitro* matriptase is a very efficient activator of pro-HGF/scatter factor (Figure 3C) and the pro-macrophage stimulating proteins (MSP/MST-1), growth factors that serve as ligands for two receptor tyrosine kinases associated with epithelial cell motility, migration and proliferation, MET/HGF receptor and RON/MST-1 receptor respectively, [116–118]. Interestingly, HGF and MSP are inactive serine protease analogues, with substitutions in two of the three residues of the catalytic triad, but require processing by conversion of a single chain precursor into the bioactive

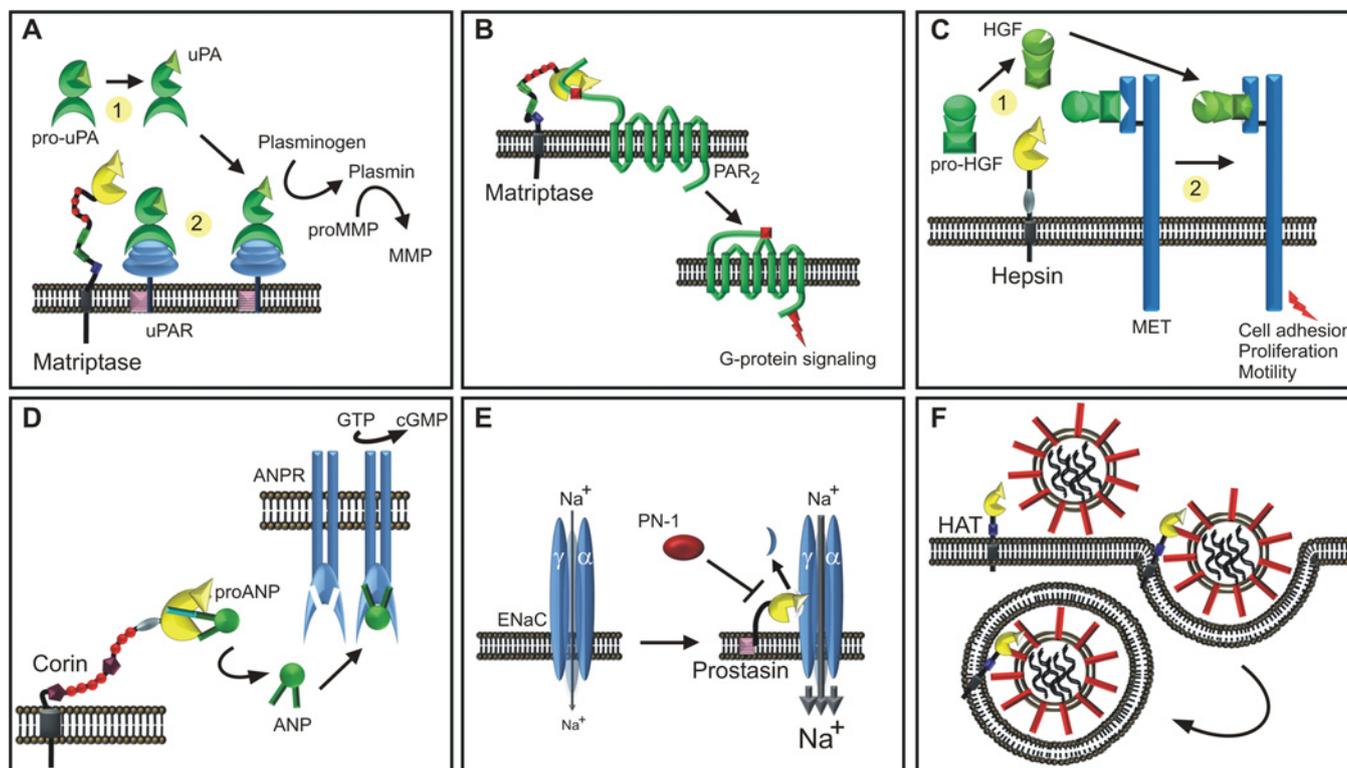


Figure 3 Proteolytic pathways associated with membrane-anchored serine proteases in pericellular microenvironments

(A) Fibrinolysis. The membrane-anchored serine proteases, matriptase, hepsin and serase-1B may participate in the initiation of the plasminogen activation cascade via conversion of pro-uPA into active uPA. Matriptase is a relatively poor activator of pro-uPA in solution (1), whereas it is an effective activator of uPAR-bound pro-uPA and initiates pericellular plasminogen activation on the monocyte cell surface (2). (B) Inflammation. Matriptase, and possibly other membrane serine proteases, may participate in the activation of PARs triggering G-protein signalling pathways. (C) Growth factor activation. Hepsin and matriptase may participate in the activation of pro-HGF to initiate signalling via the HGF receptor, MET, to modulate cell adhesion, proliferation and cell motility. Whether these enzymes target soluble pro-HGF (1) or receptor bound pro-HGF (2) is not known. (D) Natriuretic peptides. Corin processes pro-ANP to an active form that bind ANP receptors (ANPR) in the kidney and vasculature, increasing guanylate cyclase activity to regulate blood pressure and blood volume. (E) Sodium homeostasis. The open probability of the ENaC is increased by prostaticin and TMPRSS4, resulting in increased cellular uptake of Na^+ , which in turn can regulate homeostasis of extracellular fluid volume, blood pressure and Na^+ reabsorption. Processing of the α or γ subunits of ENaC is predicted to release an inhibitory fragment that leads to full activation of the channel. The serpin protease nexin-1 (PN-1, serpinE2) can inhibit prostaticin-mediated activation of ENaC. (F) Viral pathogenicity. Several TTSPs, including HAT, TMPRSS2, 4, and 13 can process precursor viral proteins that facilitate virus entry and fusion into host cells.

two-chain form [94]. At the cellular level, expression of matriptase by transfection activates pro-HGF on the surface of colon and prostate carcinoma cell lines [119]. Matriptase activation of pro-HGF may further contribute to mammary gland morphogenesis [120], since RNAi (RNA interference)-mediated suppression of murine matriptase could inhibit induction of complex ductal structures induced by pro-HGF in a three-dimensional mammary epithelial model. MSP was shown to be activated by matriptase expressed on the surface of primary peritoneal macrophages, where activation of pro-MSP could be inhibited by the presence of HAI-I or an anti-matriptase antibody [117]. Similarly, the hepsin SPD was also shown to activate pro-HGF into its bioactive form *in vitro*, with an activity comparable with HGFA (HGF activator) [83,121]. The co-localization of hepsin with HGF at the cell junctions of ovarian tumour cells [33] is supportive of a potential role for hepsin in pro-HGF activation; however, the physiological relevance of this activity remains to be demonstrated. FGF-2 (fibroblast growth factor-2) is another growth factor reported to be cleaved into several fragments by the catalytic domain of HAT-L3 [102].

In addition to activation of growth factors, several of the membrane-anchored serine proteases have been reported to modulate the EGFR (epidermal growth factor receptor). In PC3 prostate cells, prostaticin expression induced down-regulation of EGFR at

the mRNA level, an activity that required protease catalytic activity [122]. At a post-translational level, both matriptase and hepsin were found to cleave the extracellular domain of the EGFR in co-transfection studies [123,124]. Although they cleaved the extracellular domain of the EGFR at different sites, only matriptase processing resulted in a constitutively active membrane-anchored form of the receptor that was capable of initiating intracellular signalling. The physiological relevance of EGFR modifications by membrane-anchored serine proteases warrants further investigation. Interestingly, matriptase has also been shown to cleave the VEGFR-2 [VEGF (vascular endothelial growth factor) receptor] *in vitro* at a site that is predicted to induce its shedding from the cell surface, and addition of recombinant matriptase to HUVECs (human umbilical vein endothelial cells) inhibited the signalling response to VEGF [125].

Activation of natriuretic peptides

Processing of pro-ANP (pro-atrial natriuretic peptide) to the cardiac hormone ANP is catalysed by corin (Figure 3D). ANP is synthesized as a cell-associated inactive pro-hormone that requires proteolytic processing for release and activity. *In vitro* studies show that corin, which is highly expressed in cardiac

tissue [126], is able to convert the cell-associated inactive pro-ANP into its active form on the surface of cardiomyocytes [127,128]. ANP plays a key role in regulating blood pressure by regulating systemic salt and water balance [129,130]. Consistent with an *in vivo* role in this conversion, corin-deficient mice show defects in the conversion of pro-ANP into ANP, and development of spontaneous hypertension and cardiac hypertrophy that is exacerbated by a high-salt diet [131]. The hypertensive phenotype of corin-deficient mice mimics that of ANP-deficient mice [132], and emphasizes that pro-ANP is an important *in vivo* corin substrate. Several corin single nucleotide polymorphisms identified in African-American populations and associated with high blood pressure and cardiac hypertrophy [77,78] result in production of corin mutants with impaired zymogen activation, leading to reduced zymogen activity [79]. Corin has also been shown to activate the pro-form of BNP (brain/B-type natriuretic peptide) [79,127], a marker of congestive heart failure [133]. Mice deficient in BNP develop cardiac fibrosis, indicating that it is involved in ventricular remodelling rather than blood pressure regulation [134]. Accordingly, a recent study has shown that patients with the corin I555(P568) allele exhibit impaired BNP processing and are at an increased risk of adverse outcomes associated with heart failure [135].

ENaC (epithelial Na⁺ channel) activation

The ENaC regulates sodium and water flux across high-resistance epithelia, such as the airway, bladder, kidney, colon and skin. During biosynthesis, ENaC α and ENaC γ subunits are proteolytically processed by the proprotein convertase furin, but are maximally activated by further processing by cellular proteases [34]. Several membrane-anchored serine proteases referred to as CAPs (channel-activating proteases) activate ENaC when co-expressed in heterologous expression systems [34,136,137]. Proastasin activation of ENaC has been the most well characterized (Figure 3E). Studies in *Xenopus* oocytes showed that co-expression of proastasin and ENaC subunits increases the amiloride-sensitive Na⁺ current [136,138] and expression of proastasin in mammalian kidney epithelial cells [139] and in a cystic fibrosis epithelial line [140] induces increased ENaC activity. At a molecular level, proastasin cleaves the ENaC γ subunit in the extracellular loop at a site (Lys¹⁸⁶) distal to the furin cleavage site, which releases a 43-amino-acid inhibitory peptide shown to inhibit ENaC activity, resulting in increased open probability of the channel and full activation [141,142]. A recent study also showed that low levels of plasmin found in urine from patients with nephritic syndrome can activate ENaCs via activation of membrane-anchored proastasin [143]. On the other hand, there is also evidence that non-catalytic protease mechanisms may be involved in ENaC activation [106,144]. For example, in the *Xenopus* oocyte expression system, catalytically inactive proastasin mutants were able to fully activate ENaC, with no evidence of proastasin-mediated intramolecular cleavage, although cell-surface expression via the proastasin GPI anchor was essential for this activity. In addition to proastasin, TMPRSS3, TMPRSS4 and matriptase are able to activate ENaC when co-expressed in *Xenopus* oocytes [80,136]. Missense TMPRSS3 mutations associated with deafness fail to activate ENaC [80]. Direct cleavage of ENaC subunits by matriptase and TMPRSS3 has not been demonstrated, but TMPRSS4 was recently shown to cleave the inhibitory ENaC γ subunit at a site distinct from that of proastasin [145]. Interestingly, expression of TMPRSS2 has been shown to decrease Na⁺ currents and ENaC protein levels [146], suggesting that the membrane-anchored proteases may both

positively and negatively regulate ENaC function. The channel-activating activity of proastasin is likely to be pathophysiologically important. Mice lacking the proastasin gene in lung epithelium demonstrate impaired ENaC-mediated alveolar fluid clearance [147]. In addition, recent findings suggest that increased ENaC activation in the airway of cystic fibrosis patients may be linked to excessive proastasin activity on the surface of lung epithelium [148,149]. Increased ENaC activation [150] is associated with hypertension and a recent independent study identified proastasin as a candidate gene implicated in the development of hypertension in youths [151]. Hypertensive patients exhibit increased levels of urinary proastasin [42], and in a rat model of hypertension, treatment with a synthetic inhibitor of proastasin was able to significantly reduce blood pressure and increase the urinary Na⁺/K⁺ ratio [152].

Regulation of iron homeostasis

Recently, matriptase-2 has emerged as an essential regulator of systemic iron levels. Mutations in this TTSP are causal for suppression of hepcidin [1], a hepatic peptide hormone that controls body iron levels by limiting iron egress from duodenal enterocytes and macrophages to serum transferrin through its degradation of the iron transporter, ferroportin [74,153]. Insight into the involvement of matriptase-2 in iron regulation has come from the identification of frameshift, splice junction and missense mutations in the encoding gene, *TMPRSS6*, which causes IRIDA in humans [154–156]. These observations are supported by studies in mice. A chemically induced *Tmprss6* mutation, eliminating the splice acceptor site of intron 14 and resulting in loss of the matriptase-2 SPD (*Mask* mice), results in anaemia, low plasma iron and depleted iron stores [156]. In addition, levels of transcription from the hepcidin-encoding gene *Hamp* were ~10-fold higher in homozygous *Tmprss6*-mutant mice following iron deprivation than in wild-type mice. Investigation of *Tmprss6* knockout mice revealed a severe iron deficiency anaemia phenotype accompanied by a marked up-regulation of *Hamp* transcription, reduced ferroportin expression in the basolateral membrane of enterocytes and accumulation of iron in these cells [157]. Further studies on the mechanism of matriptase-2 activity suggest that this protease may proteolytically process haemojuvelin [153,158], a GPI-anchored protein that is a key activator of *Hamp* transcription [159].

Viral proteins and promotion of viral pathogenicity

Increasing evidence suggests that influenza and other respiratory viruses exploit membrane-anchored proteases to promote viral spread [160]. For example, the TTSPs TMPRSS2, HAT, TMPRSS4, TMPRSS13 and TMPRSS11A support activation and replication of human influenza viruses. Influenza entry and membrane fusion with host cells is mediated by HA (haemagglutinin), which is synthesized as a precursor protein, HA₀, that must be cleaved by a host cellular protease into the covalently linked subunits HA₁ and HA₂. Mutational evolution of the HA proteolytic cleavage site is a determinant of viral pathogenicity and affects multiplication of the virus *in vivo* [161]. Highly pathogenic avian influenza viruses possess multibasic HA cleavage motifs, e.g. R-X-K/R-R and K-X-K/R-R, which are processed intracellularly and on the cell surface by the ubiquitously expressed Ca²⁺-dependent subtilisin-like proteases, furin and proprotein convertases 5/6, enabling rapid systemic spread of viral infection. Recently TMPRSS13 was shown to recognize and process HA multibasic motifs in a

Ca²⁺-independent manner. TMPRSS13 was able to process the HA of a highly virulent recombinant strain of pathogenic influenza virus *in vitro*, that is poorly susceptible to furin, and to facilitate cell fusion [161]. In contrast with these high-virulence strains, HA of low-pathogenic viruses usually contain monobasic cleavage sites, and cleavage by trypsin is required to support efficient replication of these viruses in cell culture. HAT and TMPRSS2 were found to process monobasic cleavage of HA and to support viral replication *in vitro* (Figure 3F) [162,163]. Interestingly, TMPRSS2 and TMPRSS4, but not matriptase-3, were able to cleave and activate the HA of the genetically reconstituted 1918 influenza virus [164]. The presence of these enzymes in human respiratory epithelium may suggest a pathogenic role in promoting viral spread in human airways [164]. TTSPs have also been associated with the propagation of other viruses. TMPRSS2 supports multicycle replication of human metapneumovirus *in vitro* by proteolytic cleavage of the fusion protein F [165]. In addition, hepsin interacts with the X protein of human hepatitis B virus, although proteolytic activity was not reported [166]. Recently, the spike (S) protein of the SARS-CoV (severe acute respiratory syndrome coronavirus) was found to be susceptible to cleavage by TMPRSS11A, implicating protease-mediated enhancement of SARS-CoV entry in a fashion dependent on previous receptor binding [167].

Maintenance of epithelial barriers

Several membrane serine proteases are found to be essential for epithelial barrier integrity, although the biochemical basis for this activity is not yet defined. A role for matriptase in epithelial barrier function was first identified by examining the phenotype of mice null for the *St14* gene encoding matriptase [168]. Although these mice develop to term, they die shortly after birth due to severe dehydration caused by defective epidermal barrier function [168,169]. In humans, mutations in the matriptase gene underlie ARIH (autosomal recessive ichthyosis with hypotrichosis) syndrome, a rare human skin disease characterized by congenital ichthyosis associated with abnormal hair [170,171]. One characterized missense mutation, G827R, affects access to the binding/catalytic cleft of the enzyme, thereby preventing autocatalysis of the zymogen form resulting in a catalytically inactive protease [103,172]. Affected individuals and matriptase-deficient mice show defective epidermal differentiation processes, including altered extrusion of extracellular lipids that form the lipid lamellae, defective shedding of the stratum corneum and defective processing of the epidermal structural polyprotein profilaggrin [169,172,173].

The recent generation of murine models of inducible matriptase ablation in the whole animal, and tissue-specific ablation in the gastrointestinal tract and salivary epithelium, has revealed that matriptase is essential for the maintenance of epithelial barrier integrity [12,174]. Ablation of matriptase in intestinal and salivary gland epithelium disrupts organ function, increases epithelial permeability and, in tissues colonized by microbial flora such as the large intestine, leads to major architectural breakdown of tissue integrity [174]. Matriptase hypomorphic mice, which have a 100-fold reduction in intestinal matriptase mRNA levels, display a 35% reduction in intestinal TEER (transepithelial electrical resistance), but retain histologically normal intestinal epithelium. Reduced barrier integrity is mediated at least in part by an inability to regulate the expression and localization of the permeability associated, 'leaky' tight junction protein claudin-2 both *in vivo* and in Caco-2 polarized epithelial monolayers [29]. However, the direct molecular target(s) of matriptase during the maintenance of

epithelial integrity in multiple diverse epithelial tissues remains to be established.

Prostasin has also been implicated in the regulation of epithelial barriers and tight junction function [17]. Overexpression of prostasin in M-1 epithelial monolayers decreases TEER, a sensitive measure of epithelial barrier function, and enhances paracellular permeability to inulin. Interestingly, overexpression of a form of prostasin in which the GPI anchor was replaced with the transmembrane domain of tryptase- γ 1 resulted in enhanced TEER, reflecting increased M-1 barrier integrity. It is interesting to note that these effects on TEER appeared to be unrelated to ENaC activity, suggesting that prostasin may target an alternative substrate involved in regulation of epithelial barrier permeability, although the nature of the physiological matriptase–prostasin pathway substrate(s) mediating this process remains elusive.

Roles in hearing

The TTSPs TMPRSS3, TMPRSS5 and hepsin are implicated in hearing and their dysregulation is associated with deafness [175], although the biochemical mechanisms by which these enzymes are involved in normal hearing have not been elucidated. The *TMRSS3* gene was first identified as a gene located on a chromosomal locus associated with human familial congenital deafness [176]. Mutations in the *TMRSS3* and *TMRSS5* genes that contribute to hearing loss render the respective proteases inactive [80,177,178]. Hepsin-null mice are also associated with a markedly increased hearing threshold with abnormal cochlear structures, reduced myelin protein expression in the auditory nerve by histological analysis, and reduced level of the large conductance voltage- and Ca²⁺-activated K⁺ channel [40,179]. Hearing deficiency in hepsin-null mice has been proposed to be due to a defect in thyroid hormone metabolism since these mice demonstrate low plasma levels of thyroxine, a hormone required for inner ear development [179].

Membrane-anchored serine proteases in development

Several of the membrane-anchored serine proteases are associated with mammalian development. For example, testisin is required for epididymal sperm maturation and fertilizing ability, since mouse sperm lacking testisin display several functional abnormalities [180,181]. Ablation of the gene encoding corin in mice revealed a function in the regulation of hair shaft pigmentation, acting downstream of *agouti* gene expression as a suppressor of the *agouti* pathway [182]. Inhibition of matriptase is essential for the formation of the placental labyrinth in mice, since genetic ablation of the matriptase inhibitor HAI-1 leads to deregulation of matriptase activity and disruption of the epithelial integrity of chorionic trophoblasts [183]. In a series of elegant studies, it was shown that genetic ablation of matriptase activity in HAI-1-deficient embryos restores trophoblast integrity and enables placental labyrinth formation and development to term [183]. The specific substrates targeted by these enzymes are important areas for future study.

Dysregulation in cancer

Many membrane-anchored serine proteases are aberrantly expressed in human cancers and have been suggested as biomarkers indicative of disease state. Evidence comes from expression studies in human cancers and in some cases from mouse models of carcinogenesis. As two reviews have extensively

discussed membrane-anchored serine proteases in cancer [9,10], we focus on the key findings in this area.

Several of the membrane-anchored serine proteases are overexpressed by tumour cells and are implicated in promoting tumour development and progression [184,185]. A modest increase in matriptase activity on the epidermis of a transgenic mouse model was sufficient for the induction of spontaneous squamous cell carcinomas [185,186]. In addition, matriptase overexpression in several cell lines leads to more aggressive tumours [98,185,186]. Hepsin overexpression also induces tumour growth in mice, which was shown to be dependent on hepsin catalytic activity [33]. The expression of *TMPRSS4* in human tumour cells was shown to promote invasion, migration and metastasis by inducing the loss of E-cadherin-mediated cell–cell adhesion and facilitating the EMT, a step in the metastatic process [187]. Interestingly, chromosomal rearrangements in the human *TMPRSS2* gene (21q22.3) result in gene fusions of the 5'-untranslated region of *TMPRSS2* with *ETS* transcription factor family members (e.g. *TMPRSS2-ERG*), where *ERG* (*Ets*-related gene) expression is driven by the *TMPRSS2* gene promoter, and is associated with a high rate of prostate cancer recurrence, metastasis and death after prostatectomy [188,189].

In contrast with promotion of tumour progression, several membrane-anchored serine proteases are present in normal tissues and found to be down-regulated in or absent from corresponding malignant carcinomas. When expressed in prostate and breast tumour cell lines, matriptase-2 acts as a tumour suppressor, leading to the suppression of tumour migration and metastasis, and its expression has been correlated with a better disease prognosis in breast cancer patients [190,191]. *DESC1* is expressed in normal epithelial cells of prostate, skin, testes, head and neck, whereas it is down-regulated in squamous cell carcinomas [192]. Prostatin expression is frequently down-regulated in gastric cancer [193], and loss of prostatin expression in human bladder transitional cell carcinoma cell lines has been shown to be associated with the EMT [115]. Testisin expression was reported to be lost in testicular germ cell tumours [19], and it has been shown that epigenetic gene silencing contributes to the down-regulation of both prostatin and testisin expression in tumours [193–197].

The truncated SPDs of the membrane-anchored serine proteases are found to degrade *in vitro* the extracellular matrix protein components fibronectin, fibrinogen, and denatured type-1 collagen, gelatin and casein to various extents [48,104,186,198]. In addition, hepsin expressed in prostate cancer cells was shown to cleave basement membrane laminin-322, leading to increased tumour cell migration *in vitro* [198]. The physiological significance of these activities is not known, although they may well have particular relevance for cancer and inflammation, where protease dysregulation may result in encounters with substrates not normally seen.

INHIBITION OF MEMBRANE SERINE PROTEASE ACTIVITIES

The importance of regulated membrane-anchored serine protease expression to normal homeostasis and the frequent associations between these enzymes and cancer and other diseases indicates that these enzymes must be tightly regulated in normal settings. This regulation is partly mediated by endogenous protease inhibitors, including members of the serpin family of serine protease inhibitors and Kunitz-domain containing inhibitors (Table 4). In addition, a number of small molecule inhibitors that specifically modulate the activities of these enzymes have recently been reported.

Serpins

Serpins interact with proteases via a 'pseudosubstrate' exposed binding loop known as the RCL (reactive centre loop) that, upon cleavage, covalently traps the protease by undergoing an irreversible conformational rearrangement [199,200]. The suicide nature of serpin inhibition is an effective strategy for regulation of proteolytic activity by direct removal of unwanted proteases via membrane-bound endocytic receptors [201]. Matriptase has been isolated from human milk in inhibitory complexes with the secreted serpins, anti-thrombin III (serpinC1), α 1-proteinase inhibitor (serpinA1) and α 2-antiplasmin (serpinF2) [202]. In addition, inhibitory complexes form *in vitro* between mouse *DESC1* and the serpins PAI-1 (plasminogen-activator inhibitor 1; serpinE1) and PCI (protein C inhibitor; serpinA5) [86], matriptase-3 and PAI-1, PCI, α 1-proteinase inhibitor, α 2-antiplasmin and anti-thrombin III [203], and serase-1B and PAI-1 and α 2-antiplasmin [23].

The selectivity of a serpin towards a particular serine protease is not always readily evident from the sequence of the protease-sensitive RCL sequence because of the flexible nature of the reactive segment of the serpin and possible exosite-binding interactions. For example, the RCL sequences of the serpins PAI-1 and PCI do not match the optimal docking sequence of *DESC1* predicted by the crystal structure [61], but these serpins form effective inhibitory complexes with *DESC1* [86]. Additionally, α 1-proteinase inhibitor (serpinA1) possesses a methionine residue in the P₁ substrate position, yet can inhibit trypsin-like proteases including trypsin γ 1, matriptase, hepsin and polyserase-1 [22,26,87]. Conversely, serpinA1 is a poor inhibitor of the matriptase catalytic domain in peptide assays; however, endogenous complexes of matriptase–serpinA1 have been isolated from breast milk [87,202]. Although most studies have been performed with recombinant soluble forms of the enzymes and inhibitors, anti-thrombin III (serpinC1) and protease nexin I (serpinE2) have also been shown to inhibit cell-surface-associated activities of hepsin [91] and prostatin [204] (Figure 3E) respectively. In general, inhibition studies have provided information on the ability of the membrane-anchored serine proteases to form SDS-stable protease–inhibitor complexes and have determined IC₅₀ values in peptide-based assays; however, detailed kinetic analyses of these protease–serpin interactions is lacking for the most part.

Kunitz-type inhibitors

Kunitz-type inhibitors are plasma inhibitors that contain domains that inhibit protease activity by forming very tight, but reversible, complexes with target proteases. In contrast with serpins, reversible Kunitz inhibitors may simply compete with physiological substrates (such as extracellular matrix components), to reduce the availability of the protease. The transmembrane Kunitz-type inhibitor HAI-1/SPINT1 was originally isolated as an inhibitor of HGFA [67,83,205], but has also been implicated in the expression, zymogen activation and inhibition of matriptase [206–208], hepsin [121] and prostatin [67,83,121,205]. Indeed, *HAI-1/Spint1*-deficient mice display severe growth retardation and early post-natal lethality as well as skin and hair defects [209,210], phenotypes that are rescued by matriptase deficiency [209], demonstrating that HAI-1 maintains tissue homeostasis *in vivo* through inhibition of matriptase. The closely related Kunitz-type transmembrane serine protease inhibitor, HAI-2 (SPINT2/placental bikunin), also displays potent inhibitory activity towards matriptase [211], hepsin [121] and prostatin [85,212]. Genetic inactivation of the mouse *HAI-2*

Table 4 Membrane-anchored serine protease inhibitors

Inhibitory activity was assayed by catalytic assay (CA) or complex formation by gel electrophoresis (GE). —, Inhibitory activity less than 10%; +/-, inhibitory activity less than 50%; +, inhibitory activity greater than 50%. Inhibitory profiles for HATL1/DESC3/TMPRSS11A, HATL2/DESC4, HATL4/TMPRSS11F, HATL5/TMPRSS11B, TMPRSS2, TMPRSS3 and TMPRSS4 are not known. Abbreviations: Serpins: A1, α 1-proteinase inhibitor/ α 1-antitrypsin; A3, α 1-antichymotrypsin; A5, protein C inhibitor (PCI); C1, antithrombin III; D1, heparin cofactor II; E1, plasminogen activator inhibitor-1 (PAI-1); E2, protease nexin 1; F2, α 2-antiplasmin. SLPI, secretory leucocyte proteinase inhibitor; Upub, S. Netzel-Arnett, unpublished work.

Proteases	Endogenous protein inhibitors										Assay	Reference(s)	Other protein inhibitors					Reference(s)	
	Kunitz		Serpins										SLPI	SBTI	Aprotinin	Leupeptin	Ecotin		
	HAI-1	HAI-2	A1	A3	A5	C1	D1	E1	E2	F2									
GPI-anchored																			
Prostasin	+	+	—	—								CA, GE	[85,205,234]		—	+	+		[15,85]
Testisin																+	+		Upub
Type I																			
Tryptase γ 1			+									CA, GE	[26]		+/-	+	+		[26]
Type II																			
HAT														—	+	+	+		[54]
DESC1			—	—	+	+	—	+				CA, GE	[86,87,235]			+	+		[86,235]
HATL3																+	+		[102]
Hepsin	+	+	+/-			+		+				CA	[87,91,121]		+/-	+			[91,214]
TMPRSS13			—									CA	[84]		+	+	—		[84]
TMPRSS5																	—		[220]
Enteropeptidase																			
Matriptase	+	+	+			+		+				CA, GE	[87,202,238,239]		+	+	+	+	[48,240]
Matriptase-2			—			+		+				CA	[87]			+	+		[104]
Matriptase-3	—				+	+	—	+				CA, GE	[203]			+	+		[203]
Polyserase-1			+									CA	[22]						
Serase-1B			—				+/-	+				CA, GE	[23]	+	+/-	+	+		[23]
Corin																+/-	+	+	[228]

Spint2 gene leads to defects in neural tube closure and abnormal placental labyrinth development associated with embryonic lethality [213]. These developmental defects are also caused by unregulated matriptase activity, as both placental development and embryonic survival in HAI-2-deficient embryos were completely restored by the simultaneous genetic inactivation of matriptase [213].

Exogenous inhibitors

The activities of recombinant membrane-anchored serine proteases are inhibited to varying extents by low-molecular-mass serine protease inhibitors, e.g. PMSF, AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], benzamidine, leupeptin and aprotinin (Table 4). SBTI (soya-bean trypsin inhibitor) appears to be an effective inhibitor of hepsin in peptide substrate assays, although it was shown to have minimal effect on hepsin's ability to activate FVII on the cell surface [91,214]. Ecotin, a macromolecular inhibitor of trypsin-like serine proteases isolated from *Escherichia coli* [215], was demonstrated to be a potent subnanomolar inhibitor of matriptase [215]. However, its selectivity and inhibitory profile against other membrane-anchored serine proteases is not known.

Interestingly, a number of specific, low-molecular-mass inhibitors have been developed based upon the distinct structural features of individual membrane-anchored serine proteases. The unique fine structures of the binding pockets of the membrane serine proteases have been exploited using peptidic inhibitor library screens arrayed in a positional scanning format [61,63]. This has led to the development of nanomolar inhibitors of DESC1 [61], prostasin [63] and hepsin [216], and a submicromolar inhibitor for tryptase $\gamma 1$ [217]. A second strategy has been the design and synthesis of synthetic small-molecule inhibitors based on preferred cleavage sequences of the enzymes. One of these, a selective matriptase inhibitor (CVS3893, $K_i = 3.3$ nM) was shown to suppress the growth of prostate tumour xenografts [218]. In addition, an inhibitor of uPA was optimized to selectively inhibit matriptase with K_i values below 5 nM and this inhibitor also showed tumour inhibitory activity in a mouse model [219]. Thus far, however, the selectivity of the reported inhibitors for individual membrane-anchored serine proteases relative to other family members has not been extensively characterized.

Compounds that are already in clinical use have also shown efficacy as inhibitors of membrane-anchored serine proteases. An inhibitor of trypsin-like serine proteases, camostat, is in clinical use for the treatment of chronic pancreatitis and two of the TTSPs, matriptase and prostasin, are inhibited by camostat in peptide and cell-based assays [152,212]. Camostat inhibits prostasin-dependent regulation of ENaC activity in animal models of airway epithelium [212] as well as Na^+ transport in the kidney [152]. The inhibition of prostasin-dependent activation of ENaCs is being developed as a therapeutic strategy for cystic fibrosis and salt-sensitive hypertension.

FUTURE PERSPECTIVES

The membrane-anchored serine proteases are present in a wide range of tissues and biological fluids, and play key roles as potent modifiers of the cellular microenvironment. These enzymes catalyse pericellular proteolytic processing of a range of substrates that include peptide hormones, growth and differentiation factors, receptors, enzymes, adhesion molecules and viral proteins. Although it is clear that these proteases have roles in homeostasis, increasing evidence suggests that their

aberrant expression is a hallmark of several cancers and other diseases, and their activities are hijacked by viruses to facilitate infection and propagation. Elucidation of the endogenous substrate repertoire of the membrane-anchored proteases will facilitate our understanding of their roles in normal physiology and disease, while development of specific inhibitors may provide new avenues for the treatment of a range of ailments.

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