Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation

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Summary

Fas ligand (FasL), a potent mediator of apoptosis expressed by CTL and NK cells, is sorted into the inner vesicles of secretory lysosomes for release via exosome-like vesicles. Previous studies identified a proline-rich domain in the cytoplasmic tail required for sorting FasL to secretory lysosomes, but the mechanisms by which this occurs have not been identified. Here we demonstrate that the PRD of FasL binds Fgr, Fyn and Lyn tyrosine kinases, leading to phosphorylation of FasL. Loss of phosphorylation reduces internalisation of FasL into multivesicular bodies. FasL is also directly mono-ubiquitylated at lysines flanking the

PRD and mutation of these lysines reduces MVB localisation of FasL. Phosphorylation is not required for ubiquitylation because FasL lacking all tyrosines undergoes mono-ubiquitylation. These studies show that phosphorylation and ubiquitin signals regulate the sorting of FasL to secretory lysosomes by controlling entry into multivesicular bodies.

Key words: Fas ligand, Multivesicular bodies, Sorting, Ubiquitylation, Phosphorylation

Introduction

The majority of proteins targeted to lysosomes are destined for degradation. However a small number of cell types use the lysosome not only as a degradative organelle but also as a regulated secretory organelle. In these cell types a number of secretory proteins are targeted to the lysosomes for release at the plasma membrane and efficient lysosomal targeting is important for the secretory activity of these cells.

Fas ligand (FasL) is a Type II transmembrane protein and a member of the TNF family of proteins expressed by activated CD4+ and CD8+ cytotoxic T lymphocytes (CTL), natural killer (NK) cells and a small number of non-lymphoid cells. Upon recognition of Fas, membrane-bound FasL triggers rapid apoptosis in the Fas-bearing cell. FasL plays a key role in regulation within the immune system and loss of FasL gives rise to autoimmune lymphoproliferative syndrome (ALPS1B) (Wu et al., 1996). FasL is targeted to the multivesicular bodies (MVB) of secretory lysosomes (Andreola et al., 2002; Frangsmyr et al., 2005; Smith et al., 2003) and has been isolated on secreted bioactive microvesicles (Jodo et al., 2000; Martinez-Lorenzo et al., 1999; Mincheva-Nilsson et al., 2000; Monleón et al., 2001) thought to derive from these inner membrane vesicles. Small microvesicles are released into the synaptic cleft between CTL and target (Peters et al., 1989). The release of FasL into the cleft on microvesicles provides a mechanism by which FasL is released in its membrane-bound form, which is 1000 times more biologically active than the cleaved soluble form generated by metalloprotease cleavage of FasL that occurs upon translocation to the plasma membrane (Kayagaki et al., 1995; Schneider et al., 1998). Sorting to the inner vesicles of secretory lysosomes is therefore an important step for optimal biological activity of FasL.

Studies on epidermal growth factor receptor (EGFR) have elucidated many of the steps for sorting mammalian transmembrane proteins into the MVB pathway. The sorting signal is a mono-ubiquitin modification that can be recognised by hepatocyte growth factor-regulated receptor tyrosine kinase substrate (Hrs) and the endosomal sorting complexes required for transport (ESCRTs) many of which contain ubiquitin recognition motifs. Recognition by the ESCRTs mediates the internalisation of EGFR into the lumenal vesicles of the MVBs and ultimately transport to the lysosome (Katzmann et al., 2002). Mono-ubiquitylation is dependent on autophosphorylation of EGFR (Sorkin et al., 1992), which is required for binding of the ubiquitin ligase, Cbl (Waterman et al., 2002), and subsequent ubiquitylation of EGFR (Jiang and Sorkin, 2003). Disruption of the sites of either phosphorylation or ubiquitylation prevent EGFR from being internalised into the lumenal vesicles of MVBs.

The mechanisms that regulate sorting of FasL into the inner vesicles of secretory lysosomes are not understood. In this paper we show that phosphorylation of FasL by some members of the Src family tyrosine kinases through binding to the proline-rich domain (PRD) of FasL and ubiquitylation both control the internalisation of FasL into MVBs.

Results

The haemopoietic-specific Src-family tyrosine kinase, Fgr, interacts with the PRD within the cytoplasmic tail of FasL

Previous studies have shown that the PRD of FasL is required for sorting to secretory lysosomes (Blott et al., 2001). We therefore screened for proteins in haemopoietic cells able to bind the PRD of FasL. The entire cytoplasmic tail of human FasL was fused in frame with the Gal-4 DNA binding domain from yeast and used to screen a human bone marrow derived cDNA library in a yeast two hybrid assay. Approximately 1600 transformants were screened and 200 positive clones were identified. 84 clones were sequenced and analysed for PRD interacting domains. Four SH3 domain-containing proteins were identified of, which the most frequently isolated encoded Fgr, a Src-family tyrosine kinase expressed in haemopoietic cells.

To verify biochemically that the SH3 domain of Fgr interacted with the PRD of FasL a GST fusion protein expressing the SH3 domain of Fgr was used to pull down lysates from the rat mast cell line, RBL, expressing wild-type (WT) or mutant FasL constructs, all fused to GFP (Fig. 1A). Fig. 1B shows that the SH3 domain of Fgr is able to pull down FasL only when the PRD is present. RBL cells do not express endogenous FasL and no band is seen in the mock transfected lane. GFP-tagged FasL (WT) expressed in RBL appears as a band of 60 kDa when pulled down by the SH3 domain of Fgr and probed with an antibody against FasL, but a deletion mutant lacking the PRD (Δ-Pro) is not pulled down. Our previous studies revealed that mutation of KKR at the end of the PRD (Fig. 1A) resulted in mis-sorting of FasL from the lysosomes to the cell surface in RBL (Blott et al., 2001). We therefore asked whether these lysines are critical for Fgr binding. FasL-GFP in which the KKR motif was mutated to the oppositely charged EEE motif was also able to bind the SH3 domain of Fgr. Similarly, FasL in which all three tyrosines (Y7, Y9, Y13), which form potential substrates for phosphorylation, were mutated to alanines was able to bind the SH3 domain of Fgr. In each case GST alone failed to pull down FasL and western blotting of lysates was used to confirm equivalent expression levels of all constructs.

Fig. 1. Fgr binds FasL proline-rich domain (PRD) residues 41-60. (A) Amino acid sequences of the cytoplasmic tails of WT, Δ-Pro, KKR and 3Y FasL. All constructs are tagged with GFP at the amino terminus and have wild-type transmembrane and extracellular domains at the carboxy-terminus. Amino acid numbering is shown with the PRD shaded and asterisks marking the positions of tyrosine and lysine residues. Amino acid substitutions are underlined for each construct and the deleted region in Δ-Pro is shown. Please note that Figure 2a in a related paper (Blott et al. 2001) contains a typographical error, showing the sequence of C32 in FasL cytoplasmic tail as L. The correct sequence is shown here (A), and all constructs used in both studies corresponded to this sequence. (B) Western blots of pull downs using Fgr-SH3-GST incubated with cell lysates from untransfected RBL (–) or RBL expressing wild-type (WT) or mutant FasL GFP-tagged constructs lacking the PRD (Δ-Pro), with K71, K72 and R73 all mutated to glutamic acid (KKR) or Y7, Y9, Y13 all mutated to alanine (3Y). Controls of the total lysate used in each pull down are shown. All blots were probed with anti-FasL, G247-4. Molecular mass markers are shown (kDa). (C) Different concentrations [shown above peaks (μM)] of monomeric Fgr-SH3 were passed over a random peptide (solid line), peptide 1-20 (light dotted line) and peptide 61-80 (bold dotted line) immobilised on a BIAcore^{TM} chip at 37°C. Protein injection points are indicated by horizontal bars. (D) Different concentrations [shown above peaks (μM)] of monomeric Fgr-SH3 were passed over peptides corresponding to amino acids 1-20 (dotted line) and 41-60 (bold line) immobilised on a BIAcore^{TM} chip at 37°C. Protein injection points are indicated by horizontal bars. (E) Specific equilibrium binding values were plotted (squares) and the K_D calculated by nonlinear curve fitting (line) and Scatchard analysis (see inset graph).
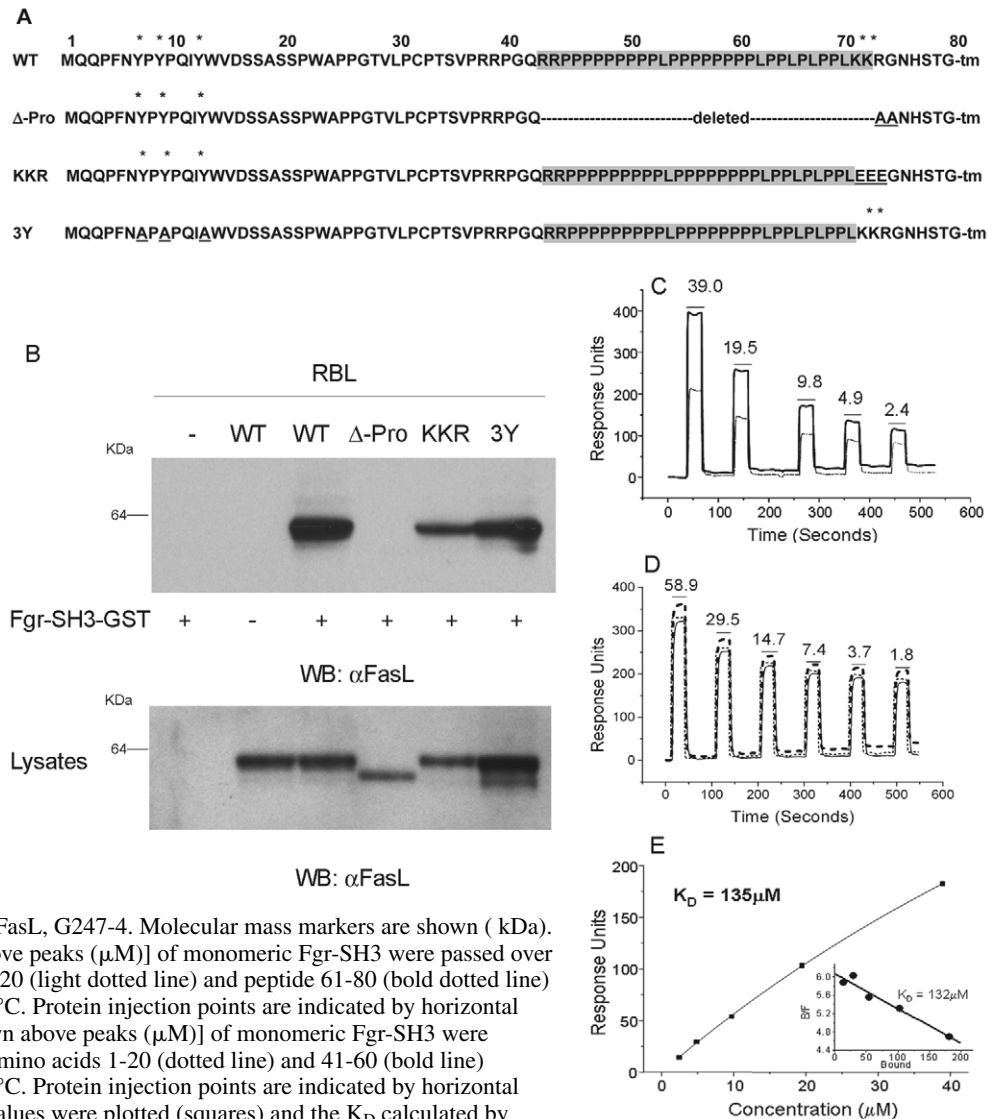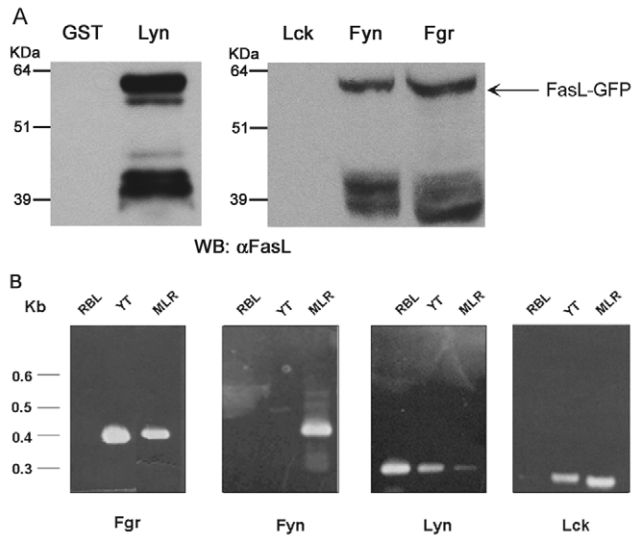
Fig. 2. FasL interacts with members of the Src family of tyrosine kinases. (A) GST alone or GST-fusion proteins of the SH3 domains of Lyn, Lck, Fyn or Fgr were used to pull-down FasL from cells lysates of RBL expressing FasL-GFP. The western blot was probed with anti-FasL, G247-4. Molecular mass markers are shown (kDa) and the arrow indicates the position of FasL-GFP. The 40 kDa band is a degradation product. (B) Agarose gel separation of RT-PCR products made with primers specific for rat Fgr, Fyn, Lyn and Lck mRNA isolated from RBL, YT and a mixed lymphocyte reaction (MLR) containing activated CD4+ and CD8+ T lymphocytes. Products were verified by sequencing. DNA ladder markers are shown on the left.

We mapped the binding site and affinity of the Fgr SH3 domain using surface plasmon resonance with 20 amino acid peptides, spanning the cytoplasmic tail of FasL, bound to the chip, and passing monomeric GST-SH3-Fgr across the bound peptides. These studies showed binding only to peptide 41-60, revealed by the difference in response units when Fgr-SH3 is passed over peptide 41-60 compared with peptide 1-20 or an irrelevant peptide (Fig. 1C) (Fig. 1C). Peptides corresponding to 1-20 and 61-80 gave no binding above that of irrelevant control peptides (Fig. 1D). Similarly, no binding above this level was detected to peptide 21-40 (data not shown). Measuring the response units of Fgr-SH3 binding to peptide 41-60 at varying protein concentrations (Fig. 1E) gave a calculated affinity of 135 μM. These results show that Fgr binds to an SH3 binding motif contained within amino acids 41-60 with a relatively weak affinity.

The SH3 domains of Fyn, Lyn and Fgr but not Lck bind to FasL

We then asked whether SH3 domains from other Src family tyrosine kinases are also able to interact with FasL by using SH3 domain fusion proteins derived from Lck, Fyn and Lyn. GST alone does not pull down FasL-GFP expressed in RBL cells. GST fusion proteins expressing the SH3 domains of Fyn, Fgr and Lyn all pull down FasL-GFP (60 kDa) as well as a degradation product of 40 kDa, however the SH3 domain of Lck does not pull down FasL (Fig. 2). Protein expression levels of all GST-SH3 domains were shown to be equivalent by Coomassie blue staining (data not shown). These results show

that some but not all Src family tyrosine kinases can bind to FasL. The finding that Lck could not bind FasL revealed that although several of the kinases can bind, the interactions are specific, confirming earlier peptide binding studies (Hane et al., 1995).

RT-PCR was used to identify expression of related Src family tyrosine kinases, Fgr, Fyn and Lyn in the NK-like cell line, YT, and cells of a mixed lymphocyte reaction (MLR) of human peripheral blood representing activated CD4 and CD8 T lymphocytes, all of which express endogenous FasL, as well as in RBL cells, which do not express endogenous FasL. The results demonstrate that tyrosine kinase expression varies between cell types (Fig. 2B). The mast cell line RBL expresses RNA encoding only Lyn and the NK cell line Fgr, Lyn and Lck. RNA from the MLR express Fgr, Fyn and Lck. This shows that cells that sort FasL to secretory lysosomes express a number of tyrosine kinases capable of binding the PRD of FasL.

Over-expression of Fgr results in mis-localisation of FasL to the early endosome

We asked whether Fgr might play a role in regulating sorting of FasL to secretory lysosomes. When expressed after transfection into RBL cells, FasL-GFP is targeted to the lysosomes very efficiently and co-localises with the lysosomal membrane marker, lgp120 (Fig. 3A) and not with the early endosomal marker EEA1 (Fig. 3B). When Fgr-GFP is expressed in RBL cells it localises both to the plasma membrane and the early endosome but not to the lysosomes, partially co-staining with EEA1 and not with lgp120 (Fig. 3C,D). When FasL is co-expressed with Fgr, a pool of FasL co-localises with the peri-nuclear pool of Fgr (Fig. 3E,F) partially overlapping with EEA1 (Fig. 3F). We used the more sensitive and quantitative technique of FACS analysis to ask whether there was a change in cell surface levels of Fas ligand expression. Co-expression of Fgr with FasL results in mis-localisation of FasL-GFP to the plasma membrane relative to cells expressing equal levels of FasL-GFP alone, as detected by FACS analysis using the NOK-1 antibody, which recognises the extracellular domain of FasL, to detect cell surface staining (Fig. 3G). By contrast, over-expression of Fgr does not increase cell surface levels of the lysosomal membrane protein, CD63 (Fig. 3H). Over-expression of Lck, which does not bind FasL (Fig. 2), does not result in mis-localisation of FasL to the plasma membrane compared to cells expressing the same level of FasL-GFP (Fig. 3I). Lck is found only at the plasma membrane when expressed in RBL (Fig. 3J), and does not co-localise with FasL (Fig. 3K). These results suggest that Fgr specifically influences the sorting of FasL to the lysosomes, with over-expression of Fgr disrupting the lysosomal sorting of FasL and resulting in mis-localisation of FasL to the plasma membrane.

Fas ligand is phosphorylated

FasL contains three potential sites of tyrosine phosphorylation at residues Y7, Y9 and Y13. To determine whether FasL is phosphorylated at these sites we mutated all three tyrosines to alanines (3Y). Wild type, Δ-Pro, KKR or 3Y FasL-GFP chimeras were expressed in RBL. As previously reported, Δ-Pro and KKR were both mis-sorted with 21% and 25% of protein appearing at the plasma membrane compared to 1.5% of wild-type FasL on the plasma membrane while the rest is targeted to the secretory lysosomes (Blott et al., 2001). The 3Y
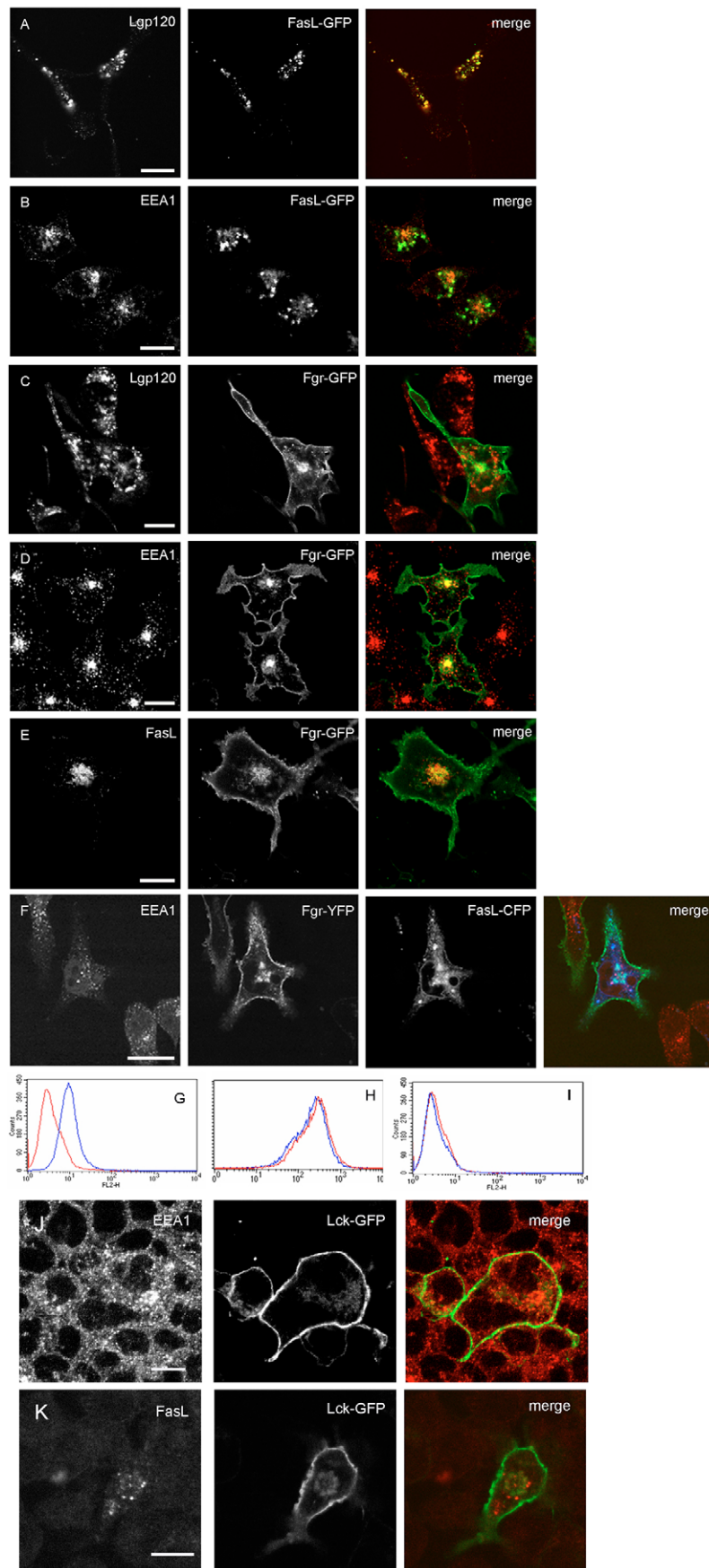
Fig. 3. Overexpression of Fgr, but not Lck, leads to mis-sorting of FasL. (A-F,J,K) Confocal images of transiently transfected RBL expressing FasL-GFP (A,B), Fgr-GFP (C-E), Fgr-YFP (F) and Lck-GFP (J,K) or untagged FasL (E,K) and stained with antibodies against Lgp120 (A,C), EEA1 (B,D,F,J) or FasL (E,K). Scale bars represent 10 μm (C,D,E,J,K) or 25 μm (A,B,F). (G-I) FACS plots showing cell surface expression of FasL (G,I) or CD63 in RBL expressing equal levels of FasL-GFP or CD63-GFP. Surface staining was measured using antibodies against the extracellular domains of FasL, or CD63 in the presence of the metalloprotease inhibitor BB3013. RBL expressing FasL-GFP alone are shown as red histograms and RBL expressing FasL-GFP and either Fgr (G,H) or Lck (J) are shown as blue histograms.

mutation disrupts lysosomal sorting and 20% of FasL appears on the plasma membrane (Fig. 4), suggesting that the three tyrosine residues may also be involved in lysosomal sorting. FasL was immunoprecipitated using the anti-FasL antibody NOK-1, and probed with either G247-4 anti-FasL, or 4G10 anti-phosphotyrosine (Fig. 5A). Probing immunoprecipitates with anti-FasL, reveals bands of 60 kDa in WT, the KKR and 3Y mutants and a band of 58 kDa for Δ-Pro, but not in untransfected RBL (–). Additional bands of 68 kDa are seen in WT and 3Y immunoprecipitates, which may correspond to a mono-ubiquitylated form of FasL. Bands of 50 kDa corresponding to Ig heavy chain from the immunoprecipitating antibodies are present in all lanes. Probing the immunoprecipitates with anti-phosphotyrosine antibodies reveals bands corresponding to FasL for WT, KKR and Δ-Pro mutants, but the FasL band is lacking in the 3Y mutant lacking all three tyrosines even though equal amounts of protein are immunoprecipitated in all samples. Additional, albeit much fainter, bands of 66 kDa are visible in immunoprecipitates from KKR and 3Y lysates with an additional band of 58 kDa also visible for 3Y. These bands are unlikely to correspond to FasL as they are not recognised by anti-FasL when the filter is stripped and re-probed. These bands may represent other phosphorylated proteins associated with FasL.

These results show that FasL is constitutively phosphorylated in RBL cells even when the PRD is absent. Since RBL express other tyrosine kinases (Fig. 2) as well as a number of potential SH3-domain binding sites in the first 40 amino acids of the cytoplasmic tail, we examined FasL phosphorylation in cells lacking expression of haemopoietic tyrosine kinases.

Fgr binds to the PRD and phosphorylates FasL

To determine whether Fgr, the tyrosine kinase expressed endogenously in CTL and NK cells, is able to phosphorylate FasL, we analysed the phosphorylation state of FasL in HeLa cells. HeLa

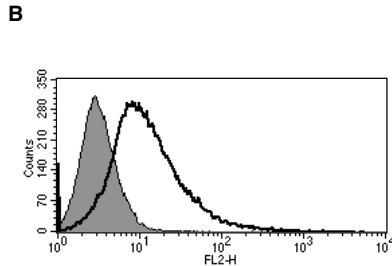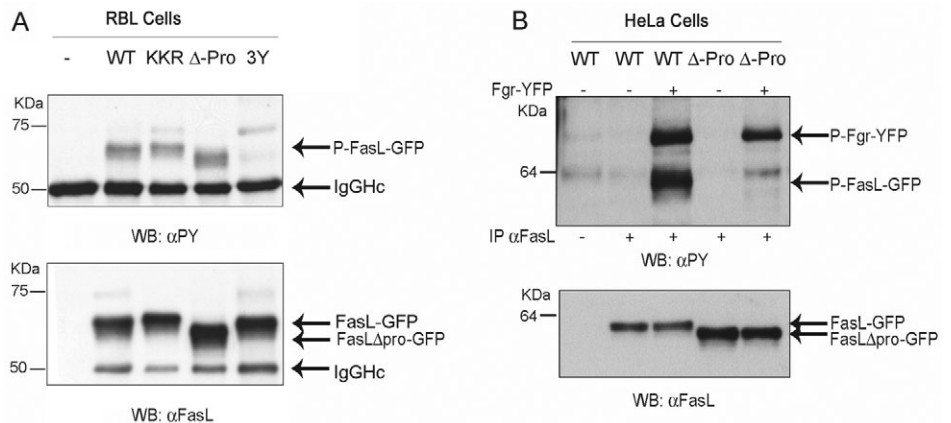| Construct	Cell surface expression	Reference
Wild-type	1.5%	Blott et al., 2001
Δ-Pro	25%	Blott et al., 2001
KKR	21%	Blott et al., 2001
3Y	20%	Present study (see B)

B

Fig. 4. Cell surface expression of FasL mutants in RBL cells. (A) Cell surface expression of Fas ligand as a percentage of total FasL expression in cells expressing equal levels of GFP (protein). (B) FACS plot showing histograms of cell surface expression of FasL in RBL transiently transfected with WT (shaded) and 3Y (unshaded) FasL-GFP constructs, gated for equal expression of GFP and stained with the NOK-1 antibody against the extracellular domain of FasL, in the presence of metalloprotease inhibitor BB3013.

cells express endogenous Src (Black, 1992), but lack expression of the haemopoietic-restricted Src family tyrosine kinases Fgr, Fyn and Lyn. FasL-GFP expressed in HeLa is not phosphorylated unless co-expressed with Fgr, when bands of 60 kDa, corresponding to FasL-GFP and 70 kDa, corresponding to autophosphorylated Fgr-YFP are seen (Fig. 5B). FasL Δ-Pro is not phosphorylated in HeLa cells and the 58 kDa Δ-Pro-GFP is absent in the anti-phosphotyrosine blot, regardless of Fgr co-expression. A faint, non-specific band of 64 kDa, is visible in WT and Δ-Pro lanes, but does not appear when re-probed with anti-FasL. These results show that FasL is phosphorylated by Fgr when the PRD, where Fgr binds FasL, is present.

FasL is mono-ubiquitylated

Studies on the EGFR have revealed phosphorylation is a pre-requisite for mono-ubiquitylation and internalisation into MVBs by the ESCRT-mediated pathway (Sorkin et al., 1992). Our previous studies showed that positively charged arginine and lysine residues at the ends of the poly-proline region in the cytoplasmic tail of FasL were likely to play an important part in SH3-mediated binding to the PRD and for this reason these positively charged residues were also deleted in the Δ-Pro mutant (Fig. 4). This mutant therefore also lacks the only potential sites of ubiquitylation in the cytoplasmic tail of FasL. Hence we asked whether K72 and K73 in FasL might also undergo ubiquitylation and, if so, whether phosphorylation is a pre-requstie for mono-ubiquitylation of FasL

FasL was immunoprecipitated using NOK-1 (Fig. 6A). Lysates and one tenth of the total immunoprecipitate were probed with anti-FasL, while the remainder of the immunoprecipitate was probed with the anti-ubiquitin antibody, P4D1. Cell lysates from FasL-GFP and FasL-KKR transfected cells, in which both potential sites of mono-ubiquitylation K72 and K73 as well as R74 have been changed to glutamic acid, were compared. In lysates, WT and 3Y reveal strong bands at 60 kDa and a weaker band at 68 kDa, whereas the KKR mutant shows only bands at 60 kDa and the Δ-Pro at 58 kDa, consistent with the deletion of the PRD. Immunoprecipitation of FasL followed by immunoblotting with P4D1 reveals that the 68 kDa band corresponds to mono-ubiquitylated FasL and is present only in WT and 3Y mutants. This was confirmed by re-probing the filters with the G247-4 anti-FasL antibody. This shows that FasL is ubiquitylated, and ubiquitylation is abolished by deletion of the only two lysines, K72 and K73, in the cytoplasmic tail of FasL, both in the KKR and Δ-Pro mutants. Phosphorylation, however, is not a pre-requisite of ubiquitylation since the 3Y mutant, which lacks all tyrosine residues in the cytoplasmic tail, is ubiquitylated.

Mutations affecting phosphorylation and ubiquitylation prevent FasL internalisation into MVBs

Over-expression of Fgr resulted in perinuclear retention of FasL in RBL, partially overlapping with Fgr and EEA1,

Fig. 5. FasL is phosphorylated by Fgr binding to the PRD. (A) Western blots of lysates from stably transfected RBL clones expressing equivalent levels of FasL-GFP (WT) and the GFP-tagged FasL mutants (KKR, Δ-Pro and 3Y), immunoprecipitated (IP) with anti-FasL antibody MIKE 1 and Protein-G-Sepharose or Protein-G-Sepharose alone (−). The blot was probed with anti-phosphotyrosine (αPY) antibody 4G10 (upper panel) and stripped and re-probed with anti-FasL antibody G247-4 (lower panel). Arrows denote the positions of bands corresponding to phosphorylated FasL-GFP (P-FasL-

GFP), FasL-GFP and the Δ-Pro mutant as well as IgG heavy chain (IgGHc). (B) Immunotyrosine blot of HeLa cells expressing untagged WT and Δ-Pro FasL and Fgr-YFP (+) as indicated probed with anti-phosphotyrosine (αPY) antibody 4G10 (upper panel) and stripped and re-probed with anti-FasL antibody G247-4 (lower panel). Arrows denote the positions of bands corresponding to phosphorylated Fgr (P-Fgr-YFP), FasL-GFP (P-FasL-GFP), FasL-GFP and the Δ-Pro mutant. Positions of molecular masses are shown (kDa).
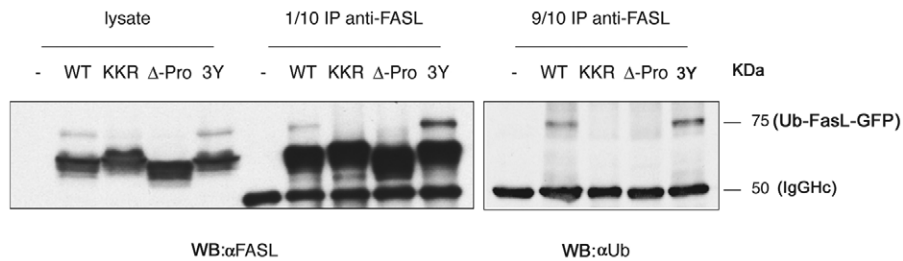
Fig. 6. FasL is monoubiquitylated in vivo. Western blots of cell lysates from stable transfectants of RBL expressing equivalent levels of WT or FasL-GFP mutants (KKR, Δ-Pro and 3Y) or 1/10 of the immunoprecipitates both probed with anti-FasL, G247-4 and 9/10 of the same immunoprecipitate probed with anti-Ub, P4D1. Arrows denote the position of bands corresponding to ubiquitylated FasL (Ub-FasL-GFP) and IgG heavy chain (IgGHc). Note that both KKR and Δ-Pro constructs lack K72 and K73 (Fig. 1). Positions of molecular masses are shown (kDa).

supporting the idea that a critical sorting step affected by phosphorylation may occur in an endosomal compartment with these markers. ESCRT mediated sorting into MVBs occurs in the early or sorting endosomes. Studies on the EGFR, the tyrosine receptor kinase, have shown that a kinase dead mutant K721A fails to undergo internalisation into MVBs (Felder et al., 1990). In addition, tyrosine auto-phosphorylation of EGFR is required for its mono-ubiquitylation (Waterman et al., 2002). We therefore examined mutations in FasL that prevented phosphorylation and ubiquitylation for their ability to internalise FasL into MVBs.

FasL localisation in MVBs of RBL was determined using immunogold labelling against the extracellular domain of FasL using the antibody NOK-1 (Fig. 7A). The number of gold particles per MVB was counted. MVBs were classified as vesicles of 200-600 nm diameter encompassing multivesicular structures. Only MVBs bearing gold particles were included in the calculations. In RBL expressing wild-type FasL-GFP, 43% of MVBs contained 1-10 gold particles with another 38% containing 11-20 gold particles per MVB and the remaining 19% more than 20 gold particles per MVB (Fig. 7B). By contrast, in RBL expressing Δ-Pro, 97% of MVBs contained 1-10 gold particles per MVB and only a single MVB was observed containing 13 gold particles. RBL expressing the KKR mutant of FasL showed 90% of MVBs containing 1-10 gold particles per MVB and only 10% with 11-20 gold particles. Similarly in RBL expressing the 3Y mutant, 89% of MVBs contained 1-10 gold particles and only 11% contained 11-20 gold particles. These results suggest that although wild-type FasL-GFP is readily internalised into MVBs, disruption of either phosphorylation or ubiquitylation motifs disrupts this sorting step.

Discussion

The packaging of FasL into small vesicles within secretory lysosomes (Andreola et al., 2002; Frangsmyr et al., 2005; Smith et al., 2003) is important for the delivery of active FasL at the immunological synapse. FasL, which is transferred to the plasma membrane is rapidly inactivated by a cell surface metalloprotease that cleaves off the extracellular domain of FasL, producing a soluble form of FasL, which is 1000 times less active than transmembrane FasL (Kayagaki et al., 1995; Mariani et al., 1995). The delivery of small vesicles into the immunological synapse allows FasL to avoid the metalloprotease and remain in the membrane-bound form, which is efficient at cross-linking Fas on the target membrane.

Previous studies revealed that FasL is packaged in secretory lysosomes in haemopoietic cells whereas in other cell types, such as epithelial cells, FasL is expressed at high levels on the plasma membrane (Blott et al., 2001). The targeting motif for secretory lysosome localisation was identified as a proline-rich region of the cytoplasmic tail of FasL and deletion of this 20 amino acid stretch results in cell surface expression of FasL in haemopoietic cells. These results suggested that the proline-rich domain of FasL might provide a cell-type-specific sorting motif for secretory lysosome localisation in haemopoietic cells.

Using a yeast two hybrid approach we identified interactions between the haemopoietic-specific Src family tyrosine kinase, Fgr. Expression of Fgr is restricted to haemopoietic cells of the myeloid lineage including monocytes, macrophages and neutrophils (Thomas and Brugge, 1997; Willman et al., 1987), as well as natural killer cells (Biondi et al., 1991) and some B cell subsets (Link and Zutter, 1995). The activation of Src family tyrosine kinases is regulated by the balance between dephosphorylation and phosphorylation (Sicheri and Kuriyan, 1997; Young et al., 2001), which would result from the cascade of signalling events that accompany receptor antigen recognition by both T cells and NK cells. FasL transcription and biosynthesis are both upregulated by T-cell receptor (TcR) recognition (Vignaux et al., 1995) and new synthesis of FasL will therefore coincide with Fgr activation.

In this paper we show that both phosphorylation and mono-ubiquitylation signals are required for efficient lysosomal targeting of FasL via the MVB pathway. We show that Fgr binding to the PRD (amino acids 43-74) is required for efficient phosphorylation of FasL in HeLa cells, which lack endogenous expression of the haemopoeitic Src family tyrosine kinases. In RBL cells, which do not express Fgr or Fyn, but do express Lyn and possibly other related kinases, we identify a critical role for phosphorylation by deletion of tyrosine residues Y7, Y9 and Y13 that reduce the efficiency of FasL entry into MVBs. In addition we find that FasL is mono-ubiquitylated and deletion of the two possible lysine substrates at amino acids 72 and 73 prevents ubiquitylation and also impairs entry of FasL into MVBs. Unlike the EGFR, where phosphorylation is a prerequisite to ubiquitylation, FasL ubiquitylation is not dependent on phosphorylation, as the 3Y mutant is still efficiently ubiquitylated. Conversely phosphorylation is not dependent on mono-ubiquitylation, as FasL lacking lysines is efficiently phosphorylated (Fig. 5). These results suggest that both phosphorylation and mono-ubiquitylation contribute to sorting FasL independently. In T cells, which express endogenous FasL and Fgr, the PRD will therefore contribute both to phosphorylation and mono-ubiquitylation, serving as a
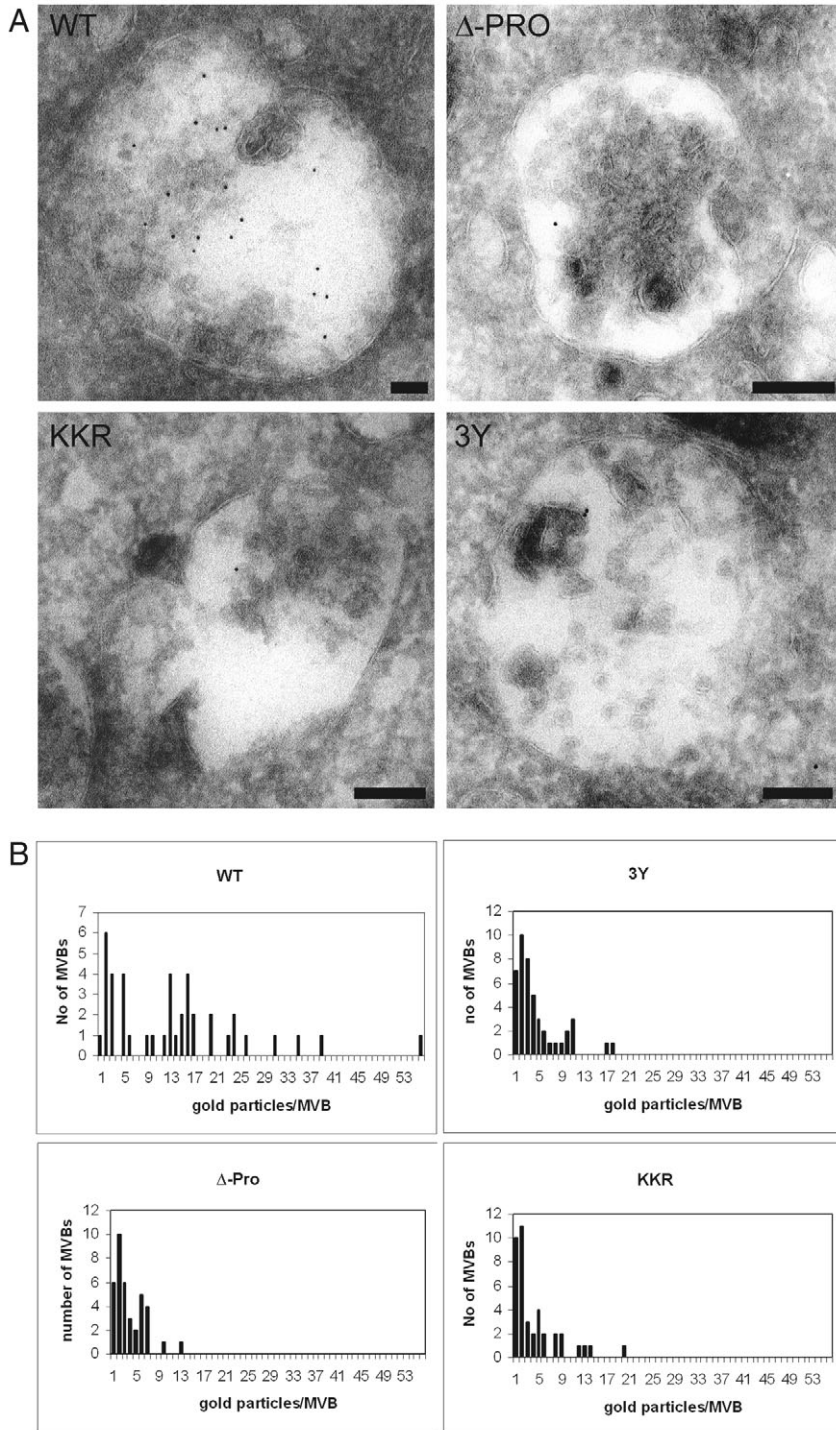
Fig. 7. Phosphorylation and ubiquitylation modifications are required for FasL sorting into MVBs. (A) Immunogold labelling of cryo-sections from stable transfectants of RBL expressing equivalent levels of WT, Δ-Pro, KKR and 3Y GFP-tagged constructs of FasL, detected with NOK-1 labelling of the extracellular domain of FasL and Protein A-gold. Bars, 100 nm. (B) Quantification of number of gold particles in MVBs when WT, Δ-Pro, KKR and 3Y FasL are expressed in RBL. Equivalent expression levels of FasL were confirmed by western blotting.

detailing the cleavage of the extracellular domain (recognised by antibodies) by cell surface metalloproteases (Tanaka et al., 1998). This suggests that FasL reaches secretory lysosomes directly via the biosynthetic pathway and that both phosphorylation and ubiquitylation are likely to play a role in the biosynthetic sorting of FasL.

We find that a number of the haemopoietic-restricted tyrosine kinases are able to bind FasL and this might explain how FasL is successfully sorted to the secretory lysosomes of a number of haemopoietic cells. Although RBL do not express Fgr, this cell line does express a number of other tyrosine kinases, including Lyn. Our finding that FasL is constitutively phosphorylated in RBL, even when lacking the PRD, suggests that other tyrosine kinases expressed in RBL can bind outside the PRD and lead to phosphorylation. It should be noted that FasL Δ-Pro lacks K72 and K73, and therefore cannot be mono-ubiquitylated and consequently is mis-sorted to the plasma membrane in RBL. Since we were unable to knock down gene expression in RBL cells we were unable to address the role of other tyrosine kinases in FasL sorting in RBL. Interestingly, in HeLa cells in which FasL localises predominantly to the plasma membrane, co-expression with Fgr reduces the cell surface localisation of FasL by approximately one third (data not shown).

Our results demonstrate that phosphorylation of FasL varies in different cell types, dependent upon the tyrosine kinases expressed. The finding that FasL binds some, but not all, of the haemopoietic-specific Src family tyrosine kinases provides a potential mechanism for the lineage specific sorting of FasL in cells with secretory lysosomes. In HeLa cells, which express Src but lack the haemopoeitic-specific tyrosine kinases, FasL is only phosphorylated when co-expressed with Fgr.

Taken together the findings presented here suggest that FasL sorting to secretory lysosomes is regulated in at least two ways by the PRD. Fgr binding to the PRD leads to phosphorylation of FasL and in addition a KKR motif at one end of the PRD forms the site of a mono-ubiquitylation modification. Our data show that both phosphorylation and

binding site for Fgr, which can phosphorylate FasL, and with K72 and K73 providing the substrate sites for mono-ubiquitylation.

Our data indicate that both phosphorylation and ubiquitylation are necessary for the appearance of FasL within the MVBs. We do not know whether phosphorylation and mono-ubiquitylation occur at different steps along the endocytic/secretory pathway. However detection of cell surface FasL requires the use of metalloprotease inhibitors (Blott et al., 2001; Bossi and Griffiths, 1999), consistent with literature

ubiquitylation control internalisation into the inner vesicles of MVBs independently. FasL sorting into MVBs is important for localisation into exosome-like vesicles that are released into the immunological synapse and provide optimally active membrane-bound FasL able to cleave Fas on target cells and trigger rapid cell death.

Materials and methods

Antibodies and cell lines

The antibodies used were: mouse anti-human FasL NOK-1 (Pharmingen), rat anti-human FasL MIKE1 (Apotech), mouse anti-human FasL G247-4 (mouse IgG1; PharMingen), mouse anti-human CD63, H5C6 (Developmental Studies Hybridoma Bank, Iowa), mouse anti-Phosphotyrosine 4G10 (Upstate), mouse anti-ubiquitin P4D1 (Santa Cruz Biotechnology), HRP-conjugated anti-phosphotyrosine RC20H (Transduction Laboratory), rabbit anti-rat Lgp 120 (Mark Marsh, UCL), mouse anti-EEA1 (Becton Dickinson), mouse anti-human Lck (Becton Dickinson), rabbit anti-Fgr (Santa Cruz Biotechnology), mouse anti-myc antibody (Covance), anti-GFP BD Living Colors™ A.v. peptide antibody (BD Bioscience), HRP-conjugated anti-mouse secondary antibody (Jackson Immunoresearch). RBL (rat basophilic leukemia), human HeLa and Jurkat cell lines were grown in DMEM medium (Gibco) supplemented with 10% foetal calf serum (FCS).

Plasmid constructs

Constructs were generated either by PCR or by direct subcloning. Wild type (WT), ΔPro-GFP and KKR FasL-GFP chimeras were constructed as previously described (Blott et al., 2001; Bossi and Griffiths, 1999; Bossi et al., 2000). 3Y was generated by Quick Change™ Site Directed Mutagenesis kit (Stratagene) using WT FasL-GFP as a template. Full-length human Lck was a kind gift from M. Marsh (UCL, London) and full-length human Fgr and Lyn were amplified by RT-PCR. Fgr-GFP, Lck-GFP and Fgr-YFP were constructed by cloning full-length human sequences into pEGFP-N3 or pEYFP-N3 (Clontech) amplified by PCR using the following primers: Fgr-5′ primer 5′- CGGGAATTCGTTATGGGCTGTGTGTTCTGC-3′, Fgr-3′ primer 5′-CCGGACGGTACCAACTGTCTGATCCCCGGG-3′; Lck-5′ primer 5′-CGGGAATTCGTTATGGGCTGTGGCTGCAGC-3′, Lck-3′ primer 5′-CCGGACGGTACCAACAGGCTGAGGCTGGTA-3′; Lyn-5′ primer 5′-CGGG-AATTCGTTATGGGATGTATAAAATCA-3′, Lyn-3′ primer 5′-CCGGACGGTAC-CAACAGGCTGCTGCTGGTA-3′. pGEX-2T-Fgr-SH3 was a kind gift from I. Gout. Lyn-SH3 was subcloned into pGEX-2T (Amersham Biosciences) using the primers: 5′-primer 5′-CGGACCGGATCCCAAGGAGACATTGTG-3′, 3′-primer 5′-GGACCGGAATTCTCAGGTGTTGAGTTT-3′, which encode a BamHI and EcoRI restriction site, respectively. Fyn-SH3 and Lck-SH3 were gifts from M. Brown.

Reverse transcriptase PCR (RT-PCR)

Full-length human Fgr and Lyn were amplified by RT-PCR from mixed lymphocyte reaction (MLR) and YT RNA, respectively, using the following primers; Fgr 5′ primer 5′-GGGAACCTGGAAATGGGCTGTGTGTTCTG-3′, Fgr 3′ primer 5′-TGCCCGGACAGGCTATGTCTGATCCCCGG-3′; Lyn 5′ primer 5′-TCACGC-GAGCGGGAAATATGGGATGTAT-3′, Lyn 3′ primer 5′-GGTCTCCCTGTGCTC-TAAGGCTGCTGCT-3′. RNA from various cell lines were screened for the expression of Fgr using the following species specific primers; human 5′ primer 5′-GTGTGTCAGGGATTGGGGTGACCCTGT-3′; human 3′ primer 5′-CACAGCC-CGTCATTCACCTCCATGTAG-3′; murine 5′ primer 5′-GCATCTCAGGGACCG-GAGTGACCATAT-3′; murine 3′ primer 5′-CACAGACCATCATTCACTTCCAT-GTAG-3′; rat 5′ primer 5′-GCGTCTCTGGGACGGGAGTGACCATAT-3′; rat 3′ primer 5′-CATAGACCGTCATTCACTTCCATGTAG-3′. These primers were designed across intron-exon boundaries. RBL RNA was screened for the presence of various tyrosine kinases using the following primers; Fyn 5′ primer 5′-GTCACG-GAATACATGAACAAAGGAA-3′, Fyn 3′ primer 3′ 5′-GGAAACTTTGCAC-CTTGTCTGGCTGT-3′; Lck 5′ primer 5′-TAGGCAGGGGACTTGTACTGA-GAAG-3′; Lck 3′ primer 5′-TCACAGGAGGCTCAGGCCAAGGCTGG-3′; Lyn 5′ primer 5′-ATTACCGAGTTCATGGCCAAGGGTA-3′; Lyn 3′ primer 5′-TCA-CAGGAGGCTCAGGCCAAGGCTGG-3′. These primers were designed to prime across areas that are divergent among the family members.

Transfection

5×10^6 RBL cells were transfected using AMAXA Nucleofactor System according to the manufacturer's instructions. 8×10^5 HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transient transfectants were analysed 24 hours post-transfection. To obtain stable transfectants, RBL cells were transfected by electroporation at 500 µF and 250 mV (Biorad, Richmond, California) and selected by supplementing the growth medium with 1 mg ml^{-1} G418 (Gibco) 24 hours post-transfection.

Cell lysates

Cells were lysed in lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl$_2$, 2%

NP40 containing 1× Complete Protease Inhibitor (Roche), 10 mM NEM (Sigma), 1 mM Phosphatase Inhibitor Cocktail 2 (Sigma) at 4×10^7 cells ml^{-1}, incubated on ice 20 minutes and centrifuged for 10 minutes at 13000 g to pellet debris.

GST-pull down assay

Lysates were pre-cleared with 15 µl GST-Sepharose beads (Pharmacia) 1 hour at 4°C and incubated over night at 4°C with GST fusion proteins immobilised onto GST-Sepharose. The samples were then washed with lysis buffer and 0.1% Triton X100, eluted with Gluthatione elution buffer (10 mM Gluthatione in 50 mM Tris-HCl) for 20 minutes at room temperature. Samples were separated by SDS-PAGE transferred to nitrocellulose and probed with anti-FasL antibody, G247-4. A small fraction of the eluate was also analysed by SDS-PAGE and stained with Coomassie blue to demonstrate equal amounts of GST fusion protein were used in each sample (data not shown).

Immunoprecipitation

Cell lysates were pre-cleared with 15 µl Protein-G Sepharose (Pharmacia Biotech) for 1 hour at 4°C, incubated over night at 4°C with 40 µl Protein-G Sepharose that had been pre-incubated with 1.5 µl of anti-FasL Mike 1 antibody (Apotech). Immunoprecipitates were washed three times with Assay Buffer (25 mM Hepes pH 7.2, 125 mM KAc, 2.5 mM MgAc, 5 mM EGTA, 1 mM DTT) supplemented with the 1 mM Phosphatase Inhibitor Cocktail 2 (SIGMA). Samples were separated by SDS-PAGE and analysed by immunoblotting. The blot was probed with anti-Phosphotyrosine 4G10 antibody (Upstate).

Western blotting

Samples from GST-pull down, immunoprecipitation experiments or cell lysates, were eluted in NuPage Sample Buffer containing Reducing Agent (Invitrogen) and separated by SDS-PAGE using NuPage Novex 4-12% Bis-Tris gel in the XCell SureLock System with MOPS SDS Running Buffer (Invitrogen). Following SDS-PAGE, the proteins were transferred to an Invitrolon-PVDF (Invitrogen) membrane and blocked in 5%milk/PBS-0.2% Tween 20 (Sigma), incubated with the designated antibody and a horseradish peroxidase (HRP)-conjugated anti mouse secondary antibody (Jackson) before detection with a Super-Signal chemiluminescence kit (Pierce). When 4G10 antibody was used, the membrane was blocked in 5%BSA/TBS-0.1% Tween20 and the primary antibody was diluted in 3%BSA/TBS-0.1% Tween20.

Ubiquitylation assay

For the in vivo ubiquitylation assay, RBL cells were lysed in JS buffer (50 mM HEPES PH 7.5, 50 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM MgCl$_2$, 5 mM EGTA) plus protease and phosphatase inhibitors (20 mM Na pyrophosphate pH 7.5, 25 µg ml^{-1} aproteinin, 25 µg ml^{-1} leupeptin, 50 mM NaF, 2 mM PMSF, 0.5 M Na vanadate in HEPES pH 7.5). 4 mg of lysates were subjected to immunoprecipitation with mouse monoclonal antibody against human FASL (NOK-1, BD Pharmingen) and protein G (Zymed). Immunoprecipitates were washed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% triton, 1% deoxycholate, 0,1% SDS) plus protease and phosphatase inhibitors as before. Anti-ubiquitin immunoblot was performed using P4D1 monoclonal antibody (Santa Cruz Biotechnology) as described previously (Avantaggiati et al., 1996). Anti-FasL immunoblots were performed using G247-4 mouse monoclonal antibody (BD Pharmingen).

Immunostaining, confocal microscopy and FACS analysis

Immunostaining and confocal microscopy have been previously described (Blott et al., 2001). For EEA1 staining cells were fixed in 2% PFA for 15 minutes at room temperature. All images were acquired sequentially to prevent 'bleed-through' between channels. FACS analysis was as described (Blott et al., 2001). RBL FasL-GFP and CD63-GFP transfectants were plated in six-well dishes 12 hours prior to FACS staining. 2 hours prior to FACS staining, the cell medium was supplemented with 10 µM BB3013 metalloprotease inhibitor (British Biotech, Oxford), after, which the dishes were placed on ice for 10 minutes. The medium was replaced with 1 µg ml^{-1} NOK-1, anti-CD63 antibody or relevant control antibody, diluted in medium plus 10 µM BB3013, and incubated for 30 minutes on ice. The cells were washed with FACS buffer (1%FCS/ PBS with 1 µM sodium azide), then fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Washington, USA) in FACS buffer for 15 minutes at room temperature. The cells were washed further, and then incubated for 30 minutes with PE-conjugated goat anti-mouse secondary antibody (final concentration 50 µg ml^{-1} in FACS buffer; Jackson Immunoresearch, West Grove, Pennsylvania, USA). The cells were washed three times in FACS buffer, and then scraped from the dishes into FACS tubes, and re-suspended in a final volume of 1 ml FACS buffer. The samples were analysed using a FACScalibur flow cytometer (Becton Dickinson). PE fluorescence was measured at 575 nm and GFP fluorescence at 525 nm. Fluorescence data was collected for 30,000 events on a four orders of magnitude log scale and analysed using CELLQuest (Becton Dickinson) software. Cells analysed for surface FasL or CD63 expression were gated so that all expressed equal levels of GFP. Cell surface expression is shown as a percentage of total FasL expression, determined by the amount of GFP.

Electron microscopy

Cells were fixed in 4% (~10 minutes) followed by 8% paraformaldehyde in 250 mM HEPES buffer (pH 7.4) for 2 hours. The cells were washed in 250 mM HEPES buffer containing 50 mM glycine for 1 hour at room temperature to quench free aldehydes and released from the plastic by scraping. Cells were pelleted in 3% gelatin, cryo-protected with 2.3 M sucrose, and cryosectioned using a Reichert Ultracut E with the FCS attachment. Cryosections of 65 nm nominal thickness were collected onto formvar-coated nickel grids using a 1:1 mixture of 2.3 M sucrose and 2% methylcellulose (Liou et al., 1996) and immunolabelled with NOK-1 (1:20 dilution) followed by goat anti-mouse IgG conjugated to 10 nm gold (British Biocell, UK). Sections were examined in either a Tecnai 12 (FEI, Eindhoven, The Netherlands) or a Zeiss Omega 912 electron microscope (Zeiss SMT, Oberkochen, Germany) equipped with a Proscan cooled slow-scan charge-coupled device camera (2048×2048 pixels). For quantification, digital images were analysed using EasiVision SIS image analysis software (Soft Imaging Software, Münster, Germany).

BIAcore analysis

Recombinant Fgr-SH3 GST was prepared and then further purified by gel filtration using Superdex 75 (Amersham Biosciences). Monomeric fractions were used on the same day. Experiments were carried out on a BIAcore 2000 (BIAcore AB), at 37°C, using HBS buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant P20) supplied by the manufacturer. Biotinylated peptides (Sigma-Genosys) were captured by streptavidin immobilised on a research grade CM5 chip. The sequence of the irrelevant peptide control, with no known SH3-binding motifs was PDNSSDSD(pTyr)DLHGAQRL. 10 μl SH3 protein was injected at 20 μl minute^{-1} over the peptides, at decreasing concentrations. Affinity data were analysed using the software SigmaPlot. Similar Kd values were obtained by non-linear fitting of the simple single site saturation binding equation to the BIAcore data, and by liner curve fitting of the Scatchard plot.

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