

Phosphatidylinositol-3 Kinase Activation Induced upon FcγRIIIA–ligand Interaction

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Summary

Induced activation of protein tyrosine kinase(s) is a central event in signal transduction mediated via the low affinity receptor for IgG (FcγRIIIA, CD16) in natural killer (NK) cells. Tyrosine phosphorylation may affect the function of several proteins directly, or indirectly by inducing their association with other tyrosine phosphorylated proteins. Here, we report that FcγRIII stimulation induces activation of phosphatidylinositol (PI)-3 kinase in NK cells. Phosphotyrosine immunoprecipitates from FcγRIII-stimulated NK cells contain PI-kinase activity and PI-3 kinase can be directly precipitated from them. Conversely, a series of tyrosine-phosphorylated proteins is coprecipitated with PI-3 kinase from the stimulated, but not from control cells. Analogous results obtained using Jurkat T cells expressing transfected FcγRIIIAα ligand binding chain in association with γ₂ or ζ₂ homodimers indicate that both complexes transduce this effect, although the FcγRIIIA–ζ₂ complexes do so with greater efficiency. Accumulation of phosphoinositide D3 phosphorylated products in stimulated cells confirms PI-3 kinase activation, indicating the participation of this enzyme in FcγRIIIA-mediated signal transduction.

Induced phosphorylation of several proteins on tyrosine residues is the earliest biochemical event detectable in NK cells upon binding of ligands to the low affinity receptor for the Fc fragment of complexed IgG (FcγRIII, CD16) (1–4). This is an obligatory event in the receptor-induced signal transduction, leading to phospholipase C (PLC)¹-γ1 and -γ2 activation, phosphatidylinositol (PI) hydrolysis, and subsequent increase in the intracellular Ca²⁺ concentration ([Ca²⁺]_i) and to the more distal events including cytotoxicity, synthesis of cytokines, and expression of IL-2 receptor α chain and other surface activation antigens (3–7). The FcγRIIIA ligand binding α chain and its associated γ and ζ chains necessary for its membrane expression (8) and signal transduction (9–11) are devoid of kinase domains (12–14) and intrinsic kinase activity, and tyrosine phosphorylation occurs via activation of the *src*-related kinase p56^{lck}, physically associated with FcγRIIIA in NK cells via its interaction with the two chains, upon FcγRIIIA–ligand binding (4, 15).

Ligand binding to receptors endowed with intrinsic tyrosine kinase activity, or associated with intracellular tyrosine

kinases, has been shown to induce activation of PI-3 kinase. This enzyme consists of a 110-kD catalytic and an 85-kD regulatory subunit (16, 17) that, through its *src* homology 2 (SH2) domains, can associate with specific phosphotyrosine-containing domains (YMXM) in other proteins (18). This association is one of the mechanisms leading to functional activation of the enzyme; association of the p85-kD subunit with the platelet-derived growth factor (PDGF) receptor is essential for the PDGF-induced mitogenic signal (19). The 110-kD PI-3 kinase subunit has been shown to be phosphorylated and to associate with the polyoma middle T-activated p60^{src} in vitro (20), and the p85-kD regulatory subunit of PI-3 kinase is phosphorylated on tyrosine residues upon stimulation of various receptors (21, 22). Phosphorylation of either subunit, however, does not seem to be essential for the activation of the enzyme. PI-3 kinase catalyzes phosphorylation of the inositol ring of PI, PI-4-P, and PI-4,5-P₂ at the D-3 position, resulting in formation of PI-3-P, PI-3,4-P₂, and PI-3,4,5-P₃ (23). These lipids are not substrates for known phospholipases. PI-3,4,5-P₃ has been reported to activate protein kinase C (PKC) ζ isozyme (24), and a role for this product in the interaction with cytoskeleton-associated proteins leading to alteration of cellular structures has been proposed (25), whereas targets for the other lipids have not been identified. PI-4 and PI-4-P5 kinase activities associated with the tyrosine phosphorylated proteins precipitated from

¹ Abbreviations used in this paper: ARH, antigen receptor homology; [Ca²⁺]_i, intracellular Ca²⁺ concentration; GaMIg, goat anti-mouse Ig; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PKC, protein kinase C; PLC, phospholipase C; SH, *src* homology.

epidermal growth factor-stimulated cells have also been detected (26). One of their products, PI-4,5-P₂, is a substrate for hydrolysis by PLC- γ leading to the synthesis of second messengers in receptor-dependent signal transduction (27).

Except for p56^{lck} (15), PLC- γ 1 and PLC- γ 2 (4, 7), and the γ and ζ chains (1, 2, 28), the identity of other proteins, either tyrosine-phosphorylated or associated with phosphoproteins, and their possible role in signal transduction via Fc γ RIIIA in NK cells has not been established. In this manuscript, we report that Fc γ RIIIA-ligand interaction in human NK cells and in T cells expressing Fc γ RIIIA α chain in association with γ or ζ chain induces activation of PI-3 kinase via its association to a number of tyrosine phosphorylated proteins, in the absence of detectable phosphorylation on tyrosine residues of the p85-kD regulatory subunit of the kinase. Stimulation of Fc γ RIIIA-transfected cells with anti-CD16 mAb also results in accumulation of D3-phosphoinositides, confirming that PI-3 kinase activation occurs upon ligand binding to Fc γ RIIIA.

Materials and Methods

Cell Lines. The human B lymphoblastoid RPMI 8866 and the murine mAb producing cell lines were maintained in culture in RPMI 1640 (Flow Laboratories, Malvern, PA) supplemented with 10% heat-inactivated FCS (Sigma Chemical Co., St. Louis, MO). Fc γ RIIIA α -transfected CD3⁻/CD2⁻ J32.65.3.1 Jurkat T cells expressing Fc γ RIIIA in association with endogenous ζ chain, and Fc γ RIIIB-transfected CD3⁺/CD2⁺ J32.10 cells were produced in our laboratory (4); the Fc γ RIIIA α + Fc ϵ R1 γ -transfected CD3⁺/CD2⁺ Jurkat cells, expressing Fc γ RIIIA α in association with γ ₂ homodimers, were a kind gift of Dr. J. V. Ravetch (Memorial Sloan Kettering Cancer Institute, New York) (11). The transfected cells were maintained in culture in RPMI 1640 supplemented with 10% heat-inactivated protein G-adsorbed FBS and geneticin (G418; Gibco Laboratories, Grand Island, NY). All cell lines were free of mycoplasma contamination.

mAbs, Polyclonal Sera, and Reagents. Production and characterization of mAb 3G8 and B73.1 (anti-CD16, IgG₁), B36.1 (anti-CD5, IgG_{2b}), OKT3 (anti-CD3, IgG_{2a}), B159.5 (anti-CD56, IgG₁), and B52.1 (anti-CD14, IgM) have been previously reported (29). Anti-phosphotyrosine mAb 1G2 was obtained from hybrid cells kindly provided by Dr. R. Frackelton (Brown University, Providence, RI) through the American Type Culture Collection (Rockville, MD). The goat anti-mouse Ig (GaMIg) used to prepare sheep E for indirect antiglobulin rosetting and for cross-linking cell surface-bound mAbs was produced in our laboratory, adsorbed on human Ig, and affinity-purified on mouse IgG-Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, Sweden). The polyclonal rabbit sera anti-phosphotyrosine and anti-p85 subunit of PI-3 kinase were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA) and Upstate Biotechnology Inc. (Lake Placid, NY), respectively. PI was from Avanti Polar Lipids Inc. (Birmingham, AL); orthophenylphosphate and adenosine were from Sigma Chemical Co.

NK Cell Preparations. PBMC were separated on Ficoll/Hypaque density gradient (Lymphoprep; Nyegaard Co., Oslo, Norway) from venous blood of healthy individuals. The majority of monocytes were depleted after adherence to plastics (45 min, 37°C). Homogeneous NK cell preparations were obtained by negative selection from short term (10 d) co-cultures of PBL with 30-Gy-irradiated RPMI 8866 cells using a mixture of anti-CD14, anti-CD3, and

anti-CD5 mAb and indirect antiglobulin rosetting (29). The purity of these preparations was always >95%, as tested in indirect immunofluorescence with a panel of mAb and human Ig-adsorbed FITC-conjugated goat F(ab')₂ anti-mouse Ig (Cappel Laboratories, Cochranville, PA) using a Profile II flow cytofluorimeter (Coulter, Hialeah, FL).

Cell Stimulation. The different cell types were washed in serum-free RPMI and resuspended in the same medium (10⁷ cells/ml). The indicated mAbs (ascites, 10⁻³ dilution) were added, and the cells were incubated for various periods of time at 37°C in the absence or presence of GaMIg (10 μ g/ml).

Immunoprecipitation and Western Blotting. After the indicated treatments, the cells were solubilized with lysis buffer (10 mM Hepes, pH 7.5, 0.15 mM NaCl, 10% glycerol, 10 μ g/ml each leupeptin and aprotinin, 1 mM PMSF, 1 mM NaVO₄, and 1% NP-40). Postnuclear supernatants were precleared (2 h, 4°C) with preimmune rabbit serum coupled to Protein A-Sepharose (LKB Pharmacia). The indicated antibodies coupled to Protein A-Sepharose were used for immunoprecipitation (2 h, 4°C). The precipitates were washed twice with lysis buffer, and twice with 0.15 mM NaCl, 10 mM Hepes, and 0.2% NP-40. After elution in sample buffer, the proteins were separated on 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose filters (MSI, Westboro, MA). These were saturated with 0.5% gelatin in Tris buffered saline (TBS) and incubated (12–16 h, 4°C) with the indicated antibodies at predetermined optimal concentrations. After five washes in TBS containing 0.25% Tween-20 and 0.25% NP-40, antibody-reactive proteins were detected using either ¹²⁵I-Protein A (ICN Biomedicals, Inc.) and autoradiography, or horseradish peroxidase (HRP)-labeled sheep anti-rabbit serum and enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL) (4). When indicated, the filters were stripped (30 min, room temperature, 0.1 M glycine/HCl, pH 2.6) and reprobbed with the indicated antibody.

PI Kinase Assays. These were performed on the material immunoprecipitated, as above, from the indicated cells using affinity-purified 1G2 anti-phosphotyrosine mAb coupled to Protein A-Sepharose via rabbit anti-mouse Ig. The immunoprecipitates were washed twice with lysis buffer, once with 20 mM Hepes, 0.5 M LiCl, and twice each with 0.15 M NaCl, 10 mM Hepes with and without 0.2% NP-40. Tyrosine phosphorylated proteins were eluted from the beads using 40 mM orthophenylphosphate in 30 mM Hepes, pH 7.5. PI kinase activity in the eluate (30 μ l) was assayed using 0.2 mg/ml PI in the presence of 40 μ M ATP, 10 μ Ci γ -[³²P]ATP (4,500 Ci/mmol sp. act; ICN), with and without added adenosine, 200 μ M, or NP-40, 0.5%, for 15 min at room temperature. The reaction was stopped with 100 μ l 1 N HCl, and the lipids were extracted with 200 μ l chloroform/methanol (1:1, vol/vol) mixture. After washing once with methanol/1 N HCl (1:1, vol/vol), lipids in the organic phase were separated by TLC on silica gel G 60 sheets (J. T. Baker Inc., Phillipsburg, NJ). The TLC was developed in chloroform/methanol/H₂O/NH₄OH (43:38:7:5, vol/vol). Radioactive PIP products were visualized by autoradiography.

Detection of Polyphosphoinositides Accumulation in Intact Cells. The indicated cells were washed in phosphate- or inositol-free RPMI 1640, as appropriate, supplemented with 1% dialyzed FBS and incubated (10⁷ cells/ml, 3 h, or 2 \times 10⁶ cells, 20 h, 37°C) in the same medium in the presence of 200 μ Ci/ml [³²P]H₃PO₄ (sp. act 285 Ci/mg P; ICN Biomedicals, Inc.) or 20 μ Ci/ml [³H]myo-inositol (sp. act 18.7 Ci/mmol; Amersham Corp.), respectively. After washing, the cells were stimulated with the indicated mAbs as described above; the lipids were extracted with 1 N HCl/chloroform/methanol (1:2:1, vol/vol), and D-3 polyphosphoinositides were

separated by anion exchange chromatography using HPLC, as described (23).

Results

Activation of PI Kinase upon FcγRIII Stimulation. PI kinase was measured in the orthophenylphosphate eluate of anti-phosphotyrosine (1G2 mAb) immunoprecipitates from post-nuclear lysates of NK cells and of FcγRIII-transfected Jurkat T cells, treated or not with anti-CD16 mAb. Minimal kinase activity was detected in the immunoprecipitates from lysates of NK cells and of three different types of transfectants (FcγRIIIAα/γ₂, FcγRIIIAα/ξ₂, and FcγRIIIB) in the absence of stimulation or after treatment with control anti-CD56 mAb (Fig. 1). Significant kinase activity was instead detected in those from NK cells and from both types of FcγRIIIA-, but not FcγRIIIB-transfected Jurkat T cells treated with mAbs directed to two different FcγRIII epitopes, 3G8 (Fig. 1), and B73.1 (not shown), and GaMIg for 5 min. On the basis of densitometric measurements, the activity detected upon CD16 stimulation in NK cells and in FcγRIIIAα/γ₂ transfectants was, on average, fourfold that in control antibody-treated cells, and approximately ninefold lower than that detected in FcγRIIIAα/ξ₂ transfectants. Phosphotyrosine precipitates from anti-CD3 mAb-treated CD3⁺ Jurkat cells transfected with FcγRIIIAα/γ₂ (Fig. 1), but not those from similarly treated NK cells or CD3⁻ Jurkat cells expressing FcγRIIIAα/ξ₂ (not shown), contained kinase activity: this was approximately threefold greater than that detected upon CD16 stimulation in the same cells, as determined by densitometry.

PIP production was detectable in the anti-phosphotyrosine immunoprecipitates from NK cells treated with anti-CD16 mAb for as short as 10 s (not shown), was maximal at 1 and 5 min, and declined afterwards; lower PIP amounts were detected, with similar kinetics, when anti-CD16 mAb was

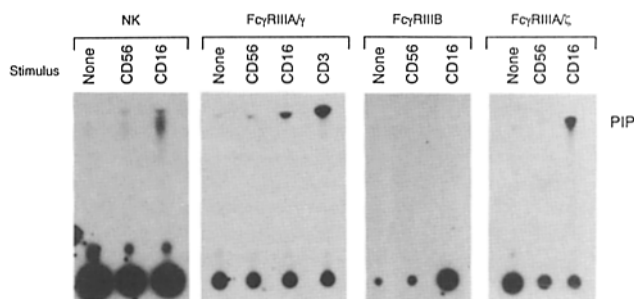


Figure 1. PI kinase activity induced upon FcγRIII stimulation. Homogeneous preparations of NK cells, CD3⁺ Jurkat cells expressing transfected FcγRIIIAα in association with γ₂, or FcγRIIIB, and CD3⁻ Jurkat cells expressing FcγRIIIAα in association with endogenous ξ₂, were incubated (10⁷ cells/ml RPMI 1640, 5 min, 37°C) in the absence (none) or presence of B159.5 (CD56), 3G8 (CD16), OKT3 (CD3) mAb (IgG, 2 μg/ml) as indicated, and GaMIg (10 μg/ml). Tyrosine phosphorylated proteins were immunoprecipitated using 1G2 mAbs. PI kinase assays were performed on the orthophenylphosphate eluate of the immunoprecipitates and analyzed in TLC as described in Materials and Methods. The position of the PIP product is indicated.

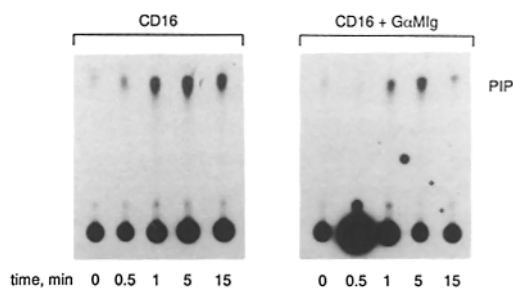


Figure 2. Kinetics of FcγRIII-induced PI-3 kinase activation in NK cells. Homogeneous preparations of NK cells were incubated, as in Fig. 1, with 3G8 mAb, with or without GaMIg added, for the indicated times. PI-3 kinase assays were performed in the presence of adenosine on the orthophenylphosphate eluate of 1G2 immunoprecipitates from the respective lysates, under the conditions described in Fig. 1.

cross-linked with GaMIg (Fig. 2). Similar results were obtained using FcγRIIIAα/ξ₂ transfectants (not shown).

Identification of PI-3 Kinase in the Anti-phosphotyrosine Immunoprecipitates upon FcγRIIIA Stimulation. PI-3 and PI-4 kinases differ in sensitivity to detergent and adenosine. Detergents, e.g., NP-40, inhibit PI-3 and stimulate PI-4 kinase, whereas adenosine inhibits only the latter (30). The products of the PI kinase formed, in the presence or absence of adenosine, by anti-phosphotyrosine immunoprecipitate from NK cells treated with anti-CD16 mAb (Fig. 3A) had similar TLC mobility and were produced in comparable amounts, as evaluated by densitometric measurements of the film. No kinase activity was present, under either condition, in the samples from nonstimulated or control mAb-treated cells. Upon addition of 0.5% NP-40 to the kinase assay mixture, the single PIP product formed in the presence or absence of adenosine was no longer detectable; instead, similar levels of a phosphorylated product that, on TLC, ran ahead of that produced in the presence of adenosine were detected in

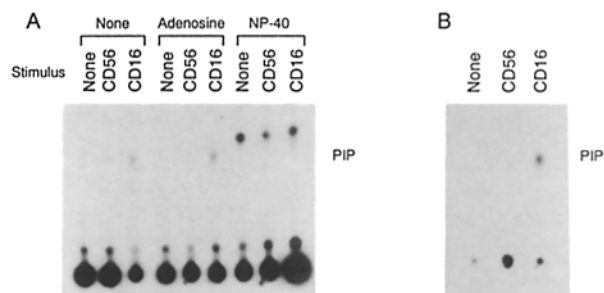


Figure 3. Identification of PI-3 kinase associated with tyrosine phosphorylated proteins upon FcγRIII stimulation. Lysates from NK cells, incubated with the indicated mAb, were subjected to anti-phosphotyrosine immunoprecipitation, orthophenylphosphate elution, and PI kinase assays, under the same conditions described in Fig. 1. (A) PI kinase assays were performed on identical aliquots of each sample in the absence (none) or presence of adenosine, 200 μM, or NP-40, 0.5%. (B) p85-kD PI-3 kinase subunit was immunoprecipitated from the orthophenylphosphate eluates of the 1G2 immunoprecipitates from NK cells treated as indicated. PI kinase assay was performed on the immunoprecipitates in the presence of adenosine, 200 μM.

all samples, irrespective of stimulation. Analogous results were obtained using the Fc γ RIII α / ζ_2 transfectants (not shown).

To confirm the presence of PI-3 kinase in the phosphotyrosine immunoprecipitates, the orthophenylphosphate eluates derived from the phosphotyrosine immunoprecipitates from control or CD16-treated NK cells were reimunoprecipitated with anti-p85 antiserum and PI kinase assays were performed on the products of the second precipitation. PI-3 kinase activity was detected only in the precipitates from the stimulated cells (Fig. 3 B).

Polyphosphoinositides Accumulation in CD16-stimulated Cells. To determine whether Fc γ RIII α -induced PI-3 kinase activation correlates with accumulation of D-3 lipids in intact cells, the levels of these products were analyzed in cells metabolically labeled with [32 P]H $_3$ PO $_4$ or [3 H]inositol and treated with anti-CD16 and control anti-CD56 mAb in the presence of GaMlg for 5 min (Fig. 4). In the Fc γ RIII α / ζ_2

transfectants (Fig. 4, top panels) significant levels of PI-3, 4-P $_2$ (panel B) and even higher levels of PI-3,4,5-P $_3$ (panel C) accumulated in the CD16-stimulated cells, as compared with nontreated or control mAb-treated cells. In the CD3 $^+$ Fc γ RIII α / γ_2 transfectants both CD16 and CD3 stimulation (Fig. 4, bottom and middle panels, respectively) induced levels of PI-3,4,5-P $_3$ low, but significantly higher than controls. In repeated experiments, we were unable to detect significant levels of D-3 phosphoinositides in NK cells upon treatment with anti-CD16 mAb in the presence or absence of GaMlg over a 15-min period (data not shown).

Tyrosine Phosphorylated Proteins Associated with PI-3 Kinase. Detection of PI-3 kinase activity in the phosphotyrosine precipitates from Fc γ RIII-stimulated cells may depend on its induced association with tyrosine-phosphorylated proteins and/or on phosphorylation on tyrosine residues of either or both subunits of the kinase. To distinguish between these possibilities, Western blot analysis was performed with anti-

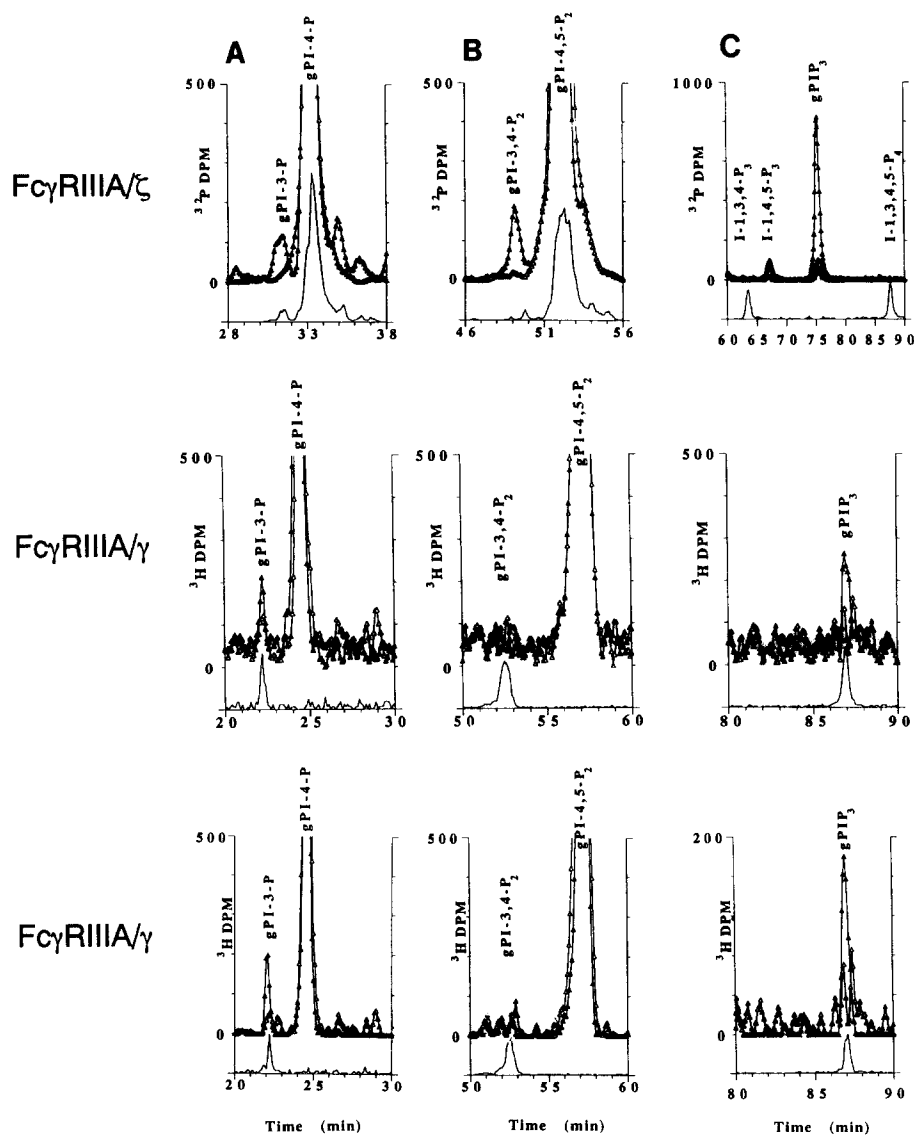


Figure 4. Fc γ RIII α -induced accumulation of polyphosphoinositides in Fc γ RIII α -transfected Jurkat cells. Fc γ RIII α / ζ_2 (top panels) and Fc γ RIII α / γ_2 (middle and bottom panels) Jurkat transfectants were incubated at 37°C with [32 P]H $_3$ PO $_4$ or [3 H]myo-inositol in phosphate- or inositol-free RPMI 1640, 1% dialyzed FBS for 3 and 20 h, respectively. The metabolically labeled cells were washed and incubated (5 min, 37°C) with: \blacktriangle , 3G8 (CD16) (top and middle panels) or OKT3 (CD3) (bottom panels), or \triangle , B159.5 (CD56) (ascites, 10 $^{-3}$ final dilution) in the presence of GaMlg (10 μ g/ml). After stimulation, the lipids were extracted and analyzed by anion exchange chromatography on HPLC, as described in Materials and Methods; x-axis, elution time, y-axis, dpm in the eluted fractions. The position of 3 H-labeled PI-4-P and PI-4,5-P $_3$ standards run on parallel columns (---) is indicated underneath each panel.

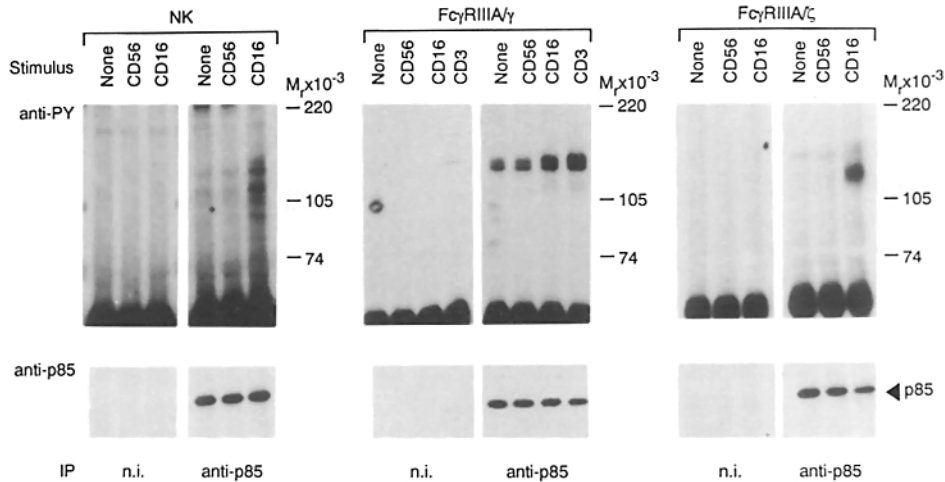


Figure 5. Tyrosine phosphorylated proteins associated with PI-3 kinase upon Fc γ RIIIA stimulation. Homogeneous preparations of NK cells, Fc γ RIIIA α / γ $_2$, and Fc γ RIIIA α / ζ $_2$ transfected Jurkat cells were incubated with the indicated antibodies and GaMlg as described in Fig. 1. Anti-p85 rabbit serum, or nonimmune (*n.i.*) serum as control, were used for immunoprecipitation (*IP*). The precipitated material was subjected to 7.5% SDS-PAGE followed by Western blotting with anti-phosphotyrosine (anti-PY, *top panels*) and anti-p85 PI-3-kinase subunit rabbit serum (*bottom panels*) used sequentially on the same blots. Reactive proteins were detected with 125 I-protein A and autoradiography. The position of molecular weight standards is indicated.

phosphotyrosine antibody on the PI-3 kinase precipitated, using an anti-85-kD subunit antibody, from the postnuclear lysates of nonstimulated, CD16-, or control CD56-treated NK cells (Fig. 5). PI-3 kinase, detected with an anti-85-kD subunit antibody after stripping and reblotting the same filter, was present at similar levels in all lysates. No tyrosine phosphorylated proteins, nor PI-3 kinase p85-kD subunits, were detected in the precipitates obtained using control nonimmune sera. Minimal traces of tyrosine-phosphorylated proteins were detected in the precipitates from untreated and control anti-CD56-treated cells, whereas four major tyrosine phosphorylated proteins of 140–135, 110, 95, and 85 kD were detected in the PI-3 kinase precipitates from anti-CD16 + GaMlg-treated NK cells. In Fc γ RIIIA α / ζ $_2$ expressing Jurkat cells the anti-phosphotyrosine antibody detected two proteins of 135 and 100 kD associated with the PI-3 kinase p85 subunit only in immunoprecipitates from cells stimulated with anti-CD16 mAb. In Fc γ RIIIA α / γ $_2$ transfectants, a tyrosine phosphorylated doublet of M_r 135–142 was precipitated also from the control cells; this, as well as an additional band at 110 kD, appeared hyperphosphorylated upon stimulation with either anti-Fc γ RIII or anti-CD3 antibody. No tyrosine phosphorylated proteins of 85 kD were detected in either cell line. The level of phosphorylation of the p85 regulatory subunit of PI-3 kinase, analyzed in p85 immunoprecipitates from 32 P-labeled NK and Fc γ RIIIA/ ζ $_2$ -transfected cells, was similar, irrespective of cell stimulation (data not shown).

Discussion

After ligand binding to Fc γ RIIIA, tyrosine phosphorylation of several proteins is induced in NK cells and in Fc γ RIII-transfected T cell lines (1, 2). For some of these proteins, namely the tyrosine kinase p56 lck (4, 14) and PLC- γ 1 and - γ 2 (4, 7, 15), this results in activation of their enzymatic activity. In this manuscript, we present evidence that activa-

tion of another enzyme, PI-3 kinase, is also induced upon Fc γ RIII stimulation. Its activation does not correlate with phosphorylation on tyrosine residues of its p85-kD regulatory subunit, but correlates with its induced association to a set of other tyrosine phosphorylated proteins.

PI-kinase activity is present in the phosphotyrosine immunoprecipitates from NK cells, and from Fc γ RIIIA α -transfected Jurkat T cells, only after their stimulation with anti-Fc γ RIII-specific mAb, indicating that Fc γ RIII-dependent tyrosine phosphorylation affects the activity of the kinase, inducing either its phosphorylation or its association with other proteins that are tyrosine phosphorylated upon receptor stimulation. The identity of the enzyme responsible for this activity as PI-3 kinase was established on the basis of two distinct criteria: (*a*) characteristic inhibition by detergents (NP-40) and resistance to adenosine (30), and (*b*) coprecipitation of the activity with PI-3 kinase p85 subunit from the phosphotyrosine immunoprecipitates. A PIP product with TLC mobility faster than that detected in the presence of adenosine was produced when the kinase assays were performed in the presence of NP-40, suggesting the expected activation of PI-4 kinase by the detergent. Although PI-3-P migrates more slowly than the other spot in the TLC system we used, unambiguous identification of these two lipids by TLC is not possible, and the presence of minimal levels of PI-4-P in the spots detected when PI kinase assays were performed in the absence of adenosine can not be excluded. However, the observation that the slower migrating spot completely disappears when NP-40 is added to the assay confirms its identification as PI-3-P. The data we obtained in T cells upon CD3 stimulation are identical to those obtained with NK cells upon CD16 stimulation, supporting reports of PI-3 kinase activation upon CD3 stimulation (31).

The kinetics of induction of PI-3 kinase activity is fast, and similar whether or not Fc γ R is cross-linked at the cell membrane, although differences in the levels of the PIP products were present in the two conditions. In other systems

(32, 33) PI-3 kinase has been reported to translocate to the cytoskeleton upon cell stimulation, and the possibility that, after cross-linking (and maximal stimulation), at least part of the PI-3 kinase is trapped within the detergent-insoluble fraction of the lysate may explain these results.

Precipitation of PI-3 kinase from stimulated Fc γ RIII, but not control cells, results in coprecipitation of tyrosine phosphorylated proteins, that appear to be at least in part different in NK and in the T cell transfectants. The identity of these proteins is unknown at present, and their differences are likely to be related to the cell types analyzed. Two of the proteins detected in NK cells have relative molecular mass corresponding to that of the PI-3 kinase 85- and 110-kD subunits. The p85-kD regulatory subunit of PI-3 kinase is a known substrate for receptor and nonreceptor protein tyrosine kinases (20, 34–37). The absence of 85-kD proteins phosphorylated on tyrosine residues in the PI-3 kinase precipitates from both Fc γ RIII α / γ ₂ and Fc γ RIII α / ζ ₂ expressing T cells, and the observation that this subunit, precipitated from [³²P]-labeled, nonstimulated or stimulated NK cells, has similar levels of phosphorylation argue against the possibility that phosphorylation of the regulatory subunit of PI-3 kinase occurs upon Fc γ RIII cross-linking. Thus, activation of the kinase upon Fc γ RIII stimulation does not correlate with its tyrosine phosphorylation and, as proposed in other receptor systems, is likely to occur upon its association with phosphotyrosine residues on other proteins (38). PI-3 kinase activation is indeed observed in vitro upon binding of phosphopeptides to the SH2 domains of the 85-kD subunit (39, 40). This, in turn, may induce structural modifications that are transmitted to the associated 110-kD subunit and may regulate PI-3 kinase enzymatic activity via an allosteric mechanism (18, 39).

PI-3 kinase activation occurred independently of the type of accessory chain (γ or ζ) associated with Fc γ RIII. However, quantitative differences were observed in the levels of in vitro kinase activity between the Fc γ RIII α / ζ ₂ and the NK cells and Fc γ RIII α / γ ₂ transfectants. The possibility that the two cell lines used for transfection (the parental CD3⁺ and its CD3⁻ mutant) differ in essential aspects of signal transduction is unlikely, because both express similar levels of surface Fc γ RIII, which mediate the same early activation events upon stimulation (4, 11, and data not shown). Moreover, stimulation of CD3, associated with the endogenous ζ chain in the Fc γ RIII/ γ ₂ transfectants, results in levels of PI-3 kinase activity in vitro similar to those detected upon CD16 stimulation in the Fc γ RIII α / ζ ₂ transfectants. A more likely explanation takes into consideration that both γ and ζ chain contain conserved sequences (antigen receptor homology 1 [ARH1] motifs), common to other receptor-associated signal-transducing molecules in several lymphocyte receptor systems, that play a role in coupling the receptors with other molecules essential in the signal transduction pathways (41, 42). Three ARH1 motifs are present in ζ , whereas only one is present in the γ chain. Such motifs are sites of interaction with tyrosine kinases and, possibly, with

other SH2 domains-containing proteins. No formal proof exists of a direct correlation with, or dependence on, these motifs for the γ and ζ chains association with tyrosine kinases. However, this possibility is suggested by the observation that coprecipitation of p56^{lck} with truncated ζ chain expressing only one or two ARH1 motifs is decreased (15). The type of the receptor-associated chain, as in the case of the two transfectants analyzed, may dictate quantitative differences in phosphorylation of proteins that associate physically and/or functionally with PI-3 kinase after receptor stimulation.

In several receptor systems, induction of PI-3 kinase activity in the tyrosine phosphorylated proteins pool in vitro correlates with activation of the enzyme in the intact cells and subsequent accumulation of D-3 phosphoinositides (23, 35). In agreement with this, significant levels of PI-3-P₁, PI-3,4-P₂, and high levels of PI-3,4,5-P₃, were detected in Fc γ RIII α / ζ ₂ transfectants treated with anti-CD16, but not control anti-CD56 antibodies. Low, but detectable levels of PI-3,4,5-P₃ were accumulated in Fc γ RIII α / γ ₂-expressing T cells, which were similar to those detected after CD3 stimulation. The observation that the levels of PI-3,4,5-P₃ induced via CD3 (associated with the endogenous ζ chain) are lower than those induced via CD16 in the Fc γ RIII α / ζ ₂ transfectants is compatible with data in primary T cells, indicating that activation of the TCR-CD3 complex and of CD2 antigen results in small increases of PI-3,4-P₂ and PI-3,4,5-P₃ (31), and suggests that differences in the molecular complexes formed by the Fc γ RIII α and TCR-CD3 with the ζ chain may result in quantitative differences in the signal transduction events. The different levels of D3-lipids detected in the γ and ζ transfectant T cells may depend on the mechanisms discussed above for the in vitro detected activation of PI-3 kinase. In NK cells, where we could not detect significant increase in D-3 lipids at any time point tested after Fc γ RIII α stimulation, a lower efficiency of the Fc γ RIII α / γ ₂ molecular complexes, that constitute the majority of the receptors (28), to activate the kinase may be responsible for the difficulty in detecting D-3 products, analogous to the situation with the Fc γ RIII α / γ ₂ transfectants. Alternatively, and in addition, phosphatases may be activated in NK cells upon Fc γ R stimulation, that quickly dephosphorylate the lipids or inactivate the kinase by reducing its association with tyrosine phosphorylated proteins. The significance of a dissociation between induced PI-3 kinase activity detectable in vitro and detection of its products in intact cells is unknown, but examples of it exist: e.g., epidermal growth factor stimulation results in accumulation of the PI-3 kinase products in NA1 Leydig tumor cells (43), but not in A431 cells (26, 44), whereas in both cell types PI-3 kinase activation is detected in vitro upon receptor stimulation.

Although a correlation between kinase activation and specific functional effects can not be established at present, the Fc γ RIII α -induced association of PI-3 kinase with tyrosine phosphorylated proteins and its consequent activation point to a role for this kinase in Fc γ RIII-mediated signal transduction.

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