The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not all, growth factors (microinjection/protein-tyrosine kinases/signal transduction)

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ABSTRACT The phosphatidylinositol 3-kinase (PI 3-K) becomes activated when quiescent cells are stimulated with a variety of growth factors. We have microinjected antibodies specific for the p110α subunit of the PI 3-K into quiescent fibroblasts and tested their effect on the ability of growth factors to stimulate exit from quiescence and entry into S phase. The antibodies inhibited platelet-derived growth factor-induced DNA synthesis, a result in keeping with previous studies using mutant platelet-derived growth factor receptors. Interestingly, functional PI 3-K was required for the first 6 hr of G1—i.e., until ~4 hr before the point at which the cells were committed to make DNA. A second tyrosine kinase receptor, the epidermal growth factor receptor, also required the PI 3-K for efficient signaling. However, colony-stimulating factor 1 (whose receptor is highly related to the platelet-derived growth factor receptor) could induce DNA synthesis in the absence of active PI 3-K, as could two growth factors (bombesin and lysophosphatidic acid) whose receptors are functionally coupled to G proteins. These data, therefore, demonstrate that some, but not all, growth factors require functional PI 3-K.

The phosphatidylinositol 3-kinase (PI 3-K) associates with several proteins, including growth factor receptors, nonreceptor tyrosine kinases of the Src family, and ckr and abl oncogene products (for review, see ref. 1). PI 3-K phosphorylates inositolphospholipids at the D3 position of the inositol ring, leading to the formation of phosphatidinositol 3-phosphate, phosphatidinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (2), which are poor substrates for any known phospholipase C (3, 4) and may form a new class of second messenger (1). PI 3-K activation and 3-phosphorylated inositophospholipid formation occur rapidly after growth factor activation (5, 6); thus PI 3-K activation and the formation of its products could be important steps in mitogenic signal transduction. PI 3-K usually consists of a heterodimer of an 85-kDa and a 110-kDa subunit. There are at least two 85-kDa proteins (p85α and p85β), which contain one Src homology region 3 (SH3) domain and two Src homology domain 2 (SH2) domains (for review, see ref. 7). The latter bind with high specificity and affinity to tyrosine phosphorylated sequences (8); indeed, p85α associates with growth factor receptors and the mT-cSrc complex via its SH2 domains (9–12). The catalytic activity resides in the 110-kDa subunit, which has similarity to a Saccharomyces cerevisiae protein, VPS34 (13), that has been implicated in targeting of proteins to the yeast vacuole and vacuole morphogenesis during budding (14), leading to speculation on a possible role of PI 3-K in protein trafficking and vesicular morphogenesis (7). A second catalytic subunit, called p110β, has recently been described (15); little is known about its biological activity.

The binding sites for the SH2 domains of PI 3-K are in the kinase insert region of the platelet-derived growth factor (PDGF) β receptor (Tyr-740 and Tyr-751 in the human receptor). Mutant receptors in which one or both of these tyrosines are replaced with phenylalanine are at least partially defective in signaling (16, 17). However, the recent demonstration that another protein, the adaptor protein and protooncogene Nck, also associates with the PDGF β receptor via Tyr-751 (18) has complicated the analysis of these data. A highly related receptor, the colony-stimulating factor 1 (CSF-1) receptor, also associates with PI 3-K (19) via a tyrosine in its kinase insert region (20, 21), but mutation of this binding site does not always affect CSF-1 signaling—at least in heterologous cell types (20–22). For other receptors, the binding site for PI 3-K is not known, and so mutational analysis has not been possible.

We recently described a microinjection approach to investigate the role of Src family protein-tyrosine kinases in PDGF signal-transduction pathways (23). We showed that microinjection of either dominant interfering forms of these kinases or an antibody specific for them inhibited DNA synthesis in response to PDGF. The dominant interfering approach, like the mutant receptor approach, cannot distinguish between the requirement for two proteins that bind to the same site. However antibody microinjection allows the function of a given protein to be assessed, without affecting other proteins that may share the same binding site. For this reason, we tested the ability of two antibodies specific for the catalytic subunit of the PI 3-K to interfere with growth factor-initiated signaling.

MATERIALS AND METHODS

Generation of PI 3-K Antibodies and Their Characterization. Synthetic peptides were generated that corresponded to amino acids 1054–1068 (p110.1) and 776–791 (p110.2) of p110α and antibodies to each raised (13, 24). For immunoperoxidase purification, ammonium sulfate-precipitated sera were loaded onto Sepharose columns to which cognate peptides were coupled (using cyanogen bromide-activated Sepharose and protocols from Pharmacia), and the column was washed with 10 mM sodium phosphate/500 mM NaCl buffer, pH 7.0. Antibody was eluted with 1 M propionic acid; fractions (0.5 ml) were then collected into 1 M ice-cold sodium phosphate buffer, pH 7.0 (0.5 ml), and concentrated by using a Minicon microconcentrator (Amicon) in phosphate-buffered saline. Premune IgGs were purified from sera collected from the rabbits before immunization using a protein G-Sepharose column.

Abbreviations: PI 3-K, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; EGFr, epidermal growth factor; LPA, lysophosphatidic acid; SH2 and SH3, Src homology regions 2 and 3, respectively; BrdU, 5-bromodeoxyuridine; CSF-1, colony-stimulating factor 1.

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column (Pharmacia). The antibodies were filtered and stored at −70°C.

To measure the effect of purified antibodies on the basal and activated forms of PI 3-K, 20 ng of the purified enzyme was incubated with purified antibodies (2 μg) in 20 mM Hepes, pH 7.5/1 mM dithiothreitol/150 mM NaCl/5 mM MgCl₂ at 4°C for 30 min. Then peptide (200 μM) corresponding to amino acids 741–756 of the PDGF receptor (75I) or the same concentration of peptide in which the tyrosine corresponding to residue 751 was phosphorylated (75IP) was added and incubated for a further 30 min at 4°C. PI 3-K activity and immunoblotting were as described (24).

**Growth Factors.** The growth factors and concentrations used were PDGF (25 ng/ml), Upstate Biotechnology (Lake Placid, NY); epidermal growth factor (EGF) (100 ng/ml), Upstate Biotechnology; CSF-1 (3000 units/ml), from Genetics Institute (Cambridge, MA); bombesin (10 nM), Sigma; and lysosphosphatidic acid (LPA; 1 μM), Sigma. These concentrations were selected to ensure that 50–70% of the cells entered S phase in response to ligand.

**Purification of PI 3-K from Insect Cells.** We have previously described a method, using phosphotyrosine affinity chromatography, to purify proteins that contain SH2 domains (25). Briefly, after a 72-hr coinfection of S9 cells with baculoviruses expressing regulatory (p85α) and catalytic (p110α) subunits of PI 3-kinase, cells were lysed, and the enzyme was purified as described for p85 (25).

**Cell Culture Techniques and Microinjection of Cells.** Cells were grown and seeded on glass coverslips as described. Medium was replaced with DMEM/0.5% fetal calf serum/ transferrin (5 μg/ml)/insulin (5 μg/ml)/antibiotics in the case of NIH 3T3 cells or with DMEM/transferrin (5 μg/ml)/ insulin (5 μg/ml) in the case of NIH 3T3 cells expressing CSF-1 receptors, and the cells were incubated for a further 24–48 hr. Hepes was then added to a concentration of 20 mM. Purified antibodies (2–4 mg/ml) were centrifuged for 15 min before use and then injected into cell cytoplasm (26). 5-Bromo-2′-deoxyuridine (BrdU; Sigma) and growth factors were added 30 min after microinjection unless stated otherwise, and the cells were incubated for a further 18 hr (for NIH 3T3) or 24 hr (for Swiss 3T3) before fixing and staining. Detection of microinjected antibodies and BrdU-positive cells was as described (23).

**RESULTS**

**Antibodies Specific for the Catalytic Subunit of PI 3-K.** Affinity-purified antibodies specific for p110α and premun IgG (from serum collected from the rabbits before immunization) were used for these experiments. The PI 3-K phosphozenzyme (p85α and p110α) was purified from insect cell lysates by binding to and salt-gradient elution from a column containing phosphotyrosine (Fig. 1A). We have previously shown that antibody ap110.1 recognizes the native mouse enzyme (24), although it works poorly in immunoblotting. Fig. 1B shows that, by immunoblotting, antibody ap110.2 recognized bovine p110 expressed in insect cells but also recognized a major band of the same size from whole-cell lysates of both NIH 3T3 and Swiss 3T3 cells. Other analyses have shown that ap110.2 recognizes the native p110 protein in mouse cells, although not as efficiently as ap110.1 (data not shown).

PI 3-K activity is stimulated by incubation with tyrosine phosphorylated peptides that bind with high affinity to the SH2 domains of p85 (27, 28). In our hands, a phosphopeptide corresponding to the Tyr-751 site of the PDGF receptor increases PI 3-K activity ∼4-fold. We incubated purified PI 3-K with the antibodies, in the presence of either peptide or phosphopeptide, and subsequently measured phosphatidylinositol kinase activity, using phosphatidyl(4,5)-bisphosphate as substrate (Fig. 1C). Neither premun IgG affected basal or stimulated PI 3-K activity. However, antibody ap110.1 inhibited both basal and phosphopeptide-stimulated kinase activity by ∼80–90%. Antibody ap110.2 had no effect on basal catalytic activity, but it prevented the phosphopeptide-stimulated activation, presumably by hindering a conformational change of the enzyme that occurs upon phosphopeptide binding.

**PDGF-Induced DNA Synthesis Requires PI 3-K.** We next tested whether the anti-p110 antibodies could inhibit PDGF-induced DNA synthesis in NIH 3T3 cells. Quiescent cells seeded onto coverslips were microinjected with the antibodies, then PDGF and BrdU were added, and cells were analyzed 18 hr later. Most anti-p110-containing cells were negative for BrdU, suggesting that the ap110.1 antibodies had an inhibitory effect on PDGF-induced DNA synthesis (Fig. 2). Similar images were obtained when the ap110.2 antibody was used (data not shown). Microinjection of the premun IgGs was not inhibitory (Figs. 2 and 3A). We quantitated the data from several such experiments (Fig. 3A). The ap110.1 antibody caused >75% inhibition when microinjected at 2 mg/ml, whereas ap110.2 antibody inhibited to ∼60% when
microinjected at 4 mg/ml. Fig. 3A also shows that PDGF-induced DNA synthesis in Swiss 3T3 cells was inhibited by antibody ap110.1, demonstrating that this effect was not cell-type specific. We therefore conclude that PI 3-K is required for PDGF-induced DNA synthesis.

We next tested how long functional PI 3-K was required for cells to enter S phase. In our NIH 3T3 cells, growth factors must be present in the medium for ≈10 hr (the "restriction point"), and DNA synthesis begins 12–14 hr after growth factor addition (M. Vittoria Barone, personal communication). Microinjection of antibody ap110.1 at any time up to 6 hr after PDGF addition inhibited subsequent entry into S phase, but microinjection at later times did not (Fig. 3B). Microinjection of the antibody into the nucleus did not inhibit, ruling out the possibility that PI 3-K was required at later times in the nucleus (data not shown).

**PI 3-K Is Required for EGF to Stimulate DNA Synthesis.**

The activated EGF receptor, like the PDGF receptor, associates with PI 3-K (10, 29, 30). However, no specific binding site on the receptor has been described, and the functional significance of binding has not been determined. Our Swiss 3T3 and NIH 3T3 cells respond well to EGF, and Fig. 4 shows that both op110.1 and op110.2 antibodies inhibited EGF-induced DNA synthesis by >60% in NIH 3T3 cells. Inhibition with antibody op110.1 was also detected in Swiss 3T3 cells (Fig. 4). These data demonstrate a requirement for PI 3-K in EGF-initiated signal-transduction pathways.

**Not All Growth Factors Require PI 3-K.**

The activated CSF-1 receptor also associates with PI 3-K when expressed in NIH 3T3 cells (19). However, mutant analyses had suggested that the association was not necessary for CSF-1-mediated growth (20, 21). To test whether functional PI 3-K was required for CSF-1-induced DNA synthesis, we used the microinjection approach. Fig. 5A shows that neither of the op110 antibodies inhibited the entry of CSF-1-stimulated cells into S phase (even though they did inhibit the PDGF response in the same cells; data not shown), confirming the results of the mutation experiments.

Finally, two growth factors, bombesin and LPA, are potent mitogens for several cell types, including Swiss 3T3. The receptors for bombesin and LPA are linked to heterotrimeric G proteins (31, 32), yet stimulation with these ligands leads to a rapid increase in tyrosine phosphorylation (33–36) and activation of tyrosine kinases—focal adhesion kinase in the case of bombesin and LPA (34–36) and Src in the case of LPA (37). Because several tyrosine kinases activate PI 3-K, we tested whether functional PI 3-K was required for bombesin- and LPA-induced DNA synthesis. As shown in Fig. 5B, neither response was inhibited, although the response to

**Fig. 2.** Antibodies specific for p110α inhibit PDGF-stimulated DNA synthesis. Quiescent NIH 3T3 cells seeded on coverslips were microinjected (into the cytoplasm) with op110.1 antibodies or preimmune IgG (Pl.1) as indicated. PDGF and BrdU were added to the cells, and 18 hr later cells were fixed and stained. Hoechst staining reveals the position of all nuclei (A). The op110.1 antibodies were visualized with a fluorescein-conjugated anti-rabbit antibody (B), and the BrdU antibodies were visualized with Texas Red-conjugated anti-mouse antibodies (C). White arrowheads in C mark positions of microinjected cells.

**Fig. 3.** Inhibition of PDGF signaling in NIH 3T3 and Swiss 3T3 cells. (A) Quiescent NIH 3T3 or Swiss 3T3 cells seeded onto coverslips were microinjected with the antibodies shown, stimulated with PDGF, and processed for immunofluorescence as described in the Fig. 2 legend. For each experiment, several coverslips were analyzed, and the percentage of cells that were BrdU-positive was calculated. Results from several experiments (n > 3) have been averaged, and the mean and SD of the mean are shown. (B) Quiescent NIH 3T3 cells were microinjected with affinity-purified op110.1 antibodies at the times shown relative to PDGF addition. All coverslips were processed at 18 hr after growth factor addition. Several coverslips were analyzed, and for each time point the extent of DNA synthesis in injected cells was calculated according to the formula: % of control = [number of BrdU-positive cells (injected)/number of BrdU-positive cells (uninjected)] × 100.

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DISCUSSION

The requirement for PI 3-K in PDGF signal transduction was previously addressed by mutagenesis. Mutant PDGFβ receptors lacking PI 3-K-binding sites are partially impaired in signaling (16, 17), whereas PDGFα receptors lacking the kinase insert domain (and therefore the PI 3-K-binding site) are only slightly defective in signaling (38). The inconsistencies may be due to different cell types or to level of receptor expression. That Nck shares a binding site on the PDGF receptor with the PI 3-K (18) also complicates analysis of the mutagenesis. With the microinjection approach, cells in which the wild-type PDGF receptors (both α and β in NIH 3T3 cells) are expressed can be used, and we show with this approach that PI 3-K was required for NIH 3T3 cells to enter S phase in response to PDGF. These data, therefore, confirm the requirement for PI 3-K that was suggested by mutagenesis experiments. We further showed that PI 3-K was required for approximately the first 6 hr after growth factor addition. This requirement is inconsistent with the brief appearance of PI 3-K products (5, 6) and suggests that a second wave of enzyme activation occurs.

How do the antibodies inhibit DNA synthesis? Because antibody αp110.1 inhibited catalytic activity by ~90% in vitro, it probably also inhibits the enzyme in vivo. Antibody αp110.2 did not affect basal activity but inhibited the allosteric activation of the enzyme in vitro and also inhibited DNA synthesis in vivo. In both cases, the in vitro data predict the antibody will cause an ~75–90% reduction in growth factor-stimulated PI 3-K activity in vivo. Our results further suggest that recruitment of the PI 3-K to the membrane is not alone sufficient and that allosteric activation via association with receptors must also occur for efficient signaling.

PI 3-K associates with the EGF receptor (refs. 10, 29, and 30 and S.R., data not shown), but no binding site is known and, indeed, the EGF receptor lacks the canonical phospho

FIG. 4. Inhibition of EGF signaling in NIH 3T3 and Swiss 3T3 cells. Coverslips seeded with quiescent NIH 3T3 and Swiss 3T3 cells were microinjected (into the cytoplasm) with affinity-purified antibodies as indicated, and 30 min later EGF was added to the medium. For each experiment, several coverslips were analyzed, and the percentage of cells that were BrdU-positive was calculated. Results from several experiments (n > 3) have been averaged, and the mean and SD of the mean are shown.

PDGF and EGF was inhibited in Swiss 3T3 cells (Figs. 3 and 4). Thus, neither bombesin nor LPA uses signaling pathways involving PI 3-K to stimulate growth.

FIG. 5. Antibodies to the PI 3-K do not inhibit CSF-1, LPA, or bombesin signaling. (A) NIH 3T3 cells that express the CSF-1 receptor (3T3 cells) were seeded onto coverslips and made quiescent. After injection with the affinity-purified antibodies indicated, recombinant CSF-1 was added to the medium. Eighteen hours later, cells were fixed and stained. Shown are the mean and SD of the mean. (B) Coverslips seeded with quiescent Swiss 3T3 cells were microinjected (into the cytoplasm) with affinity-purified antibodies as indicated, and 30 min later bombesin (bomb.) or LPA was added to the medium. For each experiment, several coverslips were analyzed, and the percentage of cells that were BrdU-positive was calculated. Results from several experiments (n > 3) have been averaged, and the mean and the SD of the mean are shown.

Tyr-Xaa-Xaa-Met PI 3-K-binding site (16, 39), making it difficult to derive appropriate mutant receptors to assess PI 3-K function. Using microinjection, we show that Swiss 3T3 and NIH 3T3 cells required PI 3-K to respond to EGF. Because all tyrosine phosphorylation sites on the EGF receptor can be removed without affecting mitogenic signaling (40), it is possible that PI 3-K does not associate with the receptor directly but instead associates with a receptor-associated protein, whose binding itself does not depend on phosphotyrosines.

Previous experiments had suggested that the PI 3-K-binding site on the CSF-1 receptor was not absolutely required for receptor function (20, 21), although recent analyses have shown that the adaptor protein GrB2 also binds to the CSF-1 receptor and that both proteins may influence receptor functioning (41). The readouts used in these experiments were long term, either cell growth or transformation assays. We used the microinjection system to analyze whether PI 3-K was required for more immediate responses that occur after growth factor addition and lead to DNA synthesis. Our data demonstrate that PI 3-K was not required for CSF-1-
stimulated entry into S phase in NIH 3T3 cells. However, we emphasize that in these experiments the CSF-1 receptor is expressed in a heterologous cell type. It remains possible that PI 3-K is required in cell types in which the receptor is normally expressed. We have also found that PI 3-K was not required for bombesin or LPA-induced DNA synthesis. Because Src family kinases are also not needed for bombesin and LPA signaling (S.R. and S.A.C., unpublished observations), it remains to be determined what the important signaling events for these mitogens are.

The cDNA cloning of another PI 3-K catalytic subunit, which has been called p110β, has recently been reported (15). This protein is expressed in NIH 3T3 cells; however, it is not yet clear what the substrate specificity of this enzyme is, nor whether it associates with activated growth factor receptors. Our anti-p110α antibodies do not crossreact with p110β (S.R., unpublished observations). The inhibitory effects of the p110α antibodies, therefore, suggest that p110β cannot substitute for p110α in PDGF-induced mitogenesis. However, it is possible that growth factors such as CSF-1, bombesin, and LPA, while not requiring p110α catalytic activity, may signal through p110β. Further analysis of p110β will be required to test this hypothesis.

What is the function of the PI 3-K in signal transduction? Some data suggest that it is required for the membrane ruffling seen shortly after growth factor addition (42). However, PI 3-K is needed for 6 hr after growth factor addition, and membrane ruffling occurs only in the first hour, suggesting that PI 3-K also has other functions. Experiments with mutant receptors suggest that PI 3-K products may activate a phosphokinase Cγ-dependent pathway (45), which may be required for mitogenesis in fibroblasts (46). Further microinjection experiments to elucidate signaling events that happen in the first hours after growth factor addition may resolve these issues.

From our previous experiments examining the role of the Src family kinases in PDGF-stimulated signal transduction (23) and from those reported here, we conclude that both Src family kinases and PI 3-K are necessary, but neither is alone sufficient, to mediate the cell’s response to PDGF. Moreover, mutant receptors that could bind only PI 3-K and Src family kinases could trigger DNA synthesis, suggesting that activation of these two pathways is sufficient for mitogenesis (43). Nevertheless, whether or not other proteins shown to associate with the activated PDGF receptor—for example, Nck, Syp, and phospholipase Cγ—are also required for signaling is yet to be answered.

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