The high affinity IgE Fc receptor (FcεRI) β chain functions as a signal amplifier and has been linked to atopy, asthma, and allergy. Herein, we report on a previously unrecognized negative regulatory role for the nonconventional β chain immunoreceptor tyrosine-based activation motif that contains three tyrosine residues (YXXY). Degranulation and leukotriene production was found to be impaired in cells expressing the mutated FcεRIβ immunoreceptor tyrosine-based activation motifs FYY, YYF, YFY, and FFF. In contrast, cytokine synthesis and secretion were enhanced in the FYF and FFF mutants. FcεRI phosphorylation and Lyn kinase co-immunoprecipitation was intact in the FYF mutant but was lost in the FYF and FFF mutants. The phosphorylation of Syk, LAT, phospholipase Cγ1/2, and Src-homology 2 domain-containing protein phosphatase 2 was intact, whereas the phosphorylation of SHIP-1 was significantly reduced in the FYF mutant cells. The FYF and FFF mutants were defective in phosphorylating all of these molecules. In contrast, the phosphorylation of ERK, p38 MAPK, IκB kinase β (IKKβ), and nuclear NFκB activity was enhanced in the FYF and FFF mutants. These findings show that the FcεRIβ functions to both selectively amplify (degranulation and leukotriene secretion) and dampen (lymphokine) mast cell effector responses.

The FcεRIβ in mast cells and basophils is a tetrameric structure consisting of three distinct polypeptides including the IgE-binding α, the tetransparent β, and the disulfide-linked γ homodimer (1). Aggregation of FcεRI by the interaction of bound IgE with multivalent antigens induces the release of histamine, leukotrienes, and inflammatory cytokines, resulting in the recruitment and activation of circulating leukocytes leading to allergic inflammation (2). Polymorphisms in the FcεRIβ have been linked to atopy, asthma, and allergy (3–5). However, studies on the role of these polymorphisms in FcεRI expression and function have not yielded an understanding of the effects on mast cell physiology (6, 7). Nonetheless, it is clear that the FcεRIβ functions as a signal amplifier in mast cells and is important for augmenting the allergic reactions (8–10). In humans, it has been demonstrated that the γ homodimer can act as an autonomous signaling molecule in various cell types, whereas the β subunit enhances early activation signals by 5–7-fold through FcεRI (reviewed in Ref. 1). Moreover, the β chain also enhances cell-surface expression of FcεRI (11), providing increased sensitivity in an allergic response.

The β- and γ-chains contain ITAMs, a conserved feature of many antigen receptors that imparts signaling competence. The ITAM consensus sequence is (D/E)XXYXXLXxxYXXL(I/1), where the tyrosine residues are phosphoacceptor sites for the action of receptor-associated protein tyrosine kinases (reviewed in Ref. 1). Phospho-ITAMs provide a docking site for cytoplasmic proteins that contain the Src-homology 2 domain (SH2). Some of these function to couple receptors to molecular signaling complexes anchored by adaptor proteins (12), which serve to coordinate and amplify intracellular signals. The structure of the β and γ chain ITAMs differ. In particular, the β chain ITAM shows a notable departure from the consensus ITAM sequence with the presence of a third tyrosine (the middle tyrosine) between the two canonical tyrosine residues and a shorter spacer region between the canonical tyrosines. The Src family protein tyrosine kinase Lyn weakly binds to β chain ITAM in resting cells and is further recruited after the receptor aggregation (13–15). Lyn is required for the initial step of activation by phosphorylating FcεRI specifically on the β and γ ITAMs (16, 17). Although the requirements for this initial event are not fully understood, it appears that the protein-protein interaction of Lyn with FcεRI and an appropriate lipid microenvironment, where this interaction may be enhanced, are probably prerequisites (18–20). Recently, we described that another Src family protein tyrosine kinase Fyn was also activated by and co-immunoprecipitated with the FcεRI (21). This kinase initiates a complementary signaling pathway that is anchored by the adapter molecule Gab2 (Grb2-associated binder protein 2) whose phosphorylation is critical for activation of phosphatidylinositol 3-OH-kinase.

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1 The abbreviations used are: FcεRI, high affinity IgE Fc receptor; ITAM, immunoreceptor tyrosine-based activation motif; BMMC, bone marrow-derived mast cell; DNP, 2,4-dinitrophenol; ERK, extracellular signal-regulated kinase; LAT, linker for activation of T-cells; IKK, IκB kinase; Ab, antibody; IL, interleukin; MAPK, mitogen-activated protein kinase; PLCγ, phospholipase Cγ; PI3K, phosphatidylinositol 3-OH kinase; SH2, Src-homology 2; SHP-2, SH2 domain-containing protein tyrosine phosphatase 2; SHIP, SH2 domain-containing inositol phosphatase-5-phosphatase; FITC, fluoroscein isothiocyanate; Syk, spleen tyrosine kinase; TNF, 2′(3′/5′)-O-(2′,4′,6-trinitrophenyl); BSA, bovine serum albumin; TNF, tumor necrosis factor; JNK, c-Jun N-terminal kinase; ELISA, enzyme-linked immunosorbent assay; LTC4, leukotriene C4.
negative role for the Fc chain of monoclonal antibody to PLC previously (26). After 4-weeks of culture, cells were stained for cell provided by Drs. Toshiaki and Yuko Kawakami (La Jolla Institute of because the requirements for Fc containing protein phosphatase 2 (SHP-2) (22). However, the FcRβ ITAM tyrosines in Lyn-dependent activation of mast cell responses. This focus was based on the recent finding that Lyn kinase is a negative regulator of mast cell responsiveness and the allergic response (23), thus raising the possibility that its interaction with FcRβ could exert negative regulatory influences. Because bone marrow-derived cultured mast cells (BMMCs) from β chain-deficient mice lack cell surface expression of FcRβ despite normal expression of FcRβ α and γ chain mRNA, we chose this system to retrovirally transduce mutant β chains that reconstituted cell surface FcRβ. Most mutant FcRβ-expressing cells showed impaired degranulation and leukotriene secretion when compared with wild type FcRβ-expressing cells. The YFY mutant was an exception, as it showed no inhibition of these responses. Strikingly, cytokine synthesis and secretion was increased in YFY mutant cells and was greatly enhanced in the FFF mutant. The increased cytokine production was associated with increased activation of the gene transcription regulators, MAPKs, and NFκB. Whereas a positive role for the FcRβ β chain in allergic responses has been rigorously demonstrated (reviewed in Ref. 1), our findings revealed a previously unrecognized negative regulatory role for the FcRβ ITAM in cytokine production.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**Anti-raft β monoclonal Ab (clone JRRK), which also recognizes mouse β chain (24), was used in these studies. Mouse monoclonal Abs to IgE (anti-TNP or anti-DNP) were also used (25). IgE was labeled with FITC (Sigma). Mouse monoclonal Abs to SHP-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal Abs to Lyn and PLCγ1 and monoclonal antibody to PLCγ2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal and mouse monoclonal Abs to Syk were kindly provided by U. Blank (Institute Pasteur, Paris, France) and P. Draber (Institute of Molecular Genetics, Prague, Czechoslovakia), respectively. Polyclonal Ab to LAT was generously given by L. E. Samelson (National Institutes of Health, Bethesda, MD). All of the phosphotyrosine and its tyrosine 203 mutants were from Cell Signaling Technology (Beverly, MA) as well as antibodies to ITAM, IL-3, and IκBα. Horseradish peroxidase-conjugated extravidin and monoclonal anti-rabbit immunoglobulin (clone RG16) were from Sigma. Horseradish peroxidase-conjugated antibodies to mouse and rabbit immunoglobulin were from Amersham Biosciences. Detergents (Nonidet P-40 and octyl glucoside) were from Pierce. Phosphatase and proteinase inhibitors were from Sigma. Naf was from Fluka (Buchs, Switzerland).

**Mice—**The β chain-deficient (β−/−) mice on the chimera BDF1 and 129 background have been described previously (10) and were bred in transgenic mice from the same background as RI despite normal expression of FcRβ chain-deficient mice lack cell surface expression of FcRβ chain in allergic responses has been initiation as described previously (27). After 2 weeks of selection, cells were analyzed for FcRβ expression. Cultures were used when >95% expressed FcRβ.

Site-specific Mutagenesis and Retroviral Constructs—The PCR-based mutagenesis described by M. P. Weiner et al. (28) was employed to create single nucleotide-mutagenized constructs. The 800-bp EcoRI fragment containing the mouse β chain coding sequence was isolated and subcloned into the EcoRI site of plbueprint (Stratagene, La Jolla, CA). Two sets of PCR primers, including the mutation site, were designed in the opposite direction of the mutation site. The sequences of sense and antisense oligonucleotide primers for each mutagenesis were as follows: wild type, 5′-GATTTCACAAATCTAGGAGGATTGT3′ and 5′-ACATTAACTTCCATATGAGCAGATAT3′; FYY, 5′-GATTTCACAAATCTAGGAGGATTGT3′ and 5′-ACATTAACTTCCATATGAGCAGATAT3′; YFY, 5′-GATTTCACAAATCTAGGAGGATTGT3′ and 5′-ACATTAACTTCCATATGAGCAGATAT3′; YYY, 5′-GATTTCACAAATCTAGGAGGATTGT3′ and 5′-ACATTAACTTCCATATGAGCAGATAT3′ and 5′-ACATTAACTTCCATATGAGCAGATAT3′. Mutation sites amino acid replacement of tyrosine to phenylalanine are underlined.

PCR was carried out by using 2.5 units of Thermococcus kodakaraensis polymerase (Toyobo, Tokyo, Japan) in a 100-μl reaction mixture containing 10 ng of plbueprint as template, 50 pm of each phosphorylated primer, 2 mM MgCl2, 0.2 mM dNTPs, 120 mM Tris-HCl (pH 8.8), 10 mM KCl, 6 mM (NH4)2SO4, 0.1% Triton X-100, and 10 μg/ml BSA. PCR primers were designed at 94°C for 45 s of amplification (94°C for 1 min, 55°C for 1 min, and 72°C for 4 min) followed by a final extension at 72°C for 10 min. The PCR products were treated with DpnI (New England Biolabs, Beverly, MA) to digest the template DNA followed by self-ligation. The integrity of all of the mutants was confirmed by sequence analysis using capillary sequencer (ABI prism 310 Genetic Analyzer, ABI, Foster City, CA). The mutant β chain cDNA were subcloned into the EcoRI site of the Moloney murine leukemia virus-based retroviral vector, pMX-puro, and the virus was produced (29). Empty vector (pMX-puro) was used as a negative control to generate the virus not expressing the FcRβ.

**Flow Cytometry—**The cells (3×106) were incubated with 5 μg/ml FITC-labeled IgE on ice for 30 min in 200 μl of phosphate-buffered saline (PBS) with 0.1% BSA and 0.05% sodium azide. The cell surface expression of IgE was measured using a flow cytometer (FACS can3B, BD Biosciences). The number of dead cells was determined by propidium iodide staining.

β-Hexosaminidase Release Assay—Degranulation via FcRβ stimulation was determined by β-hexosaminidase release. Cells (5×105 cells/ml) were incubated with TNP- or DNP-specific IgE (2 μg/ml) in Mg2+/Ca2+-free Tyrode's buffer (10 mM HEPES buffer, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, and 0.1% BSA) for 30 min at 4 °C. The sensitized cells were washed and resuspended at a concentration of 5 × 105 cells/ml in Tyrode's buffer containing 1 mM CaCl2 and 0.6 mM MgCl2. TNP-ovalbumin (1 μg/ml) or DNP-BSA (10 μg/ml) was used for maximal stimulation or at indicated concentrations. Phorbol 12-myristate 13-acetate (10 ng/ml) and sodium iomyocin (100 ng/ml) were employed for providing stimulatory concentrations of calcium ionophore A23187 at 37 °C, the reaction was terminated by centrifugation at 4 °C. The total hexosaminidase concentration was obtained by cell lysate in 1% Nonidet P-40. Aliquots of the supernatants and total cell lysates were incubated with 1.3 mg/ml p-nitrophenyl-N-acetyl-β-D-glucopyranoside (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) for 90 min at 37 °C. The reaction was terminated by the addition of 0.2 μl glycine buffer (pH 10.7). The release of the product 4-nitrophenol was monitored by optical absorbance at 405 nm. Percentage of β-hexosaminidase release was calculated as follows: (supernatant optical density value of the stimulated cells − supernatant optical density value of the unstimulated cells) × 100/(the total cell lysate optical density value − supernatant optical density value of the unstimulated cells). Percent spontaneous release was usually below 5% of the total cell lysate.

**Cytokine, LTC4, NFκB Activity, and Real-time PCR—**Cell culture supernatants after stimulation were analyzed for IL-6, IL-13, TNFα, and LTC4, by specific ELISA kits according to the manufacturers’ instruction (Genzyme Technic, Minneapolis, MN or BIOSOURCE International for IL-6, IL-13, and TNFα, Cayman Chemical, Ann Arbor, MI for LTC4). Cells (2×105 cells/ml) sensitized with IgE (2 μg/ml) were stimulated for 3 h at 37 °C, and supernatants collected and analyzed. NFκB binding activity was determined by measuring p65 binding to a NFκB consensus binding site oligonucleotide in an ELISA-based format according to the manufacturers’ instructions (TransAM™ NFκB family, Active Motif, Carlsbad, CA). Nucleic lysates (prepared per manufacturers’ instruction) of stimulated and nonstimulated (as above) cells expressing the YY, YY, FYF, and FFF ITAMs were incubated in ELISA wells containing the NFκB consensus binding site
oligonucleotide. Binding was measured by incubation with a primary antibody to p65 followed by a secondary horseradish peroxidase-conjugated antibody that recognized the primary antibody. Following a final step of multiple washes, a colorimetric reaction was performed. The positive control of stimulated Jurkat T cells and negative lysis buffer controls were also included. Absorbance was read at 450 nm.

For analysis of mRNA levels, RNA was isolated using a Qiagen RNeasy kit (Valencia, CA) and reverse-transcribed using a one-strand cDNA synthesis kit (Invitrogen). Real-time PCR (TaqMan) was performed using the ABI PRISM7700 sequence detection system (Applied Biosystems, Foster City, CA). An analysis of IL-6, TNFα, and 18 S rRNA levels was performed using commercially available primer/probe sets (Applied Biosystems). Levels of IL-6 and TNFα were determined by normalization of 18 S rRNA levels relative to the unstimulated transduction buffer (10 mM NaH2PO4, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, pH 7.4). The same 18 S rRNA level was used to normalize the stimulated and unstimulated transduced cell line, which was arbitrarily designated a value of 1.0.

**Cell Lysates, Immunoprecipitation, Immunoblotting, and Phospho-ITAM Peptide Pull-down Assays—**Cell aliquots of 3 × 10^7 were stimulated in a total volume of 1 ml of Tyrode buffer and sampled at different times or stimulated with 100 ng/ml antigen. Stimulated cells were washed twice with ice-cold PBS and lysed in either SDS sample buffer or borate-buffered saline that contained 1% Nonidet P-40, 60 mM octyl glucoside, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2 μg/ml leupeptin and pepstatin, 5 mM sodium pyrophosphate, 50 mM NaF, and 1 mM orthovanadate and were resolved for 30 min on ice. Lysates were centrifuged for 20 min (4 °C) at 14,000 rpm. For immunoprecipitation, cell lysates were incubated for 3 h with antibodies prebound to either protein G-Sepharose (monoclonal) or protein A-Sepharose (polyclonal). Proteins were recovered by denaturation with an equal volume of 2× Tris-glycine SDS sample buffer that contained 1% 2-mercaptoethanol and 1 mM orthovanadate and were resolved by 8, 10, or 12% SDS-PAGE (Invitrogen or Schleicher & Schuell). Nitrocellulose membranes were blocked with either 4% BSA or 5% dry milk. Nitrocellulose membranes were washed with radioimmune precipitation assay, and bound proteins were detected using the ABI PRISM7700 sequence detection system (Applied Biosystems). An analysis of IL-6, TNFα, and 18 S rRNA levels was performed using commercially available primer/probe sets (Applied Biosystems). Levels of IL-6 and TNFα were determined by normalization of 18 S rRNA levels relative to the unstimulated transduced cell line, which was arbitrarily designated a value of 1.0.

**Calcium Measurements—**Calcium was measured as described previously (26). Cells (2 × 10^6) were dual-loaded with 16 μM Fluo-4-AM and 16 μM Fura Red (Molecular Probes, Eugene, OR) in RPMI 1640, 2% fetal calf serum media for 45 min at 37 °C. Cells were incubated with IgE (1 μg/ml) for 1 h and brought to room temperature for 20 min. Cells were resuspended in Tyrode-BSA, and changes in dye fluorescence with time were determined by flow cytometry after stimulation with 30 ng/ml antigen at 37 °C. Calcium mobilization was reported as the ratio of Fluo-4 to Fura Red fluorescence intensity over time.

**Statistical Analysis—**A nonparametric test (Mann-Whitney) was employed to determine the statistical significance among the experimental groups. *p < 0.05* was considered significant.

**RESULTS**

**Expression of Wild Type and Mutant Forms of FcεRIβ in BMNC from β Chain-deficient Mice Reconstitutes Cell Surface Expression of FcεRI—**To investigate the functional significance of canonical and noncanonical tyrosine of the β chain ITAM in mast cell responses, we generated five mutants of the β chain with the replacement of tyrosine to phenylalanine in which the YYV (N-terminal to C-terminal sequence) was changed to YFY, YFY, YFY, YFY, and YFF. Retroviral-mediated gene transfer into β−/− mouse mast cells and cell surface expression analysis of FcεRI revealed that cells expressing the YYV, YFY, YFY, YFY, and YFF were all FcεRI-positive on their cell surface (>95%), similar to cells transduced with the wild type (YYY) ITAM virus, whereas cells transduced with the empty vector (pMX-puro) virus did not express FcεRI, similar to β−/− mast cells (Fig. 1A). The relative fluorescence intensities of FcεRI for each transduced cell culture were almost identical. The cells were shown to express FcεRIβ protein by immunoblotting with monoclonal antibody to β chain (Fig. 1B). The 31-kDa band, which corresponds to the β chain, in YYV, YFY, YFY, YFY, YFF, and wild type (YYY) cells was easily detected, whereas no protein band was observed in cells transduced with the vector-control virus (Fig. 1B). Moreover, the level of protein expression was similar to nontransduced BMNC. These results show that the mutated β chain ITAM successfully reconstituted FcεRI cell surface expression to the same extent as wild type ITAM, and expression levels were comparable among the transduced cells.

**Mutation of the FcεRI β ITAM Canonical Tyrosines but Not the Noncanonical Tyrosine Partially Impairs Mast Cell Degranulation and LTC4 Secretion—**We investigated whether the expression of the different mutant β chains affected the degranulation and LTC4 secretion responses of the transduced mast cells upon FcεRI stimulation. As shown in Fig. 2A, all of the cells expressing surface FcεRI with wild type or mutant β chains, but not pMX-puro-transduced cells, were able to degranulate in response to FcεRI stimulation. Cells expressing the YYV, YFY, YFY, and YFF forms of the β chain ITAM showed a 30–50% reduction in mast cell degranulation and LTC4 secretion responses of the transduced mast cells (Fig. 2A). A striking enhancement in IL-6, IL-13, and TNFα secretion was observed in cells expressing the FYY, YYF, FYF, and FFF mutant when compared with wild type ITAM, and expression levels were comparable among the transduced cells.

**FcεRI-mediated Cytokine Production and Secretion Is Dramatically Enhanced in the Absence of FcεRI-β ITAM Tyrosines—**IgE-dependent activation of mast cells leads to de novo synthesis and secretion of cytokines (30). We investigated whether the mutations of the β chain ITAM would influence the cytokine secretion from mast cells. Because IL-6, IL-13, and TNFα are cytokines that are potently produced and secreted from mast cells, we measured their release by ELISA (Fig. 3, A–C). As expected, the engagement of the FcεRI carrying the wild type β chain ITAM induced a significant net release of IL-6, IL-13, and TNFα when compared with pMX-puro-transduced cells (where cytokines measured from 5 to 10% of response of wild type β chain-reconstituted cells, see Fig. 3 legend). The YYV-, YFY-, and YFF-expressing cells either showed no significant change or a significant decrease in response when compared with the wild type ITAM-expressing cells (Fig. 3, A–C). Interestingly, cells expressing the mutant of the noncanonical tyrosine (YYV) showed a modestly elevated level of IL-6 and IL-13 secretion when compared with wild type cells, whereas TNFα was not significantly affected. This differed from the results for β-hexosaminidase release and LTC4 secretion where the YYV mutant showed no significant difference when compared with the wild type ITAM, suggesting that the noncanonical tyrosine could contribute to the cytokine response. A striking enhancement in IL-6, IL-13, and TNFα secretion was observed in cells expressing the FFF mutant.
Cytokine secretion was enhanced by 2–4-fold compared with cells expressing the wild type ITAM (YYY). To further elucidate whether this negative regulatory role of the FcRI/ITAM was at the level of protein secretion or mRNA expression, we examined the cytokine mRNA expression of YYY, YFY, FYF, and FFF ITAM-expressing cells by real-time PCR (TaqMan). The expression of mRNA for IL-6 and TNFα in YFY and FFF-expressing cells was increased post-FcRI stimulation when compared with the wild type (YYY) ITAM-expressing cells (Fig. 3D). Although the increase in TNFα mRNA was quite modest in YFY-expressing cells, it was significant and reproducible. In contrast, FYF-expressing cells showed reduced mRNA levels relative to the wild type ITAM. This finding suggested that the mutation of the noncanonical tyrosine in the FcRI/ITAM released a negative regulatory constraint on gene expression that is augmented by (but does not depend on) the canonical tyrosines since the FFF, but not the FYF, mutant demonstrated this phenotype.

Phosphorylation of FcRI β Chain and γ Chains, Lyn Interaction with the β Chain, and Phosphorylation of Receptor-proximal Signaling Molecules Are Partly Dependent on an Intact FcRI β Chain ITAM—To gain a better understanding on how the FcRIβ ITAM functions in mast cell effector responses, we investigated its role in signal generation. We first analyzed the effect of wild type (YYY) and YFY, FYF, and FFF mutant ITAMs on FcRI phosphorylation and interaction with Lyn kinase to understand whether there was an association between the interaction with Lyn and the observed effects on mast cell degranulation and cytokine production. This line of investigation might also allow us to differentiate the relative importance of the noncanonical and canonical tyrosines in receptor phosphorylation and Lyn interaction. Tyrosine phosphorylation of FcRIβ was reproducibly but minimally reduced in YFY cells; however, a striking inhibition of its phosphorylation was apparent in FYF and FFF cells (Fig. 4A), demonstrating that the canonical tyrosines were most important to this event. This was mirrored by a decrease in the phosphorylation of the γ chains in the same mutants, albeit not to the same extent (Fig. 4A). Because the decreased phosphorylation of the β and γ chains suggested a possible loss of interaction with Lyn, we investigated the ability of the mutant ITAMs to co-immunoprecipitate Lyn kinase. Whereas the YFY mutant reproducibly showed a slight reduction in co-immunoprecipitated Lyn kinase, Lyn was not co-immunoprecipitated by the FYF and FFF mutants (Fig. 4B), suggesting that one or both of the canonical ITAM tyrosines was required for this interaction. Lyn kinase is
also critical to the activation of Syk and thus to the phosphorylation of the Syk substrate, LAT. When we analyzed the phosphorylation of Syk and LAT, a minimal reduction of Syk, but not LAT, phosphorylation was seen in YFY cells (Fig. 4, C and D) where Lyn could still bind to the β ITAM (Fig. 4B). In contrast, the inhibition of Syk and LAT phosphorylation (ranging from 50 to 74%) was observed in cells transduced with the FYF and FFF mutants, which also failed to effectively bind Lyn (Fig. 4B).

We next explored the possibility that the activity or localization of the SHP-2 and the lipid phosphatase SHIP might be affected by mutation of the FcεRIβ ITAM based on the previous in vitro observation of their interaction with this receptor subunit (31, 32). Investigation of their phosphorylation status in the YFY, FYF, and FFF mutants revealed that SHP-2 phosphorylation was relatively unaffected by mutation of the noncanonical (YFY) tyrosine and only modestly (the observed trend was not significant) reduced by mutation of canonical (FYF) tyrosines. Only when both canonical and noncanonical tyrosines were mutated (FFF) was significant inhibition of SHP-2 phosphorylation observed (Fig. 4E). In contrast, the phosphorylation of SHIP-1 was significantly inhibited by the mutation of the noncanonical tyrosine and this defect was maintained in the FYF and FFF mutants (Fig. 4F). Thus, SHIP-1 phosphorylation, which was shown as important for SHIP localization to the membrane (33), is positively regulated by both the FcεRIβ noncanonical as well as the canonical tyrosines.

We further explored whether the mutation of the FcεRIβ canonical or noncanonical tyrosines showed selectivity in interactions with SHP-2 or SHIP-1. We used phospho-ITAM peptides to enhance these interactions and allow detection of modest changes in possible interactions. As shown in Fig. 4G, SHP-2 was found to interact with the FcεRIβ phospho-ITAM equivalent (see “Experimental Procedures”) of YYY and YFY but not with FYF and FFF. SHIP-1 also interacted with YYY and YFY and was very weakly but consistently detected with FYF. No interaction was observed with FFF. Interestingly, in this series of experiments, we also found that the p85 regulatory domain of PI3K also interacted with the FcεRIβ YYY and YFY ITAMs and was also weakly but consistently found to interact with FYF but not with FFF ITAMs. This interaction is consistent with the previous report of SHIP-1-mediated recruitment of PI3K (p85) during FcyRIlβ-mediated inhibition of the B cell antigen receptor (34). As expected, Lyn interacted with YYY and YFY but not with FYF and FFF ITAMs, confirming the co-immunoprecipitation experiments shown in Fig. 4B. However, the modest differences in the amount of Lyn binding to YYY and YFY ITAMs seen in the co-immunoprecipi-
tation experiments were not observed, suggesting that other influences on Lyn interaction with the FcRIβ are likely to account for the modest loss of Lyn interaction in the co-immunoprecipitation experiments from YFY-expressing cells. Syk failed to interact with the FcRIβ ITAM, confirming the specificity of the demonstrated interactions (Fig. 4G).

Phosphorylation of PLCγ1/2 and the Calcium Response Is Also Impaired in Cells Expressing YFY and FFF Mutant ITAMs—We recently demonstrated that LAT is required for PLCγ activation and calcium mobilization in mast cells (26). Mutation of the four distal tyrosines of LAT revealed that the PLCγ binding site (Tyr-136) is most critical for the tyrosine phosphorylation of PLCγ1 and 2, calcium responses, and degranulation (27). Thus, we explored the impact of the decreased LAT phosphorylation observed in YFY and FFF β chain ITAM mutants and the status of PLCγ1 and PLCγ2 phosphorylation and calcium responses. A minimal but not significant decrease in the phosphorylation of PLCγ1 and 2 was observed in the YFY mutant (Fig. 5A). In contrast, a marked reduction (up to 70%) of the phosphorylation of these proteins was observed in YFY and FFF mutant cells (Fig. 5A). As shown in Fig. 5B, the marked inhibition of PLCγ1 and 2 phosphorylation in the mutant YFY and FFF ITAM-expressing cells caused a significant delay in the initiation of the calcium response with the time required for half-maximal rise of calcium in response to Ag increasing by 4-6-fold (Fig. 5B). In contrast, the YFY-expressing cells showed no significant delay in the calcium response, although an effect on the extent of the calcium rise was noted in several experiments (Fig. 5B). Collectively, these findings demonstrate the contributory importance of the canonical β ITAM tyrosines in events leading to the calcium response. Future studies will examine the contribution of other ITAM tyrosines (38) and the role of other ITAM-regulated pathways in the calcium response (22).

ERK, p38 MAPK, IKKβ, and 1BPhosphorylation Is Enhanced in Mutants (YFY and FFF) with Increased Cytokine Production and Secretion—Given that many of the phenotypic traits of β ITAM mutation mirrored those of Lyn deficiency (21, 35, 36), we explored the effect of ITAM mutation on MAPK activation, because the activity of these kinases is intact or enhanced in Lyn-deficient mast cells. Strikingly, all of the ITAM mutants showed an intact activation response for ERK, JNK, and p38 MAPK, suggesting that the activation of MAPK was essentially independent of the FcRI β ITAM (Fig. 6, A–C). Moreover, a sustained and/or enhanced phosphorylation of ERK and p38 MAPK were reproducibly observed in YFY and FFF mutant cells when compared with wild type ITAM cells (Fig. 6, A and C). The phosphorylation of JNK and of protein kinase B/Akt, which is important for cell survival and gene expression, was also intact (Fig. 6, B and D). Thus, the path-
ways that lead to gene expression were intact or enhanced (ERK and p38 MAPK) in FcR\(\beta\) ITAM mutants.

IL-6 and TNF\(\alpha\) mRNA production was demonstrated to depend on NF\(\kappa\)B activity in mast cells (37, 38). Moreover, Krystal and colleagues (39) have recently shown that SHIP-1 mast cells had increased NF\(\kappa\)B activity. Given that both receptor-associated Lyn phosphorylation and SHIP-1 phosphorylation are lost in the YFY and FFF mutants, we explored the phosphorylation/degradation of the components of NF\(\kappa\)B signaling.

The binding of I\(\kappa\)B to NF\(\kappa\)B sequesters NF\(\kappa\)B in the cell cytoplasm. The phosphorylation of I\(\kappa\)B by a macromolecular kinase complex, comprised of two catalytic subunits (IKK\(\gamma\)/NEMO) with adapter function, causes the degradation of I\(\kappa\)B and releases NF\(\kappa\)B, allowing its nuclear translocation. IKK\(\beta\) has been shown as essential for both the phosphorylation of the NF\(\kappa\)B subunit p65(ReLA) and I\(\kappa\)B, whereas IKK\(\alpha\) phosphorylates p65(ReLA) (40). As seen in Fig. 7, the phosphorylation of both IKK and I\(\kappa\)B was enhanced in YFY and FFF mutant cells. In particular, a significant enhancement of IKK\(\beta\) phosphorylation was observed in the YFY mutant and the phosphorylation of this subunit was further enhanced and more sustained in the FFF mutant (Fig. 7A). Whereas some induction of IKK\(\alpha\) phosphorylation was observed, it was not as dramatic as that of IKK\(\beta\). Moreover, the phosphorylation and degradation of I\(\kappa\)B were consistent with increased IKK\(\beta\) activity (Fig. 7B). The extent of reduction in I\(\kappa\)B protein mirrored the enhanced phosphorylation of IKK\(\beta\) and I\(\kappa\)B in the YFY and FFF mutants, respectively, demonstrating the cooperation between the noncanonical and canonical tyrosine residues of the FcR\(\beta\) ITAM in negative regulation of the NF\(\kappa\)B pathway leading to cytokine production.

Whereas it is recognized that initiation of IKK signaling by a wide variety of stimuli results in NF\(\kappa\)B activation (41), we investigated whether cells transduced with FcR\(\beta\) (YYY, YFY, FYF, and FFF) showed levels of nuclear NF\(\kappa\)B binding activity that reflected their respective levels of activation of IKK\(\beta\) and cytokine production. As a control, we measured the nuclear NF\(\kappa\)B binding activity of the nonstimulated transduced cells of each genotype and found no significant difference (Fig. 7C), consistent with the requirement of FcR\(\beta\) stimulation for induction of IKK\(\beta\) and I\(\kappa\)B phosphorylation. FcR\(\beta\) stimulation resulted in increased nuclear NF\(\kappa\)B binding activity for all of the cells expressing the exogenous FcR\(\beta\). YFY and FFF mutants showed respective increases in the levels of nuclear NF\(\kappa\)B binding activity relative to the wild type (YYY) transfectant, as seen for IKK\(\beta\) and I\(\kappa\)B phosphorylation. In contrast, whereas the nuclear NF\(\kappa\)B binding activity of the FYF mutant also increases upon FcR\(\beta\) stimulation, this activity was decreased relative to the wild type (YYY) and mirrored the IKK\(\beta\) and I\(\kappa\)B phosphorylation for this mutant (Fig. 7, A and B). The increased NF\(\kappa\)B binding activity was previously demonstrated as essential for mast cell TNF\(\alpha\) and IL-6 production (37, 39), thus...
**DISCUSSION**

The FceRIβ ITAM has long been recognized as an important site of interaction for Lyn kinase (15, 42, 43), and this subunit was demonstrated to provide an amplifying function in FceRI expression, IgE-dependent signals, and cellular responses (9, 11). By employing retroviral transduction of FceRIβ-null BMMC with FceRIβ ITAM mutants, we revealed a previously unrecognized dichotomy in FceRIβ-driven cellular response. FceRI-mediated degranulation and leukotriene secretion were adversely affected by the mutation of the canonical tyrosines of the FceRIβ ITAM, whereas cytokine production and secretion were enhanced by mutation of either the noncanonical tyrosine or both the noncanonical and canonical tyrosines. Our findings demonstrate that FceRIβ ITAM tyrosine residues that control the lymphokine responses also control the extent of activation of MAPK family members and the NFκB signaling pathway. Cooperativity of the noncanonical and canonical tyrosines in control of cytokine production was apparent. This was most clearly demonstrated by the failure of the FYF mutant to enhance lymphokine responses and NFκB signaling. In contrast, the enhancing effects of the YFY mutant on these responses were obvious but modest compared with the effect of the ITAM-null mutant (FFF). Therefore, whereas the noncanonical tyrosine plays the major role in exerting negative regulation on NFκB activation and lymphokine production, it requires the canonical tyrosines to amplify this effect.

Many of the phenotypic traits observed by mutation of the FceRIβ ITAM are recapitulated in Lyn and SHIP-1-deficient mast cells. An analysis of signals in Lyn-deficient mast cells demonstrated a pronounced loss of Syk, LAT, and PLCγ1 and 2 phosphorylation (21, 35, 36). This is similar to the observed defects in FYF and FFF FceRIβ ITAM mutants, which did not associate with Lyn, arguing that these defects may largely be a consequence of the loss of Lyn-FceRIβ interaction. Similarly, calcium responses were also delayed and the extent of the response was diminished as seen in Lyn-deficient mast cells (21, 35). In contrast, Kawakami and colleagues (35) demonstrated that MAPK activation was intact (JNK) or enhanced (ERK2 and p38 MAPK) (35) in Lyn-deficient mast cells mirroring the FceRIβ ITAM-null mutant. A similar phenotype was seen with SHIP-1-deficiency, a protein whose phosphorylation requires Lyn activity (44, 45). Kristal and co-workers (46) show enhanced ERK2 activity in these mast cells. However, in contrast to the partial defect of the FceRIβ ITAM mutants on degranulation and leukotriene secretion, Lyn and SHIP-1-deficient mast cells hyperdegranulate (21, 46). This finding suggests that the hyperresponsive degranulation of Lyn- and SHIP-deficient mast cells is not a consequence of the loss of negative regulation by Lyn at the level of the receptor itself, because the binding of Lyn to the FFF-mutated β chain was not...
observed and Lyn is active in SHIP-1-deficient mast cells. The findings also imply that the negative influence of Lyn and SHIP-1 on degranulation is separate from the positive or amplifying influence of the FcεRIβ ITAM. This is consistent with the findings that Lyn and SHIP are important in controlling cellular homeostasis (23, 44, 47–49) and with the finding that Lyn deficiency increases Fyn activity that is required for degranulation (23). The resulting postulate is that activation of Lyn deficiency increases Fyn activity which is required for degranulation and with the finding that Lyn and SHIP are important in controlling cytokine production.

Tyrosine phosphorylation of inositol 5′-phosphatase SHIP-1 does not appear to be important for its activity; however, it plays an important role in membrane targeting of this phosphatase (33), probably because of its ability to interact with membrane proteins (similar to Lyn) and become stabilized in the membrane (50). Because the substrate of SHIP activity (inositol 1,4,5-trisphosphate) is membrane-restricted, the targeting of SHIP to the membrane is critical for its function in controlling the levels of inositol 1,4,5-trisphosphate and thus influencing the activity of inositol 1,4,5-trisphosphate-binding proteins such as the P13K-dependent kinase 1, PLC, Akt, and others. Interestingly, the mutation of the noncanonical tyrosine (YFY) caused a significant loss of SHIP-1 phosphorylation, although SHIP-1 interaction with FcεRIβ and Lyn recruitment to FcεRIβ was still detected. This finding suggests that the phosphorylation of SHIP-1 rather than recruitment to the FcεRIβ might play a more significant role in the suppression of cytokine production. Remarkably, SHIP-1 was demonstrated to negatively regulate FcεRI induction of IL-6 production by inhibiting NFκB activity, which drives IL-6 transcription by binding to the NFκB locus of the IL-6 promoter (39). The absence of SHIP was found to enhance NFκB phosphorylation and degradation as well as increase NFκB DNA binding activity, similar to our observations in the YFY and FFF mutants. Collectively, these findings implicate FcεRIβ-associated or -regulated SHIP-1 in promoting this phenotype. Although we exhaustively explored co-immunoprecipitation of SHIP with the FcεRIβ, we could not reproducibly establish this interaction. This is probably due to the small amount of SHIP associated with FcεRIβ. Nonetheless, our in vitro studies with phospho-ITAM peptides showed a strong SHIP-1 interaction with the canonical tyrosines and a modest, but reproducible, interaction when only the noncanonical tyrosine was phosphorylated.

We cannot formally exclude a conformational alteration of FcεRIβ engendered by ITAM tyrosine mutation. However, several of our findings argue against this possibility. First, the differential role of the FcεRIβ ITAM in degranulation and leukotriene secretion versus NFκB signaling and lymphokine production argues that the effect is not a broad consequence of conformational inactivation or activation. Second, selectivity in activation (ERK2 and NFκB) and inactivation (Syk, LAT, and PLC) was observed, demonstrating that the mutations of FcεRIβ
showed selectivity in their effects on molecules that interact with the receptor (FcεRIγ) as well as others (similar to LAT) that do not require receptor localization for their activity or function (26, 51). Third, no effects on FceRI expression were observed regardless of which tyrosine residue was mutated in the ITAM in contrast to a previous study in which FcεRIβ deletion mutants with altered conformations showed defective or variable expression (52). Finally, the phenotype observed for FceRIβ mutant mast cells showed striking similarity to Lyn- or SHIP-1-deficient mast cells (21, 23, 35, 39) and reproduced the previously demonstrated SH2 domain interaction of Lyn with receptor and the described weak signaling phenotype of the human αγγε FceRI (8, 9, 43).

It is not yet clear whether the cooperativity of the noncanonical and canonical tyrosines in negative regulation of cytokine production is entirely mediated through Lyn and SHIP-1. The possibility of another player is suggested by the FYF mutant, which did not bind Lyn (similar to FFF), showed reduced SHIP-1 phosphorylation (similar to YFY and FFF) and its reduced binding to receptor but yet failed to enhance cytokine production. Identification of other molecules that specifically bind the noncanonical tyrosine may reveal the key negative regulatory component(s). Regardless, our findings show that the FceRIβ can act not only as an amplifier of signaling leading to degranulation and leukotriene secretion but also as a negative regulator of selected signals that are important in gene expression. This presents the intriguing possibility that FceRIβ ITAM can shift the balance between the amplifying and inhibitory functions of this subunit, thus influencing the allergic response.

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The Fc?RIβ Immunoreceptor Tyrosine-based Activation Motif Exerts Inhibitory Control on MAPK and IκB Kinase Phosphorylation and Mast Cell Cytokine Production
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