syk protein tyrosine kinase regulates Fc receptor γ -chain-mediated transport to lysosomes

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B- and T-cell receptors, as well as most Fc receptors (FcR), are part of a large family of membrane proteins named immunoreceptors and are expressed on all cells of the immune system. Immunoreceptors' biological functions rely on two of their fundamental attributes: signal transduction and internalization. The signals required for these two functions are present in the chains associated with immunoreceptors, within conserved amino acid motifs called immunoreceptor tyrosine-based activation motifs (ITAMs). We have examined the role of the protein tyrosine kinase (PTK) syk, a critical effector of immunoreceptor-mediated cell signalling through ITAMs, in FcR-associated γ -chain internalization and lysosomal targeting. A point mutation in the immunoreceptor-associated γ -chain ITAM affecting syk activation, as well as overexpression of a syk dominant negative mutant, inhibited signal transduction without affecting receptor coated-pit localization or internalization. In contrast, blocking of γ -chain-mediated syk activation impaired FcR transport from endosomes to lysosomes and selectively inhibited the presentation of certain T-cell epitopes. Therefore, activation of the PTK syk is dispensable for receptor internalization, but necessary for cell signalling and for γ -chain-mediated FcR delivery to lysosomes.

Keywords: antigen presentation/Fc receptors/immune complex/lysosomes/syk protein tyrosine kinase

Introduction

Antigen recognition by cells of the immune system is mediated by a large family of membrane receptors, called immunoreceptors (Daeron, 1997). B- and T-lymphocytes express clonally distributed receptors which recognize either soluble antigens, in the case of B-cell receptors (BcRs), or peptides associated with major histocompatibility complex (MHC) molecules, for T-cell receptors (TcRs). Virtually all cells of the immune system, including granulocytes, macrophages and dendritic cells, express receptors for antigen–antibody complexes, which recog-

nize the Fc portion of immunoglobulins (FcRs) (Ravetch, 1994). TcRs, BcRs and FcRs are multimeric complexes composed of a ligand-binding module, which determines the specificity of ligand recognition, and a transducing module composed of two to six associated chains. These associated chains contain conserved amino acid motifs in their cytoplasmic tails, called immunoreceptor tyrosine-based activation motifs (ITAMs, DxxYxxL6xYxxL) (Reth, 1989; Cambier, 1995b). ITAMs couple immunoreceptors to intracellular effectors of signal transduction pathways (Cambier, 1995a). Upon multimerization, the ITAM tyrosine residues are phosphorylated by protein tyrosine kinases (PTK) of the src family (Cambier, 1995a). Phosphorylated ITAMs then recruit PTKs of the syk family (syk itself or ZAP70 in T cells), which activate several signalling effectors including the PLC γ and ras pathways, as well as the phosphatidylinositol 3' kinase (PI3K) (Agarwal *et al.*, 1993; Benhamou *et al.*, 1993; Kiener *et al.*, 1993; Kanakaraj *et al.*, 1994; Kurosaki *et al.*, 1994; Shen *et al.*, 1994).

An important step, after the engagement of immunoreceptors by their ligands, is their internalization and delivery to lysosomes. Internalization and degradation of the TcR (downmodulation) are thought to regulate T-cell activation (Valitutti *et al.*, 1997), processes which possibly also operate for other immunoreceptors (such as the BcR or various FcRs). Internalization is also critical for peptide presentation to T lymphocytes after antigen or antigen–antibody complex recognition by the BcR or different FcRs, respectively (Lanzavecchia, 1990). Immunoreceptor endocytosis generally occurs through coated pits and vesicles. However, the signals required for immunoreceptor internalization are as yet ill defined. In the case of the TcR, the associated CD3 ϵ -chain bears a double leucine internalization signal, which also mediates direct transport from the *trans*-Golgi network (TGN) to endosomes (Letourneur and Klausner, 1992). Internalization of antigen-bound BcR requires tyrosine-based internalization signals present in the cytoplasmic tail of either the heavy chain of certain membrane immunoglobulin (mIg) isotypes (Weiser *et al.*, 1994; Knight *et al.*, 1997) or the mIg-associated Ig α /Ig β heterodimer (Bonnerot *et al.*, 1995). The FcR-associated γ -chain also contains internalization signals. Mutation of either of the two tyrosine residues in the γ -chain ITAM blocked both cell activation (Bonnerot *et al.*, 1992) and efficient receptor internalization (Amigorena *et al.*, 1992), suggesting a possible link between signalling and internalization.

Thus, signal transduction and internalization/lysosomal transport are initiated simultaneously after immunoreceptor engagement. The cytosolic effectors of cell signalling have been analysed extensively, but very little is known about the pathways and effectors of immunoreceptor internalization and lysosomal transport. It is known, how-

Fig. 1. Mutagenesis analysis of the γ -chain cytoplasmic domain. IIA1.6 cells expressing Fc γ R/ic γ chimeras harbouring the indicated mutation in the γ -chain cytoplasmic tail were tested for the induction of cell activation (black bars) and for the internalization of multivalent Fc receptors ligands (grey bars). Cell activation through Fc γ R/ic γ chimeras was triggered by culturing the cells with rat anti-mouse FcR 2.4G2 (10 μ g/ml) and mouse anti-rat antiserum (30 μ g/ml). In control experiments, the cells were stimulated through endogenous mIg using F(ab')₂ fragments of anti-mouse IgG rabbit antiserum. After 18 h of incubation at 37°C, IL-2 secretion was measured in the supernatant by a CTLL2 proliferation assay. The results are presented as the ratio between induced IL-2 secretion through Fc γ R/ic γ chimera and endogenous mIgG2a by the same transfected cells. To measure receptor internalization, cells were incubated for 30 min at 4°C with iodinated 2.4G2 antibodies (10 μ g/ml). The cells were then washed and the internalization was triggered with prewarmed solution of mouse anti-rat IgG (30 μ g/ml). After 20 min of incubation at 37°C, the cells were put on ice and the rate of internalized 2.4G2 was determined after acidic elution of antibodies remaining at the cell surface. The results are presented as the rate of the cell-associated radioactivity after acidic or no treatment (bars represent the mean of three independent experiments).

ever, that the FcR-associated γ -chain and the PTK syk are involved in phagocytosis in macrophages (Greenberg *et al.*, 1994, 1996; Crowley *et al.*, 1997). Recently, the PTK lck has been shown to target TcRs for lysosomal degradation (D'oro *et al.*, 1997), suggesting that effectors of cell signalling may also determine the intracellular fate of immunoreceptors.

In order to analyse the relationships between signalling and intracellular transport of immune receptors, we performed an extensive mutagenesis of the ITAM of the FcR-associated γ -chain. One of the mutants we isolated, leucine 35 to alanine (L35A), blocked cell activation without affecting internalization. This mutant had lost the ability to activate the PTK syk completely, but localized to coated pits and was internalized with normal kinetics and efficiency. Importantly, the L35A mutation also blocked transport to the lysosomes completely, suggesting that the recruitment and/or activation of syk is required for lysosomal delivery. Furthermore, expression of a dominant negative truncated syk prevented cell activation through the γ -chain, but not receptor internalization. This mutant also blocked transport from endosomes to lysosomes and presentation of certain T-cell epitopes. Therefore, a major effector of signal transduction through immunoreceptors, the PTK syk, is also required for their lysosomal transport.

Results

Cell activation and internalization through the FcR-associated γ -chain

The γ -chain is associated with different immunoreceptors, including Fc ϵ RI, Fc γ RIII, Fc γ RI, Fc γ RIIA and some TcRs (Daeron, 1997; Ravetch and Kinet, 1991). To define precisely the signals required for γ -chain-mediated signalling and internalization, we prepared a large number of γ -chain mutants. A chimeric receptor composed of the two extracellular and the transmembrane domains of

Fc γ RII and the cytosolic tail of the γ -chain was used to individually mutate amino acids 18–37 of the γ -chain ITAM to alanine (excluding amino acids 26–29; Figure 1). The recombinant mutated chimeric receptors were expressed stably by cDNA transfection in the Fc γ R negative IIA1.6 B lymphoma cell line. Bulk cell populations expressing Fc γ R chimeras were obtained after selection and used directly for testing the ability of the different receptors to induce cell activation and ligand internalization after the cross-linking of receptors with the rat anti-mouse Fc receptor mAb, 2.4G2 and F(ab')₂ fragments of mouse IgG anti-rat IgG (Bonnerot and Daeron, 1994). Cell activation was assessed by measuring the induction of interleukin (IL)-2 secretion in the cell culture supernatant; receptor internalization was assessed by measuring the proportion of intracellular radioactive ligand (iodinated 2.4G2).

As shown in Figure 1, mutations at amino acid sites (Y21, Y32, L24 and K36) inhibited the activity of ITAM in both activation and internalization assays. The first three amino acids are highly conserved in different ITAMs and are known to be required for ITAM function. Lysine 36 is not conserved, but nevertheless was essential for both signalling and receptor-mediated internalization. Two mutants were found to selectively affect cell activation or receptor internalization. Mutation of aspartic acid at position 18 to alanine (D18A) blocked receptor internalization without affecting cell activation. Mutation of leucine at position 35 to alanine (L35A) had the opposite effect: cell activation was blocked, whereas internalization was not affected. Therefore, in the case of the FcR-associated γ -chain, receptor internalization and cell activation were not necessarily dependent on each other. Cell activation may occur in the absence of internalization (D18A mutant) and, conversely, internalization can proceed without effective signalling (L35A mutation).

To further characterize the effect of the L35A mutant

Fig. 2. L35 mutation prevents syk phosphorylation induced by FcγR/icy chimera. IIA1.6 cells expressing FcγRIIb2 receptors, FcγR/icy chimera or L35A mutant were stimulated, or not stimulated, through endogenous BcR with F(ab')₂ fragments of rabbit anti-mouse IgG antibodies (15 μg/ml) or through Fc receptors with the rat anti-mouse FcR antibody 2.4G2 (20 μg/ml) then F(ab')₂ fragments of mouse anti-rat IgG (30 μg/ml). The cells were then washed and lysed with 0.5% Triton X-100. Cell lysates were immunoprecipitated with antiphosphotyrosine antibodies (A) or rabbit anti-syk antibodies (B). (A) Phosphoproteins were detected by Western blotting using HRP-coupled antiphosphotyrosine antibody, PY20, (upper panel) or a rabbit antiserum specific for the N-terminal end of the syk tyrosine kinase (lower panel). (B) The syk tyrosine kinase activity was determined by measuring the phosphorylation of a specific substrate, the GST-HS1 fusion protein, by Western blotting using HRP-coupled antiphosphotyrosine antibody, PY20, (upper panel). The amount of syk tyrosine kinase was determined, for each point on the same filter, by using a rabbit antiserum specific for the N-terminal end of the syk tyrosine kinase (lower panel).

on receptor-mediated cell activation and ligand internalization, cells expressing the L35A chimeric receptors were cloned and three individual clones expressing similar levels of the mutant receptors were isolated and analysed. Results obtained with one of the clones are shown; similar results were obtained with the two other clones. We first tested whether the L35A mutant activated PTK syk. Wild-type and L35A FcγR/icy-expressing cells were stimulated for 2 min at 37°C through FcRs [using the anti-FcR monoclonal antibody (mAb), 2.4G2] or through endogenous mIgG2a [using F(ab')₂ fragments of rabbit anti-mouse IgG]. Cell lysates were prepared and phosphotyrosine-containing proteins were immunoprecipitated with agarose-coupled anti-phosphotyrosine PT66 antibody and analysed by immunoblotting with HRP-coupled antiphosphotyrosine mAb Py20 or rabbit anti-syk antisera.

As shown in Figure 2A, B-cell stimulation through endogenous BcR or FcγR/icy chimera induced the phosphorylation of similar pattern cellular substrates and PTK syk (Figure 2A, lower panel). Engagement of the BcR or FcγR/icy chimera also increased the kinase activity of syk, as measured by the phosphorylation of a specific substrate, a glutathione *S*-transferase (GST) fusion protein containing HS1 polypeptide. In cells expressing the L35A-mutated FcγR/icy chimera or the FcγRIIb2 (which contains no ITAM), FcR engagement did not induce either phosphorylation of cellular proteins and syk (Figure 2B, middle panels) or activation of syk PTK activity (Figure 2B). Endogenous mIg induced PTK activity and syk phosphorylation efficiently in both cell lines (Figure 2A and B).

Therefore, L35A mutation prevents phosphorylation and activation of syk tyrosine kinase.

Activation of syk is dispensable for receptor internalization

To examine the role of syk activation in γ-chain-mediated internalization, we next assessed, by electron microscopy, the ability of wild-type and L35A-mutated FcγR/icy chimera to localize to coated pits. We have reported previously that the internalization of heterotrimeric type III Fc receptor or FcγR/icy chimera, in contrast to type II Fc receptor, is only triggered by efficient receptor cross-linking (Bonnerot and Daeron, 1994). Therefore, to test whether L35 mutation affects the coated-pit localization of the FcR/icy chimera, cells were sequentially incubated for 30 min at 4°C with the mAb 2.4G2 and a rabbit polyclonal anti-rat IgG and protein A coupled to 10 nm gold particles. After 2 min of receptor engagement at 37°C, both receptors were found in clathrin-coated pits and vesicles (Figure 3A–D). The frequencies of localization to these structures were not affected by the mutation, since 70.7 and 70% of gold-labelled receptors were found in clathrin-coated structures in cells expressing wild-type or L35A-mutated FcγR/icy chimera, respectively.

Next, we evaluated the kinetics and efficiency of immune complex internalization. Horse radish peroxidase (HRP)-containing immune complexes were bound to cells expressing the chimeric wild-type or mutant receptors at 4°C. The cells were then incubated at 37°C for different times and the amounts of internal HRP were measured.

Fig. 3. Recruitment of Fc γ R/icy and L35A mutant in clathrin-coated pits. Cells were incubated sequentially for 30 min at 4°C with the mAb 2.4G2 and a rabbit polyclonal anti-rat IgG and protein A coupled to 10 nm gold particles. The cells were then incubated for 2 min at 37°C. In both Fc γ R/icy- (A and B) and L35A- (C and D) expressing cells, the gold particles were present at the cell surface, in coated-pits and in coated-vesicles, bar = 100 nm.

As shown in Figure 4A, no differences in the kinetics or efficiency of HRP immune complex internalization were found between the wild-type type III Fc receptor, Fc γ R/icy chimera and the L35A mutant receptors. Together, these results show that the L35A mutation did not affect the early steps of receptor endocytosis and suggest that efficient induction of syk PTK activation is not required for coated-pit-mediated, ligand-induced uptake through the immunoreceptor-associated γ -chain.

L35A mutation prevents transport from endosomes to lysosomes

Next, we tested the effect of L35A mutation on the lysosomal transport of the chimeric receptor, first by assessing its ability to mediate degradation of internalized radiolabelled bovine serum albumin (BSA), and secondly by directly measuring transport to lysosomes by subcellular fractionation. Radiolabelled BSA immune complexes were incubated for various times at 37°C with cells expressing either the wild-type or the L35A mutant receptors. Degradation of the internalized immune complexes was estimated by measuring the TCA-soluble fraction of iodinated-immune complexes. As shown in Figure 4B, the type III FcR or Fc γ R/icy chimera allowed immune complex degradation at similar amounts, whereas the L35A muta-

Fig. 4. L35A mutation prevents immune complex degradation through the Fc γ R/icy chimera but does not affect ligand internalization. (A) Internalization of the HRP/anti-HRP immune complexes by Fc γ R/icy and L35A mutant or heterotrimeric type III Fc γ R (Fc γ RIII α . γ) was measured as described in Materials and methods. (B) Degradation of iodinated DNP-BSA/anti-DNP immune complexes was measured as described in Materials and methods.

tion inhibited the generation of TCA soluble counts by >90% during the first 2 h of culture, suggesting that lysosomal transport was blocked by the mutation.

The delivery of HRP-containing immune complexes by wild-type or L35A mutant chimeras to lysosomes was measured directly by subcellular fractionation. Lysosomal fractions were isolated in continuous self-forming 25% Percoll gradients. Dense Percoll fractions contained most of the β -hexosaminidase activity (a lysosomal resident enzyme), whereas light Percoll fractions contained the majority of the alkaline phosphodiesterase activity (APDE, a marker of the plasma membrane), as well as early and late endosomes detected by the presence of rab5 and rab7 (Figure 5A).

HRP immune complexes bound at 0°C to cells expressing the wild-type or L35A mutant chimeric receptors were found exclusively in Percoll light fractions (Figure 5B, upper panel). When the cells were incubated for 1 h at 37°C, a significant proportion of the HRP was found in dense Percoll fractions in the case of cells expressing the wild-type chimeric receptors (40–50%, Figure 5B and C). In contrast, in the case of cells

Fig. 5. L35A mutation prevents immune complexes lysosomal transport through FcγR/icγ chimera. (A) Cells were fractionated on Percoll gradients. Fractions containing lysosomes were identified by measuring β-hexosaminidase activity and fractions containing plasma membranes were identified by measuring APDE activity. The content of each fraction was characterized by Western blotting using anti-rab5, anti-rab7 and anti-lamp1 specific antibodies. Each blot was quantified after scanning the films with a photometer. (B) HRP/anti-HRP immune complexes were bound for 2 h at 4°C on cells expressing FcγR/icγ or L35A mutant then fractionated directly (time 0) or after 1 h incubation at 37°C (time 60 min). HRP activity was measured in each fraction using an enzymological assay. The results are presented as the percentage of the HRP activity contained in each fraction versus the total of the HRP activity contained in all the fractions. (C) The experiment was performed three times and the mean value of HRP activity was calculated in the four fractions containing β-hexosaminidase (dense fractions in black) or in the four fractions containing APDE (light fractions in grey).

expressing the L35A mutant chimeras, delivery to dense Percoll fractions was inhibited, with only 10–20% of the HRP activity found in dense Percoll fractions after 60 min (Figure 5B and C). These results show that the L35A mutation prevented γ-chain-mediated, ligand-induced lysosomal transport.

Syk mutant prevents efficient syk kinase activation

Different mechanisms might account for the effect of the L35A mutation on immunoreceptor lysosomal transport. One possibility was that overall cell activation through the ITAMs increased the efficiency of lysosomal transport and degradation of immune complex by indirect means. This possibility was excluded by co-activation experiments where cell activation (and syk activation) were induced through surface immunoglobulins (which are also ITAM-dependent). We found that co-activation through mIg did not modify the efficiency of immune complex degradation or transport to lysosomes in cells expressing the L35A mutant chimeras (not shown). A second possibility was that downstream effectors of cell activation through the ITAMs might be involved directly in endosome to lysosome transport of γ-chain-associated immunoreceptors. Indeed, a direct connection between PTK activation and receptor internalization has been established for PTK receptors (PTKR), such as the epidermal growth factor receptor (EGFR), since EGFR kinase activity was shown to be required for receptor recruitment into clathrin-coated pits (Lamaze and Schmid, 1995).

To determine whether syk is involved directly in the lysosomal transport of immunoreceptors, we stably expressed a kinase-deficient syk mutant [corresponding to the two syk SH2 domains tagged with a hemagglutinin (HA) epitope] in FcγR/icγ- or FcγRIIb2-expressing B lymphoma cells. Individual clones were isolated by intracellular staining with anti-HA mAb (not shown) and expression of the dominant negative Syk was measured by Western blot using a rabbit anti-sera specific for a peptide contained in the N-terminal portion of the syk SH2 domains. As shown in Figure 6A, the syk mutant (34 kDa) was expressed in a 3- to 5-fold excess as compared with endogenous syk (72 kDa), in two independent clones expressing FcγR/icγ chimeras and in two clones expressing FcγRIIb2.

The effect of the syk mutant on the activation of the endogenous syk through FcγR/icγ chimera was analysed first. The cells were stimulated, or not, through chimera or endogenous mIgG2a, and phosphoproteins were immunoprecipitated and analysed by Western blotting using PY20 or anti-syk antibodies. Overexpression of the syk dominant negative mutant prevented the phosphorylation of intracellular proteins (Figure 6B, upper panels), as well as the phosphorylation of endogenous syk kinase (Figure 6B, lower panels), through the FcγR/icγ chimera. Induction of phosphorylation of endogenous proteins and syk by engagement of the mIg was also decreased, but not abolished (Figure 6B). This is possibly because of the high level of expression of sIgG2a in these cells. In addition, using *in vitro* kinase assays, we found that the syk mutant prevented autophosphorylation of syk after engagement of the FcγR/icγ chimera (Figure 6C). We also found that overexpression of syk SH2 domains led to a substantial inhibition of kinase activity after cell stimulation through the FcγR/icγ chimera and endogenous mIg measured by phosphorylation of the GST-HS1 fusion protein *in vitro* (not shown). Similar results were obtained with another independent clone (not shown).

Together, these results show that expression of a domin-

Fig. 6. Syk mutant inhibits the activation of endogenous syk tyrosine kinase. (A) Stable overexpression of a cDNA encoding the two SH2 domains of the rat syk tyrosine kinase in Fc γ R/IIb2 receptors or Fc γ R/ic γ chimera expressing cells was analysed by Western blotting using a rabbit antiserum specific for the N-terminal end of syk. The clones G4 and C2 (Fc γ R/IIb2) or C3 and C7 (Fc γ R/ic γ) are representative of a series of 40 independent clones characterized. The arrow heads indicate the endogenous syk tyrosine kinase (70 kDa) and the dominant negative mutant (34 kDa), respectively. (B and C) Fc γ R/ic γ chimera cells overexpressing the dominant negative syk (G2 and C2 clones) mutant were stimulated or not through endogenous BcR or Fc chimera as described in Figure 4. The cells were then washed and lysed with 0.5% Triton X-100. Cell lysates were immunoprecipitated with antiphosphotyrosine antibodies (B) or rabbit anti-syk antibodies (C). (B) Phosphoproteins (upper panel) or syk tyrosine kinase (lower panel) were detected by Western blotting as in Figure 4. (C) The phosphorylation of syk kinase was detected after a kinase assay and products were analysed by 10% SDS-PAGE. The quantity of immunoprecipitated syk was controlled for each point by Western blotting (not shown).

ant negative truncated syk PTK inhibits Fc γ R/ic γ chimera-mediated activation of endogenous wild-type syk.

Involvement of the PTK syk in γ -chain-mediated lysosomal transport

Stable expression of the syk SH2 domains, and observed inhibition of the γ -chain-mediated activation of the endo-

ogenous syk, provided us with an experimental model to determine the involvement of this PTK in receptor internalization and lysosomal transport. We compared the effect of syk mutant overexpression on internalization and degradation of immune complexes Fc γ R/ic γ -chimera- and Fc γ R/IIb2-expressing cells. Internalization of HRP immune complexes and degradation of radiolabelled BSA immune complexes were determined as before.

As shown in Figure 7A, Fc γ R/ic γ - and Fc γ R/IIb2-mediated immune complex internalization with the same kinetics and efficiencies in cells expressing, or not expressing, the syk mutant. In contrast, overexpression of the syk mutant strongly decreased the degradation of iodinated BSA immune complexes internalized through the Fc γ R/ic γ chimera, whereas no effect was observed on Fc γ R/IIb2-mediated degradation (Figure 7B). These results suggest that the syk mutant specifically inhibited transport of ITAM-containing immunoreceptors from endosomal to lysosomal compartments.

Lysosomal transport of HRP immune complexes through the Fc γ R/ic γ chimera was analysed using subcellular fractionation on Percoll gradients as described above. After 1 h of incubation at 37°C, the percentage of HRP activity in the lysosomes was decreased slightly in clones expressing the syk mutant, as compared with non-transfected Fc γ R/ic γ -expressing cells (Figure 7C). Therefore, overexpression of dominant negative syk mutant prevented γ -chain-mediated lysosomal transport of immunoreceptors. This suggests that γ -chain-containing receptors need to activate syk in order to be efficiently targeted to lysosomes for efficient degradation.

Involvement of PTK syk in MHC class II restricted antigen presentation

We recently reported that internalization of antigen-antibody complexes through Fc γ R/ic γ chimeras induced the efficient presentation of a larger number of peptides on MHC class II molecules (Amigorena *et al.*, 1998). In contrast, the L35A mutant receptors and Fc γ R/IIb2 only presented a subset of these MHC class II-peptide complexes. In the case of the CI λ phage repressor, the same peptide (12–24) may be presented to two different T-cell hybrids in association with IAd or IE^d. In this particular case, CI immune complex internalization through Fc γ R/ic γ chimeras induced the presentation of these two T-cell epitopes, whereas internalization through the L35A mutant receptors or through Fc γ R/IIb2, only induced presentation of the IAd-restricted T-cell epitope (IAd12–26).

We therefore evaluated whether the alteration of lysosomal transport of immune complexes through Fc γ R/ic γ chimeras by overexpression of the syk mutant would also affect presentation of the IAd12–26 or IE^d12–26 T-cell epitopes. To test this, cells were incubated with various concentrations of CI immune complexes in the presence of one of the two T-cell hybridomas.

As shown in Figure 8A, the IAd12–26 epitope was presented efficiently after immune complex internalization in Fc γ R/ic γ - or Fc γ R/IIb2-expressing cells. Similarly, two independent clones expressing the truncated SH2 domains of syk still presented this T-cell epitope efficiently (Figure 8A). As we have shown previously, the IE^d12–26 T-cell epitope was also presented efficiently after immune complex internalization in Fc γ R/ic γ -expressing cells (Figure

Fig. 7. syk mutant selectively affected lysosomal transport and immune complex degradation through γ -chain cytoplasmic tail. Fc γ RIIb2 receptors (triangles) or Fc γ R/IC γ chimera (circles) positive cells, overexpressing (black) or not (white) syk dominant negative mutant were tested for the internalization of the HRP/anti-HRP immune complexes (A), and for the degradation of iodinated DNP-BSA/anti-DNP immune complexes (B). (C) Lysosomal transport of HRP/anti-HRP immune complexes through Fc γ R/IC γ was compared in cells overexpressing (C3 clone) or not overexpressing syk mutant. Cell fractionation was performed using Percoll gradients as in Figure 4. The results correspond to the percentage of the HRP activity contained in each fraction/total of the HRP activity contained in all the fractions after 1 h incubation at 37°C of the C3 clone overexpressing the syk dominant negative mutant. The same experiment was performed twice and the mean value of HRP activity was calculated in the four fractions containing β -hexosaminidase (dense fractions in black) or in the four fractions containing APDE (light fractions in grey).

8A). In contrast, in cells overexpressing the syk SH2 domains, no presentation of the IE^d12–26 epitope was detected after internalization of the immune complex through Fc γ R/IC γ chimeras (Figure 8A). Nevertheless, these cell lines presented the 12–24 peptides on IE^d or IA^d with similar efficiencies (Figure 8B).

Therefore, overexpression of the syk dominant negative mutant selectively inhibited the presentation of a T-cell epitope specifically generated after γ -chain-mediated internalization. The syk tyrosine kinase, a major effector of the FcR- and BcR-induced signalling pathway, therefore possibly influences the selection of T-cell epitopes presented by MHC class II molecules.

Discussion

Internalization and lysosomal transport of immunoreceptors are critical steps for both cell signalling (receptor downmodulation) in haematopoietic cells and MHC class II-restricted antigen presentation in antigen presenting cells. The signals required for cell activation through immunoreceptors lie within conserved amino acid motifs, the ITAMs, which are present in the cytosolic tails of immunoreceptor-associated chains. These motifs are involved directly in the activation of cytosolic PTKs. ITAMs also bear the information required for the internalization of immunoreceptors. In most cases, these internalization signals are different from the conventional internalization and lysosomal targeting motifs described in non-haematopoietic cells, suggesting that haematopoietic cells may have unique pathways for immunoreceptor

membrane traffic. Indeed, several reports have recently shown that cytosolic effectors of cellular signalling may control the endocytosis or phagocytosis of immunoreceptors. Here, we show that a point mutation, which prevents syk activation via the γ -chain ITAM, and the expression of a syk dominant negative mutant inhibit FcR lysosomal transport without affecting receptor internalization. Therefore, a major cytosolic effector of signal transduction via immunoreceptors, the PTK syk, is selectively required for the γ -chain-dependent sorting of FcRs from endosomes to lysosomes.

However, syk activation *per se* does not account for lysosomal targeting of immune complexes, since activation by surface immunoglobulins (which induces activation of syk) did not restore immune-complex degradation in L35A mutant-expressing cells (not shown). These results suggest that the receptors interacting with the ligand through their luminal domains must also interact individually with syk through the cytosolic tails of their associated chains to be efficiently routed to lysosomes. This system would allow the selective degradation of receptors that have transduced signals (and not of those that were not engaged) in the case of receptor downmodulation, and in the exclusive delivery of receptor–ligand complexes to lysosomes (and not of free receptors), in the case of antigen presentation.

Our mutagenesis analysis of the γ -chain ITAM indicates that lysosomal transport of immunoreceptors is not controlled at the level of receptor internalization. The cytoplasmic tail of the γ -chain contains two YxxL motifs which, unphosphorylated, might mediate receptor internalization by interacting with AP2 complexes (Ohno

Fig. 8. Overexpression of syk dominant negative mutant selectively affected the presentation of T cell epitope. (A) Fc γ RIIb2 receptors (squares), Fc γ R/icy chimera (circles) or L35 mutated Fc γ R/icy chimera (triangles) positive cells, overexpressing (black and grey) or not overexpressing (white) syk dominant negative mutant were cultured in the presence of increasing concentrations of CI-IgG complexes (FcR-mediated endocytosis). T-cell hybridomas specific for a dominant epitope (IA^d12-26) or a cryptic epitope (IE^d12-26) from the CI λ repressor were also included in the cultures. The secretion of IL-2 by the T-cell hybrids was measured after overnight incubation. (B) All the transfected cell lines presented the 12-26 peptide on IA^d or IE^d with identical efficiencies. T-cell stimulation was evaluated by a CTLL2 proliferation assay (similar results were obtained in two independent experiments).

et al., 1995, 1996). However, we have shown previously that mutation of either tyrosine residue in the γ -chain ITAM prevents internalization (Amigorena *et al.*, 1992). Either of these two mutations also prevents phosphorylation of the other ITAM tyrosine and activation of PTKs (Bonnerot *et al.*, 1992), suggesting that internalization is somehow related to signalling. In contrast, the L35A mutant, suggests that ITAM tyrosine phosphorylation is not an absolute requirement for receptor internalization: γ -chain localization to coated pits and endocytosis occurred in the absence of syk activation. Furthermore, the D18A mutation, which prevented internalization but not signalling, suggests that phosphorylation of the YxxL motif is also not sufficient for internalization. Therefore, activation of syk is neither necessary nor sufficient for γ -chain-mediated immunoreceptor internalization.

In contrast, transport from endosomes to lysosomes was critically dependent upon activation of syk. L35A mutation blocked immune complex degradation and lysosomal transport suggesting that leucine 35 is part of a lysosomal targeting signal. It is unlikely, however, that this leucine is part of a dileucine signal (Letourneur and Klausner, 1992) (a well-characterized lysosomal targeting signal), since this residue is surrounded by charged or polar amino acids (T34 and K36). Since the L35A mutation prevented activation of syk, we postulated that activation of syk is required for lysosomal transport of immunoreceptors.

However, our results do not exclude the possibility that a downstream effector activated by syk, and not syk itself, is the actual direct effector controlling lysosomal transport. If syk was involved directly in lysosomal transport, it should be present on endosomal membranes after receptor engagement. By confocal and electron microscopy, we found that after immune complex internalization in cells expressing Fc γ R/icy chimeras, the PTK syk is detected in transferrin-receptor-containing compartments (unpublished results). The phosphorylated ITAM γ -chain may therefore activate and recruit syk either at the cell surface, and then follow the internalized receptors, or directly in endosomal compartments.

The molecules linking syk recruitment to the sorting for lysosomal transport are yet to be defined. Recently, syk was shown to associate with and activate the regulatory subunit of the PI3K, p85 (Deckert *et al.*, 1996) (which modulates the activity of the catalytic subunit, p110). The target of this enzyme is the inositol ring bound to the fatty acids of most of cell membranes. A role of PI3K in intracellular transport has been demonstrated by analysis of yeast mutants (vps 34, yeast equivalent of the PI3K) deficient for transport to the vacuole (yeast equivalent of lysosomes) (Liscovitch and Cantley, 1995). Furthermore, wortmanin, an inhibitor of PI3K, blocks endosomal transport to lysosomes. PI3K therefore represents a good candidate for being the effector of syk in endosomal γ -chain sorting towards lysosomes.

Supporting this possibility, PI3K was shown to be involved in the lysosomal transport of EGFR and of platelet growth factor receptor (PGFR). In the case of PGFR, recruitment of PI3K onto endosomal membranes was found (Joly *et al.*, 1995). By analogy with the model proposed for tyrosine kinase growth factor receptors, we propose that syk may act as an 'adaptor' between the phosphorylated ITAM in immunoreceptors and PI3K. This would allow the recruitment of the PI3K onto endosomal membranes and immunoreceptor sorting into lysosomes.

Why, then, would lysosomal transport be coupled to cell signalling? First, lysosomal degradation may control the function of immunoreceptors by removing them from the cell surface (receptor downmodulation). Indeed, in the case of T lymphocytes, activation of the PTK Lck is involved in TcR lysosomal degradation (D'oro *et al.*, 1997). Since syk is expressed in subpopulations of intra-epithelial T cells, our results suggest that the activation of syk (or ZAP70 in other T cells) could be the effector of the Lck-induced transport of TcR to lysosomes. Secondly, syk is also a major effector of several FcRs, including IgE FcRs (Fc ϵ RI) in mast cells (Scharenberg *et al.*, 1995) and different IgG FcRs (such as Fc γ RI or Fc γ RIII) in macrophages, NK cells and granulocytes

(Ravetch, 1994). In these cell types, syk activation and lysosomal transport of FcRs might participate in the regulation of specific cell functions, such as IgE-induced mast cell degranulation, antibody-dependent cell cytotoxicity (ADCC) or phagocytosis of IgG-coated particles by macrophages.

Finally, the PTK syk is also a cytoplasmic effector of several receptors involved in antigen uptake at the surface of antigen-presenting cells. Indeed, binding of immune complexes (to FcγRI/III) or soluble antigens (to the BcR) induces both syk activation and receptor-mediated antigen uptake for lysosomal degradation. We have shown that the optimal antigen presentation of a variety of T-cell epitopes requires the efficient activation of syk. Since syk activation is also required for lysosomal transport, it is tempting to speculate that lysosomal transport is required for efficient antigen presentation, at least of some epitopes. However, the results obtained with cells expressing FcγRIIb2 suggest that the situation may be more complex. Indeed, FcγRIIb2-expressing cells presented the same epitopes as the L35A mutant-expressing cells or as cells expressing the syk dominant negative mutant. However, in contrast to these two latter cell lines, FcγRIIb2-expressing cells efficiently mediated lysosomal transport and degradation of immune complexes.

Therefore, there is not a simple correlation between lysosomal transport of immune complexes and the presentation of different T-cell epitopes. Efficient antigen presentation may thus be due to transport to another, non-lysosomal compartment, similar to the class II vesicles described previously (Amigorena *et al.*, 1994), transport into which may also be syk dependent. Alternatively, different sub-populations of lysosomes may exist, all heavy in Percoll gradients and of high proteolytic activity, which would be differentially accessed by syk-dependent and syk-independent pathways.

Materials and methods

B lymphoma cell lines

The B lymphoma IIA1.6 is a FcγR-defective variant of A20 B lymphoma cells (Jones *et al.*, 1986). The cell lines were cultured in RPMI 1640 containing 10% fetal calf serum (FCS), 10 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol and 5 mM sodium pyruvate (Gibco-BRL, Paisley, UK). FcγRII/icy chimeras were constructed by adding the sequences encoding the cytoplasmic domain of the γ-chain to cDNA encoding the extracellular and transmembrane domains of mouse FcγRII (Bonnerot *et al.*, 1992). Mutated-chimeras were constructed by polymerase chain reaction (PCR) using two complementary oligonucleotides as described previously (Bonnerot *et al.*, 1992) thereby introducing an alanine as shown in Figure 1. The resulting constructions were inserted into expression vectors bearing neomycin resistance, sequenced and stably expressed by transfection in the mouse B-cell line IIA1.6 as described previously (Bonnerot *et al.*, 1992). After selection with G418 (1 mg/ml), positive cells were enriched by panning using plastic plates coated with the mAb 2.4G2 (Cassard *et al.*, 1996). Cell-surface expression of the FcR chimera was measured with the rat anti-mouse FcγR mAb 2.4G2 and detected by FITC-coupled mouse anti-rat antibodies. The samples were analysed using a FACScan flow cytometer (Becton Dickinson). After one or two rounds of panning, 90% of the transfected cells were positive for FcR expression. The cloning of L35A FcγRII/icy chimeras was as described previously (Amigorena *et al.*, 1998).

Stable expression of syk dominant negative mutant

The syk dominant negative mutant was constructed by joining the *EcoRI*-*KpnI* fragment of rat syk cDNA (Benhamou *et al.*, 1993) to a *KpnI*-*XbaI* PCR fragment amplified from an HA-tag-containing plasmid.

The resulting truncated syk construct was verified by sequencing. It corresponds to the first 260 amino acids of the rat syk cDNA fused to GSGYSYDVPDYA (HA-tag). This construct was then inserted in an *Srα*-driven expression vector bearing puromycin resistance. After linearization with *ScaI*, 50 µg of DNA was used to electroporate B cells expressing either the FcγRicy chimera or FcγRIIb2 as described previously (Bonnerot *et al.*, 1992). After 48 h, the transfected cells were resuspended at 10³ cells/ml in culture media containing 2 µg/ml puromycin (Sigma). The cell suspension was distributed in 96-well plates at 100 µl/well and incubated at 37°C for several weeks. Cells were selected for the expression of the truncated syk using FITC-coupled 12CA5 anti-HA-tag antibody (Boehringer Mannheim). Cells were fixed with 3% paraformaldehyde in PBS then incubated for 10 min in 100 mM glycine in PBS. Cells were permeabilized with 0.05% Saponin (Sigma) in PBS then incubated for a further 30 min at room temperature with 20 µg/ml FITC-coupled 12CA5 in 0.05% Saponin, 0.2% BSA in PBS. After washing, the cells were resuspended in PBS and samples were analysed by FACScan flow cytometer (Becton Dickinson). Positive cells were further characterized by Western blotting using an affinity-purified rabbit antibody raised against amino acids 13–31 within the first SH2 domain of human syk (Santa Cruz Biotechnology). The sequence of this peptide is identical in rat and mouse syk. A quantity of cells (5 × 10⁵) were lysed with 0.5% Triton X-100, separated in a 10% polyacrylamide gel and transferred on PVDF membrane (Millipore). The membranes were incubated for 1 h with 0.1 µg/ml of the anti-syk antibody, washed three times then further incubated with an HRP-coupled goat anti-rabbit antiserum (Amersham). Chemiluminescence was detected using a commercial kit (Boehringer Mannheim). The C3 and C7 clones, (for the FcγR/icy-expressing cells) and the C2 and G4 clones, (for the FcγRIIb2-expressing cells) were selected on the basis of high levels of truncated-syk expression (34 kDa) and similar levels of FcR chimera (detected with the rat anti-mouse FcR antibody 2.4G2) as the parental cells.

Assays for antigen presentation

T-cell hybridomas and cells used in antigen presentation assays were cultured in RPMI 1640 containing 10% FCS, 1 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2-ME (5 × 10⁻⁵ M). The CI λ-repressor-specific hybridomas 24.4 and 26.1 have been characterized previously (Guillet *et al.*, 1986). Antigen presentation was assessed by culturing transfected IIA1.6 cells together with specific T-cell hybridomas for 18–20 h in the presence of various concentrations of antigens either complexed or not complexed with specific antibodies: λ repressor was complexed with the two mAbs, 22D and 51F (Amigorena *et al.*, 1992). Complexes were preformed by incubating different concentrations of purified λ-repressor (from 30 000 to 0.5 ng), with 15 µg/ml of the mAbs at 37°C for 15 min. The release of IL-2 by the T-cell hybridoma was determined by a CTL.L2 proliferation assay. Each point represents the average of duplicate samples, which varied by <5%.

Detection of tyrosine kinase activation

Cells were preincubated at 4°C with or without 20 µg/ml of 2.4G2 for 30 min and then washed twice with RPMI. A quantity of cells (5 × 10⁶) was then stimulated with prewarmed F(ab')₂ fragments of mouse anti-rat antiserum (50 µg/ml) at 37°C for 2 min. As a positive control, untreated cells were stimulated with prewarmed F(ab')₂ fragments of anti-IgG antibodies (30 µg/ml) for 2 min at 37°C. The stimulated cells were washed and resuspended in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% TX-100) containing a cocktail of protease inhibitors and tyrosine phosphatase inhibitors (NaF 5 mM, EDTA 5 mM, Orthovanadate 1 mM). Phosphoproteins were immunoprecipitated with agarose-coupled antiphosphotyrosin antibodies PT-66 (Sigma) or a rabbit antiserum raised against the 11 C-terminal amino acids from human syk coupled to keyhole limpet haemocyanin. The phosphoproteins were then analysed using 10% SDS-PAGE gels and Western blotting with the antiphosphotyrosine mAb Py20 coupled to HRP (ICN Flow, France). Chemiluminescence was detected with a commercial kit (Boehringer Mannheim) by exposure of the filters to X-omat films (Kodak). The filters were then stripped by incubation at 50°C in 50 mM Tris pH 6.8, 2% SDS, 100 mM β-2-mercaptoethanol for 30 min. The filters were then incubated for 1 h with 0.1 µg/ml of the anti-syk antibody (Santa Cruz Biotechnology), washed three times and then incubated further with an HRP-coupled goat anti-rabbit antiserum. Two kinase assays were performed with syk immunoprecipitates. A GST-HS1 fusion protein, constructed by joining a *Bam*HI-*Eco*RI PCR fragment containing the HSI peptide motif (EQEDEPEGDYEEVLE-Stop) in-frame into the polylinker of the pGEX-2TK vector, served as a specific substrate for syk (Brunati *et al.*, 1995). Phosphorylation on tyrosine was detected by

Western blotting using HRP-coupled anti-phosphotyrosine antibody PY20 as above. In another set of experiments, autophosphorylation of the syk tyrosine kinase was detected using ^{32}P -labelled ATP (Scharenberg *et al.*, 1995). For these two assays the same kinase buffer was used and the reaction mixture were analysed by SDS-PAGE (Scharenberg *et al.*, 1995).

Immune complexes internalization

The cells were washed once with internalization buffer (RPMI, 5% FCS, 10 mM glutamine, 5 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 20 mM HEPES, pH 7.4) and incubated with HRP anti-HRP immune complexes for 2 h at 4°C (10⁷ cells/ml). Immune complexes were prepared as a 10 $\times$ solution with internalization buffer (HRP 50 $\mu\text{g}/\text{ml}$ and a polyclonal rabbit anti-HRP antibody at 400 $\mu\text{g}/\text{ml}$) for 30 min at 37°C. After fixation of HRP-ICs, the cells were washed three times with internalization buffer and incubated at 37°C for various times (2 $\times$ 10⁶ cells/ml). Internalization was stopped by adding cold internalization buffer and the cells were washed once with PBS. Duplicates of each time point were either left in PBS at 4°C to measure cell surface HRP-ICs or incubated in Triton X-100 (0.1%) for 5 min at room temperature to measure the total amount of HRP-ICs. HRP was revealed by adding substrate buffer (0.5 mg/ml OPD, Sigma, and 0.12% H₂O₂ in 50 mM phosphocitrate buffer pH 5.0) at 4°C (Drake *et al.*, 1989). The reaction was stopped with 6N HCl and the change in colour was determined spectrophotometrically at 492 nm.

Immune complex degradation

BSA was coupled to TNP (2,4,6-trinitrophenyl) as described previously (Little and Eisen, 1967) to yield 32 mol of TNP/mol of BSA. The TNP-BSA was iodinated using Iodo-Beads (Pierce, USA) as described by the manufacturer. Free Na¹²⁵I was separated by a Sephadex G25 column. Immune complexes were prepared by incubating iodinated TNP-BSA (20 $\mu\text{g}/\text{ml}$) and affinity-purified rabbit anti-TNP antibodies (20 $\mu\text{g}/\text{ml}$) (Molecular Probes) for 30 min at 37°C in internalization buffer (see above) to give a 2 $\times$ solution. The TNP-BSA immune complexes were fixed on cells by incubation with 2 $\times$ 10⁷ cells/ml at 4°C for 2 h. After extensive washing in internalization buffer, cells were incubated for various times at 37°C and then left at 4°C. TCA was added to 20% final concentration for 1 h at 4°C to precipitate proteins. The solution was then centrifuged for 30 min at 14 000 r.p.m. in an Eppendorf centrifuge to pellet proteins. Radioactivity in the cell pellet and in the supernatant was determined using a gamma-counter. The amount of degraded TNP-BSA was calculated as the percentage of TCA insoluble (supernatant) of total (supernatant and pellet) c.p.m. The average of three independent experiments is shown.

Cell fractionation

Preparation, fixation and internalization of HRP immune complexes were performed as described above. After internalization, cells were washed once with PBS and then resuspended in homogenization buffer (10 mM Triethanolamine, 10 mM acetic acid, 1 mM EDTA and 250 mM sucrose, pH 7.4) at 3 $\times$ 10⁷ cells/ml and passed through a ball-bearing homogenizer 8–10 times. Intact cells and nuclei were removed by centrifugation at 1200 r.p.m. for 10 min. The post-nuclear supernatant (500 μl) was mixed with homogenization buffer and Percoll to give 5 ml of a 23% Percoll solution, which was then fractionated by centrifugation at 33 000 r.p.m. for 30 min in a Beckman ultracentrifuge using a TLA-100.4 rotor. Sixteen fractions were collected from the bottom of the gradient. β -hexosaminidase and APDE enzymological assays were performed as described previously (Green *et al.*, 1987) to determine the subcellular fractions containing lysosomes and plasma membranes, respectively. Briefly, 75 μl of each fraction were incubated with 100 μl of the APDE substrate for 1 h at 37°C; a colorimetric assay was then performed by measuring absorbance at 405 nm. For the β -hexosaminidase assay, 5 μl of each fraction were incubated for 30 min with 50 μl of the enzyme substrate buffer. The reaction was stopped by adding 2 ml of stop-buffer and the quantity of enzyme was measured using a fluorimeter (Hoefer) at 365 nm excitation wavelength, emission was read at 450 nm. The content of each fraction was also characterized by Western blotting using anti-rab5, anti-rab7 and anti-lamp1 specific antibodies. Each blot was quantified after scanning the films with a photocalera.

Electron microscopy

Internalization of immunogold labelled-receptors was performed as described (Raposo *et al.*, 1987). Fc γ RII/icy- and Fc γ RIII/icy L35-expressing cells were incubated for 30 min at 4°C in RPMI 5% FCS first with the primary antibody directed against mice Fc receptors, 2.4G2,

then with polyclonal rabbit anti-rat IgG antibodies (Dako), and finally with protein A–gold conjugates. Cells were then incubated for 2 min at 37°C then fixed for 1 h at 20°C with 1.5 % glutaraldehyde in 0.1 M cacodylate buffer, and then for 90 min with 1.5% glutaraldehyde, 1% tannic acid in 0.1 M cacodylate buffer. After 2 h of post-fixation at 4°C with 0.5% osmium tetroxide, 1.5% ferrocyanure in H₂O, cells were dehydrated in ethanol and embedded in Epon. Ultrathin sections were prepared and counterstained with 2% uranyl acetate in 50% methanol. To test for specificities, cells were incubated with either the rabbit anti-rat IgG and protein A–gold, or with protein A–gold alone. In both cases, no gold particles were detected. Gold-labelled receptors were counted directly from images of 30 cell profiles for Fc γ RII/icy-expressing cells (416 gold particles) and 50 cell profiles for Fc γ RIII/icy-L35-expressing cells (304 gold particles).

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