Roles and regulation of membrane-associated serine proteases

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Abstract

Pericellular proteolytic activity affects many aspects of cellular behaviour, via mechanisms involving processing of the extracellular matrix, growth factors and receptors. The serine proteases have exquisitely sensitive regulatory mechanisms in this setting, involving both receptor-bound and transmembrane proteases. Receptor-bound proteases are exemplified by the uPA (urokinase plasminogen activator)/uPAR (uPAR receptor) plasminogen activation system. The mechanisms initiating the activity of this proteolytic system on the cell surface, a critical regulatory point, are poorly understood. We have found that the expression of the TTSP (type II transmembrane serine protease) matriptase is highly regulated in leucocytes, and correlates with the presence of active uPA on their surface. Using siRNA (small interfering RNA), we have demonstrated that matriptase specifically activates uPAR-associated pro-uPA. The uPA/uPAR system has been implicated in the activation of the plasminogen-related growth factor HGF (hepatocyte growth factor). However, we find no evidence for this, but instead that HGF can be activated by both matriptase and the related TTSP hepsin in purified systems. Hepsin is of particular interest, as the proteolytic cleavage sequence of HGF is an 'ideal substrate' for hepsin and membrane-associated hepsin activates HGF with high efficiency. Both of these TTSPs can be activated autocatalytically at the cell surface, an unusual mechanism among the serine proteases. Therefore these TTSPs have the capacity to be true upstream initiators of proteolytic activity with subsequent downstream effects on cell behaviour.

Introduction

Pericellular and membrane-associated proteases are involved in regulating cell behaviour both in normal physiology and development and in a wide variety of pathologies, encompassing all the major degenerative diseases, including cancer, atherosclerosis, arthritis and neurodegeneration. In many of these, the proteases are involved in either initiation or progression of the disease and represent potential therapeutic targets. The well-publicized failure of relatively broad-specificity synthetic inhibitors of MMPs (matrix metalloproteinases) in Phase III clinical trials of advanced cancer has clearly dampened enthusiasm for proteases in general as therapeutic targets. However, it has also made it clear that a much more comprehensive understanding of the basic biology and biochemistry of these and other proteases is necessary to identify valid targets and discriminate them from anti-targets, i.e. those proteases that have beneficial effects. One powerful approach being adopted to address these issues is the use of proteomic screening to identify both direct and downstream substrates of individual proteases [1,2]. An alternative, more targeted, approach is to identify the proteases that are responsible for known proteolytic events using biochemical and enzyme kinetic approaches.

Membrane-associated proteases influence cell behaviour by essentially two fundamental mechanisms [3–5]. First, by proteolytic modification of the ECM (extracellular matrix) influencing communication between the cell and its surrounding matrix environment and, secondly, by the proteolytic activation of latent growth factors, cytokines and hormones. The proteases involved in these processes are not limited to a single mechanistic class, with the best studied examples belonging to the SP (serine protease) and metalloproteinase families. The membrane association of these proteases can either be a consequence of soluble proteases binding to specific binding sites or receptors on the plasma membrane, as exemplified by the uPA (urokinase plasminogen activator)/uPAR (uPA receptor) system of plasminogen activation, or by the anchorage of true transmembrane enzymes, for example, the MT-MMPs (membrane-type MMPs) and the ADAM (a disintegrin and metalloproteinase) family of metalloproteinases. In recent years an unexpected subfamily of SPs with membrane-anchorage has emerged, the TTSPs (type II transmembrane SPs) [6,7].

In this article, we briefly review a number of recent advances in our understanding of the regulation of the activity of the uPA/uPAR system, its functional connection with members of the TTSP family and roles of these proteases in the activation of latent growth factors and the regulation of protease cascades.

Key words: plasminogen, plasminogen activator, tetraspanin, transmembrane serine protease, type II transmembrane serine protease (TTSP), urokinase plasminogen activator receptor (uPAR).
Abbreviations used: ECM, extracellular matrix; GPI, glycosylphosphatidylinositol; HAI-1, hepatocyte growth factor activator inhibitor-1; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase; SF, scatter factor; SP, serine protease; TTSP, type II transmembrane serine protease; uPA, urokinase plasminogen activator; uPAR, uPA receptor.
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Regulation of uPA/uPAR activity by tetraspanins and integrins

The GPI (glycosylphosphatidylinositol)-anchored protein uPAR binds uPA with high affinity (K_d of approx. 0.1 nM) via the N-terminal EGF (epidermal growth factor)-like domain of uPA. The crystal structure of uPAR has recently been solved independently by several groups, both in complex with a peptidomimetic antagonist [8] and the N-terminal fragment of uPA [9]. These studies appear to confirm predictions from biochemical and enzymological studies that the catalytic domain of uPA is not involved in or affected by receptor binding, and that uPAR has no direct cofactor effect on the activity of uPA [10,11]. Nevertheless, binding of uPA to uPAR on the cell surface greatly enhances plasminogen activation, both in cell culture systems [12–14] and in *in vivo* models [15–17]. The interaction between uPA and uPAR is therefore critical for the generation of pericellular plasmin activity. If the function of the uPA/uPAR system is to generate proteolytic activity to modify the ECM, it might be expected that it communicates with the systems involved in cell adhesion and there has been much interest in potential interactions between uPAR and integrins, the major class of cell-adhesion receptors. In various cell types, uPAR has been shown to associate with different integrins, and, in some cases, to modulate integrin function (reviewed in [18]). Modulation of uPAR function by cell-adhesion systems is an equally intriguing prospect.

We have shown that expression of CD82, a member of the tetraspanin family of transmembrane proteins, profoundly affects uPAR-dependent plasminogen activation. Tetraspanins have roles in organizing cell-adhesion molecules on the cell surface by direct interactions with integrins and other tetraspanins, to form large multimeric complexes. Transfection of normal breast epithelial cells with CD82, a tetraspanin with metastasis-suppressing activity, has no effect on the expression of uPA, uPAR or other components of the plasminogen activation system, but nevertheless causes a 50-fold reduction in plasminogen activation [19]. This reduction was shown to be due to uPAR losing its ability to bind uPA in the presence of CD82, despite no covalent alterations in the receptor, e.g. proteolysis. Therefore, in these cells, the presence of CD82 renders uPAR 'cryptic', and may provide a novel mechanism for dynamically down-regulating the activity of this system.

Surprisingly, CD82 and uPAR did not co-localize on the cell surface, although uPAR was found to be redistributed to focal adhesions by CD82 where it co-localized with the integrin $\alpha5\beta1$, and uPAR and $\alpha5\beta1$ could be co-immunoprecipitated under these conditions. This led to the hypothesis that an interaction with $\alpha5\beta1$ was responsible for the altered behaviour of uPAR and subsequent effects on plasminogen activation [19].

We have used cell lines transfected with the $\alpha5$ integrin subunit to confirm that it does influence uPAR-mediated plasminogen activation, but have been unable to demonstrate a physical interaction between uPAR and $\alpha5\beta1$ using soluble recombinant proteins (R. Bass and V. Ellis, unpublished work). Although this observation does not exclude the existence of interactions on the cell surface, where they could be promoted by proximity effects, they do hamper attempts to define the mechanism involved. The various crystal structures of uPAR suggest that there is significant conformational mobility between its three constituent domains, all of which co-operate to form the ligand-binding cavity [8,9]. Therefore it could be postulated that interaction with an partner protein, such as $\alpha5\beta1$, alters the conformational relationship between the three uPAR domains such that uPA binding is abolished, as we observe in the presence of CD82.

Initiation of uPAR-mediated plasminogen activation

uPAR-mediated plasminogen activation is inherently efficient due to the amplification of proteolytic activity resulting from reciprocal zymogen activation. In this feedback activation process, any amount of plasmin initially generated can activate the zymogenic pro-uPA form, leading to further plasmin generation and subsequent amplification. However, the source of the proteolytic activity responsible for initiating this process is a matter of controversy. Essentially two mechanisms are possible, one involving only the proteolytic activities of uPA and/or plasmin, the other requiring an exogenous protease to activate pro-uPA. In model systems, the very low intrinsic activity of pro-uPA is sufficient to efficiently initiate plasminogen activation, but only in kinetically favourable situations where the apparent K_m for plasminogen activation is lowered, such as the cell surface [20]. However, although plasmin can efficiently activate pro-uPA, there is little specificity in this activation, and *in vitro* many other proteases with trypsin-like activity can activate pro-uPA. Initiation of plasminogen activation by this relatively non-specific mechanism seems unlikely, given the potentially wide-ranging downstream consequences. Nevertheless it is possible that in specific situations, other proteases do have a role in the initiation of uPAR-mediated plasminogen activation.

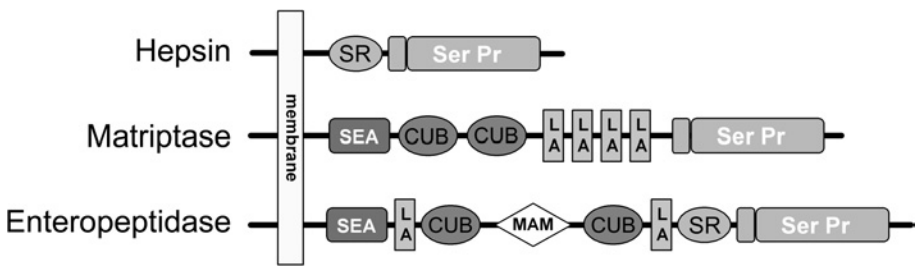
Strong evidence that this could be an important mechanism came from our comparison of two closely related, uPAR-expressing, monocytic cell lines. In the presence of pro-uPA, U937 cells accumulated pro-uPA on their surface, whereas THP-1 cells accumulated active uPA, suggesting constitutive activation of pro-uPA on the latter [21,22]. This activity was shown to be specific for uPAR-bound pro-uPA, with no activation detected in the conditioned medium; the activating protease was associated with the plasma membrane and appeared to be constitutively active. These characteristics are close to ideal for a specific initiation mechanism. The protease responsible for pro-uPA activation on THP-1 cells was identified as the TTSP matriptase [21].

TTSPs

SPs are historically the most well-studied family of proteolytic enzymes, so it came as some surprise when the presence of a previously unsuspected subfamily was identified, characterized by the presence of an N-terminal transmembrane

Figure 1 | TTSP domain structures

The unique modular structures of the TTSPs discussed are shown, using the following module name abbreviations: SEA, SEA (sea urchin sperm protein, enteropeptidase, agrin) domain; CUB, CUB [complement, UEGF (urchin embryonic growth factor), BMP-1 (bone morphogenetic protein 1)] domain; LA, LDL (low-density lipoprotein) receptor type-A repeat; MAM, MAM (meprin, A5, protein phosphatase mu) domain; SR, Group A scavenger receptor domain; Ser Pr, serine protease domain. Each of the TTSPs has a short cytoplasmic tail of typically 20–50 residues, although some extend to 160 residues, and are likely to play a role in targeting the proteases to discrete membrane localizations.



extension [6]. The TTSPs, estimated to contain more than 20 human members, are mosaic proteins in common with the soluble proteases of the plasminogen activation, blood coagulation and complement systems. However, they utilize a completely distinct set of protein modules in their assembly (Figure 1), including some known to be protein–protein interaction domains in other proteins [6,7]. The majority of these proteases have yet to have their biological function and potential substrates identified. Interestingly, the family includes enteropeptidase, the existence of which was first demonstrated by Pavlov over 100 years ago, and known for many years as the highly specific activator of trypsinogen, but only recently characterized as a transmembrane protein [23,24]. Other TTSPs with known physiological roles are corin and TMPRSS3. Corin activates the cardiac hormone atrial natriuretic peptide and is involved in blood pressure regulation [25], and mutations in TMPRSS3 are associated with congenital deafness, although the mechanism involved is unknown [26].

Two of the most interesting TTSP family members in terms of pathology are matriptase and hepsin, both of which are up-regulated in a variety of cancers and correlate closely with disease progression [27]. Hepsin is among the smallest of the TTSPs, containing only one additional protein module (Figure 1), and the first to be cloned [28]. Mice with targeted deletion of the hepsin gene display no overt phenotypic abnormalities [29]. Overexpression of hepsin in prostatic epithelium of transgenic mice leads to disruption of basement membrane, and promotes disease progression in a model of prostate cancer [30]. Matriptase-deficient mice develop normally, but do not survive postnatally owing to an epidermal barrier defect [31]. Transgenic mice with a very modest overexpression of matriptase in the skin become remarkably susceptible to carcinogen-induced tumour formation [32]. The molecular targets of hepsin and matriptase leading to these effects on tumour initiation and progression have yet to be identified.

Proteolytic activation of HGF (hepatocyte growth factor)/SF (scatter factor)

HGF/SF is one of two growth factors closely related to plasminogen. Both have mutations in the catalytic triad residues, rendering them inactive as enzymes, but retain the canonical proteolytic activation site of the SPs. Cleavage at this site is an absolute requirement for activation of the cognate receptor, which, in the case of HGF/SF, is the proto-oncogene c-Met [33], making this a key step in the regulation of the c-Met signalling pathway.

uPA has been proposed to be an efficient activator of HGF/SF [34–36]. However, despite the similarity of HGF/SF and plasminogen, the proteolytic activation sequences are dissimilar, and uPA is known to be an extremely specific protease. In our own laboratory, we have been unable to corroborate these findings (K. Owen and V. Ellis, unpublished work), leading us to investigate other potential pericellular activators. In biochemical studies, both matriptase [37] and hepsin [38,39] have been shown to activate HGF/SF. Our own quantitative data show that matriptase is the most efficient of the known HGF/SF activators, with hepsin having approx. 10% relative activity and both being at least three orders of magnitude more efficient than uPA (K. Owen and V. Ellis, unpublished work). These studies utilized truncated soluble forms of the proteases, but, using both siRNA (small interfering RNA) and overexpression approaches, we have also demonstrated that the transmembrane forms of both proteases efficiently process HGF/SF to its biologically active form (D. Qiu, K. Owen and V. Ellis, unpublished work). Although this has yet be demonstrated *in vivo*, it may provide a mechanism for the involvement of these TTSPs in the development of cancer. At the biochemical level, it remains to be determined whether membrane localization of the proteases confers them with any kinetic advantage and whether their N-terminal domains are involved in substrate recognition of HGF/SF by exosite interactions.

A general role for TTSPs as initiators of protease cascades?

Our observation that matriptase can activate uPAR-bound pro-uPA, and therefore the plasminogen activation cascade, raises the question of whether matriptase, and potentially other TTSPs, could have a more general role in the initiation of protease cascades. An observation that supports this hypothesis is the demonstration that matriptase is responsible for the activation of a previously unsuspected protease cascade. The epidermal defect observed in matriptase-null mice [31] is due to an inability to process the keratinocyte polyprotein filaggrin in the absence of matriptase [40]. However, although profilaggrin was assumed to be a direct substrate for matriptase, mice deficient in prostasin, a GPI-anchored SP, were subsequently found to have a similar phenotype and lack of filaggrin processing [41]. One interpretation of the observation that both proteases are necessary is that they form part of the same proteolytic cascade. Both biochemical and genetic studies demonstrate this to be the case, with matriptase responsible for activating prostasin [42]. A very interesting feature of matriptase that further supports this hypothesis is that it has the ability to undergo autocatalytic activation [43,44]. The majority of SP zymogens are unable to do this, i.e. the sequence of their proteolytic activation sites do not match the substrate specificity of their active site, and zymogen activation requires an upstream activating protease or other mechanism. Although the TTSPs as a family have not been studied in detail, it is nevertheless significant that several of those that have been studied can be autoactivated *in vitro*. In addition to matriptase, its close homologues matriptase-2 [45] and matriptase-3 [46], TMPRSS2 [47] and TMPRSS3 [26] have all been demonstrated to autoactivate when expressed as truncated soluble proteins, and hepsin also when expressed in its transmembrane form (D. Qiu and V. Ellis, unpublished work). Although this critical characteristic in most cases needs to be demonstrated more rigorously, these observations clearly point to many of the TTSPs having an ability not common among the highly regulated multidomain SPs. Therefore, as with matriptase, they could act as true initiators of proteolytic cascades. It is worth mentioning that enteropeptidase, although the initiating protease of the digestive cascade, does not undergo autoactivation and needs to be activated by trypsin.

Although the autoactivation of these TTSPs can be readily detected *in vitro*, it appears unlikely that it would not be a highly regulated process *in vivo*. As these are transmembrane proteins, it could be envisaged that a potential mechanism for its regulation could be membrane localization and clustering. Another important consideration is the potential role of the accessory extracellular domains of these proteins. If these are involved in homotypic protein–protein interactions, autoactivation could be facilitated. There is some evidence for this, as several disease-associated mutations in the N-terminal domains of TMPRSS3 inhibit its activation [26], and both point and deletion mutations in the N-terminal domains of matriptase have the same effect [44]. The interpretation of the observations with matriptase is not straightforward, as they may be due to an impaired interaction of matriptase with its physiological inhibitor, the Kunitz-family member HAI-1 (HGF activator inhibitor-1), which has been proposed to be involved in the activation of matriptase in some cell culture models [44]. Although much needs to be learned regarding the regulation of autoactivation, it has been clearly demonstrated that HAI-1 is not necessary for matriptase activation, either *in vivo* [48] or in the *in vitro* activation of pro-uPA by leucocytes [21].

Conclusions

The regulation of proteolytic activity at the cell surface is clearly complex, as befits its key role in dictating cellular behaviour. Recent evidence has highlighted unexpected complexity in the functional regulation of the pericellular plasminogen activation system, which can be influenced by components of the cell-adhesion machinery. The TTSP matriptase also has a potential role by initiation of the plasminogen-activation cascade. An argument has been made here for a more general role of the TTSPs in initiating protease cascades, although they can clearly also have direct effects, such as in the proteolytic activation of HGF/SF. The TTSPs, in particular those that are highly up-regulated in cancer, such as matriptase and hepsin, represent exciting future therapeutic opportunities, as evidence accumulates that they may have functional roles in disease development and progression. However, it is clear that, before then, much more needs to be known about the activities, natural substrates and functional regulation of these proteases in biochemical and cellular studies as well as their physiological roles in animal models.

The work described here from our laboratory was supported by grants and research fellowships from the British Heart Foundation, with additional support from the Norfolk and Norwich Big C Appeal.

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Received 19 March 2007