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### Fcε Receptor I-Associated *lyn*-Dependent Phosphorylation of Fc γ Receptor IIB During Negative Regulation of Mast Cell Activation

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#### References

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# Fcε Receptor I-Associated *lyn*-Dependent Phosphorylation of Fcγ Receptor IIB During Negative Regulation of Mast Cell Activation<sup>1</sup>

Odile Malbec,\* Dana C. Fong,<sup>†</sup> Martin Turner,<sup>‡</sup> Victor L. J. Tybulewicz,<sup>‡</sup> John C. Cambier,<sup>†</sup> Wolf H. Fridman,\* and Marc Daëron<sup>2\*</sup>

FcγRIIB are low-affinity receptors for IgG whose intracytoplasmic domain contains an immunoreceptor tyrosine-based inhibition motif (ITIM). FcγRIIB inhibit cell activation triggered by receptors that signal via immunoreceptor tyrosine-based activation motifs. This inhibition requires ITIM tyrosyl phosphorylation and is correlated with the binding of SH2 domain-containing phosphatases that may mediate the inhibitory signal. In the present work, we investigated the mechanism of FcγRIIB phosphorylation and its consequences in mast cells. We demonstrate that the phosphorylation of FcγRIIB requires coaggregation with FcεRI and that, once phosphorylated, FcγRIIB selectively recruit the inositol polyphosphate 5 phosphatase SHIP, *in vivo*. *In vitro*, however, the phosphorylated FcγRIIB ITIM binds not only SHIP, but also the two protein tyrosine phosphatases, SHP-1 and SHP-2. We show that the coaggregation of FcγRIIB with FcεRI does not prevent FcεRI-mediated activation of *lyn* and *syk*. Both kinases can phosphorylate FcγRIIB *in vitro*. However, when coaggregated with FcεRI, FcγRIIB was *in vivo* phosphorylated in *syk*-deficient mast cells, but not in *lyn*-deficient mast cells. When FcεRI are coaggregated with FcγRIIB by immune complexes, FcεRI-associated *lyn* may thus phosphorylate FcγRIIB. By this mechanism, FcεRI initiate ITIM-dependent inhibition of intracellular propagation of their own signals. *The Journal of Immunology*, 1998, 160: 1647–1658.

Receptors for the Fc portion of IgG expressed by mouse B cells, FcγRIIB, have long been known to inhibit B cell activation when coaggregated with BCR<sup>3</sup> by immune complexes (1, 2). FcγRIIB are single-chain low-affinity IgG receptors (3–5) encoded by a single gene in which a separate exon encodes the transmembrane domain and three other exons the intracytoplasmic (IC) domain (6). This enables three membrane isoforms to be generated by alternative splicing of corresponding sequences. FcγRIIB1 retain sequences encoded by all four exons; FcγRIIB2 lack sequences encoded by the first IC exon; FcγRIIB1' are generated by the use of a cryptic splice donor site located in the murine first IC exon (7). Mouse B cells express FcγRIIB1 (8) and FcγRIIB1' (7). A 13-amino acid IC sequence containing a tyrosine residue, followed by a leucine at position +3, was found to be necessary (9) and sufficient (10) for inhibition. This sequence, present in all three murine FcγRIIB isoforms (7, 9), is conserved

in the two human FcγRIIB isoforms, FcγRIIB1 and FcγRIIB2, and both human isoforms were reported to inhibit mouse (11) and human (12) B cell activation. FcγRIIB were subsequently shown to negatively regulate not only BCR-mediated B cell activation, but also TCR-mediated T cell activation (13) and high-affinity IgE receptor (FcεRI)-mediated mast cell activation (14). FcγRIIB also inhibited cell activation by chimeric molecules whose IC domain consisted of that of the Igα (10), TCR-ζ, or FcRγ (13) transduction subunits, providing evidence that FcγRIIB functions as a general negative coreceptor for all receptors containing an immunoreceptor tyrosine-based activation motif (ITAM). The same sequence was found to account for the regulatory effects of FcγRIIB on cell activation by the three ITAM-containing immunoreceptors, and mutation of the tyrosine residue was sufficient to abrogate inhibition (10, 13).

Because the YxxL motif present in this sequence was reminiscent of the double YxxL motif constitutive of ITAMs, this sequence was referred to as an immunoreceptor tyrosine-based inhibition motif (ITIM), and occurrence of ITIMs was searched for in other negative coreceptors. ITIMs were found in the IC domain of human (15) and murine (16) killer cell inhibitory receptors (KIRs), which inhibit the cytotoxic activity of NK cells when they bind to MHC class I molecules on target cells (17); in the IC domain of CD22 (18) and of CTLA-4 (19), expressed on B and T cells, respectively, and which inhibit B cell (20) and T cell activation (21); in the IC domain of gp49B1 (22) and MAFA (23), expressed on mast cells, and which inhibit IgE-induced mediator release (24, 25); and in the IC domain of ILT3, expressed on monocytes and dendritic cells, and which can inhibit the activation of these cells (26).

One general property of ITIMs is their tyrosine phosphorylation and recruitment of SH2 domain-bearing phosphatases. FcγRIIB was shown to be phosphorylated both in B cells (10) and in mast cells (27, 28). FcγRIIB was reported to recruit the tyrosine phosphatase SHP-1

\*Laboratoire d'Immunologie Cellulaire et Clinique, INSERM U.255, Institut Curie, Paris, France; <sup>†</sup>Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206; and <sup>‡</sup>Division of Cellular Immunology, National Institute for Medical Research, London, United Kingdom

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<sup>2</sup>Address correspondence and reprint requests to Dr Marc Daëron, Laboratoire d'Immunologie Cellulaire et Clinique, INSERM U.255, Institut Curie, 26 rue d'Ulm, 75005 Paris, France. E-mail address: Marc.Daeron@curie.fr

<sup>3</sup>Abbreviations used in this paper: BCR, B cell receptor; BMMC, bone marrow-derived mast cell; GST, glutathione S-transferase; IC, intracytoplasmic; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; IVKA, *in vitro* kinase assay; KIR, killer cell inhibitory receptor; MAR, mouse anti-rat; PTK, protein tyrosine kinase; PY, phosphotyrosine; RAM, rabbit anti-mouse; GAR, goat anti-rabbit; TNP, trinitrophenyl; IMDM, Iscove's modified Dulbecco's medium; SHIP, SH2-bearing inositolphosphate phosphatase; SHP, SH2-bearing protein tyrosine phosphatase.

in B cells (29), and the inositol phosphatase SHIP in both B cells (27, 30, 31) and mast cells (27, 28). In the present work, we devised an experimental model to study the mechanism of inhibition of mast cell activation by FcγRIIB. We show that the coaggregation of FcεRI with FcγRIIB not only does not inhibit the activation of protein tyrosine kinases (PTK) associated with FcεRI, but enables one of these kinases, *lyn*, to phosphorylate FcγRIIB ITIM. Phosphorylated FcγRIIB then selectively recruit SHIP. We propose a model according to which FcεRI contribute to the extinction of the intracellular propagation of their own signals.

## Materials and Methods

### Cells

RBL-2H3 cells (32) were cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Only adherent cells, recovered with trypsin-EDTA, were used. All culture reagents were from Life Technologies (Paisley, Scotland, U.K.). Previously described cDNAs encoding murine FcγRIIB1, FcγRIIB1', FcγRIIB2, and FcγRIIB(IC1) (7, 33) were inserted into an expression vector under the control of the SRα promoter in pBR322 (34) and in which a resistance gene to zeocin was introduced (NT-zeo). Transfectants were selected by culture with 500 μg/ml of zeocin (Cayla, Toulouse, France). RBL transfectants recovered after selection were cloned with steel cylinders, as described (33). The expression of recombinant receptors by cloned cells was assessed by indirect immunofluorescence. The expression of recombinant receptors on clones remained stable over the duration of experiments. Several clones of each transfectant expressing comparable amounts of recombinant receptors were selected and used for experiments. They gave similar results. Transfectants expressing FcγRIIB2(Y26G) were previously described (35).

Mast cells were derived by culturing fetal liver cells from wild-type and *syk*-deficient 16.5-day embryos in culture medium supplemented with 10% WEHI 3B-conditioned medium, as previously described (36). Bone marrow-derived mast cells (BMMC) were obtained by culturing bone marrow cells from wild-type and *lyn*-deficient mice under the same conditions. After 4 wk, the cultures were found to contain more than 90% mast cells, as assessed by microscopic examination. Based on indirect immunofluorescence, all cells expressed FcεRI and FcγRIIB, and the deficient cells expressed comparable levels of both receptors as wild-type counterpart cells.

### Abs and immunoadsorbents

The mouse IgE mAb 2682-I was used as culture supernatant of a subclone of DNP-H1-e-26 hybridoma cells (37). The rat anti-mouse FcγRIIB 2.4G2 mAb (38) was purified by affinity chromatography on protein G-Sepharose (Pharmacia, Uppsala, Sweden) from ascitic fluid of nude mice inoculated with 2.4G2 hybridoma cells i.p. F(ab')<sub>2</sub> fragments were obtained by pepsin digestion for 48 h. The purity of IgG and F(ab')<sub>2</sub> fragments was assessed by SDS-PAGE analysis. F(ab')<sub>2</sub> fragments of polyclonal mouse anti-rat Ig (MAR), and F(ab')<sub>2</sub> fragments and intact IgG of polyclonal rabbit anti-mouse Ig (RAM) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). MAR F(ab')<sub>2</sub> were trinitrophenylated by incubation for 2 h at room temperature with trinitrobenzene sulfonic acid (Eastman Kodak, Rochester, NY) in borate-buffered saline, pH 8. TNP<sub>10</sub>-MAR F(ab')<sub>2</sub> obtained were purified on Sephadex G25 (Pharmacia). Rabbit Abs against recombinant extracellular domains of mouse FcγRIIB (39) were gifts from Dr. Catherine Sautès (Institut Curie, Paris, France). Horseradish peroxidase-conjugated anti-phosphotyrosine (PY) mouse mAbs PY-20 were purchased from Chemicon (Temecula, CA). Mouse monoclonal anti-SHP-1 and anti-SHP-2 Abs were purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-SHIP Abs were a gift from Dr. Gerald Krystal (University of British Columbia, Vancouver, Canada). Rabbit Abs against FcRγ were a gift from Dr. Jean-Pierre Kinet (Harvard Medical School, Boston, MA). Rabbit anti-*lyn* Abs used for immunoprecipitation were purchased from Upstate Biotechnology (Lake Placid, NY), and mouse anti-*lyn* Abs used for Western blotting were purchased from Transduction Laboratories. Rabbit anti-*syk* Abs used for immunoprecipitation were gifts from Dr. Ulrich Blank (Institut Pasteur, Paris, France), and rabbit anti-*syk* Abs used for Western blotting were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Horseradish peroxidase-conjugated polyclonal goat anti-mouse (GAM) Ig Abs and polyclonal goat anti-rabbit (GAR) Ig Abs were purchased from Santa Cruz Biotechnologies.

Protein G-Sepharose (50 μl beads diluted 1/2) was used to precipitate 2.4G2-bound FcγRIIB. Synthetic nonphosphorylated or tyrosine-phosphorylated peptides with the FcγRIIB ITIM sequence EAENTIT(p)YSLKKH

were purchased from Macromolecular Resources (Fort Collins, CO). They were coupled to CNBr-activated Sepharose beads (Pharmacia) at 1 mg/ml. Anti-FcRγ, anti-*lyn*, and anti-*syk* Abs were adsorbed onto protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) (5 μl serum or 5 μg purified Abs/50 μl beads diluted 1/2) by an overnight incubation at 4°C. Washed beads were used as immunoadsorbents to precipitate FcεRI complexes, *lyn* or *syk*.

### GST fusion proteins

cDNA encoding the IC domain of FcγRIIB1' (ICIIB1') was generated by PCR and inserted in the pGEX-4T-2 vector (Pharmacia). The sequence was determined on the two strands by dideoxynucleotide sequencing. GST-ICIIB1' was produced in DH5-α bacteria by isopropylthiogalactopyranoside induction, purified on glutathione agarose (Sigma Chemical Co.), and analyzed by SDS-PAGE. Soluble GST-ICIIB1' was obtained by incubating the beads with 50 mM Tris, 25 mM glutathione, pH 8. GST-HS1 was a gift from Dr. Ulrich Blank. GST-p62 (amino acids 331–443 of the mouse GTPase-activating protein-associated p62) was purchased from Santa Cruz Biotechnologies.

### Serotonin release and assay

Transfected RBL cells, resuspended in RPMI medium supplemented with 10% FCS (RPMI-FCS) at  $1 \times 10^6$  cells/ml, were incubated at 37°C for 1 h with 2 μCi/ml [<sup>3</sup>H]serotonin (Amersham, Les Ulis, France), washed, resuspended in RPMI-FCS, incubated for another hour at 37°C to remove excess [<sup>3</sup>H]serotonin, washed again, resuspended in the same medium, distributed in 96-well microculture plates at  $2 \times 10^5$  cells/well, and incubated for 1 h at 37°C with IgE and 2.4G2 F(ab')<sub>2</sub> or not in a final volume of 50 μl. Adherent cells were washed four times with 200 μl HBSS; 25 μl culture medium was added to each well and cells were warmed at 37°C for 15 min before challenge. Cells were challenged for 30 min at 37°C with 25 μl TNP-MAR F(ab')<sub>2</sub> or RAM F(ab')<sub>2</sub> or intact RAM IgG, previously warmed at 37°C for 15 min. Reactions were stopped by adding 50 μl ice-cold medium and by placing plates on ice. Fifty microliters of supernatants were mixed with two hundred microliters of Aqualuma-Plus scintillation fluid (Lumac, The Netherlands) and counted in a beta plate counter (Pharmacia). The percentage of [<sup>3</sup>H]serotonin release was calculated, using as 100% cpm in 50 μl harvested from wells containing the same number of cells that were lysed in 100 μl of 0.5% SDS and 0.5% Nonidet P-40.

### Measurement of intracellular Ca<sup>2+</sup> concentration

Transfected RBL cells were resuspended at  $1 \times 10^6$  cells/ml in IMDM containing 5% FBS, 5 μM Indo-1 AM (Molecular Probes, Eugene, OR), and IgE, incubated for 1 h at 37°C, and washed twice. Cells were resuspended in IMDM containing 5% FBS and stimulated at  $1 \times 10^6$  cells/ml/sample with either 10 μg/ml RAM F(ab')<sub>2</sub> or 15 μg/ml intact RAM IgG. Intracellular Ca<sup>2+</sup> was monitored for 15 min using a flow cytometer (model 50H; Ortho Diagnostic Systems, Raritan, NJ). The percentage of cells responding was calculated using the data acquisition system ACQ-CYTE and MTIME software (Phoenix Flow Systems, Phoenix, AZ).

### [<sup>35</sup>S]methionine labeling

RBL cells were washed twice in methionine-free DMEM (Sigma Chemical Co.), resuspended at  $1 \times 10^6$  cells/ml in methionine-free DMEM supplemented with 5% dialyzed FBS, and incubated for 4 h with 0.5 mCi/sample of [<sup>35</sup>S]methionine (DuPont NEN, Wilmington, DE) at 37°C. Cells were washed twice in PBS and lysed, as described below.

### Immunoprecipitation and Western blot analysis

Transfected RBL cells, resuspended at  $5 \times 10^6$ /ml, were incubated for 1 h at 37°C with IgE anti-DNP (culture supernatant diluted 1/10) and 2.4G2 F(ab')<sub>2</sub> (3 μg/ml) in complete culture medium, washed three times, resuspended in the same medium at  $10^7$  cells/ml, and challenged for 2 min or for the indicated periods of time at 37°C with 10 μg/ml or indicated concentrations of TNP-MAR F(ab')<sub>2</sub>. They were centrifuged, and pellets containing  $10^7$  cells for FcγRIIB immunoprecipitation or  $3 \times 10^7$  cells for FcεRI immunoprecipitation were lysed for 10 min at 0°C at  $2 \times 10^8$  cells/ml in lysis buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 5 mM NaF, 5 mM sodium pyrophosphate, 0.4 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM PMSF. Lysates were centrifuged at 10,000 rpm for 10 min at 4°C, and supernatants were incubated with protein G-Sepharose for 30 min at 4°C or with anti-FcRγ-bound protein A-Sepharose for 1 h at 4°C. Immunoadsorbents were washed three times in 1 ml lysis buffer and boiled for 3 min in reducing sample buffer. Eluted material was submitted to SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA).

Membranes were saturated with either 5% BSA or 5% skimmed milk (Régilait, Saint-Martin-Belle-Roche, France) diluted in a buffer containing 10 mM Tris, 150 mM NaCl, pH 7.4, and 0.5% Tween-20 (Merk, Schuchardt, Germany) (Western buffer), and blotted with appropriate dilutions of either horseradish peroxidase-conjugated anti-PY Abs or anti-Fc $\gamma$ RIIB, anti-SHP-1, anti-SHP-2, and anti-SHIP Abs, followed by horseradish peroxidase-conjugated GAR or GAM IgG. Peroxidase-labeled Abs were detected using the Amersham enhanced chemoluminescence kit.

#### Immunoprecipitation and in vitro kinase assay

RBL cells or transfectants sensitized with IgE anti-DNP and 2.4G2 F(ab')<sub>2</sub> were washed three times, resuspended at 10<sup>7</sup> cells/ml, and challenged for 2 min at 37°C with 10  $\mu$ g/ml TNP-MAR F(ab')<sub>2</sub> or 1  $\mu$ g/ml DNP-BSA. Pellets containing 10<sup>7</sup> cells for anti-*lyn* and anti-*syk* immunoprecipitations or 3  $\times$  10<sup>7</sup> cells for anti-Fc $\gamma$  immunoprecipitation were lysed for 10 min at 0°C at 2  $\times$  10<sup>8</sup> cells/ml in TNE buffer containing 50 mM Tris, pH 8, 1% Nonidet P-40, 20 mM EDTA, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM PMSF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Lysates were centrifuged at 10,000 rpm for 10 min at 4°C, and incubated for 1 h at 4°C with protein A-Sepharose coated with the indicated Abs. Immunoabsorbents were washed three times for anti-Fc $\gamma$  immunoprecipitation or seven times for anti-*lyn* and anti-*syk* immunoprecipitations in 1 ml TNE buffer and once in kinase buffer containing 30 mM HEPES, pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, and 0.1 mM Na<sub>3</sub>VO<sub>4</sub>. Immunoabsorbents were incubated in 40  $\mu$ l reaction buffer containing 1  $\mu$ M ATP, 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP or not, and 1  $\mu$ g of the indicated GST fusion proteins for 3 min at 30°C. Reactions were stopped by boiling for 3 min with 20  $\mu$ l 3 $\times$  concentrated reducing sample buffer. Eluted material was subjected to SDS-PAGE and either stained with Coomassie blue and autoradiographed or transferred onto Immobilon-P membranes. Membranes were saturated with 5% BSA diluted in Western buffer and blotted with an appropriate dilution of horseradish peroxidase-conjugated anti-PY Abs. In these experiments, the controls for immunoprecipitation were treated in the same conditions, but were not submitted to in vitro kinase assay (IVKA). Eluted material was run on SDS-PAGE, transferred onto Immobilon-P. Membranes were saturated with 5% skimmed milk diluted in Western buffer and blotted with the appropriate dilutions of anti-*lyn* or anti-*syk* Abs, followed by horseradish peroxidase-conjugated GAM or GAR IgG.

## Results

### The coaggregation of Fc $\gamma$ RIIB with Fc $\epsilon$ RI inhibits IgE-dependent mast cell activation

To analyze the mechanism of Fc $\gamma$ RIIB-mediated inhibition in mast cells, we used the rat RBL-2H3 mastocytoma cells, which express endogenous Fc $\epsilon$ RI and which we stably transfected with cDNA encoding wild-type mouse Fc $\gamma$ RIIB of the three isoforms or Fc $\gamma$ RIIB without IC domain.

In a first set of experiments, transfectants expressing Fc $\gamma$ RIIB were sensitized with mouse IgE and challenged with F(ab')<sub>2</sub> fragments of polyclonal RAM Ig Abs to aggregate Fc $\epsilon$ RI, or with intact RAM IgG to coaggregate Fc $\epsilon$ RI with Fc $\gamma$ RIIB. As expected, RAM F(ab')<sub>2</sub> induced an IgE dose-dependent serotonin release. Serotonin release of a lower magnitude was observed when cells were challenged with RAM IgG (Fig. 1A). Likewise, RAM F(ab')<sub>2</sub> induced a rapid increase of intracellular Ca<sup>2+</sup> concentration that persisted for several minutes. RAM IgG induced a rapid increase of intracellular Ca<sup>2+</sup> with a similar magnitude, but which declined sharply within a few seconds. By contrast, the same sustained Ca<sup>2+</sup> response was observed when RBL transfectants expressing Fc $\gamma$ RIIB whose IC domain was deleted (Fc $\gamma$ RIIB(IC1)) were challenged with RAM F(ab')<sub>2</sub> or IgG. The sustained Ca<sup>2+</sup> response was restored in Fc $\gamma$ RIIB1-expressing transfectants sensitized with mouse IgE, if the IgG binding site of Fc $\gamma$ RIIB1 was blocked with 2.4G2 before challenge with RAM IgG (Fig. 1B). When coaggregated with Fc $\epsilon$ RI, Fc $\gamma$ RIIB can therefore inhibit IgE-induced serotonin release and extracellular Ca<sup>2+</sup> influx.

To analyze early events leading to inhibition of mast cell activation, we devised another system in which each receptor could be ligated separately or coligated. RBL transfectants expressing Fc $\gamma$ RIIB1 were sensitized with murine IgE anti-DNP and incu-

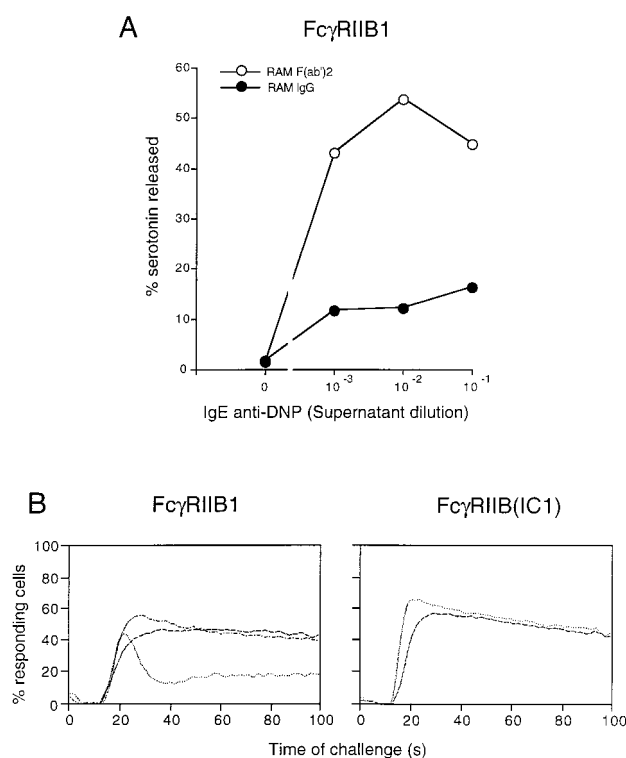


**FIGURE 1.** Inhibition of IgE-mediated mast cell activation by Fc $\gamma$ RIIB induced by the Fc portion of IgG. **A**, Inhibition of serotonin release. Transfectants expressing Fc $\gamma$ RIIB1 were sensitized with dilutions of IgE anti-DNP indicated on abscissa before they were challenged for serotonin release with 30  $\mu$ g/ml RAM F(ab')<sub>2</sub> or 45  $\mu$ g/ml RAM IgG. The figure represents the percentage of serotonin released as a function of the concentration of IgE. **B**, Inhibition of Ca<sup>2+</sup> influx. Transfectants expressing Fc $\gamma$ RIIB1 or Fc $\gamma$ RIIB(IC1) were sensitized with IgE anti-DNP and challenged for Ca<sup>2+</sup> mobilization with 10  $\mu$ g/ml RAM F(ab')<sub>2</sub> (---) or 15  $\mu$ g/ml RAM IgG (····). IgE-sensitized transfectants expressing Fc $\gamma$ RIIB1 were also preincubated with 3  $\mu$ g/ml 2.4G2 F(ab')<sub>2</sub> before challenge with RAM IgG (----). The figure represents the percentage of responding cells as a function of time.

bated or not with 2.4G2 F(ab')<sub>2</sub> before they were challenged with TNP-conjugated F(ab')<sub>2</sub> fragments of polyclonal mouse anti-rat Ig Abs (TNP-MAR F(ab')<sub>2</sub>). This reagent was recognized as a multivalent Ag by Fc $\epsilon$ RI-bound mouse IgE anti-DNP and recognized Fc $\gamma$ RIIB-bound 2.4G2 F(ab')<sub>2</sub>. It therefore aggregated Fc $\epsilon$ RI in cells sensitized with IgE, it aggregated Fc $\gamma$ RIIB in cells incubated with 2.4G2 F(ab')<sub>2</sub>, and it coaggregated Fc $\epsilon$ RI with Fc $\gamma$ RIIB in cells sensitized with IgE and incubated with 2.4G2 F(ab')<sub>2</sub>. Unless otherwise specified, these conditions were used in all experiments and, whenever possible, they are referred to as aggregation of Fc $\epsilon$ RI, aggregation of Fc $\gamma$ RIIB, and coaggregation of Fc $\epsilon$ RI with Fc $\gamma$ RIIB, respectively. We first assessed the kinetics of Fc $\gamma$ RIIB-dependent inhibition of mast cell activation. Serotonin release in response to Fc $\epsilon$ RI aggregation was complete after 10 min of stimulation. The coaggregation of Fc $\gamma$ RIIB1 with Fc $\epsilon$ RI induced an inhibition of serotonin release that was visible as soon as serotonin was detectable and that persisted over a 30-min stimulation period (Fig. 2A). The coaggregation of Fc $\gamma$ RIIB with Fc $\epsilon$ RI, therefore, does not affect the kinetics of mediator release, but induces an early and long-lasting inhibition of mast cell activation. We next compared the inhibition induced by the three isoforms of murine Fc $\gamma$ RIIB, Fc $\gamma$ RIIB1, Fc $\gamma$ RIIB1', or Fc $\gamma$ RIIB2, expressed in RBL cells. Serotonin release induced by Fc $\epsilon$ RI aggregation was inhibited when Fc $\epsilon$ RI were coaggregated with Fc $\gamma$ RIIB1, with

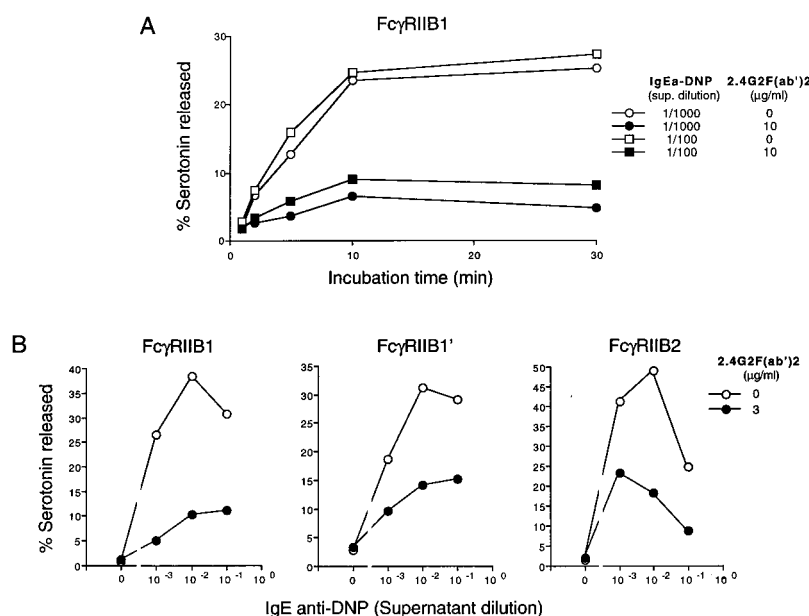


**FIGURE 2.** Inhibition of IgE-dependent mast cell activation by FcγRIIB induced by 2.4G2 F(ab')<sub>2</sub>. **A**, Kinetics of FcγRIIB-induced inhibition of serotonin release. Transfectants expressing FcγRIIB1 were sensitized with IgE anti-DNP, preincubated or not with 2.4G2 F(ab')<sub>2</sub> before they were challenged for serotonin release with 10 μg/ml TNP-MAR F(ab')<sub>2</sub> for indicated periods of time. The figure represents the percentage of serotonin released as a function of time. **B**, Inhibition of serotonin release by the three isoforms of FcγRIIB. Transfectants expressing FcγRIIB1, FcγRIIB1', or FcγRIIB2 were sensitized with dilutions of IgE anti-DNP indicated on abscissa and incubated with or without 2.4G2 F(ab')<sub>2</sub> before they were challenged for serotonin release with 10 μg/ml TNP-MAR F(ab')<sub>2</sub>. The figure represents the percentage of serotonin released as a function of the concentration of IgE.

FcγRIIB1', or with FcγRIIB2 (Fig. 2B). All three isoforms of FcγRIIB can therefore inhibit IgE-mediated mast cell activation.

*The coaggregation of FcγRIIB with FcεRI induces the phosphorylation of FcγRIIB ITIM, enabling the selective recruitment of SHIP by phosphorylated FcγRIIB*

FcγRIIB were found previously to become tyrosine phosphorylated when coaggregated with BCR in B cells (10, 29) or with FcεRI in BMMC (27, 28). To determine conditions required for

FcγRIIB phosphorylation, transfectants expressing FcγRIIB1 were incubated with 2.4G2 F(ab')<sub>2</sub> and sensitized or not with IgE anti-DNP before they were challenged with TNP-MAR F(ab')<sub>2</sub> for various periods of time. FcγRIIB were immunoprecipitated and analyzed by Western blotting with anti-PY and anti-FcγRIIB Abs. The weak phosphorylation of resting (data not shown) or aggregated FcγRIIB1 increased dramatically upon coaggregation with FcεRI (Fig. 3A). The increase of FcγRIIB1 phosphorylation was maximal at 15 s and persisted for several minutes. To identify

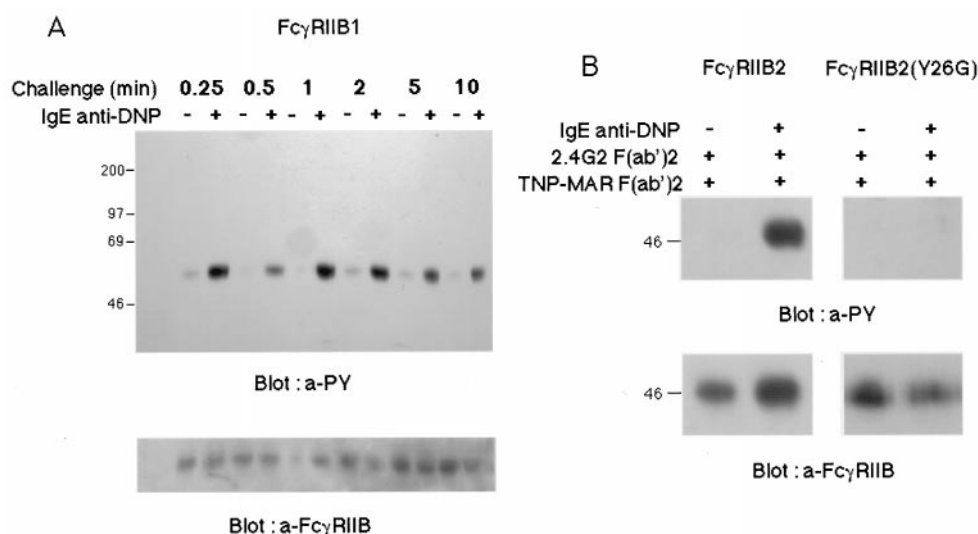


**FIGURE 3.** Phosphorylation of FcγRIIB upon coaggregation with FcεRI. **A**, Kinetics of FcγRIIB phosphorylation. Transfectants expressing FcγRIIB1 were preincubated with 2.4G2 F(ab')<sub>2</sub>, sensitized or not with IgE anti-DNP and challenged with TNP-MAR F(ab')<sub>2</sub> for indicated periods of time. Cells were lysed and FcγRIIB were precipitated with protein G-Sepharose. Immunoprecipitates were run on SDS-PAGE, transferred onto Immobilon, and sequentially blotted with anti-PY and anti-FcγRIIB Abs. **B**, Effect of the mutation of the ITIM tyrosine on the phosphorylation of FcγRIIB2. Transfectants expressing FcγRIIB2 or FcγRIIB2 in which the ITIM tyrosine was replaced by a glycine (FcγRIIB2(Y26G)) were incubated with 2.4G2 F(ab')<sub>2</sub>, sensitized or not with IgE anti-DNP, challenged with TNP-MAR F(ab')<sub>2</sub> for 2 min, and lysed. Lysates were incubated with protein G-Sepharose. Immunoprecipitated material was submitted to SDS-PAGE, transferred onto Immobilon, and sequentially Western blotted with anti-PY and anti-FcγRIIB Abs.

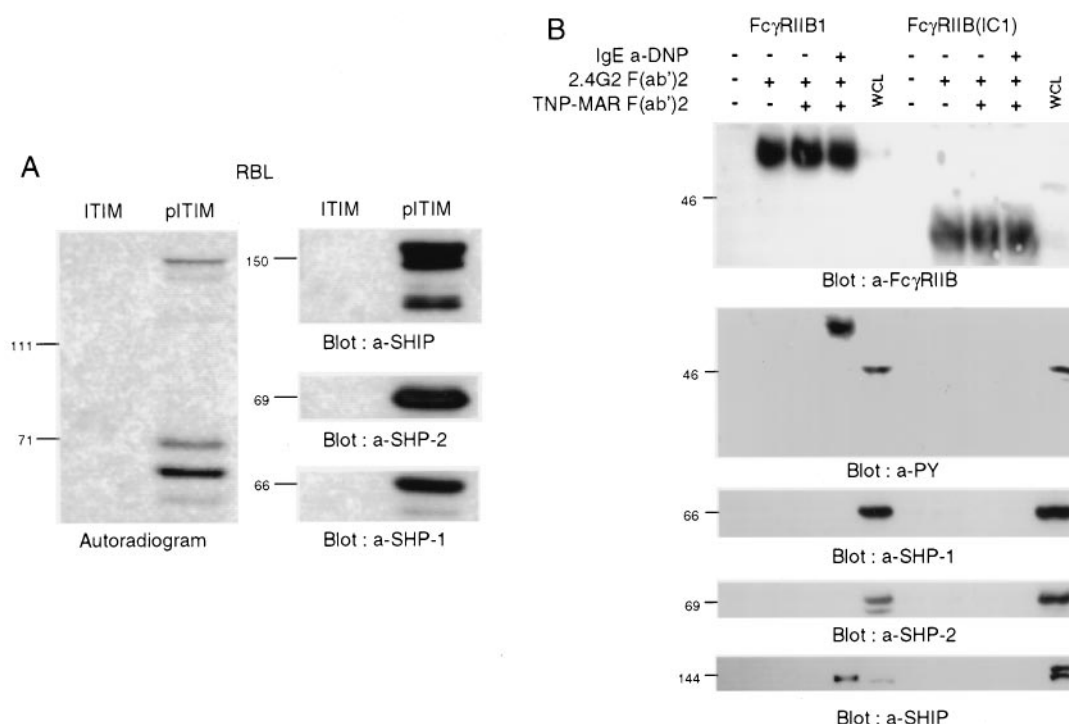


**FIGURE 4.** In vitro and in vivo binding of SHP-1, SHP-2, and SHIP to phosphorylated ITIM peptides or FcγRIIB. **A**, Precipitation of the three phosphatases by phosphorylated ITIM peptides in cell lysates from [<sup>35</sup>S]methionine-labeled RBL cells. Lysates from metabolically labeled RBL cells were incubated with Sepharose coupled to nonphosphorylated or phosphorylated ITIM peptides. Material eluted from immunoadsorbents was submitted to SDS-PAGE and transferred onto Immobilon. *Left panel*, Autoradiogram; *right panel*, Western blot analysis. Blots were incubated sequentially with anti-SHP-2, anti-SHP-1, and anti-SHIP Abs. **B**, Coprecipitation of SHIP with phosphorylated FcγRIIB. Transfectants expressing FcγRIIB1 or IC domain-deleted FcγRIIB (FcγRIIB(IC1)) were sensitized or not with IgE anti-DNP, incubated with or without 2.4G2 F(ab')<sub>2</sub>, challenged or not with TNP-MAR F(ab')<sub>2</sub> for 2 min, and lysed. Lysates were incubated with protein G-Sepharose. Immunoprecipitated material was submitted to SDS-PAGE, transferred onto Immobilon, and Western blotted with anti-FcγRIIB, anti-PY, anti-SHP-1, anti-SHP-2, and anti-SHIP Abs. Whole cell lysates (WCL) were used as positive controls for Western blotting with anti-phosphatase Abs.

FcγRIIB tyrosine residues that become phosphorylated upon coaggregation with FcεRI, we used RBL transfectants expressing FcγRIIB2 bearing a point mutation of the ITIM tyrosine. FcγRIIB1 IC domain contains four tyrosine residues, and FcγRIIB2 IC domain only two. Like FcγRIIB1, FcγRIIB2 became strongly tyrosine phosphorylated upon coaggregation with FcεRI. There was, however, no detectable tyrosine phosphorylation in FcγRIIB2 whose tyrosine residue, in the ITIM (Y26), was replaced by a glycine (Fig. 3B). When coaggregated with FcεRI, FcγRIIB are therefore rapidly phosphorylated, and phosphorylation is abolished when the ITIM tyrosine is mutated.

Phosphorylated ITIM is a potential ligand for molecules containing SH2 domains, and phosphorylated FcγRIIB were shown previously to bind to the SH2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2, and to the inositol polyphosphate 5-phosphatase SHIP. These three phosphatases, however, were differentially recruited in B cells (29, 30) and in BMBC (28), as judged by their ability to coprecipitate with phosphorylated FcγRIIB. To determine whether phospho-ITIM could bind to SHP-1, SHP-2, SHIP, and/or to other SH2-containing molecules in RBL cells, synthetic peptides consisting of the ITIM sequence, phosphorylated or not on the tyrosine residue and coupled to Sepharose beads, were used to precipitate lysates from [<sup>35</sup>S]methionine-labeled RBL cells. As detected by autoradiography, no material was precipitated by nonphosphorylated ITIM peptides. Phosphorylated ITIM peptides precipitated three major proteins, whose apparent m.w. were 150, 69, and 66 kDa. These proteins were iden-

tified, by Western blot, as the various isoforms of SHIP, SHP-2, and SHP-1, respectively (Fig. 4A).

We next examined whether these phosphatases could be coprecipitated with phosphorylated FcγRIIB, after coaggregation with FcεRI. Transfectants expressing FcγRIIB1 were incubated with or without 2.4G2 F(ab')<sub>2</sub> and sensitized, or not, with mouse IgE anti-DNP, before they were challenged with TNP-MAR F(ab')<sub>2</sub> for 2 min. FcγRIIB immunoprecipitates were analyzed by Western blotting with anti-FcγRIIB, anti-PY, anti-SHP-1, anti-SHP-2, and anti-SHIP Abs. FcγRIIB1 was not phosphorylated when aggregated, but became strongly tyrosine phosphorylated when coaggregated with FcεRI. Neither SHP-1 nor SHP-2 was coprecipitated with phosphorylated FcγRIIB1. However, SHIP was coprecipitated with phosphorylated FcγRIIB1 (Fig. 4B). It was reported recently that SHIP can bind several cytoplasmic molecules involved in cell activation via FcεRI (40). To determine whether SHIP seen in FcεRI-coaggregated FcγRIIB immunoprecipitates was associated with FcγRIIB, RBL transfectants expressing FcγRIIB whose IC domain was deleted were used under the same conditions in the same experiment. No SHP-1 and no SHP-2, but also no SHIP, were coprecipitated with IC domain-deleted FcγRIIB (Fig. 4B). SHIP is therefore selectively recruited by phosphorylated FcγRIIB, and recruitment requires the IC domain of FcγRIIB.

These results altogether indicate that the coaggregation of FcγRIIB with FcεRI induces the phosphorylation of FcγRIIB on the ITIM tyrosine and this phosphorylation leads to the selective recruitment of SHIP.

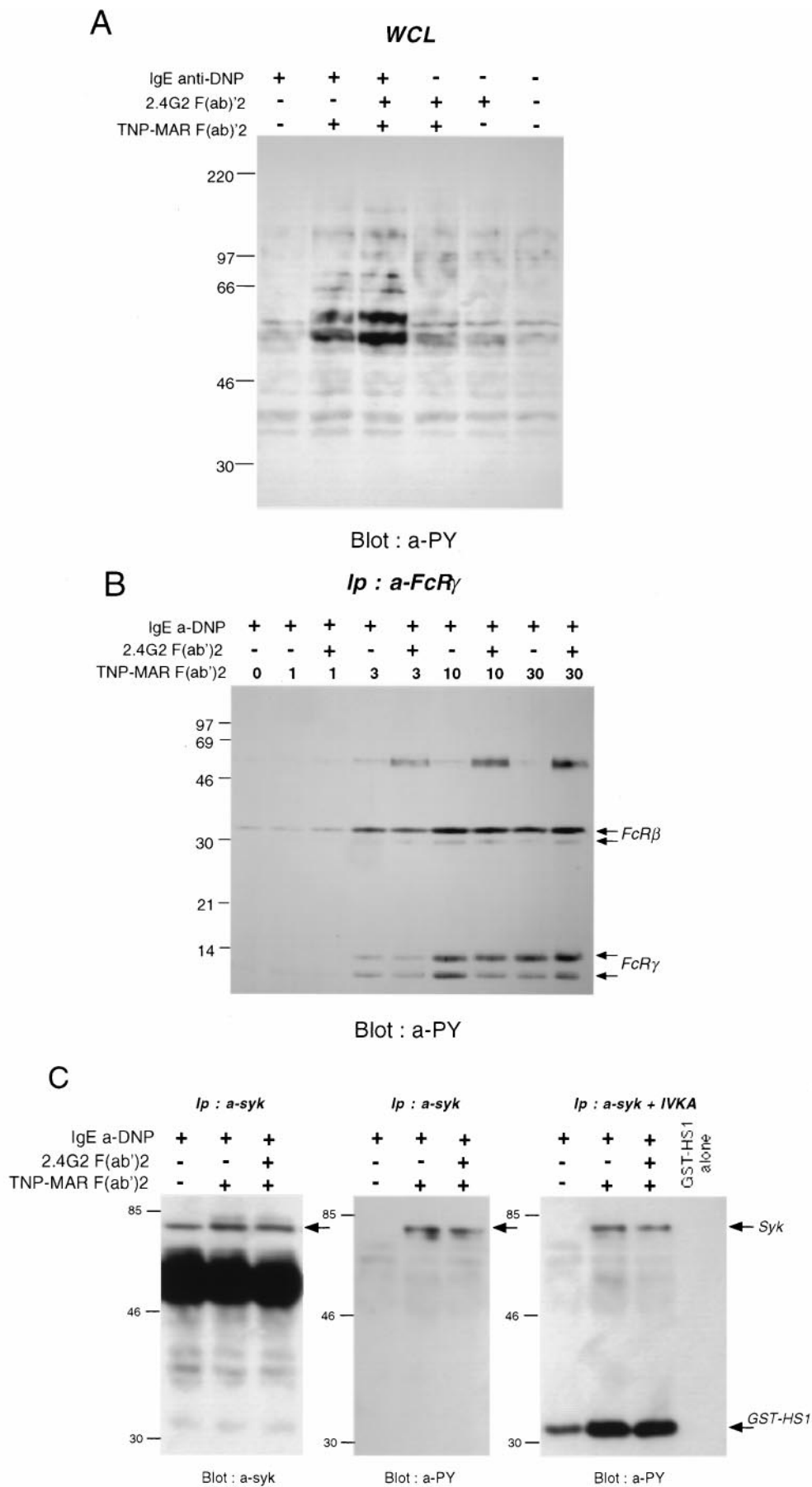


**FIGURE 5.** Phosphorylation events induced by FcεRI, upon coaggregation with FcγRIIB. A, Tyrosine phosphorylation of intracellular proteins. Transfectants expressing FcγRIIB1 were sensitized or not with IgE anti-DNP, incubated with or without 2.4G2 F(ab')<sub>2</sub>, and challenged or not with TNP-MAR

*The coaggregation of FcεRI with FcγRIIB does not affect the phosphorylation of FcεRI ITAMs and the activation of syk*

To assess the effects of FcγRIIB on signals transduced by FcεRI, we compared the pattern of tyrosine phosphorylation in whole cell lysates after aggregation of FcγRIIB, aggregation of FcεRI, or coaggregation of the two receptors in transfectants expressing FcγRIIB1. No tyrosine phosphorylation was induced after FcγRIIB1 aggregation, whereas FcεRI aggregation induced the tyrosine phosphorylation of a number of intracellular proteins. A comparable pattern of phosphorylation was induced when FcεRI were coaggregated with FcγRIIB (Fig. 5A). These results indicate that FcγRIIB neither induce phosphorylation, when aggregated, nor inhibit IgE-induced phosphorylation, when coaggregated with FcεRI.

To analyze the first steps of signal transduction, we next examined whether coaggregation of FcεRI with FcγRIIB would affect the tyrosine phosphorylation of ITAMs in the FcRγ and FcRβ subunits of FcεRI. RBL transfectants expressing FcγRIIB1 were sensitized with mouse IgE anti-DNP, incubated or not with 2.4G2 F(ab')<sub>2</sub>, and challenged with increasing concentrations of TNP-MAR F(ab')<sub>2</sub>. FcεRI complexes were immunoprecipitated with anti-FcRγ Abs and analyzed by Western blotting with anti-PY Abs. The aggregation of FcεRI induced a TNP-MAR F(ab')<sub>2</sub> dose-dependent tyrosine phosphorylation of FcRβ and FcRγ. Neither phosphorylation of FcRβ nor that of FcRγ was reduced significantly upon coaggregation of FcεRI with FcγRIIB (Fig. 5B). ITAM phosphorylation was therefore not prevented by coaggregation, suggesting that the activity of PTK activated following FcεRI aggregation was not affected by coaggregation.

To analyze the next steps of signal transduction, we examined the phosphorylation of *lyn* and *syk* and their kinase activities. Transfectants expressing FcγRIIB1 were sensitized with mouse IgE anti-DNP and incubated or not with 2.4G2 F(ab')<sub>2</sub> before they were challenged or not with TNP-MAR F(ab')<sub>2</sub> for 2 min. *lyn* and *syk* were immunoprecipitated with anti-*lyn* or anti-*syk* Abs, and immunoprecipitates were submitted or not to an IVKA in the presence of ATP and, as exogenous substrates, GST-p62 and GST-HS1, respectively. GST-HS1 was phosphorylated by *syk*, but not by *lyn*, whereas GST-p62 was phosphorylated by both kinases (data not shown). Reaction mixtures were analyzed by Western blotting with anti-PY, anti-*lyn*, or anti-*syk* Abs. As previously reported (41, 42), *lyn* was phosphorylated constitutively in anti-*lyn* immunoprecipitates, and *lyn* phosphorylation was neither significantly increased upon FcεRI aggregation nor significantly decreased upon coaggregation of FcεRI with FcγRIIB. Likewise, the in vitro kinase activity of *lyn* on GST-p62 neither increased after FcεRI aggregation nor decreased after FcεRI-FcγRIIB coaggregation (data not shown). Therefore, no conclusion can be drawn regarding inhibition of *lyn* activation by FcγRIIB. By contrast, no tyrosine phosphorylation of *syk* was seen in nonstimulated cells, but *syk* phosphorylation was induced after the aggregation of

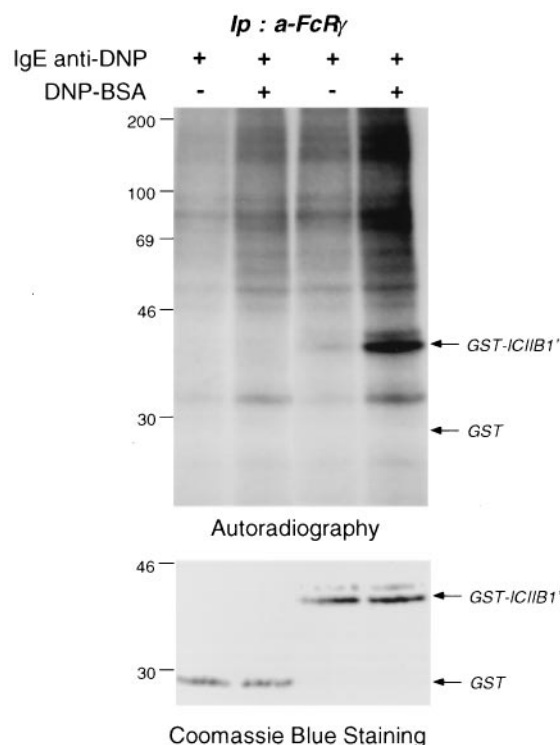


**FIGURE 6.** In vitro phosphorylation of GST-ICIB1' by FcεRI-associated kinases. RBL cells were sensitized with IgE anti-DNP, challenged or not with DNP-BSA for 2 min, and lysed. Lysates were incubated with protein A-Sepharose coated with anti-FcRγ Abs. Immunoprecipitates were submitted to an IVKA in the presence of GST or GST-ICIB1' and [ $\gamma$ -<sup>32</sup>P]ATP. Reaction mixtures were fractionated by SDS-PAGE, and proteins were visualized by Coomassie blue staining and by autoradiography.

FcεRI. *syk* phosphorylation was also observed when FcεRI were coaggregated with FcγRIIB, and this phosphorylation, although appearing slightly reduced in this experiment, was not reproducibly decreased (Fig. 5C). GST-HS1 was in vitro phosphorylated by *syk* immunoprecipitated from nonstimulated cells. The aggregation of FcεRI strongly increased the in vitro phosphorylation of GST-HS1. A comparable in vitro phosphorylation of GST-HS1 was mediated by *syk* immunoprecipitated from transfectants whose FcεRI were coaggregated with FcγRIIB1 (Fig. 5C). The coaggregation of FcγRIIB with FcεRI therefore does not detectably affect either the phosphorylation or the kinase activity of *syk* induced upon FcεRI aggregation by IgE and Ag.

Taken together, these results indicate that FcγRIIB-mediated inhibition does not prevent FcεRI-mediated transduction of signals leading to receptor phosphorylation and *syk* activation.

F(ab')<sub>2</sub> for 5 min. Cells were lysed with SDS and proteins were precipitated with cold acetone. Whole cell lysates were submitted to SDS-PAGE, transferred onto Immobilon, and Western blotted with anti-PY Abs. **B.** Tyrosine phosphorylation of FcεRI ITAMs. Transfectants expressing FcγRIIB1 were sensitized with IgE anti-DNP, incubated or not with 2.4G2 F(ab')<sub>2</sub>, challenged with the indicated concentrations (in μg/ml) of TNP-MAR F(ab')<sub>2</sub> for 2 min, and lysed. Lysates were incubated with protein A-Sepharose coated with anti-FcRγ Abs. Immunoprecipitates were submitted to SDS-PAGE, transferred onto Immobilon, and Western blotted with anti-PY Abs. **C.** Tyrosine phosphorylation and activation of *syk*. Transfectants expressing FcγRIIB1 were sensitized with IgE anti-DNP, incubated with or without 2.4G2 F(ab')<sub>2</sub>, challenged or not with TNP-MAR F(ab')<sub>2</sub> for 2 min, and lysed. Lysates were incubated with protein A-Sepharose coated with anti-*syk* Abs. Immunoprecipitates were separated into three parts. One part was used for Western blot with anti-*syk* Abs, one part was used for Western blot with anti-PY Abs, and one part was submitted to an IVKA in presence of 1 μg GST-HS1 for 3 min. All samples were run on SDS-PAGE, transferred onto Immobilon, and Western blotted as indicated. One microgram of GST-HS1, not incubated with immunoprecipitates, was used as a negative control for the IVKA.

*The coaggregation of FcγRIIB with FcεRI enables FcεRI-associated lyn to phosphorylate FcγRIIB*

Since FcγRIIB: 1) does not become phosphorylated when aggregated, but only when coaggregated with FcεRI, and 2) does not prevent FcεRI-associated kinases from being activated when coaggregated with FcεRI, we hypothesized that FcγRIIB might be a substrate for FcεRI-associated PTK.

To test this hypothesis, we made a GST fusion protein consisting of the IC domain of FcγRIIB1' (GST-ICIIB1'), which was used as an exogenous substrate for FcεRI-associated kinases in IVKA. Nontransfected RBL cells were sensitized with mouse IgE anti-DNP and challenged or not with DNP-BSA for 2 min. FcεRI complexes were precipitated with anti-FcRγ Abs and submitted to an IVKA in the presence of [ $\gamma$ - $^{32}$ P]ATP and GST-ICIIB1' or, as a negative control, GST. Reaction mixtures were fractionated by SDS-PAGE and analyzed by Coomassie blue staining and by autoradiography. The aggregation of FcεRI increased the in vitro phosphorylation of a number of proteins that coprecipitated with FcεRI, including a protein with the same apparent m.w. as FcRβ. GST-ICIIB1' was weakly phosphorylated when added to FcεRI complexes precipitated from nonstimulated cells and heavily phosphorylated when added to FcεRI complexes precipitated from stimulated cells. Under the same conditions, GST was not phosphorylated (Fig. 6). Active kinase(s) that associates with FcεRI complexes following receptor aggregation can therefore phosphorylate the IC domain of FcγRIIB in vitro.

*lyn* and *syk* are among the PTKs that are activated upon FcεRI aggregation (41, 42). Indeed, when cell lysates from RBL sensitized with IgE and challenged with Ag were immunoprecipitated with anti-FcRγ and analyzed by IVKA on GST-p62 and GST-HS1 used as exogenous substrates for *lyn* and *syk*, respectively, both substrates were phosphorylated (data not shown). To investigate whether *lyn* and/or *syk* may phosphorylate GST-ICIIB1' in vitro, RBL cells were sensitized with IgE anti-DNP and challenged with DNP-BSA for 2 min. *lyn* and *syk* were immunoprecipitated and submitted to IVKA with GST-ICIIB1' as an exogenous substrate. GST-p62 and GST-HS1 were used as positive control substrates for *lyn* and *syk*, respectively. Reaction mixtures were analyzed by Western blotting with anti-PY, anti-*lyn*, and anti-*syk* Abs. Equivalent in vitro phosphorylation of GST-ICIIB1' was observed when GST-ICIIB1' was added to anti-*lyn* and anti-*syk* immunoprecipitates (Fig. 7). Both *lyn* and *syk* can therefore phosphorylate GST-ICIIB1' in vitro.

To evaluate the respective roles of the two kinases in vivo, we examined the phosphorylation of FcγRIIB, when coaggregated with FcεRI in mast cells derived from *syk*-deficient or *lyn*-deficient mice. Mast cells derived from both knockout mice expressed similar amounts of FcεRI and FcγRIIB as mast cells derived from wild-type mice, as assessed by indirect immunofluorescence (data not shown). As expected, no *syk* was detected in *syk*-deficient mast cells (Fig. 8A), and no *lyn* was detected in *lyn*-deficient mast cells (Fig. 8B), as assessed by Western blotting in whole cell lysates. To analyze kinase activities present in *syk*-deficient and *lyn*-deficient mast cells, cells were sensitized with mouse IgE and challenged with RAM F(ab')<sub>2</sub>. *syk* and *lyn* were immunoprecipitated and submitted to IVKA in the presence of GST-HS1 or GST-p62, respectively. Reaction mixtures were analyzed by Western blotting with anti-PY Abs. The same phosphorylation of GST-p62 was observed when added to anti-*lyn* immunoprecipitates from wild-type and *syk*-deficient mast cells, whereas GST-HS1 was phosphorylated when added to anti-*syk* immunoprecipitates from wild-type mast cells, but not from *syk*-deficient mast cells (Fig. 8A). The same phosphorylation of GST-HS1 was observed when added to anti-

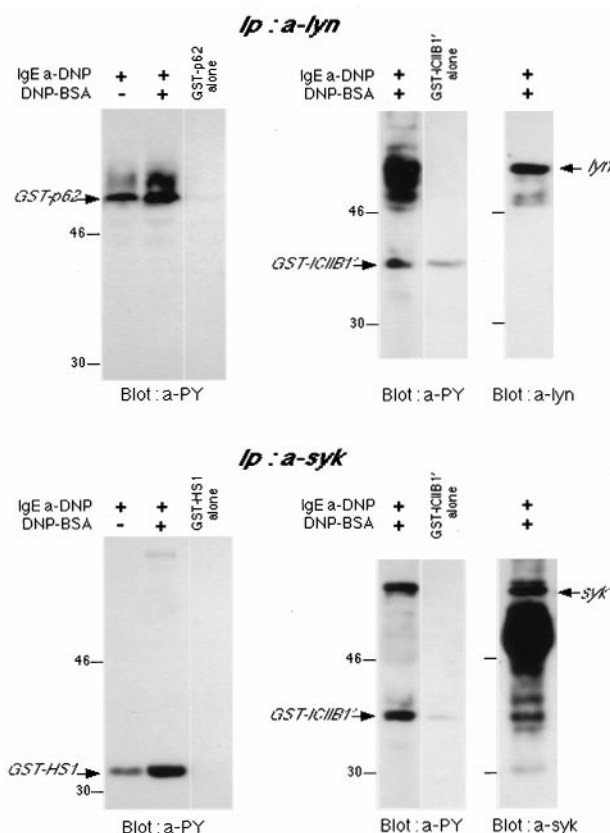


**FIGURE 7.** In vitro phosphorylation of GST-ICIIB1' by *lyn* and *syk*. RBL cells were sensitized with IgE anti-DNP, challenged or not with DNP-BSA, and lysed. Lysates were incubated with protein A-Sepharose coated with anti-*lyn* or anti-*syk* Abs. Immunoprecipitates were submitted or not to IVKA in the presence of GST-ICIIB1', GST-p62, or GST-HS1. Samples were run on SDS-PAGE, transferred onto Immobilon, and Western blotted with anti-PY or anti-kinase Abs. GST fusion proteins, not incubated with immunoprecipitates, were used as negative controls for IVKA.

*syk* immunoprecipitates from wild-type and *lyn*-deficient mast cells, whereas GST-p62 was phosphorylated when added to anti-*lyn* immunoprecipitates from wild-type mast cells, but not from *lyn*-deficient mast cells (Fig. 8B). To study the in vivo phosphorylation of FcγRIIB, *syk*-deficient and *lyn*-deficient mast cells were sensitized with mouse IgE and challenged or not with RAM F(ab')<sub>2</sub> or RAM IgG. FcγRIIB were immunoprecipitated and analyzed by Western blotting with anti-PY and anti-FcγRIIB Abs. FcγRIIB phosphorylation was seen in immunoprecipitates from wild-type and *syk*-deficient mast cells, and the level of phosphorylation was correlated with the amount of immunoprecipitated FcγRIIB (Fig. 8A). FcγRIIB phosphorylation was seen in immunoprecipitates from wild-type mast cells, but not in immunoprecipitates from *lyn*-deficient mast cells, although comparable amounts of FcγRIIB were precipitated from wild-type and *lyn*-deficient mast cells (Fig. 8B). The phosphorylation of FcγRIIB was therefore not impaired in *syk*-deficient mast cells, but completely inhibited in *lyn*-deficient mast cells.

## Discussion

In the present work, we examined the consequences and the mechanism of the phosphorylation of murine FcγRIIB, following coaggregation with FcεRI. We found that: 1) phosphorylated FcγRIIB selectively recruit the inositol polyphosphate 5 phosphatase, SHIP, in vivo; 2) the phosphorylation of FcγRIIB requires the

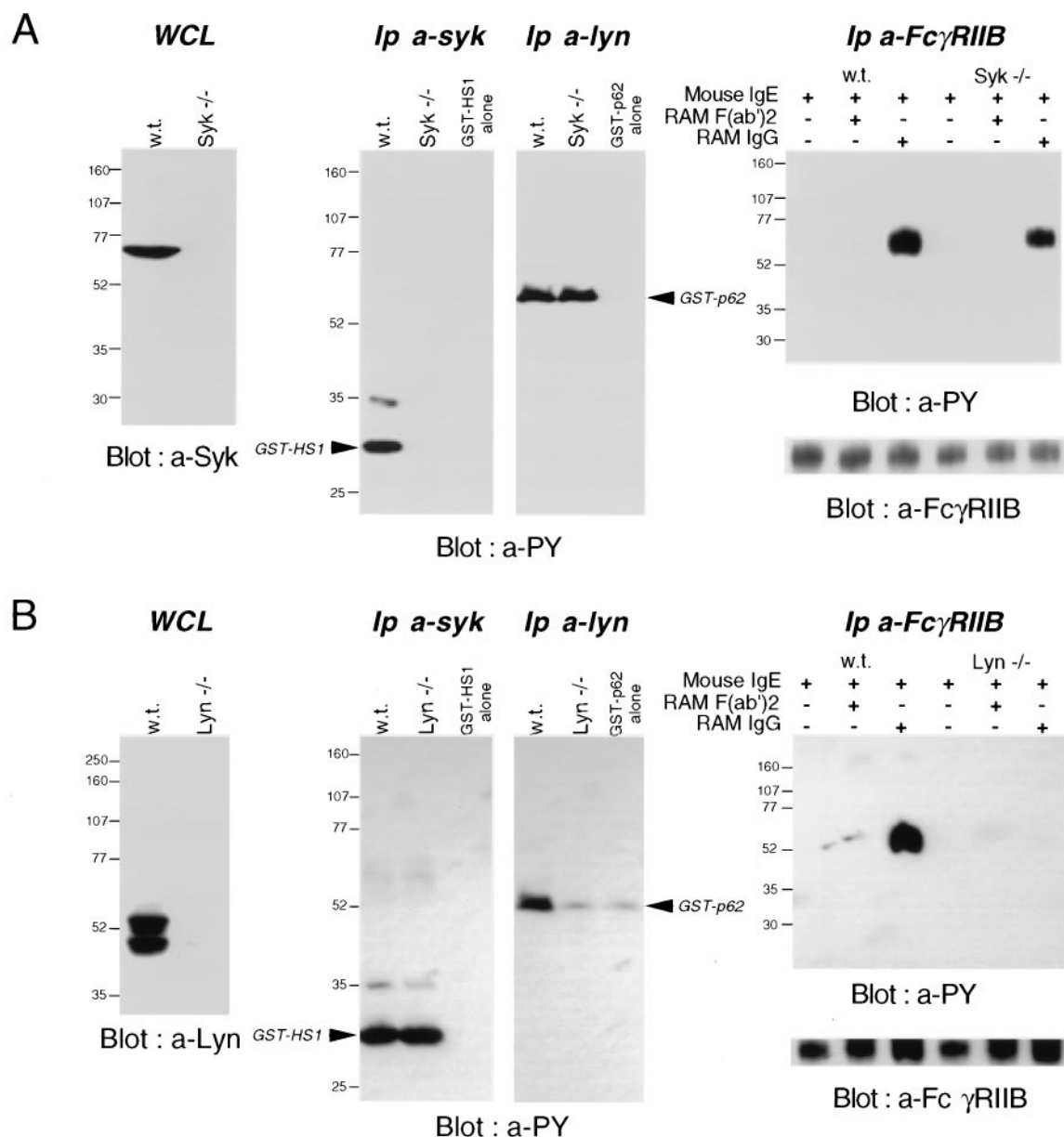


**FIGURE 8.** In vivo phosphorylation of FcγRIIB in syk-deficient (A) and lyn-deficient (B) mast cells. *Left panel*, Expression of syk or lyn. Whole cell lysates from wild-type and syk<sup>-/-</sup> mast cells (A) or from wild-type and lyn<sup>-/-</sup> mast cells (B) were run on SDS-PAGE, transferred onto Immobilon, and Western blotted with anti-syk (A) or anti-lyn (B) Abs. *Central panels*, Kinase activities of syk and lyn. Wild-type and syk<sup>-/-</sup> mast cells (A) or wild-type and lyn<sup>-/-</sup> mast cells (B) were sensitized with mouse IgE and challenged with RAM F(ab')<sub>2</sub> for 2 min. syk and lyn were immunoprecipitated with anti-syk or anti-lyn Abs bound to protein A-Sepharose. Immunoprecipitates were submitted to IVKA in the presence of GST-HS1 or GST-p62. Samples were run on SDS-PAGE, transferred onto Immobilon, and Western blotted with anti-PY Abs. *Right panel*, Phosphorylation of FcγRIIB. Mast cells from wild-type and syk<sup>-/-</sup> mice (A), or mast cells from wild-type and lyn<sup>-/-</sup> mice (B) were sensitized with mouse IgE and challenged or not with RAM F(ab')<sub>2</sub> or RAM IgG for 2 min. FcγRIIB were immunoprecipitated with 2.4G2 bound to protein G-Sepharose. Immunoprecipitates were run on SDS-PAGE, transferred onto Immobilon, and Western blotted sequentially with anti-PY and with anti-FcγRIIB Abs.

coaggregation with FcεRI; 3) the coaggregation of FcγRIIB with FcεRI does not prevent FcεRI from transducing signals leading to FcεRI phosphorylation and syk activation; 4) when activated by the aggregation of FcεRI, lyn and syk can both phosphorylate FcγRIIB in vitro; but 5) lyn, but not syk, is required for in vivo phosphorylation of FcγRIIB.

To study the mechanism of FcγRIIB-mediated inhibition of IgE-induced mast cell activation, we devised an experimental model in which FcεRI and murine recombinant FcγRIIB, expressed in RBL-2H3 cells, could be independently aggregated, or coaggregated at the cell surface. Using this model, we confirmed

that the three mouse FcγRIIB isoforms, which all contain an ITIM, are able to inhibit IgE-induced serotonin release. Inhibition was observed as soon as serotonin release was detectable, and it did not result from a delay in secretion.

An early event observed when coaggregating FcεRI with FcγRIIB was the tyrosine phosphorylation of FcγRIIB. FcγRIIB phosphorylation occurred within 15 s, i.e., as early as that of the ITAM-containing FcεRI subunits (43). FcγRIIB phosphorylation required the conservation of the ITIM tyrosine. This result does not formally prove that phosphorylation occurs on the ITIM tyrosine since FcγRIIB2 contains two tyrosines. It nevertheless indicates

that this residue is mandatory for FcγRIIB phosphorylation. Supporting the assumption that the ITIM tyrosine is indeed phosphorylated, FcγRIIB was found to recruit SH2 domain-bearing phosphatases (27–30). We observed that, when incubated with lysates from [<sup>35</sup>S]methionine-labeled RBL cells, synthetic peptides corresponding to FcγRIIB phospho-ITIM bound in vitro to the same three phosphatases as when incubated with B cell (29) or BMMC (27, 28) lysates. These were two tyrosine phosphatases, SHP-1 and SHP-2, and the inositol polyphosphate 5 phosphatase, SHIP. Remarkably, only SHIP was coprecipitated with phosphorylated FcγRIIB in RBL cells. The same was observed in BMMC (28). The structural bases and biologic significance of selective recruitment remain to be established. Supporting a selective in vivo recruitment of SHIP in mast cells, FcγRIIB were found to inhibit IgE-mediated activation in mast cells derived from bone marrow of the SHP-1-deficient motheaten mice (27, 28), indicating that SHP-1 is not required for inhibition of serotonin release. Whether SHIP is directly responsible for FcγRIIB-mediated inhibition of mast cell activation remains to be demonstrated. The coprecipitation of SHIP with phosphorylated FcγRIIB is simply correlated with inhibition of secretion. We observed that the coaggregation of FcεRI with FcγRIIB in RBL cells inhibits the late Ca<sup>2+</sup> flux, without affecting the early phase of Ca<sup>2+</sup> mobilization. The same was observed in B cells (44) and in BMMC (28). How SHIP can possibly block the influx of extracellular Ca<sup>2+</sup> is unclear. SHIP, indeed, dephosphorylates 5'-phosphate groups in inositol (1, 3, 4, 5)-tetraphosphate and in phosphatidylinositol (3, 4, 5)-trisphosphate (45). These substrates are not known to be directly involved in the opening of Ca<sup>2+</sup> channels. SHIP can however interfere with the phospholipid metabolism: phosphatidylinositol (3, 4, 5)-trisphosphate was suggested to enhance the translocation of PLCγ-2 to the plasma membrane (46), where it hydrolyzes phosphatidylinositol (4, 5)-biphosphate into inositol (1, 4, 5)-trisphosphate. SHIP may thus ultimately decrease the sustained production of inositol (1, 4, 5)-trisphosphate, which is needed for generating the signals leading to the entry of extracellular Ca<sup>2+</sup> (47), as it was observed in B cells (48). Alternatively and not exclusively, SHIP can bind, directly or indirectly, to other molecules implicated in signal transduction, such as *shc* (49) or SHP-2 (50), respectively. Finally, one cannot exclude that not yet identified molecules may contribute to FcγRIIB-mediated inhibition.

Compatible with our finding that no tyrosine phosphatase is recruited by phosphorylated FcγRIIB in mast cells, we observed that the coaggregation with FcγRIIB did not prevent FcεRI from activating PTK. The pattern of phosphorylation of intracellular substrates was not affected by coaggregation. The phosphorylation of FcRβ and FcRγ ITAMs was not inhibited, and the activation of *syk* was not decreased, as judged by its in vivo phosphorylation and by its in vitro kinase activity on a *syk*-specific substrate. This indicates that the initial steps of signal transduction following FcεRI aggregation are not impaired by FcγRIIB, and that inhibition acts on second messengers rather than on early phosphorylation events.

FcγRIIB must be phosphorylated to inhibit mast cell activation: the point mutation of the ITIM tyrosine was sufficient to abrogate inhibition of mediator release (13), and this mutation prevented FcγRIIB phosphorylation. Inhibition of serotonin release required that FcεRI be coaggregated with FcγRIIB (14), and FcγRIIB became tyrosine phosphorylated only when coaggregated with FcεRI. This suggests that FcγRIIB may not be constitutively associated with a tyrosine kinase. Supporting this hypothesis, FcγRIIB aggregation induced no phosphorylation of intracellular substrates. We therefore hypothesized that FcγRIIB phosphorylation depends on FcεRI-associated kinases that become activated and/or recruited in the receptor complex. We provide evidence

supporting this possibility. PTK that were coprecipitated with activated FcεRI could phosphorylate in vitro a GST fusion protein consisting of the FcγRIIB IC domain. *lyn* and *syk* were shown previously to coprecipitate with aggregated FcεRI under these conditions (41, 42), and we found in vitro kinase activities on both *lyn* and *syk* substrates in immunoprecipitates of activated FcεRI (data not shown). When immunoprecipitated from RBL cells sensitized with IgE and challenged with Ag, both *lyn* and *syk* phosphorylated GST-FcγRIIB IC in vitro. To validate these in vitro data, we examined whether FcγRIIB could be phosphorylated in vivo when coaggregated with FcεRI in mast cells derived from *syk*-deficient mice or *lyn*-deficient mice. We found that FcγRIIB became phosphorylated in *syk*-deficient mast cells. This indicates that *syk* is not mandatory for FcγRIIB phosphorylation. By contrast, FcγRIIB phosphorylation was abrogated in *lyn*-deficient mast cells, although the kinase activity of *syk* was conserved in these cells. This indicates that *lyn* is required for FcγRIIB phosphorylation in vivo, and that it cannot be replaced by another kinase. Whether *lyn* directly phosphorylates FcγRIIB or whether it activates another kinase that phosphorylates FcγRIIB cannot be determined. Our data altogether suggest that, upon coaggregation of the two receptors, *lyn* is normally activated and brought by FcεRI in the vicinity of the FcγRIIB ITIM, enabling FcγRIIB to be phosphorylated, to recruit SHIP and, ultimately, to inhibit mast cell responses. Such a mechanism seems to be unique, in view of previous studies on FcγRIIB-mediated inhibition of B cell activation and on inhibition mediated by other ITIM-bearing negative coreceptors such as human KIRs.

In B cells, phosphorylated FcγRIIB were reported to recruit SHP-1, and FcγRIIB-mediated inhibition of cell activation was abrogated in B cells from motheaten mice (29). CD19 was proposed to be a potential substrate of SHP-1, as its phosphorylation was inhibited upon coaggregation of BCR with FcγRIIB. As a consequence, the association of CD19 with phosphatidylinositol 3-kinase seen upon BCR aggregation was prevented (51, 52). No equivalent of CD19 is known in mast cells. Neither the phosphorylation pattern of intracellular substrates nor the phosphorylation of specific proteins involved in signal transduction, including Igα, Igβ, *lyn*, *syk*, and PLC-γ, was affected (51, 52) upon coaggregation of BCR with FcγRIIB. Kinases associated with BCR might therefore phosphorylate FcγRIIB in B cells, like we found for kinases associated with FcεRI in mast cells, as suggested by the recent observation that FcγRIIB-mediated inhibition is impaired in B cells from *lyn*<sup>-/-</sup> mice (53). Finally, the apparent discrepancy between studies made in B cells and in mast cells may be reconciled by the recent finding that phosphorylated FcγRIIB could recruit SHIP also in B cells (30, 31). By contrast with FcγRIIB, phosphorylated KIR ITIM peptides were shown to bind in vitro to SHP-1 (54) and SHP-2, but not to SHIP (55) and, when coaggregated with FcεRI in RBL cells (56), p58 KIRs recruited SHP-1, whereas FcγRIIB recruited SHIP (our unpublished results). The recruitment of tyrosine phosphatases by phosphorylated KIRs was correlated with the dephosphorylation of ζ, ZAP-70, and PLCγ-1/2 in NK cells (57).

In conclusion, this study provides evidence that FcεRI are involved in FcγRIIB-mediated inhibition of IgE-dependent mast cell activation. FcγRIIB (14) and KIRs (56) both must be coaggregated with ITAM-bearing receptors to inhibit cell activation. The biologic significance of coaggregation seems, however, to be different for the two ITIM-bearing receptors. In KIR-mediated inhibition, coaggregation makes phosphorylated tyrosines of cell-activating receptors and/or kinases the substrates of protein tyrosine phosphatases recruited by phosphorylated KIRs. In FcγRIIB-mediated

inhibition, coaggregation makes the ITIM a substrate for Fc $\epsilon$ RI-associated *lyn*, enabling Fc $\gamma$ RIIB to recruit SHIP. As a consequence, cell-activating receptors may be passive targets in KIR-mediated inhibition, whereas they actively participate to their own inhibition, by enabling ITIM phosphorylation, in Fc $\gamma$ RIIB-mediated inhibition.

## Acknowledgments

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