Fcγ Receptor Activation Induces the Tyrosine Phosphorylation of Both Phospholipase C (PLC)-γ1 and PLC-γ2 in Natural Killer Cells

By Adrian T. Ting,* Larry M. Karnitz,* Renee A. Schoon,* Robert T. Abraham,*‡ and Paul J. Leibson*

*From the Departments of *Immunology and ‡Pharmacology, Mayo Clinic and Foundation, Rochester, Minnesota 55905*

Summary

Crosslinking of the low affinity immunoglobulin G (IgG) Fc receptor (FcγR type III) on natural killer (NK) cells initiates antibody-dependent cellular cytotoxicity. During this process, FcγR stimulation results in the rapid activation of phospholipase C (PLC), which hydrolyzes membrane phosphoinositides, generating inositol-1,4,5-trisphosphate and sn-1,2-diacylglycerol as second messengers. We have recently reported that PLC activation after FcγR stimulation can be inhibited by a protein tyrosine kinase (PTK) inhibitor. Based on the paradigm provided by the receptor tyrosine kinases, we investigated whether PLC-γ1 and/or PLC-γ2 are expressed in NK cells, and whether the PLC-γ isoforms are tyrosine phosphorylated in response to FcγR stimulation. Immunoblotting analyses with PLC-γ1– and PLC-γ2–specific antisera demonstrate that both isoforms are expressed in human NK cells. Furthermore, FcγR crosslinking triggers the tyrosine phosphorylation of both PLC-γ1 and PLC-γ2 in these cells. Phosphorylation of both isoforms is detectable within 1 min, and returns to basal level within 30 min. Pretreatment with herbimycin A, a PTK inhibitor, blocked the FcγR-induced tyrosine phosphorylation of PLC-γ1 and PLC-γ2, and the subsequent release of inositol phosphates. These results suggest that FcγR-initiated phosphoinositide turnover in human NK cells is regulated by the tyrosine phosphorylation of PLC-γ. More broadly, these observations demonstrate that nonreceptor PTK(s) activated by crosslinkage of a multisubunit receptor can phosphorylate both PLC-γ isoforms.

The presence of foreign antigens in an immunocompetent host elicits the generation of antigen-specific antibodies. The binding of these antibodies to antigen-bearing cells marks them for subsequent destruction by various immune effector cells, including NK cells. NK cells represent a distinct subpopulation of lymphocytes that expresses low affinity receptors for IgG (FcγR type III) on their surfaces (1). The binding of FcγR type III (hereafter referred to as FcγR) to its ligand, the Fc portion of IgG antibodies present on the target cell's surface, initiates a cascade of intracellular biochemical signals in the NK cells that ultimately leads to the exocytosis of preformed cytolytic granules (2–4). Our group and others have characterized proximal signal transduction events initiated by FcγR activation in NK cells (for reviews see references 5, 6) and have shown that FcγR crosslinking results in the rapid induction of phosphoinositide-specific phospholipase C (PLC) activity (7). The activated PLC hydrolyzes membrane phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate and sn-1,2-diacylglycerol, which, in turn, mediate the mobilization of intracellular calcium and the activation of protein kinase C,

respectively (8). Experiments with calcium ionophores and phorbol esters (1, 5, 6, 9) suggest critical roles for PLC-derived second messengers in the development of antibody-dependent cellular cytotoxicity. However, the mechanism that couples FcγR stimulation to PLC activation remains unknown.

Transmembrane receptors can be coupled to PLC either via heterotrimeric G proteins or protein tyrosine kinases (PTK) (for reviews see references 10–12). For example, receptors with seven transmembrane regions such as the m1 muscarinic receptor can utilize $G_{q/11}$ to activate the PLC-β family (13). In contrast, growth factor receptors with intrinsic PTK activity, such as the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors, can directly tyrosine phosphorylate members of the PLC-γ family (14–16). More recently, activation of the TCR, which does not contain an intrinsic PTK domain, has also been shown to induce the tyrosine phosphorylation of PLC-γ1 (17–19).

Using exogenous guanine nucleotides and permeabilized NK cells, we have recently demonstrated that the FcγR-PLC coupling does not involve a G protein (20). However, the PTK inhibitor, herbimycin A, inhibits the FcγR-induced PLC

activity, implicating a PTK-dependent event as critical in FcγR-PLC coupling (20). In this study, we investigated which members of the PLC-γ family are expressed in human NK cells, and whether any of these are tyrosine phosphorylated after FcγR stimulation. Our initial characterization of the PLC-γ family in NK cells showed that both isoforms, PLC-γ1 and PLC-γ2, were expressed. Subsequent experiments demonstrated that crosslinking of the FcγR rapidly induced the tyrosine phosphorylation of both PLC-γ isoforms. Finally, pretreatment of NK cells with the PTK inhibitor, herbimycin A, blocked both FcγR-induced tyrosine phosphorylation of PLC-γ and the receptor-mediated increase in inositol phosphate formation. Thus, our results demonstrate that both PLC-γ1 and PLC-γ2 are expressed in human NK cells and strongly suggest that these isoforms participate in FcγR-induced phosphoinositide hydrolysis.

Materials and Methods

Cell Lines. Human CD16$^+$ NK cell lines were isolated and passaged as previously described (7). The cell surface phenotype of these NK cell lines was monitored by flow cytometry. All clonal NK cell lines used in these studies were 100% CD16$^+$/CD3$^-$; polyclonal cell lines were >90% CD16$^+$. In addition, all CD16$^+$ NK cells expressed the following additional phenotypic markers: CD56$^+$, CD11b$^+$, CD2$^+$, and HLA-DR$^+$. The human leukemic T cell line, Jurkat, was obtained from the American Type Culture Collection (Rockville, MD), and PJL-EBV is an EBV-transformed human B cell line.

Chemical Reagents and Antibodies. The PTK inhibitor, herbimycin A, was generously provided by Dr. Y. Uehara (Department of Antibiotics, National Institute of Health, Tokyo, Japan). All other chemicals and drugs, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorescein and PE-conjugated mAb were obtained from Becton Dickinson Monoclonal Center (Mountain View, CA). PLC-γ1 antisera was obtained by immunizing New Zealand White rabbits with a PLC-γ1 peptide (bovine PLC-γ1 amino acid 1249–1262) chemically coupled to KLH (Calbiochem Corp., La Jolla, CA) by a m-maleimido-benzoyl-N-hydoxysuccinimide ester (Pierce Chemical Co., Rockford, IL). Similarly, PLC-γ2 antisera was obtained by immunizing New Zealand White rabbits with a KLH-coupled PLC-γ2 peptide (human PLC-γ2 amino acid 461–481). The anti-PLC-γ1 and anti-PLC-γ2 sera immunoprecipitated the predicted 150- and 140-kD molecular mass isoforms, respectively. Furthermore, anti-PLC-γ1 or anti-PLC-γ2 immunoprecipitated protein was detectable by immunoblotting with the same antiserum, but not by the antiserum to the other isoform.

*PLC Immunoblotting.* 5–10 × 10^6 cells were washed, pelleted, and lysed with buffer containing 10 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM Na$_4$P$_2$O$_7$, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100, pH 7.4. Insoluble material was removed by centrifugation at 15,000 *g* for 15 min, and the protein content of the detergent-soluble fraction determined by the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Protein (150 μg) was then resolved by discontinuous SDS-PAGE in 8.5% acrylamide gels and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked overnight in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 2% milk and 0.2% polyoxyethylene sor-

bitan monolaurate (Tween 20) and incubated for 1 h with PLC-γ1- or PLC-γ2-specific antiserum (1:2000 dilution of anti-PLC-γ1 or 1:400 dilution of anti-PLC-γ2) in TBS containing 2% BSA, 0.2% Tween 20, and 0.05% NaN$_3$. After three washes with 0.2% Tween 20 in TBS, immunoreactive proteins were detected with protein A–horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system (Amersham Corp., Arlington Heights, IL).

PLC-γ Immunoprecipitation and Antiphosphotyrosine Immunoblotting. Aliquots of NK cells (2–4 × 10^7/sample) were resuspended in 200 μl of HBSS containing 10 mM Hepes, pH 7.2, and 1 mg/ml BSA. Cells were stimulated by adding 50 μl of anti-FcγRIII mAb (3G8) (final concentration, 10 μg/ml) and goat F(ab')$_2$ fragment anti–mouse IgG (Organon Teknika Corp., West Chester, PA). After the indicated time, reactions were terminated by the addition of 4 volumes of ice-cold lysis buffer containing 20 mM Tris-HCl, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM Na$_4$P$_2$O$_7$, and 500 μM Na$_3$VO$_4$, 1 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100, pH 7.4. After 10 min at 4°C, the samples were centrifuged (15,000 *g*, 15 min) to remove nuclear and cellular debris. Postnuclear supernatants were immunoprecipitated for 2 h at 4°C with PLC-γ1- or PLC-γ2–specific antisera bound to protein A–Sepharose beads. The immunoprecipitates were washed three times, bound proteins eluted with 50 μl of SDS sample buffer, and resolved by SDS-PAGE as described above. The proteins were electrophoretically transferred to nitrocellulose membranes and antiphosphotyrosine immunoblotting was performed essentially as previously described (20). Briefly, nitrocellulose membranes were blocked overnight in TBS containing 4% BSA, 0.5% Tween 20 and 0.05% NaN$_3$, and were then probed for 2 h with antiphosphotyrosine mAb 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) (1 μg/ml in TBS with 0.5% Tween 20). The membranes were washed three times with 0.2% Tween 20 in TBS, and incubated for 45 min with rabbit anti–mouse IgG (H + L) (1 μg/ml) (Pierce Chemical Co.) in TBS containing 2% BSA and 0.5% Tween 20. After three washes, immunoreactive proteins were detected with protein A–horseradish peroxidase and the ECL detection system from Amersham Corp.
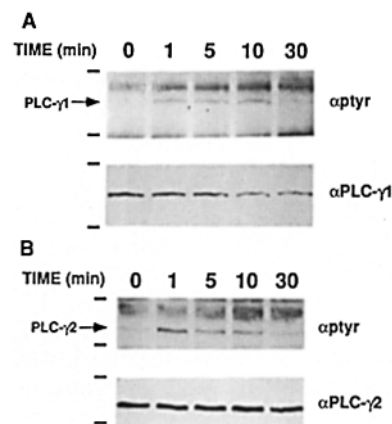
Results and Discussion

Expression of PLC-γ Isoforms in Human NK Cells. We first examined whether PLC-γ1 and/or its closely related isoform, PLC-γ2, are expressed in human NK cells. Fig. 1 shows PLC-γ1 and PLC-γ2 immunoblots of equivalent amounts of detergent-soluble proteins from Jurkat cells (a human leukemic T cell line), EBV-transformed human B cells (PJL-EBV), and three different clonal human NK cell lines. Consistent with prior reports (17–19, 21), Jurkat cells expressed relatively high levels of PLC-γ1, migrating at an apparent molecular mass of 150 kD (Fig. 1 *A*, lane *1*). The PLC-γ1 antiserum also detected the presence of PLC-γ1 in the three human NK clones (Fig. 1 *A*, lanes *3–5*), albeit at lower levels than Jurkat. The same blot was stripped and reprobed with the PLC-γ2 antiserum (Fig. 1 *B*). Consistent with prior reports (22–24), relatively high levels of PLC-γ2 (migrating at 140 kD) were detected in EBV-transformed human B cells (Fig. 1 *B*, lane *2*), whereas little PLC-γ2 was present in Jurkat (Fig. 1 *B*, lane *1*). In each of the NK cell lines, significant amounts of PLC-γ2 were detected (Fig. 1 *B*, lanes *3–5*). Our data suggest that both PLC-γ1 and PLC-γ2 are present in
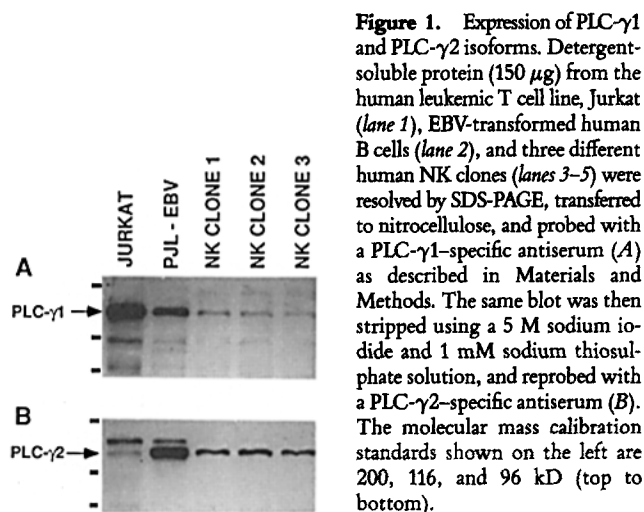
Figure 1. Expression of PLC-γ1 and PLC-γ2 isoforms. Detergent-soluble protein (150 μg) from the human leukemic T cell line, Jurkat (*lane 1*), EBV-transformed human B cells (*lane 2*), and three different human NK clones (*lanes 3–5*) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with a PLC-γ1–specific antiserum (*A*) as described in Materials and Methods. The same blot was then stripped using a 5 M sodium iodide and 1 mM sodium thiosulphate solution, and reprobed with a PLC-γ2–specific antiserum (*B*). The molecular mass calibration standards shown on the left are 200, 116, and 96 kD (top to bottom).

human NK cells, with PLC-γ2 being the more prominent immunoreactive isoform.

FcγR Crosslinking Induces the Tyrosine Phosphorylation of PLC-γ1 and PLC-γ2. We next investigated whether PLC-γ1 and PLC-γ2 are direct substrates for FcγR-induced tyrosine phosphorylation. NK cells were stimulated with crosslinked anti-FcγR mAb (3G8), and then either PLC-γ1 or PLC-γ2 was immunoprecipitated with their respective antiserum. The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the antiphosphotyrosine mAb, 4G10. As shown in Fig. 2, FcγR crosslinkage resulted in a rapid increase in the phosphotyrosine contents of both PLC-γ1 and PLC-γ2. Kinetic analyses indicated that tyrosine phosphorylation of both isoforms followed
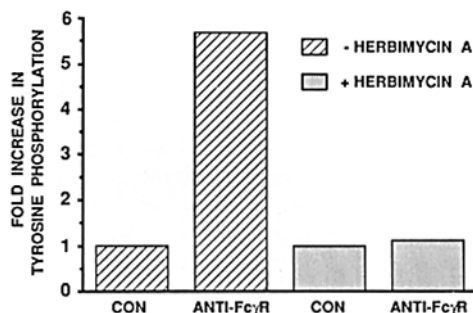
a similar time course, being detectable within 1 min after stimulation, remaining elevated for at least 10 min, and returning to basal level by 30 min (Fig. 2). Reanalyses of the nitrocellulose with the antiserum specific for each PLC-γ isoform indicated that the kinetic changes were not due to FcγR-induced alterations in the level of PLC protein expression. Sultzman et al. (25) have reported that in Rat-2 fibroblasts transfected with PLC-γ2, PDGF stimulation resulted in the tyrosine phosphorylation of both PLC-γ1 and PLC-γ2. Our data extend that study by demonstrating that in a cell type that endogenously expresses both PLC-γ1 and PLC-γ2, non-receptor PTK(s) activated by crosslinkage of a multisubunit receptor can phosphorylate both PLC-γ isoforms.

Herbimycin A Inhibits FcγR-induced Tyrosine Phosphorylation of PLC-γ. To examine further the notion that the PTK-mediated phosphorylation of both PLC-γ is a critical event in FcγR-induced activation of PLC, we tested the effect of herbimycin A, a PTK inhibitor (26), on the FcγR-induced tyrosine phosphorylation of the PLC-γ isoforms. NK cells were treated with herbimycin A under conditions that we have previously shown to inhibit tyrosine phosphorylation and phosphoinositide hydrolysis in NK cells (3 μM herbimycin A for 16 h) (20, 27). These same condition leave intact other protein kinase-dependent cellular responses such as the phorbol ester-induced phosphorylation of p56[lck] (20, 27). Pretreatment with herbimycin A effectively blocked the FcγR-induced tyrosine phosphorylation of both PLC-γ1 (data not shown) and PLC-γ2 (Fig. 3). These results are consistent with a model in which FcγR stimulation in NK cells rapidly activates an intracellular PTK, which, in turn, induces the tyrosine phosphorylation and activation of PLC-γ.

Although none of the identified components of the multimeric FcγR complex have been shown to possess intrinsic PTK activity, the FcγR must be associated with an intracel-

Figure 2. Kinetics of FcγR-induced tyrosine phosphorylation of PLC-γ1 and PLC-γ2 in NK cells. Polyclonal NK cells (3.5–4 × 10⁷ per sample) were stimulated with crosslinked anti-FcγR mAb (3G8) for the indicated times. Anti-PLC-γ1 (*A*) or anti-PLC-γ2 (*B*) immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose. (*A*) The nitrocellulose was sequentially probed with antiphosphotyrosine antibody (*top*), and after stripping, with the PLC-γ1 antiserum (*bottom*). (*B*) The membrane was sequentially probed with antiphosphotyrosine antibody (*top*) and after stripping, with the PLC-γ2 antiserum (*bottom*). The molecular mass standards are 200 and 116 kD.

Figure 3. Herbimycin A inhibits the FcγR-induced tyrosine phosphorylation of PLC-γ2. NK cells were pretreated for 16 h either with (*shaded bars*) or without (*hatched bars*) 3 μM herbimycin A, and then stimulated for 1 min with either media (control) or crosslinked anti-FcγR mAb (3G8). PLC-γ2 immunoprecipitates were immunoblotted with antiphosphotyrosine antibody as described in Fig. 2. After detection of the immunoreactive protein with the ECL reagent, the developed films were subjected to densitometric analysis using the Image version 1.28 software (Wayne Rasband, National Institutes of Health, Research Services Branch, NIMH, Bethesda, MD) on a MacIntosh IIci (Cupertino, CA) and Hewlett-Packard ScanJet Plus (Sunnyvale, CA). The levels of tyrosine phosphorylation shown here are normalized to their respective amount of PLC-γ2 protein.

lular PTK. Candidate tyrosine kinases include members of the src-family (NK cells express P56^{lck}, p62^{yes}, p59^{fyn}, and p56^{lyn} [28]), and other nonreceptor PTK, such as p72^{syk} (29) and PTK72 (30). The information provided in this report provides a foundation for evaluating the potential role of these intracellular PTK in the development of antibody-dependent cellular cytotoxicity. For example, it has become increasingly clear that the amino acid sequences of the SH2 domains of

specific enzymes can directly influence their association with phosphotyrosine-containing transmembrane or intracellular proteins (31). Since PLC-γ1 and PLC-γ2 differ in their SH2 domains, these differences may dictate unique associations with specific PTK, and raise the possibility that PLC-γ1 and PLC-γ2 may be differentially regulated in lymphoid cells through either intermolecular associations or phosphorylation.

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