Expression in mammalian cell cultures reveals interdependent, but distinct, functions for Star and Rhomboid proteins in the processing of the *Drosophila* transforming-growth-factor-α homologue Spitz

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We report here distinct interdependent functions for two proteins, Star and Rhomboid, that are key determinants of the epidermal-growth-factor (EGF)-receptor signalling pathway in *Drosophila*. When we expressed the *Drosophila* EGF-receptor ligand Spitz in mammalian cells, the protein failed to traffic to the plasma membrane, as assessed by either cell-surface protein biotinylation or immunocytochemical staining. However, when we co-expressed Star with Spitz, trafficking of Spitz to the cell surface could be demonstrated. Only when we co-expressed Spitz, Star and Rhomboid could the release of soluble Spitz protein into the medium be shown. Taken together, our results indicate that Star is required for the intracellular trafficking of Spitz, and that Rhomboid is essential for the release of soluble Spitz protein from cells.

Key words: epidermal growth factor, protease.

INTRODUCTION

The epidermal-growth-factor (EGF) family comprises a group of structurally related proteins that bind and activate ErbB receptor tyrosine kinases [1,2]. The family includes EGF, transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin [1,3], epiregulin [4] and multiple neuregulins [5]. Nearly all these factors are initially synthesized as glycosylated transmembrane precursor proteins that comprise an extracellular region (ectodomain), a hydrophobic membrane-spanning region and a cytoplasmic tail. The proteins' ectodomains contain a conserved six cysteine motif of 36–40 residues (EGF-like domain) that is required for binding to ErbB receptor tyrosine kinases [6]. In some cases, the intact membrane-anchored proteins appear to be able to activate EGF receptors on neighbouring cells, a so-called 'juxtacrine' mode of action [3]. However, soluble forms of the growth factors that act in a paracrine or autocrine manner can be released from cells by limited proteolytic cleavage of the extracellular region of the precursor proteins. Several studies have highlighted the importance of the release of soluble ligands by this so-called ectodomain shedding for the *in vitro* and *in vivo* activity of EGF-family factors [7–9].

In *Drosophila*, at least three EGF-related ligands, acting via a single EGF receptor, control numerous cell fate decisions throughout fly development [10–12]. The most widely used of these ligands is the Spitz protein, which, like most members of the mammalian EGF family, is a membrane protein with an EGF-like motif in its predicted ectodomain [13]. In several assays, the transmembrane form of Spitz has little activity compared with the effects of expression of a Spitz construct encoding a protein that lacks the cytoplasmic and membrane domains and is consequently secreted [14–16]. These results have led to the proposal that Spitz activation may normally occur by regulated proteolytic release of the protein's extracellular domain [10,11,17].

Drosophila EGF receptor signalling is positively regulated by the membrane proteins Star and Rhomboid [18]. Mutations in the *Spitz*, *Star* or *Rhomboid* genes give rise to similar phenotypes [19], and genetic analyses indicate that Star and Rhomboid act interdependently upstream of Spitz, leading to the proposal that their principal role is to promote Spitz-protein activation [10,11]. Recently, Bang and Kintner [16] used a *Xenopus* animal cap assay to obtain additional biochemical support for this proposal. They found that 'presentation' of cell-surface Spitz to its receptor required both Rhomboid and Star, although the function of each protein was not defined. Here we present evidence, based on expression of Spitz protein in mammalian cells, for distinct, interdependent functions of Rhomboid and Star; Star facilitates the intracellular trafficking of Spitz, and Rhomboid is additionally required for the release of soluble Spitz protein to the medium.

EXPERIMENTAL

DNA constructs

Plasmid CS2 Spitz^{myc}, which contains an insert encoding full-length Spitz protein containing five copies of a myc tag sequence [16], was a gift from Dr Anne Bang, Salk Institute, San Diego, CA, U.S.A. Plasmid pSpitz^{myc} was derived by transferring the insert from CS2 Spitz^{myc} to pcDNA3. Plasmid p(1–161)Spitz^{myc} was derived from pSpitz^{myc} by PCR insertion of a stop codon immediately downstream of Arg^{161}. Plasmid p(1–139)Spitz^{myc} was similarly derived by inserting a stop codon downstream of

Abbreviations used: EGF, epidermal growth factor; ER, endoplasmic reticulum; HB-EGF, heparin-binding EGF; TGF-α, transforming growth factor-α; DMEM, Dulbecco's modified Eagle's medium; ECL®, enhanced chemiluminescence detection system from Amersham Pharmacia Biotech (now Amersham Biosciences).

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Ser[139]. Plasmid pRHB-EGF contains an insert encoding the full-length rat HB-EGF precursor cloned into pcDNA3. pHB-EGF^{myc} was derived from pRHB-EGF by isolation of the pentamyc tag from pCS2 Spitz^{myc} by PCR and insertion of the tag into the *Pvu*II site within the rat HB-EGF cDNA between amino acids Gln[49] and Leu[50]. Plasmid pSpi^{myc}/HB encodes a fusion protein comprising Spitz (amino acids 1–140) followed by a glycine-serine linker sequence joined to amino acids 162–208 of rat HB-EGF and was derived from pHB-EGF^{myc} and pSpitz^{myc} by PCR. Plasmids containing inserts encoding *Drosophila* Rhomboid and Star proteins were a gift from Dr Matthew Freeman (MRC Laboratory of Molecular Biology, Cambridge, U.K.) and were transferred by PCR to pcDNA3 to derive plasmids pRhomboid and pStar respectively.

Cell culture and transfections

COS-7 and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal-calf serum. The day before transfection, cells were trypsin-treated and transferred to 35-mm-diameter culture dishes (for HEK293T cells, the dishes were pretreated with poly-L-lysine). After 24 h, when the cells had reached confluence, the cultures were transfected with plasmid DNA using LIPOFECTAMINE™ (Invitrogen) in serum-free, antibiotic-free medium. Cells were transfected with a total of 2 μg of plasmid DNA generally comprising myc-tagged protein construct (1 μg) and either pcDNA3 (1 μg) or pStar (1 μg). Where indicated, pRhomboid (0.1 μg) was added to the transfection mix and, in these experiments, an additional amount (0.1 μg) of pcDNA3 was added to the control transfections. At 5 h after transfection, the medium was supplemented with serum (10%, v/v). HEK-293T cells were analysed 24–48 h after the start of transfection. COS-7 cells were harvested from dishes 24 h after transfection, transferred to eight-well chamber slides (Permanox; Nunc) and maintained for a further 24 h in complete DMEM before immunocytochemical staining.

Cell-surface protein biotinylation and analysis

Transfected HEK293T cells were cooled to 4 °C and rinsed with 2 × 2 ml of ice-cold biotinylation buffer [50 mM Hepes (pH 7.5)/150 mM NaCl]. Cells were incubated in biotinylation buffer containing 0.2 mg/ml sulpho-*N*-hydroxysuccinimidobiotin ('sulfo-NHS-biotin'; EZ-Link™; Pierce) for 20 min at 4 °C with gentle agitation. The reaction was quenched by washing the cells with 3 × 2 ml of biotinylation buffer containing glycine (0.1 M). Cells were lysed into 1 ml of RIPA (radioimmunoprecipitation) 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 for 15 min at 4°C. The cell lysates were centrifuged at 13000 ***g*** for 5 min at 4 °C. A sample (50 μl) was removed and added to an equal volume of 2 × SDS/PAGE sample buffer [160 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol]. The samples were heated to 100 °C for 3 min, and then centrifuged (13000 ***g*** for 1 min). The residual cell-lysate samples and the media samples were transferred to tubes containing 20 μl (packed volume) of streptavidin–agarose beads. The samples were rotated at 4 °C for 1–2 h, the beads were recovered by brief centrifugation (13000 ***g*** for 30 s) and washed with 3 × 1 ml of RIPA buffer to remove non-biotinylated proteins. Bound proteins were recovered into 50 μl of SDS/PAGE sample buffer by heating at 100 °C for 3 min.

Aliquots of the cell lysates and biotinylated samples were subjected to SDS/PAGE [12.5% (w/v) resolving gel]. Proteins were then transferred to Immobilon-P membranes (Millipore). Membranes were incubated for 1 h at room temperature in PBS_a (138 mM NaCl/ 2.8 mM KCl/ 8 mM Na_2HPO_4/1.5 mM KH_2PO_4) containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat-milk powder. The membrane was incubated overnight at 4 °C in the same buffer containing a 1:10 dilution of a hybridoma culture supernatant of monoclonal antibody (9E10) to the myc epitope. Immunoreactive proteins were detected with a 1:2500 dilution of sheep anti-mouse Ig conjugated with horseradish peroxidase and the enhanced chemiluminescence (ECL®) detection system (Amersham Pharmacia Biotech, now Amersham Biosciences) according to the manufacturer's instructions.

Immunoprecipitation of myc-tagged proteins

Transfected cultures were cooled to 4 °C, the medium was removed to tubes and PMSF (100 μg/ml) and EDTA (1 mM) were added to the media samples. Cells were lysed into RIPA buffer containing PMSF (100 μg/ml) and EDTA (1 mM) and cell lysates and media samples were centrifuged at 13000 ***g*** for 5 min at 4 °C. The samples were then incubated with antibody 9E10 supernatant (50 μl/ml) at 4 °C for 2 h. The tubes were then centrifuged at 13000 ***g*** for 5 min at 4 °C and the supernatants were incubated for 2 h at 4 °C with 25 μl (packed volume) of Protein A–Sepharose beads (Sigma). Beads were recovered by brief centrifugation (13000 ***g*** for 30 s) and washed once with RIPA buffer. The immunoprecipitated proteins were recovered into 50 μl of SDS sample buffer by heating at 100 °C for 3 min. Aliquots of immunoprecipitated material were then analysed by SDS/PAGE and Western blotting as described above.

Immunocytochemical staining of Spitz protein

Transfected COS-7 cells were washed with PBS_a and fixed with 4% (w/v) paraformaldehyde in PBS_a for 25 min at room temperature. Cells were then rinsed in PBS_a, and permeabilized with 1% (v/v) Triton X-100 for 15 min at 4 °C. The cells were then rinsed with PBS_a and blocked with 5% (v/v) normal horse serum in PBS_a for 40 min at room temperature. The slides were incubated for 2 h at room temperature with 9E10 monoclonal antibody supernatant, either undiluted or diluted 1:2 in PBS_a. Cells were washed twice in PBS_a and incubated with a 1:50 dilution of FITC-conjugated goat anti-mouse IgG in PBS_a for 40 min at room temperature. The slides were washed with PBS_a, mounted with coverslips using a UV-free aqueous mountant obtained from TAAB Laboratories Equipment Ltd (Aldermaston, Berks., U.K.) and viewed using a Zeiss Axiophot2 UV fluorescence microscope.

RESULTS

Spitz protein does not traffic to the plasma membrane of HEK293T cells

We transfected a rat HB-EGF cDNA, with a myc tag in the protein's extracellular domain, into HEK293T cells. The presence of the expressed HB-EGF at the cell surface was demonstrated following biotinylation of cell-surface proteins using a membrane-impermeable reagent (Figure 1A, lanes 3 and 4). This result is consistent with previous findings that ectopically expressed HB-EGF is efficiently delivered to the plasma membrane [20,21]. In contrast, when cells were transfected with a construct encoding a similarly tagged Spitz protein, no cell-surface protein was detectable by biotinylation (Figure 1B, lanes 1 and 2). We were concerned that the failure of Spitz^{myc} to traffic to the plasma membrane might be due to an effect of an elevated

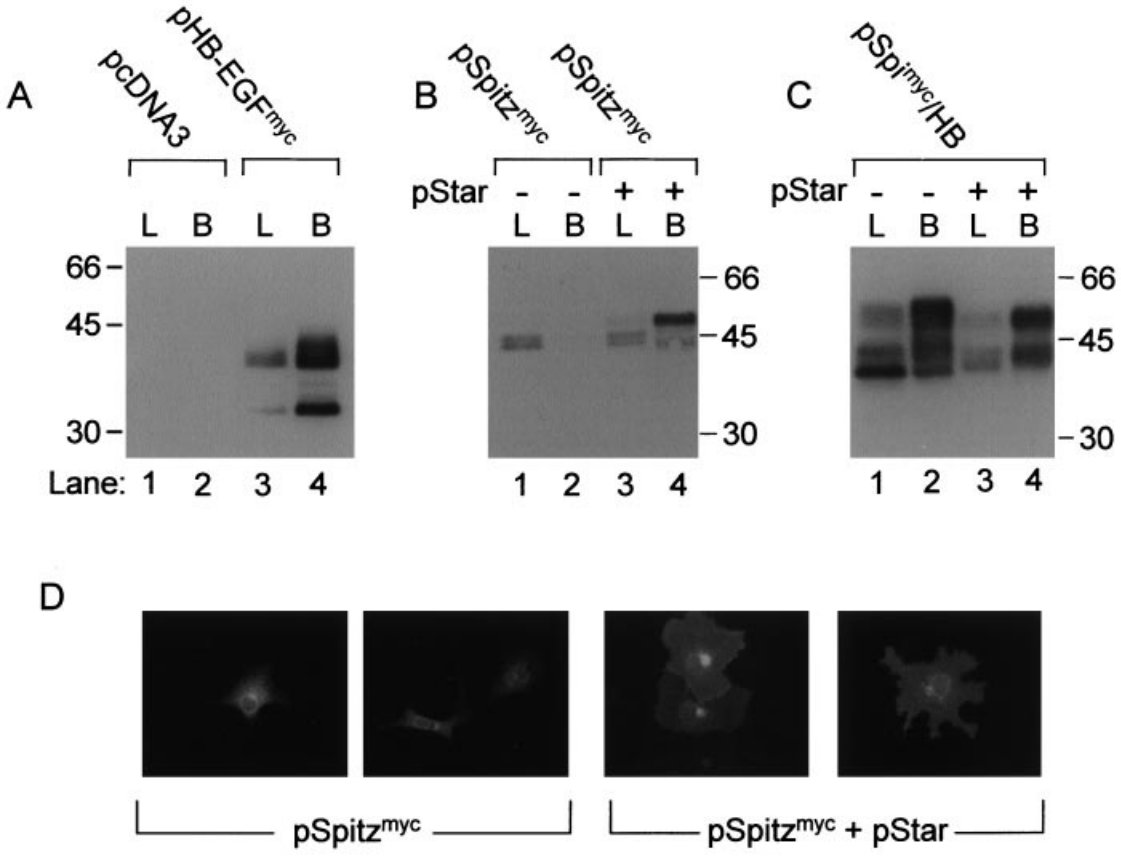

Figure 1 Star increases the delivery of Spitz to the plasma membrane

(**A**–**C**) HEK293T cells were transfected with pHBEGF^{myc}, pSpitz^{myc} or pSpi^{myc}/HB, with or without co-transfection of pStar as indicated. After 48 h (**A**) or 24 h (**B** and **C**), cell-surface proteins were biotinylated. Cell lysates were prepared and biotinylated proteins were recovered using streptavidin–agarose beads. Proteins in the cell lysates (L) and the biotinylated protein samples (B) were separated by SDS/PAGE and analysed by Western blotting using the 9E10 myc monoclonal antibody. The migration positions of molecular-mass marker proteins (kDa) are indicated. (**D**) COS-7 cells were transfected with pSpitz^{myc} together with either pcDNA3 or pStar. Cells were fixed and the Spitz^{myc} protein was detected by immunostaining as described in the Experimental section.

temperature on the *Drosophila* protein. We therefore performed an experiment in which cells were incubated at 28 °C (instead of 37 °C) following transfection, but again failed to detect Spitz^{myc} at the cell surface (results not shown).

Star promotes the delivery of Spitz to the plasma membrane of transfected cells

Genetic experiments have demonstrated that the Rhomboid and Star proteins play an important role in *Drosophila* EGF receptor signalling [10,11,17]. Although the precise mode of action of these two proteins remains unknown, it is likely that they are in some way required for the activation or 'presentation' of the Spitz ligand [16,22]. We therefore tested the effect of co-expression of Star or Rhomboid on the delivery of Spitz^{myc} to the plasma membrane of HEK293T cells. Biotinylated Spitz^{myc} was shown to be present on the surface of cells co-transfected with Star (Figure 1B, lanes 3 and 4), but not on the surface of cells co-transfected with Rhomboid (results not shown), suggesting that Star is required for the efficient trafficking of Spitz^{myc} to the plasma membrane. Since HB-EGF^{myc} and Spitz^{myc} contrast markedly in their requirement for Star for delivery to the cell surface, we expressed a chimaeric protein comprising the extracellular domain of Spitz and the transmembrane and cytoplasmic tail of HB-EGF. Like wild-type HB-EGF, the chimaeric protein was delivered to the plasma membrane independently of Star expression (Figure 1C).

The effect of Star on the trafficking of Spitz to the plasma membrane does not appear to be due to an increase in Spitz^{myc} expression levels, because the amount of Spitz^{myc} protein present in cell lysates was not markedly affected by co-expression of Star (Figure 1B, lanes 1 and 3; results not shown). However, expression of Star leads to the appearance of additional Spitz^{myc} proteins with increased molecular mass that appear to accumulate at the cell surface, as indicated by their preferential biotinylation (Figure 1B, lanes 3 and 4). Since Spitz is a glycoprotein [14], it seems likely that this increase in mass is due to glycosylation during Spitz trafficking.

To further investigate the effects of Star on the cellular localization of Spitz^{myc}, we performed immunostaining on transfected COS-7 cells. When cells were transfected with Spitz^{myc} cDNA alone, the expressed protein appeared to be localized to the endoplasmic reticulum (ER), with no detectable staining of the plasma membrane (Figure 1D, left panels). In contrast, in cells co-transfected with Star, the Spitz^{myc} staining pattern was markedly altered, with staining extending to the cell periphery indicative of plasma membrane protein (Figure 1D, right panels). Taken together, the biotinylation and immunostaining results indicate that very little Spitz^{myc} protein is delivered to the plasma membrane in the absence of Star.

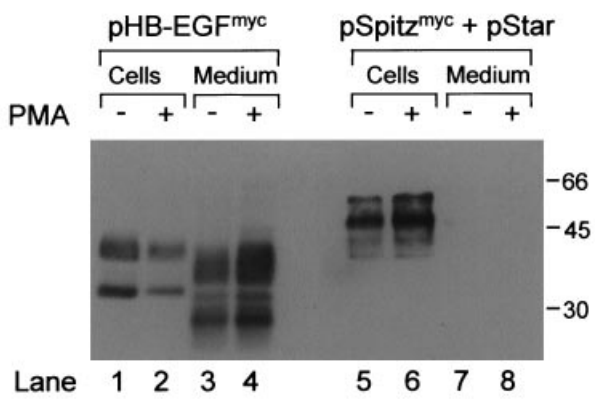

Figure 2 Cell-surface Spitz^{myc} does not undergo ectodomain shedding

HEK293T cells were transfected with pHB-EGF^{myc} or pSpitz^{myc} and pStar as indicated. After 48 h, cell-surface proteins were biotinylated. The cells were incubated in serum-free DMEM at 37 °C in the presence or absence of PMA (500 nM) for 60 min. The medium was collected, cell lysates were prepared and biotinylated proteins were recovered from both medium and lysate samples using streptavidin–agarose. Biotinylated proteins were separated by SDS/PAGE and analysed by Western Blotting using the 9E10 myc monoclonal antibody.

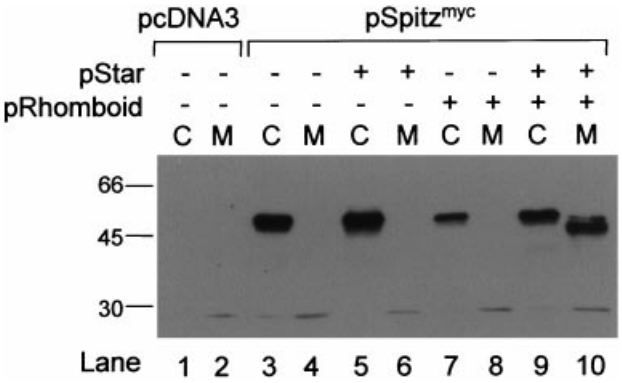

Figure 3 Rhomboid and Star are both required for the release of soluble Spitz from cells

Cells were transfected with pSpitz^{myc}, pStar and pRhomboid as indicated. After 24 h, cell lysates and conditioned-medium samples were immunoprecipitated with the 9E10 myc antibody. Immunoprecipitated proteins were analysed by Western Blotting using the same antibody.

Star and Rhomboid are required for the release of soluble Spitz protein from cells

Multiple forms of soluble HB-EGF are released from the cell surface by ectodomain shedding [20,23]. We observed high basal levels of soluble HB-EGF^{myc} in the bathing medium of transfected HEK293T cells and, consistent with previous findings [20,23], shedding was increased in cells treated with PMA (Figure 2, lanes 1–4). In contrast, we were unable to detect soluble Spitz^{myc} protein in medium from cells transfected to express Spitz^{myc} and Star. Furthermore, PMA treatment did not stimulate the release of soluble Spitz^{myc} from these cells. This result suggests that, although Star facilitates the trafficking of Spitz to the plasma membrane, cell-surface Spitz, in contrast with mammalian EGF-family proteins, is not susceptible to protein kinase C-mediated ectodomain shedding.

Since Rhomboid is known to act, together with Star, as a key regulator of EGF receptor signalling in *Drosophila*, we tested to see whether Rhomboid activated the release of soluble Spitz^{myc} protein. Cells were transfected to express Spitz^{myc} alone, or with Star, or with Rhomboid and Star. Spitz^{myc} protein was immunoprecipitated from transfected cell lysates and from cell-conditioned medium and detected by Western blotting (Figure 3). Soluble Spitz^{myc} was detected only in the medium of cultures transfected with Spitz^{myc}, Star and Rhomboid (Figure 3, lane 10). Rhomboid-mediated release of soluble Spitz was not inhibited by a metalloprotease inhibitor (BB-3103 from British Biotech, Oxford, U.K.) that potently inhibits HB-EGF shedding (results not shown).

Requirement for Star in Spitz protein release

Since we found that replacing the membrane and cytoplasmic domains of Spitz with the corresponding region of HB-EGF allowed the chimaeric protein to traffic in the absence of Star (Figure 1C), we tested the effect of simply removing the Spitz cytoplasmic domain on delivery of the protein to the cell surface. In cells transfected to express Spitz^{myc}-(1–161)-peptide, biotinylated protein was detected at the cell surface in the absence of Star co-expression (Figure 4A, lane 6). Since the chimaeric or truncated proteins traffic in the absence of Star, we predicted that expression of Rhomboid alone would be sufficient to cause the release of soluble Spitz from cells expressing these proteins. We therefore tested the effects of Rhomboid, in the presence or absence of Star, on the release of soluble Spitz from cells transfected with p(1–161)Spitz^{myc} or pSpi^{myc}/HB. Surprisingly, little soluble Spitz was released in response to expression of Rhomboid alone; both Star and Rhomboid expression were necessary to mediate the efficient release of Spitz protein from the transfected cells (Figure 4B).

DISCUSSION

Genetic studies in *Drosophila* have demonstrated a requirement for the Star and Rhomboid proteins in signalling through the EGF-receptor pathway. Whilst the precise functions of these proteins are unclear, recent work by Bang and Kintner [16] using a *Xenopus* cap assay has provided evidence that they facilitate the 'presentation' of the Spitz ligand to its receptor. In the present study we have identified distinct functions for Star and Rhomboid. Star facilitates Spitz trafficking through the cell from an apparently ER location, and Rhomboid, together with Star, is required for Spitz release from the cell.

Our results are consistent both with those obtained in the studies in *Drosophila* [18], and the findings of Bang and Kintner [16], that both Star and Rhomboid are required for maximal activation of the EGF receptor pathway by Spitz ligand. However, Bang and Kintner [16] found that Spitz was present at the surface of *Xenopus* cells in the absence of Star, and that the amount expressed at the cell surface was not affected by the presence of Star and Rhomboid. In contrast, in the absence of Star, we found little if any Spitz protein present at the cell surface, and that Star mediates the delivery of Spitz to the plasma membrane. A possible explanation for this difference is that a protein is present in *Xenopus* cells that substitutes for *Drosophila* Star, whereas no functional protein is present in mammalian cells.

Removal of the cytoplasmic domain of Spitz (Figure 4), or substitution of the transmembrane and cytoplasmic domains of Spitz by the corresponding regions of HB-EGF (Figure 1), allows Spitz to traffic to the plasma membrane in the absence of Star. This implies that the intracellular domain of Spitz mediates

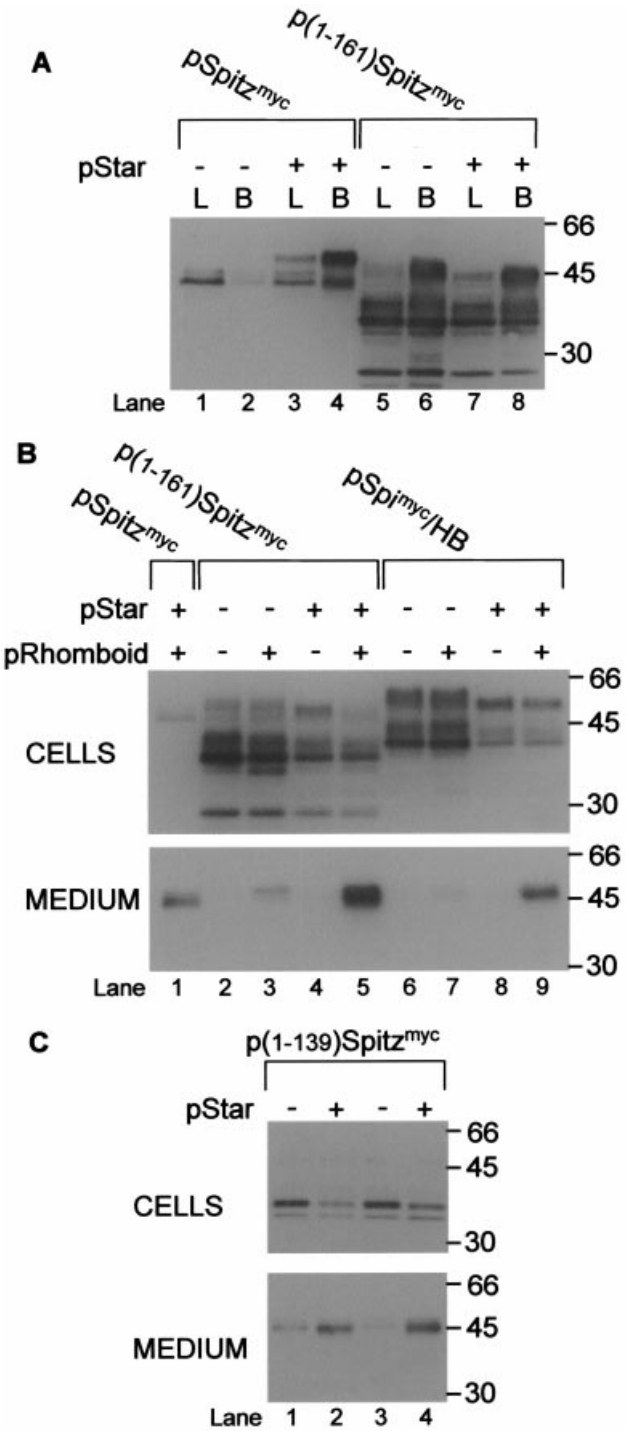

Figure 4 Requirement for Star in Spitz protein release

(**A**) HEK293T cells were transfected with pSpitz^{myc} or p(1–161)Spitz^{myc} with or without co-transfection of pStar as indicated. After 48 h, cell-surface proteins were biotinylated. Cell lysates were prepared and biotinylated proteins were recovered using streptavidin–agarose beads. Proteins in the cell lysates (L) and the biotinylated protein samples (B) were separated by SDS/PAGE and analysed by Western blotting using the 9E10 myc monoclonal antibody. (**B** and **C**) HEK293T cells were transfected with the indicated Spitz constructs with or without pStar and/or pRhomboid as indicated. After 24 h, cell lysates and conditioned-medium samples were immunoprecipitated with the 9E10 myc monoclonal antibody. Immunoprecipitated proteins were analysed by Western Blotting using the same antibody.

its retention in the ER, although the location of the motif responsible for retention remains to be determined. A recent study has reported that Star interacts with a region of the extracellular domain of Spitz [24]. It is difficult to envisage how this interaction could facilitate Spitz release from the ER, unless the interaction of Star with the extracellular domain of Spitz causes a structural change in its intracellular domain. Alternatively, there may be additional sites of interaction between the two proteins.

During the preparation of this manuscript, two papers were published that addressed the functions of Star and Rhomboid. Urban and co-workers [25] presented persuasive evidence that Rhomboid is a novel serine protease that is directly responsible for the intramembrane cleavage of Spitz. In an associated paper, Lee et al. [26] demonstrated that Rhomboid is localized in the Golgi apparatus. These authors analysed the effects of Rhomboid and Star on the intracellular trafficking, proteolytic processing and release of Spitz protein in transfected COS-7 and S2 cells. On the basis of their findings, they proposed the following model for the mechanisms of action of Star and Rhomboid. In the absence of Star, membrane-anchored Spitz is retained in the ER. The role of Star is to promote the relocalization of Spitz from the ER to the Golgi. In the Golgi, Rhomboid induces Spitz cleavage releasing a soluble luminal fragment which is then secreted from the cell (see Figure 7G in [26]).

Our results are consistent with the proposed model except for one important difference: we observed a requirement for Star for the efficient release of soluble Spitz from cells expressing membrane-anchored Spitz proteins that traffic through the cell in the absence of Star, and would thus be expected to be exposed to Rhomboid in the Golgi (Figure 4B). Two possible mechanisms, that are not mutually exclusive, can be envisaged to explain this observation. Star might be a cofactor required for the efficient processing of Spitz by the Rhomboid protease. In this respect, it is noteworthy that nicastrin is essential for presenilin-mediated intramembrane proteolysis in *Drosophila*, although the mechanism has not been determined [27,28]. Alternatively, Star might be required for the efficient secretion of Spitz following its cleavage by Rhomboid in the Golgi. Our findings with an ectopically expressed, soluble Spitz protein support this latter mode of action. This protein, truncated at Ser^{139}, lacks both cytoplasmic and membrane domains and would not require Rhomboid-mediated cleavage. Nevertheless, the release of this protein from transfected cells was substantially increased by co-expression of Star (Figure 4C). These findings suggest that the model for Spitz processing proposed by Lee et al. [26] requires revision to include an additional role for Star in facilitating the secretion of soluble Spitz following intracellular cleavage.

Rhomboid is a member of a family of proteins that shows high sequence conservation within their transmembrane domains. Database analysis has identified over 75 members in bacteria, plants and animals [25]. There are seven known *Drosophila* members [29,30], and a similar number of human Rhomboids have been identified ([25,31,32]; J. C. Pascall, unpublished work). The amino acids shown to be critical for the activity of *Drosophila* Rhomboid [25] are conserved throughout the family, raising the possibility that Rhomboids have a general function as intramembrane proteases. In this regard it is noteworthy that the human family member RHBDL2 efficiently cleaves Spitz [25]. In addition, studies in the bacterium *Providencia stuartii* have suggested a role for aarA, a Rhomboid family member, in the synthesis or release of uncharacterized extracellular-signalling molecules [33,34]. Thus a reasonable speculation is that Rhomboid family members may have a general role in the release of signalling peptides or proteins by mediating the cleavage of

precursor proteins. Important goals for future work will be to confirm the proteolytic activity of Rhomboids by *in vitro* reconstitution, and to identify the putative substrate proteins of the mammalian Rhomboids.

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