Different Roles for the FcεRI γ Chain as a Function of the Receptor Context

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Summary

The high affinity immunoglobulin E receptor (FcεRI) and the B and T cell antigen receptors (TCR) are multimeric complexes containing subunits with cytoplasmic antigen recognition activation motifs (ARAMs). The presence of multiple motifs may be a way to amplify a single signal or provide independent activation modules. Here we have compared the signaling capacity of the same FcεRI γ motif in the context of two different receptors, FcεRI and TCR/CD3, simultaneously reconstituted on the surface of the same ζ-deficient T cell line. Both reconstituted receptors mediate early (phosphorylation) and late (interleukin [IL]-2 release) signals. Mutation of the two tyrosine residues of ARAM γ alters early signaling by both receptors, but the set of substrates phosphorylated via ARAM γ is different for each receptor and is thus dependent on the receptor context. Furthermore, the mutations prevent FcεRI- but not TCR/CD3-mediated IL-2 release. These data demonstrate that ARAM γ is necessary for allowing both receptors to phosphorylate the complete set of substrates, and that the CD3 complex, unlike the FcεRI β chain, contains activation modules capable of compensating for the absence of a functional ARAM γ in generating late signals such as IL-2 release.

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The B and T cell antigen receptors (TCR/CD3), the high (FcγRI) and low (FcγRIII/CD16) affinity Fc receptors for IgG, and the high affinity Fc receptor for IgE (FcεRI) belong to a class of multimeric receptors associated with nonreceptor tyrosine kinases (1–12). The activation of these kinases is one of the first steps in the signaling cascade, allowing the multimeric receptors to initiate various specialized functions. FcεRI is a tetrameric complex formed by an IgE-binding α chain, a β chain, and a homodimer of γ chains (13, 14). The TCR/CD3 complex is also a multichain structure composed of the two antigen-binding α and β chains, the three invariant chains γ, δ, and ε constituting the CD3 complex, and one of the six possible dimers formed by disulfide linkage between TCR ζ, η, and FcεRI γ chains (15–17). The homodimers γγ and ζζ and the heterodimer γζ are also part of one Fcγ receptor (FcγRIII/CD16) and are therefore subunits common to Fc and T cell receptors (16–25). The high level of homology between the transmembrane domains of γ and ζ (26) may explain why these dimers associate equivalently with various receptor complexes and appear interchangeable. In addition, the presence of these γ- or ζ-containing dimers is required for efficient surface expression of the multimeric complexes (18, 21–23, 27, 28).

The cytoplasmic tails of the FcεRI γ, ζ, and η chains contain a common 18–amino acid motif named antigen recognition activation motif (ARAM)[2] (29–31), which, when fused to the extracellular and transmembrane part of an unrelated transmembrane protein, confers receptor-like activity to the chimeric molecule (32–38). Signaling through this motif is absolutely dependent on the presence of two intramotif tyrosines (34, 36–38). ARAMs are also present in the γ, δ, and ε chains of CD3 and in the β chain of FcεRI (29–31). The fact that ARAM ζ or ARAM γ by itself is able to generate the various signals observed with the entire receptor raises the question of the function of the other ARAMs present in the same receptor. In the TCR it has been shown that two autonomous transduction modules exist: the ζ chain and the γδε chains (39). A TCR with a truncated ζ chain devoid of ARAMs is able to induce IL-2 release via the CD3 com-

[1] Both authors contributed equally to this work.

[2] *Abbreviations used in this paper:* ARAM, antigen recognition activation motif; CTLL, cytotoxic T lymphocyte leukemia; DNP, dinitrophenyl; FBS, fetal bovine serum; HSA, human serum albumin; NEPHGE, nonequilibrium pH gel electrophoresis.

plex. In the B cell antigen receptor, the ARAMs of Ig-α and Ig-β also control distinct signals (4). Similarly, the β and γ chains of FcεRI control different tyrosine kinases: β binds Lyn of the Src family and γ activates Syk of the Syk/ZAP-70 family (40).

The FcεRI γ chains are part of at least four different types of receptors: FcγRI (41, 42), FcγRIII (18, 21, 22), FcεRI (13, 14), and TCR (17). Some of these γ chain–containing receptors are expressed concomitantly on the surface of the same cell. For example, FcεRI and FcγRIII are both expressed on mast cells, and FcγRIII and TCR are expressed on subsets of T cells. Therefore we wanted to address whether FcεRI γ, and the FcεRI γ motif in particular, could function differently when part of different multimeric complexes. We have reconstituted two different γ chain–containing complexes, FcεRI and TCR, on the surface of the same cell, and have compared the signaling capacity of each γ chain motif in the natural environment of their respective receptor. To avoid any interference from endogenous subunits, we have chosen to reconstitute these receptors in a T cell line deficient in ζ, γ, and η chains (28).

Materials and Methods

Cell Lines and Media. 2.M2 cells, a variant of the 2B4 mouse T cell line that lacks the ζ chain of the TCR (28) (kindly provided by R. Klausner, National Institutes of Health, Bethesda, MD), were cultured in RPMI-1640 with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 50 μM β-ME and 2 mM L-glutamine. An IL-2–dependent T cell, CTLL (a gift of H. Rigger, National Cancer Institute, National Institutes of Health, Frederick, MD), was maintained in RPMI-1640 with 10% FCS, 2 mM L-glutamine, and 20 U/ml rhIL-2. The BWδ cells were previously described (39).

Reagents and Antibodies. Dinitrophenyl (30) human serum albumin (DNP-HSA, 30 molecules of DNP per molecule of HSA) was from Sigma Chemical Co. (St. Louis, MO). Radiochemicals were purchased from Amersham Corp. (Arlington Heights, IL). The chemiluminescent substrate for Western blotting, AMPPD, was purchased from Tropix, Inc. (Bedford, MA).

Anti-DNP monoclonal mouse IgE was purified from the supernatant of hybridoma Hi-DNP ϵ 26.82 grown in a Miniflo-path bioreactor (Amicon, Beverly, MA) by ion exchange chromatography on diethylaminoethyl-Trisacryl (IBF Biotechnics, Columbia, MD). The goat anti–hamster IgG and the goat anti–mouse IgG were purchased from Organon Teknika (Durham, NC). Fluorescein-conjugated rabbit anti–hamster IgG was from Pierce Chemical Co. (Rockford, IL). The goat anti–mouse and goat anti–rabbit antibodies conjugated to alkaline phosphatase were from Jackson ImmunoResearch Labs, Inc. (West Grove, PA); the antiphosphotyrosine antibody 4G10 was from UBI (Lake Placid, NY); the anti-FcεRI β subunit antibody JRK and the anti-FcεRI γ subunit antiserum were described previously (43).

^{125}I-iodination of BC4, an anti–rat FcεRI α monoclonal antibody (kind gift of Reuben Siraganian, National Institute of Dental Research, National Institutes of Health) and 2C11, a monoclonal anti-CD3ϵ (kind gift of Jeffrey Bluestone, University of Chicago, Chicago, IL) was performed by use of chloramine T.

Mutants and Transfections. Point mutations in rat FcεRI γ cDNA were created via PCR. The full-length cDNA was amplified with a 5′ primer (bp 16–37) including the first ATG and a 3′ primer (bp 289–172) starting after the stop codon, converting tyrosine (Y) residues to phenylalanine (F). 2.M2 cells were cotransfected by electroporation with rat FcεRI α and β cDNAs subcloned in pcDL-SRα 296 (44) and with either the wild-type or the mutated form of rat γ cDNA subcloned in pBJ1neo (45). Cells were grown under G418 (GIBCO BRL, Gaithersburg, MD) selection, and resistant clones were selected for FcεRI and CD3 surface expression. BWδ cells were transfected with either the wild-type or the mutated form of rat γ cDNA subcloned in pBJ1neo.

Flow Cytometry Analysis. Flow cytometric analysis was performed by use of a FACScan® (Becton Dickinson & Co., San Jose, CA) after staining of the 2.M2 cells (5×10^5) with 10 μg/ml of FITC-conjugated mouse IgE or 10 μg/ml of monoclonal anti-CD3ϵ (2C11) followed by FITC-conjugated rabbit anti–hamster antibody (Organon Teknika). For FcεRI, the background staining was assessed by preincubation with 200 μg/ml of mouse IgE and for CD3/TCR by staining with the second antibody only.

In Vivo Phosphorylation, Immunoprecipitation, Two-dimensional Nonequilibrium pH Gel Electrophoresis (NEPHGE)-PAGE, SDS-PAGE, and Western Blotting. 2.M2 cells were incubated in phosphate-free medium for 4 h at 37°C and then labeled at 3×10^7 cells/ml with 37 MBq/ml [^{32}P]orthophosphate for 2 h at 37°C. The cells (2×10^7/ml) were incubated at 4°C for 30 min with 10 μg/ml of anti-FcεRI α (BC4) or anti-CD3ϵ (2C11) monoclonal antibodies, and then stimulated for 2 min with medium alone or with 50 μg/ml of the appropriate second antibody, as indicated for individual experiments. The cells were then immediately washed and lysed as previously described (43). The postnuclear supernatants were precleared with protein A–Sepharose beads, and then immunoprecipitated with antiphosphotyrosine monoclonal antibody bound to protein A–Sepharose. For two-dimensional analysis, the tyrosine-phosphorylated proteins were loaded at the anode end of 6% acrylamide, 2% ampholine (pH 3.5–10) tube gels. Separation was performed for 6 h at 500 V. NEPHGE gels were equilibrated for 1 h with 1× SDS sample buffer and then frozen at −70°C or applied directly on top of the second-dimension gels, which were run at 20 mA/gel. To show the tyrosine phosphorylation of the γ chains, the proteins precipitated by antiphosphotyrosine antibody were eluted with phenylphosphate and the γ chains immunoprecipitated with anti-γ antiserum. The samples were analyzed by SDS-PAGE (13.5% gel under reducing conditions) and autoradiography.

For Western blotting, 10^8 cells were lysed in a buffer containing 1% digitonin, 150 mM NaCl, 50 mM Hepes, pH 7.5, and protease and phosphatase inhibitors as previously described (43). The postnuclear supernatants were immunoprecipitated and analyzed by SDS-PAGE as above, and the proteins were transferred electrophoretically to membranes (Immobilon-P; Millipore Corp., Bedford, MA) for 6 h at 50 V. Membranes were then incubated overnight in TBS (20 mM Tris, pH 7.5, 500 mM NaCl) containing 5% BSA before a 2-h incubation in the same buffer containing 1:200 dilution of anti-γ serum. After two washes in TTBS (TBS containing 0.1% Triton X-100 and 2.5% BSA), the membranes were incubated with 0.2 μg/ml goat anti–rabbit antibody conjugated to alkaline phosphatase and developed with the chemiluminescence substrate AMPPD according to the manufacturer.

IL-2 Production and Biological Assay. For stimulation with 2C11, flat-bottom 96-well microtiter plates (Costar Corp., Cambridge, MA) were coated with 50 μl of PBS containing 10 μg/ml of 2C11 (0.5 μg/well). For stimulation with BC4, microtiter wells were first precoated overnight at 4°C with 2.5 μg/well of goat anti–mouse and then, after a 2-h saturation with 10% FBS in RPMI-1640, with 1 μg/well of BC4. After 2 h at room temperature and 1 h at 4°C, the wells were washed three times with PBS, and 10^5 cells in 250 μl of RPMI were added to each well. In all assays,

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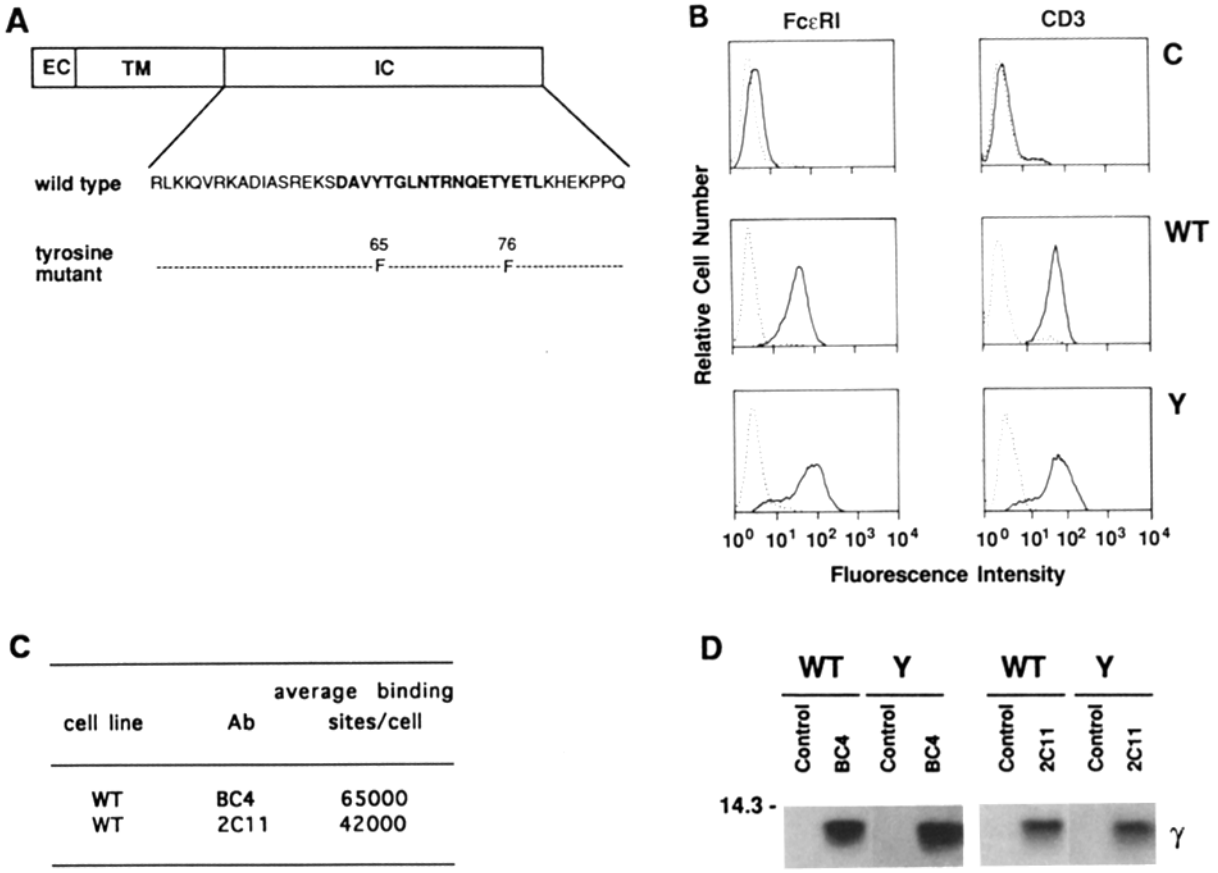
after 24 h of culture, supernatants were collected and assayed for IL-2 content by measuring the proliferation of IL-2–dependent murine T cells (CTLL). CTLL cells were incubated with IL-2–free medium for 3 h at 37°C, and then extensively washed with the same medium; 50-μl aliquots (3×10^5 cells/ml) were plated in 96-well microtiter plates with serial dilutions of supernatants. After 18 h at 37°C, 1 μCi of [^{3}H]thymidine was added to each well for 6 h. [^{3}H]Thymidine incorporation was quantitated by harvesting onto glass fiber filters by use of a semiautomated cell harvester (LKB Wallac, Gaithersburg, MD) and by placing the filters in a scintillation counter (Beta plate™ liquid scintillation counter; LKB Wallac) (NIH laboratory) or by direct β counting by use of gas ionization (Matrix 96 direct beta counter; Packard Instrument Co., Meriden, CT) (INSERM laboratory) (39). The two different methods of quantitation explain the differences in the overall ^{3}H counts observed with 2.M2 transfectants (NIH laboratory) and BWδ transfectants (INSERM laboratory).

Results

Simultaneous Expression of FcεRI and TCR/CD3 Complexes Containing Wild-type and Mutated γ Chain Motifs on 2.M2 Cells. The cDNAs coding for FcεRI α, β, and γ chains were cotransfected into 2.M2 cells, a variant of the 2B4 mouse T cell line which lacks TCR ζ, TCR η, and FcεRI γ and, as a result, does not express TCR/CD3 complexes at the cell surface (28). To identify γ motif–controlled functions, we mutated the two intramotif tyrosines, which are required for activity (37, 38), and we cotransfected the FcεRI γ mutant cDNA with FcεRI α and β cDNAs (Fig. 1 *A*). Cotransfection of FcεRI α and β cDNAs with either wild-type or tyrosine-mutated γ cDNA results in cell surface expression of FcεRI receptors capable of binding IgE (Fig. 1 *B*, *left*). Because the FcεRI γ chain can substitute for the absent TCR ζ chain (27), the cotransfected cells also express surface TCR/CD3 complexes (Fig. 1 *B*, *right*). Two clones with wild-type γ (WT23 and WT66) and three clones with tyrosine-mutated γ (Y1, Y5, and Yc) were used for further analysis.

The levels of the two reconstituted complexes containing wild-type γ chains on clone WT23 are approximately equivalent, as shown by a binding assay using iodinated monoclonal



cell line	Ab	average binding sites/cell
WT	BC4	65000
WT	2C11	42000

Figure 1. Simultaneous expression of FcεRI and TCR/CD3 complexes containing wild-type or mutated γ chain. (*A*) Schematic representation of the wild-type and tyrosine-mutated FcεRI γ chain. The sequence of ARAM (29) is indicated in bold. The two tyrosine residues that were replaced with phenylalanine are indicated by F and their respective amino acid numbers (and single-letter code of the amino acid that was substituted). (*B*) Flow cytometry analysis of FcεRI and TCR/CD3 surface expression in transfected 2.M2 cells. Solid lines indicate specific staining; dotted lines indicate background staining. Untransfected cells (*C*); cells cotransfected with FcεRI α, β and either the wild-type γ (*WT*) or the tyrosine-mutated γ (*Y*) cDNAs. (*C*) Average antibody-binding site numbers were calculated from saturation binding data of ^{125}I-labeled BC4 and ^{125}I-labeled 2C11. Depending on the stoichiometry of binding of BC4 to FcεRI α and 2C11 to TCR ϵ, the receptor numbers are either the ones reported in the table or twice as high. (*D*) Anti-γ immunoblotting of FcεRI complexes immunoprecipitated from total extract with the BC4 monoclonal antibody and TCR/CD3 complexes immunoprecipitated with the 2C11 monoclonal antibody. M_r calibration is shown on the left.

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antibodies directed against FcεRI α (BC4) or CD3ε (2C11) (Fig. 1 *C*) or by FACS analysis (data not shown).

To demonstrate that both FcεRI and TCR/CD3 complexes contain FcεRI γ chains, total cell extracts from WT or Y transfectants were incubated with BC4 and 2C11. The immunoprecipitates were then analyzed by SDS-PAGE followed by immunoblotting with anti-γ or anti-β. The FcεRI complexes in both transfectants contain equivalent amounts of γ (Fig. 1 *D*) and β subunits (data not shown). Similarly, anti-γ immunoblotting of the anti-CD3ε precipitates shows that the TCR/CD3 complexes associate equivalently with wild-type and tyrosine-mutated γ chains (Fig. 1 *D*). As expected, an anti-FcεRI β immunoblot and an anti-TCR ζ immunoblot of the anti-CD3ε precipitates did not detect any reactive bands (data not shown).

Engagement of TCR/CD3 and FcεRI Induces Tyrosine Phosphorylation of the γ Chain. To determine whether stimulation through reconstituted receptors activates early signaling events, we first studied whether cross-linking could induce the tyrosine phosphorylation of γ. After in vivo labeling of the transfected 2.M2 cells with [^{32}P]orthophosphate, FcεRI or TCR/CD3 complexes were engaged (or not) with relevant mAbs and then cross-linked with second antibodies. Phosphorylated proteins were eluted from an antiphosphotyrosine immunoprecipitate and reprecipitated with an anti-γ antiserum before SDS-PAGE (Fig. 2). Phosphorylation of both β and wild-type γ chains occurred in activated FcεRI (lane *2*), as previously described in a mast cell line (43). The wild-type γ chain is also phosphorylated after TCR/CD3 engagement (lane *4*). As expected, the phosphorylation of γ is lost in both receptors when the tyrosines of ARAM-γ are mutated (lanes *6* and *8*). Thus, each receptor complex is able to activate a tyrosine kinase capable of phosphorylating the γ chain. In addition, the fact that the mutation of the γ chain does not affect the phosphorylation of the FcεRI β chain (compare the 35-kD band in lanes *2* and *6*) indicates that the kinase responsible for β phosphorylation is not under the control of ARAM-γ. This kinase is likely to be the Src family kinase Fyn because in vitro kinase assays after immunoprecipitation with anti-Fyn show an increase in kinase activity after aggregation of either FcεRI or TCR/CD3 (data not shown).

The γ Motif Associated with TCR/CD3 and FcεRI Controls the Phosphorylation of Different Substrates in 2.M2. To further our understanding of the role of the γ motif in the activation of tyrosine kinases, we analyzed the tyrosine phosphorylation of the various substrates induced by the two receptor complexes. After engagement of FcεRI or TCR/CD3 complexes, the ^{32}P-labeled phosphorylated proteins were immunoprecipitated with antiphosphotyrosine antibody and resolved

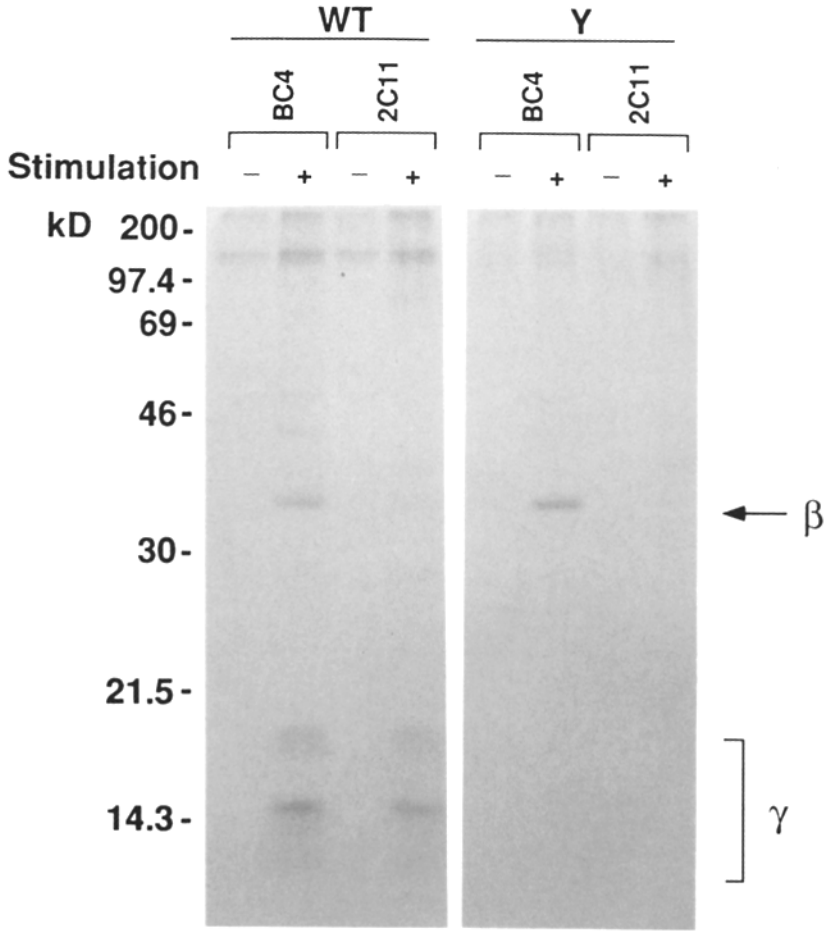

Figure 2. Activation-induced phosphorylation of γ chains from TCR/CD3 and FcεRI. ^{32}P-labeled wild-type γ (*WT*) and tyrosine-mutated γ (*Y*) transfectants were incubated at 4°C for 30 min with BC4 or 2C11, and then stimulated for 2 min with medium alone or the specific second antibody, as indicated for individual lanes. The tyrosine-phosphorylated proteins were immunoprecipitated with antiphosphotyrosine antibody, eluted with phenylphosphate and the γ chains immunoprecipitated with anti-γ antiserum, and separated by SDS-PAGE and autoradiography as before (43). The positions of molecular weight standards and the β and γ chains of FcεRI are indicated.

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by two dimensional gels, the first dimension being NEPHGE and the second SDS-PAGE (Fig. 3). Under resting conditions, a number of substrates are phosphorylated (*A*). At least two prominent (*a*,*b*) and a few fainter substrates disappear when the tyrosines of γ are mutated (compare *A* and *C*). This indicates that the γ motif directly or indirectly controls the resting level of phosphorylation of these substrates. Engagement of wild-type FcεRI with anti–FcεRI α cross-linked with a second antibody (*B*) or with IgE and multivalent antigen (not shown) increases the phosphorylation of several substrates and results in the appearance of an acidic 42-kD phosphorylated species (*d*) and another around 150 kD (*c*). Other phosphorylated species, although faint, are also reproducibly seen around 32, 40 and 48 kD, which correspond to the positions of phosphorylated and phosphorylated/ubiquitinated FcεRI β chains, already described (46). Among these substrates only phosphorylated after FcεRI engagement, substrate *d* is the only one to disappear after mutation of the tyrosines in γ (compare *B* and *D*). Therefore activation through the γ motif in the context of FcεRI controls the phosphorylation of substrate *d*.

Engagement of wild-type CD3 also results in the tyrosine phosphorylation of various substrates (Fig. 3, *E* and *F*). Most of these substrates are common to both CD3 and FcεRI engagement (compare *B* and *F*). However, at least three substrates around 120 kD (*e*), 60 kD (*f*) and 140 kD (*g*) are reproducibly seen only after CD3 stimulation. Moreover, phosphorylation of these three substrates is completely abolished when the tyrosines of γ are mutated (compare *F* and *H*). Therefore, the γ motif in the context of TCR/CD3 controls the phosphorylation of these three substrates. The 42-kD acidic substrate *d* is also phosphorylated by the CD3 pathway, but its phosphorylation is not affected by tyrosine mutation of γ and is therefore not under the control of the γ motif in the context of TCR/CD3. Thus, the γ motif in the context of the two receptors controls the phosphorylation of different substrates.

The γ Motif Associated With FcεRI but Not TCR/CD3 Is Necessary to Mediate the Release of IL-2. We next investigated whether the γ motif plays a role in a later activation event, the production of IL-2. Cross-linking of CD3 in both 2.M2 WT and Y transfectants induces IL-2 production. Two WT clones and 3 Y clones were analyzed two to three times each. Clone WT66 was always a higher producer than any other clone (Fig. 4 *A*). This was not due to a higher level of expression of TCR/CD3, since these levels, which were tested by FACS before each experiment, were found comparable (Fig. 1 *B* and data not shown). Those results seem to indicate a slightly higher production of IL-2 by WT compared with Y, with a marked heterogeneity between clones.

The same experiments were performed with the BWδ transfectants. Three WT clones and 3 Y-mutated clones were analyzed for IL-2 production after TCR engagement. Comparable levels of IL-2 were induced in the different clones (Fig. 4 *B*).

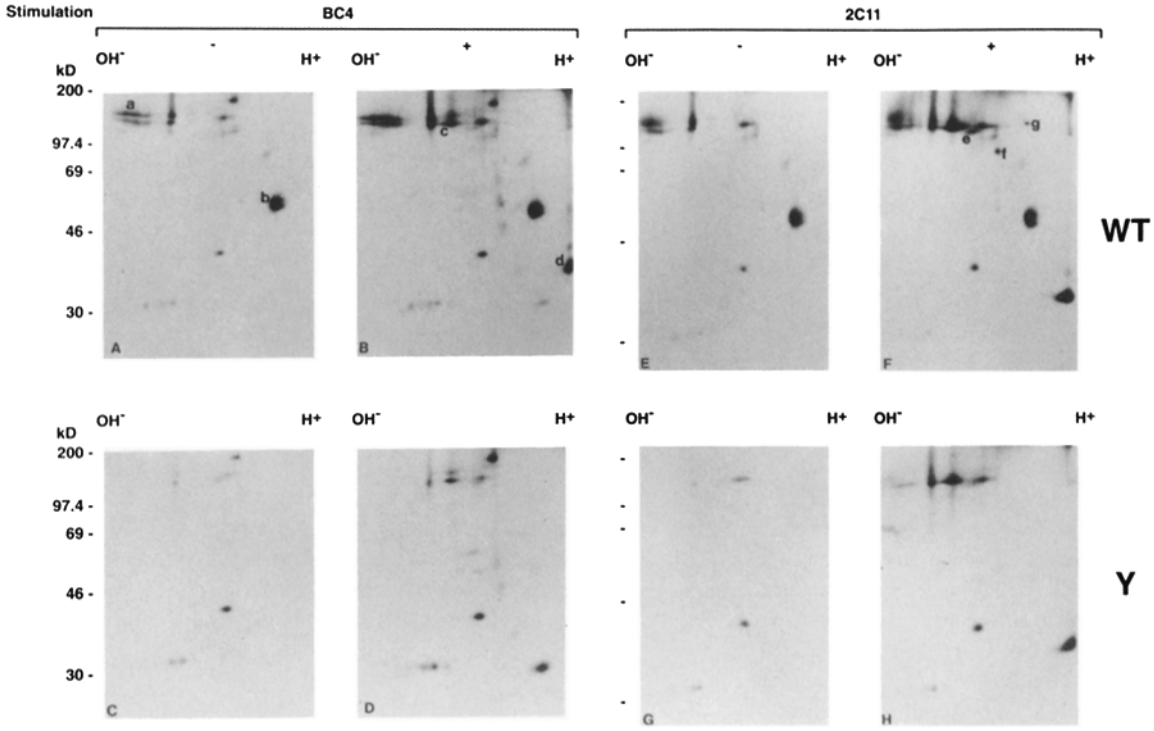

Figure 3. The γ motif in association with TCR/CD3 and FcεRI mediates the phosphorylation of distinct substrates. ^{32}P-Labeled cells (10^8) were stimulated as in Fig. 2 in the absence (*A*, *C*, *E*, and *G*) or presence (*B*, *D*, *F*, and *H*) of the first specific antibody and in both cases with the corresponding second antibody. Lysates were immunoprecipitated with the antiphosphotyrosine antibody and resolved by NEPHGE-PAGE (10% slab gels) as described (46). M_r calibration is shown on the left. *WT*, wild-type γ; *Y*, tyrosine-mutated γ.

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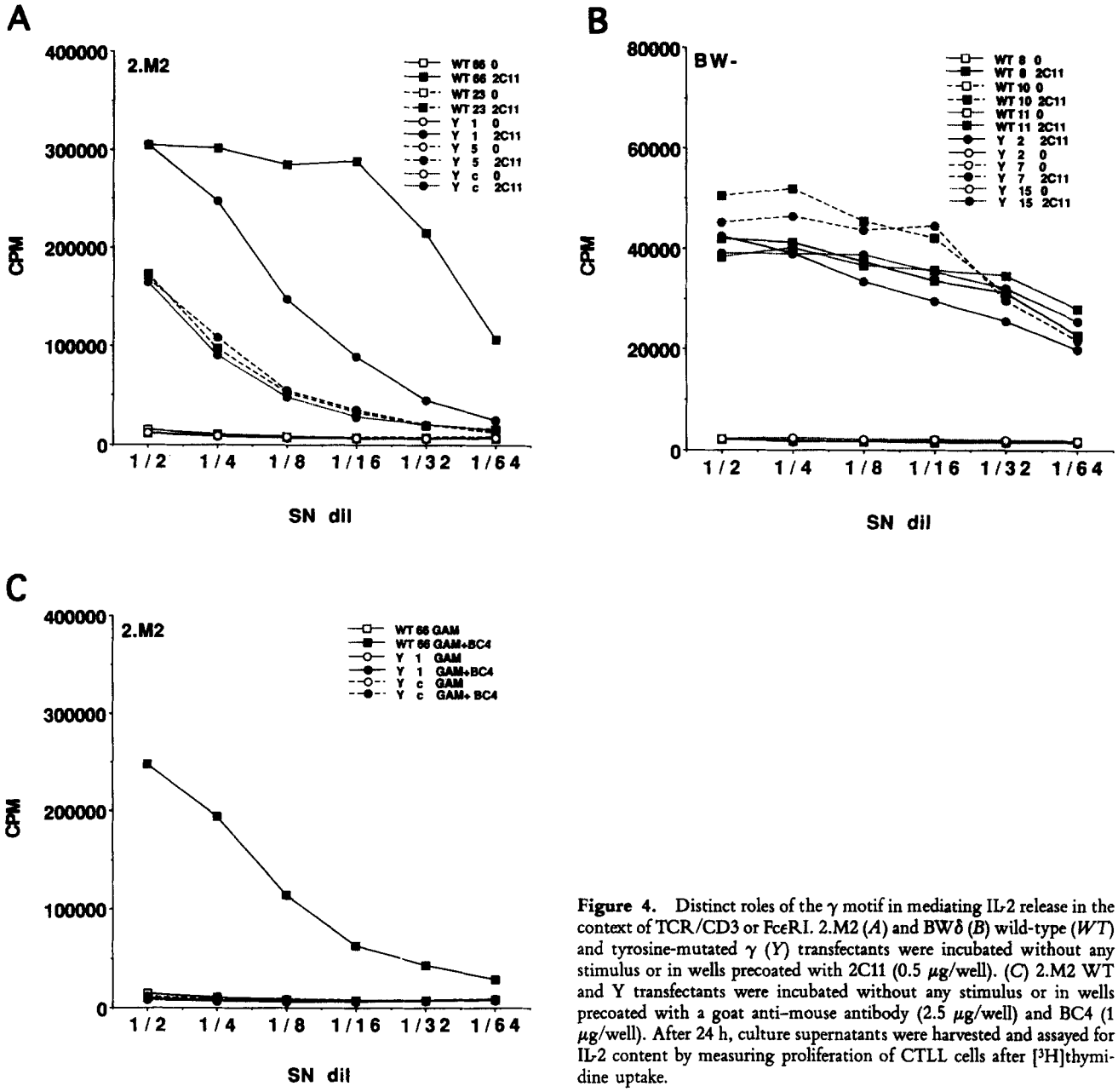

Figure 4. Distinct roles of the γ motif in mediating IL-2 release in the context of TCR/CD3 or FcεRI. 2.M2 (*A*) and BWδ (*B*) wild-type (*WT*) and tyrosine-mutated γ (*Y*) transfectants were incubated without any stimulus or in wells precoated with 2C11 (0.5 μg/well). (*C*) 2.M2 WT and Y transfectants were incubated without any stimulus or in wells precoated with a goat anti-mouse antibody (2.5 μg/well) and BC4 (1 μg/well). After 24 h, culture supernatants were harvested and assayed for IL-2 content by measuring proliferation of CTLL cells after [^{3}H]thymidine uptake.

Statistical calculations applied to similar experiments confirmed that no significant difference was present between the wild-type and tyrosine-mutated clones.

To test the significance of the heterogeneity in the response of WT and Y in 2.M2, which was not observed in BWδ, we applied the same statistical calculations to the results from 2.M2. When the comparison was performed between clones, that is, the various results from one clone obtained in various experiments were combined, there was no significant difference between WT and Y. We therefore conclude that the production of IL-2 after CD3 engagement does not require the integrity of the γ motif.

When FcεRI was engaged with multivalent antigen or with BC4 alone, no IL-2 secretion was observed (data not shown). However, reproducible IL-2 release was induced after FcεRI engagement with high doses of BC4 bound to a cross-linking specific antibody coated to the 96-well plates (see Materials and Methods) (Fig. 4 *C*). The IL-2 secretion was completely abolished when the tyrosines of γ were mutated. Therefore, in the context of FcεRI complexes, the presence of the γ motif was necessary for initiating the signal cascade leading to IL-2 production.

Discussion

In this study, we have cotransfected a ζ-deficient T cell line with FcεRI α, β, and γ cDNAs to induce the surface expression of two different FcεRI γ-containing receptors,

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FcεRI and TCR/CD3. The use of a wild-type FcεRI γ chain and a mutated form, in which ARAM-γ had been inactivated by mutating its tyrosine residues to phenylalanine, has allowed us to specifically study the function of ARAM-γ in the context of the two different receptors. Both reconstituted TCR/CD3 and FcεRI with the wild-type FcεRI γ chain are functional, as shown by their capacity to induce IL-2 release and to activate kinase(s).

The reconstituted FcεRI with a tyrosine-mutated FcεRI γ loses its capacity to induce IL-2 release even though the β chain is phosphorylated and ubiquitinated upon triggering. Like the FcεRI γ chain, the β chain contains an ARAM in its COOH-terminal cytoplasmic tail. We have recently demonstrated that the FcεRI γ chain activates the kinase Syk, while β binds the Src family tyrosine kinase Lyn and controls receptor phosphorylation. This led us to propose a model in which the full signaling capacity of FcεRI is achieved by cooperation between β and γ. This model is supported by the results presented here. When the tyrosines of ARAM-γ are mutated, the β chain cannot initiate the full signaling repertoire of the entire receptor. However, by itself it can activate a tyrosine kinase, presumably the Src family kinase Fyn, responsible for the phosphorylation of β and other substrates. Taken together, the results presented here suggest a new classification for ARAMs: those able to control late signals, like the ones in CD3, TCR ζ, and FcεRI γ, and those that do not, like the one in FcεRI β. This distinction should help in determining how ARAMs control the various arms of the signaling machinery.

In contrast with FcεRI, the TCR/CD3 complex with a tyrosine-mutated FcεRI γ is still able to induce IL-2 release upon aggregation. Therefore, the other ARAM-containing subunits of CD3 are capable of inducing IL-2 release independently. This could also explain the fact that one phosphorylated substrate, substrate *d* in Fig. 3, requires an intact ARAM-γ when FcεRI is activated but not when TCR/CD3 is activated. An alternative module in TCR/CD3 could substitute for the mutated ARAM-γ and promote the phosphorylation of substrate *d*. These results are in agreement with the findings that both CD3 ε and ζ are capable of autonomously activating cells (39), and that a TCR/CD3 complex can still induce IL-2 production despite the truncation of the cytoplasmic tail of ζ (36). These previous reports have led to the concept of two parallel transducing units in the TCR/CD3 complex, the TCR ζ on the one hand and the CD3 complex on the other hand (39). In this scenario, when FcεRI γ is mutated, the CD3 complex, in particular CD3 ε, would simply take over.

While the concept of two parallel transducing units in TCR/CD3 seems reasonable to explain the results discussed above, it does not satisfactorily explain some other results. At least three substrates, which are phosphorylated exclusively after TCR/CD3 engagement, disappear after mutation of FcεRI γ (Fig. 3). Therefore, CD3 ε is not capable of replacing FcεRI γ to mediate the phosphorylation of these substrates. The presence of FcεRI γ seems necessary. However, FcεRI γ does not mediate the phosphorylation of these three substrates after FcεRI triggering. Therefore, FcεRI γ mediates the phosphorylation of these substrates, but only in the context of the TCR/CD3 complex. We propose that the environment of the FcεRI γ motif influences its capacity to mediate phosphorylation of various substrates. If one assumes that a substrate must associate, even temporarily, with the receptor complexes before phosphorylation, then the difference in substrates could be due to differences in recruitment by the two phosphorylated receptors. This, in turn, could be explained by the difference in subunit composition of the two receptors. Regardless of the mechanism that might explain the differences in substrate phosphorylation, our results clearly demonstrate that two different receptors with a common FcεRI γ ARAM expressed on the same cells can signal differently.

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