

# A unique insert in the linker domain of Syk is necessary for its function in immunoreceptor signalling

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**Accumulating data indicate that the ‘linker’ region of Syk, which lies between its tandem Src homology 2 (SH2) domains and kinase region, provides a critical function for the biological activity of Syk. This importance has been ascribed to the presence of tyrosine phosphorylation sites capable of mediating the recruitment of cellular effectors. We and others previously identified an alternatively spliced variant of Syk, termed SykB, which lacks a 23 amino acid sequence in the linker domain. As this ‘linker insert’ is also not present in the closely related enzyme Zap-70, it seems plausible that Syk possesses this unique sequence for functional reasons. To understand its role better, we have compared the abilities of Syk and SykB to participate in immunoreceptor-triggered signal transduction. The results of our experiments revealed that, unlike Syk, SykB was inefficient at coupling stimulation of FcεRI on basophils or the antigen receptor on T cells to the early and late events of cellular activation. Further studies showed that the functional defect in SykB was not caused by the absence of crucial tyrosine phosphorylation sites, or by a reduced intrinsic kinase activity. Rather, it correlated with the reduced ability of SykB to bind phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) *in vitro* and *in vivo*. In combination, these results demonstrated that the unique insert in the linker domain of Syk is crucial for its capacity to participate in immunoreceptor signalling. Furthermore, they provided evidence that the linker region can regulate the ability of Syk to bind ITAMs, thus identifying a novel function for this domain.**

**Keywords:** linker/hemopoietic cells/Syk/tyrosine protein kinase

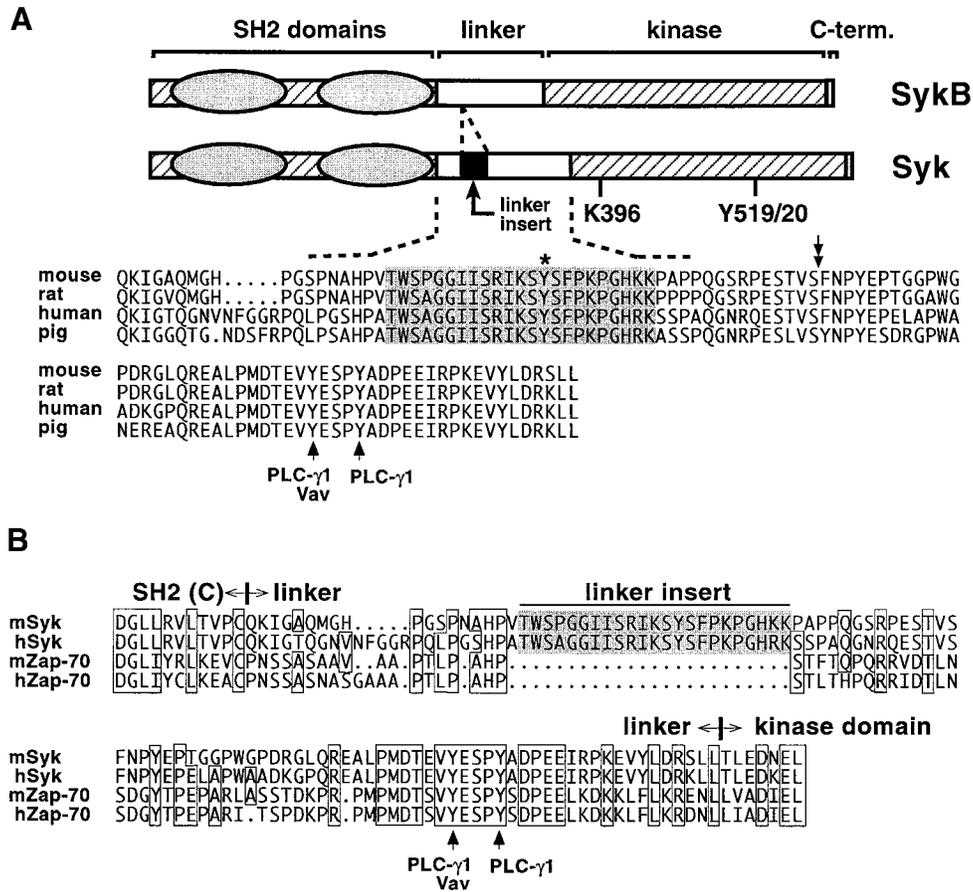
## Introduction

Stimulation of the antigen receptor on T and B lymphocytes, or the high affinity receptor for immunoglobulin (Ig) E on mast cells, leads to a rapid increase in tyrosine protein phosphorylation (reviewed in Perlmutter *et al.*, 1993; Weiss and Littman, 1994; Wange and Samelson,

1996; Daëron, 1997; Reth and Wienands, 1997). This signal predicates all subsequent events of cell activation, including lymphokine release, proliferation, differentiation, antibody production and degranulation. Even though these ‘immunoreceptors’ do not possess intrinsic tyrosine protein kinase (TPK) activity, they contain subunits capable of recruiting cytoplasmic TPKs. This capacity is mediated by an intracytoplasmic sequence termed immunoreceptor tyrosine-based activation motif (ITAM), which includes two YXXL motifs typically separated by 6–8 residues. There is mounting evidence that the two tyrosines of the ITAM become phosphorylated upon immunoreceptor engagement, primarily as a result of the action of Src family TPKs (reviewed in Chow and Veillette, 1995). This phosphorylation permits the recruitment of Src homology 2 (SH2) domain-containing molecules such as Syk and Zap-70, thus triggering the signalling cascade that leads to cell activation.

The Syk/Zap-70 family of cytoplasmic TPKs comprises two known members termed Syk and Zap-70 (Taniguchi *et al.*, 1991; Chan *et al.*, 1992; reviewed by van Oers and Weiss, 1995). While Syk is present in most hemopoietic cell types, including B cells and mast cells, Zap-70 is restricted to T cells and natural killer cells. Syk is also expressed in selected T cell types, including all major thymocyte populations and intra-epithelial  $\gamma\delta$  T lymphocytes (Chan *et al.*, 1994; Mallick-Wood *et al.*, 1996). Lower Syk expression levels are encountered in mature  $\alpha\beta$  T cells. The structure of Syk and Zap-70 is highly conserved. It includes, from the N- to the C-terminus (Figure 1A): (i) two SH2 domains, which bind doubly phosphorylated ITAMs; (ii) a ‘linker’ region, containing sites of tyrosine phosphorylation mediating the recruitment of downstream effectors like phospholipase C- $\gamma$  (PLC- $\gamma$ ) and Vav (Deckert *et al.*, 1996; Law *et al.*, 1996; Wu *et al.*, 1997); (iii) a catalytic domain, including sites for ATP-binding and tyrosine phosphorylation; and (iv) a short C-terminal extension of yet undetermined function.

Analyses of Syk-deficient variants of the chicken B-cell line DT-40 and the rat basophilic leukemia cell line RBL-2H3 have provided compelling indication that Syk is essential for signalling through the B-cell antigen receptor (BCR) and the Fc receptor for IgE (FcεRI) (Takata *et al.*, 1994; Zhang *et al.*, 1996). Furthermore, even though Syk is not necessary for antigen receptor signalling in most T lymphocytes, recent findings indicated that it plays a role distinct from that of Zap-70 in these cells (Chu *et al.*, 1996; Latour *et al.*, 1997; Williams *et al.*, 1997). This property is at least in part the result of the greater intrinsic TPK activity of Syk (Latour *et al.*, 1996; Zoller *et al.*, 1997). The creation of Syk-deficient mice by homologous recombination has also highlighted the importance of Syk in developmental processes (Cheng *et al.*, 1995; Turner *et al.*, 1995). Most importantly,



**Fig. 1.** Structure and sequence of Syk, SykB and Zap-70. (A) Structure of Syk and SykB. The structure of Syk and SykB, as well as the location of the 23 amino acid linker insert in Syk, are depicted at the top. The positions of the ATP-binding site, lysine 396 (K396) and the tyrosine phosphorylation sites in the kinase domain (tyrosines 519 and 520; Y519/520) are indicated. The sequence of the Syk linker domain in various species is shown at the bottom. Dots indicate missing amino acids. The conserved linker insert is shadowed and the position of tyrosine 290 is highlighted by an asterisk. The putative site of the proteolytic cleavage is shown by a double arrow, whereas those of binding of PLC- $\gamma$ 1 and Vav are indicated by single arrows. (B) Linker domain of Syk and Zap-70. The sequences of the linker domain of mouse Syk (mSyk), human Syk (hSyk), mouse Zap-70 (mZap-70) and human Zap-70 (hZap-70) are compared. Areas of identity are boxed, and the linker insert in Syk is shadowed. The putative binding sites for PLC- $\gamma$ 1 and Vav are indicated by single arrows.

Syk-deficient embryos were shown not to be viable as a result of a marked hemorrhagic diathesis. Moreover, they exhibited defects in B-cell development, Fc receptor-mediated activation of mast cells and macrophages (Costello *et al.*, 1996; Crowley *et al.*, 1997) and  $\gamma\delta$  T-cell maturation (Mallick-Wood *et al.*, 1996).

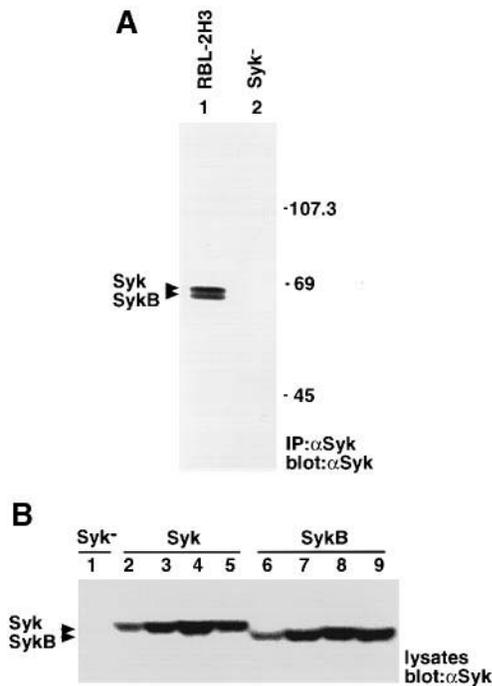
SykB is a naturally occurring isoform of Syk that was identified in mouse, rat and human cells (Yagi *et al.*, 1994; Rowley *et al.*, 1995; Latour *et al.*, 1996). As a consequence of alternative splicing of a 69 nucleotide-exon in the *syk* gene (Rowley *et al.*, 1995), it lacks a 23 amino acid insert in the linker region (hereafter termed 'linker insert'; Figure 1A). It is noteworthy that this sequence is highly conserved in mouse, rat, human and pig Syk. However, it is not present in Zap-70, even though the rest of the Zap-70 linker domain is significantly homologous to that of Syk (Figure 1B). On this basis, it could be supposed that Syk 'gained' this unique sequence for functional purposes. To better understand its role, we have compared the capacity of Syk and SykB to participate in immunoreceptor-mediated signal transduction. The results reported in this paper showed that, in comparison with Syk, SykB was much less effective at mediating immunoreceptor signal-

ling. This defect seemed to be related to the reduced capacity of SykB to bind tyrosine phosphorylated ITAMs.

## Results

### *Syk is more efficient than SykB at mediating Fc $\epsilon$ RI-induced activation of RBL-2H3 cells*

RBL-2H3 is a rat basophilic leukemia cell line used extensively to study Fc $\epsilon$ RI-mediated signal transduction. The importance of Syk in this cell line was emphasized by the finding that a Syk-deficient variant of RBL-2H3 failed to exhibit most of the Fc $\epsilon$ RI-induced tyrosine protein phosphorylation and subsequent release of inflammatory mediators (Zhang *et al.*, 1996). Hence, this system appeared ideal to study the importance of the unique linker insert in the ability of Syk to mediate immunoreceptor signalling. Since a previous report showed that RBL-2H3 contained both Syk and SykB (Rowley *et al.*, 1995), we first wanted to examine the expression of these two isoforms in the RBL-2H3 cells used for our studies. To this end, unstimulated cells were lysed in detergent-containing buffer, and Syk polypeptides were recovered by immunoprecipitation using a rabbit antiserum directed



**Fig. 2.** Expression of Syk and SykB in RBL-2H3 cells. **(A)** Detection of two Syk polypeptides in parental RBL-2H3 cells. Lysates were immunoprecipitated with anti-Syk ( $\alpha$ Syk) antibodies as detailed in Materials and methods. Syk polypeptides were then detected by anti-Syk immunoblotting. The migrations of molecular weight markers are shown on the right, while those of the two polypeptides consistent with Syk and SykB are indicated on the left. Exposure: 12 h. **(B)** Expression of Syk and SykB after transfection of Syk-deficient RBL-2H3 cells. The expression of Syk and SykB in total cell lysates was determined by immunoblotting with anti-Syk ( $\alpha$ Syk) antibodies. Lane 1, Syk-deficient RBL-2H3 cells; lane 2, Syk.25; lane 3, Syk.42; lane 4, Syk.49; lane 5, Syk.57; lane 6, SykB.2; lane 7, SykB.11; lane 8, SykB.37; and lane 9, SykB.46. The migrations of Syk and SykB are indicated on the left. Exposure: 14 h.

against the linker domain. Polypeptides were then resolved by SDS-PAGE and revealed by immunoblotting with anti-Syk antibodies (Figure 2A). Preliminary studies had indicated that these antibodies reacted equally well with Syk and SykB, implying that the absence of the 23 amino acid insert in the linker domain did not affect the immunoreactivity of SykB (data not shown). This experiment revealed that RBL-2H3 (Figure 2A, lane 1) expressed approximately equal amounts of two immunoreactive products consistent with Syk and SykB. By contrast, the previously reported Syk-deficient variant of RBL-2H3 cells (Figure 2A, lane 2) lacked expression of both products.

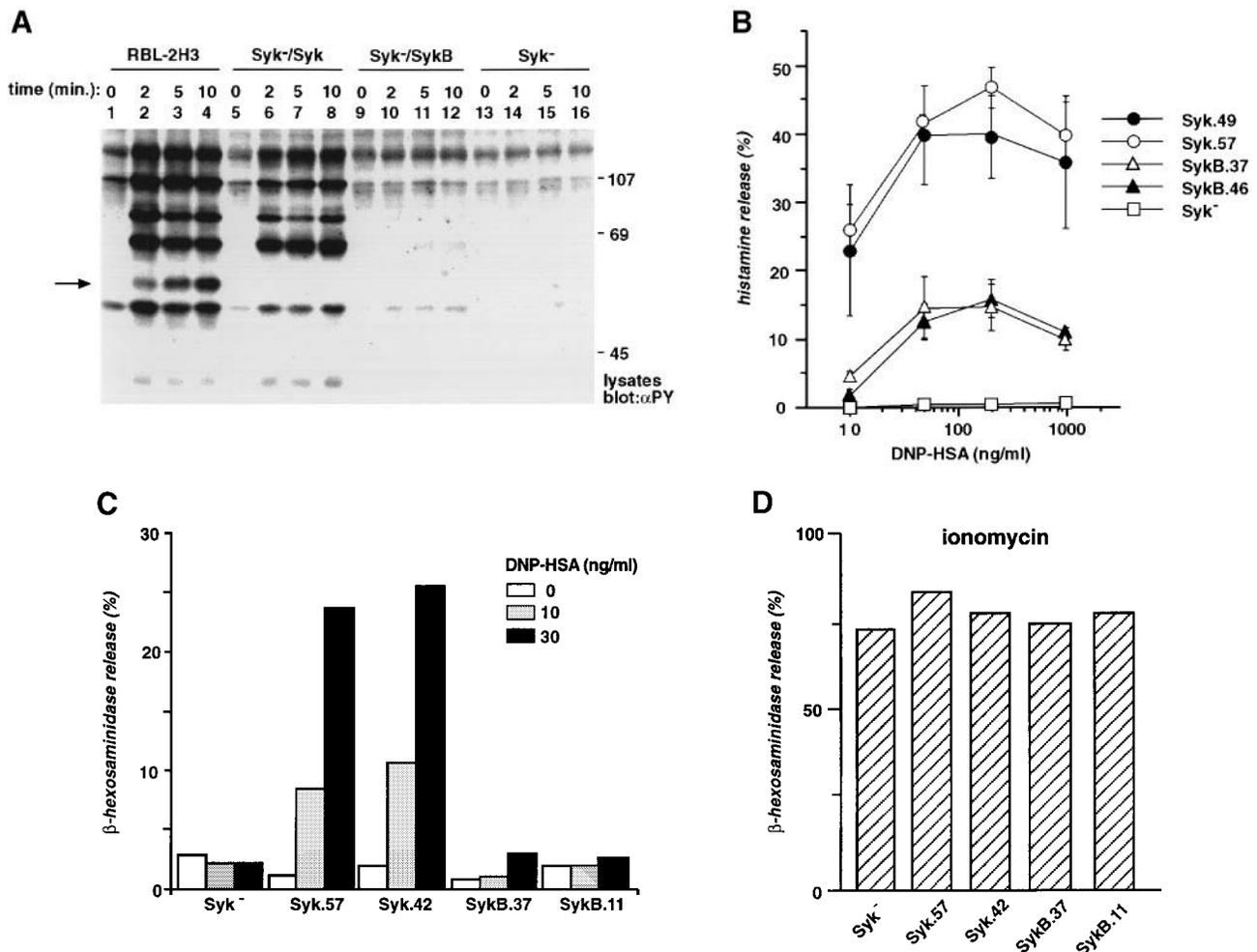
To compare the ability of Syk and SykB to mediate Fc $\epsilon$ RI signalling, mouse cDNAs coding for these proteins were individually introduced into Syk-deficient RBL-2H3 cells by electroporation, as detailed in Materials and methods. Representative clones stably expressing either of the two Syk isoforms are depicted in Figure 2B. Using these cell lines, the impact of Syk and SykB on Fc $\epsilon$ RI-mediated tyrosine protein phosphorylation was examined. Cells were activated for various periods of time with anti-Fc $\epsilon$ RI monoclonal antibody (mAb) BC4, and the accumulation of phosphotyrosine-containing proteins was monitored by immunoblotting of total cell lysates with anti-phosphotyrosine antibodies (Figure 3A). Fc $\epsilon$ RI stimulation

induced a rapid increase in tyrosine protein phosphorylation in wild-type RBL-2H3 cells (Figure 3A, lanes 1–4). However, it failed to provoke tyrosine protein phosphorylation in Syk-deficient cells (Figure 3A, lanes 13–16), in keeping with an earlier report (Zhang *et al.*, 1996). As previously documented for rat Syk (Zhang *et al.*, 1996), expression of mouse Syk (Figure 3A, lanes 5–8) restored Fc $\epsilon$ RI-induced tyrosine protein phosphorylation in Syk-deficient cells. This improvement affected most Fc $\epsilon$ RI-regulated substrates, with the exception of an ~60 kDa polypeptide, which was tyrosine-phosphorylated in wild-type RBL-2H3 cells (Figure 3A, lanes 1–4), but not in the mutant cell line expressing Syk (Figure 3A, lanes 5–8). In comparison with Syk, the SykB protein (Figure 3A, lanes 9–12) was inefficient at re-establishing Fc $\epsilon$ RI-mediated tyrosine protein phosphorylation in Syk-deficient cells. However, it was not fully inert in these cells, as it allowed Fc $\epsilon$ RI-mediated tyrosine phosphorylation of some substrates, especially of a product migrating at ~54–56 kDa (Figure 3A, lanes 10–12). Moreover, SykB itself underwent a reproducible, albeit small, increase in tyrosine phosphorylation after Fc $\epsilon$ RI stimulation (Figure 5). It should be pointed out that contrary to most other Fc $\epsilon$ RI-regulated substrates, the extent of tyrosine phosphorylation of the ITAM-containing  $\beta$  and  $\gamma$  chains of Fc $\epsilon$ RI was not affected by the expression of Syk in RBL-2H3 cells (data not shown). A similar observation was made by others using mast cells derived from Syk-deficient mice (Costello *et al.*, 1996).

Engagement of Fc $\epsilon$ RI ultimately results in the release of inflammatory mediators by RBL-2H3 cells. To ascertain the effects of Syk and SykB on this late activation-related event, RBL-2H3 derivatives were sensitized with anti-trinitrophenyl (TNP) IgE and challenged with increasing concentrations of the antigen dinitrophenyl-human serum albumin (DNP-HSA). The exocytosis of inflammatory mediators was monitored by measuring either histamine (Figure 3B) or  $\beta$ -hexosaminidase (Figure 3C) in the culture supernatant. As described elsewhere (Zhang *et al.*, 1996), Syk-deficient RBL-2H3 cells did not release mediators in response to antigen stimulation, in striking contrast to wild-type RBL-2H3 cells. Furthermore, the responsiveness of Syk-deficient cells could be rescued by enforced expression of Syk. In contrast to Syk however, SykB was significantly less efficient at restoring degranulation. This was especially evident at the lower concentrations of antigen (10 and 30 ng/ml), at which SykB failed to mediate Fc $\epsilon$ RI-triggered degranulation (Figure 3C). Despite these differences, all clones exhibited reactions comparable with calcium ionophores (Figure 3D).

#### **Syk, but not SykB, can enhance TCR-mediated signals in the antigen-specific T-cell line BI-141**

The unique impact of Syk on T-cell receptor (TCR)-mediated signals has been exemplified by the finding that Syk expression caused a marked increase in TCR-induced tyrosine protein phosphorylation and lymphokine release in the antigen-specific T-cell line BI-141 (Latour *et al.*, 1997). Surprisingly, Syk also enhanced tyrosine phosphorylation of the ITAMs of the TCR complex in this system, a property previously reserved for Src family kinases. In a related manner, others have observed that Syk was a potent positive regulator of TCR signalling in the T-cell



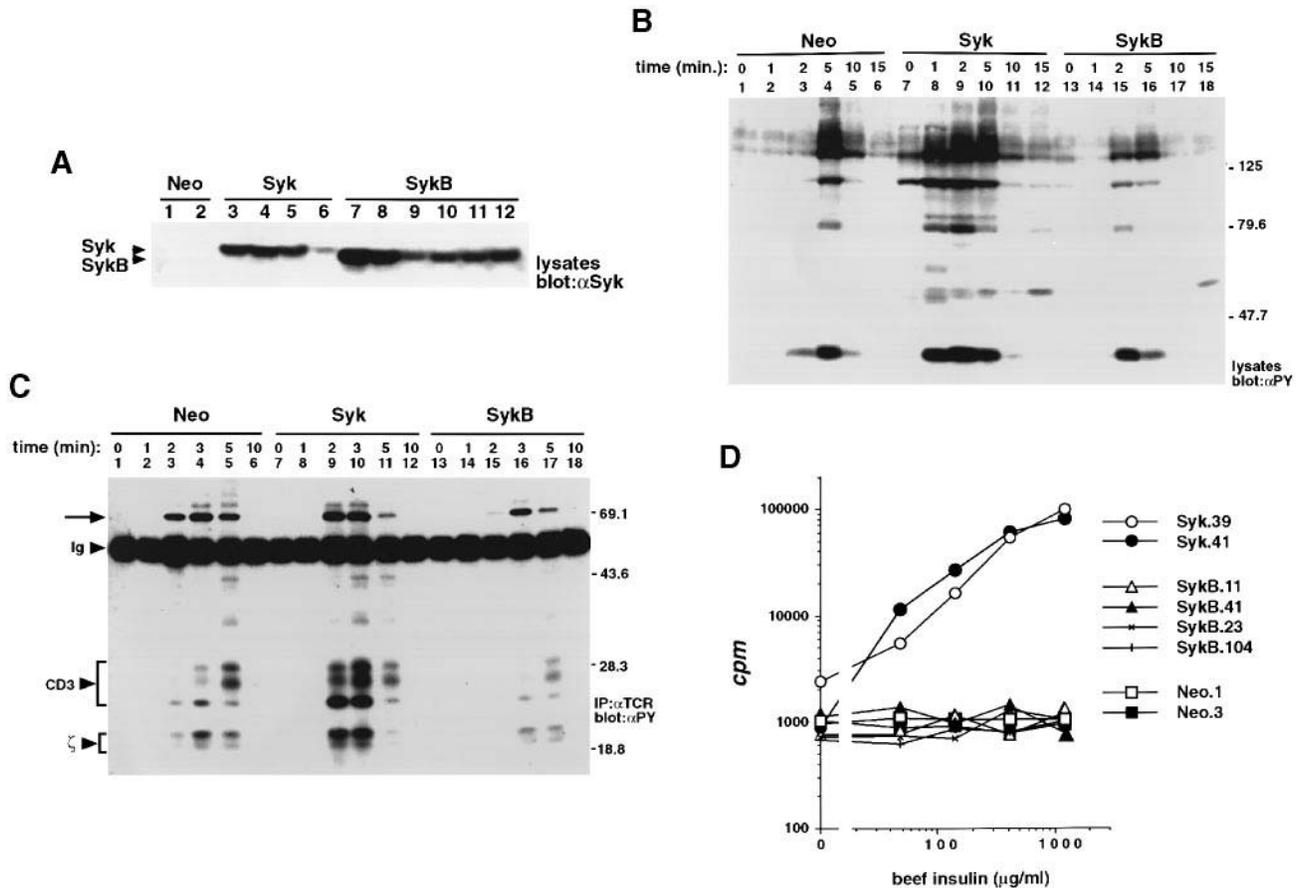
**Fig. 3.** Impact of Syk and SykB on FcεRI signalling in RBL-2H3 cells. **(A)** Tyrosine protein phosphorylation. Cells were stimulated for the indicated periods of time with anti-FcεRI mAb BC4, and changes in tyrosine protein phosphorylation were assessed by immunoblotting of total cell lysates with anti-phosphotyrosine (αPY) antibodies. Lanes 1–4, wild-type RBL-2H3 cells; lanes 5–8, Syk.49; lanes 9–12, SykB.46; and lanes 13–16, Syk-deficient RBL-2H3 cells. The migrations of prestained molecular mass markers are shown on the right, whereas that of an ~60 kDa-substrate phosphorylated in parental RBL-2H3 cells, but not in derivatives of Syk-deficient RBL-2H3 cells, is indicated by an arrow on the left. Exposure: 24 h. **(B)** Histamine release. Cells were stimulated with IgE and the indicated concentrations of the antigen DNP-HSA, or with the calcium ionophore A23187. The antigen-induced release was normalized by expression as a percentage of that induced with 1 μM A23187. The results depicted represent the mean values ± SD from three experiments. **(C)** Hexosaminidase release. Cells were activated with anti-TNP IgE mAb TIB-142 and the indicated concentrations of DNP-HSA. The release of hexosaminidase in the supernatant was measured as outlined in Materials and methods. The results shown are representative of at least three independent experiments. **(D)** Hexosaminidase release in response to calcium ionophore. Cells were stimulated for 45 min with the calcium ionophore ionomycin (1.5 μM). Hexosaminidase release was measured as for (C).

line Jurkat (Chu *et al.*, 1996; Williams *et al.*, 1997), and that it could rescue TCR signalling in cells lacking Lck or CD45 (Chu *et al.*, 1996).

In order to further examine the importance of the linker insert in the function of Syk, we wanted to compare the abilities of Syk and SykB to improve the responsiveness of BI-141 T cells. To this end, BI-141 derivatives expressing SykB were generated by transfection, as described above for RBL-2H3 cells. Clones expressing SykB (Figure 4A, lanes 7–12) in amounts similar to the levels of Syk in previously described BI-141 derivatives (Figure 4A, lanes 3–6) were chosen for further studies. BI-141 cell lines expressing the neomycin phosphotransferase alone, and lacking Syk expression, were also included as controls (Neo; Figure 4A, lanes 1 and 2).

The influences of Syk and SykB on TCR-induced tyrosine protein phosphorylation were compared. Cells were stimulated with mouse anti-TCR mAb F23.1 and

sheep anti-mouse (SAM) IgG, and the accumulation of phosphotyrosine-containing proteins over time was detected by immunoblotting of total cell lysates with anti-phosphotyrosine antibodies (Figure 4B). When contrasted with control neomycin-resistant cells (Figure 4B, lanes 1–6), Syk-expressing BI-141 cells (Figure 4B, lanes 7–12) exhibited a pronounced and global increase in TCR-mediated tyrosine protein phosphorylation, in agreement with our past findings (Latour *et al.*, 1997). Notably though, cells containing SykB (Figure 4B, lanes 13–18) showed no improvement in the TCR-induced tyrosine phosphorylation response. We also analysed the capacity of Syk and SykB to promote tyrosine phosphorylation of the ITAMs of the TCR complex (Figure 4C). Cells were stimulated as in Figure 4B, except that rabbit anti-mouse (RAM) IgG was used as a second step antibody. After lysis, TCR complexes were immunoprecipitated according to the protocol detailed in Materials and methods, and



**Fig. 4.** Effects of Syk and SykB on TCR-mediated signal transduction in BI-141 cells. (A) Expression of Syk and SykB in BI-141 T cells. The expression of Syk and SykB in BI-141 cells was ascertained by immunoblotting of total cell lysates with anti-Syk ( $\alpha$ Syk) antibodies. Lane 1, Neo.1; lane 2, Neo.3; lane 3, Syk.29; lane 4, Syk.34; lane 5, Syk.39; lane 6, Syk.41; lane 7, SykB.11; lane 8, SykB.17; lane 9, SykB.23; lane 10, SykB.38; lane 11, SykB.41; and lane 12, SykB.104. The positions of Syk and SykB are shown on the left. Exposure: 14 h. (B) Tyrosine protein phosphorylation. BI-141 derivatives were stimulated for the indicated periods of time with anti-TCR mAb F23.1 and SAM IgG. The accumulation of phosphotyrosine-containing proteins was monitored by immunoblotting of total cell lysates with anti-phosphotyrosine ( $\alpha$ PY) antibodies. Lanes 1–6, Neo.1; lanes 7–12, Syk.34; and lanes 13–18, SykB.17. The positions of molecular weight standards are shown on the right. Exposure: 12 h. (C) Tyrosine phosphorylation of CD3 and  $\zeta$ . As in (B), except that cells were stimulated with anti-TCR mAb F23.1 and RAM IgG. TCR complexes were recovered from non-ionic detergent-containing cell lysates by immunoprecipitation and probed by anti-phosphotyrosine ( $\alpha$ PY) immunoblotting. Lanes 1–6, Neo.3; lanes 7–12, Syk.41; and lanes 13–18, SykB.41. The migrations of pre-stained molecular mass markers are indicated on the right, while those of CD3 and  $\zeta$ , as well as heavy chain of Ig, are shown on the left. The position of TCR-associated Zap-70/Syk is highlighted by an arrow. Exposure: 36 h. (D) Antigen-induced lymphokine secretion. Cells were stimulated with the antigen beef insulin and the appropriate antigen-presenting cells. Lymphokine secretion was then measured using a bioassay. The concentration of antigen is represented on the abscissa, whereas the incorporation of [ $^3$ H]thymidine in IL-2-dependent HT-2 cells is shown on the ordinate in c.p.m. The results shown are representative of at least three independent experiments.

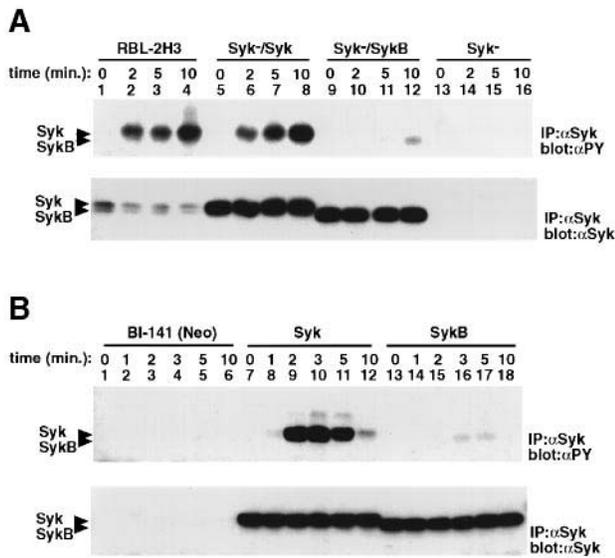
tyrosine phosphorylation of the CD3 and  $\zeta$  subunits of TCR was determined by anti-phosphotyrosine immunoblotting. Upon TCR stimulation, BI-141 derivatives expressing Syk (Figure 4C, lanes 7–12) exhibited a greater increase in tyrosine phosphorylation of CD3 and  $\zeta$ , when compared with Neo cells (Figure 4C, lanes 1–6). A similar increase was not noted in cells containing SykB (Figure 4C, lanes 13–18).

The effects of Syk and SykB on antigen-induced lymphokine secretion were also evaluated. Cells were incubated with increasing concentrations of the antigen beef insulin in the presence of the appropriate antigen-presenting cells, and lymphokine secretion was monitored using a bioassay (Figure 4D). In accordance with an earlier report (Latour *et al.*, 1997), Syk caused a marked enhancement of antigen-induced lymphokine secretion in BI-141 cells. In striking contrast though, SykB failed to improve this response. Coupled with the data obtained

with RBL-2H3 cells, these results clearly indicated that the presence of the linker insert dramatically enhanced the ability of Syk to couple engagement of ITAM-containing receptors to the early and late events of cellular activation.

#### **Differential tyrosine phosphorylation of Syk and SykB in response to immunoreceptor stimulation**

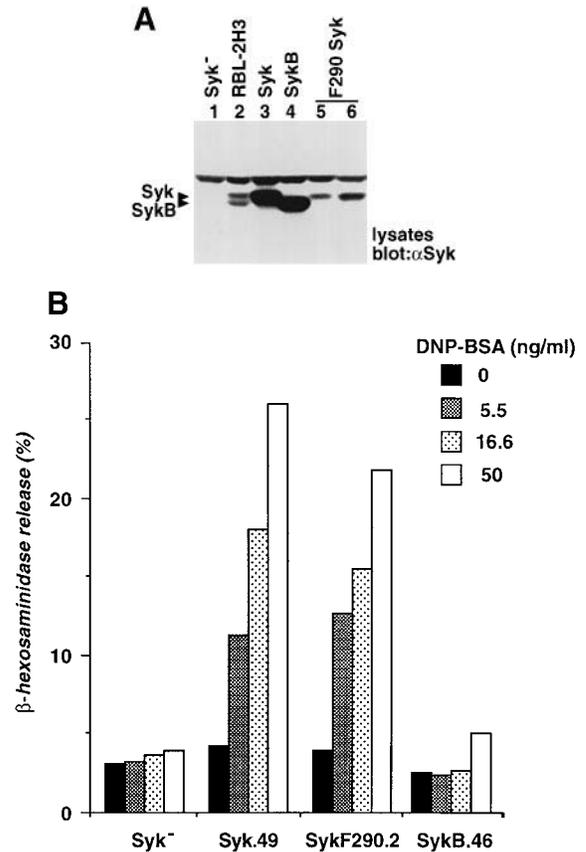
Engagement of Fc $\epsilon$ RI or TCR triggers the binding of Syk and/or Zap-70 to phosphorylated ITAMs (reviewed in Perlmutter *et al.*, 1993; Weiss and Littman, 1994; Wange and Samelson, 1996; Daëron, 1997). As a result, Syk and Zap-70 undergo tyrosine phosphorylation at multiple sites, which mediates an increase in their kinase activity and the recruitment of SH2 domain-containing effectors such as PLC- $\gamma$  and Vav. To begin to understand the mechanism of action of the linker insert, the effect of Fc $\epsilon$ RI stimulation on tyrosine phosphorylation of Syk and SykB was examined (Figure 5A). Wild-type RBL-2H3 and its derivatives



**Fig. 5.** Tyrosine phosphorylation of Syk and SykB in RBL-2H3 cells and BI-141 cells. (A) RBL-2H3 cells. The different cells were stimulated for the indicated amounts of time with anti-FcεRI mAb BC4. Syk polypeptides were subsequently recovered from cell lysates with anti-Syk (αSyk) antibodies, and were immunoblotted either with anti-phosphotyrosine (αPY) antibodies (top panel) or anti-Syk antibodies (bottom panel). The migrations of Syk and SykB are shown on the left. Exposures: top panel, 12 h; bottom panel, 6 h. (B) BI-141 cells. As in (A), except that clones were activated with anti-TCR mAb F23.1 and SAM IgG. The migrations of Syk and SykB are shown on the left. Exposures: top panel, 12 h; bottom panel, 6 h.

were stimulated for the indicated periods of time with anti-FcεRI mAb BC4. After lysing in boiling sample buffer to minimize protein degradation (see below), Syk polypeptides were immunoprecipitated with anti-Syk antibodies and probed by anti-phosphotyrosine immunoblotting (Figure 5A, top panel). While Syk (Figure 5A, lanes 5–8) underwent a prompt and marked increase in tyrosine phosphorylation upon FcεRI stimulation, SykB (Figure 5A, lanes 9–12) exhibited only a meagre increase in its phosphotyrosine content. Tyrosine phosphorylation of SykB was also delayed, being noticeable only after 10 min of stimulation (Figure 5A, lane 12). Importantly, these differences were not due to variations in the amounts of Syk and SykB recovered by immunoprecipitation, as documented by reprobing of the immunoblot membrane with anti-Syk antibodies (Figure 5A, bottom panel). It is noteworthy that the abundance of tyrosine phosphorylated Syk in wild-type RBL-2H3 cells (Figure 5A, top panel, lanes 1–4) was similar to that observed in Syk-expressing transfectants (Figure 5A, lanes 5–8), even though the former expressed lower amounts of Syk protein (Figure 5A, bottom panel). It is possible that the extent of tyrosine phosphorylation of Syk was not limited by its abundance, but rather was dictated by the degree of ITAM phosphorylation, which was comparable in both cell lines (data not shown).

Similar experiments were conducted with BI-141 cells (Figure 5B). After stimulation of cells, the Syk polypeptides were immunoprecipitated and probed by immunoblotting with anti-phosphotyrosine antibodies (Figure 5B, top panel). As was the case in RBL-2H3 cells, Syk (Figure 5B, lanes 7–12) exhibited a pronounced increase in its phosphotyrosine content in activated BI-141 cells. By

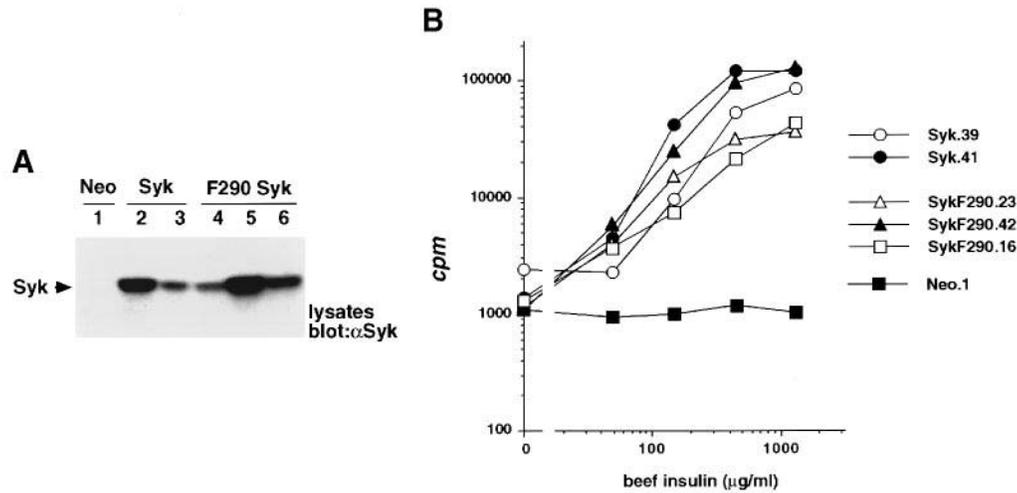


**Fig. 6.** Role of tyrosine 290 in RBL-2H3 basophil leukemia cells. (A) Expression of F290 Syk in Syk-deficient RBL-2H3 cells. Cells were stably transfected with a cDNA coding for F290 Syk. Polyclonal G418-resistant populations were used in these analyses. Lane 1, Syk-deficient cells; lane 2, wild-type RBL-2H3 cells; lane 3, Syk.49; lane 4, SykB.46; lane 5, SykF290.1; and lane 6, SykF290.2. The migrations of Syk and SykB are shown on the left. The immunoreactive species seen in all lanes, and migrating above the Syk proteins, is due to non-specific cross-reactivity of the antibodies (Latour *et al.*, 1997). It was not observed with other anti-Syk sera (data not shown). Exposure: 16 h. (B) β-hexosaminidase release. Cells were triggered as outlined for Figure 3C.

contrast, the tyrosine phosphorylation of SykB (Figure 5B, lanes 13–18) was only minimally augmented by TCR stimulation.

**Tyrosine 290, a potential site of phosphorylation in the linker insert, is dispensable for the impact of Syk on immunoreceptor signalling**

On the basis of these findings, we wished to examine the possibility that the linker insert contained a site of tyrosine phosphorylation crucial for the function of Syk. This idea was further supported by the previous demonstration that tyrosine 290, a highly conserved tyrosine residue in the linker insert (Figure 1A), was a site of Syk autophosphorylation *in vitro* (Furlong *et al.*, 1997). To address the importance of this residue, it was replaced by phenylalanine through site-directed mutagenesis. The resulting mutant (F290 Syk) was stably introduced in RBL-2H3 cells by electroporation (Figure 6). An anti-Syk immunoblot of total cell lysates from two independent polyclonal G418-resistant populations (Figure 6A, lanes 5 and 6) confirmed that F290 Syk was expressed in RBL-2H3 cells, although the overall levels of its expression were lower than those



**Fig. 7.** Role of tyrosine 290 in BI-141 T cells. **(A)** Expression of F290 Syk in BI-141 cells. The abundance of Syk molecules was verified by immunoblotting of total cell lysates with anti-Syk ( $\alpha$ Syk) antibodies. Lane 1, Neo.3; lane 2, Syk.39; lane 3, Syk.41; lane 4, SykF290.16; lane 5, SykF290.23; and lane 6, SykF290.42. The migration of Syk is indicated on the left. Exposure: 12 h. **(B)** Antigen stimulation assay. As in Figure 4D.

of Syk (Figure 6A, lane 3) and SykB (Figure 6A, lane 4) in selected monoclonal cell lines. Nevertheless, we found that F290 Syk was efficient at rescuing Fc $\epsilon$ RI-mediated degranulation in RBL-2H3 cells, in a manner analogous to Syk (Figure 6B). Identical results were obtained with the two F290 Syk-expressing polyclonal populations (data not shown).

Similar transfections were performed in BI-141 T cells (Figure 7). An anti-Syk immunoblot (Figure 7A) of representative monoclonal cell lines showed that F290 Syk (Figure 7A, lanes 4–6) was expressed in a manner comparable with that of Syk (Figure 7A, lanes 2 and 3). Moreover, in an antigen stimulation assay (Figure 7B), BI-141 derivatives containing F290 Syk responded to antigen as robustly as cells expressing wild-type Syk molecules. Similar results were observed in analyses of TCR-induced tyrosine protein phosphorylation (data not shown). Therefore, based on findings with RBL-2H3 and BI-141 cells, we concluded that phosphorylation at tyrosine 290 was not required for the function of Syk in immunoreceptor signalling.

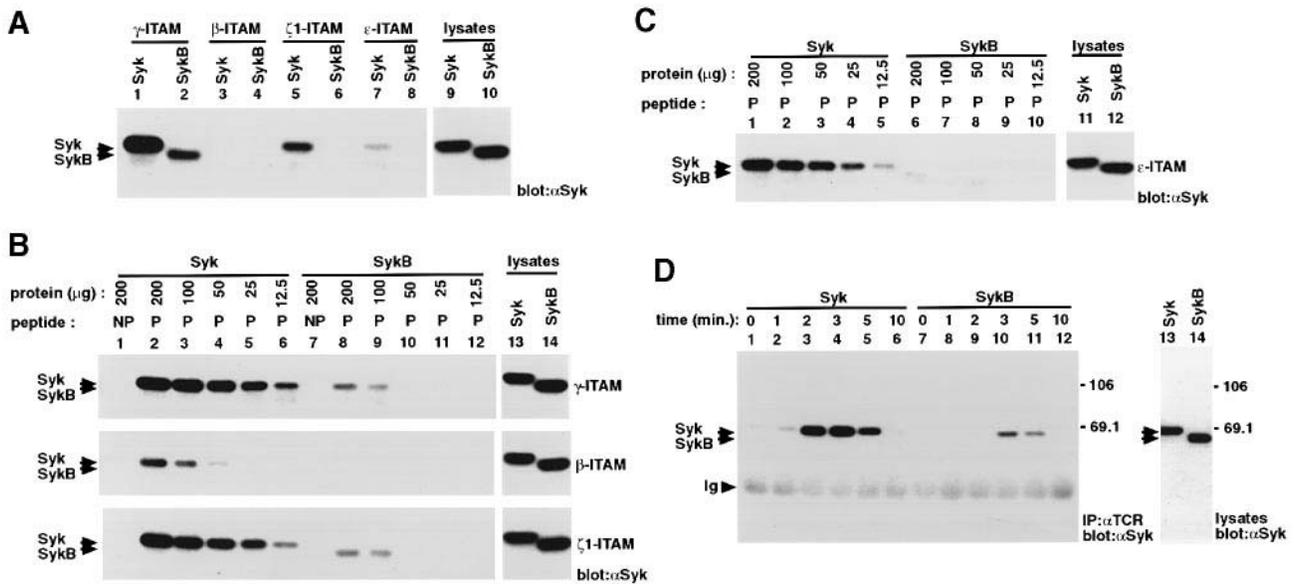
#### **Syk and SykB bind differentially to tyrosine phosphorylated ITAMs *in vitro* and *in vivo***

Given the marked differences in the extent of tyrosine phosphorylation of Syk and SykB in stimulated RBL-2H3 and BI-141 cells, it seemed likely that the phosphorylation of the majority, if not all, of the Syk tyrosine phosphorylation sites was diminished as a result of the absence of the linker insert. Two scenarios emerged as plausible. First, this effect could be explained by a reduction in intrinsic kinase activity as a result of removal of the 23 amino acid sequence. This possibility was unlikely, however, as we have shown previously that Syk and SykB possess comparable abilities to phosphorylate substrates such as Cbl in transiently transfected Cos-1 cells (Latour *et al.*, 1996). Moreover, they were equally efficient at phosphorylating erythrocyte band III *in vitro*. Secondly, the distinct impacts of Syk and SykB on Fc $\epsilon$ RI and TCR signalling could be attributed to differences in their binding to the tyrosine phosphorylated ITAMs, a known requirement for activa-

tion of Syk during immunoreceptor-mediated signal transduction.

To address this possibility, the capacity of Syk and SykB to associate with ITAM-containing peptides *in vitro* was studied. Biotinylated peptides (3 ng) corresponding to diphosphorylated versions of the ITAMs of the  $\gamma$  and  $\beta$  chains of Fc $\epsilon$ RI, the  $\epsilon$  chain of TCR and the first ITAM of the  $\zeta$  subunit of TCR were incubated with BI-141 lysates expressing Syk or SykB. After 30 min, the peptides were captured on avidin-coupled agarose beads and the association of Syk molecules was detected by immunoblotting with anti-Syk antibodies (Figure 8A). This analysis revealed that Syk bound strongly to the  $\gamma$  peptide (Figure 8A, lane 1) and, to a lesser extent, the  $\zeta$  (Figure 8A, lane 5) and  $\epsilon$  (Figure 8A, lane 7) peptides. Small amounts of Syk were also seen in association with the  $\beta$  peptide (Figure 8A, lane 3), although their detection required longer autoradiographic exposures (data not shown). The lower binding of Syk to the ITAM of  $\beta$  was in keeping with an earlier report (Kimura *et al.*, 1996). In comparison with Syk, SykB bound much less efficiently to all four ITAM peptides (Figure 8A, lanes 2, 4, 6 and 8; data not shown), even though it was present in cell lysates in quantities comparable with those of Syk (Figure 8A, lanes 9 and 10).

To obtain a better quantitation of the differences between Syk and SykB, these assays were also conducted using serial dilutions of cell lysates (Figure 8B and C). Greater amounts (30 ng) of the  $\beta$ ,  $\zeta$  and  $\epsilon$  peptides were also used to help detection of the associated Syk polypeptides. In these experiments, both Syk (Figure 8B, lanes 2–6; Figure 8C, lanes 1–5) and SykB (Figure 8B, lanes 8–12; Figure 8C, lanes 6–10) bound in a concentration-dependent manner to the diphosphorylated peptides. However, the binding of Syk was ~16-times more efficient than that of SykB, independent of the ITAM peptide studied. No binding of either Syk (Figure 8B, lane 1) or SykB (Figure 8B, lane 7) occurred when non-phosphorylated ITAM peptides were used. The differences between Syk and SykB in these assays were not due to differences in their reactivity with the anti-Syk serum, since identical results were



**Fig. 8.** Association of Syk and SykB with ITAMs *in vitro* and *in vivo*. **(A)** *In vitro* binding to ITAM peptides. Biotinylated peptides (3 ng) corresponding to various diphosphorylated ITAMs were incubated with 200  $\mu$ g of lysates from BI-141 cells expressing either Syk or SykB. After 30 min, peptides were recovered with avidin-coupled agarose beads. Syk proteins bound to the peptides were revealed by immunoblotting with anti-Syk ( $\alpha$ Syk) antibodies. The positions of Syk and SykB are shown on the left. Exposure: 13 h. **(B)** Differential binding of Syk and SykB to the ITAMs of the  $\beta$  and  $\gamma$  subunits of Fc $\epsilon$ RI and  $\zeta$  chain of TCR. As for (A), except that the ITAM peptides (3 ng for  $\gamma$ ; 30 ng for  $\beta$  and  $\zeta$ ) were mixed with various quantities of the cell lysates expressing Syk or SykB. Binding of the Syk proteins to the peptides was revealed by immunoblotting with anti-Syk ( $\alpha$ Syk) antibodies (lanes 1–12). The abundance of Syk and SykB in the lysates was also verified by immunoblotting of aliquots of cell lysates with anti-Syk antibodies (lanes 13 and 14). Lanes 1 and 7: non-phosphorylated peptides (NP), and lanes 2–6 and 8–12: diphosphorylated peptides (P). The positions of Syk and SykB are indicated on the left. The ITAMs used are indicated on the right. Exposures: 13 h. **(C)** Differential binding of Syk and SykB to the ITAM of the  $\epsilon$  subunit of TCR. ITAM peptides corresponding to  $\epsilon$  (30 ng) were used as outlined for (B), except that a non-phosphorylated peptide was not used. The positions of Syk and SykB are indicated on the left. Exposures: 13 h. **(D)** Association of Syk and SykB in anti-TCR ( $\alpha$ TCR) immunoprecipitates was detected by immunoblotting with anti-Syk ( $\alpha$ Syk) antibodies (lanes 1–12). The presence of Syk and SykB in anti-TCR ( $\alpha$ TCR) immunoprecipitates was detected by immunoblotting with anti-Syk ( $\alpha$ Syk) antibodies (lanes 1–12). The abundance of Syk and SykB in the two cell lines used was examined by immunoblotting of total cell lysates with anti-Syk antibodies (lanes 13 and 14). The migrations of molecular mass markers are depicted on the right, while those of Syk, SykB and heavy chain of Ig are indicated on the left. Exposures: lanes 1–12, 12 h; lanes 13 and 14, 6 h.

obtained when an antibody directed against the C-terminal portion of Syk was used for immunoblotting (data not shown).

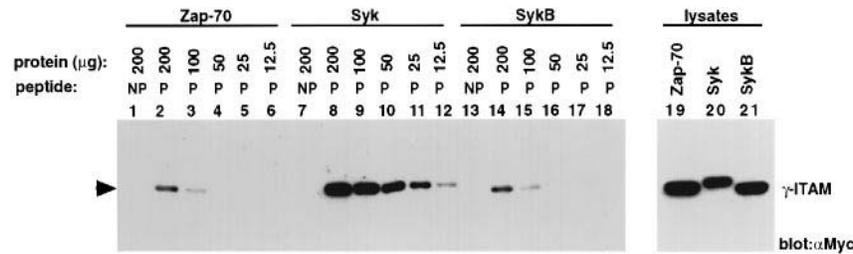
We also wished to examine the ability of Syk and SykB to associate with ITAMs *in vivo*. Unfortunately, these analyses could not be performed in RBL-2H3 cells, because of the differential sensitivity of Syk and SykB to proteolysis in RBL-2H3 lysates, even in the presence of protease inhibitors (Figure 10). As a result, they were conducted using BI-141 cells (Figure 8D). After stimulation with anti-TCR antibodies, the TCR complex was immunoprecipitated as outlined in Figure 4C, and the association of Syk and SykB was revealed by immunoblotting with anti-Syk antibodies. Under these conditions, Syk (Figure 8D, lanes 1–6) became rapidly and prominently associated with the TCR complex in TCR-stimulated BI-141 cells. However, only small quantities of SykB (Figure 8D, lanes 7–12) underwent binding to the antigen receptor in activated BI-141 cells.

As Zap-70 also lacks the linker insert (Figure 1B), we wanted to evaluate whether this characteristic might correlate with a reduction in binding of Zap-70 to tyrosine phosphorylated ITAMs (Figure 9). To this end, it was important to select an ITAM for which the intrinsic affinity of the isolated tandem SH2 domains of Zap-70 and Syk was identical. Hence, the ITAM of

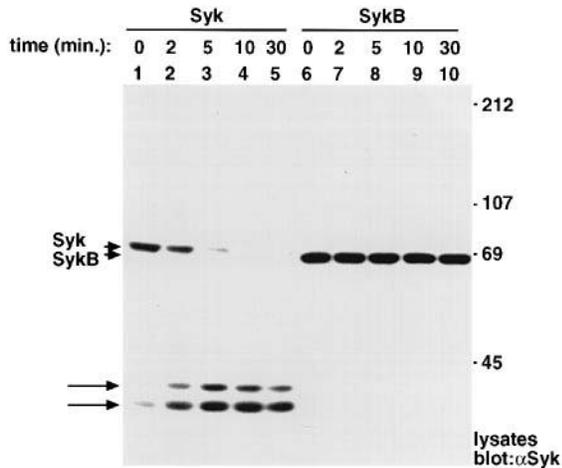
the  $\gamma$  chain of Fc $\epsilon$ RI was chosen, as it was recently shown to bind with equal affinity to tandem SH2 domains of Zap-70 and Syk (Ottinger *et al.*, 1998). To allow adequate comparison of the binding of these kinases, we also used BI-141 derivatives expressing versions of Zap-70, Syk and SykB carrying a Myc-derived epitope at the N-terminus. This experiment showed that, in comparison with Syk (Figure 9, lanes 7–12), the Zap-70 kinase (Figure 9, lanes 1–6) bound 12-times less efficiently to the diphosphorylated  $\gamma$  peptides. Strikingly, the degree of binding of Zap-70 to these peptides was similar to that of SykB (Figure 9, lanes 13–18). None of the kinases bound to non-phosphorylated peptides (Figure 9, lanes 1, 7 and 13). Moreover, all were expressed in comparable amounts in cell lysates (Figure 9, lanes 19–21).

#### **Unlike Syk, SykB is resistant to proteolysis in lysates of RBL-2H3 cells**

During these experiments, it was noted that Syk was frequently degraded in lysates of RBL-2H3 cells. To characterize this observation further, we wanted to compare the sensitivity of Syk and SykB with endogenous proteases. For this purpose, Syk-deficient RBL-2H3 derivatives expressing either Syk or SykB were lysed in non-ionic detergent-containing buffer, in the absence of protease inhibitors. Cell lysates were then incubated



**Fig. 9.** Differential binding of Zap-70, Syk and SykB to a tyrosine phosphorylated ITAM. As in Figure 8B, except that lysates from BI-141 cells expressing Myc-tagged versions of Zap-70, Syk or SykB were used. Bound proteins were detected by immunoblotting with anti-Myc mAb 9E10. The position of the Myc-tagged kinases is indicated by an arrowhead on the left. Exposure: 16 h.



**Fig. 10.** Sensitivity of Syk and SykB to degradation by proteases in lysates of RBL-2H3 cells. Derivatives of Syk-deficient RBL-2H3 cells expressing either Syk or SykB were lysed in non-ionic detergent-containing buffer in the absence of protease inhibitors. Equivalent amounts of lysates were then incubated at 37°C for the indicated periods of time, and the abundance and apparent molecular mass of the Syk isoforms in these lysates were assessed by immunoblotting with anti-Syk ( $\alpha$ Syk) antibodies. The positions of prestained molecular mass markers are indicated on the right, whereas those of Syk and SykB are shown on the left. The migrations of the presumed proteolytic fragments of Syk are highlighted by two arrows on the left. Exposure: 24 h.

at 37°C for various periods of time, and Syk polypeptides were detected by anti-Syk immunoblotting (Figure 10). This experiment showed that intact Syk (Figure 10, lanes 1–5) rapidly disappeared from cell lysates under these conditions. This loss coincided with the accumulation of two immunoreactive products of ~35–40 kDa. In striking contrast, SykB (Figure 10, lanes 6–10) remained intact over the same period of time. The 35–40 kDa-species detected in lysates of Syk-expressing cells (Figure 10, lanes 2–5) were reminiscent of the degradation products of Syk previously noted in bovine thymus and porcine spleen lysates (Zionchek *et al.*, 1988; Taniguchi *et al.*, 1991). Seemingly, these fragments result from post-lysis proteolytic cleavage of Syk in the linker region (Figure 1A).

## Discussion

We wished to elucidate the role of a unique 23 amino acid insert present in the linker region of Syk (Figure 1). To this end, the activity of Syk in immunoreceptor signalling was contrasted with that of SykB, a naturally occurring variant lacking this sequence (Yagi *et al.*, 1994;

Rowley *et al.*, 1995; Latour *et al.*, 1996). Using a Syk-deficient variant of RBL-2H3 cells, we observed that, in contrast to Syk, SykB was very inefficient at coupling stimulation of Fc $\epsilon$ RI to intracellular tyrosine protein phosphorylation and degranulation. SykB was also incapable of augmenting TCR-mediated tyrosine protein phosphorylation and lymphokine secretion in the antigen-specific T-cell line BI-141. This was in opposition to the strong, positive regulatory effect of Syk in this system. Finally, we also noted that SykB was inefficient at rescuing BCR-induced tyrosine protein phosphorylation in a Syk-deficient variant of the DT-40 B-cell line (S.Latour, T.Kurosaki and A.Veillette, unpublished results). Hence, in three different hemopoietic cell systems, the absence of the linker insert drastically interfered with the ability of Syk to couple engagement of ITAM-containing receptors to cellular activation.

To understand the basis of this phenomenon, we first examined the amino acid composition of the insert. Notably, it contains a highly conserved tyrosine, tyrosine 290, which was shown previously to be a site of Syk autophosphorylation *in vitro* (Furlong *et al.*, 1997). As other tyrosine phosphorylation sites in the linker region can provide crucial functions by allowing the recruitment of cellular effectors (Deckert *et al.*, 1996; Law *et al.*, 1996; Wu *et al.*, 1997), we evaluated whether mutation of tyrosine 290 could recapitulate the phenotype of SykB. However, this alteration did not affect the function of Syk in either RBL-2H3 or BI-141 cells, indicating that phosphorylation at tyrosine 290 was not critical for its function.

Combined with the observation that the defect in tyrosine protein phosphorylation observed in SykB-expressing cells affected the majority of cellular substrates (including SykB itself), these results implied that the 23 amino acid linker insert had a global influence on the function of Syk. Along these lines, however, an earlier report demonstrated that the basal catalytic activity of SykB was not significantly different from that of Syk in immune complex kinase reactions (Latour *et al.*, 1996). Like Syk, SykB was also efficient at causing tyrosine phosphorylation of the Cbl protein (Latour *et al.*, 1996), as well as of a variety of other substrates such as PLC- $\gamma$ 1 and  $\zeta$  (our unpublished data) in transiently-transfected Cos-1 cells. Together, this information provided compelling evidence that the linker insert did not affect the intrinsic TPK activity of Syk.

In light of this notion, we tested the possibility that the unique insert influenced the binding of Syk to tyrosine phosphorylated ITAMs, a step required for its participa-

tion in immunoreceptor signalling. Experiments in BI-141 T cells revealed that greater amounts of Syk became associated with the TCR complex in activated cells, in comparison with SykB. However, the interpretation of this finding was somewhat complicated by the fact that Syk was also more apt at promoting tyrosine phosphorylation of the ITAMs in activated BI-141 cells. Nonetheless, using synthetic peptides corresponding to various diphosphorylated ITAMs in *in vitro* binding assays, it was revealed conclusively that Syk bound much more efficiently than SykB to phosphorylated ITAMs.

Therefore, it seemed likely that the linker insert augmented the impact of Syk in immunoreceptor signalling by improving its ability to bind phosphorylated ITAMs. It is plausible that this sequence modified the conformation of Syk, thereby enhancing the accessibility of its SH2 domains to the ITAMs. In keeping with this idea, it has been postulated that the linker region may function as a molecular 'hinge', regulating intramolecular interactions between the N- and C-terminal portions of Syk (Kimura *et al.*, 1996). The linker insert may enhance the flexibility of this hinge, thereby augmenting the capacity of Syk to 'open' and associate with ITAMs. Alternatively, it is conceivable that the 23 amino acid sequence stabilized the binding of the Syk SH2 domains to the ITAMs. This effect could be direct, as a result of binding of the linker insert itself to the ITAM; or indirect, via alteration of the conformation of the Syk SH2 domains or binding to other cellular partners. Future studies, including determination of the structures of Syk and SykB complexed to phosphorylated ITAMs, are obviously needed to distinguish between these various propositions. It will also be interesting to determine whether the length or the actual amino acid composition of the linker insert is important for this effect. As the sequence of the linker insert is highly conserved across species (Figure 1A), it seems probable that its amino acid content is crucial.

Our results also showed that, like SykB, Zap-70 was less efficient at binding a tyrosine phosphorylated ITAM *in vitro*. This observation raised the possibility that part of the previously noted functional differences between Syk and Zap-70 in immunoreceptor signalling (Kolanus *et al.*, 1993; Chu *et al.*, 1996; Latour *et al.*, 1997; Taylor *et al.*, 1997; Williams *et al.*, 1997) may result from the absence of the 23 amino acid insert in Zap-70 (Figure 1B). Coupled with the lower intrinsic kinase activity of Zap-70 (Latour *et al.*, 1996), this difference may explain the more complex post-translational modifications needed for Zap-70 activation *in vivo*, including its binding to oligomerized phosphorylated ITAMs and phosphorylation by Src family kinases (Kolanus *et al.*, 1993; Neumeister *et al.*, 1995). These may ultimately compensate for the absence of the linker insert.

We were intrigued by the finding that Syk was more sensitive than SykB to the action of proteases in lysates of RBL-2H3 cells. It is plausible that, in addition to enhancing binding to ITAMs, the conformation imposed by the linker insert rendered Syk more accessible to the action of proteases. Moreover, since the site of Syk proteolysis has been mapped to the linker region (Figure 1A) (Zionchek *et al.*, 1988; Taniguchi *et al.*, 1991), it is possible that the linker insert, which is located near the predicted proteolytic cleavage site, facilitated recognition

by the proteases. Additional studies will be needed to distinguish between these two possibilities. As this phenomenon could allow SykB to be resistant to proteolysis under physiological circumstances, it may provide the shorter Syk isoform with an advantage in certain settings. As a consequence, SykB may remain functional in situations where Syk is normally degraded. However, it remains to be determined whether the degradation of Syk observed in cell lysates also takes place in intact cells.

SykB is a version of Syk that exists naturally. It is expressed in low amounts in thymus, spleen and a variety of hemopoietic cell lines (Latour *et al.*, 1996; our unpublished data). Moreover, it seemingly represents ~50% of the Syk protein in normal mouse bone marrow cells (Latour *et al.*, 1996) and RBL-2H3 cells (Rowley *et al.*, 1995; this study). Hence, it is probable that SykB can carry out some biological functions. Its reduced ability to bind ITAMs *in vitro* and *in vivo* may be viewed as an indication that it does not operate in immunoreceptor-mediated signal transduction. However, as indicated by our results in RBL-2H3 cells, SykB can mediate productive immunoreceptor signalling at higher ligand concentrations. Consequently, cells that selectively express SykB could still show a response, albeit significantly attenuated, to immunoreceptor stimulation. SykB may also mediate signals for other types of receptors, like integrins, cytokine receptors and G-protein-coupled receptors. While these receptors are devoid of ITAMs, there is evidence that they can utilize Syk for signal transduction, by a process that seems independent of its SH2 domains (Clark *et al.*, 1994; Minami *et al.*, 1995; Wan *et al.*, 1996; Gao *et al.*, 1997).

In summary, the results presented in this report provide evidence that the unique insert in the linker region of Syk is crucial for its ability to participate in immunoreceptor signalling. This requirement most likely reflects the capacity of the insert to facilitate binding of the Syk SH2 domains to tyrosine phosphorylated ITAMs. These findings also identify a novel role for the linker domain of Syk. They raise the possibility that modifications in this region, through phosphorylation or binding of cellular proteins, could also alter the ability of Syk to bind ITAMs and thus represent a potent mechanism to influence cell signalling.

## Materials and methods

### Cells

RBL-2H3 rat basophilic leukemia cells and the Syk-deficient variant of RBL-2H3 were described elsewhere (Zhang *et al.*, 1996). They were propagated in  $\alpha$  minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS), glutamine and antibiotics. Neomycin-resistant transfectants of these cells were grown in the additional presence of G418 (0.5 mg/ml). The CD4-negative, class II major histocompatibility complex (MHC)-restricted antigen-specific T-cell line BI-141 (Reske-Kunz and Rude, 1985) was maintained in RPMI 1640 containing 10% FBS, glutamine and antibiotics. BI-141 derivatives expressing either Syk, Myc-Syk, Myc-Zap-70 or the neomycin phosphotransferase alone (Neo) were reported previously (Latour *et al.*, 1997). Clones expressing a Myc-tagged version of SykB were produced as described elsewhere (Latour *et al.*, 1997). All neomycin-resistant derivatives of BI-141 were propagated in the presence of G418 (0.6 mg/ml).

### cDNA constructs

Mouse *syk* and *sykB* cDNAs were described elsewhere (Latour *et al.*, 1996). A Syk variant in which tyrosine 290 was replaced by phenylalanine (F290 Syk mutant) was created by PCR. For transfection, cDNAs were inserted in the multiple cloning site of pNT-Neo, which contains an SR $\alpha$

promoter, as well as the neomycin phosphotransferase gene (*neo*). Cells were stably transfected by electroporation (260 V, 960  $\mu$ F) and selected in the presence of G418 (0.5 mg/ml for RBL-2H3 cells and 0.75 mg/ml for BI-141 cells). Monoclonal cell lines expressing Syk, SykB or F290 Syk were produced by limiting dilution (unless specified) and identified by immunoblotting of total cell lysates with anti-Syk antibodies. All RBL-2H3 clones used in our experiments expressed unaltered levels of Fc $\epsilon$ RI (data not shown). Furthermore, their levels of surface expression of CD45 were similar to those of parental cells. All BI-141 derivatives selected for our studies expressed comparable levels of TCR, CD3, CD45 and Thy-1, and were CD4-negative (data not shown).

#### Immunoprecipitations and immunoblots

For BI-141 T cells, cells were lysed in 1 $\times$  TNE buffer (50 mM Tris pH 8.0, 1% Nonidet P-40, 2 mM EDTA pH 8.0) supplemented with protease and phosphatase inhibitors, as outlined elsewhere (Latour *et al.*, 1996). In the case of RBL-2H3, cells were disrupted in boiling SDS-containing sample buffer to minimize Syk degradation. These lysates were diluted with TNE buffer prior to immunoprecipitation. Syk was immunoprecipitated from equivalent amounts of cellular proteins using a polyclonal rabbit antiserum directed against amino acids 258–356 of mouse Syk (Latour *et al.*, 1996). Preliminary studies had indicated that the reactivity of this antiserum was not affected by deletion of the 23 amino acid insert in SykB (our unpublished data). In some experiments, we also used a polyclonal rabbit antiserum directed against amino acids 615–629 of mouse Syk (data not shown). However, this serum was not routinely used, as it gave weaker signals in immunoblots. Immune complexes were collected with *Staphylococcus aureus* protein A (Calbiochem). After several washes, they were eluted in sample buffer, boiled and resolved by SDS-PAGE. Proteins were subsequently analysed by immunoblotting with either affinity-purified rabbit anti-phosphotyrosine antibodies, anti-Syk antibodies or anti-Myc mAb 9E10 (Evan *et al.*, 1985), according to a previously detailed protocol (Veillette *et al.*, 1988). Immunoreactive products were detected with either [<sup>125</sup>I]protein A (Amersham) or [<sup>125</sup>I]goat anti-mouse IgG (ICN Biomedicals Inc.) and autoradiography, and data were quantitated using a phosphorimager (BAS2000; Fuji).

#### Immune receptor-mediated tyrosine protein phosphorylation

For stimulation of RBL-2H3 cells, 1 $\times$ 10<sup>6</sup> cells were first plated in tissue culture dishes. The next day, they were treated for the indicated periods of time at 37°C with mouse anti-rat Fc $\epsilon$ RI mAb BC4 (Zhang *et al.*, 1996). Cells were lysed with boiling SDS-containing buffer, and lysates were analyzed by anti-phosphotyrosine immunoblotting. To stimulate BI-141 T cells, 5 $\times$ 10<sup>6</sup> cells were incubated at 37°C with mouse anti-TCR mAb F23.1 (Staerz *et al.*, 1985) and SAM IgG. After the indicated periods of time, cells were disrupted in boiling sample buffer and processed for anti-phosphotyrosine immunoblotting. For immunoprecipitation of the TCR complex, BI-141 cells were stimulated with anti-TCR mAb F23.1 and RAM IgG, using a protocol described elsewhere (Latour *et al.*, 1997). Control cells were treated in a similar manner, except that they were lysed prior to the addition of RAM IgG. After stimulation, cells were disrupted with 2 $\times$  TNE buffer (100 mM Tris pH 8.0, 2% Nonidet P-40, 40 mM EDTA), supplemented with protease and phosphatase inhibitors. Anti-TCR immune complexes were collected with *Staphylococcus aureus* protein A. TCR complexes were resolved in either 12% SDS-PAGE gels to study the TCR-associated CD3 and  $\zeta$  subunits, or 8% SDS-PAGE gels to examine TCR-bound Syk molecules.

#### Histamine and $\beta$ -hexosaminidase release

RBL-2H3 cells and their derivatives were stimulated and examined for histamine release as detailed elsewhere (Zhang *et al.*, 1996). For  $\beta$ -hexosaminidase measurement, RBL-2H3 cells or their variants (2 $\times$ 10<sup>5</sup> cells) were grown overnight in 96 well plates in the presence of saturating amounts of anti-TNP IgE MAb TIB-142 (obtained from American Type Culture Collection, Rockville, MD). After 12–16 h, the excess IgE was removed by washing in Tyrode buffer (10 mM HEPES pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.6 mM glucose and 0.1% bovine serum albumin), and cells were stimulated at 37°C in Tyrode buffer containing the indicated concentrations of DNP-HSA (Calbiochem) or ionomycin (1.5  $\mu$ M; Sigma). After 45 min, the supernatant was harvested and the remaining cell monolayer was lysed in Tyrode buffer supplemented with 0.5% Triton X-100. Hexosaminidase activity was measured in both the supernatant and the cell monolayer as described elsewhere, using the substrate 4-nitrophenyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside (1.3 mg/ml; Aldrich). After

40 min at 37°C, the enzymatic reaction was stopped by addition of 3 volumes of 0.2 M glycine pH 10.7. Absorbance at 405 nm was read in an ELISA reader. The percentage of hexosaminidase released from each cell line was determined by calculating (released hexosaminidase/ released+cell monolayer hexosaminidase) $\times$ 100. All experiments were done in triplicate.

#### Antigen stimulation

BI-141 cells were stimulated with A $\alpha$ <sup>b</sup>A $\beta$ <sup>k</sup>-expressing L cells (kindly provided by Dr R.Germain, National Institutes of Health, Bethesda, MD), previously pulsed with various concentrations of the antigen beef insulin. After 24 h, supernatants were collected and assayed for lymphokine production by measuring their ability to support [<sup>3</sup>H]thymidine incorporation into HT-2 cells. Controls were without addition. All assays were done in triplicate.

#### Peptide binding experiments

Biotinylated peptides (phosphorylated or non-phosphorylated) corresponding to the ITAMs of the  $\beta$  and  $\gamma$  chains of Fc $\epsilon$ RI were described elsewhere (Kimura *et al.*, 1996). Those corresponding to the first ITAM of the  $\zeta$  subunit of TCR, or to the single ITAM of the  $\epsilon$  chain of TCR, were kindly provided by Dr Phillip Morton, Monsanto Company, St Louis, MO. Peptides (3 or 30 ng) were incubated for 30 min at 4°C with TNE lysates from BI-141 cells expressing either Syk, SykB, Myc-Syk, Myc-SykB or Myc-Zap-70, supplemented with 150 mM NaCl. After this incubation, they were recovered with avidin-coupled agarose beads (Boehringer Mannheim). Following several washes, bound proteins were eluted in sample buffer and analyzed by immunoblotting with anti-Syk antibodies.

#### In vitro degradation assays

Derivatives of the Syk-deficient variant of RBL-2H3 expressing Syk or SykB were lysed in TNE buffer, in the absence of protease inhibitors. Equivalent amounts of cell lysates were incubated at 37°C for the indicated periods of time, to allow digestion of cellular proteins by endogenous proteases. Reactions were stopped by the addition of sample buffer, and the Syk proteins were detected by anti-Syk immunoblotting.

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