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Intramembrane cleavage of ephrinB3 by the human rhomboid family protease, RHBDL2

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Abstract

Rhomboid-1 is a serine protease that cleaves the membrane domain of the *Drosophila* EGF-family protein, Spitz, to release a soluble growth factor. Several vertebrate rhomboid-like proteins have been identified, although their substrates and functions remain unknown. The human rhomboid, RHBDL2, cleaves the membrane domain of *Drosophila* Spitz when the proteins are co-expressed in mammalian cells. However, the membrane domains of several mammalian EGF-family proteins were not cleaved by RHBDL2, suggesting that the endogenous targets of the human protease are not EGF-related factors. We demonstrate that the amino acid sequence at the luminal face of the membrane domain of a substrate protein determines whether it is cleaved by RHBDL2. Based on this finding, we predicted B-type ephrins as potential RHBDL2 substrates. We found that one of these, ephrinB3, was cleaved so efficiently by the protease that little ephrinB3 was detected on the surface of cells co-expressing RHBDL2. These results raise the possibility that RHBDL2-mediated proteolytic processing may regulate intercellular interactions between ephrinB3 and eph receptors.

Keywords: Rhomboid; RHBDL2; Ephrin; Serine protease; Epidermal growth factor; Spitz

Intramembrane-cleaving proteases, or ICliPs [1], cleave substrate proteins within their membrane domains, leading to the release or relocation of intra- or intercellular signalling proteins [2–5]. Rhomboid-like proteins are a recently recognised ICliP family [6]. The family's founding member, Rhomboid-1, was identified as one of a group of genes that regulates signalling through the *Drosophila* EGF receptor (DER) [7]. Although Rhomboid-1 had been implicated in the activation of the DER ligand Spitz [8,9], the mechanism involved was not known. Recent work has shown that Rhomboid-1, together with a second protein, Star, mediates the release of a soluble form of Spitz from its membrane-anchored precursor protein [10–12]. Whereas Star enables the intracellular trafficking of Spitz, Rhomboid-1 is a Golgi-localised serine protease that cleaves the membrane domain of Spitz leading to the release of the growth factor through the secretory pathway [13]. Rhomboid-1 has seven predicted membrane domains and its catalytic triad comprises an asparagine, a serine, and a histidine residue located in membrane domains 2, 4, and 6, respectively [13].

Genes encoding rhomboid-like proteins have been identified in a wide variety of organisms including vertebrates [6]. Four mammalian rhomboids have been characterised: RHBDL1 (also known as RRP1) [14], RHBDL2, presenilins associated rhomboid-like protein (PARL) [15], and RHBDL4 (also known as ventrhoid) [16]. The biological functions of these mammalian rhomboids remain to be determined. The conservation of the principal components of the EGF receptor signalling pathway between flies and mammals has led to an assumption that rhomboid proteases will also play a role in the release of mammalian EGF-family factors. Furthermore, it has been shown that human RHBDL2, like *Drosophila* Rhomboid-1, efficiently cleaves the membrane-anchored DER ligand, Spitz [13]. In this study, we have tested the ability of RHBDL2 to cleave the membrane domains of six members of the mammalian EGF family. We found that none of these were cleaved by the protease, suggesting that these

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mammalian growth factors are not released from cells by RHBDL2-mediated processing. We demonstrate that the amino acid sequence at the luminal face of a substrate's membrane domain is important for cleavage by RHBDL2. Based on this finding, we have identified membrane-anchored B-type ephrins as candidate endogenous substrates for human RHBDL2.

Materials and methods

DNA constructs

Substrates. pMyc-Spitz comprises pcDNA3 containing an insert encoding the full-length Spitz protein with five copies of a myc tag inserted between amino acids 30 and 31 [9,11]. pSpi/HB comprises pcDNA3 containing an insert encoding amino acids 1–140 of Spitz with the 5× myc tag between amino acids 30 and 31, followed by a *Bam* HI linker sequence encoding Gly–Ser, and amino acids 162–208 of rat HB-EGF [11]. pSpi/HB-A encodes amino acids 1–133 of Spitz (including the 5× myc tag), followed by a *Bam*HI linker sequence encoding Gly–Ser, and then amino acids 156–208 of rat HB-EGF.

Other fusion proteins comprise the extracellular domain of Spitz linked to the membrane and cytoplasmic domains of candidate rhomboid substrates. These were derived from plasmid pSpi/HB-A by replacement of the HB-EGF fragment with a PCR-derived DNA sequence encoding the protein fragment of interest. These proteins encode amino acids 1–133 of Spitz (including a 5× myc tag), followed by a *Bam*HI linker sequence encoding Gly–Ser, and then one of the following: amino acids 1034–1217 of mouse epidermal growth factor (pSpi/EGF), amino acids 94–159 of mouse transforming growth factor-α (pSpi/TGF), amino acids 195–252 of human amphiregulin (pSpi/AR), amino acids 116–178 of human betacellulin (pSpi/BTC), amino acids 114–169 of human epiregulin (pSpi/EPI), amino acids 237–346 of human ephrinB1 (pSpi/EphrinB1), amino acids 224–333 of human ephrinB2 (pSpi/EphrinB2), and amino acids 219–340 of human ephrinB3 (pSpi/EphrinB3).

Parental cDNA stocks used to prepare the PCR derived fragments were from clones previously generated in our laboratory (EGF, TGF-α, HB-EGF) or were expressed sequence tags purchased from the IMAGE consortium of the MRC Geneservice, Cambridge, UK. The IMAGE numbers are as follows: amphiregulin, 4277616; betacellulin, 3952444; epiregulin, 1375478, ephrinB1, 6578335; ephrinB2, 2435541; and ephrinB3, 5286243. The betacellulin clone contained a mutation within its membrane domain (Leu^{124} to Met) compared with the previously published sequence [17]. This was reverted using PCR-based mutagenesis.

A clone expressing a form of ephrinB3 was generated using PCR. To simplify epitope tagging, the ephrinB3 signal peptide sequence was replaced with that from Spitz. Briefly, a DNA fragment encoding amino acids 1–30 of Spitz, together with the following 5× myc tag, was amplified from pMyc-Spitz. This was linked through **a** *Bam*HI linker sequence encoding Asp-Pro, to a DNA fragment encoding amino-acids 29–340 of ephrinB3 to generate pEphrinB3. To generate C-terminally truncated ephrinB3 constructs, the ephrinB3 fragment was excised from plasmid pEphrinB3 using *Bam*HI and *Eco*RI and replaced by *Bam*HI–*Eco*RI flanked PCR products encoding amino-acids 29–214, 29–226, or 29–250 of ephrin-B3 to generate pEphrinB3-R214, pEphrinB3-P226 or pEphrinB3-C250, respectively.

Rhomboids. All rhomboids were cloned into plasmid pCANHA2, a derivative of pcDNA3 with a HA tag inserted in the multi-cloning site. Plasmid pHA-Rho1 was derived by transferring the insert from plasmid pRhomboid [11] into pCANHA2. pHA-RHBDL1 encodes human RHBDL1 (also known as RRP1) [14]. This protein has a different N-terminal sequence from that which we previously reported [14], as the original sequence coded either for a minor alternatively spliced variant or, more likely, represented a partially spliced cDNA. The revised sequence has accession number CAC00640. pHA-RHBDL2 encodes human RHBDL2. The protein sequence is similar to that described [13], but is extended N-terminally based on our analysis of RHBDL2 cDNAs. The sequence is described under accession number AAM95697. pHA-$RHBDL2^{S187G}$ was derived from pHA-RHBDL2 by PCR-based mutation of Ser^{187} to Gly. pHA-RHBDL4 encodes the human rhomboid also termed ventrhoid [16].

All PCR-derived plasmid inserts were sequenced to confirm that no errors had been introduced during PCR amplification. Additional details regarding construct generation, including PCR primer sequences, can be obtained on request from the authors.

Cell culture and transfections

HEK293T cells were maintained in Dulbecco's modified Eagle's medium containing 100 i.u. penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal calf serum. Cells for transfection were seeded on 35-mm culture dishes (Nunc) that had been pretreated with poly-L-lysine. After 24–48 h, the cultures were transfected with plasmid DNA using Lipofectamine (Invitrogen) as described [11]. Cells were transfected with a DNA mixture generally containing 0.5 μg of a myc-tagged substrate protein construct, with or without 0.5 μg pStar [11] and 0.1 μg of a HA-tagged rhomboid construct as indicated in the figure legends. Plasmid pcDNA3 was included to maintain the total input DNA at 2 μg. Cells and medium were harvested for analysis of immunoreactive proteins approximately 24 h after the start of transfection.

Biotinylation of cell surface proteins

The surface proteins of transfected HEK293T cells were biotinylated using 0.4 mg/ml sulpho-*N*-hydroxysuccinimidobiotin (EZ-link; Pierce) as described [11]. Biotinylated proteins were recovered from cell lysates using streptavidin–agarose beads and myc-tagged proteins were detected by Western blotting.

Western blot analysis

Media were removed from the cells and myc-tagged proteins were immunoprecipitated, separated by SDS–PAGE (10% (w/v) resolving gel), and transferred to Immobilon P (Millipore) membranes [11]. The myc-tagged proteins were detected by Western blotting using a 1:10 dilution of a hybridoma supernatant of monoclonal antibody 9E10 as described [11]. Immediately after removal of the media samples, the transfected cells were lysed into 1 ml RIPA buffer (50 mM Tris/HCl, pH 8.0/150 mM NaCl/1% (v/v) Nonidet P40/0.5% (w/v) sodium deoxycholate/0.1% (w/v) SDS) containing 100 μg/ml PMSF and 1 mM EDTA. The lysates were centrifuged at 13,000*g* for 5 min at 4 °C. An equal volume of 2× SDS sample buffer (160 mM Tris/HCl, pH 6.8/2% (w/v) SDS/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol) was added to the supernatant. Two aliquots of each cell lysate were analysed by Western blotting. One was heated at 100 °C for 3 min, proteins were separated by SDS–PAGE on 10% resolving gels, and myc-tagged proteins were detected by Western blotting as described above. Proteins in the second aliquot were separated by SDS–PAGE and transferred to Immobilon-P, but without prior heating of the sample. To detect HA-tagged rhomboid proteins, the membrane was incubated overnight at 4 °C with a 1:10 dilution of a hybridoma supernatant of monoclonal antibody 12CA5. Immunoreactive proteins were detected using a 1:2500 dilution of sheep anti-mouse Ig conjugated with horseradish peroxidase followed by enhanced chemiluminescence (ECL, Amersham Biosciences) according to the manufacturer's instructions.

Results

Substrate determinants of cleavage by RHBDL2

We have previously shown that *Drosophila* Rhomboid-1 cleaves a chimeric substrate, Spi/HB, comprising the extracellular domain of Spitz fused to the membrane and cytoplasmic domains of the mammalian EGF receptor ligand, HB-EGF [11]. Our new results show that human RHBDL2 also cleaves the Spi/HB chimeric protein releasing the soluble Spitz extracellular domain into the medium (Fig. 1B, lanes 1 and 2). Since the extracellular domain of Spitz is highly glycosylated [8,13], the largest form of the Spi/HB protein (53 kDa) in cell lysates is presumably the uncleaved, fully glycosylated form of the chimeric protein. The virtual disappearance of this 53 kDa protein from cells expressing RHBDL2 (Fig. 1B, lanes 1 and 2) indicates the high efficiency with which membrane-anchored Spi/HB is cleaved by the protease.

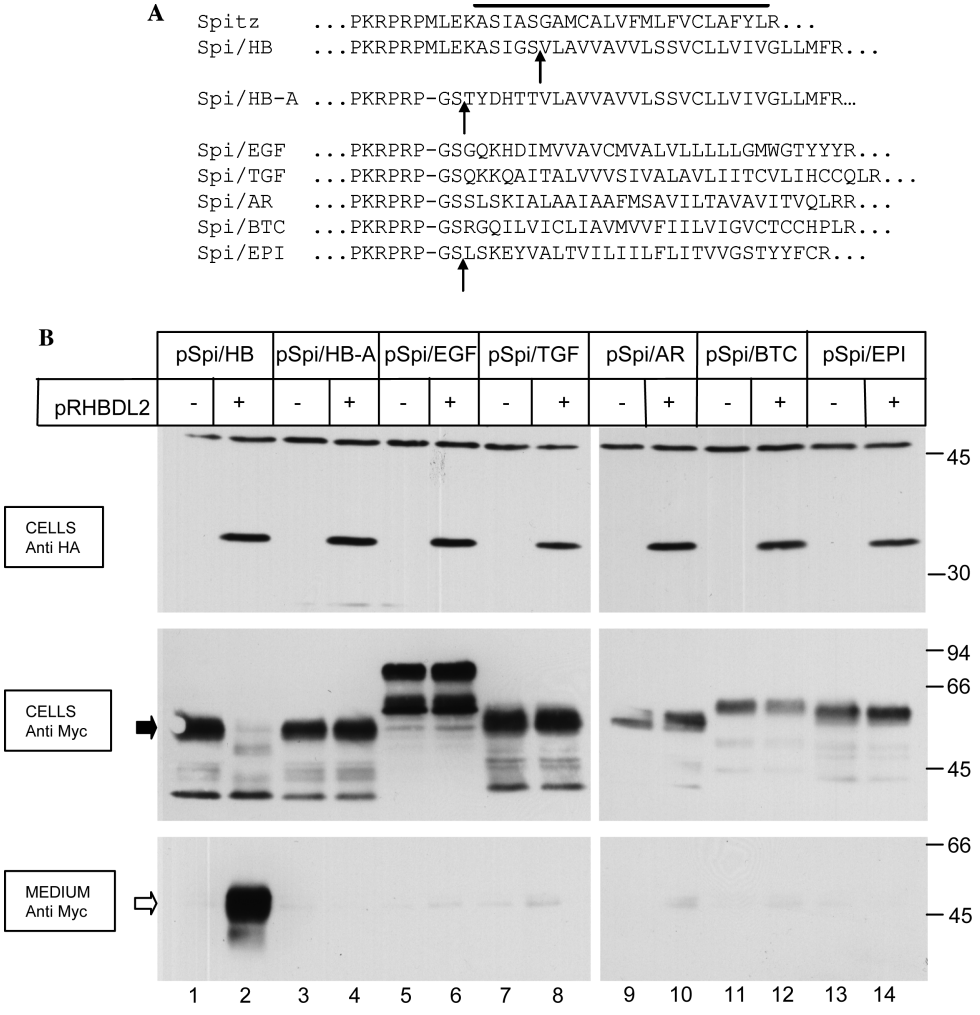

Fig. 1. The membrane domains of mammalian EGF-family members are not substrates for RHBDL2. (A) Comparison of the Spitz membrane domain (overlined) with those of fusion proteins in which the extracellular region of Spitz was linked to the membrane domain and cytoplasmic domains of mammalian EGF family proteins. Spi/HB comprises amino acids 1–140 of Spitz followed by a Gly–Ser linker and amino acids 162–208 of HB-EGF. Spi/HB-A comprises amino acids 1–133 of Spitz followed by a Gly–Ser linker and then amino acids 156–208 of HB-EGF. Details of the composition of the other chimeric proteins are given in the Materials and methods section. The fusion positions between Spitz and the EGF family proteins are indicated by arrows. All constructs contained a 5× myc tag inserted between amino acids 30 and 31 of the Spitz sequence. (B) HEK293T cells were transfected with 0.5 μg of plasmid DNA encoding the fusion proteins with or without 0.1 μg pHA-RHBDL2, which encodes HA-tagged RHBDL2. All transfections included 0.5 μg pStar. pcDNA3 was used to maintain the total input DNA at 2 μg. After 24 h, the medium was removed and immunoprecipitated with the 9E10 myc antibody. Cell lysates were prepared as described in the Materials and methods section. Proteins in the cell and medium samples were analysed by Western blotting using the 9E10 (myc) or 12CA5 (HA) antibody. The migration positions of molecular weight marker proteins are indicated in kDa. In the HA-blot, the band migrating above the 45 kDa marker corresponds to an endogenous cellular protein that reacts with the antibody. Note that the amount of the uncleaved, fully glycosylated form of Spi/HB (closed arrow) was markedly reduced in the lysates of cells co-expressing RHBDL2. The open arrow indicates the soluble product released from cells to the medium following Spi/HB cleavage.

The finding that Spi/HB is cleaved by RHBDL2 might, at first sight, suggests that the protease is able to cleave the membrane domain of HB-EGF. However, inspection of the Spi/HB sequence shows that, in addition to the Spitz extracellular domain, several amino acids predicted to be at the luminal face of the Spitz membrane domain are present in the fusion protein (Fig. 1A). Since cleavage of Spitz by Rhomboid-1 most likely occurs in this region [13], it seemed possible that cleavage of the chimeric substrate depends on the presence of these Spitz-derived residues. To test this possibility, we constructed a second fusion construct, Spi/HB-A, in which the junction between the proteins was moved further into the extracellular domain of Spitz thus eliminating any Spitz contribution to the membrane spanning domain (Fig. 1A). RHBDL2 was unable to cleave this protein as indicated by the absence of cleaved product in the medium, and by the absence of any change in the level of uncleaved 53 kDa protein in the cell lysate (Fig. 1B, lanes 3 and 4). These results strongly suggest that the amino acid sequence at the luminal face of a protein's membrane domain determines its susceptibility to cleavage by RHBDL2. During the course of this work, Urban and Freeman [18] reported a similar finding for Spitz cleavage by *Drosophila* Rhomboid-1. Based on an extensive mutational analysis, they concluded that the protease does not recognise a specific amino acid sequence, but targets a partially disordered conformation that results from the presence of several helix destabilising residues, particularly glycines and alanines, in the luminal region of the Spitz membrane domain.

The membrane domains of mammalian EGF receptor ligands are not cleaved by RHBDL2

None of the membrane domains of the mammalian EGF receptor ligands show obvious sequence similarities with the membrane domain of *Drosophila* Spitz (see Fig. 1A), Moreover, with the exception of amphiregulin, none of the membrane domains have a preponderance of glycine or alanines in their luminal region. Nevertheless, because of the ability of RHBDL2 to efficiently cleave Spitz [13], we wished to test whether the protease can cleave any of the mammalian growth factors. When we replaced the HB-EGF membrane and cytoplasmic domains in Spi/HB-A with the corresponding regions of EGF, TGF-α, amphiregulin, betacellulin, or epiregulin, we were unable to demonstrate activity of RHBDL2 towards any of the chimeric proteins (Fig. 1B, lanes 5–14). These findings suggest that these EGF family members are not endogenous substrates for RHBDL2 and emphasise the importance of the identification of substrates in seeking to understand the biological role of the protease.

Cleavage of B-type ephrins by RHBDL2

The efficient cleavage of Spi/HB, together with the lack of cleavage of Spi/HB-A, indicates that the short sequence at the luminal face of the membrane domain of Spitz determines its susceptibility to cleavage by RHBDL2. To predict candidate substrates for RHBDL2, we searched

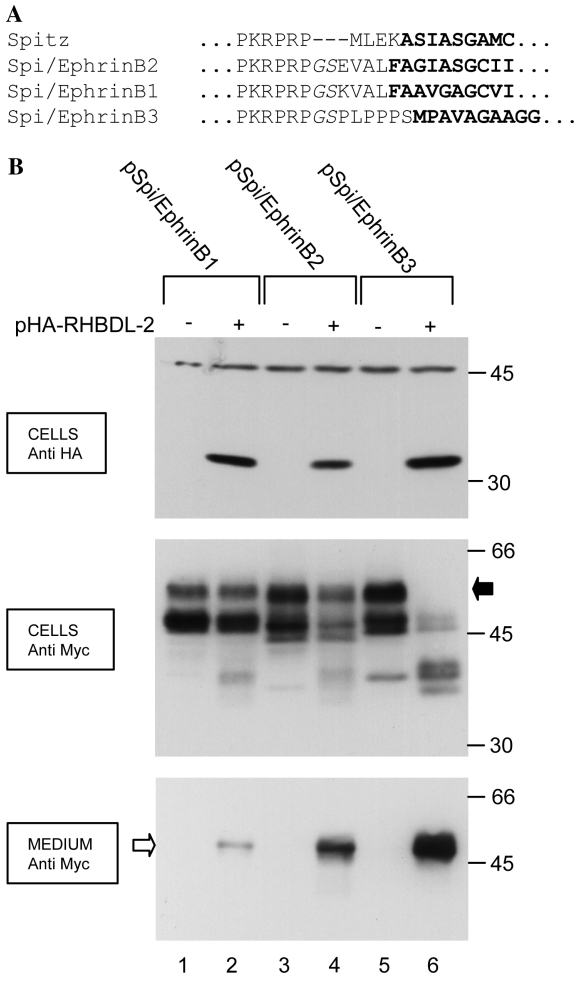

Fig. 2. Chimeric proteins containing the membrane domains of B-type ephrins are cleaved by RHBDL2. (A) Sequence at the junctions of chimeric proteins comprising the extracellular domain of Spitz linked to the membrane and cytoplasmic domains of human B-type ephrins. The chimeric proteins are composed of amino acids 1–133 of Spitz with a 5× myc tag between residues 30 and 31, followed by a Gly–Ser linker (italics), and then amino acids 237–346 of ephrinB1, or 224–333 of ephrinB2, or 219–340 of ephrin B3. The membrane domains of Spitz and the ephrins were predicted using the TMHMM programme [29] and their luminal regions are shown in bold letters. (B) HEK293T cells were transfected with 0.5 μg of the indicated myc-tagged Spi/Ephrin construct, 0.5 μg pStar, with or without 0.1 μg pHA-RHBDL2. Total transfected DNA was maintained at 2 μg with pcDNA3. After 24 h, cells and media were harvested and analysed as described in the legend to Fig. 1. Note that the level of uncleaved, fully glycosylated Spi/EphrinB3 (closed arrow) was markedly decreased in the lysates of cells co-expressing RHBDL2. The open arrow indicates the soluble products released from cells to the medium following cleavage of the Spi/Ephrin fusion proteins.

for human type-I membrane proteins with sequences at the luminal face of their membrane domain that are similar to this region of Spitz. We identified ephrin-B2, with five of six residues identical to those in Spitz (Fig. 2A). Interestingly, while the membrane domains of the other B-type ephrins (B1 and B3) do not show such a clear sequence similarity with that of Spitz, both have a preponderance of alanines or glycines in their luminal region raising the possibility that all three proteins may be cleaved by RHBDL2.

In order to determine whether RHBDL2 is able to cleave the membrane domains of the three B-type ephrins we generated chimeric proteins in which the membrane and cytoplasmic domains of the ephrins replaced the HB-EGF component of Spi/HB-A. We expressed each of these chimeric proteins with RHBDL2 in HEK cells and looked for cleaved substrate in the medium harvested from the cells. A small amount of cleaved product was detected in the medium from cells expressing Spi/ephrinB1, suggesting that this protein is cleaved by RHBDL2 with low efficiency (Fig. 2B, bottom panel, lane 2). Much larger amounts of cleaved products were present in medium from cells expressing Spi/ephrinB2 or Spi/ephrinB3, indicating that these proteins are more efficiently cleaved by the rhomboid (Fig. 2B, bottom panel, lanes 4 and 6). Indeed, the processing of Spi/ephrinB3 appears to be almost quantitative as little of the uncleaved, fully glycosylated form of this protein was detected in lysates of cells co-expressing RHBDL2 (Fig. 2B, middle panel, lane 6).

We tested the ability of several rhomboids to cleave Spi/ephrinB3. *Drosophila* Rhomboid-1 was found to cleave this substrate (Fig. 3, lane 2), consistent with the finding that Rhomboid-1 and RHBDL2 have related substrate specificities as indicated by their common ability to cleave Spitz [13]. In contrast, the human rhomboids RHBDL1 and RHBDL4 did not cleave Spi/ephrinB3 (Fig. 3, lanes 4 and 5). Interestingly, RHBDL1 and RHBDL4 conserve all of the amino acids previously shown to be necessary for cleavage of Spitz by either Rhomboid-1 or RHBDL2 [13], indicating that

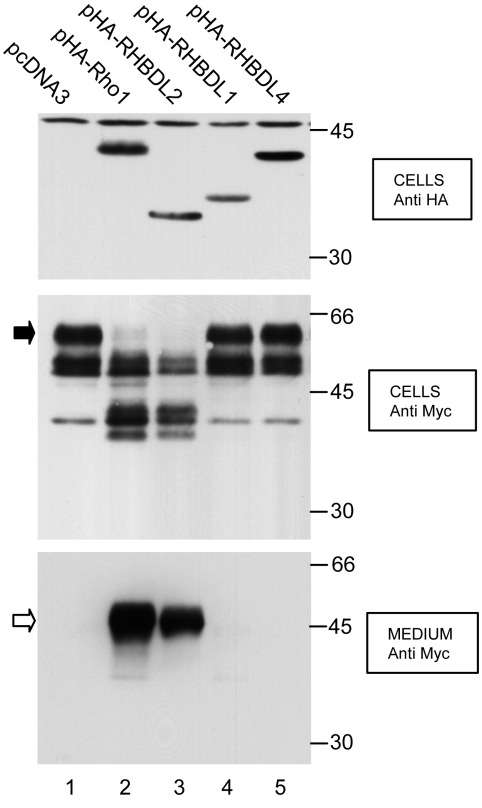

Fig. 3. Specificity of Spi/ephrinB3 cleavage by rhomboid proteases. HEK293T cells were transfected with 0.5 μg pSpi/EphrinB3, 0.5 μg pStar, with or without 0.1 μg of the indicated rhomboid constructs. pHA-Rho1 encodes *Drosophila* Rhomboid-1. pHA-RHBDL1, pHA-RHBDL2, and pHA-RHBDL4 encode human rhomboid-like proteins 1, 2, and 4, respectively. Total transfected DNA was maintained at 2 μg with pcDNA3. After 24 h, cells and media were harvested and analysed as described in the legend to Fig. 1. The closed arrow indicates the uncleaved, fully glycosylated form of Spi/EphrinB3 in cell lysates, and the open arrow indicates the soluble product released to the medium following Spi/EphrinB3 cleavage.

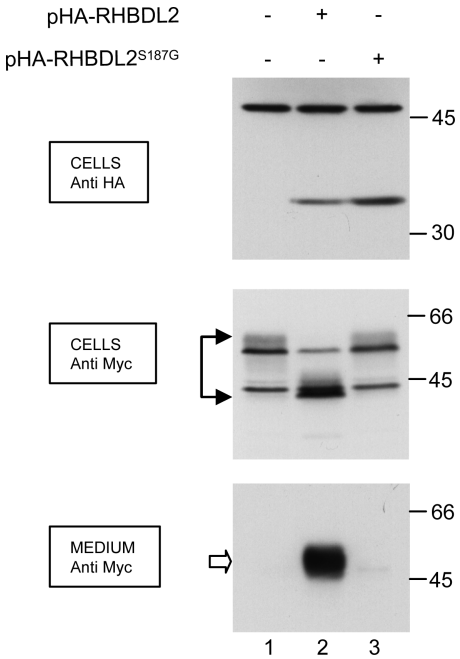

Fig. 4. EphrinB3 is a substrate for RHBDL2. HEK293T cells were transfected with 0.5 μg pEphrinB3 (see the Materials and methods section for a description of the encoded protein), with either 0.1 μg pHA-RHBDL2 or 0.2 μg pHA-RHBDL2^{S187G}, a plasmid that encodes an inactive RHBDL2 mutant. Total transfected DNA was maintained at 2 μg with pcDNA3. After 24 h, cells and media were harvested and analysed as described in the legend to Fig. 1. The bracketed arrows indicate the forms of ephrinB3 present in cell lysates. The open arrow indicates the soluble product released from cells to the medium following ephrinB3 cleavage.

additional determinants of rhomboid substrate specificity remain to be established.

Having established that the Spi/ephrinB3 chimeric protein is efficiently cleaved by RHBDL2, it was important to determine whether ephrinB3 itself was a substrate for the protease. To do this, we generated a construct encoding an essentially full-length form of ephrinB3 except that, to simplify tagging with a 5× myc epitope, the ephrinB3 signal peptide was replaced with that of Spitz. This protein was efficiently cleaved by RHBDL2 as shown by the large amount of cleaved product released to the medium, and by the marked reduction in the levels of uncleaved ephrinB3 present in cell lysates (Fig. 4, lane 2). This cleavage is dependent on RHBDL2 activity as shown by the absence of substrate cleavage in control cells (Fig. 4, lane 1), or in cells expressing an inactivated RHBDL2 in which the active site serine was mutated to glycine (Fig. 4, lane 3). Consistent with the similar substrate specificities of human RHBDL2 and *Drosophila* Rhomboid-1, the ephrinB3 protein was also cleaved by the latter protease (result not shown).

EphrinB3 appears to be cleaved in its membrane domain

In order to estimate the site of ephrinB3 cleavage, we prepared constructs encoding truncated forms of ephrinB3 terminating at R^{214}, P^{226}, and C^{250} (Fig. 5A). We compared the sizes of the proteins released from cells transfected with these constructs (in the absence of RHBDL2) with the size of the cleaved product released from cells co-expressing ephrinB3 and RHBDL2. The RHBDL2-cleaved product appears to be substantially larger than the ephrinB3-R214 protein and slightly larger than the ephrinB3-P226 protein (Fig. 5B). This result strongly suggests that ephrinB3 is cleaved in its membrane domain close to residue P^{226}, consistent with previous findings that Spitz cleavage occurs in the luminal region of its membrane domain [13,18]. The third truncated protein (ephrinB3-C250), which contains the whole of its predicted membrane domain, was not released to the culture medium in the absence of RHBDL2, indicating that the protein remains membrane-anchored. Interestingly, this protein, which lacks essentially all of its cytoplasmic domain, can undergo efficient cleavage by RHBDL2 to release a product of the same size as that released from the full-length ephrinB3 (Fig. 5C).

Many actions of ephrins are mediated by the interaction of the membrane-anchored cell surface proteins with their cognate eph receptors on adjacent cells [19,20]. The large reduction in the levels of uncleaved ephrinB3 in the lysates of cells co-expressing RHBDL2 suggests that this should lead to a marked decrease in

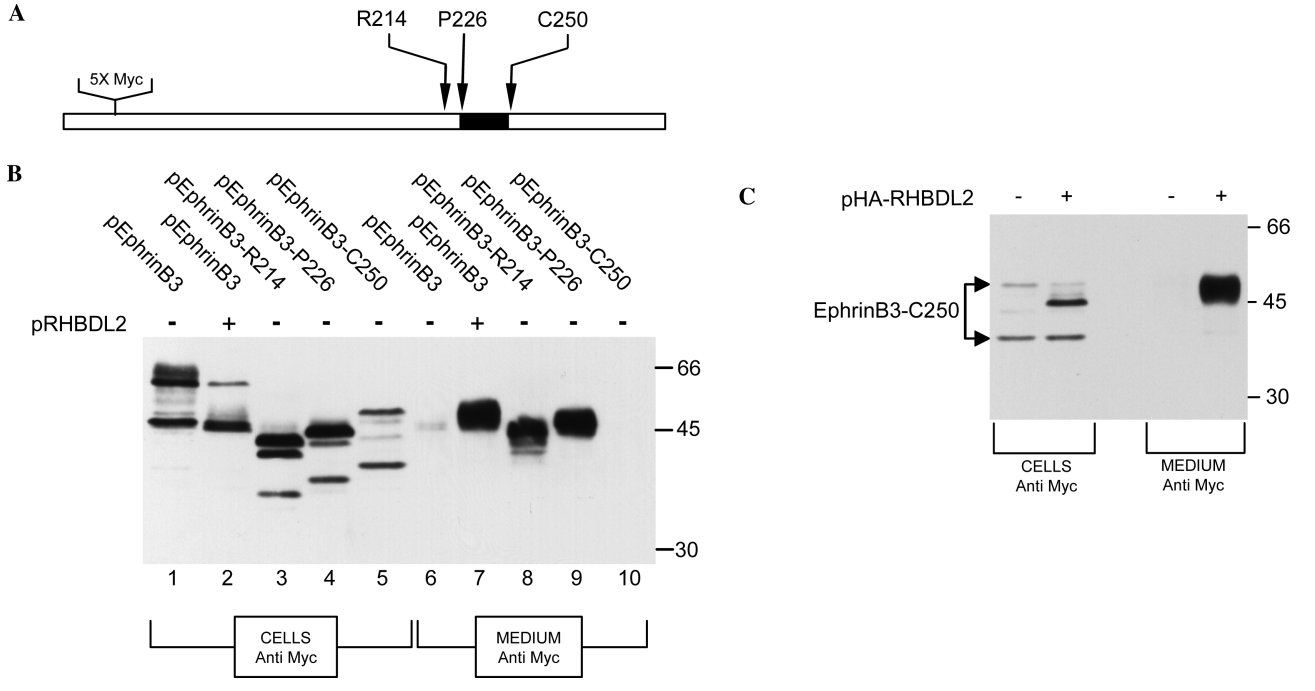

Fig. 5. Estimation of the site of ephrinB3 cleavage. (A) Diagram showing the truncation sites of the ephrinB3 derivatives terminating at amino acids R^{214}, P^{226}, and C^{250}. The ephrinB3 membrane domain, predicted by the TMHMM programme [29], spans residues 225–247 and is shown in black. (B) HEK293T cells were transfected with 0.5 μg pEphrinB3, pEphrinB3-R214, pEphrinB3-P226 or pEphrinB3-C250 with or without 0.1 μg pHA-RHBDL2. pcDNA3 was used to maintain the total input DNA at 2 μg. After 24 h, cells and media were harvested and analysed using the 9E10 myc antibody. (C) Cells were transfected with 0.5 μg pEphrinB3-C250 with or without 0.1 μg pHA-RHBDL2. pcDNA3 was used to maintain the total input DNA at 2 μg. After 24 h, cells and media were harvested and analysed using the 9E10 myc antibody.

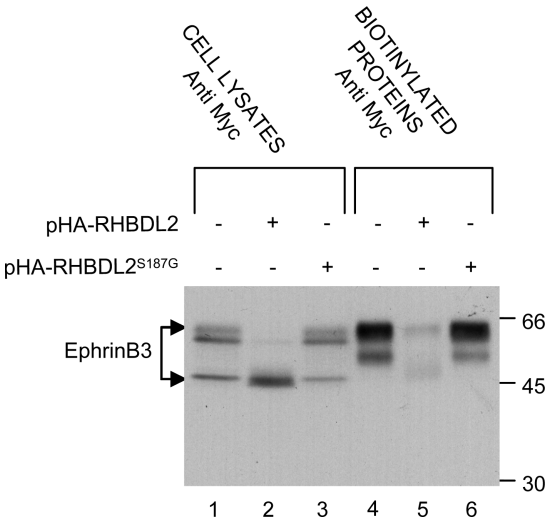

Fig. 6. EphrinB3 cleavage prevents its accumulation at the cell surface. HEK293T cells were transfected with 0.5 μg pEphrinB3, with or without 0.1 μg of pHA-RHBDL2 or 0.2 μg pHA-RHBDL2^{S187G}. pcDNA3 was used to maintain the total input DNA at 2 μg. After 24 h, cell surface proteins were biotinylated. Cell lysates were prepared and biotinylated proteins were recovered from the lysates using streptavidin–agarose beads. Myc-tagged ephrinB3 in the cell lysates and in the samples of biotinylated cell surface proteins were analysed by Western blotting using the 9E10 myc antibody.

the amount of intact ephrinB3 available on the surface of these cells. Using biotinylation analysis, we confirmed that little ephrinB3 is present at the surface of cells expressing RHBDL2 (Fig. 6). In contrast, expression of an inactive RHBDL2 mutant did not affect the levels of cell surface ephrinB3 (Fig. 6).

Discussion

Drosophila Rhomboid-1 cleaves the membrane domain of Spitz, an EGF-family protein, releasing a soluble form of the growth factor from cells [13]. Although vertebrate rhomboid-like proteins have been identified, their natural substrates remain unknown. Whilst the human rhomboid, RHBDL2, is capable of cleaving *Drosophila* Spitz, our current data suggest that this protease is unlikely to play a role in the release of mammalian EGF receptor ligands. It is, of course, possible that other mammalian rhomboids may mediate their release. However, current evidence suggests that metalloproteases of the ADAM family are the principal processing enzymes for the mammalian growth factors [21]. The ADAM proteases cleave substrates in their extracellular juxtamembrane regions rather than within their membrane domains [22].

The biological roles of the mammalian rhomboids may well, therefore, depend on the processing of substrates other than EGF-family proteins. The identification of these substrates is a critical step in elucidating the cellular processes mediated by rhomboids. Both human RHBDL2 and *Drosophila* Rhomboid-1 appear to recognise the luminal end of the Spitz membrane domain (Fig. 1 and [18]). We reasoned that mammalian proteins that have a similar motif in this region of their membrane domain might also be cleaved by RHBDL2. By this approach, we identified B-type ephrins as in vitro substrates of RHBDL2. The cleavage of one of these, ephrinB3, appears to be as efficient as the cleavage of Spitz by RHBDL2.

B-type ephrins are transmembrane ligands for Eph family receptor tyrosine kinases. Since both the ephrin ligand and Eph receptor are cell surface proteins, binding and activation depends on cell to cell interactions. Moreover, as well as activating Eph receptors, B-type ephrins are capable of reverse signalling into the cells that express them [23]. A common response to Eph/ephrin activation is the repulsion of neighbouring cells or cellular projections. Through this mechansism, ephrins guide cell migration and cell patterning and play multiple roles during development and in adult animals [19,20].

Two mechanisms have been reported to explain how cell–cell adhesion due to Eph/ephrin binding is destabilised to allow cell repulsion: first, the metalloprotease-mediated shedding of the GPI-anchored ephrinA2 [24], and second, the bi-directional endocytosis of full-length EphB/ephrinB complexes into cells [25,26]. Our results raise the possibility that RHBDL2-mediated cleavage of B-type ephrins, particularly ephrinB3, may provide an additional mechanism. This proposal presupposes that ephrin cleavage by RHBDL2 is regulated by EphB/ephrinB activation. Unfortunately, nothing is known about the signalling pathways that control either the activity or expression of RHBDL2.

During the preparation of this manuscript, Lohi et al. [27] reported that, by manual analysis of 1200 mouse proteins with predicted membrane domains, they identified 12 proteins with a putative Spitz-type recognition motif [18] in their membrane domains. The membrane domains of 11 of these proteins were not cleaved by RHBDL2. The membrane domain of one protein, thrombomodulin, was cleaved by the protease, although the authors noted that this was at significantly lower levels than Spitz cleavage [27]. Surprisingly, thrombomodulin was not cleaved by *Drosophila* Rhomboid-1, and extensive mutations in its membrane domain did not alter its cleavage by RHBDL2. Since the Spitz-like motif in the thrombomodulin membrane domain appeared not to direct its cleavage by RHBDL2, Lohi et al. [27] analysed the importance of other regions of the substrate. They found that the cytoplasmic domain of thrombomodulin was both necessary and sufficient for cleavage, and, in chimeric constructs, the cytoplasmic domain was able to confer cleavage by RHBDL2 on the membrane domains of proteins that were otherwise not

substrates. Thus, thrombomodulin is cleaved in its membrane domain by RHBDL2 by a distinct mode of substrate recognition that is directed by its cytoplasmic domain [27]. In contrast, the cleavage of ephrinB3 appears to conform to the Spitz-like mode of substrate recognition. The evidence for this is twofold: first, in contrast with thrombomodulin, ephrinB3 is cleaved not only by RHBDL2 but also by Rhomboid-1; second, the protein's cytoplasmic domain is not essential for substrate recognition by RHBDL2, as shown by the cleavage of the ephrinB3-C250 truncated protein.

The physiological relevance of the cleavage of B-type ephrins by RHBDL2 remains to be determined. In this context, it should be emphasised that, as reported here and by Lohi et al. [27], the membrane domains of many other mammalian proteins are not cleaved by RHBDL2. In addition, the cleavage of ephrinB3 is efficient and leads to a marked fall in cell surface ephrinB3 as well as to the release of soluble forms of the protein that could act as inhibitors of Eph/ephrin-mediated cell interactions [28]. Our results provide a basis for additional studies of the significance of rhomboid-mediated cleavage using various cellular and animal models of ephrin function.

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