Molecular dissection of the FcRβ signaling amplifier

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Running title: Analysis of FcRβ ITAM
Summary

Human high affinity IgE receptors are expressed as two different isoforms: the tetrameric isoform, $\alpha\beta\gamma_2$, or the trimeric isoform, $\alpha\gamma_2$. The $\alpha$ chain is the IgE binding subunit while the FcR$\beta$ and FcR$\gamma$ chains are the signaling modules. Both FcR$\beta$ and FcR$\gamma$ contain Immunoreceptor Tyrosine-based Activation Motifs (ITAM), but the $\beta$ ITAM differs from canonical ITAMs in two ways: the spacing between the two canonical tyrosines harbors a third tyrosine, and it is one-aminoacid shorter than in canonical ITAMs, making it unfit to bind the tandem SH2 of syk. We have shown that FcR$\beta$ functions as an amplifier of the FcR$\gamma$ signaling function. However, the molecular mechanism of this amplification remains unclear.

Here we show that mutation of the three tyrosines (Y219, Y225 and Y229) in the $\beta$ ITAM essentially converts $\alpha\beta\gamma_2$ into an $\alpha\gamma_2$ complex in term of lyn recruitment, FcR$\gamma$ phosphorylation, syk activation and calcium mobilization. Y219 is the most critical residue in this regard. In addition, a detailed analysis of the dynamics of calcium mobilization suggests a possible inhibitory role for Y225, which becomes apparent when Y219 is mutated. Thus, the signaling amplification function of FcR$\beta$ is mainly encoded in Y219 and in its capacity to recruit lyn. In turn, this Y219-mediated lyn recruitment enhances $\gamma$ chain phosphorylation, syk activation and calcium mobilization. The two other tyrosines appear to have a modulating function that remains to be fully assessed.

Introduction

The high affinity receptor for IgE, Fc$\varepsilon$RI, forms a high affinity cell surface receptor for the Fc region of antigen specific immunoglobulin E (IgE) molecules. Fc$\varepsilon$RI is multimeric and is a
member of a family of related antigen/Fc receptors that have conserved structural features and play similar roles in initiating intracellular signaling cascades (1). FcεRI controls the activation of mast cells and basophils, and, in humans, participates in IgE-mediated antigen presentation. Multivalent antigens bind and crosslink IgE molecules held at the cell surface by FcεRI. Receptor aggregation induces multiple signaling pathways that control diverse effector responses, including secretion of allergic mediators and the induction of cytokine gene transcription (such as IL-4, IL-6, TNFα and GM-CSF). FcεRI, therefore, is central to the induction and maintenance of an allergic response and physiologically may confer protection from parasitic infections (1).

FcεRI is a tetrameric structure made of an IgE binding α chain and a signaling module consisting of an FcRβ chain and a dimer of FcRγ chains. In humans, but not in rodents, a trimeric form lacking FcRβ, αγ2, can also be expressed. The β and γ chains contain conserved Immunoreceptor Tyrosine-based Activation Motifs (ITAM) in their cytoplasmic tails (2) and reviewed in (3,4)). The consensus sequence of these motifs is D/E-XXYXXL-(X)7-9YXXL-L/I. The tyrosine residues in these motifs are phosphoacceptor sites for the action of receptor-associated protein tyrosine kinases of the src family (5,6). Phospho-ITAMs link receptor and signal transduction cascades by virtue of the capacity of SH2 domains, present in numerous signaling molecules, to bind phosphorylated tyrosines present in an appropriate aminoacid sequence context. ITAMs are present in antigen receptors such as the T- and B-cell receptors and FcγRs. ITAMs have the capacity, when expressed independently of the rest of the receptor, to elicit the full range of activation signals observed with the complete receptor (5,7-10). In particular the γ ITAM is capable of inducing serotonin release when expressed as a chimera with the extracellular and transmembrane domains of the α chain of the IL-2 receptor in the mast cell.
line RBL-2H3 (9). In contrast, FcRβ lacks autonomous signaling capacity (11). Instead, it acts as an amplifier of signals transduced by FcRγ (12,13). This was an unexpected finding, the molecular basis of which has not been elucidated so far. The β ITAM differs from the canonical ITAM in 2 ways: 1) it contains a third tyrosine between the canonical tyrosines; and 2) it has a shorter spacing between the canonical tyrosine residues. Whether these features are responsible for the unique functional characteristics of β has not been determined. Three additional features of FcRβ further explain why it has been the focus of many studies since its cloning. Firstly, it possesses the unique capacity of amplifying receptor expression, as evidenced by the higher level of expression of αβγ₂ compared to αγ₂ (14). This expression amplification results from the capacity of FcRβ to promote intracellular processing of the α chain. Secondly, genetics studies have identified the gene for FcRβ as a candidate gene for atopy, even though the mechanism that underlies this effect is unknown (15). Thirdly, the two FcεRI isoforms, αγ₂ and αβγ₂, differ in their expression pattern and their functions. While αβγ₂ is expressed on effector cells of the allergic response, mast cells and basophiles, αγ₂ is expressed on antigen presenting cells in humans (rodents do not express αγ₂), where it participates in antigen presentation (16,17).

Protein tyrosine kinases of the src family are responsible for phosphorylating the tyrosines in the ITAMs of the antigen receptors. In the case of FcεRI this kinase is lyn (18,19). Lyn is activated upon FcεRI aggregation. Lyn is bound to β in resting cells by a mechanism that has not been identified. This association increases after FcεRI aggregation (11,20-24). It could be mediated by binding of the lyn SH2 to the tyrosine phosphorylated β, but this is debated (11,20-24). Lyn is responsible for phosphorylating the β and γ chains (19), and lyn activation is the earliest signal detected upon FcεRI aggregation. These findings highlight the critical role played by FcRβ in cell activation after FcεRI aggregation. Phosphorylation of the γ ITAM by lyn
creates binding sites for the two SH2 domains of syk, a protein tyrosine kinase of the syk/ZAP-70 family (19,25). Syk binding to FcRγ leads to lyn-dependent tyrosine phosphorylation and activation of the kinase. This step enables the productive interaction of active Syk with its many targets (26). These targets include various adaptors and enzymes (reviewed in (27,28)). One of these is phospholipase Cγ1, which catalyses the breakdown of membrane phospholipids to generate two second messengers, inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG). These signaling molecules are responsible for calcium (Ca^{2+}) release from intracellular stores and activation of various protein kinase C isoforms, respectively.

To further our understanding of how β acts as a signal amplifier we investigated the role played by each of the three tyrosines of the β ITAM in controlling early signals after FcεRI aggregation. We generated mutant human receptors in which each tyrosine individually or in combination were replaced with phenylalanine, and compared the signaling capacities of these mutated receptors to that of αβγ2 and αγ2. We found that the N terminal tyrosine plays a predominant role in controlling early signals after FcεRI aggregation. Our data also suggest a possible inhibitory role for the middle tyrosine on Ca^{2+} release.

**Experimental procedures**

**Cell Culture**

The human monocytic cell line U937 and its transfectants were maintained in RPMI 1640 as described in (11). Transfectants were selected in the presence of 0.6 mg/ml G418 (Cellgro/Mediatech, Inc., Herndon, VA).
Reagents

Anti-4-hydroxy-3-nitrophenylacetyl (NP) human IgE was from Serotec, Inc. (Raleigh, NC). For FcεRI staining it was used after biotinylation with Biotin-X-NHS (Calbiochem, La Jolla, CA). Fura-2 acetoxymethyl (AM) ester and Pluronic F-127 were from Molecular Probes (Eugene, OR). Streptavidin-phycoerythrin (SA-PE) and monoclonal anti-human Syk and lyn antibodies (Ab) were from BD Biosciences-Pharmingen (San Diego, CA). PE-conjugated goat anti-mouse IgG Ab was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Monoclonal (15-1) and polyclonal (997) anti-human FcεRI α, and polyclonal (934) anti-γ chain Ab were generated in the laboratory (29-31). Monoclonal anti-phosphotyrosine (PY) Ab 4G10 was from Dr. T. Roberts (Dana-Farber Cancer Institute, Boston). Monoclonal anti-V5 Ab was purchased from Invitrogen Life Technologies (Carlsbad, CA) and used chemically coupled to Protein A Sepharose beads with dimethylpipemelimidate (32). Monoclonal anti-PY (clone PY20) agarose was purchased from Sigma-Aldrich (Saint Louis, MO). Protein G Sepharose 4 Fast Flow was purchased from Amersham Biosciences (Piscataway, NJ). NP-bovine serum albumin (NP-BSA) at 25 NP groups per molecule of BSA on average was purchased from Biosearch Technologies, Inc. (Novato, CA).

Construction of U937 stable transfectants

Mutants were created by PCR using the Quickchange kit (Stratagene). The template was the N terminus V5-tagged human β cDNA. The three tyrosine residues in the β ITAM were replaced with phenylalanine, either individually or in combination. The following pairs of primers were used. The mutated codon is underlined. To mutate Y219, sense GGA AAC AAG GTT CCA GAG GAT CGT GTT TTT GAA GAA TTA AAC ATA TAT TCA GCT ACT TAC
and anti-sense GTA AGT AGC TGA ATA TAT GTT TAA TTC TTC AAA AAC ACG ATC CTC TGG AAC CTT GTT TCC. To mutate Y225, sense GAG GAT CGT GTT TAT GAA GAA TTA AAC ATA TTT TCA GCT ACT TAC AGT GAG TTG GAA GAC and antisense GTC TTC CAA CTC ACT GTA AGT AGC TGA AAA TAT GTT TAA TTC ATA AAC ACG ATC CTC. To mutated Y229, sense TTA AAC ATA TAT TCA GCT ACT TTC AGT GAG TTG GAA GAC GCA GGG and antisense CCC TGG GTC TTC CAA CTC ACT GAA AGT AGC TGA ATA TAT GTT TAA. To mutate Y225 in a Y219 mutated template, sense GAG GAT CGT GTT TTT GAA GAA TTA AAC ATA TTT TCA GCT ACT TTC AGT GAG TTG GAA GAC and antisense GTC TTC CAA CTC ACT GTA AGT AGC TGA AAA TAT GTT TAA TTC AAA AAC ACG ATC CTC. To mutate Y229 in a Y225 mutated template, sense TTA AAC ATA TTT TCA GCT ACT TTC AGT GAG TTG GAA GAC CCA GGG and antisense CCC TGG GTC TTC CAA CTC ACT GAA AGT AGC TGA AAA TAT GTT TAA.

Transfectants were generated with the human α and γ cDNAs subcloned into the pCDLSRα296 vector and the V5 epitope-tagged wild type (WT) or mutant human β cDNAs subcloned in the pBJ1neo vector as described in (10).

Assessment of surface FcεRI and total β expression by flow cytometry

To detect surface FcεRI expression cells (10⁶ cells/sample) were stained with 1μg of biotinylated anti-NP human IgE, followed by PE-SA (1:200 dilution), and analyzed in a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). To assess the level of total β expressed, cells (10⁶ cells/sample) were fixed with 1.8% paraformaldehyde in PBS, permeabilized in 0.1% saponin in PBS containing 1% BSA, then stained with 0.5μg anti-V5 Ab
or mouse IgG2a (as isotype control), followed by PE-conjugated goat anti-mouse Ab. Untransfected U937 cells were included in all experiments as negative control.

Assessment of surface FceRI subunit composition

In order to rule out a possible effect of the mutations in β on receptor assembly, the subunit composition of surface receptors was assessed. The various transfectants were loaded with the anti-α mAb 15-1, washed and lysed in mild conditions (0.5% Triton X-100, 50mM Tris-HCl (pH 7.6), 150mM NaCl, 5mM EDTA, 5mM NaF, 5mM Na₃P₂O₇, 1mM sodium vanadate, 10µg/ml pepstatin, 4µg/ml leupeptin, 10µg/ml aprotinin and 1mM phenylmethylsulfonyl fluoride) in order to maintain subunit association. The lysates were placed on ice for 10 min, then centrifuged at 14,000 rpm for 15 min at 4°C. 15-1 bound receptors were precipitated with protein G Sepharose. After a 1-hour incubation at 4 °C, the beads were washed in lysis buffer and the proteins eluted in SDS-PAGE sample buffer. The proteins were resolved by SDS-PAGE, transferred to Immobilon-P membrane (Millipore Corp., Billerica, MA) and blotted with anti-V5 (for β) and anti-γ Abs. Immunoreactive proteins were visualized using the ECL system (Amersham Biosciences, Piscataway, NJ). Films were scanned using the Personal Molecular Imager (BioRad, Hercules, CA) in order to obtain quantitative data (in arbitrary units) for bands on western blots. Background counts present in windows of the same size as the ones used for the bands were subtracted to obtain specific counts.

Cell activation by antigen and lysis

U937 transfectants (10⁷ cells/ml per sample) were incubated with 10 µg/ml anti-NP human IgE for 1 hour at 37°C with rocking. Cells were washed and resuspended at 2.5 x 10⁶
cells/ml in calcium (Ca^{2+}) buffer (pH 7.5) containing 135mM NaCl, 5mM KCl, 1mM MgCl_2, 1mM CaCl_2, 5.6mM glucose, 10mM HEPES (pH 7.4) and 0.1% BSA, and triggered with 25 ng/ml NP-BSA for 2 min at 37°C. Near the end of stimulation, cells were pelleted by brief centrifugation and lysed at 3 x 10^7 cells per ml in the same lysis buffer as above.

**Immunoprecipitation, SDS PAGE, Western transfer and immunoblotting**

Immunoprecipitation was performed with the Abs indicated in each case. Samples were processed as described above and blotted with the indicated Ab. In some cases blots were stripped according to the instructions provided by the manufacturer of the ECL kit and reprobed with a different Ab.

**Measurement of Ca^{2+} mobilization**

Transfected U937 cells were incubated with anti-NP IgE (10μg per 10^7 cells/ml at 37°C for 1 hour) and loaded with 2μM fura-2 AM and 0.2mg/ml Pluronic F-127 in Ca^{2+} buffer at 37°C for 30 min. Samples (5 x 10^6 cells in 2 ml of Ca^{2+} buffer) were analyzed in a cuvette-based Deltascan spectrofluorometer (Photon Technology International Inc., South Brunswick, NJ). Intracellular Ca^{2+} concentrations were recorded as the ratio of Fura-2 emission when excited at 340 and 380 nm.
Results

Mutation of the N terminal tyrosine mimics the phenotype of $\alpha g_2$ receptors on aggregation-induced receptor phosphorylation

In order to study the tyrosines of the $\beta$ chain we took advantage of a reconstitution system that has been extensively characterized in our laboratory and was used to establish that the $\beta$ chain is an amplifier (11,13,14). Stable transfectants were generated in the U937 monocytoid cell line, which does not express the $\alpha$ and $\beta$ chains, using the human $\alpha$ and $\gamma$ cDNAs alone or in conjunction with the wild type (WT) or mutated $\beta$ cDNAs (used as V5 tagged) (Fig. 1A). FcεRI surface expression was measured after staining with biotinylated human IgE and SAPE. For each mutation clones expressing similar FcεRI density at the cell surface were selected for further analysis. The flow cytometric profile of some of these clones is shown in Fig. 1B. Human FcεRI is expressed as two isoforms, $\alpha\beta\gamma_2$ and $\alpha\gamma_2$, which differ in their expression level and signaling capacity. Therefore, it is necessary to verify that mutations of the tyrosines do not affect either expression of $\beta$ or subunit association, which could result in the expression of $\alpha\gamma_2$ trimers along with $\alpha\beta\gamma_2$ tetramers at the cell surface, and would confer a lower signaling capacity upon these cells independently of a direct effect of the mutations in $\beta$. Note that it is unlikely that the receptors expressed at the cell surface on some mutants contain a significant proportion of $\alpha\gamma_2$ complexes since we selected clones with the same level of FcεRI surface expression. If a significant proportion of $\alpha\gamma_2$ complexes was present at the cell surface in certain mutants, the mean expression level of the clones of these mutants would be reduced compared to that of WT transfectants as a result of the loss of the expression amplification effect of $\beta$ (14). We did not observed such an effect. We first verified that among the clones selected for analysis the mutants did not express less $\beta$ than the $\alpha\beta\gamma_2$ clones by staining permeabilized
cells with anti-V5 directed against the V5 tag appended to the N terminus intracellular tail of β
(Fig. 1C). We then assessed the subunit composition of membrane receptors. Cells were
saturated with the anti-α mAb 15-1, washed and lysed. The membrane receptors were
immunoprecipitated with protein G Sepharose, run on SDS-PAGE, and blotted with anti–V5 (β)
and anti-γ (Fig. 1D). Although there was some clonal variability in the relative amounts of β and
γ coprecipitated with α (Fig. 1D, compare clones 1 and 2 of YFY and clones 1 and 2 of FFY), no
consistent differences were observed in the capacity of particular β mutants to interact with α
and γ in vivo, except possibly for FFF. This indicates that there is no systematic effect of the
mutations in β on receptor assembly.

FcεRI aggregation induces phosphorylation of β and γ on the tyrosine residues in the
ITAMs. The role played by individual tyrosines in FcRβ has not been defined. In RBL cells
strongly stimulated via the tetrameric FcεRI the C terminal tyrosine was found to be significantly
more phosphorylated than the N terminal tyrosine, while phosphorylation of the middle tyrosine
was the lowest (33). Whether this difference in phosphorylation intensity of the three tyrosines is
relevant for the β amplification function has not been determined. We compared aggregation
induced β and γ tyrosine phosphorylation among αβγ2, αγ2 and β mutant transfectants. Cells
were loaded with anti-NP IgE and triggered with NP-BSA for 2 min. They were lysed in
conditions (Triton X-100 0.5%) that maintain subunit association, and subjected to
immunoprecipitation with the anti-γ Ab 934 and blotting with the anti-PY Ab 4G10 (Fig. 2A and
data not shown from 2 additional experiments). The intensity of the β and γ bands on the anti-PY
and anti-V5 or anti-γ blots were quantified by scanning. Although we observed some clonal
variability in the amount of β coprecipitated with γ, this is not due to intrinsic differences in the
capacity of specific mutants of β to interact with α and γ in vivo, as we showed in Figure 1D. To
normalize the variable amounts of coprecipitated β, the specific values calculated from the phosphorylated β bands (in arbitrary units) were divided by the ratio of total β to total γ for the same clone calculated from the anti-β (V5) and anti-γ blots. The values were also normalized to the same ratio for the αβγ₂ clones. For phosphorylated γ the values were simply normalized to the value for total γ, and then to the same value for the αβγ₂ clone (Fig. 2A). As expected given the amplifier role of β, β and γ phosphorylation was strong in the αβγ₂ transfectants, and γ phosphorylation was substantially lower in the αγ₂ transfectants (12). Please, note that in the anti-PY blot FcRβ is represented by the top band in the smear between 36 and 22 kD, as demonstrated by the absence of this band in the αγ₂ lane. FcRβ phosphorylation was substantially diminished in the FYY transfectant (ratio to αβγ₂ = 0.21). Furthermore, it was abolished in the double mutants that contain Y219F (FFY and FYF) and in FFF, but not in the double mutant that does not contain Y219F, YFF (ratio = 0.14). This showed that, in this experimental system, phosphorylation of the N terminal tyrosine of β appeared to be predominant, in contrast with what had been shown previously (33). FcRγ phosphorylation was similarly affected. It was substantially diminished in FYY transfectants (ratio to αβγ₂ = 0.08), in the double mutants that contained Y219F (FFY and FYF) (ratio = 0.22 and 0.12, respectively), and in FFF (ratio = 0.19). In fact, in the mutants containing Y219F FcRγ phosphorylation was reduced to the level of αγ₂ (ratio = 0.17). In contrast, in the double mutant that does not contain Y219F, YFF, γ phosphorylation was not decreased, suggesting that the effect of this mutations on β phosphorylation did not extend to downstream events. Similar conclusions about the predominant role of Y219 on β phosphorylation can be drawn when β is directly precipitated with anti-V5 tag Ab (Fig. 2B). All together these results show that the N terminal tyrosine is the most phosphorylated of the three in intact cells, and that its mutation abolishes the β amplifier
function on γ phosphorylation. These results suggested that Y219 may play a predominant role in β function. Y225 and Y229 may contribute to this function.

The Y219F mutation destabilizes the association of lyn with β

Lyn of the src family of protein tyrosine kinases has been shown to be the major kinase responsible for FcεRI tyrosine phosphorylation. Lyn is associated with FcεRI in resting conditions and this association increases after FcεRI triggering. The mode of interaction between β and lyn has not been elucidated completely. We assessed a possible effect of the β ITAM mutations on lyn association with β by comparing the amount of lyn coprecipitated with β in the various transfectants after FcεRI triggering. Cell lysates were immunoprecipitated with anti-V5 to precipitate β and blotted with anti-lyn (Fig. 3 and data not shown from 2 additional experiments). Whereas β-associated lyn was easily detected in αβγ2 transfectants, it was substantially decreased or abolished when Y219 was mutated (FYY, FFY, FYF, FFF), even though reblotting with anti-V5 shows that the amount of immunoprecipitated β in these mutants was equivalent to, or higher than, that in the αβγ2 transfectants. The amount of lyn coprecipitated was normalized to the amount of β precipitated and quantified as a ratio between the densities of the lyn and β band (Fig. 3). The ratio in the FYY transfectant was 11% of the ratio in the αβγ2 transfectant, and 0-22% in FFY, FYF and FFF. In the mutants affecting Y225 and Y229 the ratios were moderately decreased or increased. In particular the double mutation that leaves Y219 intact, YFF, resulted only in a minor decrease in lyn association (ratio = 0.83) when compared to the double mutations that affect Y219 (FFY and FYF). These results favor a predominant role for Y219 in lyn association with β, and suggest that the effect of the Y219
mutation on β and γ phosphorylation is mainly due to its capacity to decrease lyn association with β.

The Y219F mutation decreases FcεRI-aggregation induced syk phosphorylation

Phosphorylation of the γ ITAM creates a binding site for syk, which is recruited to the aggregated receptors, and phosphorylated and activated by lyn and by itself. We assessed the effects of the β mutations on syk phosphorylation after FcεRI aggregation. Syk was immunoprecipitated with an anti-syk and blotted with an anti-phosphotyrosine, or anti-syk as a control for the amount of immunoprecipitated syk. The ratio of phosphorylated syk to total syk was calculated for each clone (Fig. 4 and data not shown from 2 additional experiments). Syk phosphorylation was decreased in the FYY transfectant compared to the αβγ2 transfectant (ratio to αβγ2 = 0.56). This was similar to the ratio seen with αγ2 (0.53). It was also decreased in FFY, FYF and FFF transfectants (ratio = 0.62-0.65). Syk phosphorylation was barely decreased in the other mutants (ratio = 0.8-0.95), in particular in the double mutant that leaves Y219 intact. Although less pronounced than the differences observed in receptor subunit phosphorylation and lyn association, the differences observed between the different β mutants in syk phosphorylation also support the conclusion that Y219 is the tyrosine principally responsible for β amplification function.

The Y219F mutation reduces and delays aggregation-induced Ca²⁺ mobilization

A critical signal downstream of lyn and syk is Ca²⁺ mobilization. We assessed the effect of the β mutations on FcεRI aggregation induced rise in intracellular Ca²⁺. Cells were loaded with fura-2 AM, triggered with antigen and analyzed in a cuvette spectrophotometer. Figure 5A shows the
Ca\textsuperscript{2+} traces obtained at various concentrations of antigen for one representative clone of each type. As expected, a more rapid and intense Ca\textsuperscript{2+} rise was observed in $\alpha\beta\gamma_2$ than in $\alpha\gamma_2$ as a result of the amplifier effect of $\beta$. This difference was more pronounced at low doses of triggering. The Ca\textsuperscript{2+} flux in $\alpha\beta\gamma_2$ was as intense and as rapid at 10 ng/mL as at 25-100 ng/mL of NP-BSA, whereas, in $\alpha\gamma_2$ the Ca\textsuperscript{2+} flux at 10 ng/mL was lower and more delayed than at 25 ng/mL. When Y219 was mutated alone or with one or two other tyrosines (FYY, FFY, FYF and FFF) the Ca\textsuperscript{2+} response was delayed and of lower amplitude compared to $\alpha\beta\gamma_2$. In FYY, FFY and FFF the Ca\textsuperscript{2+} profiles, both in terms of intensity and kinetics, resembled that in $\alpha\gamma_2$, while in FYF, the amplitude was lower and the delay greater than in $\alpha\gamma_2$. In contrast the single mutants with intact Y219 (YFY, YYF and YFF) had profiles closer to that of $\alpha\beta\gamma_2$ than that of $\alpha\gamma_2$.

In order to fully assess the impact of the $\beta$ mutations on Ca\textsuperscript{2+} mobilization we repeated the analysis using multiple clones of each type (5-11 clones per type) and a concentration of 50 ng/mL of antigen. There was no statistically significant difference in Fc\epsilonRI expression levels among the various clone types (data not shown). Two parameters were used to describe the Ca\textsuperscript{2+} responses: amplitude was measured as the difference between baseline and the top of the peak (Fig. 5B); delay was measured from the time when antigen was added to the time when the top of the peak was reached (Fig. 5C). This analysis confirmed that the Y219 mutation alone or in combination (FYY, FFY and FFF) resulted in a reduced Ca\textsuperscript{2+} peak that was similar to that observed in $\alpha\gamma_2$ clones ($p>0.05$ for all). In contrast, mutating the other tyrosines (YFY, YYF, YFF) did not affect peak height significantly compared to the $\alpha\beta\gamma_2$ clones ($p>0.05$ for all).

Analysis of the delay in Ca\textsuperscript{2+} mobilization provided a more subtle picture. All the mutants exhibited delays that were significantly longer than that of $\alpha\beta\gamma_2$ ($p<0.0001$ for each mutant vs. $\alpha\beta\gamma_2$), indicating that each tyrosine has some effects on the Ca\textsuperscript{2+} response. The delay was the
longest in the mutants containing Y219F, being significantly longer than in the αγ2 clones in FYY and FYF (p<0.001 and p<0.0005 vs. αγ2, respectively). This confirmed the predominant role of Y219 already identified with the Ca2+ peak analysis. In the mutants where Y219 was intact (YFY, YYF and YFF) the delays were significantly longer than in αβγ2 clones (p<0.0001), but significantly shorter than in αγ2 clones (p<0.0001-0.01). These results confirmed the conclusion, reached with peak amplitude, that Y225 and Y229 may contribute to Ca2+ mobilization, but by themselves do not play a major role. Taken together these results confirmed the predominant role of Y219 in FcRβ signal amplification observed with FcRγ phosphorylation and lyn association.

One additional element that needs to be taken into account is the likelihood that the function of each tyrosine is not independent of that of the others. In fact, we demonstrated this lack of independence when we could not calculate numerical values for the effect on the Ca2+ delay of each of the tyrosines in their wild-type and mutated states. Nevertheless, this analysis of Ca2+ flux kinetics provided an additional finding that was unexpected. When Y225 was mutated along with Y219 (FFY and FFF), the delay was shorter than when Y225 was intact (p<0.02 for FFY and FFF vs. FYY), and was not significantly different from that of αγ2 (Fig. 5C). Conversely, when Y219 was mutated and Y225 intact (FYY and FYF), the delay was significantly longer than in αγ2. When Y219 was intact (YFY and YFF), mutating Y225 had no effect. This suggested an inhibitory role for Y225, which was unmasked when the amplifying role of Y219 was removed. The longer delay in FYY and FYF compared to αγ2 suggested that this increase could be due to both the loss of the positive effect of Y219 and the persistence of the negative effect of Y225.
Discussion

Activation of lyn and phosphorylation of the receptor β and γ chains are the first signals detected after FcεRI aggregation. The β chain plays a critical role in recruiting and activating lyn, as demonstrated by comparing αγ2 and αβγ2 (12). We show here that mutation of the N-terminal canonical tyrosine in the ITAM has a profound negative effect on early signaling after FcεRI aggregation. Y219F substantially inhibits not only β, but also γ phosphorylation (Fig. 2), and lyn association with β (Fig. 3). As a result, signals downstream of lyn are inhibited, such as syk phosphorylation (Fig. 4) and Ca2+ mobilization (Fig. 5).

It is unlikely that the effects of the tyrosine mutations that we observed here were due to conformational alterations of β. The mutated receptors were expressed at the cell surface at the same level as WT receptors. If the mutated β chains could not associate with α and/or γ, receptor expression would be decreased, or the receptors at the cell surface would be αγ2, and would be expressed at a lower level than WT αβγ2, due to the capacity of β to amplify receptor expression (14). To confirm experimentally that receptor composition was not altered in the mutants, we analyzed subunit composition after precipitating surface receptors specifically by binding an anti-α Ab on intact cells. Figure 1D shows that there was no consistent alteration in the ratio between β and γ in the mutants.

The Y219F mutation induced a more profound impairment in β amplification capacity than did the mutation of the other tyrosines in the β ITAM. Mutating the C terminal canonical tyrosine (Y229) or the non-canonical middle tyrosine (Y225) individually or together had no consistent effect or only milder effects compared to those of Y219F. An additional argument in favor of the role of Y219 is the fact that mutant receptors containing Y219F resembled αγ2 in terms of γ phosphorylation and Ca2+ mobilization. This demonstrates a prominent role for Y219
both in \( \beta \) phosphorylation and in controlling the early signals downstream of receptor phosphorylation. This result was unexpected based on published findings showing that the C terminal canonical tyrosine was the most phosphorylated of the three in rat \( \beta \) (33). In that study the tyrosines phosphorylated after Fc\( \varepsilon \)RI aggregation were identified by comparison of HPLC profiles of digested purified \( \beta \) chains with \textit{in vitro} phosphorylated synthetic peptides. This difference between those results and ours, apart from technical issues, suggests that the high phosphorylation level of the C terminal tyrosine may not indicate an overall importance of that residue in mediating downstream signals. However, both sets of results can be reconciled in a scenario where mutation of Y219 would decrease substantially lyn binding and subsequent phosphorylation of Y225 and Y229. This would result in decreased \( \gamma \) phosphorylation and decreased syk recruitment and activation. In contrast, mutation of Y229 would not affect lyn binding and could result in a compensatory increase in phosphorylation of Y219 and Y225, which would not affect \( \gamma \) phosphorylation and syk recruitment and activation.

In addition to demonstrating the predominant and inhibitory effect of Y219F, our results suggest a mild positive effect of Y225F on Ca\(^{2+}\) flux when associated with Y219F. Some authors have suggested that FcR\( \beta \) could play an inhibitory role. Studies with synthetic peptides showed that the \( \beta \) ITAM could bind the inositol 5\(^{-}\)-phosphatase SHIP (34,35) and the protein tyrosine phosphatase SHP-2 (36). In addition, immunoprecipitation studies showed that SHP-1 and SHP–2 associated with Fc\( \varepsilon \)RI (36), and that they and SHIP were phosphorylated after Fc\( \varepsilon \)RI aggregation (35,36). Both of these phosphatase families have been implicated in Fc\( \varepsilon \)RI signaling regulation ((37) and reviewed in (38,39)). Whether the apparent negative activity associated with Y225 is mediated by the tyrosine phosphatases and inositol phosphatases that have been reported to associate with FcR\( \beta \) \textit{in vitro} remains to be determined.
The interactions between lyn and β include, among other possible mechanisms, the binding of the lyn SH2 to the phosphorylated tyrosines of the β ITAM after receptor aggregation. The interactions between SH2 and phosphorylated tyrosines have been extensively studied ((40-43) and reviewed in (44-48)). In particular, lyn SH2 has been shown to exhibit a preference for the phosphorylated tyrosines of the β ITAM (43). The question of whether both, and furthermore the three, tyrosines in the β ITAM can interact with lyn does not appear to have been addressed. Our results showing the functional importance of the N terminal tyrosine suggest that lyn SH2 interacts preferentially with this tyrosine.

In summary, we have demonstrated that the signal amplification function of FcRβ is mediated principally by N terminal tyrosine through its interaction with lyn. The other two tyrosines may play an accessory role, in particular the non-canonical tyrosine, which could inhibit Ca$^{2+}$ mobilization.
References

1. Kinet, J. P. (1999) Annu Rev Immunol 17, 931-972.
2. Reth, M. (1989) Nature 338, 383-384
3. Weiss, A. (1993) Cell 73, 209-212
4. Cambier, J. C. (1995) J Immunol 155, 3281-3285
5. Letourneur, F., and Klausner, R. D. (1992) Science 255, 79-82
6. Irving, B. A., Chan, A. C., and Weiss, A. (1993) J Exp Med 177, 1093-1103
7. Irving, B. A., and Weiss, A. (1991) Cell 64, 891-901
8. Romeo, C., and Seed, B. (1991) Cell 68, 889-897
9. Jouvin, M. H., Adamczewski, M., Numerof, R., Letourneur, O., Valle, A., and Kinet, J. P. (1994) J Biol Chem 269, 5918-5925
10. Lin, S., Cicala, C., Scharenberg, A. M., and Kinet, J. P. (1996) Cell 85, 985-995
11. Dombrowicz, D., Lin, S., Flamand, V., Brini, A. T., Koller, B. H., and Kinet, J. P. (1998) Immunity 8, 517-529
12. Donnadieu, E., Jouvin, M. H., and Kinet, J. P. (2000) Immunity 12, 515-523
13. Donnadieu, E., Cookson, W. O., Jouvin, M., -H., and Kinet, J.-P. (2000) J immunol 165, 3917-3922
14. Maurer, D., Ebner, C., Reininger, B., Fiebiger, E., Kraft, D., Kinet, J. P., and Stingl, G. (1995) J Immunol 154, 6285-6290
15. Maurer, D., Fiebiger, E., Reininger, B., Ebner, C., Petzelbauer, P., Shi, G. P., Chapman, H. A., and Stingl, G. (1998) J Immunol 161, 2731-2739
16. Eiseman, E., and Bolen, J. (1992) Nature 355, 78-80
17. Kihara, H., and Siraganian, R. P. (1994) J Biol Chem 269, 22427-22432
18. Yamashita, T., Mao, S. Y., and Metzger, H. (1994) Proc Natl Acad Sci U S A 91, 11251-11255
19. Vonakis, B. M., Chen, H., Haleem-Smith, H., and Metzger, H. (1997) J Biol Chem 272, 24072-24080
20. Vonakis, B. M., Haleem-Smith, H., Benjamin, P., and Metzger, H. (2001) J Biol Chem 276, 1041-1050
21. Pribluda, V. S., Pribluda, C., and Metzger, H. (1994) Proc Natl Acad Sci U S A 91, 11246-11250
22. Zhang, J., Berenstein, E. H., Evans, R. L., and Siraganian, R. P. (1996) J. Exp. Med. 184, 71-79
23. El-Hillal, O., Kurosaki, T., Yamamura, H., Kinet, J. P., and Scharenberg, A. M. (1997) Proc Natl Acad Sci U S A 94, 1919-1924
24. Turner, H., and Kinet, J. P. (1999) Nature 402, B24-30.
25. Rivera, J. (2002) Curr Opin Immunol 14, 688-693
26. Wang, B., Rieger, A., Kilgus, O., Ochiai, K., Maurer, D., Fodinger, D., Kinet, J. P., and Stingl, G. (1992) J Exp Med 175, 1353-1365
27. Letourneur, O., Sechi, S., Willette-Brown, J., Robertson, M. W., and Kinet, J. P. (1995) J Biol Chem 270, 8249-8256
31. Letourneur, O., Kennedy, I. C., Brini, A. T., Ortaldo, J. R., O'Shea, J. J., and Kinet, J. P. (1991) *J Immunol* 147, 2652-2656
32. (1988) in *Antibodies: A laboratory manual* (Harlow, E., and Lane, D., eds), pp. 524-525, Cold Spring Harbor Laboratory
33. Pribluda, V. S., Pribluda, C., and Metzger, H. (1997) *J Biol Chem* 272, 11185-11192
34. Osborne, M. A., Zenner, G., Lubinus, M., Zhang, X., Songyang, Z., Cantley, L. C., Majerus, P., Burn, P., and Kochan, J. P. (1996) *J Biol Chem* 271, 29271-29278
35. Kimura, T., Sakamoto, H., Appella, E., and Siraganian, R. P. (1997) *J Biol Chem* 272, 13991-13996
36. Kimura, T., Zhang, J., Sagawa, K., Sakaguchi, K., Appella, E., and Siraganian, R. P. (1997) *J Immuno* 147, 2652-2656
37. Huber, M., Helgason, C. D., Damen, J. E., Liu, L., Humphries, R. K., and Krystal, G. (1998) *Proc Natl Acad Sci U S A* 95, 11330-11335
38. Scharenberg, A. M. (1999) *Curr Opin Immunol* 11, 621-625
39. Katz, H. R. (2002) *Curr Opin Immunol* 14, 698-704
40. Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J., Overduin, M., and et al. (1992) *Nature* 358, 646-653
41. Eck, M. J., Shoelson, S. E., and Harrison, S. C. (1993) *Nature* 362, 87-91
42. Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D., and Kuriyan, J. (1993) *Cell* 72, 779-790
43. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et al. (1993) *Cell* 72, 767-778
44. Kuriyan, J., and Cowburn, D. (1997) *Annu Rev Biophys Biomol Struct* 26, 259-288
45. Bradshaw, J. M., and Waksman, G. (2002) *Adv Protein Chem* 61, 161-210
46. Waksman, G., and Kuriyan, J. (2004) *Cell* 116, S45-48, 43 p following S48
47. Pawson, T. (2004) *Cell* 116, 191-203
48. Songyang, Z., and Cantley, L. C. (2004) *Cell* 116, S41-43, 42 p following S48
Figure legends

Figure 1. Expression of human FcεRI in transfected U937

(A) Sequences of canonical ITAM, atypical β ITAM and the mutants used in this study. (B) Flow cytometric analysis of surface FcεRI in one selected transfectant of each type. Cells (transfectant, right hand peak, untransfected control, left hand peak) were stained with biotinylated IgE and SAPE. (C) Flow cytometric analysis of V5-tagged β expression in permeabilized cells from the same clones as in (B). Cells were fixed and permeabilized before staining with anti-V5 tag monoclonal antibody and PE-conjugated goat anti-mouse antibody (transfectant, right hand peak, untransfected control, left hand peak, except for αγ2 where the order is reverse) (D) Assessment of the cell surface receptor composition. Cells were loaded with the anti-α mAb 15-1, washed, lysed in non-dissociating conditions and analyzed by SDS PAGE. The β and γ bands were detected by western blotting with anti-V5 (β) and anti-γ, and quantified by scanning.

Figure 2. FcεRI-aggregation induced tyrosine phosphorylation of β and γ is substantially diminished when Y219 is mutated.

(A) Transfectants were triggered with antigen for 2 min (Ag +) or left untriggered (Ag -), lysed, immunoprecipitated with anti-γ and blotted with anti-PY. The blots were stripped and reprobed with anti-V5 and anti-γ. The intensity of the β and γ bands on the anti-PY and anti-V5 or anti-γ blots were quantified by scanning. The specific values calculated from the phosphorylated β bands (in arbitrary units) were divided by the ratio of total β to total γ for the same clone calculated from the anti-β (V5) and anti-γ blots. The values were also normalized to the same
ratio for the $\alpha\beta\gamma_2$ clones. For phosphorylated $\gamma$ the values are expressed as the ratio of phosphorylated to total $\gamma$ bands for each clone after normalization to the same ratio for the $\alpha\beta\gamma_2$ clone. The experiment shown here is representative of three similar ones. (B) Same as in (A) except that lysates were precipitated with anti-V5 instead of anti-$\gamma$.

**Figure 3. FcεRI-aggregation induced association of lyn with $\beta$ is decreased when Y219 is mutated.**

Transfectants were triggered with antigen for 2 min (Ag +) or left untriggered (Ag -), lysed, immunoprecipitated with anti-V5 and blotted with anti-lyn. The blots were reprobed with anti-V5. The intensities of the lyn and $\beta$ bands were quantified by scanning. The values are expressed as the ratio between lyn and $\beta$ for each clone after normalization to the same ratio for the $\alpha\beta\gamma_2$ clone. The experiment shown here is representative of three similar ones.

**Figure 4. FcεRI-aggregation induced tyrosine phosphorylation of syk is reduced when Y219 is mutated.**

Transfectants were triggered with antigen for 2 min (Ag +) or left untriggered (Ag -), lysed, immunoprecipitated with anti-syk and blotted with anti-PY. The blots were reprobed with anti-syk. The intensities of the syk bands on the anti-PY and anti-syk blots were quantified by scanning. The values are expressed as the ratio between phosphorylated and total syk for each clone after normalization to the same ratio for the $\alpha\beta\gamma_2$ clone. The experiment shown here is representative of three similar ones.
Figure 5. FcεRI-aggregation induced calcium mobilization is reduced and delayed when Y219 is mutated.

(A) Transfectants were loaded with anti-NP human IgE and the fluorescent calcium indicator Fura-2, and placed in the cuvette of the spectrofluorometer. Signal collection was started and baseline signal was recorded for 30 sec after which the cells were triggered with 10, 25, 50 and 100ng/mL of the multivalent antigen NP-BSA (arrow). Ca²⁺ concentrations are expressed as the ratio of fura-2 emission with excitation at 340 and 380 nm. (B) Multiple clones for each type (αβγ₁: 9, αγ₂: 10, FYY: 10, FFY: 8, FYF: 5, FFF: 11, YFY: 8, YFF: 8, YFF: 5) were analyzed after triggering with 50 ng/mL of NP-BSA. Peak height was measured from baseline to the top of the peak. Values are mean +/- SD and were compared by unpaired t test. (C) The same clones as in (B) were compared for the delay in Ca²⁺ responses. Delays were measured between the time of antigen addition and the time when the maximum of the peak was reached and are expressed in seconds. Values are mean +/- SD and were compared by unpaired t test.
Figure 1A

A

|                     | D/EX₂ | Y X₁L/I | X₇₁₁ | YX₂LL/I |
|---------------------|-------|---------|-------|---------|
| Canonical ITAM      |       |         |       |         |
| hFcRβ ITAM          | DRV   | YEEL    | NIYSAT| YSELE   |
| Y219F (FYY)         | DRV   | FEEL    | NIYSAT| YSELE   |
| Y225F (YFY)         | DRV   | YEEL    | NIFSAT| YSELE   |
| Y229F (YYF)         | DRV   | YEEL    | NIYSAT| FSELE   |
| Y225F Y229F (YFF)   | DRV   | YEEL    | NIFSAT| FSELE   |
| Y219F Y225F (FFY)   | DRV   | FEEL    | NIFSAT| YSELE   |
| Y219F Y229F (FYF)   | DRV   | FEEL    | NIYSAT| FSELE   |
| Y219F Y225F Y229F (FFF) | DRV | FEEL | NIFSAT | FSELE |

Figure 1B

B

Fluorescence Intensity
Figure 1C

C

Fluorescence Intensity

Figure 1D

D

|     | αβγ2 | FYY | YFY | YFF | YFF | FFY | FFY | FFF |
|-----|------|-----|-----|-----|-----|-----|-----|-----|
| cl. 1 | 1   | 0.99 | 0.96 | 0.8  | 0.96 | 0.78 | 0.98 | 0.94 |
| cl. 2 | 1.02| 0.96 | 0.94 | 0.81 |   |     |     |     |

β/γ
Figure 2A

A

IP: anti-γ

| Ag: | αβγ2 | FYY | YFY | YFF | YFF |
|-----|------|-----|-----|-----|-----|
| phosphorylated β | - | + | - | + | - | + |
| phosphorylated γ | - | + | - | + | - | + |
| total β | 1 | 1.35 | 1.33 | 1.91 | 3.84 | 1 |

| phosphorylated ß / total ß | 1 | 0.21 | 1.56 | 0.42 | 0.14 | 1 | 0 | 0 | 0 |

Figure 2B

B

IP: anti-ß (V5)

| Ag: | αβγ2 | FYY | YFY | YYF | YFF | αβγ2 | FYY | FYF | FFF | αγ2 |
|-----|------|-----|-----|-----|-----|------|-----|-----|-----|-----|
| anti-PY blot | - | + | - | + | - | + | - | + | - | + |
| anti-ß (V5) blot | - | + | - | + | - | + | - | + | - | + |

| phosphorylated ß / total ß | 1 | 0.24 | 0.46 | 0.67 | 0.49 | 1 | 0.01 | 0.02 | 0.01 |
**Figure 3**

**IP: Anti-β (V5)**

|        | αβγ2 | FY2 | YFY | YYF | YFF | αβγ2 | FY2 | FYF | FFF | αγ2 |
|--------|------|-----|-----|-----|-----|------|-----|-----|-----|-----|
| **Ag:**|      | -   | +   | -   | +   | -    | +   | +   | -   | +   |

**Anti-Lyn blot**

|        | αβγ2 | FY2 | YFY | YYF | YFF | αβγ2 | FY2 | FYF | FFF | αγ2 |
|--------|------|-----|-----|-----|-----|------|-----|-----|-----|-----|
| **lyn/β** | 1 | 0.11 | 0.67 | 1.25 | 0.83 | 1 | 0.00 | 0.22 | 0.03 |
Figure 4

IP: Anti-Syk

| Ag: | αβγ2 | FYY | YFY | YYF | YFF | αβγ2 | FFY | FYF | FFF | αγ2 |
|-----|------|-----|-----|-----|-----|------|-----|-----|-----|------|
| Anti-PY blot | - | + | - | + | - | + | + | - | + | - |
| Anti-Syk blot | + | - | + | - | + | + | - | - | + | - |

| phosphorylated syk /total syk | αβγ2 | FYY | YFY | YYF | YFF | αβγ2 | FFY | FYF | FFF | αγ2 |
|-------------------------------|------|-----|-----|-----|-----|------|-----|-----|-----|------|
|                               | 1    | 0.56 | 0.95 | 0.8 | 0.9 | 1    | 0.65 | 0.64 | 0.62 | 0.53 |
Figure 5B

B

|        | FYY | YFY | YFF | YFF | FYF | FYF | FFF | αγ2 |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|
| p vs. αβγ2 | <0.001 | >0.05 | >0.05 | >0.05 | <0.001 | <0.05 | <0.005 | <0.005 |
| p vs. αγ2  | >0.05 | <0.02 | <0.005 | <0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
Figure 5C

|               | FYY | YFY | YFF | YF | FF | FY | FFF | αγ<sub>2</sub> |
|---------------|-----|-----|-----|---|----|----|-----|----------------|
| p vs. αβγ<sub>2</sub> | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| p vs. αγ<sub>2</sub> | <0.001 | 0.0001 | <0.0001 | <0.01 | >0.05 | <0.0005 | >0.05 |
| p vs. FYY     |     |     | <0.001 | <0.02 |     |     | <0.02 |     |

| DELAY TIME (sec.) |
|-------------------|
| 250               |
| 200               |
| 150               |
| 100               |
| 50                |

C
Molecular dissection of the FcRβ signaling amplifier
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J. Biol. Chem. published online August 30, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404890200

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