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FcεR1-Mediated Tyrosine Phosphorylation of Multiple Proteins, Including Phospholipase Cγ1 and the Receptor $\beta\gamma_2$ Complex, in RBL-2H3 Rat Basophilic Leukemia Cells

WEI LI,[1] GRACE G. DEANIN,[2] BEN MARGOLIS,[1] JOSEPH SCHLESSINGER,[1] AND JANET M. OLIVER[2]*

Department of Pharmacology, New York University Medical Center, New York, NY 10016,[1] and the Department of Pathology, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131[2]

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In basophils, mast cells, and the RBL-2H3 tumor mast cell line, cross-linking the high-affinity immunoglobulin E receptor (FcεR1) stimulates a series of responses, particularly the activation of phospholipase C (PLC), that lead to allergic and other immediate hypersensitivity reactions. The mechanism of activation of PLC, however, is not clear. Here, we show that cross-linking FcεR1 on RBL-2H3 cells causes the tyrosine phosphorylation of at least 12 cellular proteins, including PLCγ1 (PLCγ1) and the receptor β and γ subunits. ^{32}P-labeled PLCγ1 can be detected by anti-phosphotyrosine antibody as early as 10 s after the addition of antigen. The tyrosine-phosphorylated 33-kDa β subunit and 9- to 11-kDa γ subunit of the FcεR1 are additionally phosphorylated on serine and theonine residues, respectively, and are found as complexes with other phosphotyrosine-containing proteins in antigen-stimulated cells. Our results indicate a means by which the FcεR1 may control PLC activity in RBL-2H3 cells and raise the possibility that other receptor-mediated signalling events in mast cells may also be controlled through protein tyrosine phosphorylation.

In RBL-2H3 rat basophilic leukemia cells, a model for mucosal mast cells, antigens that cross-link the high-affinity immunoglobulin E (IgE) receptor FcεR1 lead to Ca^{2+} influx, phosphatidylinositol (PI) turnover, the release of serotonin and other substances from granules, the polymerization of actin, and changes in the cell surface from a microvillous to a lamellar architecture (for reviews, see references 32 and 36). On the basis of experiments with cholera toxin in intact cells and with GTPγS in permeabilized cells, previous investigators have suggested that the FcεR1-mediated signal transduction pathway in RBL-2H3 cells is controlled through the activation of GTP-binding proteins (2, 3, 31). However, Wilson et al. (47) found that depleting RBL-2H3 cells of GTP with mycophenolic acid impairs antigen-stimulated Ca^{2+} influx without significantly affecting antigen-stimulated PI turnover. These results suggested that GTP-binding proteins may control ion channel activity but not phospholipase C (PLC) activation in RBL-2H3 cells. Subsequently, Deanin et al. (14) found that several tyrosine kinase inhibitors, including genistein (1), abolish antigen-induced secretion and membrane responses in permeabilized cells. It was also reported that genistein reduces FcεR1-mediated membrane ruffling, actin polymerization, inositol-1,4,5-trisphosphate synthesis, and secretion in intact RBL-2H3 cells (13). These results suggest that a protein tyrosine kinase is a critical component of the FcεR1-mediated signalling pathway.

These proposals arising from pharmacological studies have been supported by several biochemical studies in RBL-2H3 cells and by more extensive studies in other cells of the immune system. Although the heterotrimeric ($\alpha\beta\gamma_2$) FcεR1 does not contain intrinsic protein tyrosine kinase activity (5), it is clear from the work of Benhamou et al. (4) that FcεR1 cross-linking stimulates the tyrosine phosphorylation of several RBL-2H3 cell proteins, especially a prominent 72-kDa species. Connelly et al. (10) and Paolini et al. (37) have also observed antigen-induced protein tyrosine phosphorylation in RBL-2H3 cells. Protein tyrosine phosphorylation can also be activated by ligands that cross-link CD4, CD8, CD3, and interleukin-2 receptors on T cells, membrane immunoglobulin on B cells, and FcγRIII and tumor cell-binding sites on NK cells. In these cell types, specific members of the *src* family of cytoplasmic tyrosine kinases have been implicated in the ligand-induced phosphorylation response (6, 15–19, 22, 24, 26, 34, 35, 41, 43, 45, 46, 48). By analogy with results obtained with lymphoid cells, it has been proposed that FcεR1-stimulated protein tyrosine phosphorylation in RBL-2H3 mast cells may also be mediated by a receptor-associated *src* family kinase (4, 6, 16). Other studies have shown that the substrates for ligand-stimulated tyrosine phosphorylation in T and B cells include receptor subunits and phospholipase Cγ1 (8, 9, 24, 37, 42).

We show here that antigen binding causes the tyrosine phosphorylation of multiple proteins in RBL-2H3 cells, including PLCγ1 and the β and γ subunits of the FcεR1 complex.

MATERIALS AND METHODS

Cells. RBL-2H3 cells were cultured in minimal essential medium (GIBCO, Grand Island, N.Y.) with 15% fetal calf serum (FCS) as described previously (12–14, 38, 47). They were incubated overnight with 1 μg of monoclonal anti-dinitrophenol IgE (anti-DNP IgE [29]) per ml to saturate the cell surface FcεR1s and activated by cross-linking the resulting IgE-receptor complexes with dinitrophenol-conjugated bovine serum albumin (DNP-BSA) (Molecular Probes Inc., Junction City, Ore.).

Metabolic labeling. RBL-2H3 cells were radiolabeled with

* Corresponding author.

$^{32}P_i$ by a modification of the methods described by Li et al. (27). Briefly, IgE-primed cells were harvested and resuspended to a concentration of 2.5×10^6/ml, and 10-ml portions were incubated for 2 h in P_i-free medium (GIBCO) containing 5% dialyzed FCS and additional anti-DNP IgE. The cells were washed twice in P_i-free medium to remove unbound IgE, resuspended in 2.5 ml of P_i-free medium with 5% dialyzed FCS and 1 mCi of $^{32}P_i$ (specific activity [carrier-free], 314.5 to 337.5 TBq/mmol, New England Nuclear, Waltham, Mass.), and incubated for a further 2 h. They were harvested and resuspended in the same medium to a concentration of 10^7 cells/ml.

Cell activation and immunoprecipitation of radiolabeled proteins. Aliquots (2 ml) of IgE-primed, radiolabeled cells were stimulated for various times with DNP-BSA, and the reaction was stopped by the addition of 8.0 ml of ice-cold medium and transfer to an ice bucket. In some experiments, the cells were incubated for 10 min with 100 μM genistein (GIBCO) prior to stimulation. The cells were collected by centrifugation, immediately solubilized in 1.0 ml of ice-cold lysis buffer (10 mM Tris [pH 7.1 at room temperature], 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 2 mM iodoacetate, 5 μM $ZnCl_2$, 0.1% BSA, 1 mM freshly prepared phenymethylsulfonyl fluoride, and 0.5% Triton X-100) and centrifuged for 20 min at 13,000 × *g* in an Eppendorf microcentrifuge. Protein concentration was measured by the method of Bradford (7), and volumes were adjusted so that each supernatant contained approximately the same amount of protein.

For the isolation of tyrosine-phosphorylated proteins, supernatant extract was added to 30 μl of packed anti-phosphotyrosine (anti-PY) beads (monoclonal anti-PY antibody 1G2 covalently coupled to agarose; Oncogene Science, Manhasset, N.Y.) and rocked on a nutator (Adams) for 2 h at 4°C. The beads were then washed five times by resuspension and 10 s of centrifugation in the lysis buffer with BSA and twice by centrifugation in the lysis buffer without BSA, with 30-s vortexing between washes.

Tyrosine-phosphorylated proteins were eluted by the addition of 30 μl of elution buffer (BSA-free lysis buffer containing 0.01% ovalbumin and 1 mM phenylphosphate) and 10 min of incubation at 4°C, with gentle agitation of the tubes every 2 min. The eluate was collected by piercing the bottom of the Eppendorf tube with a 26-gauge needle and centrifuging the eluate into a second Eppendorf tube.

PLCγ1 was immunoprecipitated from unlabeled or ^{32}P-labeled cell lysates or from the anti-PY-reactive protein fractions that were eluted from anti-PY beads. Cell lysates or anti-PY-reactive fractions were incubated at 4°C for 2 h with anti-PLCγ1 antiserum (30) and incubated for an additional 1 h on a nutator with protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.) before being washed as described above.

The β and γ subunits of the FcεR1 were reprecipitated from the anti-PY-reactive protein fractions of unstimulated and stimulated cells by incubation first with mouse monoclonal anti-β subunit (40) or anti-γ subunit (37) antibodies and then with protein A-Sepharose as described above. In some experiments, the anti-receptor subunit immune complexes were washed an additional three times with a high-stringency radioimmunoprecipitation assay buffer (10 mM Tris [pH 7.6 at 4°C], 300 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) prior to their analysis by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

SDS-PAGE, Western blotting (immunoblotting), and autoradiography. Protein samples were boiled for 5 min in Laemmli sample buffer with 0.1 M β-mercaptoethanol and 1% SDS. The radiolabeled samples were resolved by SDS-PAGE on 8 to 15% polyacrylamide gradient gels and visualized by autoradiography. Unlabeled samples were resolved by SDS–8% PAGE, transferred to a nitrocellulose membrane, and blotted with anti-PLCγ1 as described previously (30).

Phosphoamino acid analysis. Phosphoamino acid analyses were carried out by using methods modified from those of Cooper et al. (11). Briefly, the bands of interest were excised from dried SDS gels, and the gel slice was rehydrated by rocking for 15 min in 4 ml of 50 mM NH_4HCO_3 and homogenized in 0.5 to 0.8 ml of NH_4HCO_3 solution. Extracted proteins were precipitated by the addition of trichloroacetic acid (TCA) to 15 to 20% in the presence of BSA (40 μg per tube) and incubation at 4°C for 1 h. The TCA precipitate was washed three times with ice-cold acetone and three times with water (1 ml, 0.5 ml, and 0.1 ml, respectively) to remove the TCA. The protein pellet was resuspended in 100 μl of 6 N HCl in a sealed tube and incubated at 110°C for 1 h. Samples were cooled to room temperature, washed three times with water (0.5 ml, 0.3 ml, and 50 μl, respectively), and dried in a Speed-Vac. They were then dissolved in 5 μl of electrophoresis buffer (pH 1.9) (11) containing 0.2 mg of phosphoamino acid standards (phosphotyrosine, phosphoserine, and phosphothreonine; Sigma), per ml. The hydrolysate was applied to a cellulose thin-layer chromatography plate and separated by electrophoresis at 1.5 kV (15 mA) for 45 min in pH 1.9 buffer and then by drying and electrophoresis in the second dimension at 1.5 kV (15 mA) for 25 min in pH 3.5 buffer. Phosphoamino acid standards on dried plates were located by ninhydrin staining, and their radioactivities were determined by autoradiography.

RESULTS

Antigen-stimulated protein tyrosine phosphorylation in RBL-2H3 cells. Previous investigators have demonstrated the antigen-stimulated tyrosine phosphorylation of RBL-2H3 proteins by immunoblotting with anti-PY antibodies (4, 10). To confirm these results and to identify additional tyrosine-phosphorylated proteins which might not have been detected by previous Western blot experiments, we carried out experiments using conditions that permit the quantitative recovery of tyrosine-phosphorylated proteins (27). Anti-DNP IgE-primed RBL-2H3 cells were metabolically labeled with $^{32}P_i$, the IgE-receptor complexes were cross-linked by incubation with specific antigen (DNP-BSA), and cell lysates were subjected to anti-PY affinity chromatography as described in Materials and Methods. The anti-PY-reactive proteins were analyzed by SDS-PAGE and autoradiography. The range of ^{32}P-labeled proteins in anti-PY immunoprecipitates of lysates of unstimulated and antigen-stimulated cells is shown in Fig. 1. Anti-PY immunoprecipitates prepared from ^{32}P-labeled unstimulated cells show radiolabeled bands at 58 kDa and within the molecular mass range of 95 to 180 kDa (lane 1). In contrast, cells incubated with 0.1 μg of antigen per ml for 1 min show at least 12 radiolabeled bands, including species with molecular masses of approximately 160, 145, 120, 117, 95, 85, 72, 60, 58, 40, 33, and 9 to 11 kDa (lane 2). In order to determine that each of these proteins is indeed tyrosine phosphorylated, radiolabeled bands (Fig. 1A, lane 2) were individually excised and subjected to

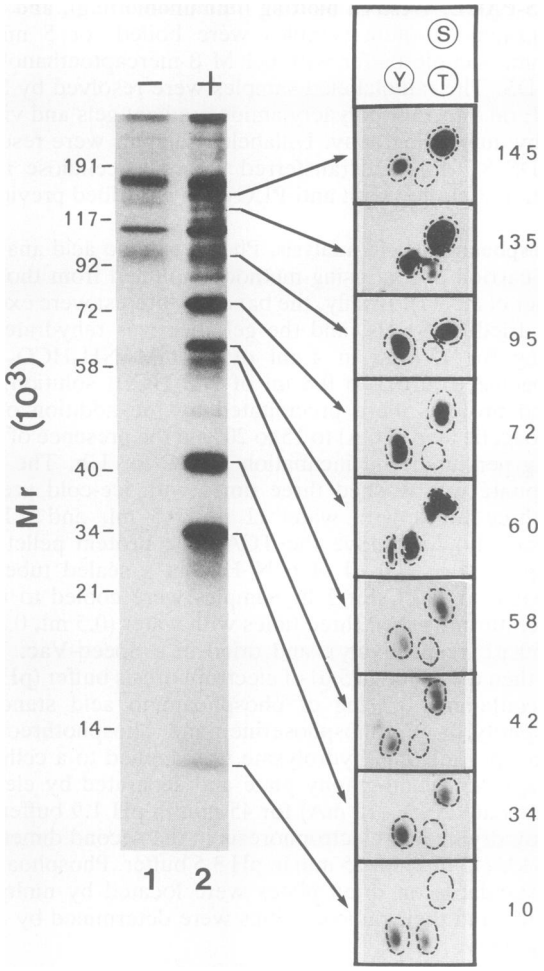

FIG. 1. FcεR1 cross-linking stimulates protein tyrosine phosphorylation. RBL-2H3 cells were primed with anti-DNP IgE, labeled with $^{32}P_i$, and incubated in the presence (lane 2) or absence (lane 1) of DNP-BSA (0.1 μg/ml) for 1 min at 37°C. Cells were solubilized, and the anti-PY-reactive proteins were isolated (see Materials and Methods) and analyzed by SDS-PAGE on 10 to 18% polyacrylamide gradient gels and autoradiography. The radiolabeled bands in the right panel were excised, and the proteins were extracted, hydrolyzed in 6 N HCl, and subjected to phosphoamino acid analysis by two-dimensional gel electrophoresis. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Exposure times: lanes 1 and 2, 4 h; right panel, 2 to 7 days.

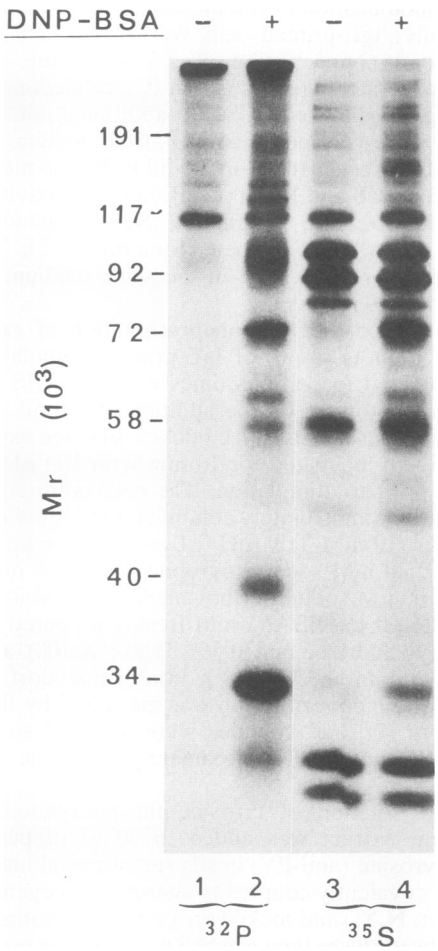

FIG. 2. Comparison of the pattern of anti-PY-reactive proteins from ^{32}P- or ^{35}S-labeled RBL-2H3 cells. Anti-DNP IgE-primed and $^{32}P_i$-labeled (lanes 1 and 2) or [^{35}S]methionine-labeled (lanes 3 and 4) cells were incubated in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of DNP-BSA (0.1 μg/ml) for 1 min at 37°C prior to solubilization and isolation of the anti-PY-reactive proteins. Samples were subjected to SDS-PAGE and autoradiography. Exposure time was 15 h without an intensifying screen.

phosphoamino acid analysis as described in Materials and Methods. It is clearly shown that all of the bands analyzed contained phosphotyrosine in addition to phosphoserine and/or phosphothreonine (Fig. 1, right panel). Since the results of the anti-PY immunoprecipitation of ^{32}P-labelled cells may reflect an increase in both tyrosine and serine phosphorylation, we have also carried out experiments in which the lysates of unstimulated or stimulated cells were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-PY antibodies (data not shown). For comparison, the anti-PY immunoprecipitates of ^{32}P-labeled cells were run in the same gel. The same pattern of proteins was seen by anti-PY blotting and anti-PY immunoprecipitation, but there were differences in the relative intensities of some of the bands. It is likely that these differences resulted from detection of tyrosine phosphorylation by anti-PY blotting and of both tyrosine and serine/threonine phosphorylation by anti-PY immunoprecipitation.

When the patterns of anti-PY-reactive proteins from $^{32}P_i$- and [^{35}S]methionine-labeled cells were compared as shown in Fig. 2, many of the bands whose phosphorylation was increased in response to antigen (lane 2) were also found to contain more protein upon antigen stimulation (lane 4). This result is consistent with the possibility that a larger fraction of the population of these proteins becomes tyrosine phosphorylated following stimulation. However, in the ^{35}S-labeled cells, bands with molecular masses of 92 to 95, 58, and 21 kDa showed little or no increase in intensity upon antigen stimulation, indicating that for these proteins there is no change in the fraction of the population that is phosphorylated at at least one tyrosine site and that antigen stimulation increased the phosphorylation (at tyrosine and/or serine) of these molecules that are already phosphorylated at tyrosine. Upon longer exposure, the 92- to 95-, 58-, and 21-kDa bands could also be detected in ^{32}P-labeled, unstimulated cells (lane 1), consistent with their detection by anti-PY antibodies from ^{35}S-labeled, unstimulated cells (lane 3).

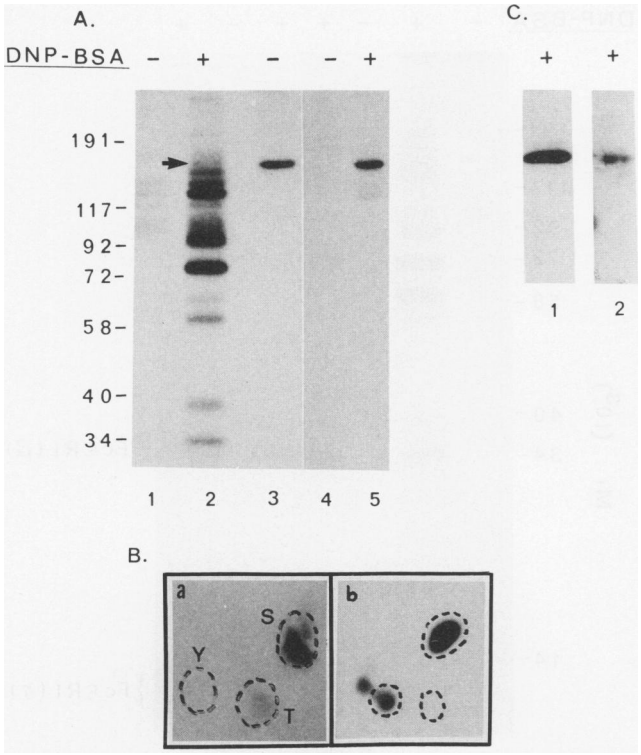

FIG. 3. FcεR1-mediated phosphorylation of PLCγ1. RBL-2H3 cells primed with anti-DNP IgE and labeled with $^{32}P_i$ (A and B) or unlabeled (C) were incubated with (lanes 2 and 5) or without (lanes 1, 3, and 4) DNP-BSA (0.1 μg/ml) for 3 min at 37°C prior to solubilization. (A) The cell lysates were immunoprecipitated with either anti-PY antibodies (lanes 1, 2, 4, and 5) or anti-PLCγ1 antibodies (lane 3). Duplicate anti-PY-reactive fractions were subjected to anti-PLCγ1 immunoprecipitation (lanes 4 and 5) prior to SDS-PAGE and autoradiography. (B) The ^{32}P-labeled PLCγ1 in lanes 3 (panel a) and 5 (panel B) were excised and subjected to phosphoamino acid analysis. (C) Lysates of approximately 3×10^7 cells were immunoprecipitated with either anti-PLCγ1 antibodies (lane 1) or anti-PY (lane 2), and the samples were resolved on SDS gels, transferred to nitrocellulose membranes, and blotted with anti-PLCγ1 antibodies. Exposure time: panel A, lanes 1 to 3, 4 h; panel A, lanes 4 and 5, 15 h; panel B, 3 to 10 days; panel C, 40 h.

PLCγ1 is tyrosine phosphorylated in antigen-stimulated RBL-2H3 cells. Since FcεR1 cross-linking increases phosphatidylinositol turnover and stimulates protein tyrosine phosphorylation of a band of 145 kDa, which corresponds to the size of PLCγ1 (Fig. 1), we tested the possibility that PLCγ1 is among the anti-PY-reactive proteins. ^{32}P-labeled and anti-PY-reactive proteins were isolated from either antigen-stimulated or unstimulated cells by anti-PY affinity chromatography and were immunoprecipitated with anti-PLCγ1 specific antibodies. The immunoprecipitates were analyzed in parallel with the total anti-PY-reactive fractions by SDS-PAGE, as shown in Fig. 3A. It can be seen that a band of 145 kDa that is barely resolved in the anti-PY-reactive fraction from antigen-stimulated (lane 2, arrow) but not from unstimulated (lane 1) cells was specifically recognized by anti-PLCγ1 antibodies (lane 5), indicating that PLCγ1 was bound, either directly or indirectly, to the anti-PY column. It is noteworthy that, except in Fig. 1, the resolution of the 145-kDa PLCγ1 in the total anti-PY-reactive fraction was often poor, possibly because of the relatively weak phosphorylation of PLCγ1. The 145-kDa bands present in the anti-PLCγ1 immunoprecipitate of the anti-PY-reactive fractions of activated cells (Fig. 3, lane 5) and in the direct anti-PLCγ1 immunoprecipitate of resting cells (lane 3) were each excised from the gels and subjected to phosphoamino acid analysis. It is clearly shown that PLCγ1 from unstimulated cells is phosphorylated mainly on serine residues (Fig. 3B, panel a), whereas the PLCγ1 from stimulated cells is phosphorylated on serine and tyrosine residues (Fig. 3B, panel b).

The extent of PLCγ1 tyrosine phosphorylation in activated cells was estimated by comparing the fraction of anti-PY-reactive PLCγ1 to the total PLCγ1 in cell lysates. Equal numbers of DNP-BSA-stimulated (0.1 μg/ml for 3 min at 37°C) cells were lysed and immunoprecipitated with either anti-PLCγ1 antibodies or anti-PY antibodies, and the immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-PLCγ1 antibodies. PLCγ1 was visualized by ^{125}I-protein A binding and autoradiography as shown in Fig. 3C. The bands corresponding to PLCγ1 were excised, and their radioactivities were measured in a gamma radiation counter. In lysates of activated cells, it was found that the amount of PLCγ1 that bound to the anti-PY affinity column (lane 2) was 17% of the amount of PLCγ1 that was immunoprecipitated by anti-PLCγ1 (lane 1). Since the recovery of anti-PLCγ1 by immunoprecipitation (lane 1) was about 70% of the total cellular PLCγ1 (based on repetitive immunoprecipitation studies [data not shown]), the anti-PY-reactive PLCγ1 in activated cell lysates was actually around 12% of the total PLCγ1.

Time course studies showed that the maximum phosphorylation detected by anti-PY antibodies occurs 1.5 min after treatment with antigen at a concentration of 0.1 μg/ml at 37°C (Fig. 4A). When the duplicate anti-PY-reactive fractions of Fig. 4A were subjected to anti-PLCγ1 immunoprecipitation prior to SDS-PAGE, as shown in Fig. 4B, the ^{32}P-labeled, anti-PY-reactive PLCγ1 was detected as early as 10 s (lane 2) after antigen stimulation, reached a maximum around 10 min later (lane 6), and was sustained for at least 60 min (lane 8) after antigen stimulation. Increasing the antigen concentration to 1.0 μg/ml, which more rapidly saturates cell surface FcεRIs, shifted the maximum PLCγ1 phosphorylation to 1 min after antigen stimulation (Fig. 4C). These results indicate that tyrosine phosphorylation of PLCγ1 is rapid and antigen concentration dependent.

Treatment of RBL-2H3 cells with genistein, an inhibitor of protein tyrosine kinase activity, blocks FcεR1-mediated IP_3 synthesis and secretion (13). To test whether genistein inhibits the tyrosine phosphorylation of PLCγ1, ^{32}P-labeled cells were incubated for 10 min with 100 μM genistein and then stimulated with 0.1 μg of DNP-BSA per ml for 1.5 min. It is shown in Fig. 4B that no anti-PY-reactive PLCγ1 is detectable in lysates of genistein-treated, antigen-stimulated cells (lanes 9 and 4). These results suggest that FcεR1 cross-linking activates PLCγ1 and stimulates PI turnover by increasing the tyrosine phosphorylation of PLCγ1.

The $\beta\gamma_2$ subunits of the FcεR1 are tyrosine phosphorylated and complexed with other PY-containing proteins in antigen-stimulated RBL-2H3 cells. The FcεR1 is a tetrameric complex of a single IgE-binding 50- to 60-kDa α subunit, a single 33-kDa β subunit, and two disulfide-linked 10-kDa γ subunits (32). cDNA sequences have revealed regions of high homology between the α subunit and FcγRIIa and between the γ subunit and the ζ chain of the T-cell receptor (23). Quarto and Metzger reported that the β and γ subunits of the FcεR1 are both substrates for tyrosine phosphorylation in an in

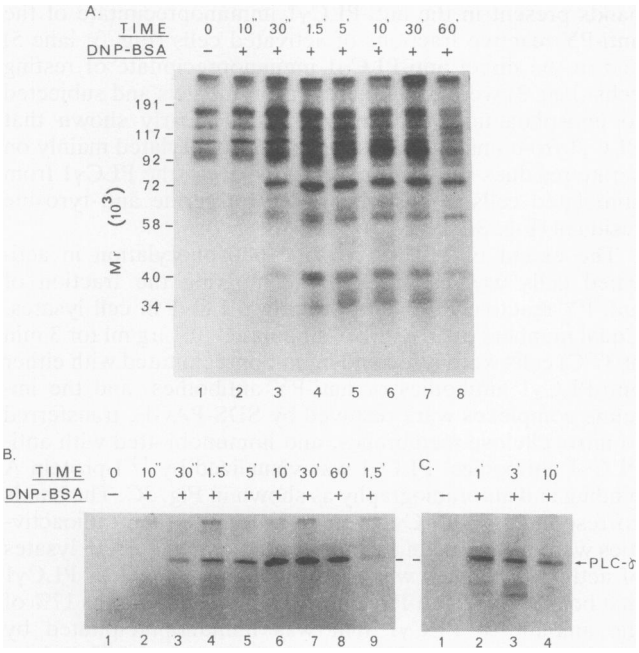

FIG. 4. Kinetics of FcεR1 cross-linking-induced protein tyrosine phosphorylation and PLCγ1 phosphorylation. RBL-2H3 cells, primed and ^{32}P labeled as described in the text, were preincubated with (panel B, lane 9) or without (panels A and B, lanes 1 to 8, and panel C) 100 μM genistein prior to incubation in the presence (lanes 2 to 8) or absence (lane 1) of 0.1 ng of DNP-BSA per ml (A and B) or 1.0 μg of DNP-BSA per ml (C) for the indicated times. Cells were solubilized, and the anti-PY-reactive proteins were isolated. (A) Kinetics of phosphorylation of the total anti-PY-reactive proteins. (B and C) Duplicate anti-PY-reactive fractions were subjected to anti-PLCγ1 immunoprecipitation prior to SDS-PAGE and autoradiography. Shown are kinetics of phosphorylation of anti-PY-bound PLCγ1 in cells stimulated with either 0.1 μg (B) or 1 μg (C) of DNP-BSA per ml. Exposure times: panel A, 4 h; panels B and C, 12 h.

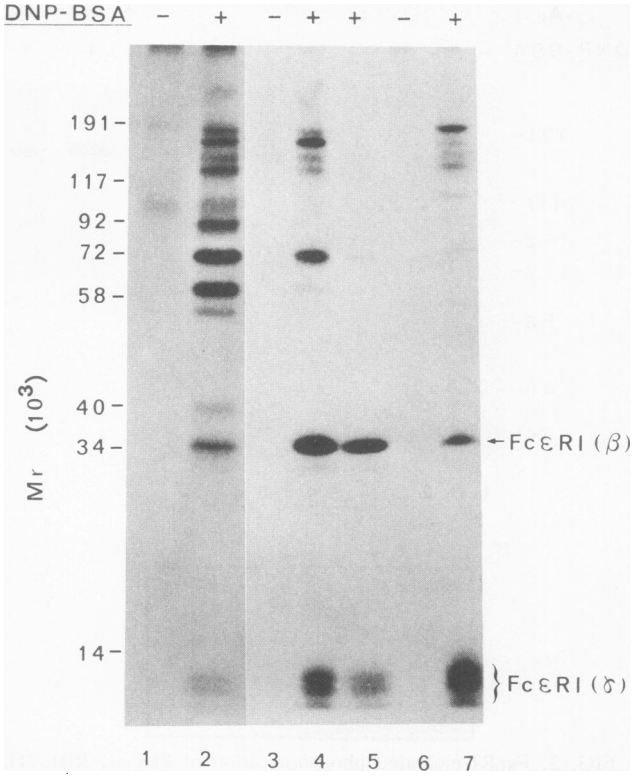

FIG. 5. FcεR1 cross-linking-mediated phosphorylation of FcεR1 β and γ subunits. RBL-2H3 cells were primed with anti-DNP IgE, labeled with $^{32}P_i$, and incubated in the absence (lanes 1, 3, and 6) or presence (lanes 2, 4, 5, and 7) of DNP-BSA (0.1 μg/ml) for 1 min at 37°C, and the anti-PY-reactive proteins were isolated as described above. The duplicate anti-PY-reactive fractions were subjected to either anti-β or anti-γ subunit antibody immunoprecipitation prior to SDS-PAGE and autoradiography. Lanes 1 and 2, total anti-PY-reactive fractions; lanes 3 to 5, anti-FcεR1 β subunit antibody immunoprecipitates of anti-PY-reactive fractions; lanes 6 and 7, anti-FcεR1 γ subunit immunoprecipitates of anti-PY-reactive fractions. Exposure times: lanes 1 and 2, 3 h; lanes 3 to 7, 8 h.

vitro kinase assay using either a cell membrane preparation or partially purified receptor subunits (39). To determine whether the in vivo-phosphorylated 33-kDa protein and the 9- to 11-kDa protein which bound to anti-PY affinity columns (Fig. 1) are the β and the reduced form (monomer) of the γ subunits, respectively, of the FcεR1, ^{32}P-labeled anti-PY-reactive fractions were isolated from antigen-stimulated or unstimulated cells, immunoprecipitated with either anti-β-subunit or anti-γ-subunit antibodies, and analyzed by SDS-PAGE (Fig. 5). It is shown that the 33-kDa protein together with the 9- to 11-kDa protein and several other PY-containing proteins, including species of 72, 117, 120, 145, and 160 kDa, were recognized by anti-β-subunit antibodies in the anti-PY-reactive fraction from stimulated (lane 4) but not from unstimulated (lane 3) cells. Only the 33-kDa and the 9- to 11-kDa species were observed when a duplicate immune complex was washed with high-stringency radioimmunoprecipitation assay buffer (see Materials and Methods) prior to SDS-PAGE (lane 5). Similar results were obtained by immunoprecipitation with anti-γ-subunit antibodies (lanes 6 and 7 and data not shown). Previous experiments (Fig. 1) showed that the 33-kDa and the 9- to 11-kDa species were phosphorylated on tyrosine and serine and on tyrosine and threonine residues, respectively. The 33-kDa and the 9- to 11-kDa species were further identified as the β and γ subunits, respectively, of FcεR1 by immunoblotting the anti-PY-reactive fractions with anti-β or anti-γ antibodies (data not shown). The bands around 9 to 11 kDa were all recognized in immunoblots by anti-γ antibodies, indicating that they correspond to different forms of the γ subunit. In addition, the 9- to 11-kDa proteins under reducing conditions migrated as 20- to 22-kDa species under nonreducing conditions, consistent with the γ subunit being a disulfide-linked dimer (32). In summary, these results demonstrate that the β and γ subunits of FcεR1 are phosphorylated on tyrosine and either serine or threonine, directly associated with each other, and form a large complex with other PY-containing proteins in cells in response to FcεR1 cross-linking.

DISCUSSION

The high-affinity IgE receptor (FcεR1) expressed on the surfaces of basophils and mast cells plays a critical role in immediate hypersensitivity reactions. The function of this receptor is to specifically bind the Fc portion of IgE and to mediate the release of preformed mediators, such as histamine, in response to specific antigens that cross-link cell surface IgE-FcεR1 complexes. Previous pharmacological studies have implicated antigen-stimulated tyrosine phos-

phorylation in the FcεR1-mediated signalling cascade. In particular, there is evidence that the tyrosine kinase-dependent activation of PI turnover may be a critical early event in the antigen-stimulated signalling pathway of RBL-2H3 cells (13, 14). Here, we show that FcεR1 cross-linking rapidly stimulates protein tyrosine phosphorylation in RBL-2H3 cells, suggesting that protein tyrosine kinases (and/or protein tyrosine phosphatases) are involved in FcεR1-mediated signal transduction. We also provide evidence that the 145, 33, and 9- to 11-kDa anti-PY-reactive proteins are PLCγ1 and the β and γ subunits of the FcεR1, respectively. We have demonstrated elsewhere that a band around the 95-kDa region is the *vav* proto-oncogene, a potential transcriptional regulator with *src* homology domains (29). Elucidation of the nature and function of the other PY-containing proteins will shed light on the pathway of FcεR1 signal transduction.

Studies of receptors with intrinsic tyrosine kinase activity, particularly the epidermal growth factor and platelet-derived growth factor receptors, have identified several cellular substrates for ligand-stimulated tyrosine phosphorylation. These include the receptors themselves, the phosphatidylinositol 3-kinase-associated protein (p85), the GTPase-activating protein of *ras* (GAP), $pp60^{src}$, and PLCγ (for reviews, see references 8, 25, and 44). Site-directed mutagenesis studies have indicated that ligand-induced tyrosine phosphorylation regulates PLCγ1 activity (23). It is therefore likely that FcεR1-mediated tyrosine phosphorylation of PLCγ1 enhances its activity, resulting in increases in PI turnover. Consistent with this hypothesis, pretreating cells with the protein tyrosine kinase inhibitor genistein blocks FcεR1-mediated PI turnover (13) and the tyrosine phosphorylation of PLCγ1 (Fig. 4B).

We have demonstrated that antibodies specific for either the β or γ subunit of the FcεR1 recognize the 33-kDa and the 9- to 11-kDa species, respectively, from the anti-PY-reactive fractions of antigen-stimulated RBL-2H3 cells. Washing the anti-β subunit immunoprecipitates at relatively high stringency does not dissociate the coprecipitated γ subunits. The resistance of the γ subunit to displacement from the anti-β subunit immunoprecipitate is consistent with previous evidence for the tight association of these two proteins in the receptor $\beta\gamma_2$ complex (32). The antigen-stimulated tyrosine phosphorylation of the β and γ subunits of FcεR1 has also been recently observed by Paolini et al. (37) and was anticipated on the basis of previous evidence for significant sequence homology between the γ chain of the FcεR1 and the ζ chain of the T-cell receptor, which is tyrosine phosphorylated during T-cell activation (24, 33).

In addition to precipitating the two receptor subunits, each antibody coprecipitates a cluster of five high-molecular-weight phosphoproteins of 72, 117, 120, 145, and 160 kDa. These results indicate that the $\beta\gamma_2$ subunits of the FcεR1 may form a large complex with other tyrosine-phosphorylated proteins in antigen-stimulated cells. The 72-kDa protein is immunoprecipitated with antibody raised against the B-cell 72-kDa protein tyrosine kinase, PTK72 (21), and the 72-kDa protein from activated cells is phosphorylated on tyrosine residues in immune complex kinase assays. These results identify the 72-kDa species as an antigen-activated protein tyrosine kinase (20). By analogy with previous studies in T and B cells, it was predicted that the associated proteins might also include members of the *src* family of tyrosine kinases. The absence of strong bands of coprecipitated proteins in the molecular weight range of most members of the *src* family of protein kinases may reflect the instability of the receptor-*src* kinase complex under the experimental conditions used here. To test whether the 145-kDa $\beta\gamma_2$-associated protein is PLCγ1, we carried out experiments in which the anti-β subunit immunoprecipitate of either anti-PY-reactive fractions or lysates of the antigen-stimulated cells was resolved by SDS-PAGE and blotted with anti-PLCγ1 antibodies. We have not been able to show that PLCγ1 is among the $\beta\gamma_2$-associated proteins.

We conclude that the tyrosine phosphorylation of PLCγ1 is likely to play a crucial role in signal transduction in RBL-2H3 mast cells. The antigen-stimulated tyrosine phosphorylation and complex formation of other RBL-2H3 proteins may also contribute to the activation of FcεR1-stimulated transmembrane signalling.

ADDENDUM

Since the submission of this paper, Park et al. (J. Biol. Chem. **266:**24237, 1991) have reported that FcεR1 cross-linking with oligomeric IgE elicits the phosphorylation of PLCγ1 on tyrosine residues, and Eiseman and Bolen (Nature (London) **355:**78, 1992) have reported that $p56^{lyn}$ and $pp60^{c\text{-}src}$ are activated after FcεR1 cross-linking and that $p56^{lyn}$ coimmunoprecipitates with receptor subunits.

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