Fc Receptor Stimulation of Phosphatidylinositol 3-Kinase in Natural Killer Cells Is Associated with Protein Kinase C-independent Granule Release and Cell-mediated Cytotoxicity

By Joy D. Bonnema, Larry M. Karnitz, Renee A. Schoon, Robert T. Abraham, and Paul J. Leibson

From the Department of Immunology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Summary

Although diverse signaling events are initiated by stimulation of multichain immune recognition receptors on lymphocytes, it remains unclear as to which specific signal transduction pathways are functionally linked to granule exocytosis and cellular cytotoxicity. In the case of natural killer (NK) cells, it has been presumed that the rapid activation of protein kinase C (PKC) enables them to mediate antibody-dependent cellular cytotoxicity (ADCC) and "natural" cytotoxicity toward tumor cells. However, using cloned human NK cells, we determined here that Fc receptor stimulation triggers granule release and ADCC through a PKC-independent pathway. Specifically, pretreatment of NK cells with the selective PKC inhibitor, GF109203X (using concentrations that fully blocked phorbol myristate acetate/ionomycin–induced secretion) had no effect on FcR-initiated granule release or ADCC. In contrast, FcR ligation led to the rapid activation of phosphatidylinositol 3-kinase (PI 3-kinase), and inhibition of this enzyme with the selective inhibitor, wortmannin, blocked FcR-induced granule release and ADCC. Additional experiments showed that, whereas FcR-initiated killing was wortmannin sensitive and GF109203X insensitive, natural cytotoxic activity toward the tumor cell line K562 was wortmannin insensitive and GF109203X sensitive. Taken together, these results suggest that: (a) PI 3-kinase activation induced by FcR ligation is functionally coupled to granule exocytosis and ADCC; and (b) the signaling pathways involved in ADCC vs natural cytotoxicity are distinct.

Critical event in the development of cell-mediated cytotoxicity is the receptor-stimulated release of granule-derived proteins (1). Before cytotoxic lymphocyte activation, preformed granules containing an array of cytotoxic and proteolytic molecules reside beneath the cell surface. Upon receptor recognition of susceptible targets, intracellular signals direct the transport of the granules toward the effector cell–target cell interface where they fuse with the plasma membrane and release their mediators. Previous work has focused on the role of phospholipase C (PLC)–derived signals in regulating NK cell and CTL secretory responses (for reviews, see references 2–5).

Specifically, protein tyrosine kinase (PTK)-dependent activation of PLC is initiated after stimulation of NK cells through their FcR (6–9) or of T lymphocytes through their TCR complex (10–14). The subsequent PLC-catalyzed hydrolysis of phosphoinositides generates sn-1,2-diacylglycerol and inositol-1,4,5-trisphosphate, which in turn mediate the activation of PKC and the mobilization of intracellular calcium, respectively. Because PKC-activating phorbol esters, acting in conjunction with calcium ionophores, can induce granule release from NK cells and CTL (15–17), it has been presumed that secretion initiated by cross-linking of multichain immune recognition receptors on these cell types is mediated via PKC-dependent pathways. Furthermore, it has been presumed that the proximal signals controlling FcR- or TCR-initiated secretion would be similar to those inducing granule release after NK cell recognition of susceptible tumor targets. Direct evidence for either of these assumptions is lacking.

Recently, it has been shown that FcyRIIIF- or TCR-initiated activation of PTK also results in the induction of a phosphatidylinositol 3-kinase (PI 3-kinase)–dependent pathway (18–20). PI 3-kinase is a heterodimer composed of a catalytic p110 subunit and a regulatory p85 subunit (21–26). Upon activation, PI 3-kinase phosphorylates phosphoinositides at the D-3 position of the inositol ring to generate such prod-
ucts as phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (27). These phosphoinositides are distinct from those used in the classical PLC-dependent signaling pathway, and it is therefore presumed that the lipids themselves are important molecules in signal transduction pathways. Specific attention has recently focused on the effects of PI 3-kinase on the sorting of vacuoles in yeast (28), platelet-derived growth factor (PDGF) receptor endocytosis (29), histamine release from mast cells (30), and cytoskeleton changes in thrombin-stimulated platelets (31). Taken together, these experimental observations suggest a potential general role for PI 3-kinase in the regulation of vesicular transport.

To investigate whether PKC- and/or PI 3-kinase-dependent pathways regulate granule release from cytotoxic lymphocytes, one would like to selectively interrupt each pathway and determine the subsequent effects on receptor-initiated secretion. With the recent identification of the bisindolylmaleimide G109203X (32) and the microbial metabolite wortmannin (30) as selective PKC and PI 3-kinase inhibitors, respectively, this experimental strategy is now possible. Using cloned human NK cells, we report here that FcyRIII-initiated granule release and antibody-dependent cellular cytotoxicity (ADCC) are, in fact, regulated by PKC-independent pathways. Rather, the profound inhibition of these functions by wortmannin suggests that FcR-initiated activation of PI 3-kinase modulates granule release from these cells. In contrast, the inability of wortmannin to inhibit direct antitumor NK cell-mediated cytotoxicity suggests that alternative signaling pathways are used during this mode of killing.

Materials and Methods

Chemical Reagents and Antibodies. Ionomycin and G109203X were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Streptolysin-O was purchased from Wellcome Diagnostics (Dartford, England). All other chemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO). The anti-FcγRIII mAb 3G8 (anti-CD16) (33) were purified by affinity chromatography over protein A-Sepharose beads. After 45 min of incubation, FcγRIII antibody-coated platelets were washed twice with wash buffer (20 mM Tris, pH 7.4, 10 mM MgCl2, 50 mM potassium glutamate, 5 mM NaCl, 0.1 mM di-thiothreitol, 0.5 mg/ml BSA, and 3 mM ATP, pH 7.2). The amount of Ca2+ required to yield the indicated concentration of free Ca2+ in the permeabilized buffer was calculated with the free calcium algorithm. For each sample (total reaction volume, 250 µl), 7.5 x 10^6 NK cells were mixed with the indicated stimuli and streptolysin O (0.4 µM). The reaction mixtures were incubated at 37°C for 30 min and then centrifuged at 500 g for 10 min. 200 µl of the supernatant was collected from each sample for subsequent quantitation by the secretion assay.

Secretion Assay. As previously described (17), a fluorescence assay was used to quantitate exocytotic release of the granule-derived enzyme, hexosaminidase, from NK cells. Briefly, duplicate sample supernatants (50 µl each) were diluted 1:2 in 250 mM sucrose, 3 mM imidazole, 0.1% ethanol, pH 7.4, and then incubated for 20 min at 37°C with 100 µl of 0.5 mM 4-methylumbelliferyl-2-acetamide-2-deoxy-β-d-glucopyranoside (Research Products International Corp., Prospect, IL) in 0.1 M sodium citrate and 0.2% Triton X-100. Reactions were terminated by the addition of 2 ml 50 mM glycine–5 mM EDTA, pH 10.4. Fluorescence was measured with a digital fluorometer (model 450; Sequoia-Turner, Mountain View, CA) at excitation and emission wavelengths of 365 and 450 nm, respectively. For each experiment, total intracellular hexosaminidase content was measured after cell lysis with 1% Triton X-100. Percent secretion was calculated as: 100 x [(stimulated release – spontaneous release)/(maximum release – spontaneous release)].

Cytotoxicity Assay. The 31Cr-release assays measuring either direct NK cell–mediated cytotoxicity or ADCC were performed as previously described (6). Spontaneous release was <15% of the maximum release for all experiments. For results expressed as lytic units, 1 lytic unit is the number of cells required to give 20% maximum release. The range of duplicate samples was <10% of the mean.

Results

PKC–independent Regulation of FcR-induced Secretion. It remains unclear which signals generated after FcR ligation

1428 FcR Stimulation of PI 3-Kinase Is Associated with Secretion and Killing
pharmacologic activation of PKC can induce secretion from NK cells. Because direct pharmacologic activation of PKC can induce secretion from NK cells (16, 17), we first evaluated whether PKC plays a similar role after FcR stimulation. We have previously shown that stimulation of NK cells with a variety of secretagogues induces the coordinate release of hexosaminidase and N-α-benzoyl-L-arginine esterase (Ble; esterase (17)). Because the fluorescence assay for hexosaminidase has proven to be the more sensitive method for measuring low levels of granule exocytosis from human NK cells (17), results from the hexosaminidase assays are reported in this study. Cloned CD16+, CD3- NK cells were first exposed to varying concentrations of the selective PKC inhibitor GF109203X (32), and then stimulated with either 3G8 mAb (anti-FcγRIII)-coated polystyrene beads or a combination of the PKC-activating phorbol ester, PMA, and the calcium ionophore, ionomycin. As shown in the top panel of Fig. 1, pretreatment of NK cells with concentrations of GF109203X that fully inhibited PMA/ionomycin–induced secretion did not alter FcR-induced granule release. These results suggest that although PKC activation can result in granule exocytosis from NK cells, FcR-stimulated granule release is regulated by PKC-independent signaling pathways.

FR-dependent Granule Release from NK Cells Is Blocked by the PI 3-Kinase Inhibitor, Wortmannin. The novel finding that FcR-induced granule exocytosis is not regulated by PKC prompted us to investigate whether PI 3-kinase, a potential modulator of vesicular transport (28–31), might influence secretion from NK cells. Because the p85 regulatory subunit of PI 3-kinase can be tyrosine phosphorylated during cellular activation and also has an SH2 domain that binds to tyrosine phosphorylated proteins during cellular activation (27, 36–39), antiphosphotyrosine immunoprecipitates from FcR-stimulated NK cells were assayed for PI 3-kinase activity. Consistent with the recent report by Kanakaraj et al. (18), we found that FcγRIII stimulation of NK cells increased phosphotyrosine-associated PI 3-kinase activity (Fig. 2). Specifically, PI 3-kinase activity increased within 2 min of FcR ligation, peaked at 10 mins (sixfold increase), and declined by 30 min.

To evaluate the functional consequences of this PI 3-kinase activation, we characterized the effects of a recently described PI 3-kinase inhibitor, wortmannin (30), in this experimental system. Wortmannin, a fungal metabolite, has been shown to specifically and irreversibly inhibit PI 3-kinase activity at nanomolar concentrations by binding to the p110 catalytic subunit of PI 3-kinase (30). To evaluate its effects on cloned human NK cells, lysates from drug-treated cells were immunoprecipitated with anti-p85 antibodies and the immunoprecipitates were assayed for PI 3-kinase catalytic activity (Fig. 3). Consistent with concentration–inhibition relationships observed in other cell types (30), wortmannin inhibited PI 3-kinase with an IC50 of ~3 nM. Concentrations of wortmannin that fully inhibited PI 3-kinase activity in these NK cells had no effect on conjugate formation with susceptible targets (as quantitated by flow cytometry) or on FcR-initiated tyrosine kinase activation (as detected by antiphosphotyrosine immunoblotting) (data not shown). In parallel studies, pretreatment of NK cells with wortmannin potentiated FcR-induced secretion in a concentration–dependent manner with an IC50 of ~2 nM (Fig. 1, bottom). It is important to note that although wortmannin blocked FcR-induced granule release from NK cells, PKC-dependent release from either intact cells stimulated with PMA/ionomycin or permeabilized cells stimulated with PMA in buffer containing high free Ca2+ concentration was not inhibited by wortmannin (Fig. 1, bottom). These data, together with the differential effects of GF109203X on NK cell secretion (Fig. 1, top) suggest that FcR-induced secretion is regulated by a PI 3-kinase–dependent, PKC-independent pathway. Conversely, pharmacologic activation by phorbol esters induces secretion via a separate PKC-dependent, PI 3-kinase–independent pathway.

**Figure 1.** Selective inhibition of FcR-induced secretion by wortmannin. Cloned CD16+, CD3- human NK cells (7.5 x 10⁶ cells/sample) were preincubated for 15 min at 37°C with the indicated concentrations of either GF109203X (top) or wortmannin (bottom). (Top) GF109203X-treated cells were then incubated for an additional 3 h with either anti-FcγRIII mAb (3G8)-coated, 6-μM-Diameter polystyrene beads (■) or PMA (1 ng/ml) and ionomycin (1 μM) (●). Hexosaminidase activity released into the supernatants was quantitated by measuring the conversion of 4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucopyranoside into the fluorescent 4-methylumbelliferyl product. Without GF109203X pretreatment, the NK cells stimulated with anti-FcγRIII-coated beads or PMA/ionomycin released 6.4 and 20.2% of their total intracellular hexosaminidase content, respectively. (Bottom) Wortmannin-treated cells were stimulated with either anti-FcγRIII mAb (3G8)-coated beads (■), PMA (2 ng/ml), and ionomycin (1 μM) (●), or PMA (1 ng/ml) and streptolysin-O (0.4 U/ml) (▲). For streptolysin-O-permeabilized cells, free calcium concentration was maintained at 10 μM. Intact cells and streptolysin O-permeabilized cells were incubated at 37°C with the agonists for 3 and 1 h, respectively. Without wortmannin pretreatment, the NK cells stimulated with anti-FcγRIII-coated beads, PMA/ionomycin, or PMA/streptolysin-O released 6.4, 11.4, and 38.4% of their total intracellular hexosaminidase content, respectively.
Figure 2. FcR stimulation induces activation of PI 3-kinase in NK cells. Aliquots (10^7 cells/sample) of cloned NK cells that had been incubated without Ib2 for 16 h were stimulated at 37°C for the time indicated with anti-FcγRIII mAb (3G8; 10 μg/ml) cross-linked with goat F(ab')2 fragments of anti-mouse IgG. Antiphosphotyrosine (IG2 mAb) immunoprecipitates were assayed for PI 3-kinase activity, using phosphatidylinositol as substrate. Phosphoinositides were separated by TLC and radiolabeled products were visualized by autoradiography. (Top arrow) Position of a PIP standard that was chromatographed in a parallel lane.

PI 3-kinase Inhibitor, Wortmannin. Because FcR-induced granule exocytosis is believed to deliver the “lethal hit” during ADCC, we performed parallel analyses evaluating the potential roles of PI 3-kinase and PKC in regulating FcγRIII-dependent, cell-mediated killing. Pretreatment of cloned NK cells with wortmannin inhibited killing of both the anti-FcγRIII mAb-secreting hybridoma, 3G8 (Fig. 4, top), and the P815 mastocytoma cell line that is normally rendered sensitive to NK cell-mediated cytotoxicity by coating with 3G8 mAb (“reverse ADCC”) (Fig. 4, bottom). Similar to the IC50 for wortmannin’s inhibition of FcR-induced granule release, wortmannin inhibited FcR-dependent killing of each target cell.

Figure 3. Inhibition of PI 3-kinase in wortmannin-treated NK cells. Cloned NK cells (10^7 cells/sample) were incubated for 30 min at 37°C with the indicated concentrations of wortmannin. Anti-p85 immunoprecipitates from cell lysates were then assayed for PI 3-kinase activity. Radiolabeled phosphoinositides were separated by TLC, visualized by autoradiography, and quantitated using an Ambis 4000 Radioanalytic Imaging System. Radiolabel in phosphatidylinositol phosphate products from NK cells treated with 300, 30, 3, 0.3, 0.03, or 0 nM wortmannin was 39, 190, 535, 1,005, 1,443, or 1,250 cpm, respectively.

Figure 4. Wortmannin inhibits FcR-dependent NK cell-mediated cytotoxicity. Cloned NK cells were preincubated for 15 min at 37°C with the indicated concentrations of wortmannin, washed, and then incubated for 1 h at 37°C with either 51Cr-labeled 3G8 (anti-FcγRIII) hybridoma cells (top) or 51Cr-labeled P815 cells bound to 3G8 mAb (bottom). Results are expressed as specific 51Cr release in the insets ([■] no wortmannin, [▲] 0.03 nM wortmannin, [★] 0.3 nM wortmannin, [□], 3 nM wortmannin, [◇] 30 nM wortmannin, and [○] 300 nM wortmannin) or as lytic units per 10^6 cells, where 1 lytic unit is the number of cells required to give 20% specific 51Cr release.
Figure 6. FcR-dependent cytotoxicity and direct antitumor cytotoxicity are regulated by separate signaling pathways. Cloned NK cells were preincubated for 15 min at 37°C with the indicated concentrations of either wortmannin (A) or GF109203X (B). Drug-treated cells were then incubated for 1 h with either 51Cr-labeled 3G8 (anti-FcγRIII) hybridoma cells (top) or 51Cr-labeled P815 cells bound to 3G8 mAb (bottom).
be critical for FcR-induced granule release and ADCC, a separate PKC-dependent pathway appears to regulate direct NK cell-mediated antitumor killing.

Discussion

Cytotoxic lymphocyte recognition of susceptible target cells elicits a diverse array of second messengers and the subsequent development of pleiotropic responses, including effects on granule release, cellular cytotoxicity, cytokine gene transcription, and cell surface receptor expression (2–5, 40, 41). The heterogeneity of these biological responses has complicated attempts to link distinct proximal signaling events with specific downstream functional responses. In this study, we selectively inhibited separate signaling pathways generated after FcR ligation in NK cells and characterized the resulting effect on granule release and FcR-dependent cytotoxicity. Our results suggest that FcR stimulation of PI 3-kinase in NK cells is associated with both granule exocytosis and the generation of cellular cytotoxicity.

Early studies focused on the PTK-catalyzed activation of PLC as a central event during lymphocyte activation (for reviews, see references 2–5). With the subsequent demonstration that PKC-activating phorbol esters together with calcium ionophores could induce granule release from NK and T cells (15–17), the consensus view was that FcR- and TCR-initiated secretory responses are controlled by PKC-dependent pathways. This notion was further bolstered by demonstrations that PKC could regulate granule exocytosis in other secretory cell types (e.g., mast cells, chromaffin cells, etc.) and could phosphorylate granule-associated proteins (42–45).

To determine whether FcR- or TCR-initiated granule release is, in fact, mediated via a PKC-dependent pathway, one would like to assess the effects of selective and potent PKC inhibitors on this process. However, to date, most of the reported PKC inhibitors display poor selectivity both in vitro and in vivo. For example, H7 inhibits with similar potency PKC, cAMP- and cGMP-dependent protein kinase (IC₅₀ values of 3–6 μM) (46). In addition, staurosporine, a potent PKC inhibitor (IC₅₀ = 10 nM) also effectively inhibits various PTK activities (47).

Discussion continues...

Increasing information implicating potential role for PI 3-kinase in vesicular transport: (a) VPS34, a homolog of the catalytic subunit of PI 3-kinase, has been identified in the yeast Saccharomyces cerevisiae (28). Yeast strains with VPS34 deletions or containing VPS34 point mutations exhibit severe defects in the sorting of vacuolar proteins (b) during thrombin-stimulated secretion from platelets, PI 3-kinase associates with the membrane cytoskeleton (31); (c) mutation of the PI 3-kinase binding site on the PDGF receptor alters endocytic trafficking of this receptor after growth factor binding (29); (d) pretreatment of the rat basophilic leukemia cell line, RBL-2H3, with wortmannin inhibits FceRI-initiated histamine release (30). Our results extend these analyses by demonstrating that ADCC, which requires the formation of a microtubule-organizing center (49) and subsequent granule release at the effector–target interface, appears to require PI 3-kinase activation.

PI 3-kinase activity can be altered by multiple factors. PI 3-kinase associates with receptor proteins and other signaling molecules that contain a tyrosine phosphorylated consensus sequence, thus allosterically modulating its potential activity (36–39, 50–52). Tyrosine and/or serine-threonine phosphorylations may also modulate its enzymatic activity (52–54). In addition, recent reports indicate that p85, the regulatory subunit of PI 3-kinase, associates with the SH3 domain of src family PTK (55, 56) and this interaction can increase PI 3-kinase catalytic activity (57). Because FcR ligation results in the rapid activation of p56⁺⁺ (9, 58–60), and PTK activation appears to be requisite for granule release and ADCC (61, 62), future work will need to focus on the role of p56⁺⁺ itself or the proximal substrates of this PTK in coupling FcR stimulation to PI 3-kinase activation.

Finally, data presented here indicate that signaling events regulating granule release after recognition of Ab-coated targets (i.e., ADCC) vs susceptible tumor targets (i.e., direct NK cell-mediated cytotoxicity) are distinct. Early studies on NK cell activation focused on similarities in signaling initiated during these alternative modes of killing, i.e., early PTK activation, inositol phosphate release, and increase in intracellular free Ca²⁺ (4, 6–9, 61–64). However, we previously reported that pretreatment of NK cells with PKC-activating...
phorbol esters had differential regulatory effects on these alternative forms of NK cell activation (65). In this report, we extend this analysis by comparatively evaluating the effects of wortmannin and GF109203X on secretion and killing induced by these separate modes of NK cell activation. These data suggest that although FcR-initiated effects on these processes proceed through PI 3-kinase–dependent pathways, direct recognition of susceptible tumor targets initiates granule release and killing through PKC-dependent pathways. These results point out that: (a) ADCC and direct natural cytotoxicity are triggered by fundamentally different biochemical events; and (b) it may be feasible to differentially modulate the effector functions of NK cells in patients.

The authors thank Christopher J. Dick for his excellent technical assistance and Theresa Lee for her skillful preparation of this manuscript.

This research was supported by the Mayo Foundation and by National Institutes of Health grants CA-47752, CA-52995, and GM-47286.

Address correspondence to Dr. P. J. Leibson, Department of Immunology, Mayo Clinic and Foundation, Rochester, MN 55905.

Received for publication 11 April 1994 and in revised form 13 June 1994.

References

1. Henkart, P.A., M.P. Hayes, and J.W. Shiver. 1993. The granule exocytosis model for lymphocyte cytotoxicity and its relevance to target cell DNA breakdown. In Cytotoxic Cells: Recognition, Effector Function, Generation, and Methods. M.V. Sitkovsky and P.A. Henkart, editors. Birkhaeuser Boston, Inc., Cambridge, MA. 153-165.

2. Weiss, A., B.A. Irving, L.K. Tan, and G.A. Koretzky. 1991. Signal transduction by the T cell antigen receptor. Seminars in Immunology. 3:313.

3. Siegel, J.N., M. Egerton, A.F. Phillips, and L.E. Samelson. 1991. Multiple signal transduction pathways activated through the T cell receptor for antigen. Seminars in Immunology. 3:325.

4. Leibson, P.J. 1992. Signal transduction during NK cell activation. In NK Cell Mediated Cytotoxicity: Receptors, Signaling and Mechanisms. E. Lotzova and R.B. Herberman, editors. CRC Press, Inc., Boca Raton, 130-146.

5. Abraham, R.T., L.M. Karnitz, J.P. Secrist, and P.J. Leibson. 1992. Signal transduction through the T-cell antigen receptor. Trends Biochem. Sci. 17:434.

6. Windebank, K.P., R.T. Abraham, G. Powis, R.A. Olsen, T.J. Barna, and P.J. Leibson. 1988. Signal transduction during human natural killer cell activation: inositol phosphate generation and regulation by cyclic AMP. J. Immunol. 141:3951.

7. Cassatella, M.A., I. Anegón, M.C. Cuturi, P. Griskey, G. Trinchieri, and B. Perussia. 1989. FcR(CD16) interaction with ligand induces Ca\(^{2+}\) mobilization and phosphoinositide turnover in human natural killer cells. Role of Ca\(^{2+}\) in FcR(CD16)-induced transcription and expression of lymphokine genes. J. Exp. Med. 169:549.

8. Ting, A.T., L.M. Karnitz, R.A. Schoon, R.T. Abraham, and P.J. Leibson. 1992. Fcy receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-\(\gamma 1\) and PLC-\(\gamma 2\) in natural killer cells. J. Exp. Med. 176:1751.

9. Azzoni, L., M. Kamoun, T.W. Salcedo, P. Kanakaraj, and B. Perussia. 1992. Stimulation of FcRIII(A) results in phospholipase C-\(\gamma 1\) tyrosine phosphorylation and p56\(^{ck}\) activation. J. Exp. Med. 176:1745.

10. Imboden, J.B., and J.D. Stobo. 1985. Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. J. Exp. Med. 161:446.

11. Secrist, J.P., L. Karnitz, and R.T. Abraham. 1991. T-cell antigen receptor ligation induces tyrosine phosphorylation of phospholipase C-\(\gamma 1\). J. Biol. Chem. 266:12135.

12. Park, D.J., H.W. Rho, and S.G. Rhee. 1991. CD3 stimulation causes phosphorylation of phospholipase C-\(\gamma 1\) on serine and tyrosine residues in a human T-cell line. Proc. Natl. Acad. Sci. USA. 88:5453.

13. Weiss, A., G. Koretzky, R.C. Schatzman, and T. Kadlecek. 1991. Functional activation of the T-cell antigen receptor induces tyrosine phosphorylation of phospholipase C-\(\gamma 1\). Proc. Natl. Acad. Sci. USA. 88:5484.

14. Grana, C., L.-L. Lin, E.J. Yunis, V. Relias, and J.D. Dasgupta. 1991. PLC\(\gamma 1\), a possible mediator of T cell receptor function. J. Biol. Chem. 266:16277.

15. Sitkovsky, M.V. 1988. Mechanistic, functional and immunopharmacological implications of biochemical studies of antigen receptor-triggered cytolytic T-lymphocyte activation. Immunol. Rev. 103:127.

16. Atkinson, E.A., J.M. Gerrard, G.E. Hildes, and A.H. Greenberg. 1990. Studies of the mechanism of natural killer (NK) degranulation and cytotoxicity. J. Leukocyte Biol. 47:39.

17. Ting, A.T., R.A. Schoon, R.T. Abraham, and P.J. Leibson. 1992. Interaction between protein kinase C-dependent and G protein–dependent pathways in the regulation of natural killer cell granule exocytosis. J. Biol. Chem. 267:23957.

18. Kanakaraj, P., B. Duckworth, L. Azzoni, M. Kamoun, I.C. Cantley, and B. Perussia. 1994. Phosphatidylinositol-3 kinase activation induced upon FcRIIIA–ligand interaction. J. Exp. Med. 179:551.

19. Ward, S.G., S.C. Ley, C. Macphee, and D.A. Cantrell. 1992. Regulation of D-3 phosphoinositides during T cell activation.
via the T cell antigen receptor/CD3 complex and CD2 antigens. *Eur. J. Immunol.* 22:45.

20. Reif, K., I. Gout, M.D. Waterfield, and D.A. Cantrell. 1993. Divergent regulation of phosphatidylinositol 3-kinase p85α and p85β isoforms upon T cell activation. *J. Biol. Chem.* 268:10780.

21. Carpenter, C.L., B.C. Duckworth, K.R. Auger, B. Cohen, B.S. Schaffhausen, and L.C. Cantley. 1990. Purification and characterization of phosphoinositide 3-kinase from rat liver. *J. Biol. Chem.* 265:19704.

22. Morgan, S.J., A.D. Smith, and P.J. Parker. 1990. Purification and characterization of bovine brain type I phosphatidylinositol 3-kinase. *Eur. J. Biochem.* 191:761.

23. Shibasaki, F., Y. Hamma, and T. Takenawa. 1991. Two types of phosphatidylinositol 3-kinase from bovine thymus. *J. Biol. Chem.* 266:8108.

24. Otsu, M., I. Hiles, I. Gout, M.J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, et al. 1991. Characterization of two 85 kD proteins that associate with receptor tyrosine kinases, middle T/pp 60 c-src complexes, and PI 3-kinase. *Cell.* 65:91.

25. Escobedo, J.A., S. Navankasattus, W.M. Kavanagh, D. Milify, V.A. Freid, and L.T. Williams. 1991. cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI 3-kinase to the PDGF β-receptor. *Cell.* 65:75.

26. Hiles, I.D., M. Otsu, S. Volinia, M.J. Fry, I. Gout, R. Dhand, G. Panayotou, F. Ruiz-Larrea, A. Thompson, N.F. Totty, et al. 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kD catalytic subunit. *Cell.* 70:419.

27. Fry, M.J., and M.D. Waterfield. 1993. Structure and function of phosphatidylinositol 3-kinase: a potential second messenger system involved in growth control. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 340:337.

28. Schu, P.V., K. Takegawa, M.J. Fry, J.H. Stack, M.D. Waterfield, and S.D. Emr. 1993. Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. *Science (Wash. DC).* 260:88.

29. Joly, M., A. Kazlauskas, F.S. Fay, and S. Corvera. 1994. Disruption of PDGF receptor trafficking by mutation of its PI-3 kinase binding site. *Science (Wash. DC).* 263:684.

30. Yano, H., S. Nakashima, K. Kimura, N. Hanai, Y. Saitoh, Y. Fukui, Y. Nonomura, and Y. Matsuda. 1993. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J. Biol. Chem.* 268:25846.

31. Zhang, J., M.J. Fry, M.D. Waterfield, S. Jaken, L. Liao, J.E.B. Fox, and S.E. Rittenhouse. 1992. Activated phosphoinositide 3-kinase associates with membrane skeleton in thrombin-exposed platelets. *J. Biol. Chem.* 267:4686.

32. Toulec, D., P. Pianetti, H. Coste, P. Bellevague, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Bourrier, F. Loricelle, et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 266:15771.

33. Perussia, B., and G. Trinchieri. 1984. Antibody 3G8, specific for the human neutrophil Fc receptor, reacts with natural killer cells. *J. Immunol.* 132:1410.

34. Augustine, J.A., J.W. Schlager, and R.T. Abraham. 1990. Differential effects of interleukin-2 and interleukin-4 on protein tyrosine phosphorylation in factor-dependent murine T cells. *Biochim. Biophys. Acta.* 1052:313.

35. Augustine, J.A., S.L. Sutor, and R.T. Abraham. 1991. Interleukin 2- and polyomavirus middle T antigen–induced modification of phosphatidylinositol 3-kinase activity in activated T lymphocytes. *Mol. Cell. Biol.* 11:4431.

36. Hu, P., B. Margolis, E.Y. Skolnick, R. Lammers, A. Ulrich, and J. Schlessinger. 1992. Interaction of phosphatidylinositol 3-kinase–associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol. Cell. Biol.* 12:981.

37. Klippel, A., J.A. Escobedo, W.J. Pantl, and L.T. Williams. 1992. The C-terminal SH2 domain of p85 accounts for the high affinity and specificity of the binding of phosphatidylinositol 3-kinase to the platelet-derived growth factor β receptor. *Mol. Cell. Biol.* 12:1451.

38. McGlade, C.J., C. Ellis, M. Reedijk, D. Anderson, G. Mbalu, A.D. Reith, G. Panayotou, P. End, A. Bernstein, A. Kazlauskas, et al. 1992. SH2 domains of the p85α subunit of phosphatidylinositol 3-kinase regulate binding to growth factor receptors. *Mol. Cell. Biol.* 12:991.

39. Myers, M.G., Jr., J.M. Backel, X.J. Sun, S. Shoelson, P. Hu, J. Schlessinger, M. Yoakim, B. Schaffhausen, and M.F. White. 1992. IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85. *Proc. Natl. Acad. Sci. USA.* 89:10350.

40. Keegan, A.D., and W.E. Paul. 1992. Multichain immune recognition receptors: similarities in structure and signaling pathways. *Immunol. Today.* 13:63.

41. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187.

42. Summer, T.A., and C.E. Creutz. 1985. Phosphorylation of a chromaffin granule–binding protein by protein kinase C. *J. Biol. Chem.* 260:2437.

43. Naor, Z., H. Dan-Cohen, J. Hermon, and R. Limor. 1989. Induction of exocytosis in permeabilized pituitary cells by α- and β-type protein kinase C. *Proc. Natl. Acad. Sci. USA.* 86:4501.

44. Isosaki, M., T. Nakashima, and Y. Kuroguchi. 1991. Role of protein kinase C in catecholamine secretion from digitonin-permeabilized bovine adrenal medullary cells. *J. Biol. Chem.* 266:16703.

45. Otsawa, K., Z. Szallasi, M.G. Kazanietz, P.M. Bumberg, H. Mischak, J.F. Mushinski, and M.A. Beaven. 1993. Ca²⁺- and Ca²⁺-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. *J. Biol. Chem.* 268:1749.

46. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry.* 23:5036.

47. Secrist, J.P., I. Sehgal, G. Pwiss, and R.T. Abraham. 1990. Preferential inhibition of the platelet-derived growth factor receptor tyrosine kinase by staurosporine. *J. Biol. Chem.* 265:20394.

48. Nakashima, S., S. Kakita, K. Takahashi, K. Wakahara, T. Sano, K. Yamada, M. Yoshida, H. Kase, and Y. Matsuda. 1992. Wortmannin, a microbial product inhibitor of myosin light chain kinase. *J. Biol. Chem.* 267:2157.

49. Kupfer, A., S.J. Singer, and G. Dennert. 1986. On the mechanism of unidirectional killing in mixtures of two cytotoxic T lymphocytes. Unidirectional polarization of cytoplasmic organelles and the membrane-associated cytoskeleton in the effector cell. *J. Exp. Med.* 163:489.

50. Backer, J.M., M.G. Myers, Jr., S.E. Shoelson, D.J. Chin, X.J. Sun, M. Mirauleu, P. Hu, B. Margolis, E.Y. Skolnik, J. Schlessinger, and M.F. Waite. 1992. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimula-
51. Carpenter, C.L., K.R. Auger, M. Chanudhuri, M. Yoakim, B. Schaffhausen, S. Shoelson, and L.C. Cantley. 1993. Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. J. Biol. Chem. 268:9478.

52. Hayashi, H., S. Kamohara, Y. Nishioka, F. Kansai, N. Miyake, Y. Fukui, F. Shibasaki, T. Takenawa, and Y. Ebina. 1992. Insulin treatment stimulates the tyrosine phosphorylation of the alpha-type 85-kDa subunit of phosphatidylinositol 3-kinase in vivo. J. Biol. Chem. 267:22575.

53. Carpenter, C.L., K.R. Auger, B.C. Duckworth, W.-M. Hou, B. Schaffhausen, and L.C. Cantley. 1993. A tightly associated serine/threonine protein kinase regulates phosphoinositide 3-kinase activity. Mol. Cell. Biol. 13:1657.

54. Dhand, R., I. Hiles, G. Panayotou, S. Roche, M.J. Fry, I. Gout, N.F. Truong, P. Vicendo, K. Yonezawa, et al. 1994. PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. EMBO (Eur. Mol. Biol. Organ.). J. 13:522.

55. Prasad, K.V., O. Janssen, R. Kapeller, M. Raab, L.C. Cantley, and C.E. Rudd. 1993. Src-homology 3 domain of protein kinase p59 fyn mediates binding to phosphatidylinositol 3-kinase in T cells. Proc. Natl Acad. Sci. USA. 90:7366.

56. Prasad, K.V., R. Kapeller, O. Janssen, H. Repke, J.S. Duke-Cohan, L.C. Cantley, and C.E. Rudd. 1993. Phosphatidylinositol (PI) 3-kinase and PI 4-kinase binding to the CD4-p561ck complex: the p561ck SH3 domain binds to PI 3-kinase but not PI 4-kinase. Mol. Cell. Biol. 13:7708.

57. Pleiman, C.M., W.M. Hertz, and J.C. Cambier. 1994. Activation of phosphatidylinositol-3' kinase by src-family kinase SH3 binding to the p85 subunit. Science (Wash. DC). 263:1609.