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Aggregation of the human high affinity immunoglobulin G receptor (FcγRI) activates both tyrosine kinase and G protein-coupled phosphoinositide 3-kinase isoforms

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ABSTRACT **Phosphoinositide 3-kinases (PI3-kinases) play an important role in the generation of lipid second messengers and the transduction of a myriad of biological responses. Distinct isoforms have been shown to be exclusively activated either by tyrosine kinase-coupled or G protein-coupled receptors. We show here, however, that certain nonclassical receptors can couple to both tyrosine kinase- and G protein-dependent isoforms of PI3-kinase: thus, aggregation of FcγRI, the human high affinity IgG receptor, on monocytes unusually leads to activation of both of these types of PI3-kinase. After aggregation of FcγRI, phosphatidylinositol 3,4,5-triphosphate (PIP_3) levels rise rapidly in interferon γ-primed cells, reaching a peak within 30 sec. Moreover, and in contrast to the situation observed after stimulation of these cells with either insulin or ATP, which exclusively activate the tyrosine kinase- and G protein-coupled forms of PI3-kinase, respectively, PIP_3 levels remain elevated up to 15 min after receptor aggregation. We show here that although the initial peak results from transient activation of the p85-dependent p110 isoform of PI-3kinase, presumably through recruitment of tyrosine kinases by the γ chain, the later sustained rise of PIP_3 results from activation of the G protein βγ subunit-sensitive isoform, p110γ. This finding indicates that receptors lacking an intrinsic signaling motif, such as FcγRI, can recruit both tyrosine kinase and G protein-coupled intracellular signaling molecules and thereby initiate cellular responses.**

Fc receptors (FcγRs) specific for IgG are expressed on the surface of many different cell types of the immune system and play an important role in linking the cellular and humoral arms of the immune response (1–3). On myeloid cells aggregation of FcγRs leads to a number of cellular responses, including the internalization of immune complexes by endocytosis or opsonized particles through phagocytosis, degranulation, and the release of proteases, activation of the respiratory burst, and the release of cytokines. These processes can lead to targeted cell killing through antibody-directed cellular cytotoxicity (4, 5), which is important for the clearance of virus-infected cells and in cancer surveillance (6).

Three classes of FcγRs have been identified and cloned in mammals (FcγRI, FcγRII, and FcγRIII), each of which has a variety of isoforms with differing affinities for IgG and tissue distributions (1, 2). FcγRI is the human high affinity receptor (7) and although its cytoplasmic tail contains no obvious signaling motif, FcγRI has been shown to associate physically with the immunoreceptor tyrosine activation motif (ITAM)-containing γ chain (8, 9), and FcγRI crosslinking results in signal transduction as evidenced by tyrosine phosphorylation events (10–13) and tyrosine kinase-dependent calcium transients (14, 15).

Phosphoinositide 3-kinases (PI3-kinases) catalyze the phosphorylation of inositol phospholipids at the 3-position of the inositol ring (16). The phospholipids produced by the actions of these enzymes: PtdIns(3)P, PtdIns(3,4)P_2 (PIP_2), and PtdIns(3,4,5)P_3 (PIP_3), act as second messengers that are increasingly implicated in regulating a number of cellular responses. For example, PI3-kinase activity has been implicated as being involved in insulin-induced glucose transport (17), platelet-derived growth factor, and insulin-induced actin rearrangement (18, 19) and in the regulation of neuronal survival (20).

There are three classes of PI3-kinases that are determined by their *in vitro* substrate specificities. Class I PI3-kinases phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2. This class of enzymes can be further subdivided into class IA and class IB. Class IA PI3-kinases consist of p110 catalytic subunits that are regulated by the Src homology 2 (SH2)/SH3-domain containing the p85 family of adaptor proteins and consist of the isoforms p110α, p110β, and p110δ (21–23). The p85 family of adaptor proteins (24–26) facilitate PI3-kinase interactions with other proteins through their SH2 and SH3 domains. Class IB consists of p110γ, an enzyme that associates with a p101 adaptor protein and is stimulated by G protein βγ subunits (27–30). Class II PI3-kinases phosphorylate PtdIns and PtdIns(4)P but not PtdIns(4,5)P_2 (31, 32), and class III PI3-kinases have a substrate specificity restricted to PtdIns and are homologous to yeast Vps34p (vacuolar protein sorting defective), which is involved in the trafficking of proteins from the Golgi to the yeast vacuole (33–35).

Previous studies have shown that receptors that are coupled to tyrosine kinases, such as insulin, exclusively activate class IA PI3-kinases through the p85 adaptor molecule. In contrast, receptors that engage heterotrimeric G proteins, such as ATP, exclusively activate class IB PI3-kinases through the generation of βγ subunits. No crosstalk between the coupling of these two receptor to PI3-kinases has been shown. We report here an example of a single receptor, FcγRI, which has the capacity to activate both class IA and class IB PI3-kinases.

MATERIALS AND METHODS

Materials. Human IgG and sheep-anti-human IgG (γ chain specific) were obtained from Serotec. Potassium oxalate and all phospholipids were from Sigma. [^{32}P]orthophosphate (HCl

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Abbreviations: FcγRI, high affinity Ig G receptor; ITAM, immunoreceptor tyrosine activation motif; PI3-kinase, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol 3,4,5-triphosphate; IFN-γ, interferon γ; SH, Src homology; IPTG, isopropyl β-D-thiogalactoside.

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free) was obtained from Amersham. Wortmannin, lavendustin A, and tyrphostins A23 and A25 were obtained from Calbiochem, and genistein was from Sigma. Cells were treated with inhibitors at concentrations specified for 30 min at 37°C before receptor aggregation or stimulation.

Antisense oligonucleotides were purchased from Oswell DNA Services (Southampton, UK). Anti-p110γ oligonucleotides corresponded to the first eight (24 mer) or 10 (30 mer) amino acids of p110γ. The sequences of these oligonucleotides were 5′-CTGTTTATAGTTCTCCAGCTCCAT-3′ and 5′-CACGGGCTGTTTATAGTTCTCCAGCTCCAT-3′. A control oligonucleotide (24 mer) of random sequence also was made, the sequence of which was 5′-CTGGTGGAAGAAGAGGACGTCCAT-3′. The 24 mers were capped at either end by phosphothiorate linkages (first two and last two linkages) whereas each linkage of the 30 mer consisted of a phosphothiorate. Cells were incubated with 10 μM of oligonucleotide for 1 hr before, and then for the duration of culture with interferon γ (IFN-γ).

Cell Culture. U937 cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, 2 mM glutamine, 10 units/ml of penicillin, and 10 mg/ml of streptomycin at 37°C, 6.8% carbon dioxide in a water-saturated atmosphere. U937:Δp85 cells (a generous gift from L. Stephens, Babraham Institute, Cambridge, UK) were similarly cultured, but in addition were cultured in the presence of 0.6 mg/ml of G418 and 0.1 mg/ml of hygromycin B (both from Calbiochem). Expression of Δp85 was induced with 15 mM isopropyl β-D-thiogalactoside (IPTG), 5 nM phorbol 12-myristate 13-acetate, and 100 μM zinc chloride for 10 hr. All cells were primed with 200 ng/ml of IFN-γ (a kind gift from Bender Wein Ltd., Vienna, Austria) for 24 hr before experimentation.

FcγRI Crosslinking. Cells were harvested by centrifugation and then incubated at 4°C with 1 μM human monomeric IgG (Serotec) to occupy surface FcγRI. Excess unbound ligand was removed by dilution and centrifugation of the cells. Cells were resuspended in ice-cold RPMI 1640 medium/10 mM Hepes/0.1% BSA, and crosslinking antibody (goat anti-human IgG; 1:50 dilution) was added. Cells then were warmed to 37°C for the times specified in the assays.

Insulin and ATP Stimulation. Cells were harvested by centrifugation, resuspended in ice-cold RPMI 1640 medium/10 mM Hepes/0.1% BSA, and incubated with either 10 μg/ml of insulin or 100 μM ATP. Cells then were warmed to 37°C for the times specified in the assays.

Pertussis Toxin Activation and Culture. Pertussis toxin (90 μg/ml) was activated by incubation in 25 mM DTT for 30 min at 37°C. The concentration then was adjusted to 60 μg/ml with 75 mM Tris·HCl, pH 7.5 containing 1 mg/ml BSA. Pertussis toxin was then incubated overnight with cells at a final concentration of 1 μg/ml (36).

Measurement of PI3-Kinase Activity. U937 cells (2×10^7 cells/ml) were treated with IFN-γ as described and harvested by centrifugation. Cells then were washed in phosphate-free RPMI and resuspended in phosphate-free RPMI containing 10% dialyzed fetal calf serum. Cells were labeled with 500 μCi/ml [^{32}P]PO_4 for 90 min at 37°C. After labeling, cells were washed in ice-cold RPMI 1640 medium/10 mM Hepes/0.1% BSA. Cells were stimulated with either insulin or ATP, or FcγRI crosslinked, and the reactions stopped at specified times with ice-cold PBS. Cells were permeabilized with methanol, and the lipids were extracted with chloroform. Aliquots from the organic phase were used to analyze total label incorporation, and duplicate aliquots were dried down under vacuum, resuspended in chloroform/methanol (19:1), PIP_2 (10 μg/ml) standard added, and spotted onto a silica TLC G60 plate (20 × 20 cm × 250 μm; Whatman) precoated with potassium oxalate. Plates were developed in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14), and the standards were visualized with iodine vapors. Radiolabeled bands were located by autoradiography or PhosphorImaging, and the PIP_3 band was scraped into scintillation vials, scintillant was added, and the associated radioactivity was determined by liquid scintillation counting. Quantification of labeled PIP_3 by PhosphorImaging or scintillation counting always yielded the same ratio of PIP_3 generation.

Western Blot. U937:Δp85 cells were induced to express Δp85 as described earlier. Noninduced or induced cells (10^7) were harvested by centrifugation and solubilized in sample buffer (37) containing 50 mM DTT. Samples were boiled for 15 min and run in a 10% SDS/polyacrylamide gel (38). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.2 μM pore size) as described (39). The blots were tested for the overexpression of Δp85 by Western blotting with a polyclonal anti-p85α antibody (Santa Cruz Biotechnology). Western blots were developed by using the ECL system (Amersham).

Immunoprecipitation. For immunoprecipitations, cells were lysed with ice-cold RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of CLAP (1 μg/ml each chymostatin, leupeptin, antipain, and pepstatin) 1 mM sodium orthophosphate, and 1 mM sodium fluoride for 30 min. Cellular debris was removed by centrifugation at 13,000 rpm for 15 min, and the cell lysates were precleared with protein-A-agarose. Cell lysates then were either incubated with 1 μg anti-p110 γ (Santa Cruz Biotechnology) followed by 10 μg protein A-agarose (Santa Cruz Biotechnology) or 10 μg of anti-p85-PI3 kinase conjugated to 10 μg of protein A-agarose (Upstate Biotechnology, Lake Placid, NY) to immunoprecipitate p85 and p110γ.

For the studies of tyrosine phosphorylation of p85, IFN-γ-treated cells were loaded with human IgG and crosslinked as described. Cells were harvested at specified times and lysed, and p85 was immunoprecipitated from the cell lysates by incubating 500 μl of cell lysate with 10 μg of anti-p85 PI3 kinase conjugated to protein A-agarose overnight. Proteins were harvested by centrifugation of the agarose beads and dissociated from the beads by boiling in sample buffer (37) containing 50 mM DTT for 15 min and run in a 10% SDS/polyacrylamide gel (38). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.2 μM pore size) as described (39). The presence of tyrosine phosphorylated proteins then was detected by Western blotting with a monclonal antiphosphotyrosine antibody (clone 4G 10; Upstate Biotechnology). Western blots were developed by using the ECL system (Amersham).

For the quantitation of the effect of antisense oligonucleotides on p110γ levels, cells were incubated with the relevant oligonucleotides as described. Cells were harvested and lysed, and p110γ was immunoprecipitated from the cell lysates. Proteins were harvested and subjected to SDS/PAGE as above. Blots were probed for the presence of p110γ and visualized by probing with radiolabeled (^{125}I) donkey anti-goat Ig. Specific bands corresponding to p110γ were quantified by using a PhosphorImager (Fuji, FUJIX BAS 1000).

RESULTS

Crosslinking FcγRI Stimulates PI3-Kinase Activity. After the formation of surface immune complexes in IFN-γ-primed U937 cells, the cellular levels of PIP_3 rose rapidly, reaching a peak 30 sec after receptor aggregation (Fig. 1*A*). After this initial peak, PIP_3 levels were reduced but remained elevated about 2-fold above control (noncrosslinked cells) up to 2 min after crosslinking (Fig. 1*A*). This 2-fold stimulation above basal levels was sustained and remained elevated 15 min after receptor crosslinking (data not shown). All of this stimulated increase in PIP_3 levels could be abolished by treating the cells

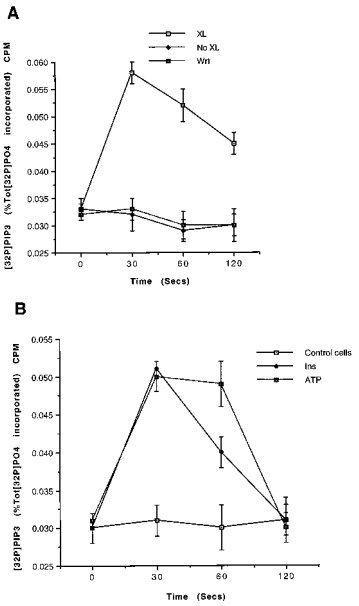

FIG. 1. PI3 kinase is activated by FcγRI aggregation in IFN-γ U937 cells. The time course of activation differs from that observed for either tyrosine kinase or G protein-stimulated PI3-kinase. (*A*) PIP_3 production in IFN-γ primed U937 cells after aggregation of FcγRI (XL). Concentrations were compared with cells in which no crosslinking antibody (No XL) was added and in cells treated with 50 nM wortmannin (Wrt). (*B*) PIP_3 concentrations in IFN-γ-primed U937 cells after stimulation of tyrosine kinase activated PI3-kinase with insulin (10 μg/ml) or G protein-activated PI3-kinase with ATP (100 μM). Data are the mean ± SD of triplicate measurements for each time point and are derived from three separate experiments.

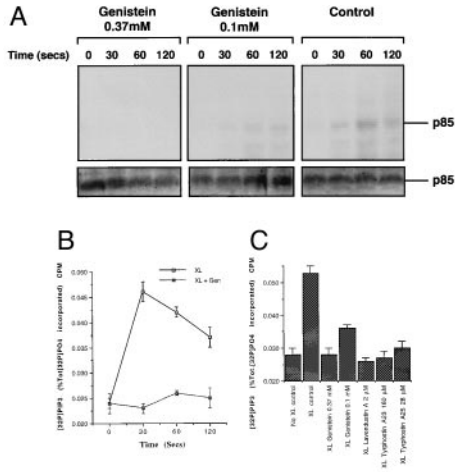

FIG. 2. Inhibition of tyrosine kinase activity blocks FcγRI-stimulated PI3 kinase activity. (*A*) Western blot analysis of immunoprecipitates of p85 probed with antiphosphotyrosine (4G10) antibody. Cells were cultured in the absence or presence of 0.1 mM genistein or 0.37 mM genistein for 30 min before aggregation of FcγRI for 0, 30, 60, or 120 sec. p85 immunoprecipitates were resolved by SDS/PAGE and probed for presence of phosphotyrosine (*Upper*). The blot then was stripped and probed with anti-p85 (*Lower*). (*B*) PIP_3 production was measured in cells pretreated with genistein (0.37 mM) for 30 min before aggregation of FcγRI (XL + Gen) and compared with untreated cells (XL). Data are the mean ± SD of triplicate measurements for each time point and are derived from five separate experiments. (*C*) Peak PIP_3 production (30 sec) was measured in cells pretreated for 30 min with various tyrosine kinase inhibitors and compared with control levels (XL control). Genistein was used at two concentrations (0.37 mM and 0.1 mM); lavendustin A (2 μM), tyrphostin A23 (160 μM), and tyrphostin A25 (28 μM). Data are the mean ± SD of triplicate measurements.

before receptor aggregation with the PI3-kinase specific inhibitor wortmannin (50 nM) (Fig. 1*A*).

Previous studies have shown that insulin and ATP stimulate distinct PI3-kinases in U937 cells (27, 40), insulin being coupled to the p85-dependent PI3-kinase whereas ATP activates the G protein βγ subunit-stimulated PI3-kinase. The response observed for FcγRI aggregation therefore was compared in these same cells to that observed for insulin and ATP. The sustained rise in PIP_3 observed after FcγRI aggregation was not observed for either insulin or ATP. Thus, insulin stimulated a rapid, transient increase of PIP_3, which quickly returned almost to basal levels within 2 min of receptor stimulation (Fig. 1*B*). The time course for PIP_3 levels after ATP stimulation were similar to that for insulin although the reduction after the peak was less rapid than that observed for insulin but, unlike FcγRI aggregation, levels reached basal within 2 min.

FcγRI Crosslinking Stimulates Tyrosine Kinase-Dependent PI3-Kinase Activities. As FcγRI has been shown to signal through the recruitment of nonreceptor tyrosine kinases (41, 42), the relationship of PI3-kinase to tyrosine kinase activation was investigated. The p85 subunit was rapidly tyrosine-phosphorylated after FcγRI aggregation as phosphotyrosine could be detected within 30 sec of adding crosslinking antibody to the cells. This phosphorylation was attenuated in cells pretreated with 0.1 mM genistein and was abolished in cells treated with 0.37 mM genistein (Fig. 2*A*).

The effect of inhibiting tyrosine kinases on PI3-kinase activation was measured. Preincubation of cells for 30 min with genistein (0.37 mM) completely blocked the increase of PIP_3 after receptor aggregation (Fig. 2*B*). Consistent with the phosphotyrosine data, the lower concentration of genistein (0.1 mM) did not abolish activity but reduced peak PI3 kinase activity by 68 ± 4%. Three other tyrosine kinase inhibitors (2 μM lavendustinA, 160 μM tyrophostin A23, or 28 μM tyrphostin A25) all inhibited peak PI3 kinase activity (Fig. 2*C*).

These data indicate that all of the increase in PI3-kinase activity after FcγRI aggregation is downstream of the activation of tyrosine kinases.

FcγRI Crosslinking Stimulates p85-Dependent and p85-Independent PI3-Kinase Activities. To investigate whether the PI3-kinase activity detected on FcγRI stimulation was caused by the activation of a p85-dependent PI3-kinase, the type usually associated with tyrosine kinase activation, a U937 cell line was used that had been stably transfected with an IPTG-inducible dominant-negative form of p85 (U937:Δp85). This dominant-negative protein lacks the binding site for the p110 catalytic subunit of PI3-kinase and so when overexpressed will oblate p85-mediated PI3-kinase associations with other signaling components (40).

First, the kinetics of PIP_3 production were compared between the wild-type U937 cells and the noninduced U937:Δp85 cells. This comparison showed that in both cell types the temporal pattern of stimulation of PIP_3 levels in the cells was identical. Thus, peak levels were observed at 30 sec but values in both cell types remained elevated over 2 min (Fig. 3). However, after overexpression of Δp85, the pattern of PI3-kinase stimulation was significantly altered (Fig. 3). In U937:Δp85 cells induced to express Δp85, the peak level of PIP_3 was delayed; significant stimulation was detectable only after 1 min (Fig. 3). However, 2 min after receptor aggregation similar levels of PIP_3 levels were measured for both Δp85-expressing and wild-type cells (Fig. 3). Overall, the amount of PIP_3 generated after receptor aggregation was reduced, and this reduced level of PI3-kinase was commensurate with the loss of the early, rapid peak of PI3-kinase activity observed in p85-functional cells.

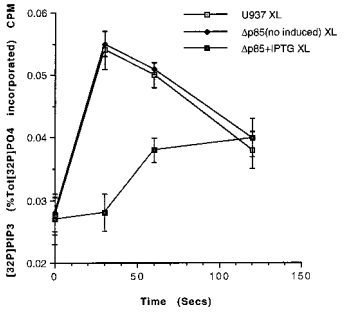

FIG. 3. Expression of a dominant-negative form of p85, Δp85, abolishes only the early peak of PIP_3 observed after FcγRI aggregation. The time course of FcγRI-stimulated PIP_3 concentrations were measured in cells induced to express to dominant-negative form of p85 (Δp85 + IPTG XL) and compared with the same transformed cells not induced to express [Δp85 (no induced) XL] and wild-type U937 cells (U937 XL). Data are the mean ± SD of triplicate measurements for each time point and are derived from three separate experiments.

The correct functioning of the dominant-negative Δp85 protein was shown in two ways. First, overexpression of the truncated Δp85 was verified by Western blot analysis, which showed the truncated form of p85 only after induction with IPTG (Fig. 4*A*). The functional dominant-negative role for this truncated p85 was verified by assaying PI3-kinase activity after stimulation of the cells by insulin or ATP. In keeping with the known requirement of insulin for p85 to couple to PI3-kinase, induction of the dominant-negative Δp85 in these cells completely abolished the expected rise in PIP_3 after insulin treatment (Fig. 4*B*). In contrast, the response to ATP was entirely unaffected by the presence of the truncated p85 (Fig. 4*C*). This finding is consistent with the fact that insulin exclusively stimulates p85-mediated PI3-kinase in response to tyrosine phosphorylation events whereas ATP exclusively stimulates p110γ, the G protein βγ-stimulated (p85-independent) PI3-kinase isoform. The data also clearly support previous observations on the exclusive coupling of these two types of receptors to distinct PI3-kinase isoforms.

Taken together, these results suggest that FcγRI aggregation stimulates both a p85-dependent PI3-kinase and a p85-independent PI3-kinase. The temporal relationship of the response in the Δp85 cells suggests that the p85-dependent activity is responsible for the rapid and transient peak of PI3-kinase activity, and the p85-independent activity is responsible for the delayed activity observed 2 min after crosslinking.

FcγRI Crosslinking Activates p110γ, a G Protein βγ Subunit-Dependent PI3-Kinase. Because it appeared that aggregation of FcγRI stimulated both p85-dependent- and p85-independent activities, we next investigated whether the p85-independent activity could be caused by the stimulation of the G protein βγ subunit-dependent PI3-kinase, p110γ. This enzyme previously has been purified and cloned from U937 cells (27, 28) and pig neutrophils (29, 30). To do this, antisense oligonucleotides were designed complementary to the N terminal-encoding region of p110γ mRNA to knock out the expression of this enzyme. Two oligonucleotides were designed; a 30-mer that included a phosphothiorate group at each linkage and a 24-mer that had only phosphothiorate links at either end. Both of these oligonucleotides significantly inhibited ATP-stimulated PI3-kinase activity in U937 cells, an activity known to be attributed to p110γ (Fig. 5*A*; refs. 27 and 43). In cells loaded with the short oligonucleotide, peak ATP stimulated PI3 kinase activity was reduced to 52 ± 6% of control, whereas in cells loaded with the 30-mer peak kinase activity was reduced to 25 ± 2% of control. Consistent with this observation, the level of p110γ was reduced by equivalent amounts in cells pretreated with the antisense oligonucleotides

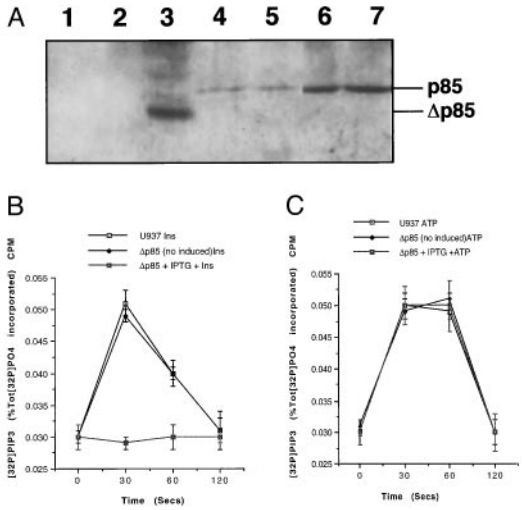

FIG. 4. Verification that Δp85 is expressed and functionally active. (*A*) Western blot analysis of cells induced to overexpress Δp85 compared with noninduced cells. Whole-cell extracts were run in a 10% SDS/polyacrylamide gel. Proteins then were transferred to nitrocellulose and blotted with an anti-p85 polyclonal antibody as described in *Materials and Methods*. Lane 1, IFN-γ-primed U937 cells. Lane 2, IFN-γ-primed U937:Δp85 cells noninduced for the expression of Δp85. Lane 3, IFN-γ-primed U937:Δp85 cells induced to overexpress Δp85. Lanes 4 and 5, loading of twice amount of protein compared with lanes 1–3 (lane 4, IFN-γ-primed U937 cells; lane 5, IFN-γ-primed U937:Δp85 cells noninduced for the expression of Δp85); Lanes 6 and 7, loading of four times amount of protein compared with lanes 1–3 (lane 6, IFN-γ-primed U937 cells; lane 7, IFN-γ-primed U937:Δp85 cells noninduced for the expression of Δp85). Bands corresponding to wild-type constitutive p85 and Δp85 are indicated. (*B*) PI3-kinase coupled to tyrosine kinase activation was inhibited in cells expressing the dominant-negative, Δp85. Production of PIP_3 in induced (Δp85 + IPTG + Ins), uninduced cells [Δp85 (no induced) Ins] and wild-type cells (U937 Ins) after treatment with insulin (10 μg/ml). Data are the mean ± SD of triplicate measurements for each time point and are derived from four separate experiments. (*C*) Activation of G protein-coupled PI3-kinase was unaffected by the expression of the dominant-negative p85. Production of PIP_3 in induced (Δp85 + IPTG + ATP), uninduced cells [Δp85 (no induced) ATP], and wild-type cells (U937 ATP) after treatment with ATP (10 μM). Data are the mean ± SD of triplicate measurements for each time point and are derived from four separate experiments.

(cells treated with short oligonucleotide 57% of control; cells treated with 30-mer 28% of control). In contrast, a control oligonucleotide of random DNA sequence had no effect on ATP-stimulated PI3-kinase activity (Fig. 5*A*) or protein expression levels (cells treated with control oligonucleotide 105% of control). The longer of the two p110γ antisense oligonucleotides was found to be the slightly more effective of the two oligonucleotides, probably because it was more resistant to nuclease attack. Neither oligonucleotide influenced the rise in PIP_3 observed after insulin stimulation (Fig. 5*B*), findings consistent with the fact that the insulin receptor couples exclusively to the p85-dependent PI3-kinase.

Treatment of the IFN-γ-primed U937cells with p110γ antisense oligonucleotides altered the pattern of PI3-kinase stimulated activity after FcγRI aggregation (Fig. 5*C*). The initial rapid increase in PIP_3 levels previously observed 30 sec after receptor aggregation remained unaltered. However, in marked contrast to control oligonucleotide treated and untreated cells, PIP_3 levels had returned to basal levels within 1 min; kinetics reminiscent of the exclusive coupling of the insulin receptor to the p85 system. Indeed, these results essentially are the inverse of those obtained with the Δp85-expressing cells where the initial peak of activation was lost but stimulation remained elevated after 1 min (Fig. 3).

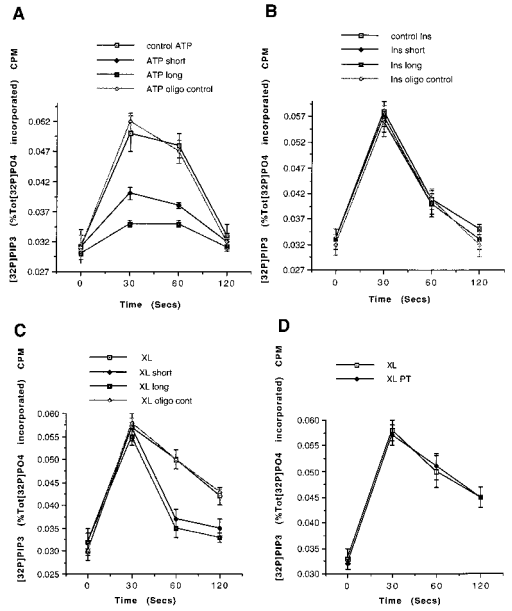

FIG. 5. Treatment of cells with an antisense oligonucleotide to p110γ selectively abolishes ATP-stimulated PI3-kinase and inhibits the delayed PI3-kinase activity after FcγRI aggregation. (*A*) Treatment of cells with either antisense oligonucleotide to p110γ (ATP short and ATP long) significantly reduced PIP_3 after stimulation with ATP (100 μM) (control ATP). A control jumbled oligonucleotide had no effect on ATP-stimulated PIP_3 levels (ATP oligo control). Data are the mean ± SD of triplicate measurements for each time point and are derived from three separate experiments. (*B*) Treatment of cells with either antisense oligonucleotide to p110γ (Ins short and Ins long) had no influence on the PIP_3 concentrations achieved after stimulation with insulin (10 μg/ml). A control jumbled oligonucleotide had no effect on insulin-stimulated PIP_3 levels (Ins oligo control). Data are the mean ± SD of triplicate measurements for each time point and are derived from three separate experiments. (*C*) The initial peak in PIP_3 observed 30 sec after FcγRI aggregation was unaffected in cells treated with the p110γ antisense oligonucleotides. The delayed sustained rise in PIP_3 observed 2 min after FcγRI aggregation (XL) was abolished by treating cells with this antisense oligonucleotide (XL short and XL long). The control jumbled oligonucleotide had no effect on any aspect of PIP_3 levels after FcγRI aggregation (XL oligo cont). Data are the mean ± SD of triplicate measurements for each time point and are derived from three separate experiments. (*D*) Treatment of cells with pertussis toxin (XL PT) had no influence on PIP_3 concentrations observed after FcγRI aggregation (XL). Data are the mean ± SD of triplicate measurements for each time point and are derived from three separate experiments.

Taken together these results indicate that FcγRI crosslinking results in initial activation of a p85-dependent PI3-kinase that lasts less than 1 min, and this action is followed by the stimulation of a G protein $\beta\gamma$ subunit-dependent PI3-kinase (p110γ). This latter activity was found to be insensitive to pertussis toxin (Fig. 5*D*) and so was unlikely to have been activated by G proteins of the G_i type.

DISCUSSION

We show that ligation of a single receptor can induce coupling to two distinct classes of PI-3-kinase: levels of PIP_3 rise very rapidly after aggregation of FcγRI in IFN-γ-primed U937 cells because of the rapid and transient activation of a p85-dependent PI3-kinase. In contrast, the sustained elevation of PIP_3 observed after receptor aggregation appears to result from a delayed, longer-lasting, p85-independent PI3-kinase activity. This latter activity was shown to be caused by the activation of p110γ, a G protein $\beta\gamma$ subunit-sensitive form of PI3-kinase (28, 29). This finding demonstrates a single receptor stimulating both tyrosine kinase and G protein-regulated PI3-kinases.

Activation of PI3-kinase appears necessary to mediate some of the effector functions for Fc receptors. Thus, inhibition of PI3-kinase with wortmannin inhibits antibody-dependent phagocytosis in U937 cells and COS cells (44, 45). It is interesting to note that although wortmannin inhibits Fc receptor-mediated phagocytosis in U937 cells, it will not block endocytosis of immune complexes in these cells (data not shown). Previous studies have shown that PI3-kinase activity is increased in immunoprecipitates by using anti-p85 and antiphosphotyrosine antibodies in U937 cells after aggregation of FcγRI and FcγRII. This activation seemed to be secondary to tyrosine phosphorylation. Consistent with this, the data presented in this paper showed that the tyrosine kinase inhibitor, genistein, was able to block completely PI3-kinase activation (Fig. 2). Similarly inhibition of tyrosine kinases with genistein previously has been shown to inhibit FcγRI-mediated phagocytosis in transiently transfected COS cells, but not FcγRI-mediated endocytosis in the same cells and in U937 cells (41, 46). Taken together, these results are consistent and suggest that PI3-kinase activation as a result of tyrosine phosphorylation events is essential for FcγRI-mediated phagocytosis, but that endocytosis of immune complexes by FcγRI is independent of these signaling events.

The question relating to how FcγRI couples to both a p85-dependent and a G protein subunit-sensitive PI3-kinase remains to be answered. FcγRI contains no known signaling motif but has to recruit an accessory signaling molecule. In IFN-γ-treated U937 cells, FcγRI recruits the γ chain, which contains a cytoplasmic ITAM (8, 9). It therefore seems likely that the p85-dependent PI3-kinase activity is a result of a direct interaction between the SH2 domain of p85 and the ITAM of the γ chain, probably after tyrosine phosphorylation of the ITAM by a src-type kinase or Syk (for review see ref. 47). Indeed, the p85 regulatory subunit of PI3-kinase has been shown to bind with phosphopeptides of the γ chain ITAMs (48) but there is also evidence to suggest that p85 can directly interact with phosphorylated Syk via a YxxM motif on the Syk protein. It has been proposed for high affinity IgE receptor signaling that Lyn becomes activated on receptor clustering and tyrosine phophorylates the ITAMs of the γ chain, initiating the recruitment of Syk through its SH2 domain. Phosphorylated Syk then can activate other downstream signaling molecules, including PI3-kinase (49). Recent studies have shown that Lyn is associated with both FcγRI and the γ chain independently of receptor crosslinking and that the level of the phosphorylation of this src-type kinase increases upon receptor crosslinking (42). These studies also showed that Syk associates with the γ chain after FcγRI aggregation and the resultant phosphorylation of the γ chain. The p85 regulatory subunit of PI3-kinase has been shown to bind with phosphopeptides of the γ chain ITAMs (48). Therefore it is probable that the p85-dependent PI3-kinase activity is recruited as a result of FcγRI recruiting the γ chain and subsequent tyrosine kinases, notably Lyn and Syk.

How FcγRI might recruit a heterotrimeric G protein is less clear. The results presented here show that all PI3-kinase activity stimulated after FcγRI aggregation was abolished by pretreating cells with genistein (Fig. 2). This finding suggests that the activation of p110γ also occurs downstream of the activation of tyrosine kinases. It is possible that members of the Tec family of tyrosine kinases, for example Tec, Itk, or Btk, could play a role in the activation of p110γ via a heterotrimeric G protein because this family of enzymes contains SH2 and Pleckstrin homology domains. It is feasible that the SH2 domain of a Tec-type tyrosine kinase could associate with the γ chain (or a tyrosine-phosphorylated protein such as Syk) and recruit a heterotrimeric G protein while at the plasma mem-

brane. This G protein then could activate p110γ via its $\beta\gamma$ subunits.

In conclusion we have shown in the present paper that two distinct PI3-kinase isoforms are activated after FcγRI aggregation in IFN-γ-primed U937 cells; both p85-regulated and G protein $\beta\gamma$ subunit-sensitive PI3-kinase isoforms are activated. This suggests that receptors such as FcγRI, which do not have an intrinsic signaling motif, can recruit both tyrosine kinase and G protein-coupled intracellular signaling molecules to initiate distinct cellular responses. It has yet to be elucidated as to how FcγRI is able to recruit these different regulatory molecules and whether there are distinct physiological roles for the two PI3-kinases and their products.

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