The Journal of Immunology

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J Immunol 1999; 162:176-185; ;
http://www.jimmunol.org/content/162/1/176

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Lyn Dissociation from Phosphorylated FcεRI Subunits: A New Regulatory Step in the FcεRI Signaling Cascade Revealed by Studies of FcεRI Dimer Signaling Activity[1]

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Cross-linking the heterotrimeric ($\alpha\beta\gamma 2$) IgE receptor, FcεRI, of mast cells activates two tyrosine kinases: Lyn, which phosphorylates β and γ subunit immunoreceptor tyrosine-based activation motifs, and Syk, which binds γ-phospho-immunoreceptor tyrosine-based activation motifs and initiates cellular responses. We studied three FcεRI-dimerizing mAbs that maintain similar dispersed distributions over the surface of RBL-2H3 mast cells but elicit very different signaling responses. Specifically, mAb H10 receptor dimers induce very little inositol 1,4,5-trisphosphate synthesis, Ca^{2+} mobilization, secretion, spreading, ruffling, and actin plaque assembly, whereas dimers generated with the other anti-FcεRI mAbs induce responses that are only modestly lower than that to multivalent Ag. H10 receptor dimers activate Lyn and support FcεRI β and γ subunit phosphorylation but are poor Syk activators compared with Ag and the other anti-FcεRI mAbs. H10 receptor dimers have two other distinguishing features. First, they induce stable complexes between activated Lyn and receptor subunits. Second, the predominant Lyn-binding phospho-β isoform found in mAb H10-treated cells is a less tyrosine phosphorylated, more electrophoretically mobile species than the predominant isoform in Ag-treated cells that does not coprecipitate with Lyn. These studies implicate Lyn dissociation from highly phosphorylated receptor subunits as a new regulatory step in the FcεRI signaling cascade required for Syk activation and signal progression. ***The Journal of Immunology,*** **1999, 162: 176–185.**

In primary and cultured basophils and mast cells, cross-linking the high affinity IgE receptor, FcεRI,[3] activates a signaling sequence that leads within minutes to degranulation and membrane/cytoskeletal responses, including actin polymerization, ruffling, spreading, integrin activation, and actin plaque assembly, and within hours to increased cytokine synthesis (reviewed in Ref. 1). Recent studies have provided insight into the sequence of early events by which cross-linking this multichain ($\alpha\beta\gamma 2$) immune system receptor leads to functional responses. RBL-2H3 rat tumor mast cells contain two FcεRI-associated protein tyrosine kinases, the Src-related enzyme, Lyn (2), whose principal substrates are the receptor's β and γ subunits (3), and PTK72/Syk (4), which phosphorylates a wide range of downstream signaling molecules, including phospholipase Cγ isoforms, the p85 subunit of phosphatidylinositol 3-kinase, Vav, Grb2, and others (5–9), and is essential for all known FcεRI-mediated responses (3). In resting RBL-2H3 cells, a proportion of Lyn associates with the FcεRI β subunit (10, 11) in an SH4 domain-dependent fashion (12). FcεRI cross-linking permits Lyn associated with one receptor to phosphorylate tyrosines located within immunoreceptor tyrosine-based activation motifs (ITAMs) in the β and γ subunits of the adjacent receptor. Some of the resulting β subunit phosphotyrosines serve as binding sites for the recruitment and activation of more Lyn molecules that, in turn, support more subunit phosphorylation (10). Other phosphotyrosines bind other signaling molecules containing SH2 domain motifs.

Analyses of subunit cytoplasmic domain sequences have shown that the FcεRI γ subunit contains a typical ITAM, with 10 amino acids, including three threonines, between the two critical tyrosine residues (reviewed in Ref. 13). It has been established that both γ-ITAM tyrosines are phosphorylated to approximately similar levels in activated cells (14) and that the doubly phosphorylated FcεRI γ-ITAMs serve as binding sites for the tandem SH2 domains of Syk, resulting in its autophosphorylation and activation (15). These and other data identify Syk as the principal γ-ITAM ligand. In contrast, the FcεRI β-ITAM has only nine amino acids, including two serines and a tyrosine between the typical ITAM tyrosines, and its three tyrosines are phosphorylated to different extents (membrane distal > membrane proximal > internal) (14). Ligands for the phosphorylated β subunit ITAM include Lyn and the negative signaling molecule, inositol polyphosphate 5-phosphatase (16). As well as stimulating tyrosine phosphorylation, FcεRI cross-linking stimulates β-ITAM serine and γ-ITAM threonine phosphorylation (7, 14, 17). The identities of the kinases and the contributions of these ITAM phosphorylation events to signal transduction are not yet established (discussed in Ref. 17).

ITAM motifs are found in the cytoplasmic tails of other members of the multichain immune recognition receptor family that

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Received for publication January 29, 1998. Accepted for publication September 10, 1998.

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[1] This work was supported by National Institutes of Health Grant RO1GM49814 and Fogarty International Research Collaboration Award RO3TW00440. Work in the U.S. laboratory was additionally supported by National Institutes of Health Grants RO1GM50562 and P50HL56384. Work in the Mexican laboratory was additionally supported by Consejo Nacional de Ciencia y Technología Grant 3154P-N9607 and Universidad Nacional Autónoma de Mexico Grant IN204696.

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[3] Abbreviations used in this paper: FcεRI, the high affinity IgE receptor; ITAM, immunoreceptor tyrosine-based activation motif; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate.

0022-1767/99/$02.00

includes, in addition to the FcεRI, the TCR, the B cell receptor, and several Fcγ receptors. Engagement of all these receptors results in ITAM tyrosine phosphorylation mediated by receptor-associated Src kinases, creating sites for the binding and activation of Syk kinases that, in turn, phosphorylate and activate enzymes that initiate a variety of response pathways (reviewed in Refs. 13, 18, and 19). Thus, the model of signal initiation by sequential kinase activation described above is not limited to the FcεRI signaling cascade, but applies to a family of related receptors.

In 1988, Ortega and colleagues reported the development of a series of mAbs, designated F4, J17, and H10, specific for the α subunit of the FcεRI expressed on RBL-2H3 cells (20). All three of these anti-FcεRI mAbs induced secretion, supporting previous evidence (reviewed in Ref. 21) that the FcεRI dimer is the minimal unit capable of activating FcεRI-coupled responses. Importantly, comparison of the secretory dose-response curves with the extent of FcεRI dimerization demonstrated that not all dimers have equivalent signaling activities. In particular, anti-FcεRI mAb H10-induced dimers elicited substantially less secretion than anti-FcεRI mAbs F4 and J17. The differences discovered between these anti-FcεRI mAbs in studies of the kinetics of FcεRI dimerization induced by each Ab and in biophysical studies of dimerized receptor properties, including rotational mobility and dimer lifetime, could not unambiguously explain the low signaling activity of mAb H10 (20–23). Thus, it was proposed that configurational differences between dimers induced by mAb H10, compared with mAbs F4 or J17, might contribute to their different signaling activities.

We have used this battery of anti-FcεRI mAbs to explore the initial events in the FcεRI signaling cascade. The experiments reported here support previous evidence that all three mAbs generate FcεRI dimers but not higher oligomers and substantially extend previous evidence for the limited signaling activity of H10-induced dimers in comparison with dimers induced with mAbs F4 and J17. Importantly, they link the weak signaling activity of H10-induced dimers to the formation of stable complexes between Lyn and incompletely phosphorylated FcεRI.

Materials and Methods

Reagents

The preparation and characterization of three anti-FcεRI mAbs, H10 (IgG2b), F4 (IgG1), and J17 (IgG1) were described previously (20). Polyclonal anti-phosphotyrosine Ab was produced by G. Deanin and J. Potter, University of New Mexico, and purified as described previously (24). DNP-specific anti-IgE mAb (anti-DNP-IgE) (25) was purified from ascites as previously described (26). Rabbit anti-IgE Ab was prepared as described previously (27). mAb to the FcεRI β subunit was a gift from Dr. J. Rivera, National Institute of Health (28). Rabbit anti-Syk Ab raised against a Syk-specific peptide was a gift from R. Geahlen (Purdue University). Mouse anti-phosphotyrosine mAb, PY20, was from Transduction Laboratories (Lexington, KY), and rabbit anti-Lyn Ab was obtained from Santa Cruz Laboratories (Santa Cruz, CA). Biotinyl anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), and protein A and protein A/G-conjugated Sepharose were obtained from Oncogene (Cambridge, MA). Fifteen-nanometer protein A-gold- and streptavidin-conjugated colloidal gold particles and Abs and solutions for ECL were purchased from Amersham (Arlington Heights, IL). DNP_{24}-BSA (DNP-BSA), fura-2, and fura-2/AM were obtained from Molecular Probes (Eugene, OR). [^{3}H]serotonin and [^{3}H]Ins(1,4,5)P_3 were obtained from DuPont-New England Nuclear (Boston, MA).

Cell activation

Conditions for RBL-2H3 cell culture were previously described (26). For studies of the signaling activity of FcεRI oligomers, cells were incubated overnight with 1 μg/ml anti-DNP-IgE, then washed with modified Hanks' buffer (29) containing 0.1% BSA and activated at 37°C by the addition of either 0.1 or 1.0 μg/ml of DNP-BSA or 1 μg/ml rabbit anti-IgE. For studies of the signaling activities of anti-FcεRI mAbs, cells were simply activated by the addition of mAb (0.1 μg/ml unless otherwise stated).

Secretion

Secretion was measured from the Ag- or anti-FcεRI mAb-induced release of preloaded [^{3}H]serotonin from cells grown as monolayers on 24-well tissue culture dishes as previously described (26). All measurements were performed in duplicate and corrected for spontaneous release of [^{3}H]serotonin during the standard 20-min assay period. To determine the percent degranulation, total cell-associated [^{3}H]serotonin was measured by Triton X-100 lysis of nonincubated cells.

Ins(1,4,5)P_3 levels

Cells (8×10^6/assay) were activated in suspension with Ag or anti-FcεRI mAbs. Ins(1,4,5)P_3 levels were determined in the supernatant fractions of neutralized TCA extracts using the isomer-specific radioreceptor assay of Challis and colleagues (30) as modified previously (31). Data were expressed as picomoles of Ins(1,4,5)P_3 per milligrams of TCA-insoluble protein.

Ca^{2+} mobilization

Ag- and anti-FcεRI mAb-induced changes in intracellular Ca^{2+} were measured in individual, fura-2/AM-loaded RBL-2H3 cells using fluorescence ratio imaging microscopy as previously described (32).

Microscopy

To observe Ag- or anti-FcεRI mAb-induced membrane ruffling and spreading, cell monolayers on glass coverslips were activated, then fixed either with 2% glutaraldehyde for scanning electron microscopy or with 2% paraformaldehyde/0.5% saponin followed by rhodamine-phalloidin for fluorescence microscopy (27, 33). Cells were examined using a Hitachi S800 scanning electron microscope or a Zeiss Photomicroscope III equipped for epifluorescence microscopy.

Receptor mapping

For receptor mapping studies, cells were activated with 1 μg/ml anti-IgE Ab or 0.1 μg/ml anti-FcεRI mAb on glass coverslips. The cells were then fixed for 10 min at room temperature in 10% paraformaldehyde, 0.075% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2, as previously described (27). After fixation, IgE-primed, anti-IgE-activated cells were rinsed and incubated for 30 min in PBS-1% BSA containing a 1/17 dilution of 15-nm protein A-gold particles (27). After further rinsing, the cells were postfixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and processed for scanning electron microscopy. For these samples, inherent receptor distributions were determined by conducting the first fixation before the addition of anti-IgE, and nonspecific gold labeling was observed by anti-IgE labeling without prior IgE priming. Anti-FcεRI mAb-labeled cells were incubated after the initial fixation with biotinyl anti-mouse IgG followed by 15-nm streptavidin-gold particles. For these samples, inherent receptor distributions were determined by anti-FcεRI mAb incubation at 4°C, and nonspecific gold labeling was observed by biotinyl anti-mouse IgG-streptavidin gold labeling without prior incubation with anti-FcεRI mAb.

Immune complex kinase assays

Cell suspensions (6×10^6 cells/ml; 0.5 ml/assay) were activated with Ag or anti-FcεRI mAb, then lysed in ice-cold 50 mM HEPES (pH 7.2), 150 mM NaCl, 1% Brij-96, and 1 μg/ml each of leupeptin, antipain, and pepstatin. In most experiments the lysis supernatants were cleared of any protein A- or A/G-reactive proteins by incubation for 2 h at 4°C with protein A- or protein A/G-Sepharose beads. After preclearing (omitted when immunoprecipitation was with directly bead-coupled Abs), they were incubated for 2 h at 4°C with specific Abs prebound to protein A-Sepharose (polyclonal anti-Lyn and anti-Syk), protein A/G-Sepharose (monoclonal anti-phosphotyrosine), or anti-phosphotyrosine-agarose or anti-Lyn agarose beads. After washing four times, kinase activity was determined from the incorporation of ATP into specific proteins during a 2-min incubation at 30°C with 10 μCi of [γ-^{32}P]ATP as previously described (26).

Immunoblotting

Cells were activated, lysed, and precleared (if appropriate), and specific proteins were immunoprecipitated with Abs as described above. Ab-protein complexes were released from the washed beads by boiling, separated by 10% SDS-PAGE, and transferred to nitrocellulose. After overnight incubation at 4°C in 3% BSA to block nonspecific binding, blots were probed with specific Ab for 1 h at room temperature and washed again. For autoradiography, blots were incubated for an additional hour with ^{125}I-labeled anti-mouse IgG, and dried membranes were exposed to x-ray film.

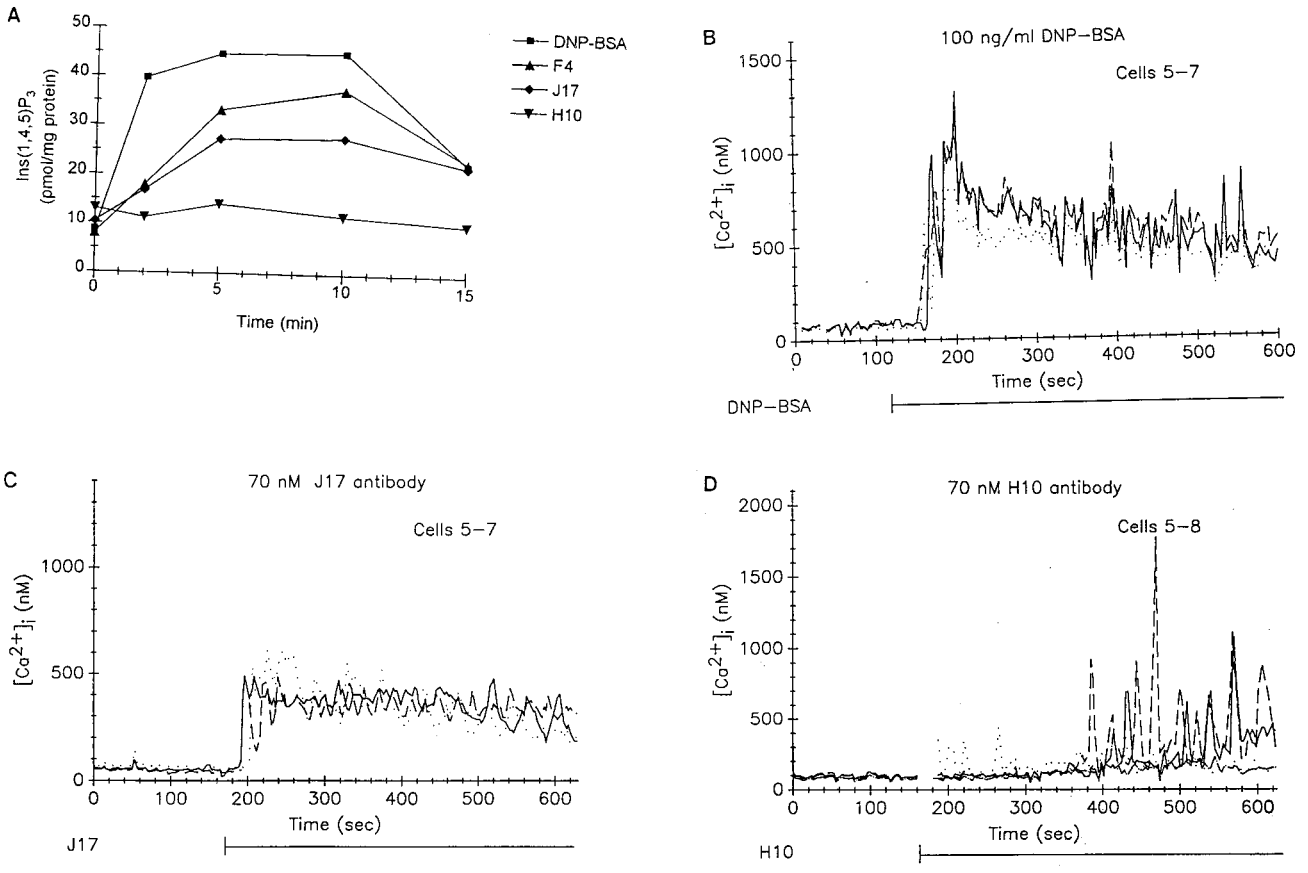

FIGURE 1. Ins(1,4,5)P_3 synthesis and Ca^{2+} mobilization induced by FcεRI dimers and oligomers. In *A*, RBL-2H3 cells were incubated for various times with 1 μg/ml DNP-BSA (IgE-primed cells) or with 0.1 μg/ml anti-FcεR1 mAbs (unprimed cells). Reactions were stopped with TCA, and levels of Ins(1,4,5)P_3 in the resulting supernatants were measured. Each point is an average from two separate experiments, each performed in duplicate. In *B–D*, cytoplasmic Ca^{2+} levels were measured in individual fura-2-loaded cells by ratio imaging microscopy. Ag (0.1 μg/ml DNP-BSA) or mAbs (0.1 μg/ml J17 or H10) were added at the time points indicated. Results show the Ca^{2+} responses of three or four cells per experiment.

For chemiluminescence detection, blots were incubated with horseradish peroxidase-conjugated anti-mouse Abs, washed, immersed in enhanced chemiluminescence solution, and exposed to x-ray film.

Results

Secretion

The anti-FcεRI mAbs used here were originally selected for their ability to induce secretion from RBL-2H3 cells. Further characterization showed that they compete with each other and with IgE for binding to the FcεRI α subunit, that they bind in a stoichiometry of 1 Fab:1FcεRI, and that they elicit quantitatively different secretory responses (20). Originally, 2–20 nM (~3–30 ng/ml) anti-FcεRI mAb elicited optimal secretion, and the order of activity in degranulation assays was Ag = F4 > J17 ≫ H10. With current anti-FcεRI mAb preparations and RBL-2H3 cells, 70-nM (0.1 μg/ml) concentrations of all three mAbs are necessary to induce optimal secretion, and the order of activity has changed somewhat. mAb H10 remains a poor secretagogue, consistently inducing the release of 15–20% of the total [^{3}H]serotonin under conditions where optimal Ag (0.1 or 1.0 μg/ml DNP-BSA) causes the release of 40–70% of the total mediator, depending on cell culture density and passage number. Current preparations of mAb J17 are strong secretagogues, inducing only 5–10% less secretion than Ag in a 20-min assay. mAb F4 receptor dimers have been less consistent, originally inducing more secretion than mAb J17 (20) but recently inducing responses that are usually smaller than J17-induced responses but consistently greater than the responses induced by mAb H10. Based on all degranulation assays performed during the course of this work, the relative activities as secretagogues of current anti-FcεRI mAb preparations are: multivalent Ag > or = J17 > or = F4 ≫ H10.

Synthesis of Ins(1,4,5)P_3

The activation of phospholipase Cγ isoforms, leading to the synthesis of Ins(1,4,5)P_3, is one of the earliest responses of RBL-2H3 cells to FcεRI cross-linking. The results in Fig. 1*A* show that multivalent Ag and anti-FcεRI mAbs F4 and J17 induce an increase in cytoplasmic Ins(1,4,5)P_3 levels that is detectable after 1 min and persists for about 10 min before returning toward baseline. In contrast, mAb H10 induces very little Ins(1,4,5)P_3 synthesis in RBL-2H3 cells.

Ca^{2+} mobilization

Secretion depends on Ca^{2+} mobilization that is initiated by the Ins(1,4,5)P_3-mediated release of intracellular stores and maintained by Ca^{2+} influx. Ratio imaging microscopy was used to compare the Ca^{2+} mobilization responses of RBL-2H3 cells to anti-FcεRI mAb-induced receptor dimers and to DNP-BSA-induced receptor oligomers. Typical results are shown in Fig. 1, *B–D*. In duplicate experiments, 100% of cells mobilized Ca^{2+} in response to 0.1 μg/ml concentrations of DNP-BSA and of anti-FcεRI mAbs J17 and H10. However, there were characteristic differences in the lag time to the initial Ca^{2+} spike response, previously attributed to the release of Ca^{2+} from intracellular stores

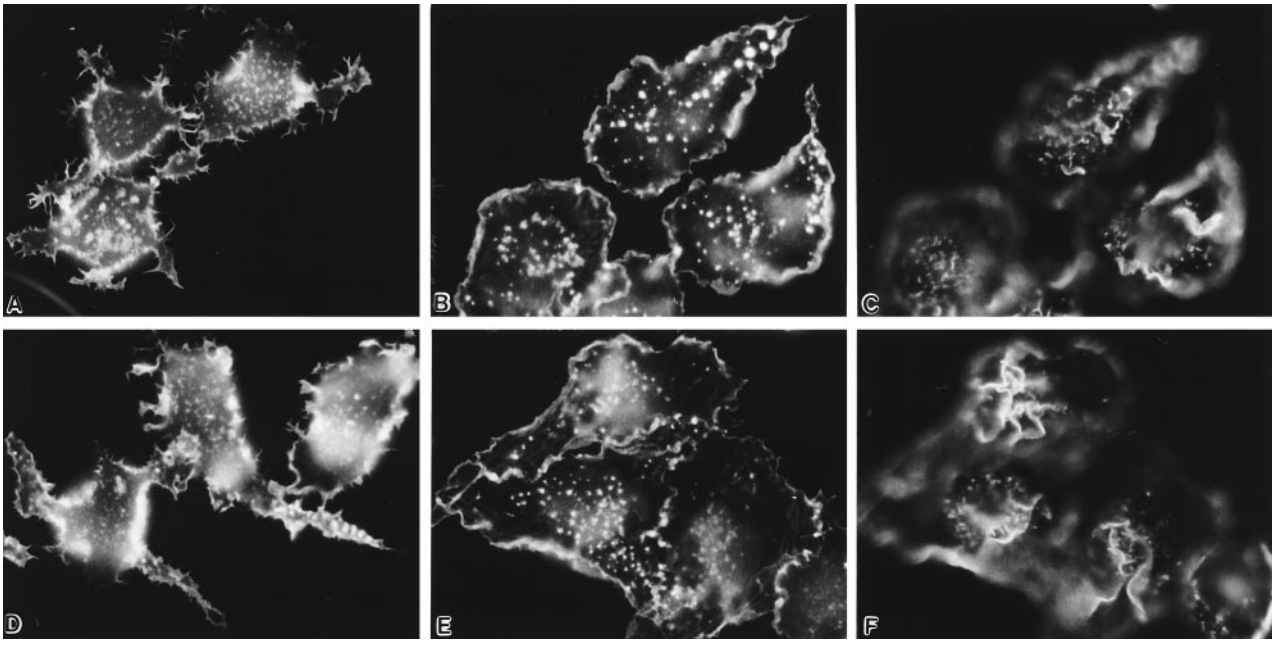

FIGURE 2. Cytoskeletal and adhesive responses induced by FcεRI dimers and oligomers. Monolayers of RBL-2H3 cells on glass coverslips were incubated for 10 min at 37°C with no addition (*A*), 1 μg/ml DNP-BSA (*B* and *C*), 0.1 μg/ml anti-FcεR1 mAb H10 (*D*), or 0.1 μg/ml anti-FcεR1 mAb J17 (*E* and *F*). Cells were fixed, and filamentous actin was labeled with rhodamine-phalloidin as described in *Materials and Methods*. Unstimulated cells are relatively rounded with short actin-containing extensions (*A*). Incubation with Ag or anti-FcεR1 mAb J17 induces a marked spreading responses that is accompanied by the assembly of actin plaques at the ventral cell surface (*B* and *E*) and of lamellae on the dorsal cell surface (*C* and *F*). Anti-FcεR1 mAb H10 induces a modest spreading response with very little cytoskeletal reorganization (*D*). Magnification, ×630.

(32): 42 ± 15 s ($n = 7$ cells) for DNP-BSA-stimulated cells, 70 ± 12 s ($n = 7$ cells) for J17-stimulated cells, and 170 ± 109 s ($n = 8$ cells) for H10-stimulated cells. Additionally, Ag and anti-FcεRI mAb J17 induced a persistent increase in cytoplasmic Ca^{2+} levels, attributable to Ca^{2+} influx (32). In contrast, anti-FcεRI mAb H10 induced a series of Ca^{2+} spikes, presumably resulting from the periodic release and reuptake of Ca^{2+} stores, but failed to support a sustained elevation in cytoplasmic Ca^{2+} levels. The supply of anti-FcεRI mAb F4 was too limited for extensive studies of Ca^{2+} mobilization. However, in a single experiment, anti-FcεRI mAb F4 receptor complexes induced responses similar to those to J17 (not shown).

Membrane and cytoskeletal responses

Cross-linking IgE-FcεRI complexes with multivalent Ag induces striking membrane and cytoskeletal responses, including filamentous actin polymerization, membrane ruffling, spreading, integrin up-regulation, and the assembly of specialized adhesion structures called actin plaques (1, 33, 34). We used fluorescence and scanning electron microscopy to examine a subset of these responses, including spreading, actin plaque assembly, and membrane ruffling. Resting cells adhere loosely to glass coverslips, and filamentous actin, localized with rhodamine-phalloidin, is distributed as a cortical meshwork that outlines pseudopodia as well as in amorphous cytoplasmic aggregates (Fig. 2*A*). These cells maintain a microvillous surface morphology (Fig. 3*A*, inset). Cross-linking with DNP-BSA for 10 min induces a strong spreading response, accompanied by the assembly of actin plaques at sites of cell-substrate interaction (Fig. 2*B*) and by a transformation of the upper cell surface to a lamellar topography (Figs. 2*C* and 3*B*, inset). Cross-linking with anti-FcεRI mAbs F4 (not shown) and J17 (Fig. 2, *E* and *F*, and Fig. 3*C*, inset) induces spreading, actin plaque assembly, and ruffling responses that are essentially the same as those induced by Ag. In contrast, H10-activated cells show a modest spreading response, with little or no actin plaque assembly (Fig. 2D) or membrane ruffling (Fig. 3*D*, inset).

Indistinguishable membrane topography of mAb-induced FcεRI dimers

Ortega et al. (20) showed previously that the binding stoichiometry of anti-FcεRI mAb Fab fragments was one Fab to one FcεRI, implying that all three mAbs can cross-link receptors only into dimers. Independently, Baird and colleagues demonstrated that small receptor oligomers induced with chemically cross-linked IgE molecules can induce large scale receptor clustering by a process that is independent of interoligomer cross-linking (35). Further studies by the Baird group suggested that the underlying mechanism may involve the segregation of membrane lipids to create local environments that are especially favorable for receptor clustering (36). Extending this concept, Stauffer and Meyer (37) suggested recently that IgE receptor signaling requires the transient association of cross-linked receptors with punctate plasma membrane microdomains where they induce the spatially restricted activation of SH2 domain-containing proteins such as Syk and phospholipase Cγ1. Based on these results, it seemed possible that the stronger signaling activity of anti-FcεRI mAb F4- and J17-induced dimers could reflect their ability to be drawn into clusters or membrane domains that might engage kinases and other signaling molecules more effectively than mAb H10 receptor dimers. To test this, we localized anti-FcεRI mAb-FcεRI complexes on the surfaces of resting and activated cells by immunogold labeling and backscattered electron imaging in the scanning electron microscope. For comparison, we looked at the effects of cross-linking IgE-receptor complexes on the same cell population with polyclonal anti-IgE Ab that elicits strong signaling activity (27). FcεRI oligomers induced on IgE-primed cells by polyclonal anti-IgE were detected by postfixation labeling with 15-nm protein A-gold particles as previously described (27). Anti-FcεRI mAb-receptor

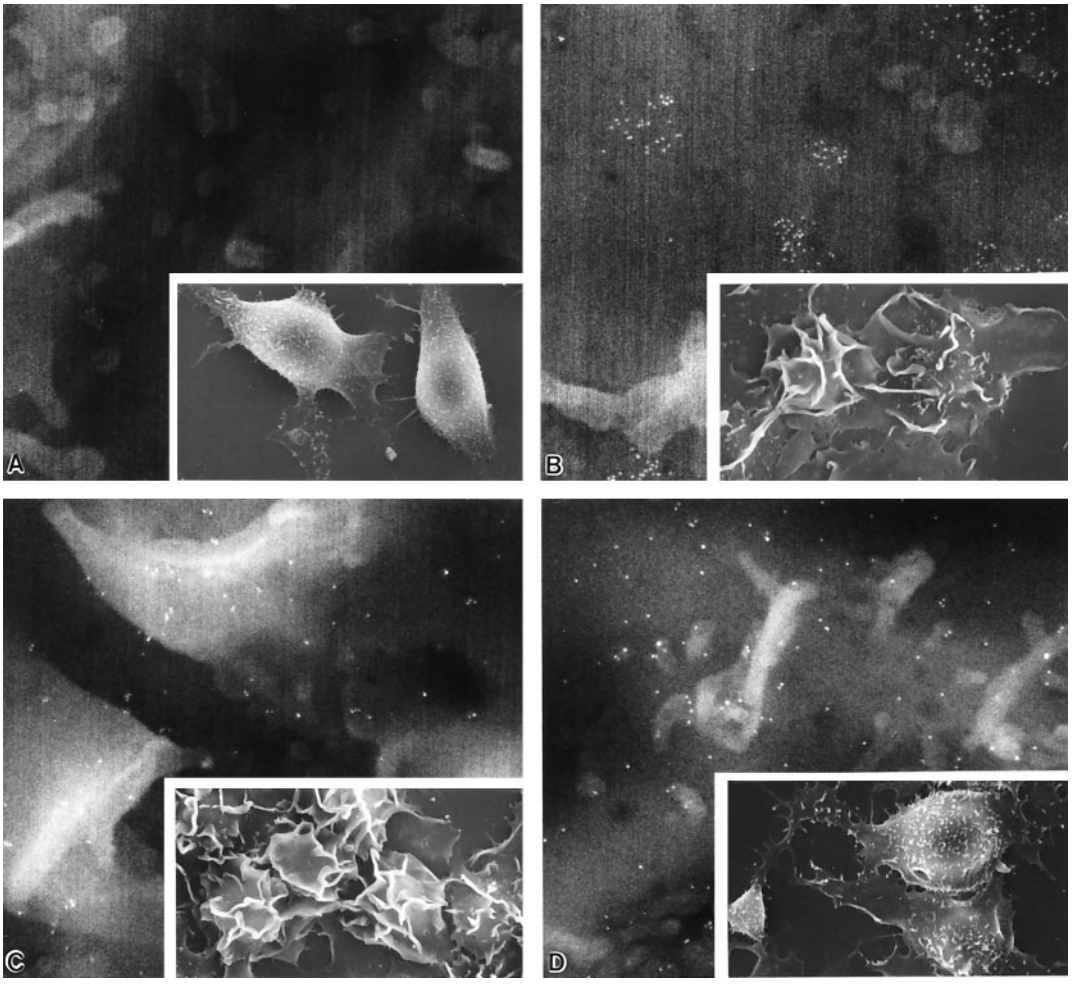

FIGURE 3. FcεRI dimers maintain a dispersed cell surface distribution. In *A–D*, RBL-2H3 cell monolayers were incubated for 10 min with no addition (*A*), with polyclonal anti-IgE Ab (1.0 μg/ml; *B*), or with anti-FcεRI mAbs (0.1 μg/ml J17 and H10; *C* and *D*). After fixation, receptors on IgE-primed cells that bound anti-IgE (*B*) were labeled with 15-nm protein A-gold particles, while receptors on unprimed cells incubated with no addition (*A*) or with anti-FcεRI mAbs (*C* and *D*) were labeled sequentially with biotinyl anti-mouse IgG and 15-nm streptavidin-gold particles. The distribution of membrane-bound gold particles marking cross-linked receptors was observed by backscattered electron imaging in the scanning electron microscope. Very little colloidal gold binds to untreated cells (*A*). FcεRI aggregates induced by polyclonal anti-IgE Ab are extensively clustered on the cell surface (*B*). Anti-FcεRI mAb-induced receptor dimers maintain a dispersed cell surface distribution (*C* and *D*). Conventional scanning electron microscope images generated from the same samples are inset to illustrate the microvillous morphology and limited spreading of untreated (*A*) and anti-FcεRI mAb H10-treated (*D*) cells and the lamellar surface topography and extensive spreading of anti-IgE mAb-treated (*B*) and anti-FcεRI mAb J17-treated (*C*) cells. Magnification, ×20,000; inset magnification, ×500.

complexes were detected by postfixation labeling with biotinylated anti-mouse IgG followed by streptavidin-conjugated 15-nm gold particles.

No gold particles bound to cells that were incubated with secondary reagents without prior exposure to cross-linking Abs (Fig. 3*A*). Typical receptor aggregates induced on IgE-primed cells by 5-min cross-linking with polyvalent anti-IgE at 37°C are illustrated in Fig. 3*B*. In contrast, gold particles localizing anti-FcεRI mAb-receptor complexes remained dispersed, mostly as singlets and doublets, over the entire membrane of cells that were incubated for 10 min at 4°C (not shown), for 10 min at 37°C (illustrated for mAbs J17 and H10 in Fig. 3, *C* and *D*), or for 20 min or longer (not shown) with all three anti-FcεRI mAbs. To detect patterns of receptor redistribution that might not be apparent by eye, photographic negatives from cells labeled at 4 or 37°C and on cells that were fixed before labeling were digitized, and gold particle distributions were determined by an image analysis system developed by C. Wofsy, M. Sanders, and G. Donohoe at the University of New Mexico (38). Image analysis also failed to reveal consistent differences in aggregate size or distribution between the different mAbs or labeling conditions. Transmission electron microscopic analyses of similar samples showed no internalization of receptor-mAb-colloidal gold complexes during incubation for up to 20 min at 37°C (not shown).

Different anti-FcεRI mAbs induce different phosphoprotein profiles in anti-phosphotyrosine kinase assays

The abilities of the different anti-FcεRI mAbs to activate protein tyrosine phosphorylation was tested by anti-phosphotyrosine immune complex kinase assays. Typical results are shown in Fig. 4. Anti-phosphotyrosine immune complexes from resting cells support relatively little incorporation of [γ-^{32}P]ATP into proteins. Anti-phosphotyrosine immune complexes from cells that were activated for 2 min with 0.1 μg/ml multivalent Ag phosphorylate a series of proteins, including a 53/56-kDa doublet known from previous studies (4, 26) to represent phosphorylated Lyn and bands at around 33 and 10–12 kDa shown previously (7) to correspond to

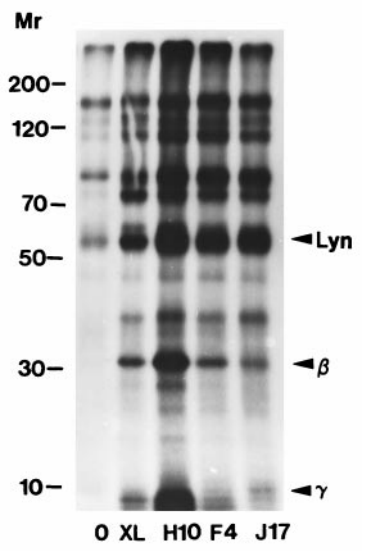

FIGURE 4. Effects of FcεRI cross-linking on anti-phosphotyrosine immune complex kinase activities. RBL-2H3 cells were incubated for 2 min at 37°C with no addition (0) or with 1 μg/ml DNP-BSA (XL) or for 5 min with 0.1 μg/ml anti-FcεRI mAbs (H10, F4, and J17). Cells were lysed, and tyrosine-phosphorylated proteins were precipitated from precleared lysates by incubation with anti-phosphotyrosine mAb PY20-conjugated to protein A/G beads. The immune complexes were incubated for 5 min with [γ-^{32}P]ATP, and the resulting phosphoproteins were separated by SDS-PAGE and detected by autoradiography. The migration of m.w. markers is indicated, and three known proteins among the principal phosphorylated bands are identified. Signal intensity from the FcεRI β and γ subunits is much stronger in immune complexes from anti-FcεRI mAb H10-treated cells than in those from cells treated with Ag or other anti-FcεRI mAbs. Results are typical of three replicate experiments.

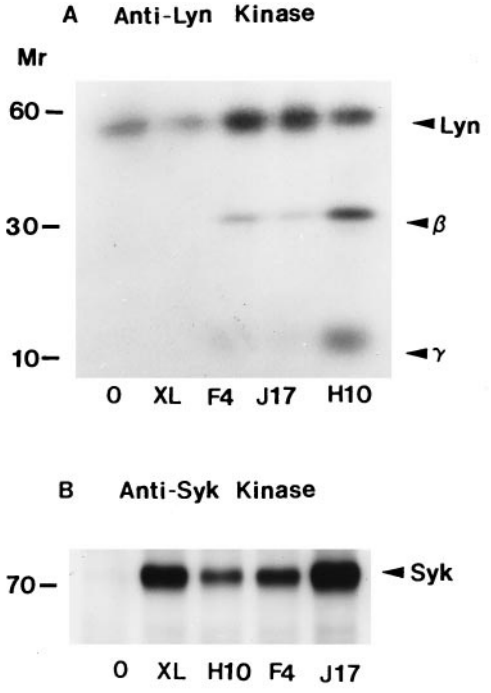

FIGURE 5. Effect of FcεRI cross-linking on anti-Lyn and anti-Syk immune complex kinase activities. The experiment was performed as described in Fig. 4, except that DNP-BSA and anti-FcεRI mAbs were added for 5 min, and immunoprecipitation from cell lysates was performed with anti-Lyn (*A*) or anti-Syk (*B*) mAbs. Anti-Lyn immune complexes (*A*) from resting and activated cells could all autophosphorylate Lyn in vitro. FcεRI β and γ subunits coprecipitated with Lyn from lysates of mAb H10-treated cells and were detected by their in vitro phosphorylation. There is vanishingly little Syk activity in anti-Syk immune complexes from resting cells (*B*). Syk activation was greatest in Ag- and anti-FcεRI mAb J17-treated cells and least in H10-treated cells. Results with anti-Syk immune complexes are typical of three replicate experiments. Additional anti-Lyn immune complex kinase assays are reported in Fig. 6.

the phosphorylated FcεRI β and γ subunits. The signal from phosphorylated Lyn was stronger when anti-phosphotyrosine immune complexes were from cells treated with any of the three anti-FcεRI mAbs than when they were from Ag-treated cells. These data suggest that all anti-FcεRI mAb receptor dimers are fully competent to initiate Lyn-mediated β and γ subunit ITAM phosphorylation, the event that launchs the FcεRI signaling sequence. Importantly, the signals from bands corresponding to the phosphorylated β and γ subunits were strikingly stronger when anti-phosphotyrosine immune complexes were generated from H10-activated cells than when they were from cells activated with Ag or the signaling-competent anti-FcεRI mAbs, F4 and J17. Results were the same regardless of whether lysates were cleared with protein A/G-Sepharose beads before the addition of anti-phosphotyrosine-coated beads. Thus, the excess βγ signal in lysates of H10-treated cells could not be explained by the nonspecific interaction of detergent-solubilized mAb receptor complexes with protein A/G beads.

H10 receptor complexes induce stable complexes between activated Lyn and FcεRI receptor subunits

The results of anti-Lyn immune complex kinase assays are given in Figs. 5*A* and 6. In Fig. 5*A*, cells were incubated for 5 min with either 0.1 μg/ml DNP-BSA or 0.1 μg/ml of all three anti-FcεRI mAbs. In Fig. 6, incubation was for 2, 5, or 10 min with the same concentrations of DNP-BSA and of two anti-FcεRI mAbs, H10 and J17 (Fig. 6*A*), or for 5 min with a 100- to 500-fold range of Ag, H10, and J17 concentrations (Fig. 6*B*). In all cases, cells were lysed, and the lysis supernatants were clarified by incubation with protein A-Sepharose beads. Lyn and Lyn-associated proteins were then precipitated from the clarified lysis supernatants using anti-Lyn-protein A-Sepharose beads. Kinase activity was again determined from the incorporation of [γ-^{32}P]ATP into proteins.

An autophosphorylated doublet of Lyn is present under all incubation conditions. In experiments using older lots of polyclonal anti-Lyn Ab (as in Fig. 5*A*) (4) there was typically an increase in autophosphorylated Lyn when immune complexes were prepared from lysates of activated cells. This is apparent in Fig. 5*A*, where Lyn autophosphorylation is substantially greater in cells treated with all three anti-FcεRI mAbs than in resting cells (the low signal from Ag-treated cells in this experiment is unusual). With more recent lots of polyclonal anti-Lyn Ab (as in Fig. 6) (26), Lyn typically shows strong autophosphorylation in anti-Lyn in vitro kinase assays whether the initial immunoprecipitation with polyclonal anti-Lyn Ab is from resting or activated cells.

In cells activated with Ag or anti-FcεRI mAbs F4 and J17, the autophosphorylated doublet of Lyn is the only strong band seen in anti-Lyn immune complex kinase assays over a range of incubation times (Figs. 5*A* and 6*A*) and concentrations of stimulus (Fig. 6*B*). In contrast, anti-Lyn immune complexes from H10-treated cells, when assessed in in vitro kinase assays, always showed substantial incorporation of [γ-^{32}P]ATP into the FcεRI β and γ subunits regardless of the incubation time (Figs. 5*A* and 6*A*) or concentration of stimulus (Fig. 6*B*). These results provided the first evidence that FcεRI cross-linking with mAb H10 induces the formation of stable complexes between Lyn and its principal endogenous substrates, the receptor's β and γ subunits. This Lyn-bound

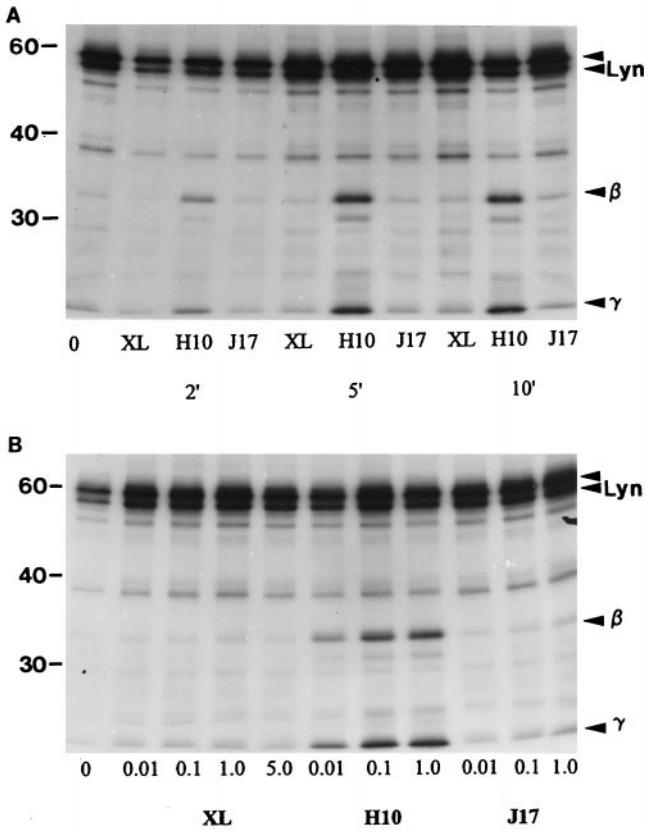

FIGURE 6. Anti-FcεRI mAb 10-receptor complexes induce stable complexes between Lyn and receptor subunits. In *A*, cells were incubated for 2, 5, or 10 min at 37°C with either 0.1 μg/ml DNP-BSA (XL) or 0.1 μg/ml anti-FcεRI mAbs H10 and J17. In *B*, incubation was for 5 min at 37°C, but concentrations of DNP-BSA or anti-FcεRI mAbs H10 and J17 ranged from 0.01–5 μg/ml. Cells were lysed, proteins were immunoprecipitated from precleared lysates using polyclonal anti-Lyn Ab coupled to protein A-Sepharose beads, and the immune complexes were incubated for 5 min with [γ-^{32}P]ATP. Phosphoproteins were separated by SDS-PAGE and detected by autoradiography. Autophosphorylated 53/56-kDa Lyn is present in all experiments under all incubation conditions. There are additional strong signals from phosphorylated FcεRI β and γ subunits in anti-Lyn immune complexes from anti-FcεRI mAb H10-treated cells. In H10-treated cells, this strong signal from coprecipitated substrate persists over a range of incubation times (*A*) and concentrations of stimulus (*B*). No signal from coprecipitated receptor subunits of other kinase substrate is induced in cells treated with Ag or anti-FcεRI mAb J17.

receptor is a likely source of some or all of the strong phospho-β and phospho-γ signals detected by anti-phosphotyrosine immune complex kinase assays shown in Fig. 4.

H10 receptor complexes are poor Syk activators

In Fig. 5*B*, anti-Syk immune complexes were incubated with [γ-^{32}P]ATP to measure cross-linker-induced Syk autophosphorylation, an index of Syk activity. As previously reported (26), there is little or no Syk activity in resting cells. Syk is strongly activated by Ag and anti-FcεRI mAb J17. There is an intermediate level of Syk activation in anti-FcεRI mAb F4-treated cells. Consistent with their impaired signaling activity, anti-FcεRI mAb H10 receptor complexes induce the least Syk activation in kinase-specific assays.

Different cross-linking agents induce different phospho-β isoforms in RBL-2H3 cells

In Fig. 7, *A* and *B*, anti-phosphotyrosine-reactive proteins were immunoprecipitated from variously activated cells, separated by SDS-PAGE, and probed with either anti-β subunit mAbs (Fig. 7*A*) or anti-phosphotyrosine mAbs (Fig. 7*B*). The results of anti-β blotting showed that anti-phosphotyrosine immune complexes from resting cells (*left lane*) are essentially free of β subunits. It thus appears that there is little intrinsic tyrosine phosphorylation of receptor subunits and of proteins that bind and coprecipitate these subunits in RBL-2H3 cells. Anti-phosphotyrosine immune complexes from cells activated for 5 min with Ag or anti-FcεRI mAbs contained three anti-β-reactive bands, designated β1, β2, and β3. β3 (~33 kDa) was readily detected in cells treated with Ag and anti-FcεRI mAb J17, was less abundant in cells treated with anti-FcεRI mAb F4, and was virtually undetectable in cells treated with anti-FcεRI mAb H10. β2 (~30 kDa) was the predominant band in cells treated with anti-FcεRI mAbs H10 and F4. There was also a substantial signal from β2 in cells treated with Ag and with anti-FcεRI mAb F4. β1 (~27 kDa) was readily detected in mAb H10-treated cells, but contributed very little signal in cells treated with Ag or with anti-FcεRI mAbs F4 and J17 (see Fig. 7).

All three anti-β-reactive bands were detected by anti-phosphotyrosine blotting of anti-phosphotyrosine immune complexes (Fig. 7*B*), indicating that they are all tyrosine phosphorylated in activated cells. However, the distribution of signal intensities between isoforms was strikingly different when detection was with anti-phosphotyrosine compared with anti-β mAbs. In particular, anti-phosphotyrosine reacted weakly with the β1 isoform, more strongly with the β2 isoform, and most strongly with the β3 isoform (see Fig. 7). Thus, the different electrophoretic mobilities of these phosphorylated β subunit isoforms are most likely explained by their different extents of tyrosine phosphorylation.

Lyn does not associate with the FcεRI β3 isoform

In Fig. 7, *C* and *D*, anti-Lyn immune complexes were precipitated from lysates of Ag- or anti-FcεRI mAb-activated cells and separated by SDS-PAGE, and Western blots of replicate gels were probed as described above with either anti-β or anti-phosphotyrosine mAbs. The two less phosphorylated β subunit isoforms, β1 and β2, discovered in highest amounts in anti-phosphotyrosine immune complexes from H10-activated cells, were also prominent in anti-β blots of anti-Lyn immune complexes from H10-activated cells (Fig. 7*C*). These results support evidence from anti-Lyn immune complex kinase assays (Figs. 5*A* and 6) that a substantial amount of Lyn exists in a stable complex with FcεRI subunits in H10-treated cells. They identify H10-induced β1 and β2 as Lyn-binding isoforms. In contrast, the highly phosphorylated β3 isoform, found particularly in anti-phosphotyrosine immune complexes from Ag- and J17-treated cells, is completely absent from anti-Lyn immune complexes and is thus identified as having very little binding activity for Lyn. Ag- and anti-FcεRI mAb J17-induced β2 also showed very little association with Lyn. The simplest explanation is that the β2 band may consist of proteins that are phosphorylated on different tyrosines or on a different combination of tyrosine and serines, resulting in their similar electrophoretic mobilities but different Lyn-binding activities and potential for further phosphorylation to the β3 isoform.

In many replicate experiments, the β2 and β3 isoforms were consistently resolved by immunoblotting antiphosphotyrosine immune complexes from activated cells (as in Fig. 7), while immune complex kinase assays usually revealed a single band (as in Figs.

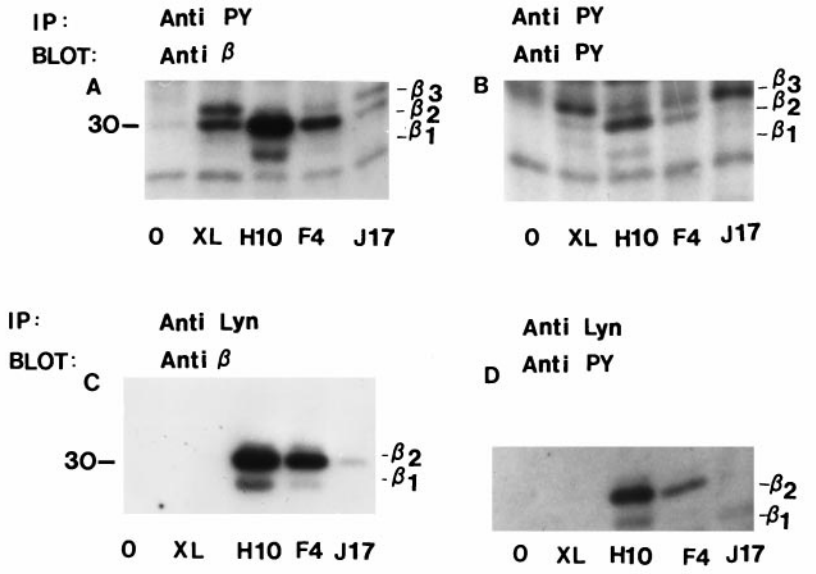

FIGURE 7. Three phospho-β isoforms are induced by FcϵRI cross-linking, but only two associate with Lyn. Cells were activated for 2 min with 0.1 μg/ml DNP-BSA or for 5 min with 0.1 μg/ml anti-FcϵRI mAbs. Anti-phosphotyrosine (*A* and *B*) or anti-Lyn (*C* and *D*) immune complexes were generated from precleared cell lysates as described in Fig. 4. Proteins were separated on duplicate 10% SDS gels and transferred to nitrocellulose for Western blotting using anti-β (*A* and *C*) or anti-phosphotyrosine (*B* and *D*) mAbs followed by ^{125}I-labeled donkey anti-mouse IgG. After blotting, the gels were dried, and radiolabeled FcϵRI β subunits, designated β1, β2, and β3, were detected by autoradiography. The experiment illustrated here (one of three with very similar results by autoradiography) was also analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Comparison of the amounts of various β subunit isoforms in this single experiment revealed β3:β2:β1 ratios of 42:57:1 in Ag-treated cells, 46:52:2 in J17-treated cells, 21:72:7 in F4-treated cells, and 9:76:15 in H10-treated cells. Comparison of signal intensities between corresponding bands in *A* and *B* showed ratios of anti-phosphotyrosine signal:anti-β signal of 0.96 for β3, 0.25 for β2, and 0.15 for β1.

4–6). We speculate that this difference reflects the release of constraints to reaching the most phosphorylated state under the conditions of our in vitro assays. In contrast, a band resembling β1 was detected in lysates of anti-FcϵRI mAb H10-treated cells both by anti-β immunoblotting (Fig. 7) and by anti-phosphotyrosine and anti-Lyn immune complex kinase assays (Figs. 4 and 6). The small amounts of β1 even in cells treated with anti-FcϵRI mAb H10 raises uncertainty about its identity. β1 may be a third phospho-β subunit isoform that cannot be phosphorylated to completion by Lyn in vitro. Alternatively, it may be a product of limited proteolysis of the β2 or β3 isoform.

Discussion

A series of older reports suggested that receptor dimers are the minimal unit capable of transducing signals from FcϵRI cross-linking to secretory responses (21). The work of Ortega and colleagues with two signaling-competent anti-FcϵRI mAbs, F4 and J17, provided definitive support for this proposal. A third mAb to the FcϵRI α subunit, H10, was found to induce very limited secretory activity. The differences in secretory activity of FcϵRI dimers induced by different mAbs have been postulated to arise from different configurational constraints of the dimers (20, 22, 23). Here, we have extended the characterization of signaling differences among the three anti-FcϵRI mAbs to include not only secretion, but also Ins(1,4,5)P_3 synthesis, Ca^{2+} mobilization, ruffling, spreading, and actin plaque assembly. The effectiveness of cross-linker-induced cell activation in these studies was Ag $>$ or $=$ J17 $>$ or $=$ F4 $\gg$ H10, somewhat different from that originally reported (20).

We considered the possibility that the different anti-FcϵRI mAb-induced receptor dimers may redistribute by a process that is independent of interdimer cross-linking into differently sized aggregates with correspondingly different signaling activities. In particular, recent evidence that cross-linked IgE receptors may redistribute to punctate plasma membrane domains visible at the resolution of the fluorescence microscope (37) led to the hypothesis that FcϵRI dimers induced by anti-FcϵRI mAb J17 might redistribute into larger clusters than FcϵRI dimers induced by anti-FcϵRI mAb H10. Fluorescence microscopy (not shown) resolved receptor clusters in cells treated with anti-IgE but not with anti-FcϵRI mAbs. The dispersed distributions of receptor complexes induced by all three anti-FcϵRI mAbs were confirmed at the higher resolution of the scanning electron microscope. Based on these results, it is clear that formation of large receptor clusters is not required for signaling. It remains possible that the signaling-competent mAbs induce very small clusters of FcϵRI dimers that cannot be distinguished from fully dispersed H10-induced dimers by our gold-labeling procedures, which tag only a small proportion of total receptors.

Contemporary models suggest that the FcϵRI signaling cascade is initiated by the Lyn-mediated transphosphorylation of tyrosines in the receptor subunit cytoplasmic tails, creating β subunit phosphotyrosine binding sites for the SH2 domains of additional Lyn molecules that, in turn, catalyze further FcϵRI β and γ subunit phosphorylation (10, 12, 39). The results of anti-phosphotyrosine immune complex kinase assays revealed strong signals from phosphorylated Lyn in H10-activated cells. The signals from phosphorylated receptor subunits were substantially greater when anti-phosphotyrosine immune complexes were prepared from H10-activated cells than when they were from Ag-activated cells. These data indicate that H10 receptor dimers are fully competent to support the initial events of Lyn-mediated subunit transphosphorylation that launch the FcϵRI signaling sequence.

Importantly, we detected strong signals from phosphorylated FcϵRI β and γ subunits when lysates of H10-treated cells were used as a source of Lyn (and Lyn-associated proteins) for anti-Lyn

immune complex kinase assays. In contrast, signals from phosphorylated receptor subunits were weak or absent when Lyn was immunoprecipitated from lysates of cells that were activated with Ag or with anti-FcεRI mAbs J17 or F4. These experiments established that mAb H10, but not Ag or the more signaling-competent anti-FcεRI mAbs, induces stable complexes between activated Lyn and receptor subunits.

There was less Syk phosphorylation in anti-Syk immune complex kinase assays when lysates were from H10-activated cells than when they were from cells activated with Ag or with anti-FcεRI mAbs F4 and J17. It thus appeared that the presence of stable Lyn-receptor complexes may be incompatible with strong Syk activation and signal propagation in intact cells.

We discovered that anti-phosphotyrosine immune complexes from activated cells contain two principal phosphorylated FcεRI β subunit isoforms, designated β2 and β3, each with its own characteristic electrophoretic mobility, resulting at least in part from differences in the extent of β subunit tyrosine phosphorylation. (Differences in serine phosphorylation might also contribute to these distinct mobilities). A third phospho-β band, β1, detected in mAb H10-treated cells may represent an additional phospho-β isoform but could also be a degradation production from the principal isoforms. Importantly, different cross-linking agents induced different distributions of total FcεRIβ between these phospho-β isoforms. Specifically, the most phosphorylated, least mobile phospho-β form, β3, was most prominent in Ag- and J17-stimulated cells, occurred in modest levels in F4-stimulated cells, and was barely detectable in H10-treated cells. The moderately phosphorylated, moderately mobile phospho-β form, β2, was found at highest levels in H10-treated cells and in substantial amounts in cells stimulated with Ag and anti-FcεRI mAbs F4 and J17. Finally, the least tyrosine-phosphorylated, most mobile phospho-β species, here called β1, was found almost exclusively in H10-treated cells. These phospho-β isoforms can be distinguished not only by their phosphorylation levels, but also by their ability to bind Lyn. Thus, the incompletely phosphorylated β1 and β2 bands that predominate in mAb H10-treated cells coprecipitate with Lyn in anti-Lyn immune complexes. In contrast, β3, the highly phosphorylated phospho-β isoform found in cells treated with Ag and the signaling-competent anti-FcεRI mAbs, does not coprecipitate with Lyn.

These results are consistent with previous evidence that Lyn binding to partially phosphorylated FcεRIβ promotes further β and γ subunit phosphorylation (10, 40). They suggest for the first time that Lyn dissociation is a prerequisite for Syk recruitment to γ subunit phospho-ITAMs and for signal propagation. They indicate that Lyn dissociation depends on the successful progression of a Lyn-mediated FcεRI β subunit phosphorylation sequence. We cannot as yet propose a definitive mechanism for the blockade of β subunit phosphorylation and Lyn dissociation in H10-treated cells. However, Ortega et al. (20) concluded that configurational differences between FcεRI dimers induced by H10 and other mAbs might explain their different signaling properties. Our data raise the possibility that these configurational constraints make a phosphorylation site(s) on the β subunit inaccessible to activated Lyn in H10-treated cells.

Recent work by Pribluda et al. (14) established that FcεRI cross-linking with multivalent Ag causes the phosphorylation of three tyrosines, all located within the β subunit ITAM region. Two are typical ITAM tyrosines, and the third is an atypical internal tyrosine. The extent of phosphorylation of these tyrosines is variable. Two serines located within the β-ITAM sequence are also variably phosphorylated following FcεRI cross-linking. In the simplest case, it is conceivable that the three phospho-β isoforms represent mono-, di-, and trityrosine-phosphorylated species and that one of the three β-ITAM tyrosines remains inaccessible to Lyn in H10-treated cells, preventing complete subunit phosphorylation and Lyn dissociation. However H10-induced β2 binds Lyn, whereas Ag-induced β2 has little Lyn-binding activity. Thus, it is perhaps more likely that the β2 band includes several species that are phosphorylated on different tyrosines or on a different combination of tyrosines and serines, resulting in their similar electrophoretic mobilities but distinct Lyn-binding activities and potential for further phosphorylation to the β3 isoform in intact cells. Further work is also needed to decide whether β1 participates in the normal phosphorylation sequence.

The occurrence of multiple phospho-β subunit isoforms in intact, Ag-stimulated cells is not a new observation. In 1992, we demonstrated that FcεRI cross-linking causes a time- and Ag concentration-dependent shift in the electrophoretic mobility of anti-phosphotyrosine-reactive FcεRI β in [γ-^{32}P]orthophosphate-labeled Ag-treated RBL-2H3, resembling the β2 to β3 transition (7). Paolini et al. (41) also demonstrated a mobility shift in response to 1 min of receptor cross-linking in RBL-2H3 cells and showed that the less mobile form of phosphorylated β induced by Ag could be returned to its original higher mobility form by the addition of monovalent hapten for 30 s. Other work by Kinet and colleagues has demonstrated a cross-linker- and Lyn-dependent β subunit mobility shift, resembling the β2 to β3 transition, in both P815 and NIH-3T3 cells induced to express wild-type and mutant FcεRI subunits (11, 42). The β1 isoform has not been reported previously in Ag-stimulated RBL-2H3 cells. However, Jouvin et al. (11) showed in transfected P815 cells that mutagenized FcεRI β lacking all three β-ITAM tyrosine phosphorylation sites but still capable of serine phosphorylation had a distinctive faster mobility than the wild-type β isoform. The similar behaviors of H10-induced β1 in RBL-2H3 cells and mutated β in P815 transfectants encourage phosphopeptide sequence analyses to determine whether β1 in RBL-2H3 cells is a third phospho-β subunit isoform with a low level of tyrosine phosphorylation, possibly supplemented by serine phosphorylation. As noted, we cannot exclude the alternative possibility that β1 is simply a product of limited proteolysis of the β2 or β3 isoforms.

Previous investigators have had difficulty in demonstrating Lyn-receptor complexes in Ag-stimulated cells (10). Our data predict this result. For signaling-competent cross-linking agents, the speed at which Lyn is recruited to Ag-receptor complexes, mediates the FcεRI subunit phosphorylation sequence, and then dissociates from the receptor implies that only a small proportion of Lyn is likely to be in the receptor-bound form during active signaling. It is clear from recent studies that Lyn is limiting for signaling (39). Thus, the transient interaction of Lyn with subunits has at least two advantages. First, it permits Syk's access to γ-phospho-ITAMs, as required for signal propagation. Second, it maintains a supply of Lyn for signal initiation through new receptor cross-linking events.

In summary, we suggest Lyn dissociation from highly phosphorylated FcεRI subunits as a new regulatory step in the FcεRI signaling cascade that is required for Syk activation and signal propagation. We predict that a similar regulatory step will be discovered by analyses of other multichain immune recognition receptor signaling pathways. Consistent with this, two groups studying the induction of T cell anergy by altered peptide ligands found that occupying the TCR by altered peptides can in some cases induce TCR ζ-chain phosphorylation with very little ZAP70 recruitment or activation (43, 44). In both studies an unusual highly mobile, less phosphorylated ζ subunit isoform dominated over the less mobile, highly phosphorylated ζ isoform induced by immunogenic peptide. Cells exposed to the altered peptide ligands were

only weakly activated by these ligands and were resistant to subsequent challenge with immunogenic peptides. Smith et al. (45) found that a nonmitogenic anti-CD3 mAb similarly induced a less phosphorylated phospho-ζ isoform than the one found in cells treated with mitogenic anti-CD3 and was also unable to induce substantial ZAP70 activation. By analogy with our studies in mast cells, it is likely that ZAP70 activation may have been impaired in all these studies due to the formation of stable complexes between incompletely phosphorylated ζ subunits and Src family members that block the ZAP70-ζ interaction.

Acknowledgments

We thank Dr. Israel Pecht (Department of Chemical Immunology, Weizmann Institute of Science), for generous access to mAbs generated in his laboratory and Claudia Garay for excellent technical assistance.

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