

## **Stimulation of Fc $\gamma$ RIII $\alpha$ Results in Phospholipase C- $\gamma$ 1 Tyrosine Phosphorylation and p56<sup>lck</sup> Activation**

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### **Summary**

Binding of ligand to the  $\alpha$  subunit of Fc $\gamma$ RIII $\alpha$ (CD16), expressed at the natural killer (NK) cell membrane in association with homo or heterodimers of proteins of the  $\zeta$  family, results in phosphorylation of several proteins on tyrosine residues. We have analyzed the role of protein tyrosine phosphorylation in the regulation of molecular events induced upon stimulation of Fc $\gamma$ RIII $\alpha$  in NK cells and in T cells expressing the Fc $\gamma$ RIII $\alpha$  chain in association with endogenous  $\zeta_2$  homodimers and devoid of other (CD3, CD2) transducing molecules. Our data indicate that treatment of these cells with protein tyrosine kinase inhibitors prevents not only Fc $\gamma$ RIII $\alpha$ -induced protein tyrosine phosphorylation but also phosphatidylinositol 4,5 diphosphate hydrolysis and increased intracellular Ca<sup>2+</sup> concentration, indicating a primary role of tyrosine kinase(s) in the induction of these early activation events. Occupancy of Fc $\gamma$ RIII $\alpha$  by ligand results in phospholipase C (PLC)- $\gamma$ 1 tyrosine phosphorylation in NK cells and in Fc $\gamma$ RIII $\alpha$ -transfected CD3<sup>-</sup>/CD2<sup>-</sup> T cells, and induces functional activation of p56<sup>lck</sup> in Fc $\gamma$ RIII $\alpha$ / $\zeta_2$ -transfected T cells, suggesting the possibility that the receptor-induced PLC- $\gamma$ 1 activation occurs upon phosphorylation of its tyrosine residues mediated by this kinase and is, at least in part, responsible for the signal transduction mediated via CD16 upon ligand binding.

The low affinity receptor for the Fc fragment of IgG, Fc $\gamma$ RIII $\alpha$ (CD16), is an oligomeric complex composed of one Fc binding ( $\alpha$ ) chain associated with homo or heterodimers of the  $\gamma$  and/or  $\zeta$  chain, originally identified as components of the multimeric high affinity IgE receptor (Fc $\epsilon$ R1) and of the TCR/CD3 complex, respectively. These chains have a primary role to prevent degradation of Fc $\gamma$ RIII $\alpha$   $\alpha$  in the endoplasmic reticulum, thus allowing its expression at the cell membrane, and are responsible for the receptor-mediated signal transduction (for review see reference 1). Most Fc $\gamma$ RIII $\alpha$  on NK cells are associated with  $\gamma_2$ , and only a minority of them with  $\zeta_2$  homodimers (2).

Fc $\gamma$ RIII $\alpha$ / $\gamma_2$  complexes transduce both early and late activation events similar to those observed in NK cells (3). In these cells, Fc $\gamma$ RIII $\alpha$  stimulation with immune complexes or specific antibodies results in protein tyrosine phosphorylation (4–6), phosphatidylinositol 4,5 diphosphate (PIP<sub>2</sub>) hydrolysis and increased intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) (7) that depends upon both release of Ca<sup>2+</sup> from the intracellular stores and extracellular Ca<sup>2+</sup> internalization, necessary for transcriptional activation of cytokine genes (7, 8). PIP<sub>2</sub> hydrolysis, primarily a consequence of activation of specific phospholipase(s), results in IP<sub>3</sub> release and consequent [Ca<sup>2+</sup>]<sub>i</sub> increase observed upon receptor occupancy. Protein

tyrosine phosphorylation is an early event in Fc $\gamma$ RIII $\alpha$ -dependent signal transduction, and its inhibition abolishes the Fc $\gamma$ RIII $\alpha$ -induced rise in [Ca<sup>2+</sup>]<sub>i</sub> and more distal effects in NK cells (6).

In this study, we analyzed the early activation events mediated by Fc $\gamma$ RIII $\alpha$  in NK cells and in CD3<sup>-</sup>/CD2<sup>-</sup> cells expressing this receptor in association with  $\zeta_2$  homodimers. Our data using Fc $\gamma$ RIII $\alpha$ -transfected CD3<sup>-</sup>/CD2<sup>-</sup>/ $\gamma$ -Jurkat T cells indicate that Fc $\gamma$ RIII $\alpha$ / $\zeta_2$  complexes transduce signals resulting in the same early biochemical events transduced by Fc $\gamma$ RIII $\alpha$ / $\gamma_2$  or observed in NK cells upon Fc $\gamma$ RIII $\alpha$  stimulation, and that Fc $\gamma$ RIII $\alpha$  stimulation induces tyrosine phosphorylation of phospholipase C (PLC- $\gamma$ 1) in both cell types and increased p56<sup>lck</sup> activity in Fc $\gamma$ RIII $\alpha$ / $\zeta_2$ -expressing cells, suggesting a causal relationship between these events and the production of second messenger molecules.

### **Materials and Methods**

*Fc $\gamma$ RIII Expression Vectors and DNA Transfection.* The human T cell leukemia-derived Jurkat cell line clone J32.10 and its CD3<sup>-</sup>/CD2<sup>-</sup> mutant J32-65.3.1 have been described (9). They were maintained in culture in RPMI-1640 medium (Flow Laboratories Inc., Malvern, VA) supplemented with 10% fetal bovine serum

(FBS) (ICN Biomedicals, Inc., Costa Mesa, CA). Fc $\gamma$ RIII $\alpha$  cDNA, obtained as described (10), was cloned in blunt-ended pGSE1731 mammalian expression vector consisting of the human  $\beta$ -globin gene promoter inserted 1.5-kb upstream of the Amp site along with the internal and 3'  $\beta$ -globin gene enhancer elements, and of the BamHI or XbaI CD2 3'-flanking fragment inserted 3' of the  $\beta$ -globin gene (11). NotI linearized plasmid containing Fc $\gamma$ RIII $\alpha$  sense cDNA was cotransfected by electroporation, together with the pFNeo geneticin resistance gene, in J32-65.3.1 cells (9). After culture in the presence of increasing concentrations of G418 (geneticin; Gibco Laboratories, Grand Island, NY), Fc $\gamma$ RIII $\alpha$ <sup>+</sup> cells were selected with anti-CD16 mAb and goat anti-mouse Ig (GaMIg)-coated magnetic beads (DynaL Inc., Great Neck, NY), and cloned by limiting dilution. Fc $\gamma$ RIII $\alpha$ <sup>+</sup> clones, identified by indirect antiglobulin rosetting of anti-CD16 antibody-sensitized cells (12), were maintained in culture in RPMI-1640 supplemented with 10% protein G (Pharmacia Fine Chemicals, Uppsala, Sweden)-adsorbed FBS and 0.4 mg/ml G418.

**NK Cell Preparations.** Buffy coats from healthy donors were obtained from the American Red Cross (Philadelphia, PA). PBMC were collected at the interface of Ficoll-Hypaque density gradient (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). PBL were obtained after adherence to plastics. Homogeneous NK cell preparations were prepared from 10-d cocultures of PBL with RPMI-8866 B lymphoblastoid cells as described (12).

**mAb, Polyclonal Sera, and Reagents.** The mAb used have been previously characterized: anti-CD16: 3G8, B73.1; anti-CD3: OKT3; and anti-CD56: B159.5 (12). IgG were prepared using protein A-Sepharose (Pharmacia Fine Chemicals). The human Ig-adsorbed, mouse Ig-affinity purified GaMIg was produced in our laboratory. Rabbit anti- $\zeta$  serum was kindly provided by Dr. J. V. Ravetch (Memorial Sloan-Kettering Cancer Institute, New York, NY). Rabbit antiphosphotyrosine antiserum was from ICN Biomedicals, Inc. Anti-PLC- $\gamma$ 1 rabbit antiserum and affinity-purified IgG were from Upstate Biotechnology Inc. (Lake Placid, NY). An anti-p56<sup>lck</sup> antiserum was raised in rabbits immunized with a synthetic peptide corresponding to amino acids 39-64 of the murine p56<sup>lck</sup> protein sequence.

**[Ca<sup>2+</sup>]<sub>i</sub> Measurements.** Anti-CD3 (control) and anti-CD16 mAb (5  $\mu$ g/ml); GaMIg (50  $\mu$ g/ml), and ionomycin (0.5  $\mu$ g/ml) were added, as indicated, in the presence or absence of 1 mM EGTA, to cells loaded with Fura-2/AM (Molecular Probes, Inc., Eugene, OR) as described (7). [Ca<sup>2+</sup>]<sub>i</sub> variations were calculated (7) as a function of time using an LS50 spectrofluorometer and FLDM software (Perkin-Elmer Cetus Corp., Norwalk, CT). Experiments were also performed on cells cultured (5  $\times$  10<sup>6</sup>/ml, 16 h) with or without 5  $\mu$ M herbimycin A (Gibco BRL Life Technology Inc., Gaithersburg, MD) or equal concentrations of diluent (DMSO) control. After culture, the cells were >90% viable and surface levels of Fc $\gamma$ RIII and other markers were identical to those on control nontreated cells, as detected by immunofluorescence.

**Phosphatidylinositol Hydrolysis.** Duplicate (10<sup>7</sup> cells) samples of cells metabolically labeled (16 h, 37°C) with 10  $\mu$ Ci/ml <sup>3</sup>H-myoinositol (sp act 18.7 Ci/mMol; Amersham Corp., Arlington Heights, IL) in the presence or absence of 5  $\mu$ M herbimycin A were incubated for 3 min with or without the indicated antibodies (ascites, 10<sup>-3</sup> dilution in HBSS supplemented with 1 mM CaCl<sub>2</sub>, 1 g/liter glucose, and 10 mM LiCl). GaMIg (50  $\mu$ g/ml) was then added to all samples. Stimulation was stopped with TCA (10% final concentration) after 45 s incubation. Hydrosoluble lipids were extracted and fractionated on AG1-X8 ion-exchange resin (Bio-Rad Laboratories, Richmond, VA) columns using a stepwise gradient of increasing concentrations of ammo-

nium formate as described (7). [<sup>3</sup>H]-IP3 and -IP4 (Amersham Corp.) were run in parallel on separate columns as standards.

**Immunoprecipitation and Western Blotting.** When indicated, cells were metabolically labeled with <sup>32</sup>P-H<sub>3</sub>PO<sub>4</sub> (sp act 285 Ci/mg; ICN Biomedicals, Inc.). Briefly, cells were prewashed with phosphate-free RPMI-1640 medium (ICN Biomedicals, Inc.) supplemented with 2% dialyzed FBS, and incubated (10<sup>7</sup>/ml, 3 h, 37°C) in the same medium with 200  $\mu$ Ci/ml [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub>. Labeled and unlabeled cells (10<sup>7</sup>/ml RPMI-1640) were treated (10 min on ice) with or without the indicated mAb (ascites, 10<sup>-3</sup> dilution), washed, resuspended in ice-cold medium containing 10  $\mu$ g/ml GaMIg, and incubated for the indicated times at 37°C. Cells were lysed with 1% NP-40 in 10 mM Hepes, 0.15 M NaCl containing 10% glycerol, 2.5  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM PMSF, 1 mM NaVO<sub>4</sub>, and 5 mM EDTA. Postnuclear supernatants were precleared (30 min, 4°C) with nonimmune rabbit serum preadsorbed on protein A-Sepharose beads (Pharmacia Fine Chemicals) and precipitated (2 h, 4°C) with the indicated rabbit antiserum, or normal rabbit serum as control, preadsorbed on protein A-Sepharose (Pharmacia Fine Chemicals). The precipitated proteins were washed with lysis buffer, eluted from the beads, and electrophoresed in SDS-11% or -7.5% polyacrylamide gels under reducing conditions. Western blot analysis was performed with the indicated antibodies after transfer of the proteins to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, NH). Either <sup>125</sup>I-protein A (0.5  $\mu$ Ci/ml, sp act 30  $\mu$ Ci/ $\mu$ g; ICN Biomedicals, Inc.) or horseradish peroxidase (HRP)-labeled donkey anti-rabbit serum (Amersham Corp.) were used for detection by autoradiography or enhanced chemiluminescence (ECL; Amersham Corp.), respectively.

**In Vitro Kinase Assay.** Cells were stimulated and lysed, and p56<sup>lck</sup> was immunoprecipitated as described above. The immunoprecipitates were washed five times in 10 mM Hepes, 0.15 M NaCl containing 0.2% NP-40, 10% glycerol, 400  $\mu$ M NaVO<sub>4</sub>, 5 mM MnCl<sub>2</sub>, and 10 mM MgCl<sub>2</sub>. The kinase reaction (13) was performed (15 min, 20°C) in 30  $\mu$ l of the same buffer in the presence of 1  $\mu$ M ATP and 10  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]ATP (sp act 4,500 Ci/mMol; ICN Biomedicals, Inc.). The proteins were then eluted (1% SDS, 30 min, 65°C), electrophoresed in SDS-PAGE, and detected as above.

## Results and Discussion

**Fc $\gamma$ RIII $\alpha$  Expression in Transfected Jurkat T Cells.** Flow cytometry analysis of the Fc $\gamma$ RIII $\alpha$ -transfected J32-65.3.1 CD3<sup>-</sup>/CD2<sup>-</sup> Jurkat T cells indicated that the receptors expressed on the cells were resistant to PI-PLC (not shown). Expression of the transfected Fc $\gamma$ RIII $\alpha$  in these CD3<sup>-</sup> cells in the absence of other cotransfected chains occurred in association with endogenous  $\zeta$  chain, as detected in the anti-CD16 immunoprecipitates from digitonin lysates of the cells by Western blot analysis with anti- $\zeta$  antiserum (data not shown), thus reproducing the natural situation of a minor proportion of Fc $\gamma$ RIII $\alpha$  in NK cells.  $\gamma$  mRNA transcripts and protein were not detected in these cells by Northern blot analysis (data not shown), thus excluding the possibility that  $\gamma$ <sub>2</sub> or  $\gamma$ / $\zeta$  dimers were associated with Fc $\gamma$ RIII $\alpha$ .

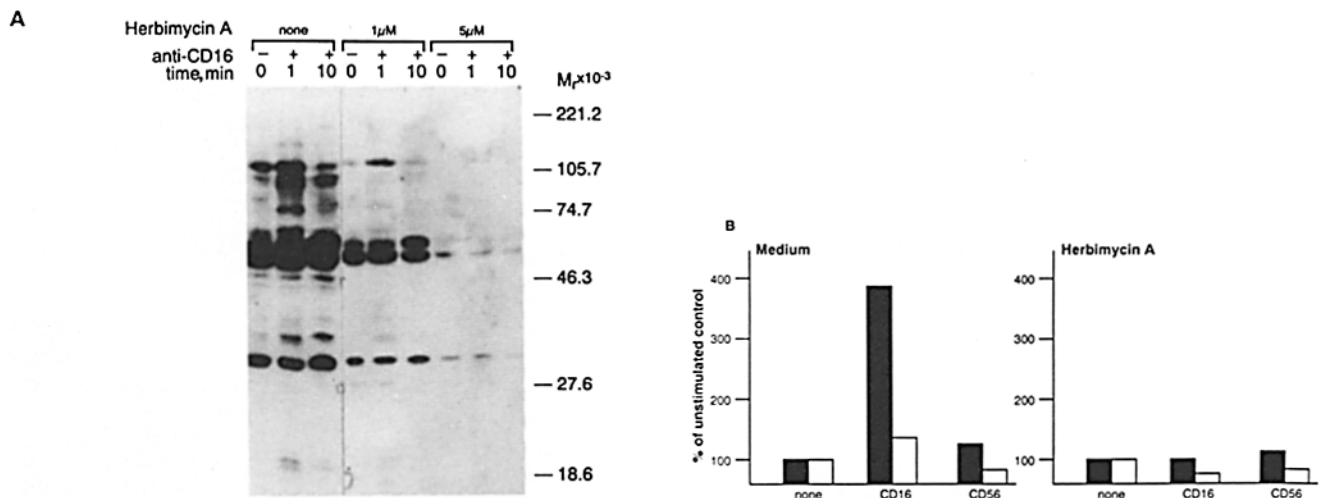
**Induction of Protein Tyrosine Phosphorylation upon Fc $\gamma$ RIII $\alpha$ / $\zeta$  Stimulation.** Western blot analysis with antiphosphotyrosine antiserum was performed on postnuclear supernatants from lysates of Fc $\gamma$ RIII $\alpha$ -transfected J32-65.3.1 cells after treatment with anti-CD16 or with control mAb. Upon stimulation of the receptor, several proteins were specifically

phosphorylated on tyrosine residues (Fig. 1 *A*), one of which was identified as the  $\zeta$  chain by immunoprecipitation (not shown). These data indicate that: (a) Fc $\gamma$ RIII $\alpha$ (CD16) stimulation induces tyrosine phosphorylation independently from coexpression of CD2 and/or CD3; (b) the complex Fc $\gamma$ RIII $\alpha$ / $\zeta$  transduces signals similar to those transduced by Fc $\gamma$ RIII $\alpha$  in association with  $\gamma$ <sub>2</sub> homodimers; and (c) the signal transduction events mediated by the  $\zeta$  chain in the presence of the cytoplasmic domain of the Fc $\gamma$ RIII $\alpha$  chain (our model) are qualitatively similar to those reported to be transduced by chimeric Fc $\gamma$ RIII $\alpha$ / $\zeta$  molecules, like what is observed in models that utilize  $\gamma$  chain (3, 14).

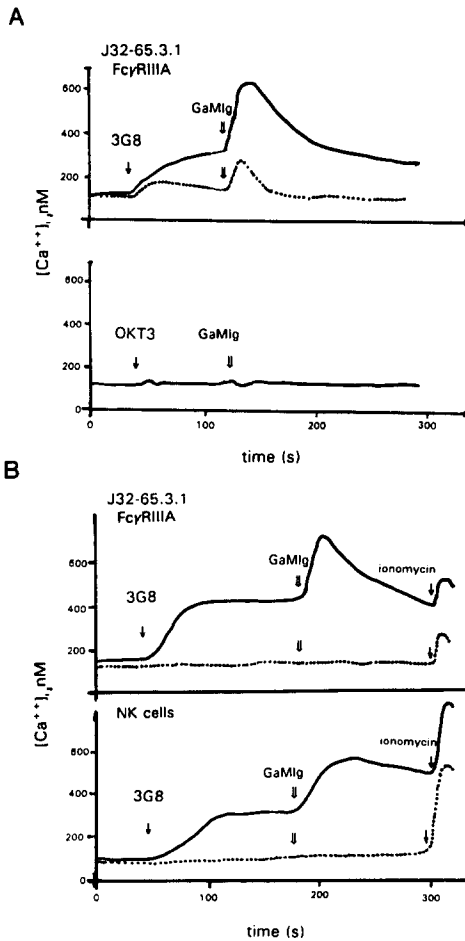
**Role of Tyrosine Phosphorylation in Fc $\gamma$ RIII $\alpha$ / $\zeta$ -induced PIP<sub>2</sub> Hydrolysis and Ca<sup>2+</sup> Mobilization.** We addressed the question of whether tyrosine kinase activation and PIP<sub>2</sub> hydrolysis with consequent Ca<sup>2+</sup> mobilization are related or independent events. Stimulation of Fc $\gamma$ RIII $\alpha$ / $\zeta$  in J32-65.3.1 cells with anti-CD16 mAb followed by crosslinking with GaMIg induced 326 ± 75% and 171 ± 54% increase (mean ± SD, six experiments) in the production of IP<sub>3</sub> and IP<sub>4</sub>, respectively, compared with that observed in control cells treated with GaMIg alone. Treatment with control anti-CD56 or anti-CD3 mAb and GaMIg did not result in increased production of IP<sub>3</sub> (109 ± 23%) or IP<sub>4</sub> (92 ± 7%). Treatment of the cells with herbimycin A (Fig. 1 *A*) or genistein (data not shown) induced inhibition of constitutive and Fc $\gamma$ RIII $\alpha$ -induced protein tyrosine phosphorylation in a dose-dependent manner. Using 5 μM herbimycin A, no significant increase in IP<sub>3</sub> or IP<sub>4</sub> over control was observed upon Fc $\gamma$ RIII $\alpha$  stimulation (Fig. 1 *B*).

A prompt increase in [Ca<sup>2+</sup>]<sub>i</sub> occurred in the cells treated with anti-CD16 mAb 3G8. This increased further upon crosslinking the Ab with GaMIg and, like in NK cells (7), was due to both Ca<sup>2+</sup> mobilization from intracellular stores and to extracellular Ca<sup>2+</sup> internalization, as detected in experiments performed in the presence of EGTA (Fig. 2 *A*). Pretreatment with herbimycin A (Fig. 2 *B*), resulted in a >90% reduction of the [Ca<sup>2+</sup>]<sub>i</sub> increase induced upon Fc $\gamma$ RIII $\alpha$  stimulation, as compared with control nontreated cells. Ionomycin induced similar levels of [Ca<sup>2+</sup>]<sub>i</sub> increase in both untreated and herbimycin A-treated cells. Identical results were obtained with NK cells (Fig. 2 *B*). These data indicate that tyrosine phosphorylation is a prerequisite for Fc $\gamma$ RIII-mediated signal transduction and suggest that PLC activation upon Fc $\gamma$ RIII $\alpha$  occupancy depends on its induced phosphorylation on tyrosine residues.

**Tyrosine Phosphorylation of PLC- $\gamma$ 1 upon Fc $\gamma$ RIII $\alpha$  Stimulation.** PLC- $\gamma$ 1 undergoes tyrosine phosphorylation and activation upon stimulation of other receptor systems that either possess intracellular tyrosine kinase domains (for review see reference 15) or associate with and activate tyrosine kinases of the *src* family (TCR/CD3, Fc $\epsilon$ R1) (16-18). Tyrosine phosphorylation of PLC- $\gamma$ 1 was analyzed in Fc $\gamma$ RIII $\alpha$ -transfected J32-65.3.1 cells. PLC- $\gamma$ 1 was immunoprecipitated from cells treated or not with anti-CD16 mAb, and GaMIg and the immunoreactive material was analyzed in Western blotting with antiphosphotyrosine antisera (Fig. 3). A tyrosine-phosphorylated protein with 145-150 kD *M<sub>r</sub>* was detected only in the PLC- $\gamma$ 1 immunoprecipitates from cells treated with anti-CD16 mAb. Using affinity-purified anti-

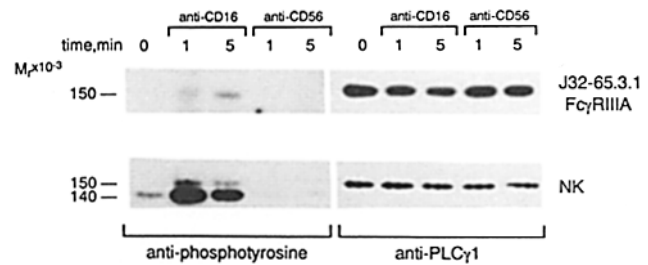


**Figure 1.** Effect of tyrosine kinase inhibition of Fc $\gamma$ RIII $\alpha$ -induced protein tyrosine phosphorylation and PIP<sub>2</sub> hydrolysis. (A) Fc $\gamma$ RIII $\alpha$ / $\zeta$ -expressing J32-65.3.1 cells were cultured for 16 h without (*none*) or with the indicated doses of herbimycin A. Cells were treated with anti-CD16 mAb 3G8 and GaMIg for the indicated times, and lysed. Postnuclear supernatants were electrophoresed in 11% SDS-PAGE. Western blot analysis was performed with antiphosphotyrosine antiserum and <sup>125</sup>I-protein A. (B) The cells were labeled with <sup>3</sup>H-myoinositol (16-h culture without (medium) or with 5 μM herbimycin A) and then treated with the antibodies indicated at the bottom and GaMIg. Levels of IP<sub>3</sub> (■) and IP<sub>4</sub> (□), analyzed by ion exchange chromatography, are reported as the percentage of those in control cells (nontreated cells: IP<sub>3</sub>, 447 cpm; IP<sub>4</sub>, 1,002 cpm; herbimycin A-treated cells: IP<sub>3</sub>, 289 cpm; IP<sub>4</sub>, 453 cpm).



**Figure 2.** Effect of tyrosine kinase inhibition on Fc $\gamma$ RIIIA-dependent  $[Ca^{2+}]_i$  increase. Cells were loaded with Fura-2/AM. mAb were added (arrows) at the indicated times to (A) Fc $\gamma$ RIIIA-expressing J32-65.3.1 cells in buffer without (—) or with (···) 1 mM EGTA, and to (B) Fc $\gamma$ RIIIA $\alpha$ / $\zeta_2$  J32-65.3.1 (top) and NK cells (bottom) cultured for 16 h in medium without (—) or with (···) herbimycin A (5  $\mu$ M) before assay.

PLC- $\gamma$ 1 IgG (Fig. 3), a single band was detected, at similar levels in all anti-PLC- $\gamma$ 1 immunoprecipitates, corresponding to a protein with  $M_r$  identical to that of the phosphoprotein, confirming the identification of the tyrosine phosphorylated

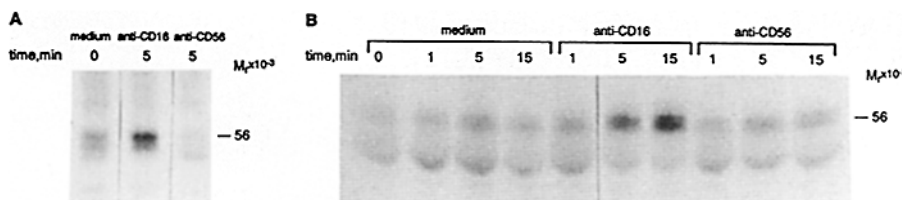


**Figure 3.** PLC $\gamma$ 1 tyrosine phosphorylation upon Fc $\gamma$ RIIIA stimulation. After incubation with or without the indicated mAb and GaMlg for the indicated times, cells were lysed and postnuclear supernatants were immunoprecipitated with anti-PLC $\gamma$ 1 antiserum. Immunoprecipitated proteins were electrophoresed on 7.5% SDS-PAGE. Western blot analysis was performed using, sequentially on the same blot, antiphosphotyrosine antiserum and  $^{125}I$ -protein A (left) and affinity-purified anti-PLC $\gamma$ 1 IgG and HRP-donkey anti-rabbit Ig serum, followed by ECL detection (right).

protein with PLC- $\gamma$ 1, and excluding that lack of its detection with antiphosphotyrosine Ab in the precipitates from control cells was due to its absence from these precipitates.

Anti-PLC- $\gamma$ 1 immunoprecipitates from lysates of NK cells treated with anti-CD16 mAb and GaMlg (Fig. 3) contained a tyrosine phosphorylated protein with the same mobility of that precipitated from the Fc $\gamma$ RIIIA-transfected cells. The same 150-kD protein was detected in antiphosphotyrosine immunoprecipitates from CD16-stimulated Fc $\gamma$ RIIIA/ $\zeta_2$ -expressing Jurkat and from NK cells after elution with  $\sigma$ -phenylphosphate, SDS-PAGE, and immunoblotting with anti-PLC- $\gamma$ 1 (not shown). However, an additional protein of 140 kD  $M_r$  was detected in all immunoprecipitates obtained from NK cells, independently from CD16 stimulation, using anti-PLC- $\gamma$ 1 serum, but not using affinity-purified IgG (not shown). The level of reactivity of this protein with the antiphosphotyrosine antibody was significantly increased, compared to controls, in the precipitates from anti-CD16-treated cells, with maximum intensity at 1-min stimulation. Reblotting the same filter with affinity-purified anti-PLC- $\gamma$ 1 IgG we detected a single band (reproducibly in four experiments), that was present with similar intensity in all lanes and corresponded to the 150-kD  $M_r$  protein.

It is unlikely that the 140-kD species not detected by the affinity-purified anti-PLC- $\gamma$ 1 IgG represents a degradation



**Figure 4.** Phosphorylation of p56 $^{kk}$  upon Fc $\gamma$ RIIIA stimulation in J32-65.3.1 cells. Fc $\gamma$ RIIIA $\alpha$ / $\zeta_2$ -expressing J32-65.3.1 cells were labeled (A) or not (B) with  $[^{32}P]H_3PO_4$  and treated with the indicated antibodies or with medium alone for the indicated periods of time. Postnuclear supernatants from the cell lysates were immunoprecipitated with anti-p56 $^{kk}$  serum. (A) The immunoprecipitated proteins were electrophoresed on 7.5% SDS-PAGE. (B) The product of the in vitro kinase assay performed on the immunoprecipitates in the presence of  $\gamma$ - $[^{32}P]$ -ATP was electrophoresed on 7.5% SDS-PAGE. The lower  $M_r$  band present at similar level in all lanes represents IgG.

product of PLC- $\gamma$ 1, specifically occurring in NK cells and detected by the immune serum. Unlike PLC- $\gamma$ 1, this protein appears phosphorylated on tyrosine residues in unstimulated cells. Instead, it is likely to represent a distinct PLC- $\gamma$ 1 homologous phosphoprotein, hyperphosphorylated upon Fc $\gamma$ RIII $\alpha$  stimulation, that crossreacts with the non-affinity purified serum and that is absent in Jurkat T cells, excluding nonspecific reactivity of the serum with a ubiquitous protein. The biochemical characteristics of the 140-kD phosphoprotein suggest its possible relation with PLC- $\gamma$ 2, reported as the major PLC species involved in PIP<sub>2</sub> hydrolysis mediated via sIg stimulation in murine B cells (19). The nature of this phosphoprotein, likely to be involved in the Fc $\gamma$ RIII $\alpha$ -mediated signal transduction in NK cells, is under investigation.

In several receptor systems PIP<sub>2</sub> hydrolysis, IP<sub>3</sub> production, and consequent release of Ca<sup>2+</sup> from the intracellular stores observed upon receptor occupancy originate from the activation of PLC- $\gamma$ 1, which depends on its phosphorylation on tyrosine residues occurring shortly after ligand-receptor interaction (17, 20). Our data demonstrate that, like in those systems, Fc $\gamma$ RIII $\alpha$  stimulation induces phosphorylation of PLC- $\gamma$ 1 on tyrosine residues both in NK and in Fc $\gamma$ RIII $\alpha$ -transfected Jurkat cells, strongly suggesting that induced PLC- $\gamma$ 1 activation may be responsible for the Fc $\gamma$ RIII $\alpha$ -induced PIP<sub>2</sub> hydrolysis observed in these cells.

*p56<sup>lck</sup> Activation upon Occupancy of Fc $\gamma$ RIII $\alpha$ / $\zeta$ 2.* The kinase(s) responsible for the protein tyrosine phosphorylation induced upon Fc $\gamma$ RIII $\alpha$  stimulation in NK cells has not been identified yet. p56<sup>lck</sup> is expressed, with other kinases of the *src* family, in both NK and T cells (13, and our unpublished data) and has been reported associated with CD4, CD8, and IL-2R p70 chain (21, 22). Its increased activity upon stimulation of these receptors results in auto- and substrate tyrosine phosphorylation. We investigated the participation of p56<sup>lck</sup> in the Fc $\gamma$ RIII $\alpha$ / $\zeta$ 2-mediated signal transduction in intact cells. p56<sup>lck</sup> was immunoprecipitated from Fc $\gamma$ RIII $\alpha$ -transfected J32-65.3.1 cells metabolically labeled with <sup>32</sup>P-orthophosphate and treated with anti-CD16 or control anti-CD56 mAb and GaMIg. Nonstimulated cells were used as control. After SDS-PAGE (Fig. 4 A), two bands of 55 and 60 kD *M<sub>r</sub>* were detected in each precipitate. In the lysates from cells treated for 5 min with anti-CD16 mAb,

these bands appeared hyperphosphorylated, compared to those from control or anti-CD56 mAb treated cells. To determine if p56<sup>lck</sup> activity was increased as a consequence of Fc $\gamma$ RIII $\alpha$  stimulation, *in vitro* kinase assays were performed on p56<sup>lck</sup> immunoprecipitates from Fc $\gamma$ RIII $\alpha$ -expressing J32-65.3.1 cells treated or not with anti-CD16 and GaMIg. The results of one experiment representative of three performed is shown in Fig. 4 B. Kinase activity and p56<sup>lck</sup> phosphorylation were evident in all precipitates but were greater in those from cells treated for 5 and 15 min with anti-CD16 mAb.

The data reported here indicate that phosphorylation of p56<sup>lck</sup> occurs both in intact cells upon Fc $\gamma$ RIII $\alpha$  stimulation and *in vitro*, supporting a functional association of this kinase with Fc $\gamma$ RIII $\alpha$ , and suggesting the possibility that p56<sup>lck</sup> is at least partially responsible for the observed protein tyrosine phosphorylation upon stimulation of the receptor. Although this may suggest a causal relationship between p56<sup>lck</sup> and PLC- $\gamma$ 1 activation, the kinetics of induction of p56<sup>lck</sup> activity upon Fc $\gamma$ RIII $\alpha$  stimulation observed in *in vitro* kinase assay is slower than that of Fc $\gamma$ RIII $\alpha$ -induced phosphorylation of most proteins and does not seem compatible with a direct effect of this kinase. However, we cannot exclude that activation of kinases other than p56<sup>lck</sup>, as well as the activity of cellular phosphatases, may modify, in the intact cells, the kinetics of protein tyrosine phosphorylation. The experiments performed using metabolically labeled cells indicate that p56<sup>lck</sup> is involved in Fc $\gamma$ RIII $\alpha$  signal transduction, but do not allow us to discriminate which amino acid residues are phosphorylated upon receptor stimulation in the intact cells. In these, phosphorylation on serine and/or threonine residues, or differential phosphorylation of negative regulatory tyrosine residues (23) could occur, and possibly modulate the kinetics of enzyme activity. Our data suggest the possibility that p56<sup>lck</sup> is physically associated, or induced to be associated upon ligand binding, with Fc $\gamma$ RIII $\alpha$ , either directly, or indirectly via  $\zeta$  and/or  $\gamma$  chains or other molecules. They do not exclude, however, that receptor-mediated p56<sup>lck</sup> activation is induced indirectly via activation and/or association of this to another kinase(s). Further analysis is needed to determine whether additional or different molecules operate and/or physically associate with the different Fc $\gamma$ RIII $\alpha$  types in NK cells and macrophages.

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