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Specific Activation of p85-p110 Phosphatidylinositol 3′-Kinase Stimulates DNA Synthesis by ras- and p70 S6 Kinase-Dependent Pathways

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We have developed a polyclonal antibody that activates the heterodimeric p85-p110 phosphatidylinositol (PI) 3′-kinase in vitro and in microinjected cells. Affinity purification revealed that the activating antibody recognized the N-terminal SH2 (NSH2) domain of p85, and the antibody increased the catalytic activity of recombinant p85-p110 dimers threefold in vitro. To study the role of endogenous PI 3′-kinase in intact cells, the activating anti-NSH2 antibody was microinjected into GRC+LR73 cells, a CHO cell derivative selected for tight quiescence during serum withdrawal. Microinjection of anti-NSH2 antibodies increased bromodeoxyuridine (BrdU) incorporation fivefold in quiescent cells and enhanced the response to serum. These data reflect a specific activation of PI 3′-kinase, as the effect was blocked by coinjection of the appropriate antigen (glutathione *S*-transferase–NSH2 domains from p85α), coinjection of inhibitory anti-p110 antibodies, or treatment of cells with wortmannin. We used the activating antibodies to study signals downstream from PI 3′-kinase. Although treatment of cells with 50 nM rapamycin only partially decreased anti-NSH2-stimulated BrdU incorporation, coinjection with an anti-p70 S6 kinase antibody effectively blocked anti-NSH2-stimulated DNA synthesis. We also found that coinjection of inhibitory anti-ras antibodies blocked both serum- and anti-NSH2-stimulated BrdU incorporation by approximately 60%, and treatment of cells with a specific inhibitor of MEK abolished antibody-stimulated BrdU incorporation. We conclude that selective activation of physiological levels of PI 3′-kinase is sufficient to stimulate DNA synthesis in quiescent cells. PI 3′-kinase-mediated DNA synthesis requires both p70 S6 kinase and the p21^{ras}/MEK pathway.

Phosphatidylinositol (PI) 3′-kinases are a family of enzymes with homologous catalytic subunits and varied regulatory domains or subunits (26). Isoforms of PI 3′-kinase include heterodimeric PI 3′-kinases that are stimulated by binding of regulatory SH2 domains to tyrosine phosphoproteins, an isoform form that is regulated by βγ subunits from trimeric G proteins, a monomeric form that is homologous to the VPS-34 yeast PI 3′-kinase, and isoforms containing C2 regulatory domains (13, 20, 22, 37, 43, 48, 57, 58, 63, 64). Substrate specificities of the PI 3′-kinases also vary: the p85-p110 and βγ-stimulated PI 3′-kinases utilize PI, PI 4-P and PI 4,5-P_2, whereas the VPS-34-like PI kinases are specific for PI (20, 58, 64) and the C2-domain-containing isoforms preferentially utilize PI and PI 4-P (37, 63). The catalytic subunit of the p85-p110 PI 3′-kinase is also homologous to a yeast PI 4-kinase, the ataxia telangiectasia gene product, the DNA-dependent protein kinase, and the TOR2/FRAP/RAFT proteins, which are upstream regulators of p70 S6 kinase and targets of the immunosuppressant rapamycin (14, 18, 30, 55, 56).

The heterodimeric PI 3′-kinase is composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (reviewed in reference 26). The p85 regulatory subunit contains multiple modular domains which mediate interactions with regulatory proteins, including two SH2 domains, one SH3 domain, two proline-rich domains, and a rho-binding domain homologous to the Bcr gene product (26). The activity of p85-p110 dimers is increased by binding of the two SH2 domains to proteins containing phosphorylated YMXM motifs (2, 7), binding of the SH3 domain to proline-rich sequences (16), binding of exogenous SH3-containing proteins to proline-rich domains in p85 (46), and binding of rho-family GTPases to the Bcr homology domain (61). It is not clear how these distinct regulatory events are integrated in intact cells. Activation of p85-p110 PI 3′-kinase also may require p21^{ras}, as expression of a dominant negative N17 ras mutant in PC12 cells blocks epidermal growth factor-stimulated accumulation of PI 3,4,5-P_3 (51) and p110 binding to ras-GTP increases activity in vitro (25).

Activation of p85-p110 PI 3′-kinase has been implicated in a wide range of cellular activities, including control of proliferation, cytoskeletal organization, apoptosis, neurite outgrowth, vesicular trafficking, and insulin-stimulated glucose transport (9, 25, 27, 29, 34, 42). However, much of the data on the role of PI 3′-kinase in particular processes is derived from experiments with inhibitors such as wortmannin and LY294002, which are only partially specific for PI 3′-kinase (11, 40). Furthermore, these inhibitors do not distinguish which PI 3′-kinase isoform is involved in a given process. Information with regard to functions of the heterodimeric p85-p110 has also been obtained from assays using mutant growth factor receptors lacking the phosphorylation sites that bind p85 SH2 domains (6). However, interpretation of these studies is complicated by the finding that the phosphotyrosine binding sites for PI 3′-kinase can also interact with other SH2-containing proteins (41).

A more specific approach to the evaluation of PI 3′-kinase signaling in intact cells is the microinjection of antibodies that

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have known effects on PI 3′-kinase activity. Thus, using an inhibitory anti-p110α antibody, Roche et al. showed that activation of p85-p110 PI 3′-kinase is required for stimulation of platelet-derived growth factor (PDGF)- and epidermal growth factor- but not colony-stimulating factor 1-stimulated DNA synthesis in NIH 3T3 cells expressing these receptors (50). In the present study, we confirm that microinjection of an inhibitory anti-p110α antibody blocks insulin-stimulated DNA synthesis in CHO cells expressing human insulin receptors. We have also developed a stimulatory anti-p85 antibody that activates p85α-p110α dimers threefold in vitro. The antibody stimulates DNA synthesis fivefold in quiescent cells and increases the response to serum. We used the antibody to assess the involvement of p70 S6 kinase and the ras/mitogen-activated protein (MAP) kinase pathway in PI 3′-kinase-induced DNA synthesis. Treatment of cells with rapamycin had a modest effect on PI antibody-stimulated DNA synthesis, whereas coinjection of an anti-p70 S6 kinase antibody blocked antibody-stimulated DNA synthesis. We also found that microinjection of anti-p21^{ras} antibodies or treatment of cells with an specific inhibitor of MEK blocked antibody-stimulated DNA synthesis. We conclude that specific activation of physiological levels of p85-p110 PI 3′-kinase is sufficient to stimulate DNA synthesis by p70 S6 kinase- and ras-dependent pathways.

MATERIALS AND METHODS

Cells lines. CHO/IR cells are derived from CHO/K1 cells, express 10^6 human insulin receptor molecules/cell, and have been previously described (3). GRC+LR73 cells are growth control revertants derived from CHO cells. They were selected for survival in suspension culture in the presence of [^{3}H]thymidine and 10% fetal bovine serum (FBS), followed by a second selection for reversion to fibroblastic morphology upon replating (47). GRC+LR73 cells show a tight G_1 arrest during serum deprivation (47). Cells were maintained in F12 medium (CHO/IR) or alpha minimal essential medium (GRC+LR73) containing 10% FBS.

Preparation and purification of antibodies and GST fusion proteins. Antibodies to the C terminus of bovine p110α (residues 1054 to 1068) or to a glutathione *S*-transferase (GST) fusion protein containing residues 321 to 724 of human p85α were raised in New Zealand White rabbits at Hazelton Research Products as previously described (3). The anti-p110 antibodies were purified by affinity chromatography, using the peptide coupled to epoxy-Sepharose (Pharmacia). The anti-p85 serum was passed multiple times over a CNBr-Sepharose-GST column to remove anti-GST antibodies. The remaining antibodies were fractionated by chromatography on columns of CNBr-Sepharose coupled to GST fusion proteins containing the N-terminal or C-terminal SH2 (NSH2 or CSH2) domain of p85 (21). Antibodies against the inter-SH2 (iSH2) domain were prepared by first passing the anti-p85 antibody two times over both the NSH2 and CSH2 columns. The flowthrough, containing antibodies that were not absorbed by the NSH2 and CSH2 columns, was then passed over a column of CNBr-Sepharose coupled to a GST fusion protein containing residues 321 to 724 of p85, which includes the NSH2, CSH2, and iSH2 domains. Reactivity of the antibodies with each domain was confirmed by Western blotting.

Anti-ras monoclonal antibodies (clone Y13-259) (15) were produced by using a CellMax hollow fiber reactor and purified on protein G-Sepharose (Pharmacia) prior to use. A second ras monoclonal antibody (Y13-238) (15) was purified from hybridoma tissue culture supernatants on protein G-Sepharose. An anti-p70 S6 kinase antiserum (66) raised against a GST fusion protein containing the C-terminal 104 amino acids of p70 S6 kinase (residues 422 to 525; provided by J. Avruch, Harvard Medical School) was purified by two passages through a CNBr-Sepharose-GST column, followed by absorption on a CNBr-Sepharose-GST-p70 (residues 422 to 525) column.

In vitro PI 3′-kinase assays. Recombinant p110 or p85-p110 dimers were produced in baculovirus-infected *Spodoptera frugiperda* Sf9 cells as previously described (53). Lysates from infected or control cells were incubated with antibody or rabbit immunoglobulin G (IgG; 2 μg/100 μl, final concentration) for 1 h at 4°C, followed by 10 min at 22°C in the presence of PI vesicles (20 μg of lipid) and 45 μM ATP containing 20 μCi of [γ-^{32}P]ATP. Lipids were extracted and analyzed by thin-layer chromatography as previously described (2). Incorporation of ^{32}P into PI was quantitated on a Molecular Dynamics PhosphorImager.

Immunoblotting. Crude lysates from Sf9 cells infected with a recombinant p85 baculovirus (53) or from GRC+LR73 cells were separated by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. The membranes were blotted with an anti-NSH2 antibody (3 μg/μl), and proteins were visualized with [^{125}I]protein-A followed by autoradiography.

Microinjection and bromodeoxyuridine (BrdU) incorporation. Cells grown on polysine-coated coverslips were made quiescent in serum-free medium containing 0.5% bovine serum albumin (for CHO/IR cells) or 1% fetal bovine serum (for GRC+LR73 cells) for 48 h prior to injection. Microinjections were conducted by using an Eppendorf semiautomated microinjection system mounted on a Nikon Diaphot microscope equipped for epifluorescence studies, using needles pulled on a Sutter P-87 micropipette puller. Antibodies or recombinant proteins (2 to 4 mg/ml) were mixed with nonspecific rabbit IgG (3 mg/ml) in 5 mM $NaPO_4$–100 mM KCl (pH 7.4). After injection, cells were kept in serum-free medium or transferred to 10% FBS or 100 mM insulin as indicated. When indicated, incubations also included wortmannin (100 nM), suramin (250 μM), rapamycin (50 nM), or the MEK inhibitor PD98059 (45) (50 μM; New England Biolabs).

Fifteen hours after injection, cells were incubated with 10 μM BrdU for 2 h and fixed in 3.7% formaldehyde. Nuclear DNA was denatured by treating the cells with 4 N HCl for 3 min, and the cells were permeabilized in methanol at −20°C and stained with rhodamine-conjugated anti-BrdU antibody (to measure DNA synthesis) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (to determine microinjected cells). The cells were photographed with a Nikon N6000 camera, and the percentage of BrdU-stained cells in each microinjected field was determined. Data from each experiment reflect the counting of approximately 100 injected cells per condition. The mean and standard error of the mean (SEM) values were generated by pooling percentages from various numbers of separate experiments.

RESULTS

Characterization of anti-PI 3′-kinase antibodies. We raised a polyclonal antibody to a peptide derived from the extreme C terminus of the PI 3′-kinase catalytic subunit p110 (residues 1054 to 1068). The antibody was affinity purified on a peptide-CNBr-Sepharose column and incubated with cell lysates from Sf9 cells expressing recombinant p110α. At a final concentration of 20 μg/ml, the anti-p110 antibody inhibited PI 3′-kinase activity by 70%, whereas no inhibition was seen with the control antibody (rabbit IgG) (Fig. 1A). The anti-p110 antibody immunoprecipitated PI 3′-kinase activity from cell lysates (data not shown) but did not detect p110 in an immunoblot. Our immunoblotting and inhibition data are consistent with those of Roche et al., who produced an anti-p110 antibody by using an identical peptide (50).

A second polyclonal antibody was raised to the C-terminal half of the PI 3′-kinase regulatory subunit p85α (residues 321 to 724), expressed as a GST fusion protein in *Escherichia coli*. This fusion protein contains the two p85 SH2 domains and the iSH2 domain. After removal of anti-GST antibodies and affinity purification, we incubated the anti-p85 antibody with lysates from Sf9 cells expressing both p85 and p110. We found that the antibody increased PI 3′-kinase activity approximately twofold (Fig. 1B, bars a and b). We then fractionated the anti-p85 antibody into pools that specifically recognized the NSH2, CSH2, and iSH2 domains, as described in Materials and Methods. When incubated with recombinant p85-p110 dimers, the anti-NSH2 antibodies increased PI 3′-kinase threefold (Fig. 1B, bar c), whereas the anti-C-SH2 and anti-iSH2 antibodies had no effect on activity (Fig. 1B, bars d and e). The anti-NSH2 antibody had no effect on recombinant p110 in the absence of p85 (data not shown). To test the specificity of the anti-NSH2 antibody, we performed immunoblot analyses with crude lysates from GRC+LR73 cells or from Sf9 cells infected with a p85 baculovirus (Fig. 1C). In both cases, a single 85-kDa band was detected.

PI 3′-kinase is required for insulin-stimulated DNA synthesis. We used the inhibitory anti-p110 antibodies to determine if PI 3′-kinase was required for the proliferative response to insulin. Quiescent CHO/IR cells were injected with anti-p110 antibodies or control IgG, incubated in the absence or presence of 100 nM insulin for 15 h, and then incubated for 2 h in 10 μM BrdU. The cells were then fixed and stained with FITC-labeled anti-rabbit IgG secondary antibodies (to locate microinjected cells) or rhodamine-labeled anti-BrdU antibodies (to

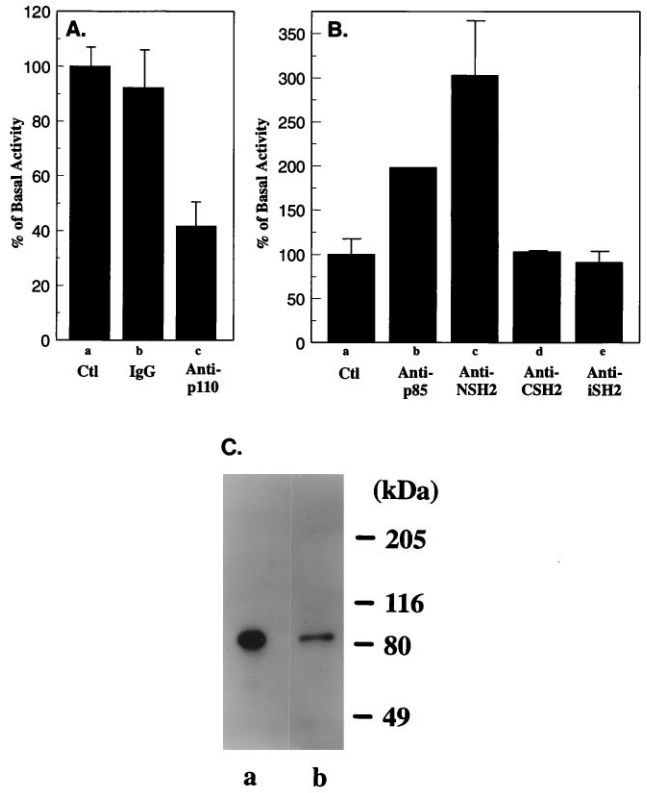

FIG. 1. Characterization of anti-PI 3′-kinase antibodies. (A) Lysates from Sf9 cells expressing p110α were incubated with buffer (Ctl) or with control IgG or anti-p110 antibody (20 μg/ml) and assayed for PI 3′-kinase activity. The data are the means of triplicates and are representative of four separate experiments. (B) Lysates from Sf9 cells expressing p85α and p110α were incubated with buffer (Ctl), unfractionated anti-p85 antibody, or affinity-purified antibodies against the p85α NSH2, CSH2, or iSH2 domain. The samples were then assayed for PI 3′-kinase activity. The data are the means of triplicates and are representative of two separate experiments. (C) Crude lysates from Sf9 cells expressing human p85 (lane a) or crude lysates from GRC+LR73 cells (lane b) were separated by SDS-PAGE, transferred to nitrocellulose, and blotted with an anti-NSH2 antibody followed by [^{125}I]protein A. The data are representative of two experiments.

detect cells which had entered S phase). Results from a typical experiment are shown in Fig. 2. In IgG-injected cells, nuclear BrdU labeling is minimal in quiescent cells (Fig. 2B and C), whereas all of the injected cells are labeled with BrdU after insulin stimulation (Fig. E and F). Injection of anti-p110 antibodies had little effect on quiescent cells (Fig. 2H and I) but markedly reduced the BrdU labeling of insulin-stimulated cells (Fig. 2K and L). In data pooled from five experiments, injection of anti-p110 antibodies inhibited insulin-stimulated DNA synthesis by 70% (Fig. 3). These data confirm the critical role of PI 3′-kinase in insulin-stimulated DNA synthesis (9, 24).

PI 3′-kinase activation is sufficient for DNA synthesis. We microinjected the stimulatory anti-NSH2 antibodies into GRC+LR73 cells, a CHO cell-derived line selected for anchorage-dependent growth (47). Unlike the case for CHO/IR cells, basal levels of BrdU incorporation were approximately 5% in the GRC+LR73 cells. Microinjection of the anti-NSH2 antibodies caused a significant stimulation of BrdU incorporation (Fig. 4A). Data pooled from four experiments indicated that BrdU incorporation in anti-NSH2-injected cells increased to 26.4% ± 3.3% from a basal level of 5.6% ± 2%. The magnitude of stimulation in individual experiments was largely dependent on variations in basal incorporation but was as high as 10- to 13-fold in two experiments. BrdU incorporation in anti-NSH2-injected cells was similar to that seen in serum-stimulated cells injected with control IgG in this series of experiments (33.9% ± 4.3%), although the response to serum was higher in some experiments. Microinjection of the anti-NSH2 antibodies actually increased the response to serum (to 46.0% ± 5.8%).

Control experiments confirmed that injection of the anti-NSH2 antibodies acted by specific activation of p85-p110 PI 3′-kinase. Coinjection of cells with a GST fusion protein containing the NSH2 domain of p85 reduced the effects of the anti-NSH2 antibody, whereas coinjection of GST-CSH2 had no effect (Fig. 4B). Similarly, stimulation of BrdU incorporation by the anti-NSH2 antibody was decreased by coinjection of the inhibitory anti-p110 antibody (Fig. 4C) or by treatment of cells with 100 nM wortmannin, an inhibitor of PI 3′-kinase (Fig. 4D). Finally, we showed that injection of the anti-CSH2 antibody, which did not activate PI 3′-kinase activity in vitro (Fig. 1B), had no effect on BrdU incorporation in intact cells (Fig. 4E).

Our data show that specific activation of p85-p110 is sufficient to stimulate the progression of quiescent cells into S phase of the cell cycle. While we presume that the observed increase in BrdU incorporation was a result of PI 3′-kinase-mediated activation of downstream signaling pathways, it is also possible that activated PI 3′-kinase induced an autocrine loop. Thus, increased secretion of a growth factor could stimulate DNA synthesis by activating cell surface receptors. To test this possibility, we injected cells with anti-NSH2 antibodies or stimulated them with insulin and then incubated the cells in suramin, a polysulfonated naphthylurea that blocks binding of a number of growth factors to their cell surface receptors (4). Treatment of the cells with suramin completely blocked insulin-stimulated BrdU incorporation yet had no effect on BrdU incorporation in cells injection with anti-NSH2 antibodies (Fig. 5). Induction of an autocrine loop by injection of anti-NSH2 antibodies was therefore unlikely.

Requirement for p70 S6 kinase during PI 3′-kinase-stimulated DNA synthesis. To study the mechanism by which activation of PI 3′-kinase stimulated DNA synthesis, we examined the role of putative downstream effectors of PI 3′-kinase. p70 S6 kinase has been suggested as a target of PI 3′-kinase, although the data remain controversial (10, 17, 33, 36, 65). We therefore tested the effect of rapamycin (50 nM), an inhibitor of p70 S6 kinase activation at the level of FRAP/RAFT-1, on BrdU incorporation (5, 54). Rapamycin treatment had no effect on BrdU incorporation in cells injected with control IgG, whereas it inhibited serum-stimulated BrdU incorporation by greater than 65% (Fig. 6A). In contrast, BrdU incorporation in cells injected with anti-NSH2 antibodies was inhibited only 35% by rapamycin.

Cheatham et al. (8) and Weng et al. (66) have recently shown that an N-terminally truncated p70 S6 kinase is insensitive to rapamycin but still sensitive to wortmannin, suggesting that FRAP/RAFT1 and PI 3′-kinase lie on distinct pathways that converge on p70 S6 kinase (8, 66). We therefore reasoned that inhibition of FRAP/RAFT1 with rapamycin might underestimate the role of p70 S6 kinase during specific stimulation of PI 3′-kinase. To test the requirement for p70 S6 kinase more directly, we used an antibody raised against residues 422 to 525 of p70 S6 kinase (provided by J. Avruch, Harvard Medical School). Injection of the anti-p70 S6 kinase antibody reduced serum-stimulated BrdU incorporation by greater than 60% and reduced anti-NSH2-stimulated BrdU incorporation to basal levels (Fig. 6B). The inhibition of anti-NSH2-mediated

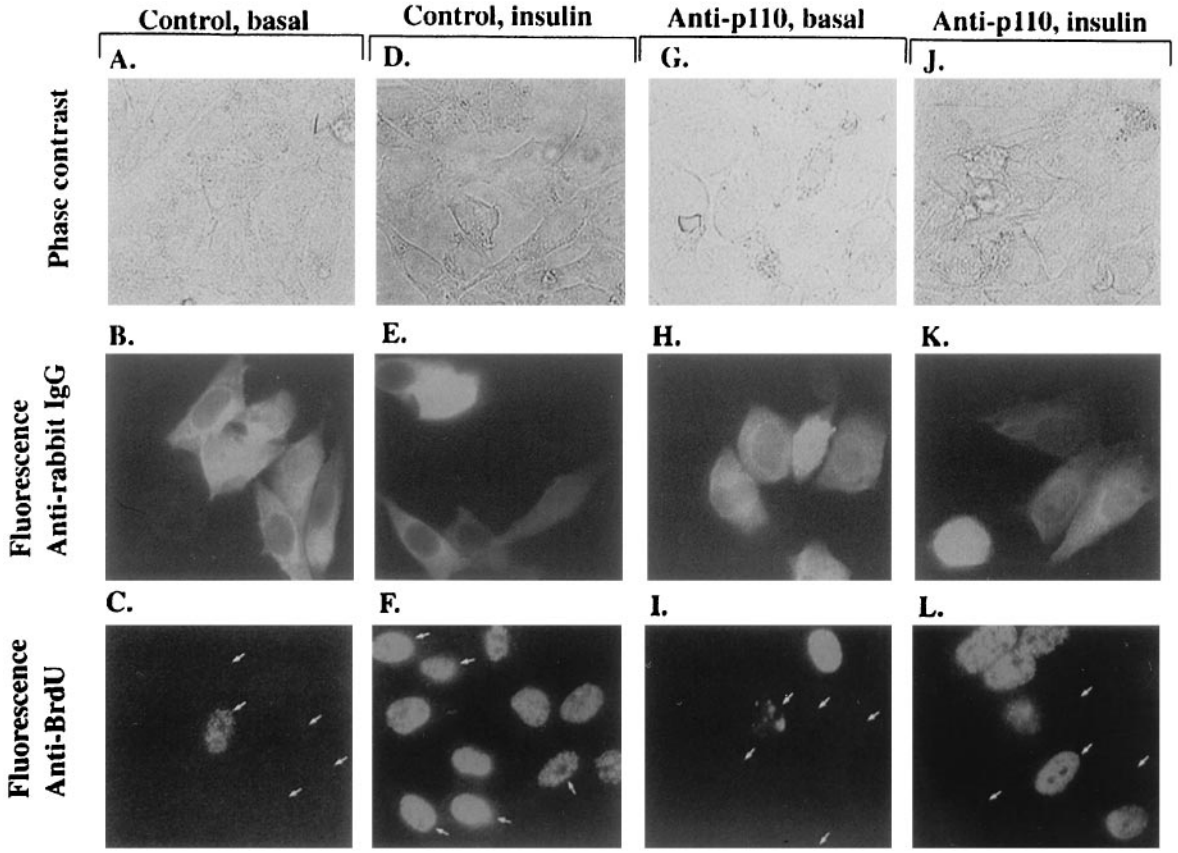

FIG. 2. Microinjection of inhibitory anti-p110 antibodies into CHO/IR cells. CHO/IR cells were injected with rabbit IgG (2 mg/ml) (A to F) or anti-p110 antibody (2 mg/ml) (G to L). After a 15-h incubation in the absence (A to C and G to I) or presence (D to F and J to L) of 100 nM insulin and an additional 2-h incubation in 10 μM BrdU, the cells were fixed. (A, D, G, and J) Phase-contrast images of four microinjected fields; (B, E, H, and K) staining of the same fields with FITC-labeled goat anti-rabbit antibodies; (C, F, I, and L) staining of the same fields with rhodamine-labeled mouse monoclonal anti-BrdU antibodies. The nuclei of microinjected cells are marked with arrows in the panels showing anti-BrdU-labeled cells.

BrdU incorporation by the anti-p70 S6 kinase antibody was reversed by coinjection of a GST fusion protein containing residues 422 to 525 of p70 S6 kinase (Fig. 6C), whereas coinjection of GST had no effect. Thus, activation of p70 S6 kinase was required for PI 3′-kinase-mediated mitogenic signaling.

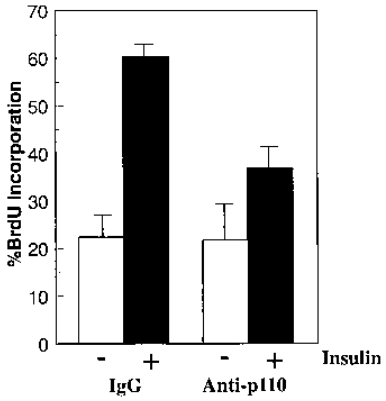

FIG. 3. Inhibition of insulin-stimulated DNA synthesis by anti-p110. Quiescent CHO/IR cells were microinjected with control IgG or anti-p110 antibodies (2 mg/ml) and incubated in the absence or presence of 100 nM insulin for 15 h, and BrdU incorporation was determined as described for Fig. 2. The data are the means ± SEM from five separate experiments.

Requirement for ras and MEK during PI 3′-kinase-stimulated DNA synthesis. We examined the requirement for activation of p21^{ras} during PI 3′-kinase-mediated signaling by coinjecting cells with an inhibitory anti-ras antibody (Y13-259) (15, 38). Injection of cells with anti-ras antibodies reduced serum-stimulated DNA synthesis by 65% (Fig. 7A). However, coinjection of anti-ras antibodies also reduced DNA synthesis in anti-NSH2-injected cells by 57% (Fig. 7A). In contrast to the effects of Y13-259, coinjection of cells with the nonneutralizing anti-ras antibody Y13-238 (15, 38) had no effect on anti-NSH2-stimulated BrdU incorporation (Fig. 7B). Therefore, the stimulation of DNA synthesis either by serum or by specific activation of PI 3′-kinase showed a requirement for p21^{ras}.

To further test whether PI 3′-kinase-mediated signaling involved activation of p21^{ras}, we treated cells with an inhibitor of MEK (45), a dual-specificity kinase that lies downstream from activated ras (12). Treatment of cells with the MEK inhibitor PD98059 (50 μM) had little effect on basal BrdU incorporation, but it inhibited serum-stimulated DNA synthesis by 50% and abolished antibody-stimulated DNA synthesis (Fig. 7C). These data suggest that PI 3′-kinase regulates DNA synthesis by a pathway requiring p21^{ras} and its downstream effectors.

DISCUSSION

Using antibodies with known effects on the activity of p85-p110 PI 3′-kinase, we have demonstrated that PI 3′-kinase

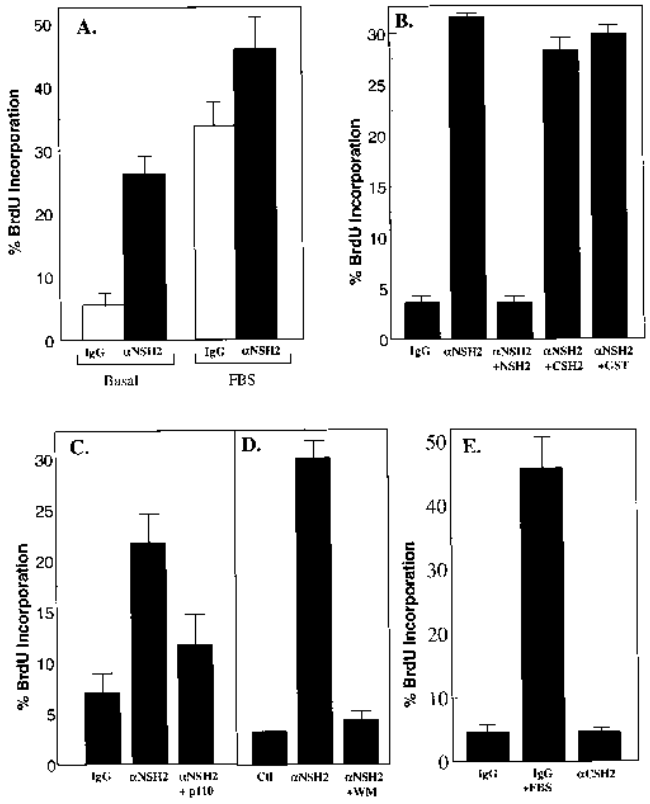

FIG. 4. Stimulation of DNA synthesis by injection of activating anti-NSH2 antibodies. Quiescent GRC+LR73 cells were injected with control IgG or anti-NSH2 antibody (αNSH2; 2 mg/ml) and incubated in the absence or presence of 10% FBS for 15 h. BrdU incorporation was then determined as described for Fig. 2. The data are the means ± SEM from four separate experiments. (B) Quiescent GRC+LR73 cells were injected with control IgG or anti-NSH2 antibody (αNSH2) in the absence or presence of GST, GST-NSH2, or GST-CSH2 fusion protein (3 mg/ml). BrdU incorporation after 15 h was determined. The data are the means ± standard deviations (SD) from two separate experiments. (C) Quiescent GRC+LR73 cells were injected with control IgG or anti-NSH2 antibody (αNSH2) in the absence or presence of inhibitory anti-p110 antibody (2 mg/ml). BrdU incorporation after 15 h was determined. The data are the means ± SEM from four separate experiments. (D) Cells were injected with control IgG or anti-NSH2 antibody (αNSH2) and incubated in the absence or presence of 100 nM wortmannin (WM) for 15 h, and BrdU incorporation was determined. The data are the means ± SD from two separate experiments. (E) Cells were injected with control IgG or anti-CSH2 antibody (αCSH2) and incubated in the absence or presence of 10% FBS for 15 h, and BrdU incorporation was determined. The data are the means ± SD from two separate experiments.

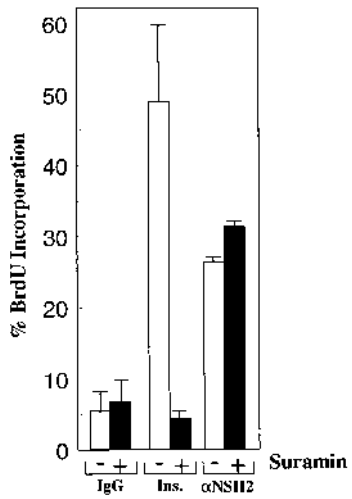

FIG. 5. Antibody-stimulated DNA synthesis is not mediated by an autocrine loop. Quiescent GRC+LR73 cells were either not injected or injected with control IgG or anti-NSH2 antibody (αNSH2) as indicated (2 mg/ml). The cells were incubated in medium in the absence or presence of suramin (250 μM) and insulin (Ins.; 100 nM) as indicated for 15 h, and BrdU incorporation was determined. The data are the means ± standard deviations from two separate experiments.

activation is both necessary and sufficient to mediate the progression of quiescent cells into S phase. Microinjection of activating anti-NSH2 antibodies increases BrdU incorporation fivefold in quiescent cells and enhances the response to serum. Antibody-stimulated DNA synthesis is unaffected by suramin and is therefore unlikely to reflect induction of an autocrine loop. Notably, our data reflect stimulation of endogenous PI 3′-kinase and show that physiological levels of activated PI 3′-kinase are sufficient to induce DNA synthesis.

Our work is in concordance with previous studies showing that activation of PI 3′-kinase is necessary for proliferative responses to mitogens. Using an inhibitory antibody similar to the one used here, Roche et al. showed that PI 3′-kinase was required for PDGF- but not colony-stimulating factor 1-stimulated DNA synthesis in NIH 3T3 cells (50). Less specific approaches, such as microinjection of p85 SH2 domains or treatment of cells with the PI 3′-kinase inhibitors, have also suggested a role for PI 3′-kinase in insulin-stimulated DNA synthesis (9, 24). Earlier studies have shown that mutation of tyrosine kinase receptors, so as to remove tyrosine phosphorylation sites known to interact with p85 and p110, blocks proliferative responses to growth factors (6). However, PI 3′-kinase and Nck share a binding site in the PDGF receptor (41), and selective removal of a given phosphorylation site may therefore affect the regulation of signaling molecules unrelated to PI 3′-kinase.

Several previous studies have addressed whether activation of PI 3′-kinase is sufficient to initiate proliferative responses. An elegant study by Valius and Kazlauskas showed that restoration of Tyr^{740} and Tyr^{751} in a mutant PDGF receptor lacking five tyrosine phosphorylation sites restored PI 3′-kinase activation and partially restored PDGF-stimulated DNA synthesis (62). However, the possibility of degeneracy in the binding of SH2 domain-containing proteins to these sites complicates the interpretation of this study (41). Alternatively, Hu et al. and Weng et al. showed that overexpression of an activated p110 construct (i) increased c-fos expression and p70 S6 kinase activation in Cos cells and (ii) stimulated raf and Erk phosphorylation and enhanced the maturation of *Xenopus* oocytes (23, 65). A membrane-targeted p110 was even more effective in activating p70 S6 kinase and also activated the Akt and JNK kinases (28). However, DNA synthesis was not measured in these studies.

Although the mechanism by which PI 3′-kinase stimulates DNA synthesis is not yet clear, four potential downstream pathways have been suggested. The first involves calcium-independent and atypical protein kinase C isoforms, which are activated in vitro and in permeabilized cells by the 3-phosphoinositide products of PI 3′-kinase (39, 59, 60, 69) or by overexpression of p110 (1). The second involves activation of rac, which is upstream of the JNK/stress-activated protein kinases that phosphorylate c-jun (19, 28, 32, 35). The third involves p70 S6 kinase, whose activation by mitogens is critical for progression from G_1 to S phase of the cell cycle (10, 17, 33, 36, 65). The fourth involves the activation of $p21^{ras}$ (23, 51). The last two pathways are addressed in this study.

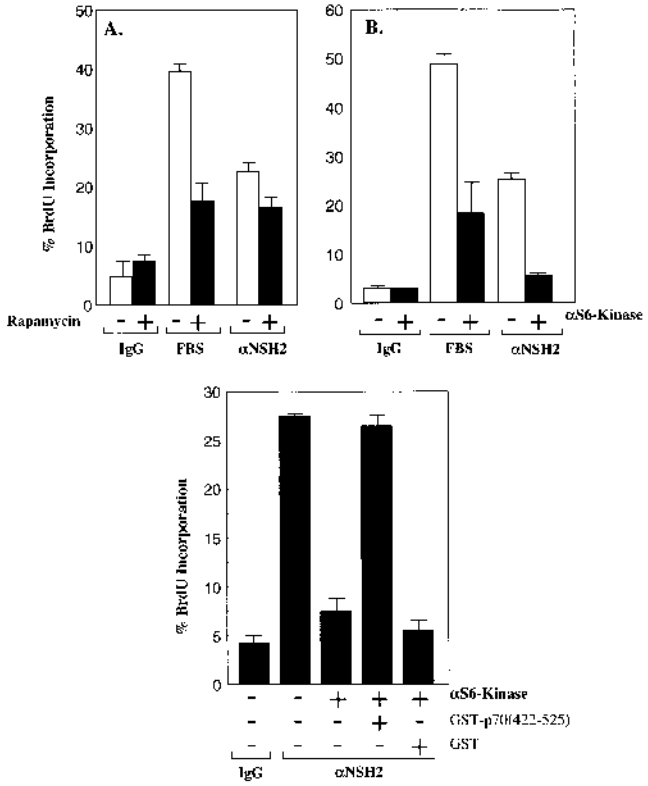

FIG. 6. Role of p70 S6 kinase in PI 3′-kinase-mediated DNA synthesis. (A) Quiescent GRC+LR73 cells were either not injected or injected with control IgG or anti-NSH2 antibody (αNSH2) as indicated (2 mg/ml). The cells were treated without or with 50 nM rapamycin for 30 min and then incubated in the absence or presence of 10% FBS for 15 h. BrdU incorporation was determined as described in the text. The data are the means ± SEM from three separate experiments. (B) Quiescent GRC+LR73 cells were injected with control IgG, anti-NSH2 antibody (αNSH2; 2 mg/ml), and anti-p70 S6 kinase antibody (αS6-Kinase; 2 mg/ml) as indicated. The cells were then incubated in the absence or presence of 10% FBS for 15 h, and BrdU incorporation was determined. The data are the means ± SEM from three separate experiments. (C) Quiescent GRC+LR73 cells were injected with control IgG, anti-NSH2 antibody (αNSH2; 2 mg/ml), and anti-p70 S6 kinase antibody (αS6-Kinase; 2 mg/ml) as indicated in the absence or presence of GST-p70 (residues 422 to 525) or GST (both at 2 mg/ml). The cells were then incubated for 15 h, and BrdU incorporation was determined. The data are the means ± SD from two separate experiments.

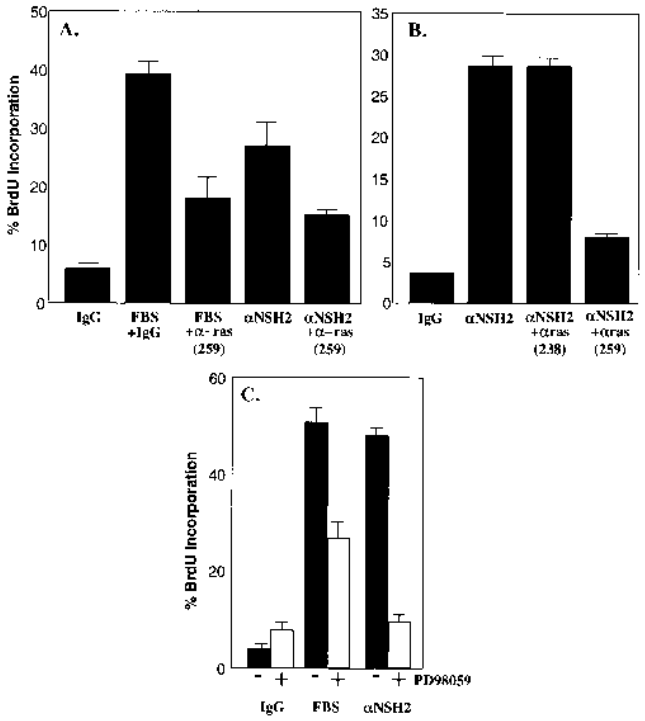

FIG. 7. Roles of p21^{ras} and MEK in PI 3′-kinase-mediated DNA synthesis. (A) Quiescent GRC+LR73 cells were injected with control IgG, anti-NSH2 antibody (αNSH2; 2 mg/ml), and neutralizing anti-ras antibody [αras(259); 0.75 mg/ml] as indicated. The cells were then incubated in the absence or presence of 10% FBS for 15 h. BrdU incorporation was determined as described in the text. The data are the means ± SEM from three separate experiments. (B) Quiescent GRC+LR73 cells were injected with control IgG, anti-NSH2 antibody (αNSH2; 2 mg/ml), neutralizing anti-ras antibody [αras(259); 0.75 mg/ml], or nonneutralizing anti-ras antibody [αras(238); 0.75 mg/ml] as indicated. BrdU incorporation was determined after 15 h. The data are the means ± SEM from three separate experiments. (C) Quiescent GRC+LR73 cells were either not injected or injected with control IgG or anti-NSH2 antibody (αNSH2) as indicated. The cells were treated without or with 50 μM PD98059 for 30 min and then incubated in the absence or presence of 10% FBS for 15 h. BrdU incorporation was determined as described in the text. The data are the means ± SEM from three separate experiments.

We find that treatment of cells with rapamycin inhibits BrdU incorporation by approximately 35% in cells injected with stimulatory anti-NSH2 antibodies, whereas coinjection of an anti-p70 S6 kinase reduces antibody-stimulated DNA synthesis to basal levels. Growth factor stimulation of p70 S6 kinase is inhibited by both rapamycin (which acts at the level of FRAP/RAFT1) and wortmannin (which acts on PI 3′-kinase) (9, 10, 31, 49). However, N-terminal truncation mutants of p70 S6 kinase are sensitive to wortmannin but not rapamycin, suggesting that these two drugs define independent inputs to p70 S6 kinase (8, 66). Consistent with these findings, rapamycin is only partially effective in blocking anti-NSH2-stimulated DNA synthesis, as it presumably reduces basal activation of p70 S6 kinase from the FRAP/RAFT1 pathway but does not affect the PI 3′-kinase-specific activation. The anti-p70 S6 kinase antibody, however, targets the enzyme itself and effectively blocks PI 3′-kinase-stimulated signaling. These data support the hypothesis that activation of p70 S6 kinase plays a major role in PI 3′-kinase-dependent mitogenic signaling.

We find that microinjections of neutralizing anti-ras antibodies have similar inhibitory effects on cells injected with an anti-NSH2 antibody or treated with serum. Furthermore, the MAP kinase kinase (MEK) inhibitor PD98059 completely blocks antibody-stimulated DNA synthesis but only partly inhibits serum-stimulated DNA synthesis. The inhibition of antibody-stimulated DNA synthesis by anti-ras antibodies could reflect a requirement for basal levels of ras function or could be due to a disruption of binding interactions between p110 and p21^{ras} (51, 52). However, the complete inhibition of PI 3′-kinase-mediated DNA synthesis by PD98059 suggests that PI 3′-kinase signaling involves not just basal ras activity but activation of the MAP kinase cascade. These findings are consistent with overexpression experiments showing that an activated PI 3′-kinase increases ras-GTP levels and raf and ERK phosphorylation in *Xenopus* oocytes (23). Yamauchi et al. have also suggested that PI 3′-kinase functions above ras during insulin-stimulated induction of c-fos in CHO cells (67). However, it is also possible that the effects of anti-ras antibodies and the MEK inhibitor on PI 3′-kinase signaling are independent. Thus, anti-ras antibodies could disrupt basal ras-p110 binding which may be required for PI 3′-kinase signaling (51), whereas activation of PI 3′-kinase could feed into the MAP kinase cascade at a level above MEK but below or independent

of ras. Additional experiments will be required to define the point at which signals emanating from PI 3′-kinase intersect with the MAP kinase cascade.

The novel activating antibody described in this study will be an powerful tool in dissecting the role of PI 3′-kinase in various cellular responses. In addition, it will be extremely useful in delineating the mechanism by which occupancy of p85 SH2 domains regulates p110. The antibody does not activate PI 3′-kinase by inducing dimerization, as antibodies against the iSH2 and CSH2 domains can also produce p85 dimers yet do not activate. It is important to note that our data do not suggest that the CSH2 domain is uninvolved in activation of p110 but merely indicate that our anti-CSH2 antibodies are not activating. In fact, we have previously shown that both N-terminal and C-terminal SH2 domains contribute to regulation of PI 3′-kinase (53). Presumably, the anti-NSH2 antibody activates p85-p110 dimers by forcing p85 into a conformation that mimics the activated, phosphoprotein-bound state. Previous studies have in fact shown that activating phosphopeptides induce conformational changes in the NSH2 domain of PI 3′-kinase (44). Determination of the epitopes recognized by our antibody will be useful in mapping region within the p85 SH2 domains whose conformation is linked to activation of the p110 catalytic subunit.

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The first two authors contributed equally to this work.

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REFERENCES

1. **Akimoto, K., R. Takahashi, S. Moriya, N. Nishioka, J. Takayanagi, K. Kimura, Y. Fukui, S. Osada, K. Mizuno, S. Hirai, A. Kazlauskas, and S. Ohno.** 1996. EGF or PDGF receptors activate atypical PKClambda through phosphatidylinositol 3-kinase. EMBO J. **15:**788–798.
2. **Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White.** 1992. The phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. **11:**3469–3479.
3. **Backer, J. M., M. G. Myers, Jr., X.-J. Sun, D. J. Chin, S. E. Shoelson, M. Miralpeix, and M. F. White.** 1993. Association of IRS-1 with the insulin receptor and the phosphatidylinositol 3′-kinase. Formation of binary and ternary signaling complexes in intact cells. J. Biol. Chem. **268:**8204–8212.
4. **Betsholtz, C., A. Johnsson, C. H. Heldin, and B. Westermark.** 1986. Efficient reversion of simian sarcoma virus-transformation and inhibition of growth factor-induced mitogenesis by suramin. Proc. Natl. Acad. Sci. USA **83:**6440–6444.
5. **Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber.** 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature **369:**756–758.
6. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64:**231–302.
7. **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:**9478–9483.
8. **Cheatham, L., M. Monfar, M. M. Chou, and J. Blenis.** 1995. Structural and functional analysis of pp70S6k. Proc. Natl. Acad. Sci. USA **92:**11696–11700.
9. **Cheatham, B., C. J. Vlahos, L. Cheatham, L. Wang, J. Blenis, and C. R. Kahn.** 1994. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. Mol. Cell. Biol. **14:**4902–4911.
10. **Chung, J., T. C. Grammer, K. P. Lemon, A. Kazlauskas, and J. Blenis.** 1994. PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. Nature **370:**71–75.
11. **Cross, M. J., A. Stewart, M. N. Hodgkin, D. J. Kerr, and M. J. O. Wakelam.** 1995. Wortmannin and its structural analogue demethoxyviridin inhibit stimulated phospholipase A_2 activity in Swiss 3T3 cells—wortmannin is not a specific inhibitor of phosphatidylinositol 3-kinase. J. Biol. Chem. **270:**25352–25355.
12. **Davis, R. J.** 1993. The mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. **268:**14553–14556.
13. **Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams.** 1991. cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β-receptor. Cell **65:**75–82.
14. **Flanagan, C. A., E. A. Schneiders, A. W. Emerick, R. Kunisawa, A. Admon, and J. Thorner.** 1993. Phosphatidylinositol 4-kinase: gene structure and requirement for yeast cell viability. Science **262:**1444–1448.
15. **Furth, M. E., L. J. Davis, B. Fleurdelys, and E. M. Scolnick.** 1982. Monoclonal antibodies to the p21 products of the transforming gene of Harvey sarcoma virus and of the cellular *ras* gene family. J. Virol. **43:**2947–304.
16. **Guinebault, C., B. Payrastre, C. Racaud-Sultan, H. Mazarguil, M. Breton, G. Mauco, M. Plantavid, and H. Chap.** 1995. Intergrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85-α with actin filaments and focal adhesion kinase. J. Cell Biol. **129:**831–842.
17. **Hara, K., K. Yonezawa, H. Sakue, K. Kotani, A. Kojima, M. D. Waterfield, and M. Kasuga.** 1995. Normal activation of p70 S6 kinase by insulin in cells overexpressing dominant negative 85kD subunit of phosphoinositide 3-kinase. Biochem. Biophys. Res. Commun. **208:**735–741.
18. **Hartley, K. O., D. Gell, G. C. M. Smith, H. Zhang, N. Divecha, M. A. Connelly, A. Admon, S. P. Lees-Miller, C. W. Anderson, and S. P. Jackson.** 1995. DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. Cell **82:**849–856.
19. **Hawkins, P. T., A. Eguinoa, R.-G. Qiu, D. Stokoe, F. T. Cooke, R. Walters, S. Wennström, L. Claesson-Welsh, T. Evans, M. Symons, and L. Stephens.** 1995. PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. Curr. Biol. **5:**393–403.
20. **Hiles, I. D., M. Otsu, S. Volinia, M. J. Fry, I. Gout, R. Dhand, G. Panayotou, F. Ruiz-Larrea, A. Thompson, N. F. Totty, J. J. Hsuan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1992. Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. Cell **70:**419–429.
21. **Hu, P., B. Margolis, E. Y. Skolnik, R. Lammers, A. Ullrich, and J. Schlessinger.** 1992. Interactions of phosphatidylinositol 3-kinase-associated p85 with epidermal growth factor and platelet-derived growth factor receptors. Mol. Cell. Biol. **12:**981–990.
22. **Hu, P., A. Mondino, E. Y. Skolnik, and J. Schlessinger.** 1993. Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. Mol. Cell. Biol. **13:**7677–7688.
23. **Hu, Q., A. Klippel, A. J. Muslin, W. J. Fantl, and L. T. Williams.** 1995. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. Science **268:**100–102.
24. **Jhun, B. H., D. W. Rose, B. L. Seely, L. Rameh, L. Cantley, A. R. Saltiel, and J. M. Olefsky.** 1994. Microinjection of the SH2 domain of the 85-kilodalton subunit of phosphatidylinositol 3-kinase inhibits insulin-induced DNA synthesis and c-*fos* expression. Mol. Cell. Biol. **14:**7466–7475.
25. **Joly, M., A. Kazlauskas, and S. Corvera.** 1995. Phosphatidylinositol 3-kinase activity is required at a postendocytic step in platelet-derived growth factor receptor trafficking. J. Biol. Chem. **270:**13225–13230.
26. **Kapeller, R., and L. C. Cantley.** 1994. Phosphatidylinositol 3-kinase. Bioessays **16:**565–576.
27. **Kimura, K., S. Hattori, Y. Kabuyama, Y. Shizawa, J. Takayanagi, S. Nakamura, S. Toki, Y. Matsuda, K. Onodera, and Y. Fukui.** 1994. Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. J. Biol. Chem. **269:**18961–18967.
28. **Klippel, A., C. Reinhard, W. M. Kavanaugh, G. Apell, M. A. Escobedo, and L. T. Williams.** 1996. Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. Mol. Cell. Biol. **16:**4117–4127.
29. **Kotani, K., K. Yonezawa, K. Hara, H. Ueda, Y. Kitamura, H. Sakaue, A. Ando, A. Chavanieu, B. Calas, F. Grigorescu, M. Nishiyama, M. D. Waterfield, and M. Kasuga.** 1994. Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling. EMBO J. **13:**2313–2321.
30. **Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N. Rao Movva, and M. N. Hall.** 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G_1 progression. Cell **73:**585–596.
31. **Kuo, C. J., J. Chung, D. F. Florentino, W. M. Flanagan, J. Blenis, and G. R. Crabtree.** 1992. Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. Nature **358:**70–73.

32. **Kyriakis, J. M., J. R. Woodgett, and J. Avruch.** 1995. The stress-activated protein kinases—a novel ERK subfamily responsive to cellular stress and inflammatory cytokines. Ann. N. Y. Acad. Sci. **766:**303–319.
33. **Lane, H. A., A. Fernandez, N. J. C. Lamb, and G. Thomas.** 1993. $p70^{s6k}$ function is essential for G1 progression. Nature **363:**170–172.
34. **Martys, J. L., C. Wjasow, D. M. Gangi, M. C. Kielian, T. E. McGraw, and J. M. Backer.** 1996. Wortmannin sensitive trafficking pathways in CHO cells: differential effects on endocytosis and lysosomal sorting. J. Biol. Chem. **271:**10953–10962.
35. **Minden, A., A. Lin, F.-X. Claret, A. Abo, and M. Karin.** 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell **81:**1147–1157.
36. **Ming, X.-F., B. M. T. Burgering, S. Wennstrom, L. Claesson-Welsh, C.-H. Heldin, J. L. Bos, S. C. Kozma, and G. Thomas.** 1994. Activation of p70/p85 S6 kinase by a pathway independent of p21-ras. Nature **371:**426–429.
37. **Molz, L., Y. W. Chen, M. Hirano, and L. T. Williams.** 1996. Cpk is a novel class of *Drosophila* PtdIns 3-kinase containing a C2 domain. J. Biol. Chem. **271:**13892–13899.
38. **Mulcahy, L. S., M. R. Smith, and D. W. Stacey.** 1985. Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature **313:**241–243.
39. **Nakanishi, H., K. A. Brewer, and J. H. Exton.** 1993. Activation of the zeta-isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. **268:**13–16.
40. **Nakanishi, S., K. J. Catt, and T. Balla.** 1995. A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. Proc. Natl. Acad. Sci. USA **92:**5317–5321.
41. **Nishimura, R., W. Li, A. Kashishian, A. Mondino, M. Zhou, J. Cooper, and J. Schlessinger.** 1993. Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. Mol. Cell. Biol. **13:**6889–6896.
42. **Okada, T., Y. Kawano, T. Sakakibara, O. Hazeki, and M. Ui.** 1994. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. J. Biol. Chem. **269:**3568–3573.
43. **Otsu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. J. Morgan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1991. Characterization of two 85 kD proteins that associate with receptor tyrosine kinases, middle-T/$pp60^{c\text{-}src}$ complexes and PI3-kinase. Cell **65:**91–104.
44. **Panayotou, G., B. Bax, I. Gout, M. Federswisch, B. Wrobolowski, R. Dhand, M. J. Fry, T. L. Blundell, A. Wollmer, and M. D. Waterfield.** 1992. Interaction of the p85 subunit of PI 3-kinase and its N-terminal SH2 domain with a PDGF receptor phosphorylation site: structural features and analysis of conformational changes. EMBO J. **11:**4261–4272.
45. **Pang, L., T. Sawada, S. J. Decker, and A. R. Saltiel.** 1995. Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. J. Biol. Chem. **270:**13585–13588.
46. **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 **263:**1609–1612.
47. **Pollard, J. W., and C. P. Stanners.** 1979. Characterization of cell lines showing growth control isolated from both the wild-type and a leucyl-tRNA synthetase mutant of Chinese hamster ovary cells. J. Cell. Physiol. **98:**571–585.
48. **Pons, S., T. Asano, E. Glasheen, M. Miralpeix, Y. Zhang, T. L. Fisher, M. G. Myers, Jr., X. J. Sun, and M. F. White.** 1995. The structure and function of $p55^{PIK}$ reveal a new regulatory subunit for phosphatidylinositol 3-kinase. Mol. Cell. Biol. **15:**4453–4465.
49. **Price, D. J., J. R. Grove, V. Calvo, J. Avruch, and B. E. Bierer.** 1992. Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase. Science **257:**973–977.
50. **Roche, S., M. Koegl, and S. A. Courtneidge.** 1994. The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not all, growth factors. Proc. Natl. Acad. Sci. USA **91:**9185–9189.
51. **Rodriguez-Viciana, P., P. H. Warne, R. Dhand, B. Vanhaesebroeck, I. Gout, M. J. Fry, M. D. Waterfield, and J. Downward.** 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature **370:**527–532.
52. **Rodriguez-Viciana, P., P. H. Warne, B. Vanhaesebroeck, M. D. Waterfield, and J. Downward.** 1996. Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. EMBO J. **15:**2442–2451.
53. **Rordorf-Nikolic, T., D. J. Van Horn, D. Chen, M. F. White, and J. M. Backer.** 1995. Regulation of phosphatidylinositol 3′-kinase by tyrosyl phosphoproteins. Full activation requires occupancy of both SH2 domains in the 85-kDa regulatory subunit. J. Biol. Chem. **270:**3662–3666.
54. **Sabatini, D. M., H. Erdjument-Bromage, M. Lui, P. Tempst, and S. H. Snyder.** 1994. RAFT1: a mammalian protein that binds to FKBP-12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell **78:**35–44.
55. **Sabatini, D. M., B. A. Pierchala, R. K. Barrow, M. J. Schell, and S. H. Snyder.** 1995. The rapamycin and FKBP12 target (RAFT) displays phosphatidylinositol 4-kinase activity. J. Biol. Chem. **270:**20875–20878.
56. **Savitsky, K., A. Bar-Shira, S. Gilad, G. Rotman, Y. Ziv, L. Vanagaite, D. A. Tagle, S. Smith, T. Uziel, S. Stez, M. Ashkenaszi, I. Peckaer, M. Frydman, R. Harnik, S. R. Patanjali, A. Simmons, G. A. Clines, A. Sartiel, R. A. Gatti, L. Chessa, O. Sanal, M. F. Lavin, N. G. J. Jaspers, M. R. Taylor, C. F. Arlett, T. Mikki, S. M. Weissman, M. Lovett, F. S. Collins, and Y. Shiloh.** 1995. A single ataxia telangiectasia gene with a product similar to PI 3-kinase. Science **268:**1749–1753.
57. **Skolnik, E. Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich, and J. Schlessinger.** 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. Cell **65:**83–90.
58. **Stoyanov, B., S. Volinia, T. Hanck, I. Rubio, M. Loubtchenkov, D. Malek, S. Stoyanova, B. Vanhaesebroeck, R. Dhand, B. Nurnberg, P. Gierschik, K. Seedorf, J. J. Hsuan, M. D. Waterfield, and R. Wetzker.** 1995. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. Science **269:**690–693.
59. **Toker, A., C. Bachelot, C. S. Chen, J. R. Falck, J. H. Hartwig, L. C. Cantley, and T. J. Kovacsovics.** 1995. Phosphorylation of the platelet p47 phosphoprotein is mediated by the lipid products of phosphoinositide 3-kinase. J. Biol. Chem. **270:**29525–29531.
60. **Toker, A., M. Meyer, K. K. Reddy, J. R. Falck, R. Aneja, S. Aneja, A. Parra, D. J. Burns, L. M. Ballas, and L. C. Cantley.** 1994. Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. J. Biol. Chem. **269:**32358–32367.
61. **Tolias, K. F., L. C. Cantley, and C. L. Carpenter.** 1995. Rho family GTPases bind to phosphoinositide kinases. J. Biol. Chem. **270:**17656–17659.
62. **Valius, M., and A. Kazlauskas.** 1993. Phospholipase C-gamma and phosphatidylinositol 3-kinase are downstream mediators of the PDGF receptor's mitogenic signal. Cell **73:**321–334.
63. **Virbasius, J. V., A. Guilherme, and M. P. Czech.** 1996. Mouse p170 is a novel phosphatidylinositol 3-kinase containing a C2 domain. J. Biol. Chem. **271:**13304–13307.
64. **Volinia, S., R. Dhand, B. Vanhaesebroeck, L. K. MacDougall, R. Stein, M. J. Zvelebil, J. Domin, C. Panaretou, and M. D. Waterfield.** 1995. A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. EMBO J. **14:**3339–3348.
65. **Weng, Q.-P., K. Andrabi, A. Klippel, M. T. Kozlowski, L. T. Williams, and J. Avruch.** 1995. Phosphatidylinositol 3-kinase signals activation of p70 S6 kinase *in situ* through site-specific p70 phosphorylation. Proc. Natl. Acad. Sci. USA **92:**5744–5748.
66. **Weng, Q.-P., K. Andrabi, M. T. Kozlowski, J. R. Grove, and J. Avruch.** 1995. Multiple independent inputs are required for activation of the p70 S6 kinase. Mol. Cell. Biol. **15:**2333–2340.
67. **Yamauchi, K., K. Holt, and J. E. Pessin.** 1993. Phosphatidylinositol 3-kinase functions upstream of Ras and Raf in mediating insulin stimulation of c-*fos* transcription. J. Biol. Chem. **268:**14597–14600.
68. **Yao, R., and G. M. Cooper.** 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science **267:**2003–2006.
69. **Zhang, J., J. R. Falck, K. K. Reddy, C. S. Abrams, W. Zhao, and S. E. Rittenhouse.** 1995. Phosphatidylinositol (3,4,5)-trisphosphate stimulates phosphorylation of pleckstrin in human platelets. J. Biol. Chem. **270:**22807–22810.