Differential Control of the Tyrosine Kinases Lyn and Syk by the Two Signaling Chains of the High Affinity Immunoglobulin E Receptor*

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Nonreceptor tyrosine kinases such as the newly described 70-kDa (ZAP-70/Syk) and Src-related tyrosine kinases are coupled to a variety of receptors, including the antigen receptors on B- and T-cells and the Fc receptors for IgE (FεRI) and IgG (FcγRI, FcγRII/CD16). Various subunits of these receptors contain homologous activation motifs which appear capable of autonomously triggering cell activation. Two forms of this motif are present in the FεRI multimeric complex: one in the β chain and one in the γ chain. Here we show that each of the two tyrosine kinases known to be involved in FεRI signaling is controlled by a distinct motif-containing chain. Lyn associates with the nonactivated β chain, whereas γ promotes the activation of Syk. We also show that neither the β nor the γ motif alone can account for the full signaling capacity of the entire receptor. We propose that, upon triggering of the tetrameric receptor, Lyn already bound to β becomes activated and phosphorylates β and γ; the phosphorylation of γ induces the association of Syk with γ and also the activation of Syk, resulting in the phosphorylation and activation of phospholipase Cγ. Cooperative recruitment of specific kinases by the various signaling chains found in this family of antigen receptors could represent a way to achieve the full signaling capacity of the multimeric complexes.

Nonreceptor tyrosine kinases of the Src and ZAP-70/Syk families are activated by engagement of various antigen receptors. This activation is the earliest detectable event in the signaling cascade initiated by these receptors and results in the tyrosine phosphorylation of cellular proteins, including receptor subunits. The antigen receptors form a family of structurally and functionally related multisubunit receptors, including the B- and T-cell antigen receptor (TCR), the high (FcγRI) and low affinity (FcγRII/CD16) Fc receptors for IgG and the high affinity IgE receptor (FεRI). A protein sequence motif DE₂EX₂LYX₂LYX₂L, which has been designated ARAM (antigen recognition activation motif) (1), has been identified in the cytoplasmic tails of some subunits within these receptors (2). When expressed independently of the other subunits, ARAMs are apparently able to elicit the full range of activation signals as observed with the complete multimeric receptors, including the activation of tyrosine kinases. Chimeras containing the cytoplasmic portions of TCR γ, CD3 ε, and FεRI γ activate early (such as calcium flux) and late (such as interleukin-2 (IL-2) production) events in T-cells (4–8). Chimeric proteins bearing the cytoplasmic portions of FεRI γ and TCR γ elicit serotonin release from transfected RBL cells. In B-cells, a γ chimera mediates antigen presentation (9). Mutation of either of the tyrosine residues in the ARAMs of CD3 ε, TCR γ, and FεRI γ abolishes signal transduction (6, 8–10). Some receptors harbor multiple forms of ARAMs. For example, within the TCR-CD3 complex, each γ chain possesses one ARAM and the δ, β, and ε chains of CD3 have one ARAM each. The functional significance of this variability and multiplicity is still unclear. Independent expression of each of the two N-terminal ARAMs of γ indicates that they are qualitatively equivalent and that the main effect of the triplication is an amplification of the signals (11). The same issue arises with FεRI, a tetrameric αβγδ complex. Two different forms of ARAM are present in FεRI, one in the C-terminal cytoplasmic part of β and one in each of the two γ chains. As mentioned above, ARAM-γ is able to induce serotonin release when expressed alone. However, the evidence for involvement of ARAM-β in signaling is contradictory. On the one hand, both rat FεRI, which requires the presence of all three types of subunits (α, β, and γ) for cell surface expression (12), and its human counterpart, for which α and γ suffice (13), elicit the same spectrum of activation reactions in transfected P815 mast cells (14). On the other hand, deletion of the C-terminal cytoplasmic tail of β containing ARAM-β, but not deletion of the N-terminal cytoplasmic domain, which does not contain an ARAM motif, abrogates activation responses in P815 (14).

In the present study, we investigated the respective roles of the β and γ subunits of FεRI in signal transduction, focusing on the recruitment of the two kinases, Lyn and Syk, already known to be involved in FεRI signaling.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies were purchased from the following sources: anti-phosphotyrosine 4G10 from Upstate Biotechnology, Inc. (Lake Placid, NY); goat anti-mouse IgG and goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase, F(ab')₂ fragment goat anti-mouse IgG (H+L), and rabbit anti-mouse IgG (H+L) antibodies from Jackson Immunoresearch (West Grove, PA); anti-Tac B 1.49.9 (15) from Amac (Westbrook, ME); anti-Fgr, anti-Hck, anti-ly, anti-Yes from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and goat anti-rabbit IgG antibodies from Jackson Immunoresearch. From the Molecular Allergy and Immunology Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852.

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Institute, Princeton, NJ) or purchased from Santa Cruz (Santa Cruz, CA).

**Cell Culture and Transfections—**The extracellular and transmembrane parts of human Tac (up to base pair 957) (21) were joined by polymerase chain reaction to the cytoplasmic C-terminal part of rat β (base pairs 652-786) (22) to give the chimeric cDNA including Tac and FcεRI (βTT). The mutated form of rat β cDNA (βTT) in which tyrosines 218, 224, and 228 are changed to phenylalanines was made by replacing the segment from base pair 680 to base pair 786 with a double-stranded oligonucleotide including the mutations. These constructs were subcloned into pCDL-SRα 296 (23). RBL-2H3 cells were cotransfected by electroporation with the TβTT construct and pSVneo to generate RBL-TβTT. P815 cells were co-electroporated with the wild type (βWT) or mutated β (βTT) cDNAs, the rat α cDNA (24) in pCDL-SRα 296 and the rat FcεRI cDNA (25) in pBluescript (26), a derivative of pCDL-SRα 296 containing a neomycin resistance cassette, to generate P815-βWT and P815-βTT. Resistant clones were selected with G418 (0.5 mg/ml for RBL transfectants and 1.0 mg/ml for P815 transfectants). One clone of RBL-TTy expressing a chimera between Tac and the cytoplasmic part of γ was obtained from François Letourneur (7). More RBL-TTy clones were generated by transfecting RBL cells with the corresponding cDNA subcloned in pCDL-SRα 296 (7). Surface expression was analyzed on a FACScan (Becton Dickinson, San Jose, CA) after staining with FITC-labeled mouse IgE or with FITC-labeled anti-Tac B1.49.9. 125I-iodination of IgE and anti-Tac 7G7BS was performed using chloramine T.

**Secretion Release—**The assay was performed as described (26).

**Calcium Flux Measurement—**Cells were cotransfected with IGE or bio-tyrinated anti-Tac B1.49.9 and then loaded with fura-2 AM (2 μM for RBL cells and 1 μM for P815 cells) for 45 min at 37°C (pH 7.4) containing 150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, 0.1% bovine serum albumin, and 2.5 mM PEG. Cells were analyzed in a cuvette on a Deltacount spectropho- rometer (Photon Technology International Inc., South Brunswick, NJ) with excitation set at 340 and 380 nm and emission at 500 nm. Calcium concentrations were calculated using the published value of 2.24 μM for the Kₐ for RBL transfectants and 1.0 μM for P815 transfectants. One clone of RBL-TTy expressing a chimera between Tac and the cytoplasmic part of γ was obtained from François Letourneur (7).

**Expression of the Transfected Molecules on RBL and P815 was assessed by flow cytometry. Staining with FITC-labeled anti-Tac antibody reveals expression of Tac chimeras on RBL-TTy and RBL-TβTT, but not on untransfected RBL (Fig. 2A). We also quantitated the number of chimeric molecules on these clones is either comparable with or twice as high as that of ARAM-β, but in comparison with RBL-TTy, RBL-TβTT clones stably expressing Tβ or TTy, respectively, were isolated. In addition, another clone of RBL-TTy was obtained from F. Letourneur.

A mutated form of full-length rat FcεRI β was generated by substituting 3 tyrosine residues with phenylalanines at positions 218, 224, and 228 within ARAM β (Fig. 1C). The cDNAs coding for rat α and γ were cotransfected into an FcεRI-negative mouse mast cell line, P815, with either the wild type or tyrosine-mutated β cDNA, to generate P815-βWT and P815-βTY, respectively.

**Generation of Transfectants—**We constructed a β chimera (TβTT) analogous to the γ chimera (TTγ) described previously (7). TβTT and TTγ contain the extracellular and transmembrane segments of the human IL-2 receptor α chain (Tac) fused to the C-terminal cytoplasmic part of rat FcεRI β and to the cytoplasmic tail of rat FcεRI γ, respectively (Fig. 1, A and B). By virtue of the Tac transmembrane segment, the chimeric molecules are expressed on the cell surface as single chain molecules. Tβ or TTy was transfected into RBL-2H3, an FcεRI-positive rat mast cell line. RBL-TTy clones and RBL-TTy clones stably expressing Tβ or TTy, respectively, were isolated. In addition, another clone of RBL-TTy was obtained from F. Letourneur.

In some experiments, after the kinase assay and the washes, the anti-phosphotyrosine immunoprecipitates were eluted from the beads with 10 mM phenylphosphate in lysis buffer at 4°C for 30 min and the anti-receptor immunoprecipitates were eluted with 1% sodium deoxycholate in 10 mM Tris (pH 7.4), 50 mM NaCl for 30 min at 37°C (30, 31). Reprecipitation was performed with an anti-Lyn, anti- Src, or control antibody. In some cases, the reprecipitation was performed in the presence of 10 μg of the peptide used to generate the corresponding antibody. The second immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Band intensities on gels were quantitated using a radioanalytic imaging system (Ambis Systems, San Diego, CA). This repro- cipitation technique allows us to identify the kinase(s) present in anti-receptor precipitates.

**RESULTS**

**Generation of Transfectants—**We constructed a β chimera (TβTT) analogous to the γ chimera (TTγ) described previously (7). TβTT and TTγ contain the extracellular and transmembrane segments of the human IL-2 receptor α chain (Tac) fused to the C-terminal cytoplasmic part of rat FcεRI β and to the cytoplasmic tail of rat FcεRI γ, respectively (Fig. 1, A and B). By virtue of the Tac transmembrane segment, the chimeric proteins are expressed on the cell surface as single chain molecules. Tβ or TTy was transfected into RBL-2H3, an FcεRI-positive rat mast cell line. RBL-TTy clones and RBL-TTy clones stably expressing Tβ or TTy, respectively, were isolated. In addition, another clone of RBL-TTy was obtained from F. Letourneur.

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**Expression of the Transfected Molecules on RBL and P815 was assessed by flow cytometry. Staining with FITC-labeled anti-Tac antibody reveals expression of Tac chimeras on RBL-TTy and RBL-TβTT, but not on untransfected RBL (Fig. 2A).**

In contrast to ARAM-γ, ARAM-β does not mediate a late signal. Serotonin Release—To study the coupling of ARAM-β to cellular activation mechanisms, we first tested the ability of the β chimera to initiate degranulation, a late event in mast cell activation. As reported previously (7), the γ chimera is able to induce degranulation as assessed by serotonin release from...
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Fig. 2. Surface expression of transfected molecules. A and B, Tac antigen expression on TTβ and TTγ chimera-transfected RBL cells. A, flow cytometry analysis: untransfected RBL (dotted line), RBL-TTβ (solid line), and RBL-TTγ (dashed line) were stained with FITC-labeled anti-Tac (B1.49.9). B, average antibody binding site numbers were calculated from saturation binding data of [125I]-IgE and [125I]-anti-Tac (7G7) on RBL-TTβ and RBL-TTγ. C, flow cytometry analysis of FceRI expression on P815 cells: transfected with FcεRI α and γ along with either wild type or mutated β. Untransfected P815 (dotted line), P815-βWT (solid line), and P815-βY (dashed line) were stained with FITC-IgE.

Table I

| Cell line          | Preincubation | Challenge | Percent serotonin release (n = 3) |
|--------------------|---------------|-----------|----------------------------------|
| RBL-TTγ            | Anti-Tac-Biotin | Avidin   | 4.7 ± 1.5                       |
| RBL-TTγ            | DNP-HSA       | Avidin   | 21.2 ± 0.1                      |
| RBL-TTγ            | IgE (saturating) | DNP-HSA | 3.8 ± 2.5                       |
| RBL-TTγ            | IgE (1/5-saturating) | DNP-HSA | 47.9 ± 0.4                      |
| RBL-TTβ            | Avidin | 48.7 ± 0.4 |
| RBL-TTβ            | Anti-Tac-biotin | Avidin | 4.6 ± 0.6                       |
| RBL-TTβ            | DNP-HSA       | Avidin   | 4.4 ± 1.0                       |
| RBL-TTβ            | IgE (saturating) | DNP-HSA | 56.0 ± 0.6                      |
| RBL-TTβ            | IgE (1/5-saturating) | DNP-HSA | 65.7 ± 0.6                      |

fully replace the tetrameric receptor and that they differ from each other in their ability to generate a late signal such as serotonin release.

The ARAM-containing Tail of β Mediates Calcium Flux, but Much Less Efficiently than ARAM-γ or Tetrameric FcεRI—We next asked whether β plays a role in an earlier activation event, the increase in free intracellular calcium, by investigating the ability of the TTβ chimera to mediate a calcium signal. RBL-TTβ and RBL-TTγ cells were saturated with biotinylated anti-Tac B1.49.9, loaded with the calcium-sensitive dye fura-2, and assayed in a spectrofluorometer. Cross-linking of chimeric molecules on RBL-TTβ with avidin leads to a small but reproducible increase in free intracellular calcium (Fig. 3A), but this increase is much smaller than that obtained by stimulation of FceRI on the same cells with 0.5 μg/ml of the anti-FcεRI α antibody BC4 (Fig. 3A). The same pattern was reproduced with 10 different clones stimulated with 15–45 μg/ml of avidin (data not shown). This small response is specific, because it is not observed either in the absence of anti-Tac antibody or on untransfected RBL cells (data not shown). It should also be noted that the response may be underestimated due to the low sensitivity of this assay. In comparison with the response through TTβ, the increase in free intracellular calcium caused by γ chimera cross-linking in RBL-TTγ is stronger, but this response, too, is slower and weaker than that obtained after stimulation through FcεRI on the same cells (Fig. 3A).

We then assessed the contribution of ARAM-β in the tetrameric receptor to the calcium signal by comparing the responses initiated by wild type and mutant receptors. Cross-linking FcεRI on P815-βWT and P815-βY transfectants with 0.5 μg/ml of the anti-FcεRI α antibody BC4 induces a comparable signal from both the wild type and the tyrosine-mutated receptor (Fig. 3B).

From the facts that TTβ generates a weak calcium flux and that mutating the tyrosine residues of β does not affect dramatically the calcium flux generated through FcεRI, we conclude that ARAM-β plays only a minimal role in calcium flux. We also find that ARAM-γ alone is less potent than tetrameric FcεRI. This again indicates that the two ARAMs are not equivalent and that the potency of either one alone is insufficient to account for the activity of the whole receptor.

The ARAM-containing Tail of β Plays a Role in Receptor Phosphorylation—We next investigated whether β plays a role in the earliest observable activation event, phosphorylation of receptor β and γ subunits, which occurs within seconds of FcεRI engagement (17). To compare phosphorylation of intact and mutated receptors in P815-βWT and P815-βY, respectively, the cells were labeled in vivo with [32P]orthophosphoric acid, incubated with anti-DNP IgE, and then activated (or not) by cross-linking with DNP-HSA. After lysis, cell surface FcεRI were
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FIG. 3. Calcium flux measurements. A, RBL-TTβ (dashed line) and RBL-TTγ (dotted line) saturated with biotinylated anti-Tac were loaded with fura-2 AM. Their capacity to respond to stimulation with avidin (30 μg/ml) or with anti-FcεRI α antibody (BC4, 0.5 μg/ml) was measured in a spectrofluorometer. Triton (0.1% final) and EGTA (18 mM final) were added for calibration (off scale). Calcium concentrations were calculated from photon counts using the published value of 2.24 × 10^(-6) for the K_d for fura-2 at 37°C. The time axis for the trace for RBL-TTγ was shifted so that the two avidin triggerings would coincide. B, P815-pWT (solid line) and P815-pY (dashed line) loaded with fura-2 were triggered with anti-FcεRI α antibody (BC4, 0.5 μg/ml). Addition of thrombin was used as a positive control.

immunoprecipitated and resolved on SDS-PAGE. Receptor phosphorylation in P815-βWT resembles that in RBL with basal level phosphorylation of receptor β and γ subunits in resting cells and an increase after triggering (Fig. 4A, lanes 1–3). Radioanalytic imaging analysis shows that the phosphorylation level on β increases by a factor 2 and on γ by 13. According to phosphoamino acid analysis, this increase is on tyrosine for both β and γ (Fig. 4B). In P815-βY there is reduced phosphorylation of β in the resting state and no increase upon activation (Fig. 4A, lanes 4 and 5). As would be expected, there is no tyrosine phosphorylation on the β subunit, demonstrating that the mutations destroyed all the tyrosine phosphorylation sites

Fig. 4. Mutating the tyrosines of β decreases the phosphorylation of the γ chain on intact cells. A, P815-βWT and P815-βY (30 × 10^4 cells/sample) were saturated with anti-DNP IgE, labeled with [γ-32P]phosphoric acid, and incubated at 37°C with medium alone (lanes 1 and 4) or with DNP-HSA (100 ng/ml for 1 min) (lanes 2, 3, and 5). Cell lysates were immunoprecipitated with normal rabbit IgG (lane 2) or rabbit anti-IgE antibody (lanes 1 and 3–5), resolved by SDS-PAGE under reducing conditions, and exposed by autoradiography (upper panel). The same immunoprecipitates were analyzed by Western blotting with a monoclonal anti-β antibody (JRK) (lower panel). B, two-dimensional phosphoamino acid analysis was performed on the β and γ bands cut out from the gel in A. (Fig. 4B). However, quite surprisingly, the phosphorylation levels of resting as well as stimulated γ are also affected by the mutation in β; although the increase in γ phosphorylation is comparable with that in the wild type receptor (a factor of 10), the overall levels are reduced to approximately half of those found in wild type FcεRI.

To examine directly whether the association of kinase activity with the receptor is influenced by the mutation in β, we performed in vitro kinase assays on immunoprecipitated FcεRI from P815-βWT and P815-βY. P815-βWT cells were incubated with different concentrations of anti-DNP IgE (5, 1, and 0.2
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Fig. 5. Mutating the tyrosines of β dramatically decreases the in vitro phosphorylation of the γ chain. P815-βY were saturated with IgE (5 μg/ml) (lanes 7 and 8); P815-βWT were loaded with 5 (lanes 1 and 2), 1 (lanes 3 and 4), and 0.2 μg/ml (lanes 5 and 6) of anti-DNP IgE. Cells were triggered (lanes 2, 4, 6, and 8) or not (lanes 1, 3, 5, and 7) as in Fig. 4, and lysates were immunoprecipitated with anti-IgE antibody. In vitro kinase assays were performed on the immunoprecipitates under the presence of γ-32P-ATP, and the products were analyzed by SDS-PAGE under reducing conditions and autoradiography (upper panel). The same products were analyzed by Western blotting with the anti-β antibody (middle panel) and with a rabbit anti-γ peptide antibody (lower panel).

μg/ml at 1 x 10^7 cells/ml) and P815-βY cells with 5 μg/ml of anti-DNP IgE. Immune complex kinase assays were performed on receptors which had been immunoprecipitated with anti-IgE from unstimulated and stimulated cells. Receptor cross-linking on P815-βWT causes an increase in phosphorylation of β and γ subunits (Fig. 5, upper panel, lanes 1–6), the intensity of which is proportional to the number of receptors engaged. In contrast, immunoprecipitates from triggered P815-βY display only a weak phosphorylation on γ, visible only after overexposure (Fig. 5, upper panel, lanes 7 and 8, and data not shown). Immunoblotting confirms that both β and γ are present in all samples, and significantly more receptor molecules from mutant cells were immunoprecipitated than are required to see increased phosphorylation on wild type receptors (Fig. 5, middle and lower panels, compare lanes 7 and 8 with 5 and 6). Together with the results of the experiments in intact cells, these results indicate that the C-terminal tail of β which contains the ARAM motif plays a critical role in the phosphorylation of FceRI γ. The mutations of the tyrosines in ARAM-β could affect the association of β with a specific kinase, or alternatively, could alter the activity of the kinase.

The ARAM-containing Tail of β Controls Phosphorylation of Other Cellular Proteins—We labeled P815-βWT and P815-βY with [32P]orthophosphoric acid and stimulated them through FceRI as above. After lysis, tyrosine phosphorylated proteins were immunoprecipitated, resolved by SDS-PAGE, and revealed by autoradiography (Fig. 6). In P815-βWT, activation induces phosphorylation of various substrates: two at approximately 35 and 55 kDa, three in the 70–80 kDa range, and additional species at higher molecular masses, as well as FceRI β and γ identified by Western blotting in a similar experiment (data not shown). Of these, FceRI β and γ are absent or reduced in activated P815-βY, as observed after receptor precipitation. In addition, the 55-kDa band is absent and the highest band in the 70–80-kDa range is reduced in the mutant, indicating that phosphorylation of additional proteins besides FceRI γ is controlled by the C-terminal tail of β.

Lyn Binds to Nonactivated FceRI β via the C-Terminal Tail of β, but Not to γ—The Src family kinase, Lyn, associates with FceRI and is activated after FceRI aggregation (29). However, the γ chimera is unable to activate Lyn (32). We therefore asked whether Lyn might interact preferentially with β. First, to verify that Lyn is expressed in P815, we performed in vitro kinase assays after immunoprecipitation with anti-Fgr, -Hck, -Lck, -Lyn, -Fyn, and -Yes antibodies. In that analysis, only Lyn is detected in P815 (data not shown). We then used an in vitro kinase assay of receptor immunoprecipitates as a way to label the kinase(s) associated with the receptor (30, 31). Surface FceRI was immunoprecipitated from nonstimulated P815-βWT and P815-βY cells and subjected to in vitro kinase assay. To identify Lyn as a kinase associated with the receptor, the labeled immune complexes were eluted from the beads and re-precipitated was performed with anti-Lyn or a control antibody. It should be noted that the presence of phosphorylated Lyn after the second immunoprecipitation would then reflect its association to surface FceRI. In fact, anti-Lyn specifically precipitates Lyn as a band of 56 kDa from wild type receptor complexes (Fig. 7A, lane 2). The intensity of this band is minimally reduced when the tyrosines in ARAM-β are mutated (lane 4).

We performed the same experiments with nonactivated RBL-TTβ and RBL-TTγ, in which the chimeras are not visibly phosphorylated (see Fig. 8). In these cells, immune complex kinase assays were performed for both the endogenous tetrameric FceRI and the chimeras. In two separate experiments Lyn is reprecipitated from the FceRI immunoprecipitates of both cell lines: the anti-Lyn antibody, though, reprecipitates Lyn only from the TTβ chimera and not from the TTγ chimera (Fig. 7B). We conclude that Lyn interacts preferentially with the C-ter-
with 1 pg/ml of anti-DNP IgE eluates were reprecipitated with a rabbit anti-Lyn peptide antibody were precipitated with rabbit anti-IgE antibody. The precipitates were labeled with IgE (5 pg/ml) by SDS-PAGE and autoradiography.

from the beads and reprecipitated with control or anti-Lyn antibody performed on immunoprecipitates, which were subsequently eluted in vitro kinase assay, then eluted from the beads. The eluates were reprecipitated with a rabbit anti-Lyn peptide antibody (lanes 2 and 4) or with a control antibody (lanes 1 and 3) and analyzed by SDS-PAGE and autoradiography. B, transfected RBL were saturated with IgE or with anti-Tac antibody and immunoprecipitated with anti-IgE or anti-mouse IgG, respectively (1st ip). In vitro kinase assays were performed on immunoprecipitates, which were subsequently eluted from the beads and reprecipitated with control or anti-Lyn antibody (2nd ip). The products from the second immunoprecipitations were analyzed by SDS-PAGE and autoradiography.

minal tail of β independently of any triggering and that this interaction does not critically require the tyrosine residues in ARAM-β. This extends the observation that the γ chimera is unable to activate Lyn (32).

The γ Tail Controls Phosphorylation of a 72-kDa Substrate, the Tyrosine Kinase Syk—We compared which proteins become phosphorylated on tyrosine after cross-linking the Tac chimeras on RBL-TTβ and RBL-TTγ. Cells were labeled with [32P]orthophosphoric acid, incubated either with anti-DNP IgE or biotinylated anti-Tac, and then activated (or not) by cross-linking with either DNP-HSA or avidin. After cell lysis, tyrosine-phosphorylated proteins were immunoprecipitated and resolved on SDS-PAGE. Cross-linking of naturally expressed FceRI on RBL-TTγ (Fig. 8, lanes 3 and 4) and RBL-TTβ (data not shown) leads to increased phosphorylation of several proteins, including the receptor β and γ subunits (17,33) and a 72-kDa protein (34, 35). When RBL-TTγ cells are stimulated through the chimera with biotinylated anti-Tac and avidin, we observe the appearance of two phosphorylated species, one at approximately 200 kDa (better visible on a shorter exposure) and another at 72 kDa (Fig. 8, lanes 1 and 2), the latter one of which comigrates with the 72-kDa band from the same cells triggered through FceRI (Fig. 8, compare lanes 2 and 4). In RBL-TTβ, triggering through the chimera also induces phosphorylation of a 200-kDa band, but we do not detect a 72-kDa band (Fig. 8, lanes 6 and 7).

In neither RBL-TTβ nor TTγ can we detect a phosphorylated band in the region corresponding to the molecular weight of the chimeras, which was determined by running iodinated chimera precipitated from the same cells on a separate gel (Fig. 8, lane 5) as well as by Western blotting (data not shown). Similarly, on an anti-Tac precipitation of the supernatants after anti-phosphotyrosine precipitation, no phosphorylated TTγ chimera is visible, even though the chimera is detected by Western blotting with an anti-γ antibody (data not shown). In addition, Triton X-100-insoluble fractions of the lysates were treated with radioimmune precipitation assay (RIPA) buffer and the supernatants, following a second centrifugation, were precipitated with the anti-phosphotyrosine antibody. A phosphorylated chimera is not detected under these conditions either (data not shown). However, we cannot exclude phosphorylation of the chimeras at a lower level than the detection limit of this technique.

Various groups have reported on the phosphorylation of a 72-kDa band induced by triggering through FceRI (34–36).

**Fig. 7. Lyn binds to nonactivated β.** A, P815-βWT were loaded with 1 µg/ml of anti-DNP IgE (lanes 1 and 2), and P815-βY were saturated with IgE (5 µg/ml) (lanes 3 and 4). Lysates from nontriggered cells were precipitated with rabbit anti-IgE antibody. The precipitates were subjected to an in vitro kinase assay, then eluted from the beads. The eluates were reprecipitated with a rabbit anti-Lyn peptide antibody (lanes 2 and 4) or with a control antibody (lanes 1 and 3) and analyzed by SDS-PAGE and autoradiography. B, transfected RBL were saturated with IgE or with anti-Tac antibody and immunoprecipitated with anti-IgE or anti-mouse IgG, respectively (1st ip). In vitro kinase assays were performed on immunoprecipitates, which were subsequently eluted from the beads and reprecipitated with control or anti-Lyn antibody (2nd ip). The products from the second immunoprecipitations were analyzed by SDS-PAGE and autoradiography.

**Fig. 8. Aggregation of TTβ and TTγ chimeras on transfected RBL induces phosphorylation of different substrates.** Transfected RBL were saturated with biotinylated anti-Tac (lanes 1, 2, 6, and 7) or with anti-DNP IgE (lanes 3 and 4), labeled with [32P]orthophosphoric acid (lanes 1–4, 6, and 7), and then incubated at 37°C with medium alone (lanes 1, 3, and 6) or with avidin (45 µg/ml for 5 min for RBL-TTβ, 30 µg/ml for 5 min for RBL-TTγ) (lanes 2 and 7), or DNP-HSA (100 ng/ml for 1 min) (lane 4). Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody (4G10) and analyzed by SDS-PAGE under reducing conditions and autoradiography. The position of the Tyr molecule was determined by running on another gel an anti-Tac immunoprecipitate from on an RBL-TTγ cell lysate after [32P]orthophosphoric acid, incubated either with anti-DNP IgE or biotinylated anti-Tac, and then activated (or not) by cross-linking with either DNP-HSA or avidin. After cell lysis, tyrosine-phosphorylated proteins were immunoprecipitated and resolved on SDS-PAGE. Cross-linking of naturally expressed FceRI on RBL-TTγ (Fig. 8, lanes 3 and 4) and RBL-TTβ (data not shown) leads to increased phosphorylation of several proteins, including the receptor β and γ subunits (17, 33) and a 72-kDa protein (34, 35). When RBL-TTγ cells are stimulated through the chimera with biotinylated anti-Tac and avidin, we observe the appearance of two phosphorylated species, one at approximately 200 kDa (better visible on a shorter exposure) and another at 72 kDa (Fig. 8, lanes 1 and 2), the latter one of which comigrates with the 72-kDa band from the same cells triggered through FceRI (Fig. 8, compare lanes 2 and 4). In RBL-TTβ, triggering through the chimera also induces phosphorylation of a 200-kDa band, but we do not detect a 72-kDa band (Fig. 8, lanes 6 and 7).
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| FcεRI | TTγ |
|-------|-----|
| **trig** | ++ | ++ |
| **syk peptide** | ++ | + |

![Graph showing the analysis of tyrosine kinases](image)

**Fig. 9. Aggregation of TTγ activates Syk.** Cells were processed as described in Fig. 8 with the omission of [32P]phosphoric acid labeling. FcεRI samples: 10 x 10⁶ cells/ lane; TTγ samples: 45 x 10⁶ cells/ lane. The anti-phosphotyrosine precipitates were subjected to an in vitro kinase assay, then eluted in the presence of 10 mM phenylphosphate. Reprecipitation was performed with an anti-Syk antibody in the presence or absence of the Syk peptide. The products from the second immunoprecipitation were analyzed by SDS-PAGE and autoradiography.

More recently, this band has been identified as the Syk kinase (46, 37). To identify the 72-kDa band present in Fig. 8 as Syk, we performed in vitro kinase assays on anti-phosphotyrosine precipitates before and after triggering through the endogenous FcεRI or through TTγ, followed by elution and reprecipitation with anti-Syk in the absence or presence of the peptide used to produce the antibody (Fig. 9). A band of 72 kDa is specifically reprecipitated by the anti-Syk antibody after FcεRI and TTγ triggering. From a radioanalysis of the counts associated with the 72 kDa, we also determined that, on a per receptor basis (see Fig. 2), TTγ triggering is about 20-fold less efficient than FcεRI triggering.

**DISCUSSION**

Various studies indicate that isolated ARAMs activate cells in the same way as the multimeric receptors that include them (4–10). However, the relationship between ARAMs and the various tyrosine kinases activated by these receptors is still debated (38–42). In addition, the question persists why, if the action of an entire receptor can be mimicked by a short motif in a single subunit, these receptors should be such complex multimeric structures. Here we have compared the signaling capacity of the ARAM-containing β and γ chains of FcεRI and have analyzed their relationship with the two kinases Lyn and Syk.

We demonstrate that the ARAM-containing tails of β or γ cannot substitute for the entire receptor complex and that they differ in their capacity to activate specific cellular functions. 1) Engagement of a chimera containing the cytoplasmic tail of γ induces calcium flux and serotonin release, but, even under optimal conditions, these signals are weaker than those obtained from the tetrameric receptor under suboptimal conditions (Table I and Fig. 3). 2) Engagement of a chimera containing the C-terminal tail of β does not induce serotonin release. Judging from the weakness of the calcium signal induced by β chimera engagement and from the fact that mutations in ARAM-β do not affect calcium flux in P815, β does not seem to play an essential role in calcium mobilization (Fig. 3). Unlike tetrameric FcεRI, the β and γ chimeras mediate the phosphorylation of few substrates (Fig. 4–6).

We also uncover a potential mechanism for these different signaling patterns of β and γ in that the two chains control different kinases. Lyn binds the β chimera, as we show by reprecipitating the kinase from anti-chimera immune complexes (Fig. 7B). Syk is phosphorylated and activated through the γ chimera (Figs. 8 and 9). Because of the critical role of early phosphorylation events in the signaling pathway of FcεRI (17, 33–35), this represents a significant difference between β and γ and demonstrates further the specificity of the relationship between β and Lyn and between γ and Syk.

Although we have shown that β binds Lyn, the precise means of this interaction is not clear. The modest reduction in the binding of Lyn to tetrameric receptors when the tyrosine residues of β are mutated, as compared with wild type β (Fig. 7A), suggests that the interaction is only partly dependent upon the tyrosines in ARAM-β. Thus, the fact that the ARAM-β contains a sequence (YEEIL) homologous to the phosphopeptide pYEEIL, which binds the SH2 domains of Src and Lck and in which the phosphorylated tyrosine is essential (43–45), does not appear to be crucial. This raises the possibility of a novel mode of interaction between at least some members of the Src family kinases and other molecules with which they associate.

The results presented here, our own previous investigations of FcεRI signal transduction, and results obtained for other antigen receptors suggest the following model. We hypothesize that β and γ synergize to give fully productive triggering. Receptor aggregation causes activation of Lyn, already bound to β, which then phosphorylates γ. The phosphorylation of the 2 tyrosines in ARAM-γ induces its interaction with the two SH2 domains of Syk, similarly what has been reported for the SH2 domains of Src and Lck (43–45). This leads to the activation of Syk followed by the phosphorylation and activation of PLCγ-1 (33, 46) and to subsequent calcium mobilization. In addition to the data presented here, several facts support this model. First, we have previously found evidence for two phosphorylation pathways activated by FcεRI, one involving phosphorylation of the receptor and the other phosphorylation and activation of PLCγ-1 (26). Taking into account our present observations, we now propose that the former is mediated by Lyn and the latter by Syk. Second, in the tetrameric receptor β plays a unique role and cannot be replaced by the dimer of γ. This is shown by the fact that deleting the C-terminal tail of β abolishes signaling, even though the dimer of γ is present (14). In addition, β can get phosphorylated independently of γ in the tetrameric receptor as demonstrated by the fact that triggering still induces phosphorylation of β when the tyrosines of ARAM-γ are mutated.²

According to our model, the question remains how the γ chimera is able to signal in the absence of β. Two mechanisms can be envisioned. In the absence of any triggering, the γ chimera could exhibit a low degree of phosphorylation, below the sensitivity of our methods of detection, allowing it to interact with Syk and initiate the corresponding activation pathway. Alternatively, in the absence of β, the γ chimera could exhibit a weak affinity for Lyn, sufficient to initiate Syk activation. We propose that the β and γ chimeras do not function exactly like the corresponding chains in the tetrameric receptor, possibly due to altered topological arrangements between these chains.

² R. Padini, K. Ochiai, M.-H. Jouvin, Y. Ueda, C. H. June, and J.-P. Kinet, submitted for publication.
and signaling molecules. This is suggested by the inability to signal of a receptor where the C-terminal tail of β is truncated, although the dimer of γ chains is still present (14).

One major finding of this study is that the two β and γ chains of FcεRI are in fact mediating different functions, even though they contain homologous ARAM signaling motifs. Similar findings have now been reported for the B-cell antigen receptor. The Ig-associated proteins Ig-α and Ig-β bind different signaling molecules (47) and only Ig-α can trigger tyrosine kinase activation, although both molecules are able to induce calcium flux independently of each other (48). Another interesting comparison is provided by the TCR. The phosphorylated ζ chain of the TCR, which is homologous to the γ chain of FcεRI, interacts with ZAP-70, a kinase homologous to Syk (39). Also, in antigen-induced T-cell activation, additional signals are provided by the CD8 coreceptor. These accessory molecules bind and, when cross-linked, activate the Src family kinase Lck (49). Activation of Lck by CD4 or CD8 may synergize with the activation of Lyn and ZAP-70 by the TCR, similar to the synergy we propose between Lyn engaged by β and Syk activated by γ.

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REFERENCES
1. Samelson, L. E., and Klausner, R. D. (1992) J. Biol. Chem. 267, 24913–24916
2. Weiss, A. (1993) Cell 73, 209–212
3. Reith, M. (1989) Nature 340, 365–366
4. Irving, B. A., and Weiss, A. (1991) Cell 64, 891–901
5. Romeo, C., and Seed, B. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 8095–8099
6. Letourneur, F., and Klausner, R. D. (1992) Science 255, 79–83
7. Amigorena, S., Salamero, J., Davoust, J., Fridman, W. H., and Bonnerot, C. (1992) Nature 355, 337–341
8. Donner, J., Amigorena, S., Choquet, D., Pavlovich, R., Choukroun, V., and Fridman, W. H. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 10733–10737
9. Chen, A. C., Iwashima, M., Tuck, C. W., and Weiss, A. (1999) Cell 97, 649–662
10. Kolasus, W. C., Romeo, C., and Seed, B. (1993) Cell 74, 171–183
11. Hall, C. S., Sancho, J., and Torsher, D. (1993) Science 261, 915–918
12. Salcedo, T. W., Kurukuri, T., Kanakaraj, P., Ravetch, J., and Perussia, B. (1993) J. Exp. Med. 177, 1475–1480
13. Eck, M. J., Shoelson, S. E., and Harrison, S. C. (1993) Nature 362, 87–91
14. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnoffof, S., Leehleider, R. J., Neel, B., Birge, R. B., Fajardo, J. E., Chou, M. M., Hahn, H., Schaffhausen, B., and Cantley, L. C. (1993) Cell 72, 767–778
15. Waskam, G., Shoelson, S. E., Past, N., Cowburn, D., and Kuriyan, J. (1993) Cell 72, 779–786
16. Park, D. J., Min, H. K., and Rhee, S. G. (1991) J. Biol. Chem. 266, 24237–24240
17. Clarke, M. R., Campbell, K. S., Kazlauscas, A., Johnson, S. A., Hertz, M., Potter, T. A., Pietman, C., and Camiller, J. C. (1992) Science 258, 223–226
18. Kim, K.-M., Alber, G., Weiser, P., and Reth, M. (1993) Eur. J. Immunol. 23, 911–916
19. Veillet, A., Bookman, M. A., Hark, E. M., Samelson, L. E., and Bolen, J. B. (1989) Nature 338, 257–259