

# Transfection of Syk Protein Tyrosine Kinase Reconstitutes High Affinity IgE Receptor-mediated Degranulation in a Syk-negative Variant of Rat Basophilic Leukemia RBL-2H3 Cells

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## Summary

Aggregation of the high affinity receptor for immunoglobulin E (FcεRI) on mast cells results in rapid tyrosine phosphorylation and activation of Syk, a cytoplasmic protein tyrosine kinase. To examine the role of Syk in the FcεRI signaling pathway, we identified a variant of RBL-2H3 cells that has no detectable Syk by immunoblotting and by in vitro kinase reactions. In these Syk-deficient TB1A2 cells, aggregation of FcεRI induced no histamine release and no detectable increase in total cellular protein tyrosine phosphorylation. However, stimulation of these cells with the calcium ionophore did induce degranulation. FcεRI aggregation induced tyrosine phosphorylation of the β and γ subunits of the receptor, but no increase in the tyrosine phosphorylation of phospholipase C-γ1 and phospholipase C-γ2 and no detectable increase in intracellular free Ca<sup>2+</sup> concentration. By transfection, cloned lines were established with stable expression of Syk. In these reconstituted cells, FcεRI aggregation induced tyrosine phosphorylation of phospholipase C-γ1 and phospholipase C-γ2, an increase in intracellular free Ca<sup>2+</sup> and histamine release. These results demonstrate that Syk plays a critical role in the early FcεRI-mediated signaling events. It further demonstrates that Syk activation occurs downstream of receptor phosphorylation, but upstream of most of the FcεRI-mediated protein tyrosine phosphorylations.

Mast cells and basophils play pivotal roles in the initiation of the allergic response. Aggregation of the high affinity receptor for IgE (FcεRI)<sup>1</sup> on these cells initiates a biochemical cascade that results in degranulation and release of inflammatory mediators (1–3).

The activation of protein tyrosine kinases is one of the earliest detectable events after FcεRI aggregation and plays a critical role in this signal transduction pathway (4–7). Since none of the FcεRI subunits possess intrinsic kinase activity, cytoplasmic protein tyrosine kinases must be involved in initiating FcεRI signaling. Among these are members of the Src and Syk protein tyrosine kinase families. Lyn, a member of the Src family of kinases, is physically associated with FcεRI in both nonstimulated and activated cells, and appears to preferentially interact with the β subunit of the receptor (8–12). These characteristics make

Lyn a good candidate for directly causing receptor tyrosine phosphorylation. Syk is a member of the Syk/ZAP-70 family of protein tyrosine kinases, has two tandem SH2 domains in the NH<sub>2</sub>-terminal half of the molecule and is expressed in hematopoietic cells (10, 13–15). The aggregation of FcεRI results in the association of Syk with the γ subunit of the receptor, an increase in its tyrosine phosphorylation, and an increase in its activity (10, 15–18).

The signal transduction pathways present in mast cells have many similarities to those in other cells of the immune system, such as T and B cells. The ligand-binding domains of the receptors on these cells lack intrinsic tyrosine kinase activity, but they associate with signal transducing subunits that contain the immunoreceptor tyrosine-based activation motif (ITAM), which is critical for cell activation (19–21). This motif is present in both the β and γ subunits of FcεRI, in the ζ subunit of the TCR, and in the Igα and Igβ of the B cell receptor (BCR).

A critical role for Lyn and Syk kinases in BCR signaling was recently demonstrated by studies of Lyn-negative and Syk-negative B cell lines. In Lyn-negative cells, BCR-

<sup>1</sup> Abbreviations used in this paper: BCR, B cell receptor; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular free Ca<sup>2+</sup> concentration; DNP, dinitrophenyl; FcεRI, high affinity receptor for IgE; ITAM, immunoreceptor tyrosine-based activation motif.

mediated tyrosine phosphorylation and activation of Syk were dramatically reduced (22, 23). In addition, cross-linking of BCR on Lyn-negative cells evoked a delayed and slow increase in intracellular free  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ), while the kinetics of inositol 1,4,5-trisphosphate formation was normal. In contrast, in Syk-negative B cells, there was no BCR-induced tyrosine phosphorylation of phospholipase C- $\gamma$ 2, inositol 1,4,5-trisphosphate generation, or  $Ca^{2+}$  mobilization (22).

Experiments suggest that Syk may also play a critical role in triggering signaling pathways that lead to the release of mediators in mast cells (15, 16, 24–26). To test this possibility and to understand the mechanism of signal transduction, we identified a Syk-deficient variant of rat basophilic leukemia RBL-2H3 cells, a model system for basophils and mast cells. We also permanently transfected these cells with the wild-type rat Syk cDNA. These experiments prove that Syk plays a critical role in Fc $\epsilon$ RI-mediated signaling in mast cells.

## Materials and Methods

**Materials.** Brij 96, Triton X-100, NP-40, protease inhibitors, and protein A-agarose beads were from Sigma Immunochemicals (St. Louis, MO). Cyanogen bromide-activated Sepharose 4B beads were from Pharmacia LKB Biotechnology Inc. The materials for electrophoresis were purchased from Novex (San Diego, CA), polyvinylidene difluoride (PVDF) transfer membrane was from Millipore Corp. (Bedford, MA), and the sources of other materials not indicated are as described previously (15).

**Antibodies.** The rabbit anti-rat p72<sup>syk</sup> antibody generated to a sequence between the second Syk SH2 and the kinase domain has been described previously (15). Anti-phospholipase C- $\gamma$ 2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phospholipase C- $\gamma$ 1 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phosphotyrosine mAb PY-20 was from ICN Immunobiologicals (Lisle, IL). Antibodies to Fc $\epsilon$ RI $\alpha$  (BC4) and Fc $\epsilon$ RI $\beta$ , rabbit anti-Fc $\epsilon$ RI $\gamma$ , and anti-Lyn were generated and purified as described previously (15, 17, 27). All other antibodies have been described previously (28, 29).

**Cell Culture.** The RBL-2H3, TB1A2, and other cell lines were maintained as monolayer cultures in Eagle's MEM supplemented with 15% heat-inactivated fetal bovine serum, penicillin, streptomycin, and amphotericin (30).

**Stable Transfection.** A 1.9-kb fragment containing the open reading frame for rat Syk (10, 15) was isolated and ligated into the SacI site of the pSVL expression vector (Pharmacia LKB). The expression construct was cotransfected with pSV2-neo vector into TB1A2 cells using electroporation (310V, 960  $\mu$ F). The stably transfected clones were selected with 300  $\mu$ g/ml of active G418 (GIBCO BRL, Gaithersburg, MD). 36 clones were transferred, expanded, and subsequently screened for Syk expression by immunoblotting using anti-Syk antibody. Two clones (3A1 and 3A5) expressing the highest level of Syk were identified and selected for further analysis. The level of Syk in these cells was similar to that in the wild-type RBL-2H3 cells.

**Cell Activation.** The cells were stimulated either with antigen after overnight culture in the presence of antigen-specific IgE, or with anti-Fc $\epsilon$ RI $\alpha$  antibodies (mAb BC4) essentially as described previously (4). Briefly,  $10^6$  cells were seeded in 9.6-cm<sup>2</sup> plates,

and after overnight culture, the cell monolayers were washed once with 3 ml of Eagle's MEM containing 0.1% BSA and 10 mM Tris (pH 7.5). The cells were then stimulated in the same medium either with the antigen dinitrophenyl (DNP) coupled to human serum albumin, or with the calcium ionophore A23187. After stimulation for the indicated times, the medium was removed for histamine analysis. For immunoprecipitation experiments,  $10^7$  or  $2 \times 10^7$  cells were seeded in petri dishes (10 or 15 cm diameter) and stimulated with 0.3  $\mu$ g/ml mAb BC4 (anti-Fc $\epsilon$ RI $\alpha$ ). All other steps were the same.

**Immunoprecipitation.** After stimulation for the indicated times, the monolayers were rinsed once with 12 ml ice-cold PBS containing protease inhibitors and vanadate (concentration as in lysis buffer), solubilized in 1 ml of Brij lysis buffer (3% Brij 96, 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM PMSF, 90 mU/ml aprotinin, 50  $\mu$ g/ml leupeptin, 50  $\mu$ g/ml pepstatin, 10 mM 2-ME). The lysates were centrifuged for 15 min at 16,000 g at 4°C, and the postnuclear supernatants were precleared 1 h at 4°C with Sepharose 4B or protein A-agarose, and then immunoprecipitated with antibodies bound to the same beads. After rotation at 4°C for 1 h, the beads were washed four times with ice-cold lysis buffer and once with 20 mM Tris, 100 mM NaCl (pH 7.5), and the proteins were eluted by boiling for 5 min with sample buffer as described previously (10).

**Immunoblotting.** Whole-cell lysates and immunoprecipitated proteins were separated by SDS-PAGE under reducing condition and electrotransferred to PVDF (Immobilon P). The blots were blocked with 4% protease-free BSA and probed with 40 ng/ml antiphosphotyrosine mAb PY-20 conjugated to horseradish peroxidase. The antiphosphotyrosine antibodies were stripped from the membranes and the membranes were reprobed with other antibodies as recommended by the manufacturer. In all blots, proteins were visualized by enhanced chemiluminescence (ECL Kit; Amersham, Arlington Heights, IL) as previously described (31).

**In Vitro Kinase Assay.** Syk and Lyn, immunoprecipitated as described above, were further washed with kinase buffer (30 mM Hepes, pH 7.5, 10 mM MgCl<sub>2</sub>, and 2 mM MnCl<sub>2</sub>) and resuspended in 50  $\mu$ l kinase buffer. The reactions were started by the addition of 3  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and 1  $\mu$ M ATP. After a 30-min incubation at room temperature, the immunoprecipitates were washed and the reactions were stopped by the addition of 50  $\mu$ l of 2  $\times$  Laemmli sample buffer and boiling for 5 min. The eluted proteins were separated under reducing conditions by SDS-PAGE (10% gels), electrotransferred to membranes, and visualized by autoradiography. The same membranes were immunoblotted with anti-Syk or anti-Lyn antibodies, as described above.

**Measurement of  $[Ca^{2+}]_i$ .** Intracellular free calcium concentration  $[Ca^{2+}]_i$  was measured using the fluorescent indicator fura-2. Monolayers of the different cell lines were detached from tissue culture flasks with 4.4 mM EDTA, washed twice with Medium 199 containing 1.8 mM CaCl<sub>2</sub> and 0.1% BSA, and resuspended at  $10^6$ /ml in the same medium. The cells were maintained at 30°C under 95% O<sub>2</sub>/5% CO<sub>2</sub> with constant agitation. For each experiment, an aliquot of  $10^6$  cells was loaded in the dark with 3  $\mu$ M fura-2 AM for 30 min at 30°C under continuous gassing. The cells were then transferred to a cuvette, washed and resuspended in 2 ml of either Ca<sup>2+</sup>-containing (1.8 mM CaCl<sub>2</sub>) or Ca<sup>2+</sup>-free (0.1 mM EGTA) Medium 199 with 0.01% BSA. They were transferred to the cuvette holder of an ARCM-MIC spectrofluorimeter (SPEX Industries, Edison, NJ) and equilibrated with constant mixing at 37°C for 2–3 min. Fura-2 fluorescence was monitored by alternating excitation wavelength between 340 and 360 nm, and measuring the emitted fluorescence at 510 nm.

## Results

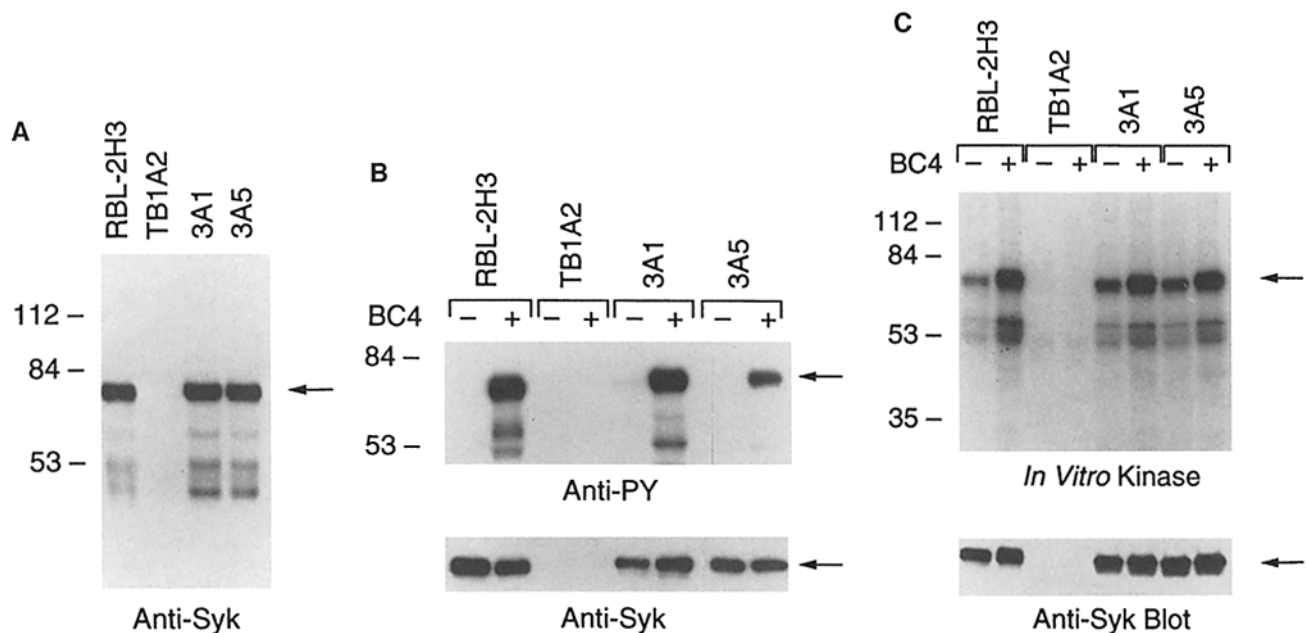
**Identification and Characterization of a Syk-deficient Variant of the RBL-2H3 Cells.** To investigate the role of Syk tyrosine kinase in FcεRI-mediated signaling, different variant cell lines derived from rat basophilic leukemia RBL-2H3 cells were tested to identify Syk-deficient cell line(s). One of the cloned cell lines, TB1A2 was found to be deficient in Syk by immunoblotting of whole-cell lysate and maintaining this phenotype in culture (Fig. 1 A). Thus, 16 different cell lines derived by recloning of the TB1A2 cells still had no detectable Syk. In vitro kinase reactions of anti-Syk immunoprecipitates from TB1A2 cells failed to detect any Syk kinase activity (Fig. 1 C). In the TB1A2 cells, there was also no detectable mRNA for Syk by Northern blotting (data not shown).

Aggregation of FcεRI in mast cells and in the cultured parental RBL-2H3 cells results in degranulation. In contrast, the TB1A2 cells did not release histamine after aggregation of FcεRI. However, the cells still maintained the capacity to degranulate by non-receptor-mediated stimulation with the calcium ionophore A23187 (see below). By FACS<sup>®</sup> analysis (Becton Dickinson & Co., Inc., Mountain View, CA), the expression of FcεRI in these cells was similar to that in the parental control RBL-2H3 cells (data not shown). Since Lyn protein tyrosine kinase associates with

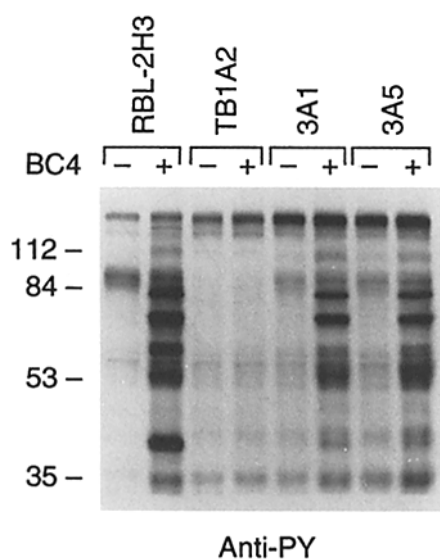
FcεRI and may play an important role in receptor-mediated signaling (8, 11, 12), we examined the expression and functional status of Lyn in TB1A2 cells. By immunoblotting of whole-cell lysates and by in vitro kinase assay of immunoprecipitates, the expression level and kinase activity of Lyn was similar in the TB1A2 and the parental RBL-2H3 cells (data not shown).

**Reconstitution of FcεRI Signaling Pathway in Syk-deficient TB1A2 Cells by Transfection with Syk.** Unlike the parental RBL-2H3 cells, the Syk-deficient TB1A2 cells did not degranulate after FcεRI aggregation. To investigate whether Syk could reconstitute this function, TB1A2 cells were transfected with wild-type rat Syk and selected in G418-containing media. 36 clones were expanded and tested for the expression of Syk. By immunoblotting, some Syk was detectable in 14 of these transfected cloned cell lines, although the level of expression in most cases was less than in the RBL-2H3 cells. However, two lines, 3A1 and 3A5, were isolated that expressed Syk at levels comparable to the wild-type RBL-2H3 cells (Fig. 1 A).

Tyrosine phosphorylation and increased kinase activity of Syk are tightly linked to FcεRI aggregation in RBL-2H3 cells (10, 15–17). To determine the functional status of expressed Syk in these transfected lines, cells were stimu-



**Figure 1.** Analysis of Syk protein tyrosine kinase in the different cell lines. (A) Immunoblot analysis of Syk expression. The cloned Syk-deficient variant cells (TB1A2) were cotransfected with cDNA encoding rat Syk in pSVL and pSV2neo vectors and selected with G418. Syk-expressing clones were expanded and analyzed by immunoblotting with an affinity-purified polyclonal anti-Syk antibody. Two Syk transfected cloned lines (3A1 and 3A5), the parental TB1A2 (Syk<sup>-</sup>), and the wild-type RBL-2H3 (Syk<sup>+</sup>) are shown for comparison. (B) Tyrosine phosphorylation of Syk in response to FcεRI aggregation. Cells ( $5 \times 10^6$ ) were either nonstimulated (BC4, -) or stimulated with the anti-FcεRI $\alpha$  mAb BC4 (BC4, +) for 20 min and lysates were immunoprecipitated with monoclonal anti-Syk antibodies coupled to Sepharose 4B beads. The immunoprecipitates were analyzed by immunoblotting with antiphosphotyrosine and affinity-purified polyclonal anti-Syk antibodies. (C) In vitro kinase assay of Syk immunoprecipitated from the different cell lines. For the in vitro kinase assay, Syk was immunoprecipitated from the different cell lines after stimulation as in B, and then incubated with [ $\gamma$ -<sup>32</sup>P]ATP in a protein autophosphorylation kinase assay. The proteins were separated by SDS-PAGE in 10% gels, electrotransferred, and radiolabeled proteins were detected by autoradiography. The same membrane was then blotted with an affinity-purified polyclonal anti-Syk antibody. Arrows indicate Syk. Molecular mass markers (in kilodaltons) represent migration of prelabeled standards.



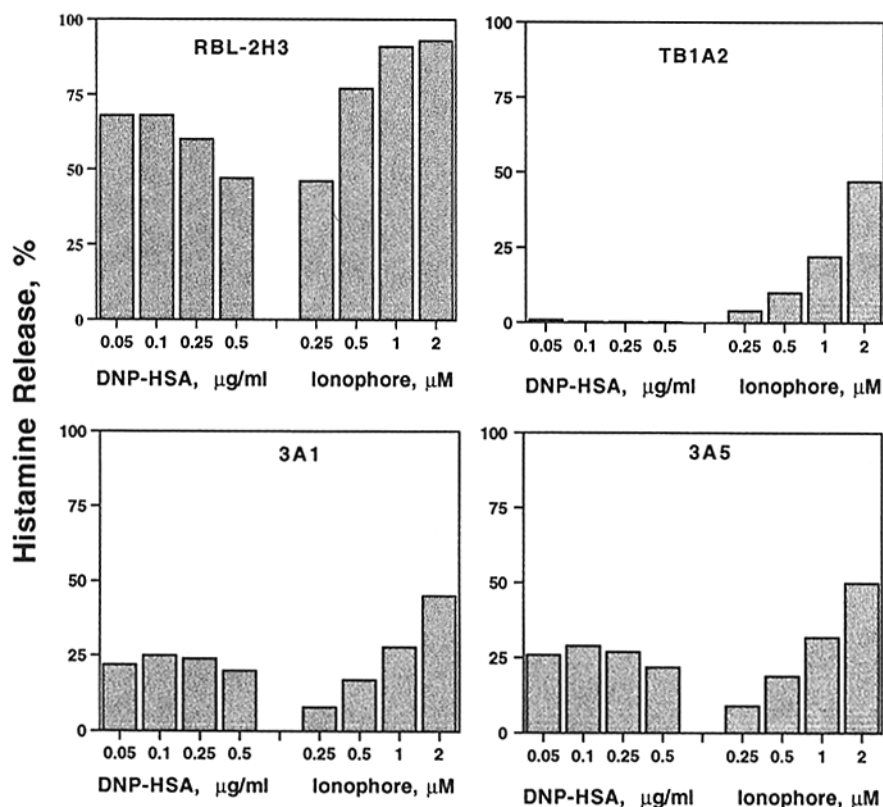
**Figure 2.** FcεRI-induced tyrosine phosphorylation of cellular proteins. RBL-2H3 (Syk<sup>+</sup>), TB1A2 (Syk<sup>-</sup>), and two Syk transfected cell lines (3A1 and 3A5) were either not stimulated (BC4, -) or stimulated with the anti-FcεRIα (BC4, +) for 45 min. Lysates prepared from  $1.5 \times 10^5$  cells were analyzed by immunoblotting with antiphosphotyrosine antibodies.

lated with the anti-FcεRI mAb BC4. As in the parental wild-type RBL-2H3 cells, FcεRI aggregation induced the tyrosine phosphorylation of Syk and increased its kinase activity in the Syk-transfected 3A1 and 3A5 cells (Fig. 1, B

and C). Therefore, transfection reconstituted functionally active Syk in these cells.

The earliest events after aggregation of FcεRI are the cellular protein tyrosine phosphorylations that are critical for signal transduction in mast cells (4, 6, 31, 32). In lysates from nonstimulated cells, the pattern of tyrosine-phosphorylated proteins was similar in RBL-2H3, TB1A2, 3A1, and 3A5 cell lines (Fig. 2). Therefore, the expression of Syk by transfection did not induce any unregulated tyrosine phosphorylation of proteins. In parental wild-type RBL-2H3 cells, aggregation of FcεRI resulted in the tyrosine phosphorylation of a number of cellular proteins. In contrast, in the Syk-deficient TB1A2 cells, receptor stimulation did not result in a significant increase in total cellular phosphorylations. In the Syk-transfected cells, the general pattern of phosphorylations was similar to that in the parental wild-type RBL-2H3 cells. Therefore, most of the FcεRI-induced cellular protein tyrosine phosphorylations require Syk.

*Transfection of Syk in the Deficient Cells Reconstitutes FcεRI-induced Histamine Release.* Stimulation of mast cells results in the release of histamine from cytoplasmic granules. As had been observed previously, both Ca<sup>2+</sup> ionophore and FcεRI aggregation induced histamine release in RBL-2H3 cells (Fig. 3). In contrast, in the Syk-deficient TB1A2 cells, FcεRI cross-linking did not induce any degranulation. The TB1A2 cells did degranulate when stimulated with the Ca<sup>2+</sup> ionophore A23187, although this was less than the response of the RBL-2H3 cells. This difference between the TB1A2 and RBL-2H3 cells was apparent both in the dose



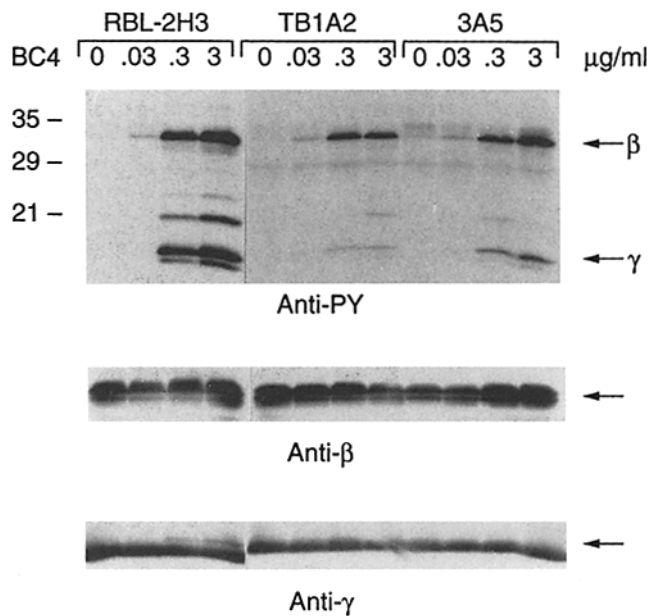
**Figure 3.** Reconstitution of FcεRI-mediated histamine release by Syk. RBL-2H3 (Syk<sup>+</sup>), TB1A2 (Syk<sup>-</sup>), and two Syk-transfected (3A1 and 3A5) cell lines were cultured overnight with antigen specific IgE, as described in Materials and Methods. After 16 h, cells were washed and either not activated or activated. Cell stimulation was for 45 min at 37°C with antigen (DNP-HSA) or with the calcium ionophore A23187 at the indicated concentrations. Supernatants were assayed for histamine. The graphs represent the average of three independent experiments.

response and in the extent of degranulation with the  $Ca^{2+}$  ionophore. The transfection of Syk into the deficient cells had no effect on A23187-stimulated histamine release. However, there was reconstitution of the FcεRI-mediated histamine release in the Syk transfected cells. In both the RBL-2H3 and the Syk-reconstituted 3A1 and 3A5 cells, the receptor-mediated histamine release was ~75–90% of the maximum that could be induced by 1 μM calcium ionophore. The results indicate that Syk reconstitutes the TB1A2 cells to the maximum that they are capable of degranulating.

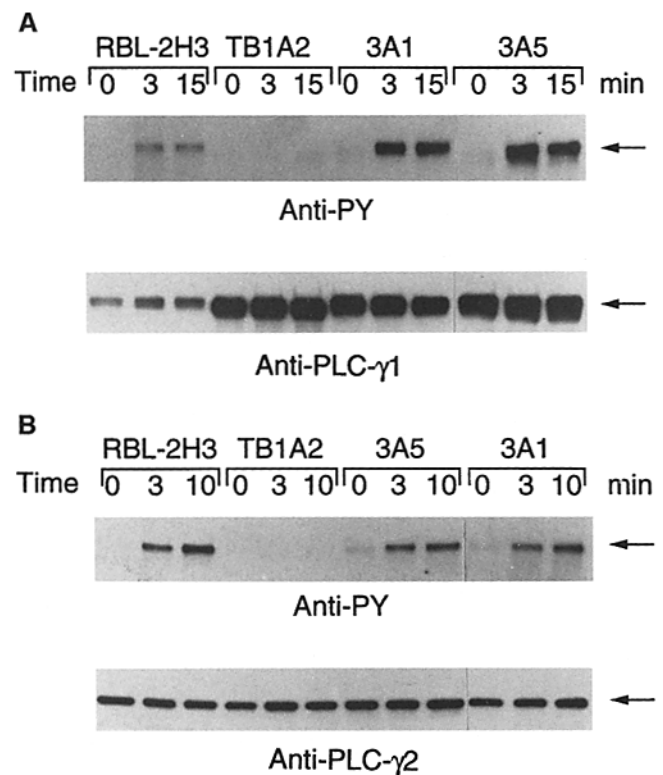
**Tyrosine Phosphorylation of the β and γ Subunits of FcεRI.** Aggregation of FcεRI in RBL-2H3 cells results in the rapid tyrosine phosphorylation of the β- and γ-receptor subunits (5, 33). As has been observed previously, FcεRI aggregation with anti-FcεRIα mAb BC4 induced strong tyrosine phosphorylations of both the β and γ subunits in the wild-type RBL-2H3 cells (Fig. 4). The results were more complicated in the Syk-deficient and Syk-transfected cells. In both the Syk-deficient and Syk-transfected cells, the β subunit became tyrosine phosphorylated to the same extent, though less than in RBL-2H3 cells. Receptor stimulation also induced detectable tyrosine phosphorylation of the γ subunit in Syk-deficient cells, and this phosphoryla-

tion was enhanced in the Syk-transfected cells. However, the phosphorylation level of γ in the Syk-transfected cells was still weaker than that in the control RBL-2H3 cells. Therefore, Syk does not influence the extent of the tyrosine phosphorylation of the β subunit, but plays a role in the phosphorylation of the γ subunit of FcεRI.

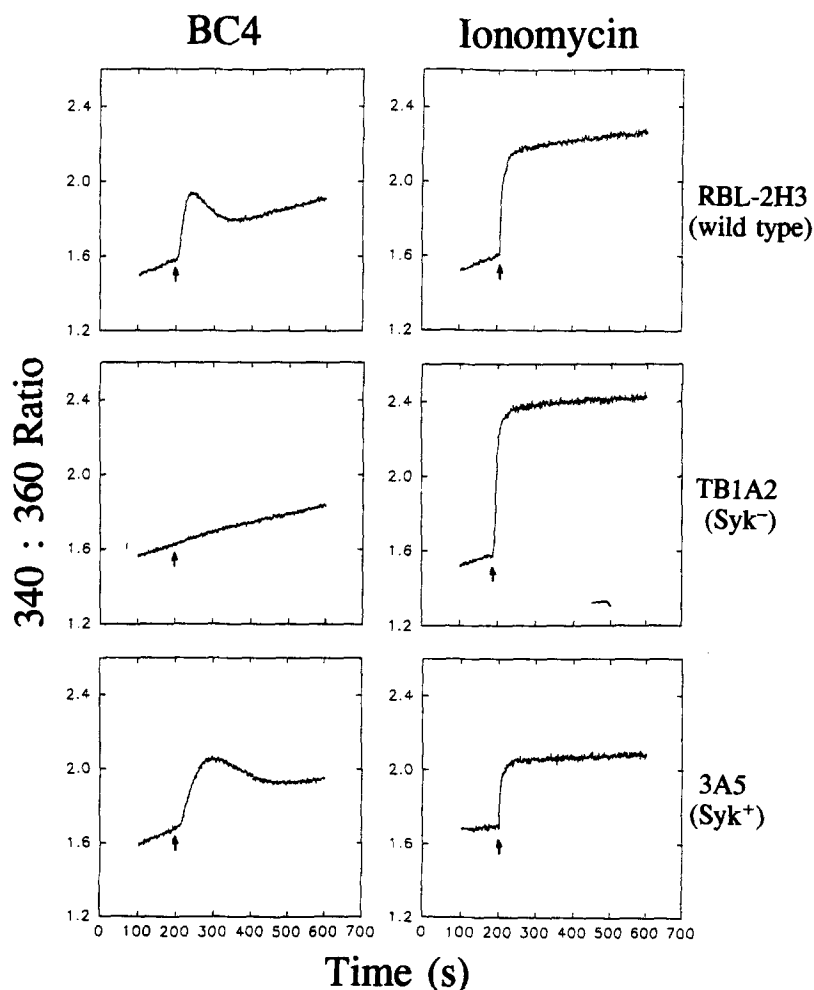
**Role of Syk in the Tyrosine Phosphorylation of Phospholipase C-γ.** One of the earliest events after receptor aggregation is the activation of phospholipase C, which results in the hydrolysis of phospholipids with the formation of inositol 1,4,5-trisphosphate and 1,2, diacylglycerol. These are then secondary signals that release  $Ca^{2+}$  from internal stores and activate protein kinase C, respectively. There is rapid phosphorylation of phospholipase C-γ1 after FcεRI aggregation (5, 34, 35). Here, we observed that in the normal RBL-2H3 cells and Syk-transfected cells, FcεRI aggregation induced tyrosine phosphorylation of phospholipase C-γ1 and phospholipase C-γ2 (Fig. 5). Interestingly there was much less phospholipase C-γ1 expressed in RBL-2H3 cells than in the variant TB1A2 and in its Syk-transfected progeny (data not shown). In contrast, receptor stimulation did not



**Figure 4.** Receptor aggregation-induced tyrosine phosphorylation of the β and γ subunits of FcεRI. The RBL-2H3 (Syk<sup>+</sup>), TB1A2 (Syk<sup>-</sup>), and the Syk transfected 3A5 cell line were either non-activated or activated with the indicated concentrations of anti-FcεRIα mAb BC4 for 30 min at 37°C. The β and γ subunits of FcεRI were immunoprecipitated with an anti-FcεRIβ and analyzed by immunoblotting with antiphosphotyrosine antibody (*Anti-PY*). The same membranes were stripped and cut into two parts. The upper half was blotted with anti-FcεRIβ (*Anti-β*) and the lower half with anti-FcεRIγ antibodies (*Anti-γ*). Arrows indicate the β and γ subunits of FcεRI. The length of exposure with the antiphosphotyrosine immunoblots were identical, but the RBL-2H3 blots were exposed after a period of 3 h.



**Figure 5.** Transfection of Syk reconstitutes FcεRI-mediated tyrosine phosphorylations. RBL-2H3 (Syk<sup>+</sup>), TB1A2 (Syk<sup>-</sup>), and two Syk-transfected (3A1 and 3A5) cell lines were stimulated by mAb BC4 (0.3 μg/ml) for the indicated times. Cell lysates from  $8 \times 10^6$  were immunoprecipitated with anti-phospholipase C-γ1 Ab (A) and anti-phospholipase C-γ2 Ab (B). Immunoprecipitated proteins were separated by SDS-PAGE (6% gels) and analyzed by blotting with anti-phosphotyrosine Ab (*Anti-PY*). After stripping, the same membranes were reprobbed with anti-phospholipase C-γ1 Ab (A, *Anti-PLC-γ1*), anti-phospholipase C-γ2 Ab (B, *Anti-PLC-γ2*).



**Figure 6.** Transfection of Syk reconstitutes FcεRI-mediated calcium mobilization. RBL-2H3 (*Syk*<sup>+</sup>), TB1A2 (*Syk*<sup>-</sup>), and Syk-transfected 3A5 (*Syk*<sup>+</sup>) cell lines were preloaded with fura-2 and then stimulated with either mAb BC4 (1.5 μg/ml; left panels) or the calcium ionophore ionomycin (0.5 μM; right panels). The traces show the 340:360 fluorescence ratio from cell suspensions stimulated at the indicated time points (arrows). The traces are representative of three other similar experiments. Results with the Syk-transfected 3A1 cells were similar (data not shown).

induce tyrosine phosphorylation of phospholipase C-γ1 nor of phospholipase C-γ2 in the Syk-deficient cells. Therefore, the tyrosine phosphorylation of phospholipase C-γ1 and phospholipase C-γ2 require Syk.

**Changes in Intracellular Ca<sup>2+</sup> Concentration.** The signals generated from phospholipase C activation result in an increase in [Ca<sup>2+</sup>]<sub>i</sub> (36). Unlike the wild-type RBL-2H3 cells, FcεRI aggregation in the Syk-deficient TB1A2 cell line did not result in an increase in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 6). In the Syk transfected 3A5 cells, however, [Ca<sup>2+</sup>]<sub>i</sub> was increased upon receptor aggregation. When extracellular Ca<sup>2+</sup> was removed, both RBL-2H3 and Syk-transfected 3A5 cells responded to receptor aggregation with a smaller, transient increase in [Ca<sup>2+</sup>]<sub>i</sub> (data not shown). All of the cell lines exhibited similar responses to the calcium ionophore ionomycin. Therefore, the FcεRI-mediated changes in [Ca<sup>2+</sup>]<sub>i</sub> correlate with the tyrosine phosphorylation of phospholipase C-γ and required Syk.

## Discussion

By screening of variants of RBL-2H3 cells, we identified the TB1A2 cells that are defective in the expression of the

Syk protein tyrosine kinase. These cells had no detectable Syk protein by immunoblotting or in vitro kinase reaction, and no detectable Syk mRNA by Northern hybridization. These TB1A2 cells that lacked Syk failed to secrete after FcεRI stimulation. The stable transfection of Syk into these cells reconstituted signal transduction and secretion. These experiments prove that Syk is critical for FcεRI signaling.

One of the earliest detectable events after FcεRI clustering is the tyrosine phosphorylation of the β and γ subunits of the receptor. Src family protein tyrosine kinase, Lyn, has been proposed to directly tyrosine phosphorylate these subunits. The expression of Syk did not affect the tyrosine phosphorylation of the β subunit, but did result in changes in the phosphorylation of the γ component. In Syk-deficient cells, the intensity of FcεRI-mediated γ tyrosine phosphorylation was very weak, and was increased after Syk-cDNA transfection into these cells. However, this does not necessarily mean that Syk directly phosphorylates the γ subunit. It is possible that the binding of Syk to the phosphorylated ITAM motif of the γ subunit may protect it from dephosphorylation. This explanation is supported by the observation that transient expression in RBL-2H3 cells of a truncated form of Syk containing the two SH2 domains enhanced

the tyrosine phosphorylation of the  $\beta$  and  $\gamma$  subunits of the receptor (26). Nevertheless, our results demonstrate that Fc $\epsilon$ RI aggregation results in tyrosine phosphorylation of the receptor subunits in the absence of Syk.

Aggregation of Fc $\epsilon$ RI results in tyrosine phosphorylation of phospholipase C- $\gamma$ 1 (5, 34, 35) and as shown here in the tyrosine phosphorylation of phospholipase C- $\gamma$ 2. Tyrosine phosphorylation of phospholipase C- $\gamma$ 1 results in increased activity that is critical for generation of the downstream signal mediators inositol 1,4,5-trisphosphate and 1,2 diacylglycerol. Chimeric proteins have been stably transfected into RBL-2H3 cells that contain extracellular and transmembrane domains of CD16 fused to Syk (24). Aggregation of these chimeric proteins by antibodies to the extracellular domain results in phosphorylation of several proteins, including phospholipase C- $\gamma$ 1, and degranulation. Similarly, in permeabilized RBL-2H3 cells, addition of a truncated Syk containing the two SH2 domains of Syk inhibits tyrosine phosphorylation of phospholipase C- $\gamma$ 1 and secretion (25). In the present experiments, we found that Fc $\epsilon$ RI aggregation did not result in tyrosine phosphorylation of phospholipase C- $\gamma$ 1 and phospholipase C- $\gamma$ 2 unless Syk was expressed in the cells. However, these results do not mean that there is direct phosphorylation of phospholipase C- $\gamma$  by Syk, since there could be intermediate molecules that are involved in this phosphorylation. Therefore, tyrosine phosphorylation of phospholipase C- $\gamma$  is downstream of Syk.

The activation of phospholipase C- $\gamma$  results in the generation of secondary mediators that result in an increase in intracellular calcium concentration. As would be predicted from the results of the tyrosine phosphorylation of phospholipase C- $\gamma$ , the changes in  $[Ca^{2+}]_i$  required the presence of Syk. In the parental TB1A2 cells, there was no rise in  $[Ca^{2+}]_i$  after Fc $\epsilon$ RI aggregation, either in the presence or absence of calcium in the extracellular medium. This rise in  $[Ca^{2+}]_i$  was reconstituted in the Syk-transfected cells. The results indicate that Syk is critical for initiating receptor-mediated calcium signals in mast cells.

The aggregation of Fc $\epsilon$ RI also induces the tyrosine phosphorylations of many cellular proteins (4, 5, 7, 31, 34). Here, we found that in the absence of Syk, there was a marked reduction in the Fc $\epsilon$ RI-induced tyrosine phosphorylations. Therefore, Syk is essential for most of the cellular phosphorylations that are detected in antiphosphotyrosine immunoblotting of total cell lysates. These results are more striking than those which would be expected from previous attempts to interfere with the function of Syk in RBL-2H3 cells. In streptolysin O-permeabilized RBL-2H3 cells, only some of the receptor-induced tyrosine phosphorylations are blocked by introducing the two tandem SH2 domains of Syk (25). Similarly, the transient expression in RBL-2H3 cells of a truncated form of porcine Syk missing the kinase domain suppressed some but not all of the Fc $\epsilon$ RI-induced tyrosine phosphorylation (26). Altogether the results of the Syk-negative and Syk-transfected cells indicate that Syk plays a critical role in the very early stages of signal transduction in mast cells.

RBL-2H3 cells provide a useful experimental model to study the signal transduction pathway for degranulation in mast cells/basophils (2, 3). The cascade of biochemical events in stimulated cells results in the degranulation and the release of various inflammatory mediators. In previous studies, protein tyrosine kinase inhibitors blocked tyrosine phosphorylation in a dose-dependent manner, and in parallel, inhibited histamine release (31, 32). There was inhibition of both receptor- and calcium ionophore-mediated release. Here, we demonstrate that Syk is involved only in the Fc $\epsilon$ RI-mediated degranulation process, with no role in the ionophore-induced release. The inhibition of ionophore-induced release with protein tyrosine kinase inhibitors could be caused by their effects on other kinases, such as pp125<sup>FAK</sup>, which function during the late steps of the release process (37, 38). The observation that calcium ionophore induced less histamine release in TB1A2 than in RBL-2H3 cells suggests that there are differences between these two cell lines that go beyond Syk and may involve late stages of the release process.

In the Syk-negative mast cells, Lyn alone was sufficient to tyrosine phosphorylate the Fc $\epsilon$ RI subunits. The Lyn SH2 domain binds to the tyrosine phosphorylated ITAM of the  $\beta$  subunit (10, 18), and this could recruit more Lyn to the aggregated receptor (11). The phosphorylation of the  $\beta$  and  $\gamma$  receptor subunits, especially the  $\gamma$  subunit, recruits Syk to the receptor. The SH2 domains of Syk bind strongly to tyrosine-phosphorylated (but not to nonphosphorylated)  $\gamma$  subunit and less efficiently to the  $\beta$  subunit of Fc $\epsilon$ RI (10, 18). This binding requires the two SH2 domains of Syk and the phosphorylation of both tyrosines in the ITAM motif that are present in both subunits of the receptor. Binding is by the two tandem SH2 domains of Syk binding the two phosphorylated tyrosines of the same ITAM motif. The binding of Syk by its two tandem SH2 domains results in a conformational change (39) that increases its kinase activity (18, 40) and propagates downstream intracellular signals. The preferential binding of Lyn and Syk to different subunits of Fc $\epsilon$ RI allows interaction between these two kinases. For example, transfection experiments in COS cells suggest that Src family tyrosine kinases are important for the tyrosine phosphorylation and activation of Syk or of the homologous kinase ZAP-70 (23, 41).

The model for signaling by Fc $\epsilon$ RI has strong parallels to that initiated by either the TCR or BCR. Signaling from all these receptors requires the cooperation between Src family and ZAP-70/Syk family kinases. Aggregation of these receptors results in activation of an Src family kinase, which results in tyrosine phosphorylation of the ITAM motifs and the recruitment of Syk/ZAP-70 to the receptor. The activated ZAP-70/Syk then tyrosine phosphorylate and activate downstream signals. Strong evidence for this model are studies of cells that lack Syk or ZAP-70. An avian B cell line that is defective in Syk has been generated (22), and human T cells have been studied from several patients with a genetic deficiency in ZAP-70 (42, 43). Our results with the Syk-negative mast cells have some similarities

ties and differences from these B and T cells. In all these deficient cells, there is a dramatic decrease in the total protein tyrosine phosphorylations induced by receptor aggregation. However, there seem to be some differences in the signals in these cells. Unlike the other cells lines, the mast cells show clear tyrosine phosphorylation of the receptor subunits in the Syk-negative cells. The receptor-mediated rise in  $[Ca^{2+}]_i$  is absent in the Syk-negative B cells and mast cells, although some ZAP-70-negative cells have a moderate increase in  $[Ca^{2+}]_i$  after receptor aggregation (42). The tyrosine phosphorylation of phospholipase C- $\gamma$  is absent in the B cells and mast cells, and either absent or markedly di-

minished in the T cells. Altogether, these results strongly support the hypothesis that Syk/ZAP-70 are critical tyrosine kinases for signaling from receptors that have ITAM motifs.

In summary, these results demonstrate that Syk is critical for signal transduction from the Fc $\epsilon$ RI. Syk is essential for propagating the downstream signals from the receptor, including the tyrosine phosphorylation of phospholipase C- $\gamma$ , the rise in  $[Ca^{2+}]_i$ , and degranulation. Therefore, blocking the interaction of this kinase with the ITAMs in Fc $\epsilon$ RI would be a possible way to inhibit the release of inflammatory mediators from mast cells.

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