

FUNCTIONAL COMPARISON OF FcεRI, FcγRII, AND FcγRIII IN MAST CELLS

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The cellular responses initiated by cross-linking rodent FcγRII-b1, FcγRII-b2, FcγRIII, and FcεRI in mast cells were compared. Individual murine FcγR isoforms were transfected into rat basophilic leukemia cells and after cross-linking the FcR, changes in the phosphorylation of protein tyrosines, in the level of intracellular Ca²⁺, in the hydrolysis of phosphoinositides, and in the release of arachidonic acid metabolites and hexosaminidase were monitored. Cross-linking of FcγRIII initiated all of these early and late biochemical functions, and although they were quantitatively somewhat smaller, the responses were qualitatively indistinguishable from those stimulated by the endogenous FcεRI. However, despite ample expression, neither FcγRII-b1 nor FcγRII-b2 stimulated these functions when cross-linked. The functional differences between FcγRII and FcγRIII were studied further by assessing the responses to cross-linking of the endogenous FcγR (FcγRII-b1, FcγRII-b2, and FcγRIII) on P815 mouse mastocytoma cells that had been transfected with normal or functionally defective FcεRI. Two types of mutant subunits had previously been observed to impair the activity of FcεRI: γ-chains missing the cytoplasmic domain, and β-chains missing the COOH-terminal cytoplasmic domain. In both types of transfectants the functional inhibition of the endogenous FcγR paralleled that of the transfected FcεRI. These results are consistent with the γ subunit being associated with the functions of FcγRIII as well as of FcεRI. The functional results also complement the recently reported evidence that FcγRIII can interact with FcεRI β-subunits (*J. Exp. Med.* 175:447, 1992).

In addition to high affinity receptors for IgE (FcεRI), rodent mast cells express distinctive receptors for IgG (FcγRII-b1, FcγRII-b2, and FcγRIII) (1). The structural characteristics of these receptors and their relationship to human FcγR and FcεRI are summarized in Figure 1. Each of these FcR contains a polypeptide whose exodomain binds the Fc region of Ig E or G, respectively. These exodomains are themselves constructed from two Ig-like domains (2, 3). For those receptors such as FcεRI and

FcγRIII that have multiple subunits, the Ig-binding polypeptides (called α-chains) have additional shared features: their transmembrane domains (but not their cytoplasmic domains) are highly homologous and they are each associated with an additional type of subunit—the γ subunit (below). The transmembrane and cytoplasmic domains of murine FcγRII-b1 and FcγRII-b2 differ from those in the α-chains of FcεRI and FcγRIII. Although homologous to each other, the cytoplasmic domain of FcγRII-b1 contains a 47 amino acid “insert.” The cytoplasmic domain of human FcγRIIC is moderately homologous to murine FcγRII-b2 but the A and B isoforms, are nonhomologous (4).

FcεRI α is associated with a β-chain and a dimer of γ-chains (5). The β-chain is a type III membrane protein that is composed of four transmembrane domains and whose COOH-terminal and NH₂-terminal ends point into the cytoplasm (6). Like the α-chain, each γ-chain is a type I transmembrane protein (7). As already noted, the same γ-chains that assemble with FcεRI associate with FcγRIII also (8), although the number of γ-chains in FcγRIII has not yet been rigorously determined. Initially, FcγRIII was not thought to be complexed with β-chains and it certainly is expressed and functional on cells lacking β-chains (9, 10). However, it has recently been observed that when both are present, β-chains can associate with FcγRIII (11). No polypeptides are known to be associated with the α-like chains of any isoform of FcγRII(9).

In our work we compared the functional responses initiated by FcεRI, FcγRII-b1, FcγRII-b2, and FcγRIII in mast cells. For one part of the analysis we transfected individual FcγRII/III isoforms into the well known RBL³ cells and compared the signals initiated by stimulation of the endogenous FcεRI with those initiated by the transfected FcγR. For a second part of the analysis, we compared the activity of the endogenous FcγR in the mouse mastocytoma P815 (10), with transfectants of the latter expressing wild-type or mutant FcεRI (12).

MATERIALS AND METHODS

Cells. RBL-2H3 cells were cultured as a monolayer in Iscove's modified Dulbecco's medium including 25 mM HEPES (Biofluids, Inc., Rockville, MD), 17% heat-inactivated FCS (Biofluids), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (medium) at 37°C in 5% CO₂ in a humidified incubator. The medium used to select stable transfectants also contained geneticin (G418, GIBCO, Grand Island, NY) at 0.5 mg/ml (complete medium). P815 HTR cells were cultured as described recently (12).

Expression vectors. Mouse FcγRII-b1 in pcEXV-3 was a gift from Dr. J. Ravetch (Memorial Sloan-Kettering Cancer Center, NY, NY).

³ Abbreviations used in this paper: RBL, rat basophilic leukemia; CHAPS, (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate); PI, phosphoinositide; DNP, dinitrophenyl.

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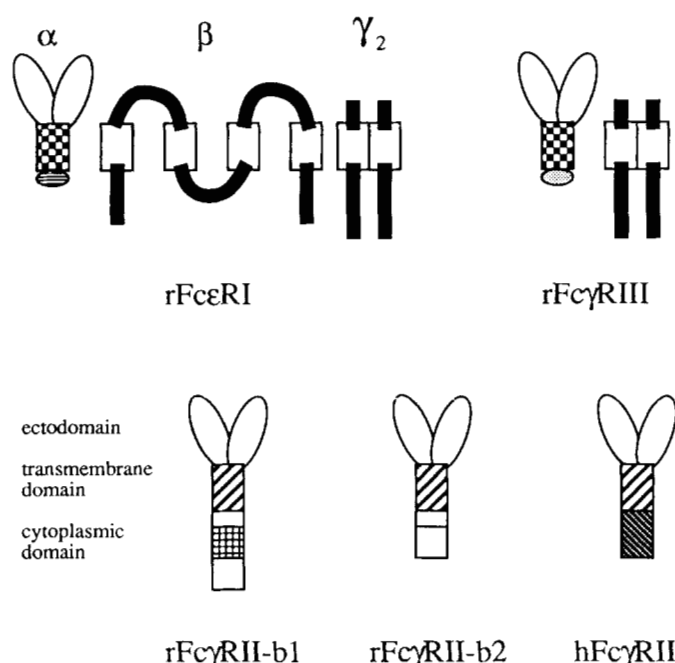


Figure 1. Schematic representation of the subunit structure of rodent (r) Fc ϵ RI, Fc γ RIII, the two different isoforms of Fc γ RII and the human (h) Fc γ RII A or B isoforms. The polypeptide marked α in the schematic of Fc ϵ RI represents the polypeptide whose exodomain (composed of two Ig-like domains depicted as ellipsoids) contains the binding site for the Fc region of IgE. The Ig-binding portions of all the other FcR depicted, show substantial homology. For each receptor, the first rectangle below the ellipsoids represents the transmembrane domain of the α - or α -like chains. They are pattern-coded to indicate the homologous regions. Below the first rectangle is a small ellipsoid or rectangle that schematically represents the cytoplasmic domain and its relative size. Again pattern coding has been used to illustrate the sequence relationships. The β -subunit and dimer of γ -subunits of the tetrameric Fc ϵ RI are more simply drawn, and their transmembrane domains (four per β and one per γ) are each distinctive. Fc γ RIII is also associated with a dimer of γ -chains identical to those that are found in Fc ϵ RI. Alternatively, Fc γ RIII may associate with the related δ -chains. As discussed here and elsewhere (11), where it is present (transfectants or mast cells), Fc γ RIII can associate with the Fc ϵ RI β subunit also. The Ig-binding polypeptides of rodent or human Fc γ RII are not known to be associated with any other subunits.

Mouse Fc γ RII-b2 and mouse Fc γ RIII in pSVL was kindly provided by Dr. J.-P. Kinet (NIAID, NIH, Bethesda, MD) and Dr. J. W. Shiver (Merck Sharpe & Dohme, West Point, PA) provided the selective marker plasmid pSV2neo. Transformed bacteria containing pSV2neo were obtained from the American Tissue Culture Collection, Bethesda, MD.

Transfections. RBL cells at subconfluent growth density were transfected by electroporation. After trypsinization, the cells were washed once in medium and then twice in ice-cold PBS. A total of 25 μ g of DNA was added to 2×10^7 cells suspended in 0.1 ml of PBS. The non-linearized Fc γ R-plasmid plus pSV3neo were used at a molar ratio of 25:1. The cell suspension with the DNA was transferred to a gene pulser cuvette (Bio-Rad, Richmond, CA), kept on ice for 10 min, and then pulsed at a voltage of 250 V and a capacitance of 250 μ F using a Bio-Rad gene pulser. After another 10 min on ice, the samples were diluted in 40 ml of medium and seeded into four 10-cm diameter tissue culture dishes (Falcon Labware, Oxnard, CA). Two days after electroporation, the cells were grown in selective medium containing 0.5 mg/ml G418. During selection with G418 the medium was changed twice a week. Two types of transfectants were examined in this study: cloned populations and uncloned sorted populations. For the former, G418-resistant colonies were isolated 2 to 3 wk after transfection with cloning cylinders (Bellco, Glass Inc., Vineland, NJ) and transferred to a 24-well cluster dish for expansion and analysis. A total of 10 to 20 clones per transfectant were screened using the fluorescein-conjugated rat monoclonal anti-mouse Fc γ R, 2.4G2 (13) and a fluorescence-activated analyzer (Coulter Electronics, Hialeah, FL, Epics Profile flow cytometer). For the uncloned populations, transfected cells were subjected to two or three rounds of cell sorting. The cells were stained with fluorescein-conjugated antibody 2.4G2 and the brightest 1% selected for expansion over a 1-wk period. An aliquot of the final population was assayed for expression of Fc γ R.

The P815 transfectants used in this study have all been described (12): Fc ϵ RIwt refers to P815 cells transfected with cDNA coding for wild-type α -, β -, and γ -subunits; Fc ϵ RI β -ct refers to P815 cells transfected with cDNA coding for wild-type α and γ , and a β -subunit with a truncated COOH-terminus; Fc ϵ RI γ -ct refers to P815 cells transfected with cDNA coding for wild-type α and β and a γ -subunit with a truncated COOH-terminus.

Ig. Antibody 2.4G2 was purified from ascites of nude mice that had received injections with 2.4G2 hybridoma cells obtained from the American Tissue Culture Collection. Purification followed basically the protocol described previously using precipitation from 45% saturated $(\text{NH}_4)_2\text{SO}_4$ and passage over DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ) (13). [^{125}I]-2.4G2 was prepared by the chloramine T method. Fluoresceinated 2.4G2 was prepared by the method of Schreiber and Haimovich (14), and such preparations averaged 4 mol of fluorophore/mol of protein. Affinity-purified goat anti-rat IgG (H+L) or rabbit Fc-specific anti-rat IgG used for cross-linking of cell-bound 2.4G2, were purchased from The Jackson Laboratories, Bar Harbor, ME. Anti-DNP monoclonal mouse IgE was the product of the hybridoma Hi-DNP ϵ 26.82 (15) and was purified as previously described (16). mAb to the rat Fc ϵ RI (mAb 5.14 (17)) was a generous gift from Dr. Zelig Eshhar (Weizmann Institute of Science, Rehovot, Israel); monoclonal anti-rat Fc ϵ RI β antibody JRK has been described (18).

Analysis of protein tyrosine phosphorylation. RBL transfectants were seeded at 2.5×10^5 cells/well overnight in complete medium. These cells were sensitized with 5 μ g/ml of either anti-DNP IgE or 2.4G2 for 1 to 2 h at 37°C. P815 cells were sensitized at 5×10^5 cells/ml with 10 μ g/ml of either anti-DNP IgE or 2.4G2 for 1 h at 37°C. After sensitization, the cells were washed twice with warm assay buffer (119 mM NaCl, 5 mM KCl, 0.4 mM MgSO_4 , 25 mM Pipes, 5.6 mM glucose, 0.1% BSA, titrated to pH 7.2 with NaOH) and stimulated for 3 min with either DNP $_{25}$ -BSA (400 ng/ml) or goat anti-rat IgG (H+L) (12.5 μ g/ml). Unstimulated cells were taken as controls for basal cellular tyrosine phosphorylation. After removal of the stimulant, RBL cells were solubilized directly with 0.2 ml hot sample buffer (25 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol) containing 1 mM Na_3VO_4 . P815 cells were first solubilized in ice cold lysis buffer (10 mM Tris/HCl, pH 7.4, 50 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 1 mM Na_3VO_4 , 5 μ M ZnCl_2 , 0.5% Triton X-100, 25 μ g/ml PMSF, 30 μ g/ml aprotinin, 10 μ g/ml leupeptin) for 15 min on ice at 2×10^7 cells/ml and then centrifuged at $16,000 \times g$ for 30 min at 4°C to remove the insoluble material. SDS-sample buffer was added to a final concentration of 25 mM Tris/HCl pH 6.8, 2% SDS, and 10% glycerol. All samples were boiled for 5 min and centrifuged before electrophoresis on a 12% SDS-PAGE. Unless otherwise indicated, 1×10^5 cell equivalents were loaded per lane. Proteins were transferred to 0.2 μ m nitrocellulose (Schleicher & Schuell, Keene, NH) for 1 h at 2.5 mA/cm ^{2} with a semi-dry "transblotter" (Millipore Continental Water Systems, Bedford, MA). Nonspecific binding sites were blocked by incubating the nitrocellulose sheets for 1 h at 37°C in blocking buffer (50 mM Tris/HCl, pH 7.5, 0.05% Tween 20, 0.9% NaCl, 4% BSA). The blots were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated antiphosphotyrosine antibody PY20 (ICN K & K Laboratories Inc., Plainville, NY). The blots were rinsed twice, followed by two 10-min washes with blocking buffer. After a final rinse with 50 mM Tris/HCl, pH 7.5, 0.9% NaCl, 0.05% Tween 20, tyrosine phosphorylated proteins were detected by chemiluminescence (Amersham Corp., Arlington Heights, IL) according to the manufacturer's directions. Blots were exposed to Hyperfilm (Amersham) for 2 to 10 min.

Immunoprecipitations. RBL cells transfected with the Fc γ RIII α were suspended at 1×10^7 cells/ml and sensitized with either 10 μ g/ml 2.4G2 or 10 μ g/ml mIgE for 1 h at room temperature. The cells were washed twice in assay buffer, suspended at 5×10^6 cells/ml/sample, and stimulated with 12.5 μ g/ml rabbit Fc specific anti-rat IgG (for cells sensitized with 2.4G2), or 400 ng/ml DNP-BSA (for cells sensitized with mIgE) for 3 min at 37°C. Sensitized but nonstimulated cells served as controls. All samples were rapidly pelleted at 4°C and solubilized in ice-cold 10 mM CHAPS, borate-buffered saline (0.2 M boric acid, 160 mM NaCl, pH 8.0), 30 mM Na pyrophosphate, 50 mM NaF, 1 mM Na_3VO_4 , 5 μ M ZnCl_2 , 175 μ g/ml PMSF, 30 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin A for 30 min on ice at 5×10^7 cells/ml. Insoluble material was removed by centrifugation at $16,000 \times g$ for 15 min at 4°C, and the supernatants were diluted to 2 mM CHAPS with buffer. IgE-sensitized samples were immunoprecipitated with 10 μ g/ml rabbit anti-mouse IgE (Fab') $_2$ for 1 h at 4°C. All samples received 50 μ l of a 50% protein A-Sepharose (Pharmacia) solution for 1 h at 4°C (IgE-sensitized samples), or 2 h at 4°C (2.4G2-sensitized samples). Protein A-Sepharose pellets were washed twice with 500 μ l 2 mM CHAPS in borate-buffered saline. A total of 100 μ l SDS-sample buffer were added and the samples were treated as described above, before analysis on 12%

SDS-PAGE. With these conditions, using 10% iodinated mIgE or 2.4G2, we found that the percent of counts solubilized and immunoprecipitated were similar regardless of whether the Fc ϵ RI or Fc γ R had been aggregated. The average values were: 74% of the IgE and 72% of the 2.4G2 counts were solubilized, and 65% of the IgE and 49% of the 2.4G2 counts were immunoprecipitated.

Calcium and phosphoinositide measurements. Intracellular calcium concentrations were measured as described by Maeyama et al. (19) using Quin2 as an indicator of the concentration of intracellular free calcium. PI turnover was examined as described (12) using the method of Beaven et al. (20).

Arachidonic acid and hexosaminidase measurements. RBL transfectants were plated into a 24-well cluster dish at 2×10^5 cells/0.5 ml/well and incubated with 0.1 μ Ci/ml [^{14} C]-arachidonic acid (Du Pont-NEN, Boston, MA) overnight in medium at 37°C and 5% CO $_2$. After 18 to 24 h the cells were washed and incubated in normal medium for an additional 1 h and sensitized with 5 μ g/ml antibody. Cells were then washed twice in assay buffer (above) including in this case 0.1% delipidated BSA and 1 mM CaCl $_2$ (21). Cells were stimulated for 30 min at 37°C (0.2 ml/well) with 10 ng/ml DNP-BSA or 0.5 μ g (2.5 μ g/ml) goat anti-rat IgG. Supernatants were quickly transferred to microtubes and placed on ice. The supernatants were spun for 2 min in a microcentrifuge at 12,000 $\times$ g. For determination of arachidonic acid release, an aliquot was removed and added directly to liquid scintillation counting vials along with 15 ml of scintillation fluid (UltimaGold, Packard Instrument Company, Meriden, CT). Samples were measured in a Tri-Carb 2500 TR liquid scintillation counting system (Packard Instrument Company). The remaining monolayer of cells was lysed by adding 0.1% Triton X-100 (0.5 ml/well). The lysate of cells was used to calculate total incorporation of [^{14} C]-arachidonic acid.

For determination of hexosaminidase release, 20 μ l of supernatant were transferred to a 96-well cluster dish (flat-bottomed wells). Supernatants were incubated with 1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in citrate buffer, pH 4.5, for 1 h at 37°C with continuous shaking. At the end of the incubation, 0.25 ml of a 1:1 mixture of 0.1 M Na $_2$ CO $_3$ and 0.1 M NaHCO $_3$ was added and the absorbance at 400 nm determined in an ELISA plate reader. A total of 20 μ l of the lysate of the cells was used to calculate the total hexosaminidase content.

RESULTS

Expression of transfected Fc γ R in RBL. Stable transfectants were generated by transfecting RBL cells with the cDNA for the α - or α -like chains of mouse Fc γ RII-b1, Fc γ RII-b2, or Fc γ RIII using SV40-based vectors. After transfection, G418-resistant colonies were obtained at a frequency of about 10^{-5} . Expression of Fc γ R was determined with mAb 2.4G2, a rat monoclonal anti-Fc γ R antibody that binds to a common epitope present on the extracellular portions of mouse Fc γ RII-b1/2 and Fc γ RIII (13). Either fluorescein-conjugated antibody and FACS analysis, or iodinated antibody and direct binding studies were used. Binding of labeled 2.4G2 to the RBL cells transfected with each of the Fc γ R was inhibited by preincubation of the cells with an excess of unlabeled 2.4G2, and untransfected RBL cells did not bind the antibody (data not shown). The results obtained with uncloned and cloned cell populations were similar. RBL cells transfected with Fc γ RII-b1 bound 2.4×10^5 molecules of 2.4G2 per cell (uncloned population). Those transfected with Fc γ RII-b2 also bound 2.4×10^5 molecules 2.4G2 per cell (uncloned, sorted population) or 4.2×10^5 molecules per cell (cloned population). RBL cells transfected with Fc γ RIII bound 2.4×10^4 to 6×10^4 molecules of 2.4G2 (uncloned, sorted populations) and 1.1×10^4 molecules of 2.4G2 (cloned population) per cell (Table I). The expression of the Fc γ R was stable during the course of this study. The expression of Fc ϵ RI appeared to be unaffected by the transfections and equaled 1.9 to 3.7×10^5 /cell in all of the transfectants.

Functional responses by RBL transfectants. Five responses were studied in the RBL transfectants. Three of

these are relatively early events: increased phosphorylation of tyrosines on selected cellular proteins (22–24), accelerated hydrolysis of PI (25), and a rise in intracellular calcium (20). However, release of arachidonic acid metabolites (26) and of hexosaminidase due to exocytosis, are relatively later events. In each case, the responses initiated by the transfected Fc γ R were compared to those triggered by the endogenous Fc ϵ RI (Table I).

Cells were "sensitized" or not with either IgE or 2.4G2, and then stimulated or not with either Ag, or a second antibody (goat anti-rat IgG), respectively. The SDS extracts of such cells were then electrophoresed on gels and finally analyzed for phosphotyrosine. The results for the unstimulated samples were the same regardless of whether they were sensitized or not. Figure 2 shows representative results of such experiments. Lanes 1 to 4 show extracts from RBL cells transfected with Fc γ RII-b1; lanes 5 to 8, extracts from cells transfected with Fc γ RII-b2; lanes 9 to 12, extracts from cells transfected with Fc γ RIII. The cells in lanes 1 and 2 had been incubated with IgE and then either without (lane 1) or with (lane 2) Ag. It is apparent that the addition of Ag has stimulated the phosphorylation of tyrosines on several proteins. Particularly prominent is the component with a molecular mass of about 72 to 75 kDa (22). We have also found that the stimulated phosphorylation of tyrosines is most reproducibly seen on this component, and a very similar component is seen in P815 cells (below). For this reason we focussed on this component for the quantitative studies. The cells transfected with Fc γ RII-b2 (lanes 5 and 6) or with Fc γ RIII (lanes 9 and 10) showed comparable stimulation when their Fc ϵ RI were aggregated. (The less pronounced phosphorylation of the 90- to 100-kDa component in the Fc γ RII-b2 transfectant (lane 6) is more apparent than real, and was not a reproducible finding.)

Addition of 2.4G2 and a second antibody failed to stimulate the RBL cells transfected with either Fc γ RII-b1 or Fc γ RII-b2 (lane 4 and lane 8, respectively) but promoted phosphorylation in the cells transfected with Fc γ RIII (lane 12). The pattern of proteins exhibiting enhanced phosphorylation of tyrosines in response to aggregation of Fc γ RIII appeared virtually identical to those whose phosphorylation was stimulated when the endogenous Fc ϵ RI were aggregated (lane 12 vs lane 10). In the unreduced samples a narrow band at about 85 kDa was seen in all the cells stimulated via Fc ϵ RI but was not clearly apparent in the cells stimulated via the Fc γ RIII. This was less apparent in samples that had been treated with reducing agents (data not shown).

The Fc γ RIII were able to initiate all of the other responses we monitored (Fig. 3; Table I) although on the RBL transfectants, all of the responses were only about one-third as great as the responses initiated by Fc ϵ RI on the same cells. However, the Fc γ RII-b1 and Fc γ RII-b2 transfectants failed to respond (Fig. 3; Table I). Except for the release of hexosaminidase (which showed greater variability), the responses triggered via the endogenous Fc ϵ RI were similar in all the transfectants (see footnotes in Table I; Fig. 3). Clearly therefore, the failure of the Fc γ RII to trigger responses was not simply due to the cells being nonspecifically inactive. Because of the negative results with Fc γ RII-b1 and Fc γ RII-b2, we believed it important to check that the cDNA we were using were

TABLE I
Responses to cross-linking of FcR on RBL and P815 transfectants^a

Cell	aFcγR/Cell ($\times 10^{-5}$) ^b	FcεRI/Cell ($\times 10^{-5}$)	Tyr-P FcγR/FcεR ^c	Ca ²⁺ FcγR/ FcεR ^d	PI FcγR/ FcεR ^e	Arachidonate FcγR/FcεR ^f	Hexosaminidase FcγR/FcεR ^g
RBL Transfectant							
FcγRII-b1	2.4 (2)	3.7 (1)	0 (1)	0 (3)	0 (1)	0 (1)	0 (1)
FcγRII-b2	3.6 (2)	1.9 (1)	0 (5)	0 (2)	0 (3)	0 (2)	0 (2)
FcγRIII	0.17 (2)	3.7 (1)	0.27 (7)	0.41 (2)	0.24 (3)	0.38 (2)	0.39 (2)
P815 Transfectant							
FcεRIwt	1.2 (3)	3.0 (8)	0.66 (4)	0.66 (3)	1.1 (4)	NT ^h	NA ⁱ
FcεRIβ-ct	1.8 (3)	2.2 (8)	0.26 (4)	0 (12)	0 (1)	NT	NA
FcεRIγ-ct	1.4 (1)	0.9 (2)	0 (4)	0 (1)	0 (2)	NT	NA

^a Average values are shown with the number of independent experiments indicated in the parentheses. In all cases the absolute increase above background was calculated for stimulation via either the FcγRI or FcεR. The ratio of the former to the latter was then calculated.

^b Expression of FcγR was determined by quantitating the number of molecules of antibody 2.4G2 (aFcγR) bound per cell. Although it is likely that each antibody bound two receptors simultaneously, it is possible that the Fc region of the antibody independently bound to a third receptor. Therefore, the actual number of receptors is likely two times, or conceivably three times greater than the number indicated.

^c The relative phosphorylation of tyrosine on the 72-kDa component was assessed by scanning autophotographs of immunoblots. The responses to aggregation of FcεRI in transfectants with FcγRII are shown in the Figure 2.

^d The absolute responses for FcεRI were 236 to 598, 30 to 1324, and 109 to 822 nM for the RBL cells transfected with FcγRII-b1, FcγRII-b2, and FcγRIII, respectively. The lower values were from RBL cells unsensitized with IgE whose receptors were aggregated with the anti-FcεRI antibody, 5.14. Correspondingly, the absolute responses for FcεRI were 42 to 130 nM, 0, and 0 nM for the P815 cells transfected with FcεRI-wt, FcεRIβ-ct, and FcεRIγ-ct, respectively; for FcγR on those cells the corresponding values were 34 to 52, 0 to 7.4, and 0 nM, respectively. Triggering of FcγR on the untransfected P815 gave an average response of 38 nM.

^e The absolute responses for FcεRI were 8.7, 2.8 to 5.3, and 3 to 6% for the RBL cells transfected with FcγRII-b1, FcγRII-b2, and FcγRIII, respectively. Correspondingly, the absolute responses for FcεRI were 1.6 to 2.2, 0.1, and 0.03% for the P815 cells transfected with FcεRI-wt, FcεRIβ-ct, and FcεRIγ-ct, respectively; for FcγR on those cells, the corresponding values were 1.9 to 3, 0 to 0.11, and 0.05%. Triggering of FcγR on the untransfected P815 gave an average response of 1.1%.

^f The absolute responses for FcεRI were 9, 5.1 to 5.3, and 2.8 to 4.3% for the RBL cells transfected with FcγRII-b1, FcγRII-b2, and FcγRIII, respectively.

^g The absolute responses for FcεRI were 34.9, 6.5 to 7, and 10 to 17.6% for the RBL cells transfected with FcγRII-b1, FcγRII-b2, and FcγRIII, respectively.

^h NT, Not tested.

ⁱ NA, Not applicable.

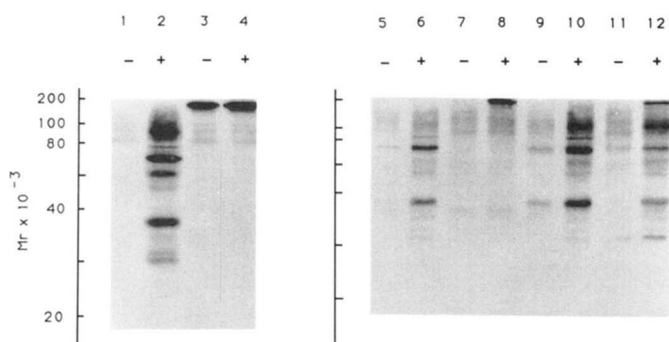


Figure 2. Changes in the phosphorylation of protein tyrosines in response to aggregation of FcR on RBL transfectants. The figure shows an autophotograph of a Western blot of SDS extracts of cells analyzed for phosphotyrosine. The m.w. scale was calculated from the mobility of a series of standards. Lanes 1 to 4, RBL transfected with FcγRII-b1; lanes 5 to 8, RBL transfected with FcγRII-b2; lanes 9 to 12, RBL transfected with FcγRIII. In each set of four lanes, the cells in the first two lanes had been reacted with IgE and subsequently without (-) or with (+) Ag; the cells in the second pair of lanes had been reacted either with (lane 3) or without (lanes 7 and 11) antibody 2.4G2 alone (-) or with both 2.4G2 and goat anti-rat IgG (+). The bands close to the origin in some of the lanes result from a cross-reaction between the 2.4G2 antibody and the goat anti-rat IgG with the mouse PY20.

correct. We, therefore, sequenced fully the cDNA constructs and verified that the sequences agreed exactly with the published sequences (2) as corrected (27).

Functional responses of P815 cells and P815 transfectants. The mouse mastocytoma P815 lacks endogenous transcripts for the α- and β-chains of FcεRI (10), but contains transcripts for FcγRII-b1, FcγRII-b2, and FcγRIII (1). Using iodinated antibody 2.4G2 (a reagent that does not distinguish between the three FcγR iso-types) we found 0.75×10^5 to 2×10^5 binding sites

expressed on the cell surface of the untransfected cells. Aggregation of FcγR on untransfected P815 cells by 2.4G2 plus anti-rat IgG triggered prompt phosphorylation of protein tyrosines, increased hydrolysis of PI, and a rise in intracellular calcium (data not shown). As with the rise stimulated by FcεRI (10), the increase initiated by cross-linking of FcγR was largely dependent on the presence of mM free Ca²⁺ in the extracellular medium.

In separate studies we reported transfection of P815 cells with wild-type (10) or variant (12) FcεRI. Aggregation of the FcεRI on transfectants expressing wild-type and certain of the FcεRI variants triggered responses similar to those observed with the RBL cells. Aggregation of the endogenous FcγR on such transfectants also initiated responses similar to those stimulated by aggregating the FcεRI on the same cells (Table I) (28). However, it was notable that transfection of P815 cells with two dysfunctional mutant FcεRI led to a parallel loss of FcγR function even though the latter were expressed in normal numbers on these transfectants (28) (Table I).

The first case involved P815 cells transfected with a combination of wild-type FcεRI α- and β-chains, and with mutant γ-chains. We previously reported that even though the P815 cells produce wild-type γ-chains endogenously, and that those chains preferentially associate with the expressed FcεRI α- and β-chains, the transfected mutant γ-chains nevertheless inhibit the normal responses to aggregation of the FcεRI (12). These transfectants also fail to respond to aggregation of FcγR induced by antibody 2.4G2 (Table I). Inasmuch as the cells responded normally to thrombin (12), it is apparent that the cells had not merely been nonspecifically inactivated. Parenthetically, it can be seen that whereas the transfec-

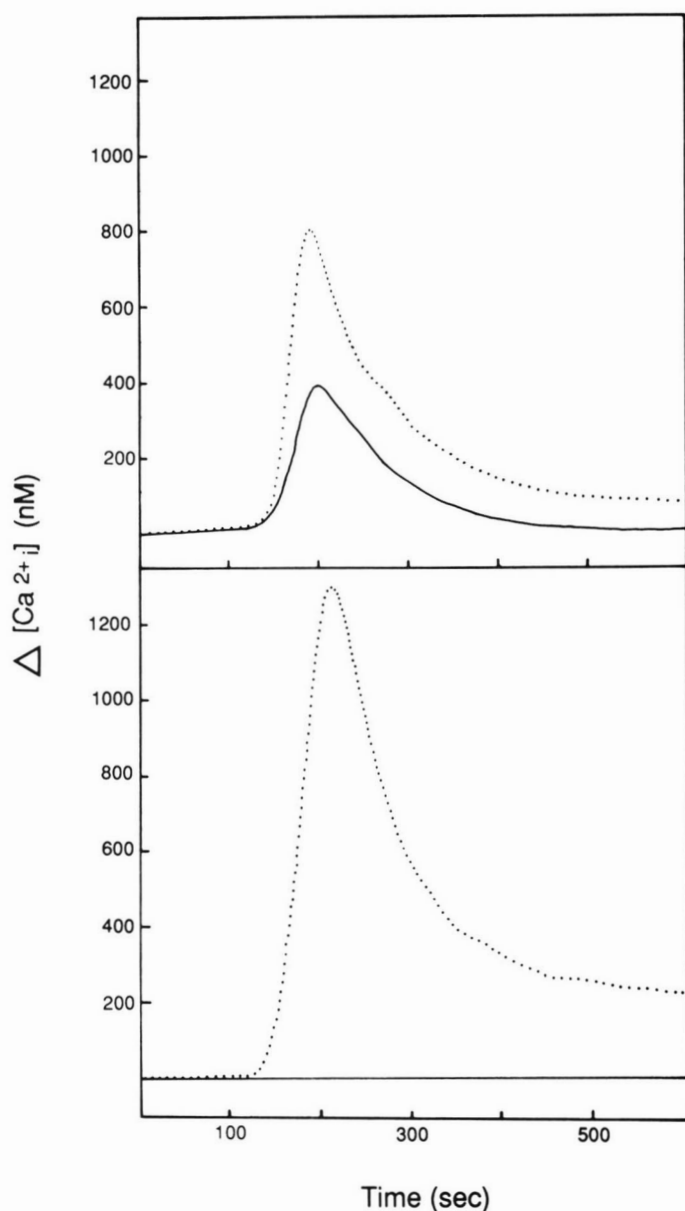


Figure 3. Changes in intracellular Ca^{2+} in response to aggregation of FcR in RBL transfectants. One million cells/ml in assay buffer containing 1 mM CaCl_2 were incubated with either 5 $\mu\text{g}/\text{ml}$ DNP-specific IgE or with 5 $\mu\text{g}/\text{ml}$ antibody 2.4G2 for 1 h, at the same time as they were loaded with 5 μM of the fluorescent indicator Quin 2. The cells were centrifuged, resuspended at 1×10^6 cells/ml in assay buffer containing 1 mM CaCl_2 and monitored in the fluorometer for 2 min (to establish a baseline) before the addition of DNP-BSA, or of goat anti-rat IgG, alternatively. The responses stimulated by FcRI are shown with dashed lines; those by FcRII with solid lines. Top panel, RBL cells transfected with FcRIII. Bottom panel, RBL cells transfected with FcRII-b2.

tant with the mutated γ -chain expressed a reduced number of FcRI (a result consistent with the effect the mutant γ has on the efficiency of expression of FcRI in COS cells (29)) no corresponding decrease in the binding of the anti-FcRI was observed. This is not surprising because only the expression of FcRIII would be expected to have been affected, and this receptor only represents a fraction of the total FcRI with which the antibody reacts on P815 cells (1).

The second case occurred in P815 cells transfected simultaneously with cDNA coding for a wild-type FcRI α - and γ -chains and for an FcRI β subunit with a truncated COOH-terminal cytoplasmic domain. We previously

reported that FcRI expressing this particular variant β -chain initiated a variety of cellular responses much less efficiently (12, 30, 31).⁴ The specificity of this diminished responsiveness was documented by the observation that the cells continued to respond normally to the addition of thrombin (12). When stimulated with antibody 2.4G2, these transfectants also responded abnormally. Figure 4 shows a representative experiment in which P815 cells transfected with the wild-type α and γ and, alternatively, with wild-type or mutant β -chains were compared with respect to the ability of their aggregated (transfected) FcRI or (endogenous) FcRI to stimulate phosphorylation of protein tyrosines. Lanes 1 to 4 show SDS extracts from cells transfected with wild-type FcRI. Addition of DNP-BSA led to stimulation of phosphorylation of protein tyrosines only in the cells that had been previously sensitized with IgE (lane 2 vs lane 1). The enhanced phosphorylation of a component corresponding to the 72- to 75-kDa component observed in RBL cells (22) has already been reported (12). Pronounced stimulation of phosphorylation of a component at around 37 kDa is also apparent.

The same transfectants were studied for their response to aggregation of their endogenous FcRI. The pattern of enhanced phosphorylation observed when the cells were reacted with 2.4G2 and second antibody (lane 4) was similar to the pattern seen when the same cells were stimulated via FcRI (lane 2).

The next four lanes show the results of a comparable experiment using cells transfected with wild-type α and γ and with the mutant β . These cells responded less effectively to aggregation of their FcRI, and a similarly

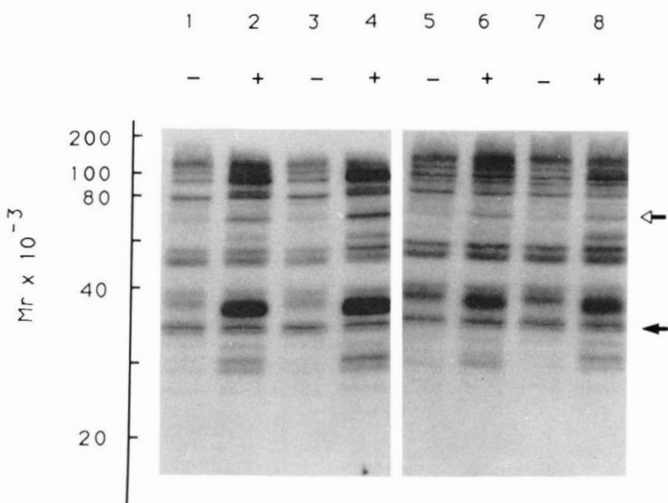


Figure 4. Changes in phosphorylation of protein tyrosines in response to aggregation of FcR on P815 transfected with wild-type and mutant FcRI. The figure shows an autoradiograph of a Western blot on which SDS extracts of cells were analyzed for phosphotyrosine. The m.w. scale was calculated from the mobility of a series of standards. Lanes 1 to 4. Extracts of cells transfected with wild-type FcRI. Lanes 5 to 8. Extracts of cells transfected with wild-type α and γ of FcRI, and with a β in which the COOH-terminal cytoplasmic domain had been truncated. In each set of four lanes the first two lanes show an unstimulated control and stimulation via FcRI (lanes 1 and 2, and 5 and 6, respectively), and the second two lanes an unstimulated control and stimulation via FcRI (lanes 3 and 4 and 7 and 8, respectively). The band indicated with the open arrow is the 72-kDa component; that indicated by the filled arrow is the component used for normalization in the analysis shown in Figure 5.

⁴ In our previously reported study (12) we used iodinated antiphosphotyrosine antibodies to detect phosphorylation of proteins, and it appeared that the FcRI on cells transfected with the mutant β -subunits were totally inactive. Using the more sensitive chemiluminescence technique it is clear that the FcRI on these transfectants are at least partially active.

reduced response was seen when the cells were stimulated via their Fc γ R. A more complete and quantitative analysis of these transfectants is given in Figure 5. In Figure 5A, we analyzed the phosphorylation of the 72-kDa component relative to a stable component at about 35 kDa (Fig. 4), after stimulating cells via Fc ϵ RI. The *solid line* is the response of the cells transfected with the wild-type Fc ϵ RI and the *dashed line* shows the response of the cells containing Fc ϵ RI with the mutant β -chains. In Figure 5B we show a comparable analysis for the same cells when their endogenous Fc γ R were aggregated. Again the *solid line* is for the cells transfected with wild-type Fc ϵ RI and the *dashed line* for the cells transfected with Fc ϵ RI containing the mutant β -chain. It was particularly striking that the relative activity of the endogenous Fc γ R in the two types of transfectants so closely paralleled the relative activity of the transfected Fc ϵ RI on the same cells (Fig. 5, B vs A; Table I). The results suggested that when present in the same cells, mutant (and likely the native) Fc ϵ RI β subunits could interact with Fc γ R.

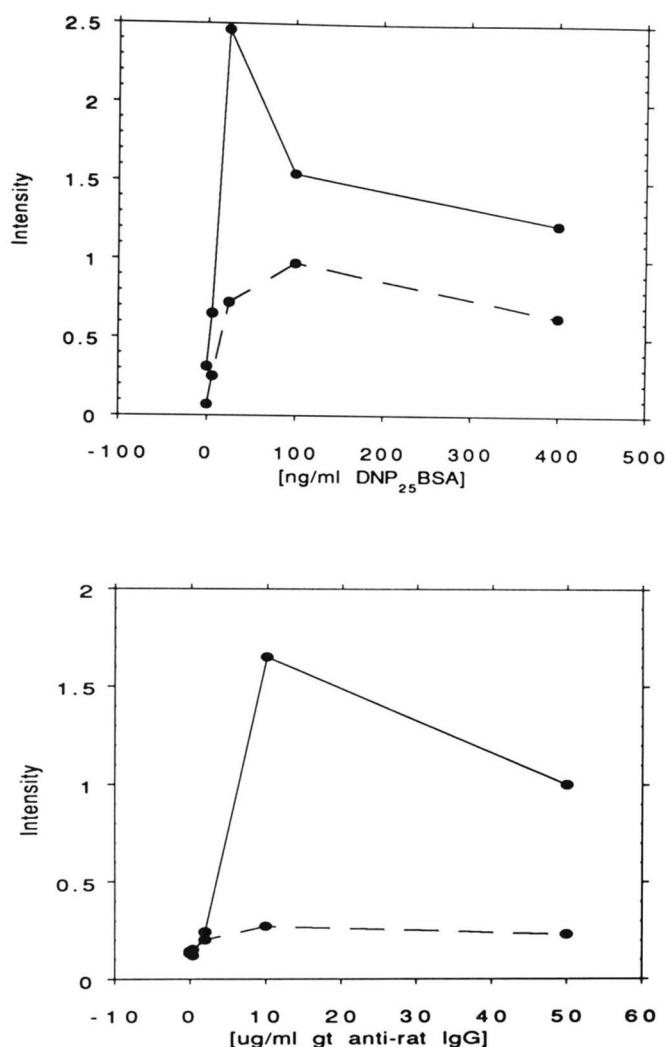


Figure 5. Quantitative analysis of the changes in phosphorylation of protein tyrosines in P815 cells transfected with Fc ϵ RI containing wild-type or truncated β -chains. The data are taken from gels similar to the one shown in Figure 4. Stimulation was quantitated by comparing the intensity of the 70-kDa component (*open arrow*, Fig. 4) relative to the component at 35 kDa [*filled arrow*, Fig. 4]. The latter does not seem to undergo any stimulation-related changes. *Solid lines*, Cells transfected with wild-type Fc ϵ RI. *Dashed lines*, Cells transfected with mutant Fc ϵ RI. A, Responses of cells to stimulation of Fc ϵ RI. B, Responses of cells to stimulation of Fc γ R.

Association of β -subunits with Fc γ RIII. We attempted to document such an association directly by a variety of experimental approaches. For example, we solubilized P815 cells that had been transfected with wild-type Fc ϵ RI under conditions that minimize the dissociation of Fc ϵ RI (32) (see *Materials and Methods*), immunoprecipitated Fc ϵ RI with anti-IgE, or Fc γ R with 2.4G2 plus rabbit anti-rat IgG, and examined the immunoprecipitated material by Western blotting with the anti- β antibody JRK. The precipitated Fc ϵ RI gave a strong reaction (12), but the immunoprecipitates of Fc γ R failed to react (data not shown). Because in RBL cells the β -chains are actively tyrosine phosphorylated after cross-linking Fc ϵ RI (33), and because the assay for phosphotyrosine is so sensitive, we repeated the immunoprecipitation protocols with the RBL cells transfected with Fc γ RIII and blotted with antiphosphotyrosine. The immunoprecipitated Fc ϵ RI from cells stimulated by cross-linking of Fc ϵ RI showed a strong reaction with antiphosphotyrosine in the position of the β -chain and a roughly equivalent reaction with the dimer of γ -chains (Fig. 6, lanes 1 and 2). The immunoprecipitated Fc γ RIII from cells stimulated by cross-linking of Fc γ RIII also showed a reaction in the position of the γ -chain dimer albeit to a lesser extent (Fig. 6, lanes 3 and 4). There was no clearly defined band at the position where the phosphorylated β -chains would have appeared, and although densitometric scans were suggestive they were not definitive. If in these transfectants β was limiting, the Fc γ RIII may have been unable to compete effectively for β -chains with the 10-fold higher numbers of Fc ϵ RI (Table I). Therefore, we repeated this experiment with the P815 cells. At least in those P815 cells transfected with the mutant β , the inhibitory effect of the truncated subunit on the responses stimulated by the Fc γ R suggests that most if not all of the Fc γ RIII must have interacted with the mutant β (Fig. 5; Table I). Nevertheless, we failed to recover a reproducible and meaningful amount of phosphorylated β -chains from the cells transfected with the wild-type β and stimulated via the Fc γ RIII. However, as these studies were being completed,

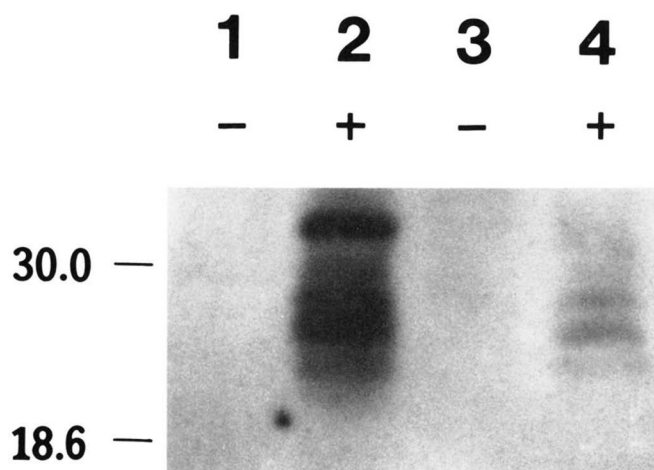


Figure 6. Analysis of phosphotyrosine in receptor subunits of RBL transfectants. The figure shows an autoradiograph of a Western blot on which immunoprecipitates were analyzed for phosphotyrosine. *Lanes 1 and 2*, Immunoprecipitated Fc ϵ RI from unstimulated cells (-) and cells stimulated by aggregating Fc ϵ RI (+). *Lanes 3 and 4*, Immunoprecipitated Fc γ RIII from unstimulated cells (-) and cells stimulated by aggregating Fc γ RIII (+). The *brace* shows the position of the phosphorylated dimers of γ -chains; the more discrete band in lane 2 is the phosphorylated β -chain.

evidence for a physical association between Fc γ RIII and β -chains was published (11).

DISCUSSION

In this study we characterized the signaling capacity of three discrete forms of Fc γ R in mast cells. RBL cells were transfected alternatively with Fc γ RII-b1, Fc γ RII-b2, or the α -chain of Fc γ RIII, and five different cellular response were monitored. Only Fc γ RIII proved to be active, and although the early and late biochemical signals stimulated by these receptors were quantitatively reduced, they were otherwise similar to those triggered by the endogenous Fc ϵ RI (Table I).

Results complementary to ours were reported by Katz et al. (34). They examined the release of hexosaminidase and three types of arachidonic metabolites in three types of murine mast cells after cross-linking of Fc γ R (with antibody 2.4G2) or Fc ϵ RI. Bone marrow-derived mast cells responded poorly to 2.4G2 relative to the responses elicited by the Fc ϵ RI. Such mast cells are known to express principally the Fc γ RII-b1 and Fc γ RII-b2 but little of the Fc γ RIII isoform (35). After coculture with fibroblasts (conditions that up-regulate the expression of Fc γ RIII) the mast cells responded considerably better (34). Their findings are consistent with ours that Fc γ RIII, but not Fc γ RII-b1 or Fc γ RII-b2 can initiate mast cell responses. Katz et al. (34) also examined serosal mast cells, cells whose phenotype resembles that of the cocultured bone marrow-derived mast cells by a variety of criteria. In particular, such cells chiefly express the Fc γ RII-b1 and Fc γ RIII isoforms (35). These cells also responded well to 2.4G2 but they secreted a distinctive pattern of arachidonic metabolites. Significantly, the responses initiated by Fc ϵ RI exhibited the same distinctive pattern of metabolites. This result compliments our findings that the early biochemical responses, particularly the patterns of tyrosine phosphorylation, stimulated by the transfected Fc γ RIII and endogenous Fc ϵ RI in RBL cells are qualitatively indistinguishable. It appears that these two types of FcR trigger similar responses, but that such responses may differ depending on the cell type.

The results of our studies are also consistent with findings recently reported in a review by Fridman et al. (36). They observed release of serotonin and TNF- α by RBL cells transfected with murine Fc γ RIII but not from cells transfected with either isoform of Fc γ RII. Thus not only do the early signals triggered by Fc ϵ RI and Fc γ RIII appear to be similar, but also the later events such as the enhanced transcription of DNA that accounts for the increase in cytokine production (37).

It has been difficult to isolate sufficiently pure rodent mucosal mast cells to analyze their spectrum of Fc γ R (H. Katz, personal communication). However, RBL cells share many of the characteristics of mucosal mast cells (38). Therefore, the failure to observe degranulation responses after cross-linking of the endogenous Fc γ R on RBL (39) combined with our observations on the transfected RBL, indicates that the predominant, if not exclusive Fc γ R expressed on mucosal-type mast cells are likely to be homologous to murine Fc γ RII rather than to Fc γ RIII.

The α -chain of the murine and human Fc γ RIII and the α -chain of Fc ϵ RI are known to associate with γ -chains (9). Recent data from several laboratories have indicated that when aggregated, the γ subunits or the related δ -

chains, are themselves able to generate both early and late signals (40–42). In contrast to those of Fc γ RIII and Fc ϵ RI, the α -like chains of Fc γ RII-b1 and Fc γ RII-b2 are not known to be associated with subunits like the γ , apparently lacking a suitable transmembrane sequence, the known site for such interactions (29). They presumably also lack suitable sequences in their cytoplasmic domains by which to interact with the necessary postreceptor molecules that account for the cellular responses we monitored.

In this regard it is interesting that although they are also thought to consist only of single α -like chains, the human Fc γ RII appear to stimulate signals similar to those described for Fc ϵ RI and Fc γ RIII. Thus, Odin et al. (43) recently reported that a murine macrophage line transfected with cDNA for human Fc γ RIIA α -like chains, responded with a rise in intracellular Ca²⁺ when aggregated. In a related study, Liao et al. (44) found that aggregation of the endogenous Fc γ RII on the human monocytic line U937 stimulated phosphorylation of tyrosines in phospholipase C- γ 1, phosphoinositide metabolism, and a rise in intracellular Ca²⁺. Although the exodomains and transmembrane segments of the human Fc γ RII isoforms are homologous to the two murine Fc γ RII isoforms, the cytoplasmic domains show much greater variations (Fig. 1) (4). In the study by Odin et al. (43) only the human Fc γ RIIA isoform was examined. Because the cytoplasmic domain of its α -like chain is substantially different than that of the murine Fc γ RII-b1 or Fc γ RII-b2 (4), the fact that it triggered responses in the macrophage transfectant is not necessarily in conflict with the results of ourselves and others on the murine Fc γ RII; Liao et al. (44), could not distinguish which of the several isoforms on the human U937 monocytic line (Fc γ RII-A,B,C) accounted for the responses they observed, because the anti-Fc γ R antibody they employed does not discriminate between these isoforms. Nevertheless, that in both instances a human Fc γ RII isoform is able to trigger responses similar to those initiated by Fc γ RIII and Fc ϵ RI in the absence of the γ -subunit that is thought to be a critical element in the responses of the latter receptors, poses an interesting problem to explore.

The characterization of Fc γ R function in RBL transfectants clarifies the results we obtained on P815 murine mast cells transfected with wild-type and mutant Fc ϵ RI. Overexpression of truncated γ -chains inhibited not only the function of Fc ϵ RI (12) but also that of the endogenous Fc γ R (28) (Table I). Based on the results with the RBL transfectants, the functional Fc γ R can now be identified as Fc γ RIII. We previously provided evidence that the failure of these transfectants to respond to aggregation of the Fc ϵ RI, was not simply related to the decreased expression of this receptor (12). However, we do not have similar direct data with respect to Fc γ RIII. As already noted above, the fact that we did not see a decrease in the binding of anti-Fc γ R on these transfectants does not rule out the possibility of a decrease in the expression of Fc γ RIII specifically. Thus, it is formally possible that these transfectants expressed few or no Fc γ RIII. However, the similarity of the α -chains of Fc ϵ RI and Fc γ RIII make this an unlikely explanation of the absent Fc γ RIII-mediated responses in these cells.

That the truncated γ -chains should impair the function of the endogenous γ -associated Fc γ RIII was less surpris-

ing than our observation that the cells transfected with β -subunits having a truncated COOH-terminal cytoplasmic domain should show a parallel decrease in activity of the Fc ϵ RI and Fc γ R (12) (Table I; Figs. 4 and 5). Again, it must have been the Fc γ RIII that were affected. At the time we first observed these unexpected results (28), Fc γ RIII was not known to be associated with β , however, Kurosaki et al. (11) recently published evidence for such an association. Given the similarity in the transmembrane regions of the α -chains of Fc γ RIII and Fc ϵ RI (3), and the fact that the transmembrane regions appear to be the principal sites where the subunits interact (29), it is not altogether surprising that the Fc γ RIII α , γ complex could interact with β -chains when the latter are present in excess. In both the P815 transfectants and the RBL cells, we had suggestive but not conclusive evidence for such an association by direct analysis. However, our functional results with the P815 cells transfected with the truncated β (Table I; Fig. 5) strongly suggest that the mutant β interacted with the majority of the Fc γ RIII. Just as we have discussed with respect to the mutant γ , we cannot formally rule out the possibility that rather than being assembled with the α - and γ -chains of Fc γ RIII, the mutant β simply prevented efficient expression of Fc γ RIII. Again, however, the fact that the mutant β allows for rather normal expression of Fc ϵ RI, and that the α of Fc ϵ RI and Fc γ RIII are so homologous in the critical transmembrane domain, makes this explanation unlikely. Even if this had been the case, because the γ -chains for Fc ϵ RI and Fc γ RIII are identical it would mean that the β -chain was interacting with the α of Fc γ RIII, and that is in principle all we are suggesting.

Assuming the receptor was expressed, our failure to observe clear physical evidence for such an association whereas using somewhat different conditions for solubilization of wild-type β transfectants, Kurosaki et al. (11) did observe such an interaction, is easily reconciled. The relative instability of the multi-subunit FcR in detergent has been well documented (32, 45) and, indeed the gels published by Kurosaki et al. (11) are consistent with dissociation of most of the β that may have been associated with Fc γ RIII α in situ.

We previously reported that P815 cells transfected only with the human Fc ϵ RI α subunit (the latter can assemble with γ even in the absence of β -chains (46)) respond fairly well when their Fc ϵ RI are aggregated (12). As discussed there, the inhibition by the mutant β could then be explained in two ways. Either the β plays no role in the signals we monitored but when mutated can interfere with normal triggering (e.g., by preventing the formation of productive aggregates) or there might be a β -like subunit in the P815 cells that is required for signaling. Such a β -like protein might have interacted with the transfected human Fc ϵ RI α and be normally associated with Fc γ RIII. If so, it would have to be so nonhomologous to authentic β that it fails to react with β -reactive reagents by Northern or Western blotting (10), and be so susceptible to dissociation from α in detergents (32), that it has so far eluded discovery. If it exists at all, such a β -like component could then have been displaced by the over-expressed mutant β .

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