The role of proteases in regulating Eph/ephrin signaling

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Abbreviations: AD, Alzheimer’s disease; ADAM, a disintegrin and metalloprotease; APP, Amyloid precursor protein; Arf1, ADP-ribosylation factor 1; BACE, β-site APP cleaving enzyme; CAM, cell adhesion molecules; CTF, cytoplasmic fragment; CNC, cranial neural crest; ECM, extracellular matrix; FLIM, fluorescence lifetime imaging microscopy; FN, fibronectin; iCLiP, intramembrane cleaving protease; LTP, long-term potentiation; MLC, myosin light chain; MMP, matrix-metalloprotease; MT1-MMP, membrane type-I matrix metalloproteinase; NCAM, neural cell adhesion molecule; NMDA, N-methyl-D-aspartate; NMJ, neuromuscular junction; NOX, NADPH oxidase; PDI, protein disulphide isomerase; PS, Presenilin; PTP, Protein Tyrosine Phosphatase; RIP, Regulated intramembrane proteolysis; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; TACE, tumor necrosis factor-α-converting enzyme; TNFα, tumor necrosis factor-α; TGFβ, transforming growth factor β.

Proteases regulate a myriad of cell functions, both in normal and disease states. In addition to protein turnover, they regulate a range of signaling processes, including those mediated by Eph receptors and their ephrin ligands. A variety of proteases is reported to directly cleave Ephs and/or ephrins under different conditions, to promote receptor and/or ligand shedding, and regulate receptor/ligand internalisation and signaling. They also cleave other adhesion proteins in response to Eph-ephrin interactions, to indirectly facilitate Eph-mediated functions. Proteases thus contribute to Eph/ephrin mediated changes in cell-cell and cell-matrix interactions, in cell morphology and in cell migration and invasion, in a manner which appears to be tightly regulated by, and co-ordinated with, Eph signaling. This review summarizes the current literature describing the function and regulation of protease activities during Eph/ephrin-mediated cell signaling.

Introduction

Proteases in regulation of cell signaling in general

Proteases are enzymes that carry out the hydrolysis of peptide bonds within proteins and polypeptides. Apart from their obvious roles in digestion and protein turnover, proteases are important in tightly regulated signaling cascades which include cell cycle regulation, blood coagulation, apoptosis, antigen presentation and protein and organelle recycling. The disruption of their strictly controlled equilibrium in the cell has been found in a number of pathologies, such as cardiovascular diseases, inflammation, neurodegenerative diseases, and cancer. There are 5 major classes of proteases known in mammals including serine, cysteine, metallo, aspartic, and threonine proteases, which are categorised according to their different catalytic mechanisms.

The cellular localization of proteases is an important factor that affects their activity and substrate selection – the greater proportion are extracellular, followed by intracellular, with a minor proportion being localized in plasma and organelle membranes. Together, all these different properties and abilities of proteases tightly regulate cellular processes throughout the body.

In early years proteases were not regarded as traditional signaling molecules, although this view is dramatically changing. Nevertheless, there are significant differences between protease signaling and other types of cellular signaling. In protease-mediated signaling, the signal is transmitted through the cleavage of protein substrates with subsequent activation, inactivation, or modulation of function, which is irreversible. Examples include activation of cytokines, inactivation of numerous repair proteins during apoptosis, exposure of cryptic sites, and shedding of various transmembrane proteins such as RTK ligands and receptors.

A number of proteases act in cascades allowing more stringent regulation and amplification of the signal. In addition to their catalytic domain, numerous additional domains or modules substantially increase the complexity of protease functions.

For many receptor families, including tyrosine kinases (RTKs), receptor activation is generally thought to require proteolytic shedding of ligands at a distance, either from the same cell or a different cell, facilitating autocrine or paracrine signaling, respectively. However, as described previously, and elsewhere in this issue, a defining feature of Eph/ephrin signaling is that
interactions occur between membrane bound ligands and receptors on adjacent cells, mediating juxtacrine signaling at cell-cell contacts, and therefore a necessary role for proteolysis is not immediately obvious. These interactions involve clusters of receptors and ligands, leading to large receptor-ligand complexes, which combined with involvement of cadherins and other cell adhesion molecules, result in tethering cells together. Yet Eph-ephrin interactions can lead either to cell spreading and adhesion, or to cytoskeletal collapse and cell retraction or segregation, necessitating disruption of these complexes.8 While trans-endocytosis of Eph-ephrin ligand complexes has been reported for EphBs,9 a number of reports show an essential role of proteases in mediating a variety of Eph-ephrin functions.

**Proteases regulating Eph/ephrin signaling**

ADAMs

ADAMs (A Disintegrin And Metalloproteases) are transmembrane metalloproteases that process and shed the ectodomains of membrane-anchored growth factors, cytokines and receptors.10 They belong to the metazincin superfamily11 which also includes the ADAM-TS, class III snake venoms and matrix metalloproteases (MMPs). ADAMs have essential roles in fertilization, angiogenesis, neurogenesis, heart development and cancer10 shown by using ADAM loss- and gain-of-function mouse models. ADAMs are widely expressed in mammalian tissues, and the observed phenotypes of ADAM knockout mice are subsequently diverse,12 although only ADAM10, 17 and 19 are essential for mouse development.13-15 ADAM10 and ADAM17 knockout leads to abnormalities in growth almost all tissues13,14 compared to ADAM19 deletion which affects only the peripheral nervous system and heart development.15 Additionally, several ADAMs play important roles in pathological situations, such as inflammation, carcinogenesis or stress-mediated angiogenic response.12 Accordingly, ADAMs are up-regulated in a wide variety of cancers, both solid and hematological tumors. They are reported to target a variety of substrates directly implicated in human disease, including ligands for receptors, and pro-inflammatory cytokines (eg. TNFα for ADAM17/TACE), motivating strong interest in ADAMs as therapeutic targets in cancer and other diseases, as reviewed elsewhere.16

A typical ADAM consists of conserved and characteristic protein domains in which an N-terminal signal sequence is followed by a pro-domain, a metalloprotease (MP) domain, a disintegrin domain (D), a cysteine-rich (C) region, an EGF-like domain (except for ADAM10 and 17), a transmembrane domain and a cytoplasmic domain (Figure 1).17 While the N-terminus signal sequence directs ADAMs into the secretory pathway18,19 the prodomain is thought to function as both a molecular chaperone, and an inhibitor of the metalloprotease domain.20,21 It was thought that removal of the prodomain at a conserved Rx(R/K)R motif by proprotein convertases was necessary to activate ADAMs, occurring in the trans-Golgi network.22-24 However, while cleavage of the ADAM10 prodomain can enhance protease activity, expression of a truncated ADAM10 lacking the prodomain results in decreased activity that is rescued by addition of recombinant prodomain, suggesting the prodomain is also necessary to assist in the correct folding of the catalytic domain.19 In support, cleaved prodomain can remain attached to the mature protease23 on the cell surface. Furthermore, removal of the prodomain is not necessary for activation, as unprocessed, cell surface ADAM17 exhibits rapid and reversible activation in response to stimuli, by alterations in catalytic site accessibility.25 Initially, it was thought the inhibitory function of the ADAM prodomain operates via a cysteine switch mechanism where a conserved unpaired cysteine residue within the prodomain preferentially coordinates with the active site zinc atom of the metalloprotease domain. However, at least for ADAM1026, ADAM1227, ADAM9 and 17 this is not the case.

The best studied domain of the ADAM proteins is the metalloprotease domain, although this is not active in all ADAMs. Only 17 of the known 23 mammalian ADAMs contain the zinc-dependent metalloprotease catalytic site sequence,11 which include consensus HEXGHXXGXXHD motif and a conserved methionine-turn in the active-site helix,28 that confer proteolytic activity to ADAMs. Peptide libraries have been used to try to understand the selectivity of ADAMs for particular substrates. Although some sequence preferences exist for particular peptide substrates, there are no clear consensus cleavage motifs for ADAMs.29 For example, while protein substrates for ADAM10 and ADAM17 are partially overlapping, each one appears to selectively target particular proteins in vivo.30

Rather, control of substrate specificity resides in the D and C domains following the MP domains, which are involved in protein-protein interactions.31 Using chimeric ADAM10-ADAM13 constructs it has been shown that ADAM13 D+C domains

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**Figure 1.** An illustration of ADAM domain structure. ADAMs consist of a prodomain (Pro), metalloprotease domain (MP), disintegrin domain (D), cysteine-rich domain (C), EGF-like domain (except for ADAM10 and 17), a transmembrane domain and a cytoplasmic domain.
cannot be replaced by that of ADAM10 to induce hyperplasia in *Xenopus laevis* embryos, indicating alternate specificity. ADAM13 C domain is also a major determinant for specific developmental events mediated by ADAM13 proteolytic shedding. Similarly, a chimeric construct of ADAM17 with the D+C domains of ADAM10 could not reconstitute ADAM17 shedding of IL-1R-II. As described below a substrate-binding pocket within the C domain of ADAM10 has been shown to define cleavage specificity in ephrin-Eph signaling.

Full length exodomain structures of mammalian ADAMs are not yet available, only for the isolated MP or D+C domains. However they share an overall domain architecture similar to class-III snake venom metalloproteases, including conserved disulfide bond patterns, so these structures can provide insight into the likely structure of ADAMs. For example the VAP1 structure shares a curved D+C domain structure with ADAM10, which confers an overall C-shape structure with the MP domain in proximity to the substrate binding region of the C domain, consistent with the idea binding to the C domain controls specificity by positioning the substrate for cleavage.

**Direct shedding of Ephs/ephrin ECDs by ADAMs**

Fundamentally, a major outcome of ephrin-Eph signaling is cell-cell repulsion following engagement, which is important for neuronal axon guidance and also for establishment of the arterial and venous vascular networks. Eph-induced retraction was first shown to require proteolytic cleavage of the Eph ligand ephrin-A2 on axons, following binding to its cognate receptor EphA3, through activity of ADAM10. As outlined below a number of studies have now shown how ADAM10, and some other ADAM family members, regulate signaling by a number of different Eph/ephrins.

**ADAM10**

ADAM10 (Kuz) is one of the better characterized in the ADAM family with respect to proteolytic function. In addition to A-type ephrins (below), it has been identified as a sheddase of cell-surface bound proteins such as the epidermal growth factor receptor ligands EGF and betacellulin, the erbB3/4 ligands neuregulins, chemokines CX3CL1 and CXCL16, E- and N-cadherin, and adhesion molecule L1. Amyloid precursor protein (APP), the cellular prion precursor protein and many others. Nevertheless, many target substrates have been identified in vitro, using inhibitors that apply to a broad-range of metzincin family members. The best characterized role of ADAM10 is in Notch signaling, where ADAM10 cleavage of both Notch receptors and ligands has been reported and, accordingly, phenotypes of ADAM10 and Notch1 deficient mice are very similar, including embryonic lethal defects in somitogenesis, neurogenesis and vasculogenesis.

In the first published study that demonstrates ADAM involvement in Eph signaling, ephrin-A2 was shown to be cleaved by ADAM10, disrupting Eph/ephrin cell contacts. Here, ephrin-A2 was found in complex with ADAM10, interacting mainly via the non-catalytic regions and ephrin cleavage was triggered by binding to its receptor EphA3. Also, a motif search to identify sequences conserved between ADAM10 substrates, including all 8 vertebrate ephrins, the Notch ligand Delta, TNF-α, and APP, revealed a consensus sequence which lies not at the cleavage site but in the ligand-binding region of ephrins, indicating this region is mostly likely bound by ADAM10. A cleavage-inhibiting mutation in ephrin-A2 delayed axon withdrawal, highlighting the biological importance of protease recognition and regulation in EphA2/ephrin-A2 mediated axon detachment.

The mechanism by which ADAM10 recognizes and cleaves Eph-bound ephrin was clarified by structure-function studies interrogating the role of ADAM10 in EphA3/ephrin-A5 signaling. ADAM10 demonstrated slight constitutive association with EphA3, rather than ephrin, but increased upon ephrin binding (Figure 2A and B). Determination of the crystal structure of the ADAM10 D+C domain identified an acidic pocket within the C domain which was required for binding to the Eph/ephrin complex, but did not effectively bind to either alone. This is consistent with the consensus recognition sequence identified by Hattori et al, lying in the Eph-binding region of ephrins (above). Furthermore, as the Eph/ephrin interaction occurs between adjacent cells, ADAM10 on the Eph-expressing cell was found to cleave ephrin from the opposing cell, in a non cell autonomous manner (in trans'). Thus only receptor-bound ligand is cleaved, breaking the molecular tethers between the opposing cell surfaces, allowing internalization of the EphA3/ephrin-A5 complexes into the Eph-expressing cell. The direct Eph-ADAM10 interaction was also subsequently shown using EphA2-expressing cells incubated on an artificial membrane displaying ephrin-A1. Supporting the importance of the substrate recognition motif, monoclonal antibodies specifically recognizing this site are able to inhibit ephrin cleavage and ephrin-induced Eph receptor internalization, phosphorylation and Eph-mediated cell-cell migration. This approach confirms the significance of the substrate-determining C domain as a potential target for therapeutic inhibition of ADAM10.

**Regulation of ADAM-mediated ephrin cleavage**

Importantly, Hattori et al found that ephrin cleavage by ADAM10 was also dependent on tyrosine kinase activity, as it was inhibited by treatment with the tyrosine kinase inhibitor genistein. This is in line with a large body of work showing that elevated Eph kinase activity promotes cell-cell repulsion, while low kinase activity is associated with adhesion. Similarly, cleavage of RTK ligands and receptors by various ADAMs increases in response to activation of PKC by phorbol esters, upon G protein coupled receptor activation, and following inhibition of protein tyrosine phosphatases (PTPs) (thereby promoting RTK activation), including ephrin-A2. However, the mechanism through which receptor tyrosine kinase (RTK) signaling regulates ADAMs remained elusive. The ADAM10 or EphA3 cytoplasmic domains are not essential for ephrin shedding, and similarly phorbol ester-stimulated shedding by ADAM17 is not effected by deletion of its cytoplasmic domain. By performing fluorescence lifetime imaging microscopy (FLIM) and electron tomography on EphA3 with or without ephrin-A5 stimulation we have shown that a conformational change known
to occur upon Eph activation⁵⁹ results in elongation of the EphA3 cytoplasmic domain, moving the kinase domain away from the plasma membrane, presumably relieving a steric hindrance or otherwise allowing productive association with ADAM10 upon activation by ephrin-A5⁵⁸ (Fig. 2A and B).

Emerging data also suggests regulation of ADAM activity by reactive oxygen species (ROS) mediated conformational changes in the extracellular domain. In this model, highly conserved cysteine sulphydryl (CxxC) motifs present in the ADAM17 (and ADAM10) D+C domain act as consensus sites for thiol disulphide exchange by protein disulphide isomerase (PDI), to induce change between inactive and active conformations. Thus, ADAM17 mutations replacing CxxC cysteine residues significantly decreased shedding of L selectin, while redox modulation by H₂O₂ treatment enhanced and reducing conditions inhibited ADAM17 activity in vitro and in vivo.⁶⁰ Subsequent studies revealed that inhibition or silencing of PDI also reduces PMA induced HB-EGF cleavage, suggesting that PDIs may control the conversion into an inactive form of ADAM17, and that PDI activity is modulated by oxidation.⁶¹,⁶² ADAM10 contains a conserved CxxC motif (C594-x-x-C597) which lies adjacent to the substrate recognition site, implying that similar to ADAM17, ADAM10 activity may also be regulated by ROS-mediated conformational changes, altering access to the substrate recognition site. During RTK signaling, cell membrane-resident NADPH oxidase generates ROS⁶³ which transiently activate RTKs by inhibiting regulatory PTPs, through oxidation of their catalytic cysteines.⁶⁴ Thus localized, NADPH oxidase-generated ROS may also trigger conformational changes controlling ADAM activity, suggesting a positive feedback loop where RTK activation causes ROS-mediated PTP inhibition and simultaneous activation of ADAM-mediated ligand shedding (Figure 2C). Considering that ROS levels are frequently elevated in tumors and other pathologies with elevated RTK and pro-inflammatory signaling,⁶⁵ and that ADAM activity is well-known to be up-regulated in such environments,⁶⁶ this mechanism may be particularly relevant for diseases such as chronic inflammation and cancer.

In addition to ephrin-As, ADAM10 also associates with and cleaves ephrin-B2 during Xenopus development, in a manner regulated by interaction with flotillin-1.⁶⁷ Thus in the absence of flotillin-1, ephrin-B2 levels are dramatically reduced, leading to defects in neural tube closure, an effect dependent on function of ADAM10. Both ephrins and flotillins are known to co-localize to similar cholesterol-rich membrane microdomains,⁶⁸ while Ephs and ephrins have previously been shown to reside in distinct microdomains.⁶⁹ Given the association of ADAM10 with EphAs within a single membrane (above), this suggests that physical segregation of ADAM10 from ephrins may provide an extra level of regulation, a notion previously proposed for other targets.⁶⁶

Other ADAMs in Eph signaling
ADAM12 modulates intracellular signaling by cleaving various membrane bound signaling receptors and their ligands,⁷⁰ as well as degrading extracellular matrix proteins such as fibronectin, at least in vitro.⁷¹ ADAM12 is highly expressed in glioblastoma multiforme, where it is linked to shedding of HB-EGF.⁷² This tumor type also over-expresses EphA2 and shows high levels of shed ephrin-A1 suggesting a possible role of ADAM12 in ephrin shedding. Indeed, ADAM12 enhances ephrin-A1 shedding in primary tumors in response to transforming growth factor β1 (TGF β1) activity, resulting in lung hyperpermeability that allows tumor cell migration into the lungs.⁷³ The receptors
EphA1 and EphA2 both colocalise with ephrin-A1 in this setting, where they are thought to maintain cell-cell contacts, since loss of either contributes to lung permeability. Soluble ephrin-A1 disrupts these contacts, and inhibition of soluble ephrin-A1 by a neutralizing antibody significantly reduced lung metastasis. This is consistent with the significant reduction in primary tumor growth and lung metastasis in ADAM12 KO mice. Interestingly, EphA1 directly bound ADAM12 by yeast 2-hybrid screening, its co-expression in cells aided in ADAM12 translocation to the plasma membrane, and in co-culture experiments EphA1/ADAM12-expressing cells caused cleavage of ephrin-A1 on adjacent cells in trans, in line with previous findings of ephrin-A5 trans cleavage by ADAM10 in complex with EphA3.

ADAM13 is also reported to cleave ephrin-B ligands, in addition to a range of other substrates, including fibronectin (FN) and Cadherin-11. ADAM13 is required for cranial neural crest (CNC) migration, a process where multipotent embryonic cells in the CNC migrate extensively to generate a large number of differentiated cell types in their target destinations, giving rise to craniofacial structures during embryonic development. Ephrin-B1 acts as a guidance cue in segmental cranial/trunk neural crest migration, and ectopic expression of ADAM13 results in disoriented migration of both cranial and trunk neural crest similar to distorted ephrin-B signaling. Both ephrin-B1 and B2 were found to be substrates of ADAM13 cleavage in cells and embryos during CNC migration. Interestingly, ADAM13 upregulated canonical Wnt signaling and early expression of the transcription factor snail2, whereas ephrin-B1 acts to inhibit this pathway, suggesting cleavage of ephrin-B1/2 by ADAM13 is required for derepression of canonical Wnt signaling and early CNC induction. In addition, the cytoplasmic domain of ADAM13 is also shed and translocated to the nucleus where it controls expression of multiple genes in CNC.

While ADAM19 cleaves various ligands including neuregulins, HB-EGF, and TNF-α, a different role has been suggested in Eph/ephrin signaling during development of the neuromuscular junction (NMJ). ADAM19 and ephrin-A5 both localize to the NMJ and participate in its formation, where ADAM19 interacts with EphA4 and blocks internalization of the ephrin-A5/EphA4 complex into the EphA4 expressing motor neurons, preventing repulsion of the axon terminal at the NMJ, independent of ADAM19 protease activity. The cytoplasmic domain of EphA4 is postulated to be less accessible when it interacts with ADAM19, thus inhibiting binding of endocytosis regulators to the EphA4 SAM domain, preventing endocytosis.

**Eph-dependent shedding of cell adhesion molecules by ADAMs**

Eph receptors not only act directly to promote cellular activities (eg. adhesion, repulsion, migration and process extension) but also co-operate with other molecules like cell adhesion molecules (CAMs) to mediate such activities. As discussed below, recently it has been shown that Eph signaling also instigates shedding of E-cadherin and the neural cell adhesion molecule (NCAM), further regulating cell adhesion and migration.

EphB signaling regulates the formation of E-cadherin based adhesions in epithelial cells to control cell migration in the intestinal epithelium. Thus, paneth cells in the intestinal crypt migrate downwards to localize at the crypt bottom resulting in compartmentalization that depends on the EphB2 and B3 expression levels of Paneth cells and ephrin-B1 expression in the transient amplifying compartment. To determine ADAM10 involvement in this process, transgenic mice expressing dominant negative ADAM10 lacking its MP domain were generated. As a result of abrogated ADAM10 activity Paneth cell segregation was impaired, diminishing the boundary with the transient amplifying compartment. This observation is reminiscent of EphB2/B3 knockout mice or intestinal ephrin-B1 null mice, and implicates the likely involvement of ADAM10 in cellular boundary formation and compartmentalization. Further experiments reveal that monolayers of MDCK (Madin-Darby canine kidney) cells expressing dominant negative ADAM10 also have impaired segregation, promoting intermingling of EphB and ephrinB positive cells and blocking cell segregation. Interestingly, ADAM10, EphB2 and E-cadherin form a complex which initiates E-cadherin proteolytic shedding at sites of EphB/ephrin-B1 interactions. However ephrin-B1 did not directly interact with ADAM10 or E-cadherin, which is compatible with a model where ADAM10 acts in trans to cleave E-cadherin from the opposing cells expressing ephrin-B1, upon substrate recognition via EphB2.

EphA/ephrinA signaling has an important role in axon guidance by controlling axon repulsion from inappropriate areas and constraining arborization in the neuronal cell populations. NCAM, a potent promoter of axon growth and synaptic plasticity, interacts with EphA3 to stimulate growth cone collapse, reduce arborization, and limit the number of synapses of cortical GABAergic interneurons. Ephrin-A5-induced growth cone collapse is ADAM10-dependent and is inhibited in cortical cultures from NCAM null mice and restored by the introduction of wild type NCAM. NCAM, ADAM10, and EphA3 co-localize in cortical neurons and, analogous to E-cadherin, EphA/ephrinA signaling triggers ADAM10 mediated NCAM shedding to promote growth cone collapse.

**MMPs**

Matrix-metalloproteases (MMPs) are a family of nearly 25 members that, like ADAMs, also belong to the metzincin superfamily. MMPs are secreted or remain membrane-anchored and subsequently cleave proteins on the membrane, secretory pathway or extracellular space. The conserved pro-domain and the catalytic domain are the common structural features to all the MMP family members. The catalytic domain includes a zinc ion in the active site and interacts with 3 conserved histidine residues (HEXXHXXGXXH). Most non-membrane bound MMPs also consist of a hemopexin domain at their C termini that mediates protein–protein interactions facilitating substrate recognition, activation of the enzyme, protease localization, internalization and degradation. A subgroup of membrane bound MMPs, the MT-MMPs, are either type I transmembrane proteins or tethered to the plasma membrane by a GPI anchor...
making them important effectors in pericellular proteolysis. A typical function of MMPs is to degrade the structural components of the extracellular matrix (ECM) to facilitate cell migration, including regulating signaling via ECM-associated membrane bound receptors such as integrins. However MMPs also participate in the release of cell-membrane-bound precursor forms of many growth factors and their receptors (eg. FGFR1, ErbB). Thus, MMPs are critical in regulation of cell migration, especially in disease settings such as cancer metastasis and angiogenesis, and in inflammation and arthritis.

Direct effects of MMPs on Ephs/ephrins

A number of MMPs are reported to cleave Eph receptors and ephrins. Ephrin-A1, which is overexpressed in multiple human malignancies along with its corresponding receptor EphA2, was reported to be released from cells by proteolytic activity, present in conditioned medium and serum, and sensitive to broad MMP and serine-protease inhibitors. Mass-spectrometry analysis and use of cleavage site mutants showed 3 cleavage sites on ephrin-A1 for MMPs. Soluble cleaved ephrin-A1 acted similar to homodimeric ephrin-A1 by activating EphA2 receptor and facilitating its internalization over time. Co-incubation of various recombinant MMPs in a non-cell-based assay showed ephrin-A5 was best cleaved by MMP1, 2, 9 and 13, although the biological relevance of this was not verified. However, MMP-specific inhibitor treatment of melanoma cells does not alter EphA2-induced invasive capacity, which is mediated rather by Rho-mediated cytoskeletal changes, conferring a shift from mesenchymal to amoeboid-like cell motility, although the same study confirmed the role of MMPs in invasion in response to other pathways, such as TNF signaling. Thus it is likely various proteases co-operate to promote cancer cell invasion, including ADAMs (as reviewed above), and interestingly, ADAM12 and MMP2 and 9 are all upregulated by TGF-B1 signaling in tumor metastasis.

EphBs and ephrin-Bs have also been reported to undergo cleavage by MMPs. EphB2-stimulated ephrin-B2 cleavage was first described in the context of subsequent intramembrane proteolysis by Presenilin (see below), and was sensitive to a broad metalloprotease inhibitor (GM6001), although as this inhibits both MMPs and ADAMs it is unclear which is responsible. However there is evidence MMP8-dependent cleavage of ephrin-B1 occurs, causing ectodomain release into the culture medium of pancreatic cancer cell lines, and hence recombinant ephrin-B1-Fc is cleaved preferentially by MMP8, compared to the other metalloproteases tested. With ephrin-B2, cleavage of ephrin-B1 was enhanced by interaction with the EphB2 receptor, which activated secretion of MMP8. This was dependent on the C-terminus of ephrin-B1, and stimulation of ephrin-B1 by EphB2 activated the ADP ribosylation factor 1 (Arf1) GTPase, a critical regulator of membrane trafficking, via the C-terminus of ephrin-B1. Therefore it is speculated that ephrin-B1 regulates the exocytosis of MMP-8 by activation of Arf1, a process implicated in invasion of collagen by prostate cancer cells, and in scirrhous gastric carcinoma cells. Moreover, there was also dependence on ephrin-B1 association with the adapter protein Dishevelled, which activates the small GTPase RhoA, mediating cell-cell repulsion in EphB reverse signaling.

Transient expression of ephrin-B1 increased the level of GTP-bound RhoA activation, which was reduced by introduction of an ephrin-B1 C-terminus peptide. Thus the ephrin-B1 C-terminus is suggested to activate both RhoA, to regulate cytoskeletal changes, and Arf1, to facilitate MMP8 mediated invasion.

The EphB2 receptor has also been reported to be cleaved by MMPs in response to ligand stimulation in primary hippocampal neuron cultures. EphB2-Fc fusion protein is cleaved in vitro by MMP9, and mutation of a potential MMP cleavage site in EphB2 inhibited cleavage, and prevented cell-cell repulsion upon interaction with ephrin-B2 stably expressing cells. EphB2 mediated growth cone collapse and withdrawal in hippocampal neurones was also significantly inhibited by mutation of the EphB2 MMP cleavage site and partially reduced by an MMP 2/9 inhibitor. Interestingly, inhibition of MMP 2/9, or expression of the cleavage-resistant EphB2 mutant, reduced phosphorylation and recruitment of signaling proteins Src and FAK and activation of RhoA, events previously described to promote ephrin-B/EphB-induced actin myosin contractility of the cytoskeleton.

The membrane type-I matrix metalloproteinase (MT1-MMP) also has a reported role in promoting cancer cell migration and invasion via cleaving the EphA2 receptor. Analysis of a panel of breast carcinoma cell lines shows co-overexpression of MT1-MMP with EphA2. EphA2 clusters recruit MT1-MMP via a mechanism independent of its catalytic domain, reminiscent of recruitment of MT1-MMP to CD44H, a major hyaluronan receptor that plays an important role in migration. Subsequently, EphA2 is proteolytically processed by MT1-MMP, as MT1-MMP depletion and inhibition resulted in reduced EphA2 receptor cleavage. Mass spectrometry studies indicate MT1-MMP mediated cleavage of EphA2 receptor occurs at the FN type-III domain, likely in cis on the cell surface, as there was some constitutive shedding in the absence of added ligand, which was enhanced by soluble ephrin-A1. SiRNA knockdown of MT1-MMP and expression of EphA2-cleavage site mutant effectively reduced intracellular accumulation of EphA2, cell invasion and Src signaling indicating interconnection of these processes. Moreover, EphA2 association with RhoA lead to cell retraction, which decreased in the presence of inactive EphA2 and EphA2 MT1-MMP cleavage-site mutant. Thus MT1-MMP/Src activity-dependent intracellular translocation of EphA2 receptor and cytoskeleton contractility via RhoA activation contribute to Eph/ephrin-mediated cell repulsion and invasion.
Effects of Ephs on MMPs and cell migration/invasion

In addition to the above mechanisms describing direct Eph/ephrin cleavage, Eph signaling can regulate other MMP functions such as degradation of extracellular matrix facilitating cell migration and invasion. In gastric carcinoma cells, silencing of EphA2 expression inhibits cell proliferation, invasion and expression of MMP 9 in vitro and in vivo, while active EphA2 promotes MMP9 expression so that it can cleave type-IV collagen, a major scaffolding molecule in the basement membrane, assisting invasion of metastatic cancer cells. Overexpression of EphA2 in pancreatic adenocarcinoma also increases MMP2 expression and FAK phosphorylation, which in turn leads to cellular invasion, although stimulation with recombinant ephrin-A1-Fc decreased cell invasion. Similarly, MMP2 is downregulated in ephrin-B2-Fc mediated EphB4 signaling in breast cancer cells, inactivating an oncogenic pathway involving Abl family tyrosine kinases and the Crk adaptor protein, and exerting a tumor suppressive effect by restraining cell motility and invasion. In this context MMP2 is most likely to degrade extracellular matrix facilitating cell invasion, rather than directly shedding ephrinB ligands. Conversely, a previous study on endothelial cells demonstrated that EphB4/ephrin-B2 signaling mediates migration via the PI3K/Akt pathway, in which case MMP2/9 expression was increased and thought to facilitate migration by degrading collagen within the basement membrane.

Regulated intramembrane proteolysis

Regulated intramembrane proteolysis (RIP) describes a processing event that often follows ectodomain shedding in which an integral membrane protein is cleaved within its transmembrane domain to yield a soluble protein fragment. The initial shedding, generally mediated by MMPs or ADAMs, or by the aspartyl proteases BACE1 and BACE2 (β-site APP cleaving enzymes), is a prerequisite for the second cleavage. The proteases that catalyze RIP are known as

Figure 3. MMPs in Eph and/or ephrin shedding. (A) Ephrin-B1 ectodomain cleavage by MMP8, which is enhanced upon interaction with the EphB2 receptor. Stimulation of ephrin-B1 by EphB2 activates the Arf1 GTPase, a critical regulator of membrane trafficking, most likely increasing the exocytosis of MMP8. The ephrin-B1 C-terminus also activates RhoA by binding to the adapter protein Dishevelled (Dsh), facilitating cell-cell repulsion and invasion. (B) Ephrin-B1-induced EphB2 receptor activation and its cleavage by MMP 2/9 cause RhoA activity via recruitment and subsequent activation of FAK, regulating growth cone withdrawal and collapse. (C) Clusters of overexpressed EphA2 recruit MT1-MMP via its non-catalytic domains, mediating cleavage of EphA2 in cis at the fibronectin repeat type-III domain, stimulated by binding to ephrin-A1, and activating Src/Rho-mediated invasion.
intramembrane cleaving proteases (iCLiPs), and are currently represented by 3 distinct and evolutionarily conserved families: the aspartyl protease-like (including presenilin-dependent γ-secretase, signal peptide peptidase, and signal peptide peptidase-like), the zinc metalloproteinase site-2 protease, and the serine protease rhomboid family (rhomboid and PARL). There are 2 types of RIP; type 1 RIP utilizes the aspartyl protease-like family enzyme (eg. γ-secretase) to processes proteins whose carboxy terminus is in the cytosol; whereas type 2 RIP employs a zinc metalloproteinase site-2 protease to process proteins with their N-terminus in the cytosol.\(^{118}\)

RIP is employed by a large number of membrane proteins, implicated in a wide range of biological processes. To date, over 60 substrates have been identified, including growth factors, cytokines, receptors, cell adhesion proteins, signal peptides and viral proteins. These substrates include Notch, EpCAM, N-cadherin, E-cadherin, and the p75 neurotrophin receptor.\(^ {114,119}\) The canonical Notch signaling pathway is a prime example of RIP, where ligand binding to the ectodomain of the Notch receptor on neighboring cells triggers sequential cleavage of the ectodomain and the transmembrane domain by metalloproteases and γ-secretase, respectively.\(^ {113}\) The released intracellular domain is then translocated to the nucleus where it regulates gene transcription by binding to transcription factors.\(^ {120}\) RTK signaling can also involve RIP, including the ErbB4 receptor.\(^ {121}\) There is some evidence suggesting Eph receptors/ligands undergo intramembrane processing, although sometimes atypical of standard, ligand-induced RIP.

\section*{γ-secretase}

γ-secretase is a membrane-embedded proteolytic complex consisting of 4 subunits; Presenilin (PS), Nicastrin, Aph1, and Pen-2. Presenilin is endoproteolytically cleaved to form 2 fragments, one of which assembles with the other 3 subunits to make the γ-secretase complex, with Presenilin residing at the catalytic core of the protease complex.\(^ {122}\) Together this complex manages the intramembranous cleavage of transmembrane proteins such as APP and Notch, following ectodomain cleavage.\(^ {123}\)

Ephrin-B2 was first identified to undergo RIP-like processing, where binding to EphB2 stimulates metalloprotease cleavage of ephrin-B2, yielding a membrane bound cytoplasmic fragment (CTF1) which is subsequently cleaved by PS1-dependent γ-secretases to produce a soluble CTF (CTF2) of ephrin-B2.\(^ {98}\) Thus PS1 Wt cells show less accumulation of membrane bound-ephrin-B2-CTF1 compared to PS1 knockout cells, and inhibiting γ-secretase accumulates membrane bound ephrin-B2 CTF. Stimulation of ephrin-B2 by EphB2 lead to detectable soluble ephrin-B2 CTF2 only in Wt fibroblasts, in which it also activated Src kinase, previously shown to mediate EphB-induced sprouting of endothelial cells, by inhibiting association with the inhibitory Csk kinase. This in turn facilitates phosphorylation of ephrin-B2 tyrosine residues.\(^ {98}\) A later study showed involvement of the Csk binding protein (PAG/Cbp), an adaptor protein that controls the activity of Src kinases.\(^ {123}\) Ephrin-B2-soluble CTF2 forms a complex with PAG/Cbp promoting Src activation,\(^ {124}\) which in other contexts has been shown to control various cellular events such as cell proliferation, survival, and migration\(^ {125}\) most likely via remodelling of the actin cytoskeleton.\(^ {126}\) (Fig. 4A; Table 1). Furthermore, PS1 mutants found in familial Alzheimer’s disease (AD) inhibit ephrin-B2 CTF cleavage as well as activation of Src, suggesting a potential role in disease.\(^ {98}\) Indeed, Src kinases play an important role in neuronal function and degeneration since their loss results in neuronal defects, abnormalities in the hippocampus, and long-term potentiation (LTP) impairment.\(^ {127,128}\)

The same group also found the EphB2 receptor to be processed by γ-secretase, via both ligand dependent and independent pathways. Interestingly, during ligand induced signaling (Fig. 4A), while the ephrin is sequentially processed by metalloprotease and γ-secretase (above), the receptor ectodomain cleavage occurs in endosomes, leads to receptor degradation, and is metalloprotease-independent. Cleavage was blocked by the BACE inhibitor peptide ZVLL but not other BACE inhibitors, so it is not clear what protease activity is responsible. In contrast, the ligand independent pathway (Fig. 4B) occurs in response to calcium influx and NMDA receptor activation, where broad metalloprotease and more selective ADAM10 inhibition prevents generation of the EphB2 CTF.\(^ {106}\) Analogous to ephrin-B2, brain extracts from PS1 knockout mice have increased accumulation of membrane-bound EphB2-CTF1, as do EphB2 transfected fibroblasts from PS1 -/- versus Wt mice, while the soluble EphB2 CTF2 is only detectable in Wt fibroblasts.\(^ {106}\) Thus ligand-independent and -dependent EphB2 signaling pathways involve distinct EphB2 ectodomain cleavage mechanisms, leading to cleavage by γ-secretase in distinct compartments.\(^ {106}\) Since neuronal ephrin-B/EphB interactions promote tyrosine phosphorylation of the NMDA receptor (NMDAR) and regulate NMDAR-dependent calcium influx and synaptic plasticity,\(^ {129}\) the authors suggest the EphB2-CTF may function in signaling cascades initiated both by EphB/ephrin-B binding or by activation of the NMDAR. Interestingly, unlike ephrin-B2, the effect of EphB2 CTF on NMDAR phosphorylation is apparently direct and independent of Src.\(^ {130}\) NMDA receptor signaling is also implicated in Alzheimer’s disease (AD), and these potential functions may be impaired by familial AD mutations in PS1 that inhibit production of both ephrin-B and EphB-soluble CTxFB.\(^ {131,132}\)

EphA4 was also identified as a γ-secretase target, by mass spectrometry analysis of proteins associated with γ-secretase purified from synaptic lipid raft membranes.\(^ {133}\) Indeed, EphA4 also contains a lysine/arginine motif preferred by γ-secretase, and production of EphA4-soluble CTF was blocked by γ-secretase inhibitors or siRNA depletion. γ-secretase and EphA4 co-localized at the synaptic lipid raft membrane, where synaptic activity increased formation of EphA4-soluble CTF. Like EphB2, the intramembrane cleavage followed EphA4 ectodomain cleavage which was ligand independent, but stimulated by calcium influx, and dependent on metalloprotease activity (Fig. 4B). In this situation, processing of EphA4 CTF by γ-secretase activates the Rac signaling pathway, contributing to the formation and maintenance of dendritic spines,\(^ {133}\) typical of a ligand-independent adhesive response.\(^ {8}\) Although soluble CTF was found in the nucleus, nuclear
Localization was not required for Rac signaling. Importantly, this process is also affected by PS1 mutation found in familial AD, reducing EphA4 CTF cleavage, and inhibiting dentritic spine formation. Interestingly, the occipital lobes of AD patients show significantly attenuated EphA4-soluble CTF levels and Rac1 signaling, indicating likely physiological importance of γ-secretase EphA4 cleavage.

**Serine Proteases**

Members of the serine protease super family have a conserved serine residue in their active site. Originally they were recognized for the presence of the Asp—His—Ser “charge relay” system or “catalytic triad,” although new catalytic triads and dyads have been discovered. Serine proteases reside in a wide range of tissues and biological fluids and participate in diverse biological activities such as blood coagulation, wound healing, digestion and immune responses. In addition, deregulated activation of this protease family contributes to disease, including tumor growth, invasion and metastasis.

**Rhombooids**

Rhombooids are intramembrane serine proteases that have been reported throughout the eukaryotes. Three major groups of Rhombooids have been described: Rhomboid-like proteases (active rhombooids), iRhoms (inactive rhombooids) and other inactive rhomboid-like proteins. Unlike most of the other serine proteases, Rhombooids use a catalytic dyad consisting of serine and histidine. Drosophila Rhomboid-1 cleaves the EGF-like growth factor Spitz, and similarly, human EGF is a substrate of the mammalian rhomboid RHBDL2, cleaving just outside its transmembrane domain, thereby facilitating the activation of the EGF receptor. Increased activity of RHBDL2 in tumors over-expressing the EGF receptor implicates its involvement in RTK receptor signaling and tumor growth. Based on a sequence motif at the luminal face of the membrane domain of Spitz, which determines its susceptibility to cleavage by RHBDL2, analysis of other type-I membrane proteins was performed to determine potential substrates. This identified ephrin-B1, with ephrin-B2 and B3 also potential candidates due to abundance of alanines or glycines in the luminal region, preferred by RHBDL2. Spitz/ephrin-B chimeric protein cleavage assays suggested that only ephrin-B3 is cleaved by RHBDL2, and overexpression of mammalian Rhomboid-1 or Rhomboid-2 together with ephrin-B3 resulted in efficient ephrin-B3 cleavage. The authors suggest that RHBDL2 cleavage...
of ephrin-B3 may represent an additional mechanism of Eph/ephrin mediated cell signaling.142

Neuropsin

Neuropsin is a secreted, multi-domain serine protease that belongs to the kallikrein-related endopeptidase 8 (KLK8) family,143 present in the limbic areas of the brain. Neuropsin exhibits the catalytic triad typical of other serine proteases and the overall structure is homologous to that of the chymotrypsin-type.144 Enzymatically active neuropsin has important functions in maintaining synaptic plasticity, by establishing the early phase of long-term potentiation (LTP). Since neuropsin deficiency completely inhibits the early phase of LTP, associations of Neuropsin between early and persistent-LTP synapses are speculated to be related to mammalian working memory and consequently integration in learning and memory.145

A recent report shows that neuropsin in the mouse brain is important for stress-related plasticity in the amygdala (a region involved in processing emotions).146 Stress initiates neuropsin-dependent cleavage of EphB2 in the amygdala, resulting in dissociation of EphB2 from the NR1 subunit of the NMDA receptor, thus inducing membrane turnover of EphB2 receptors. Usually, EphB2–NR1 interaction increases NMDA receptor signals, inducing the expression of the glucocorticoid receptor sensitivity controlling protein Fkbp5, which in turn leads to behavioral signatures of anxiety. Neuropsin-deficient mice do not show EphB2 cleavage and EphB2 dissociation from the NR1 subunit under stress, resulting in static EphB2-NR1 interaction that attenuates production of Fkbp5 and anxiety. Anxiety can be restored by introduction of Neuropsin into the amygdala of Neuropsin deficient mice. In addition, anxiety levels of wild-type mice can be suppressed by silencing the Fkbp5 gene in the amygdala, or remarkably, by injection of anti-EphB2 antibodies.146

Conclusion

It is clear from the reviewed literature that protease activity is becoming increasingly recognized as an important aspect in the regulation of Eph/ephrin signaling, beyond classical signal termination by proteosomal degradation. Thus different proteases contribute in diverse ways to facilitate both the opposing responses of cell-cell adhesion and retraction mediated by Eph/ephrin signaling. How these functions are regulated is only emerging, but it is clear that ligand-receptor interaction and ensuing bi-directional signaling are key to coordinate protease activity with changes in morphology and migration that follow Eph-driven cytoskeletal remodelling. ADAM10 is a prime example, where regulation likely occurs at multiple levels. Thus formation of a ligand-receptor complex across cell-cell junctions enables recognition by the ADAM10 substrate-binding domain, while the ensuing receptor activation likely leads to conformational changes in both Eph...
and ADAM that promote their association. This results in cleavage of ephrins and associated molecules from the opposing cell, breaking the tether between cells, while at the same time allowing internalisation of signaling complexes driving cytoskeletal collapse and cell retraction. These co-ordinated events are likely de-regulated in cancer and inflammatory diseases, where ADAMs and Ephs are often over-expressed, and high RTK and inflammatory signaling leads to oxidative conditions that favor protease activity. Similarly Eph/ephrin interaction appears to regulate their cleavage by MMPs, as well as cleavage of the extracellular matrix, although in the latter case ligand stimulation of Ephs can either promote or inhibit MMP-mediated cancer cell invasion. Lastly, ectodomain cleavage is commonly followed by intramembrane cleavage by gamma-secretase, releasing the cytoplasmic domain. This can occur in response to Eph/ephrin interaction, or to other stimuli, for example in neuronal cells where this function is implicated in Alzheimer’s disease. Thus protease regulation of Eph/ephrin signaling occurs at multiple levels and has broad significance for disease, and as these mechanisms become better understood, targeted inhibition of specific protease activities may provide a useful approach for the development of new therapies to block aberrant Eph/ephrin function.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
This review is dedicated to the memory of Martin Lackmann, an inspirational mentor and colleague who contributed much to the understanding of Eph/ephrin signaling, including key insights into the role and regulation of protease function.

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