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Membrane Localization of Phosphatidylinositol 3-Kinase Is Sufficient To Activate Multiple Signal-Transducing Kinase Pathways

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Phosphatidylinositol (PI) 3-kinase is a cytoplasmic signaling molecule recruited to the membrane by activated growth factor receptors. The p85 subunit of PI 3-kinase links the catalytic p110 subunit to activated growth factor receptors and is required for enzymatic activity of p110. In this report, we describe the effects of expressing novel forms of p110 that are targeted to the membrane by either N-terminal myristoylation or C-terminal farnesylation. The expression of membrane-localized p110 is sufficient to trigger downstream responses characteristic of growth factor action, including the stimulation of pp70 S6 kinase, Akt/Rac, and Jun N-terminal kinase (JNK). These responses can also be triggered by expression of a form of p110 (p110*) that is cytosolic but exhibits a high specific activity. Finally, targeting of p110* to the membrane results in maximal activation of downstream responses. Our data demonstrate that either membrane-targeted forms of p110 or a form of p110 with high specific activity can act as constitutively active PI 3-kinases and induce PI 3-kinase-dependent responses in the absence of growth factor stimulation. The results also show that PI 3-kinase activation is sufficient to stimulate several kinases that appear to function in different signaling pathways.

Phosphatidylinositol (PI) 3-kinase activity has been implicated in the regulation of a number of different cellular responses, including the regulation of cell growth. Oncogenic transformation or the stimulation of cells with growth factors results in an increased level in the phospholipid products of PI 3-kinase (for reviews, see references 4, 23, and 50). Mutants of platelet-derived growth factor (PDGF) receptor or certain oncogenes which fail to activate PI 3-kinase are deficient in triggering either mitogenic responses or oncogenic transformation, respectively. These data suggest that PI 3-kinase is an important mediator of signaling events that regulate cell growth and cellular transformation.

PI 3-kinase is a heterodimeric complex consisting of 85- and 110-kDa subunits (p85 and p110) (23). The p85 subunit consists of multiple domains including a Src homology 3 (SH3) domain, a breakpoint cluster region domain, and two SH2 domains. The two SH2 domains bind tyrosine-phosphorylated receptors and in this manner recruit the p85-p110 complex to activated receptors. The two SH2 domains are separated by the inter-SH2 (iSH2) region. The iSH2 domain mediates the interaction of p85 with p110, and this interaction is required for the enzymatic activity of p110 (28) (Fig. 1). It is possible that the role of p85 is to target p110 to the membrane, where its lipid substrates reside (23).

PI 3-kinase has been implicated in the regulation of many other cellular processes, including the reorganization of the actin cytoskeleton (24, 30, 40, 59), receptor internalization (21), histamine secretion (63), neutrophil activation (56), platelet activation (64), cell migration (31), glucose transport (41), and vesicular sorting (48). PI 3-kinase was implicated in the regulation of many of these processes, because they were inhibited by wortmannin, a fungal metabolite that was thought to be a specific inhibitor of p110 (55). However, recent studies indicate that a number of PI 3- and PI 4-kinases are sensitive to wortmannin (37, 51, 57). The study of PI 3-kinase-regulated processes has also become more complicated, because additional members of this family of enzymes have been discovered (see references 20 and 27 for reviews). For these reasons, a direct way of examining the cellular responses induced by PI 3-kinase is needed.

We have recently described a constitutively active PI 3-kinase molecule, p110*, which can activate signaling pathways independent of growth factor stimulation (19). p110* is a chimeric protein in which the iSH2 region of p85 was covalently linked to its binding site at the N terminus of p110, using a flexible hinge region. The iSH2 region of p85 is required for high lipid and protein kinase activities of p110, suggesting that p85 is part of the catalytic complex or regulates it. Expression of p110* caused the Ras-dependent activation of the *fos* promoter in mammalian fibroblasts and the activation of Ras, Raf, and mitogen-activated protein (MAP) kinase in *Xenopus* oocytes. In addition, p110* expression resulted in the activation of pp70 S6 kinase (58) and the stimulation of Rab5-mediated endosome fusion in vitro (33).

The use of activated forms of PI 3-kinase enables the direct study of cellular processes regulated by PI 3-kinase without prior growth factor activation. The use of activated PI 3-kinase molecules also allows the determination of whether PI 3-kinase activation alone is sufficient for the induction of a signaling event. The direct study of PI 3-kinase-induced signaling events eliminates the problems associated with the use of PI 3-kinase inhibitors such as wortmannin (see above).

In this report, we describe the construction of a new class of constitutively active PI 3-kinases. We have selectively targeted p110 to the membrane, using either N-terminal myristoylation or C-terminal farnesylation signals. To assess the function of membrane-localized PI 3-kinase, we selected several serine/threonine kinases, including pp70 S6 kinase, Akt, and c-Jun N-terminal kinase (JNK). pp70 S6 kinase has already been shown to be activated by p110* (58). Akt/Rac is activated by growth factors, and this activation seems to be dependent on PI 3-kinase (2, 13). However, the activation of Akt kinase by PI 3-kinase has never been shown directly. Finally, JNK, which is

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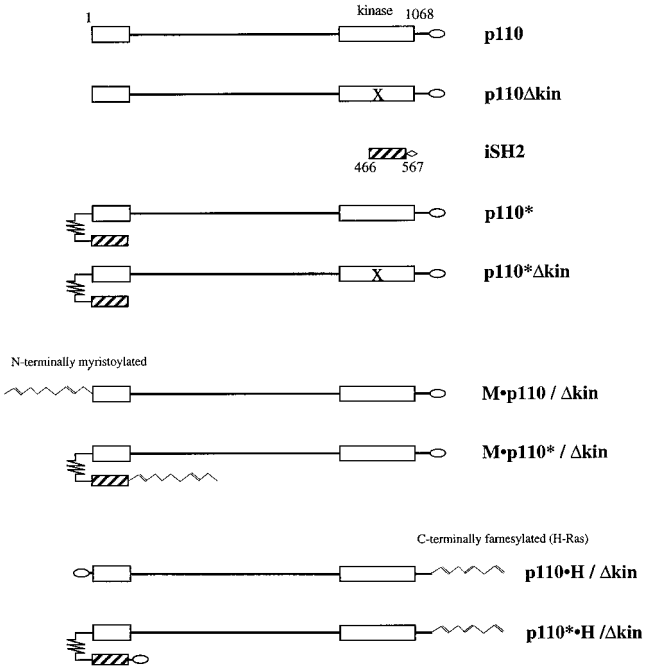

FIG. 1. Schematic representation of the PI 3-kinase derivatives used in this study. p110 constructs were tagged at either the N- or C-terminal end with the Myc epitope (oval); the iSH2 fragment of p85 (hatched bar) contained a C-terminal influenza virus HA epitope tag (diamond). The p110 region with homology to the catalytic domain of protein kinases is depicted by a box labeled "kinase." The domain responsible for the interaction with the iSH2 domain of the p85 subunit is shown as a small box at the p110 N terminus. p110Δkin is a kinase-deficient p110 in which the arginine at position 802 was mutated to a lysine residue as indicated by an asterisk within the catalytic domain. The first and last amino acids of fragments are numbered with respect to their positions in the wt p85 or p110 sequence. p110* is a constitutively active chimera that contains the iSH2 domain of p85 fused to the N terminus of p110 by a flexible "glycine-kinker" (19). Myr · p110 and Myr · p110* as well as their kinase-deficient versions were modified at their respective N-terminal ends with the myristoylation sequence of pp60 c-Src (8, 26, 46). The C-terminal ends of p110 · H, p110* · H, and their kinase-defective derivatives were extended by the farnesylation and palmitoylation sequences of H-Ras (3). Similarly, p110 molecules containing the farnesylation sequence and the polybasic stretch of K-Ras (3) were generated (not shown).

thought to be activated through pathways independent of S6 kinase and Akt kinase, has not yet been linked to PI 3-kinase. We found that all three of these pathways can be activated by either membrane-localized p110 or p110*, although a fourth signaling kinase, MAP kinase, is not. We also show that small G proteins mediate PI 3-kinase-induced signaling in some but not all pathways. We furthermore demonstrate that expression of the various constitutively active forms of PI 3-kinase results in elevated intracellular levels of PI 3-phosphatides.

MATERIALS AND METHODS

Cell culture. COS-7 cells were obtained from the American Type Culture Collection and cultured at 37°C in Dulbecco's modified Eagle medium containing 10% bovine calf serum, penicillin (50 μg/ml), and streptomycin (50 μg/ml).

Antibodies. The murine monoclonal anti-p110 antibodies U3A and I1A have been described previously (19, 28). Ascites fluid with the murine anti-influenza virus hemagglutinin 1 (HA1) monoclonal antibody 12CA5 (62) was kindly provided by Qianjin Hu (University of California, San Francisco). Hybridoma 9E10 (10) was obtained from M. Bishop (University of California, San Francisco), and mouse ascites fluid containing murine monoclonal anti-Myc antibody was prepared. Rabbit polyclonal anti-Akt/Rac1 kinase antibody was a generous gift from Brian A. Hemmings (Friedrich Miescher Institute, Basel, Switzerland). Rabbit polyclonal anti-Erk and anti-JNK antibodies and mouse monoclonal anti-Ras antibodies were obtained from Santa Cruz Biotechnology. Rabbit anti-pp70 S6 kinase antibodies were purchased from Upstate Biotechnology Inc. or Santa Cruz Biotechnology.

Plasmid constructions. The mammalian expression vector for recombinant HA-tagged pp70 S6 kinase was described recently (58). The construction of vectors directing the expression of HA-tagged iSH2 (iSH2 · HA), Myc-tagged p110 and p110* (p110 · myc and p110* · myc), RasV12, c-Ras, and RasN17 in mammalian expression vectors was previously described in detail (19, 28). The cDNA for Erk2 was kindly provided by Akira Kikuchi and cloned into a vector that directs expression from the human cytomegalovirus (CMV) promoter after modification with an N-terminal HA tag. The HA-tagged cDNA for Akt/Rac1 kinase was a generous gift of Brian A. Hemmings and cloned into a mammalian expression vector directing expression from the human CMV promoter/enhancer region (34). The cDNA for JNK was kindly provided by Roger Davis (Howard Hughes Medical Institute, Worcester, Mass.) and cloned into a vector that directs expression from the CMV promoter after modification with an N-terminal HA tag. Alan Hall generously supplied expression vectors for Myc-tagged RacV12, Cdc24 V12, wild-type (wt) Cdc42, and Cdc42 N17 as well as glutathione *S*-transferase (GST)–RacN17 DNA. The coding sequence for wt Rac was obtained by PCR from HeLa cell cDNA (Chiron Corporation, Emeryville, Calif.) and cloned into a vector that directs expression of Myc-tagged wt Rac from the CMV promoter. Using the GST-RacN17 DNA as the template, we generated an analogous mammalian expression vector for Myc-RacN17. GST-Jun(1-89) was obtained after cloning of a PCR fragment overlapping the N-terminal 89 amino acids of c-Jun (human c-Jun cDNA was supplied by Klaus Giese, Chiron Corporation) and into suitable restriction sites of pGEX-KG (16). Overexpression and purification of the GST fusion protein were performed on glutathione-agarose beads (Pharmacia) according to the manufacturer's instructions.

The p110 N terminus was modified by the pp60 c-Src myristoylation sequence (8, 26, 46), using primers SrcM -sense- (5′ C ATG GGG AGC AGC AAG AGC AAG CCC AAG GAC CCC AGC CAG CGC GGG GGA CA 3′) and SrcM -antisense- (5′ TAT GTC CCC CGC GCT GGC TGG GGT CCT TGG GCT TGC TCT TGC TGC TCC C 3′) flanked by *Nco*I and *Nde*I restriction sites (A at position 2 is the c-Src start codon). The annealed DNA fragment was fused in frame via the respective restriction sites to the N terminus of the p110 cDNA extended by a C-terminal Myc tag (28) in a mammalian expression vector that directs expression from the SRα promoter (52). To modify the C-terminal end of p110 with the H-Ras farnesylation and palmitoylation sequence (1, 3, 17), a C-terminal fragment of the p110 cDNA was amplified by using primer p110-3′ HindIII (5′ CTG AGC AAG AAG CTT TGG 3′), consisting of nucleotides 3092 to 3109 of the coding strand overlapping a *Hin*dII site, and primer p110 · H (5′ GGA TCC TCA GCT CAG CAC GCA CTT GCA GCT CAT GCA GCC GGG GCC GCT GGC GCC CCC GAG CTC GTT CAA AGC ATG CTG 3′), overlapping nucleotides 3190 to 3204 of the noncoding strand (A of the start codon is designated nucleotide 1; nucleotides that are changed with respect to the wild-type sequence are underlined). This extended the p110 C-terminal end by a sequence encoding amino acids DLGGA as a hinge region (overlapping restriction sites *Sac*I-*Ecl*136II and *Kas*I-*Nar*I), which precedes the coding region for the H-Ras CAAX box, a stop codon, and a *Bam*HI restriction site. The C-terminal end of p110 was modified with the K-Ras farnesylation sequence plus polybasic region (3, 17) by PCR using primer p110 3′ HindIII and primer p110 · K (5′ GGA TTC TCA CAT GAT CAC GCA CTT GGT CTT GGA CTT CTT CTT CTT TTT GCC ATC TTT GGA GGC GCC GAG CTC GTT CAA AGC ATG CTG. This extended the p110 C-terminal end by a sequence encoding amino acids DLGGA as a hinge region (overlapping restriction sites *Sac*I-*Ecl*136II and *Kas*I-*Nar*I), which precedes the coding region for the K-Ras farnesylation and polylysine sequence, a stop codon, and a *Bam*HI restriction site. The Myc-tagged C-terminal end of p110 (28) was substituted by the H-Ras or K-Ras CAAX box modified sequence, using *Hin*dIII and *Bam*HI. For the C-terminally farnesylated p110 constructs, the N-terminal end of the p110 coding region was modified with the 10-amino-acid Myc epitope (EQKLISEEDL [10]), using primer p110 5′ Myc -sense- (5′ CT AGA ATG GAT GAG CAG AAG CTG ATT TCC GAG GAG GAC CTG AAC GGG GGA CA 3′) and primer p110 5′ Myc -antisense- (5′ T ATG TCC CCC GTT CAG GTC CTC CTC GGA AAT CAG CTT CTG CTC ATC CAT T 3′) flanked by restriction sites for *Xba*I and *Nde*I. The Myc-coding region was fused in frame to the wt p110 N terminus by ligating the annealed oligonucleotide via *Xba*I-*Nde*I ends into pCG-p110 (28). The kinase-deficient plasmid p110Δkin, in which lysine at position 802 was changed to an arginine residue, was generated by site-specific mutagenesis using the gapped duplex DNA method (49) with primer p110-KR802 (5′ C GTC GCC ATT TCT AAA GAT GAT CTC 3′), annealing to nucleotides 2392 to 3016 of the p110 coding region. The correct sequence of the p110 fragments modified by PCR or oligonucleotides was confirmed by DNA sequence analysis. The N-terminal myristoylation or C-terminal H- or K-Ras farnesylation sequences were furthermore used to modify the coding regions for p110Δkin, p110*, and p110*Δkin by using the restriction sites described above for p110. For expression of p110 molecules in COS-7 cells, the respective DNA fragments were cloned into mammalian expression vector pCG via *Xba*I-*Bam*HI ends (28). pCG is a derivative of the vectors described by Matthias et al. (34) and directs expression in mammalian cells from the human CMV promoter/enhancer region.

Transient expression of recombinant p85 and p110 derivatives in COS-7 cells. COS-7 cells (60 to 70% confluent on a 10-cm-diameter plate) were transfected with mammalian expression vectors by the DEAE-dextran method (15). Cells

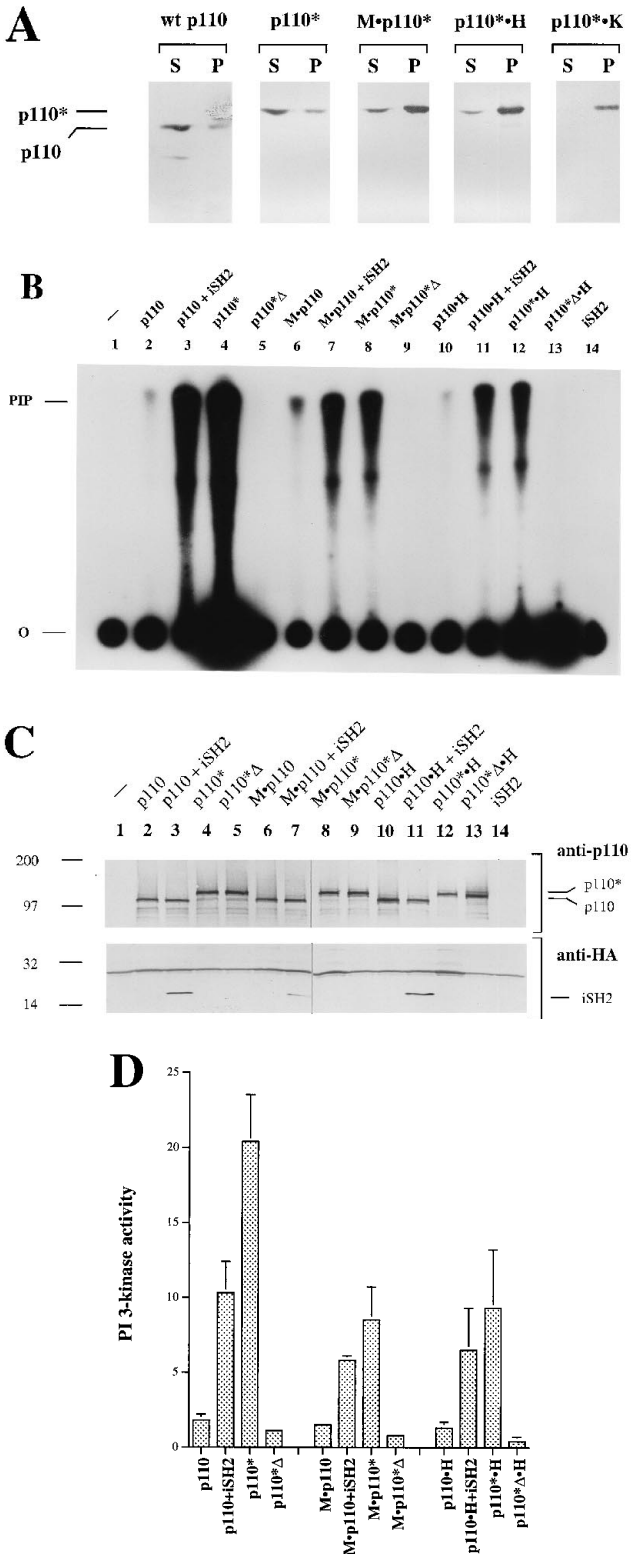

FIG. 2. (A) Subcellular localization of wt p110 and p110 derivatives. Lysates from COS-7 cells that were either untransfected or transfected with expression vectors for p110 molecules with membrane-targeting signals were separated into S100 (S) and P100 (P) fractions. Equivalent proportions were analyzed for protein distribution by SDS-PAGE. p110 and p110* molecules were detected by immunoblotting with anti-p110 antibody U3A (28). (B) PI 3-kinase activities of wt p110 and p110 derivatives. Myc-tagged p110 derivatives were expressed with or without the HA-tagged iSH2 region of p85 in COS cells and precipitated with an anti-Myc antibody. In control samples, either no molecules (lane 1) or HA-tagged (lane 14) iSH2 fragments were precipitated. One-third of each precipitate was subjected to a PI 3-kinase activity assay. The origin (O) of the chromatogram and the position of PI 3-phosphate (PIP) are indicated. (C) The protein levels were analyzed in parallel by SDS-PAGE, using the remaining two-third of the beads. After transfer to a nitrocellulose membrane, p110 or iSH2 molecules were detected by using an anti-p110 (upper panel) or anti-HA (lower panel) antibody, respectively. p110*Δ, M · p110*Δ, and p110*Δ · H designate the kinase-deficient versions of the respective p110* molecules. Positions of molecular size markers (in kilodaltons) are shown at the left. The immunoglobulin G light chain (32 kDa) of the antibodies used for the immunepreciptations was also recognized by the alkaline phosphatase-conjugated anti-mouse antibody. (D) Specific activities of wt p110 and membrane-targeted p110 derivatives. PI 3-kinase activities as shown for panel B were quantitated with a Molecular Imager (Bio-Rad) and normalized for the relative amounts of p110 protein as shown in panel C. The relative amount of PI 3-kinase activity is given in arbitrary units. Each bar represents the mean of three experiments ± standard deviation.

were starved for at least 30 h in medium containing 0.5% dialyzed fetal bovine calf serum and then treated with or without platelet-derived growth factor (PDGF) (2 nM) for 10 min at 37°C. In experiments in which the effect of wortmannin (50 nM) or rapamycin (20 ng/ml) was analyzed, the cells were pretreated for 15 min before the addition of growth factor; otherwise, they were pretreated for 2 h before lysis. COS-7 cells were washed twice with cold phosphate-buffered saline (PBS) and lysed at 4°C in mammalian cell lysis buffer containing 20 mM Tris (pH 7.5), 137 mM NaCl, 15% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 mg of aprotinin per ml, 20 mM leupeptin, 2 mM benzamidine, 1 mM sodium vanadate, 25 mM β-glycerolphosphate, 50 mM NaF, and 10 mM NaPPi. Lysates were cleared by centrifugation at 14,000 × *g* for 5 min, and aliquots of the lysates were analyzed for protein expression and enzyme activity (see below).

To investigate the intracellular distribution of p110 molecules, hypotonic lysates were prepared as described previously (3). COS-7 cells were scraped in ice-cold PBS into microcentrifuge tubes and collected at 400 × *g* for 2 min. The cells were lysed by Dounce homogenization on ice in 500 μl of 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM phenylmethylsulfonyl fluoride, 10 mg of aprotinin per ml, 20 mM leupeptin, 2 mM benzamidine, 1 mM sodium vanadate, 25 mM β-glycerolphosphate, 50 mM NaF, and 10 mM NaPPi for 10 min. After removal of the nuclei and unbroken cells at 1,500 × *g* for 5 min, the membranes were pelleted for 30 min at 120,000 × *g* in a TLA 120.2 rotor (Beckman). The supernatant (S100) and pellet (P100) fractions were collected, and equal proportions were analyzed for protein distribution.

In vitro protein kinase assays. Cell lysates containing HA-tagged pp70 S6 kinase, Akt, MAP kinase, or JNK were incubated with monoclonal anti-HA antibody 12CA5 for 1 h at 4°C. Protein A-Sepharose beads (Sigma) were used to precipitate the immune complexes. The beads were washed with 50 mM Tris-HCl (pH 7.5)–0.5 M LiCl–0.5% (vol/vol) Triton X-100, twice with PBS, and once with 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–1 mM dithiothreitol, all containing 0.1 mM sodium vanadate and 20 mM β-glycerolphosphate. For analyzing the immune complexes in an S6 kinase assay, the beads were divided in three aliquots. Two aliquots were subjected to an S6 kinase activity assay using [γ-^{32}P]ATP (5,000 Ci/mmol) based on a peptide substrate (53) in 30 μl; one aliquot was analyzed for the amount of recombinant pp70 S6 kinase in the precipitate. After 25 min at 22°C, the reaction was stopped by the addition of 10 μl of 500 mM EDTA. A 22-μl aliquot of the supernatant was applied to phosphocellulose paper (Whatman P81) and washed four times in 75 mM H_3PO_4. The relative amounts of incorporated radioactivity were determined in a liquid scintillation counter. Specific phosphorylation of the S6-derived peptide was obtained after subtraction of counts with protein A-Sepharose beads in the absence of the anti-HA antibody from counts of label incorporated in the presence of the anti-HA antibody.

For all other kinase assays, one-third of the immunobeads were subjected to an in vitro kinase reaction, and two-thirds were analyzed for the amount of the respective recombinant kinase protein. For analyzing Akt kinase activity, histone H2B was used as a substrate (13) as described previously (22). JNK activity was determined with GST-Jun(1-89) as a substrate as described previously (9). For MAP kinase activation, the phosphorylation of myelin basic protein was analyzed as described previously (44). The in vitro protein kinase reactions were carried out in 30 μl in the presence of [γ-^{32}P]ATP, and the mixtures were incubated at 22°C for 25 min. The reactions were stopped by the addition of 8 μl of Laemmli sample buffer, and 22-μl aliquots of the reaction mixtures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (32). The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Molecular Imager system (Bio-Rad). The complexes were analyzed by immunoblotting with the indicated antibodies.

Immunoblotting. Immunoprecipitates were boiled in Laemmli sample buffer (32), separated by SDS-PAGE, and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked in TBST buffer (10 mM Tris-HCl [pH 7.5],

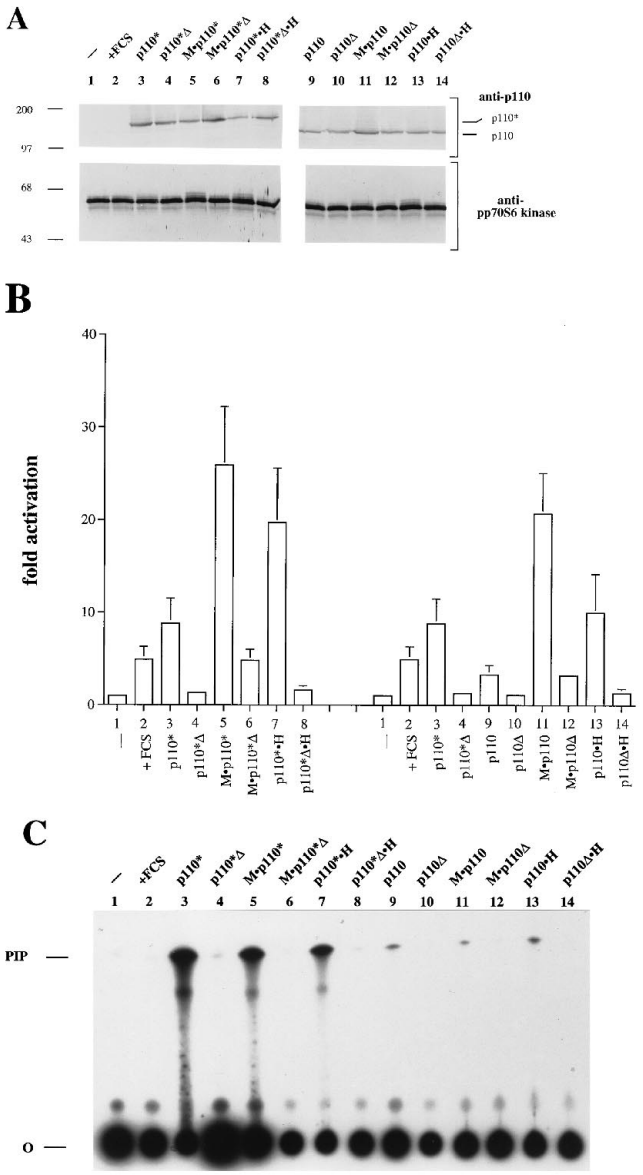

FIG. 3. pp70 S6 kinase activation by coexpression of membrane-localized p110 derivatives. The indicated Myc-tagged p110 molecules were coexpressed with HA-tagged pp70 S6 kinase. p110Δ and p110*Δ designate the respective kinase-deficient versions. (A) COS cell lysates were analyzed for p110 and pp70 S6 kinase expression levels by blotting with anti-p110 antibody U3A (upper panel) or an anti-pp70 S6 kinase antibody (lower panel). Positions of size markers are indicated in kilodaltons at the left. (B) pp70 S6 kinase was precipitated from lysates with an anti-HA antibody and subjected to an S6 kinase activity assay. The increase in S6 kinase activity is expressed relative to that of unstimulated pp70 S6 kinase coexpressed with empty vector. Each bar represents the mean of three transfection experiments ± standard deviation. A mobility shift of pp70 S6 kinase observed on the filter correlated with high S6 kinase activity and is most likely due to stimulating phosphorylation (58). (C) p110 proteins were precipitated by using an anti-Myc antibody and tested for PI 3-kinase activity. The production of radiolabeled PI 3-phosphate (PIP) was analyzed by thin-layer chromatography; the origin (O) of the chromatogram is indicated.

150 mM NaCl, 0.05% [vol/vol] Tween 20, 0.5% [wt/vol] sodium azide) containing 5% (wt/vol) dried milk. The respective antibodies were added in TBST at appropriate dilutions. Bound antibody was detected with anti-mouse or anti-rabbit conjugated to alkaline phosphatase (Promega) in TBST, washed, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Promega). Alternatively, horseradish peroxidase-conjugated anti-mouse antibody was used and developed by enhanced chemiluminescence (Amersham).

Determination of PI 3-kinase activity in p110 precipitates. The presence of PI 3-kinase activity in immune complexes was determined by incubating the beads with 30 mM HEPES, 30 mM $MgCl_2$, 50 μM ATP, 200 μM adenosine, 0.2 mg of sonicated PI per ml, and 10 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol) for 20 min at 25°C. Adenosine was added to inhibit any contaminating PI 4-kinase activity (60). Reactions were stopped by adding 100 μl of 1 M HCl, and the phospholipids were extracted with 200 μl of a 1:1 mixture of chloroform-methanol. The reaction products were separated by thin-layer chromatography as previously described (25). The conversion of PI to PI 3-phosphate was determined by autoradiography.

Analysis of intracellular levels of 3′-phosphorylated phosphoinositides. The generation of PI 3-phosphoinositides in vivo was determined essentially as described previously (47). Briefly, COS-7 cells transiently transfected with p110 derivatives were starved for 12 h in serum-free medium. The cells were metabolically labeled in phosphate-free medium for 12 h with 1 mCi of $^{32}P_i$ (8,500 to 9,120 Ci/mmol; New England Nuclear) per 10-cm-diameter dish. The phospholipids were extracted, deacylated, and subjected to anion-exchange high-pressure liquid chromatography (HPLC) analysis (47). Fractions were analyzed in a scintillation counter. Peak fractions containing glycerophosphoinositides derived from PI 3,4-P_2 or PI 3,4,5-P_3 were identified by cochromatography with deacylated ^{32}P-labeled standards produced by p110* in an in vitro PI 3-kinase reaction (see above). ^{3}H-labeled PI 4,5-P_2 (New England Nuclear) was deacylated and used as an additional standard to calibrate the column.

RESULTS

p110 derivatives with myristoylation or farnesylation signals are targeted to the cell membrane. To test whether membrane localization of p110 had an effect on PI 3-kinase-dependent processes, we constructed forms of p110 that were modified by the addition of membrane targeting signals. M · p110 contains the N-terminal myristoylation sequence of pp60$^{c\text{-}Src}$; p110 · H and p110 · K contain C-terminal farnesylation sequences of H-Ras or K-Ras, respectively (Fig. 1). Membrane-targeting signals were also used to modify the constitutively active p110*, thereby generating M · p110* and p110* · H. To confirm that the membrane-targeting strategy was successful, we tested by subcellular fractionation whether myristoylated or farnesylated p110 derivatives were localized to the membrane (Fig. 2A). In contrast to endogenous p110 and recombinant p110* molecules, p110 molecules containing lipid modification signals localized predominantly to the membrane fraction, suggesting that the respective targeting signals were functional. Since p110 molecules with an H-Ras-derived farnesylation signal were expressed to higher levels than molecules with the K-Ras-derived signal, p110 · H was used in the subsequent experiments. To assess whether the lipid modifications affected the enzymatic activity of p110, approximately equal amounts of myristoylated or farnesylated p110 proteins were precipitated from COS cell lysates and tested for PI 3-kinase activity (Fig. 2B and C). The specific activities of the myristoylated and farnesylated p110 proteins were similar to that of wt p110 (Fig. 2D). As observed for wt p110, the basal activities of the membrane-targeted p110 versions were greatly enhanced when the iSH2 fragment of p85 was coexpressed. Membrane-targeted derivatives of p110*, M · p110*, or p110* · H all displayed high specific activities. Finally, mutant forms, which were constructed by changing a conserved lysine residue in the catalytic domain of p110 to arginine, were inactive. These results indicate that the enzymatic properties of p110 molecules with N- or C-terminal lipid modifications were not changed.

Membrane-localized PI 3-kinase induced pp70 S6 kinase activity. We determined the abilities of various membrane-targeted p110 derivatives to activate a known effector of PI 3-kinase, pp70 S6 kinase. The modified proteins were transiently coexpressed with recombinant S6 kinase (58). pp70 S6 kinase was precipitated from COS cell lysates. One-third of each precipitate was subjected to an S6 kinase activity assay (Fig. 3B) (53), and the remaining two-thirds were analyzed for protein levels (Fig. 3A) and P13-kinase activities (Fig. 3C).

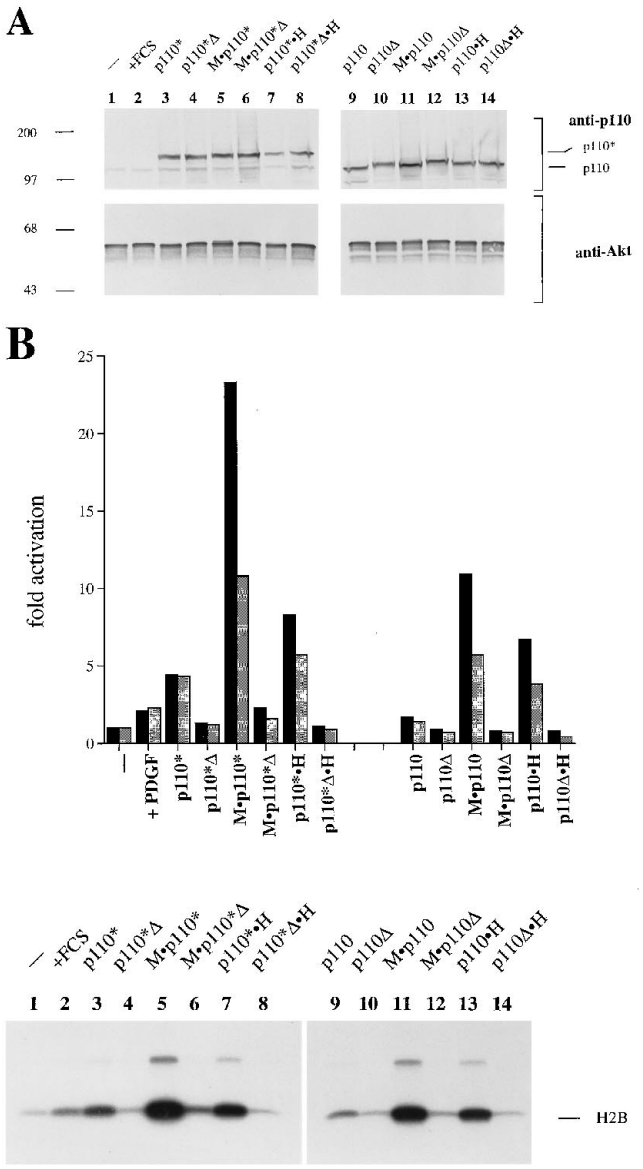

FIG. 4. Activation of the kinase activity of Akt by coexpression of membrane-localized p110. (A) The expression levels of the p110 derivatives and Akt were determined by blotting with an anti-p110 antibody (upper panel) or an anti-Akt antibody (lower panel). The positions of p110 molecules are indicated at the right. The band above the 97-kDa marker in lanes 1 to 8 represents endogenous p110 protein detected by the anti-p110 antibody. (B) Recombinant Akt was precipitated with an anti-HA antibody, and its kinase activity was determined by using histone H2B as a substrate. The incorporation of radiolabeled phosphate into H2B was quantitated with a Molecular Imager. The increase in Akt kinase activity is shown relative to that of unstimulated Akt coexpressed with empty vector DNA (upper panel). Black bars show the results of an experiment in which 3 μg of Akt vector DNA was transfected per plate; shaded bars show data obtained after transfection of 0.8 μg of Akt DNA per plate. The autoradiograph of a representative experiment is shown in the lower panel.

Remarkably, M · p110 and p110 · H caused a more than 10-fold increase in S6 kinase activity compared with wt p110, which could not substantially activate S6 kinase (Fig. 3B), although M · p110 and p110 · H exhibited approximately the same basal PI 3-kinase activity as wt p110 (Fig. 3C). p110*, which exhibited high PI 3-kinase activity, caused a 5- to 10-fold increase in S6 kinase activity over the background level. However, M · p110* and p110* · H showed a more than 20-fold activation of pp70 S6 kinase. This strong activation is also reflected by a mobility shift of the pp70 molecules in the gel as a result of phosphorylation (Fig. 3A) (5, 36, 58). Kinase-deficient versions of p110 or p110* were not able to activate S6 kinase substantially, which indicates that a functional kinase domain is required to induce the signaling event. The activation of pp70 S6 kinase by M · p110 or p110 · H was higher than that caused by the cytoplasmic p110*, although p110* had much higher specific activity (Fig. 3C). These data demonstrate that low amounts of PI 3-kinase activity are sufficient to activate pp70 S6 kinase when targeted to the membrane.

Membrane-targeted PI 3-kinase efficiently stimulates Akt/Rac. It was recently shown that stimulation of cells with PDGF activates the kinase activity of Akt and that PDGF receptor mutants which are defective in associating with PI 3-kinase fail to activate Akt (2, 13). These data suggest that PI 3-kinase is

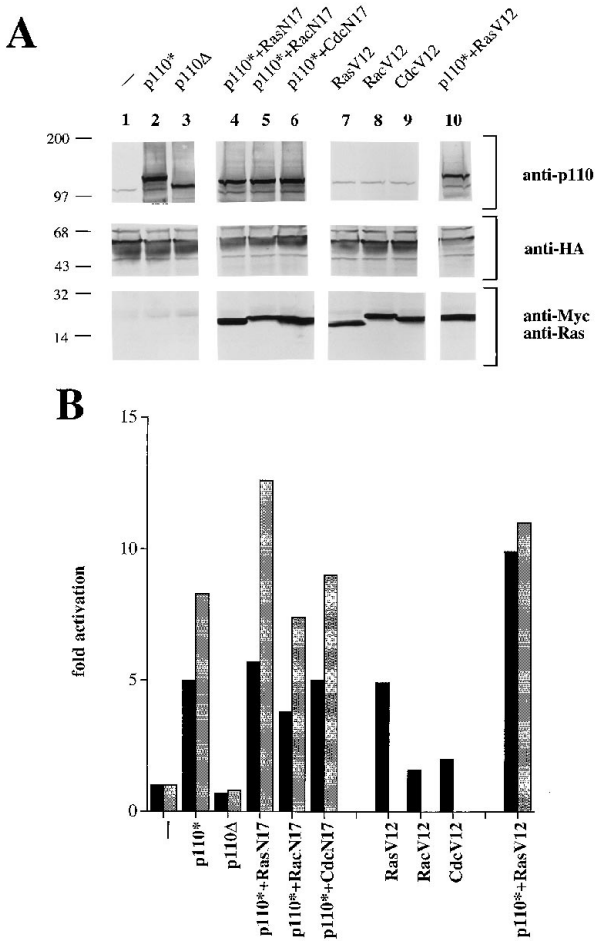

FIG. 5. Dominant negative mutants of the small G proteins Ras, Rac, and Cdc42 do not affect PI 3-kinase induced activation of Akt. p110* or p110* · H was coexpressed either with or without the indicated dominant negative or activated mutants of Ras, Rac, or Cdc42 and Akt. (A) Aliquots of the COS cell lysates were analyzed for expression of p110 and HA-tagged Akt by immunoblotting with an anti-p110 antibody U3A (upper panel) and an anti-HA antibody (middle panel), respectively. The expression of mutant Ras or Myc-tagged Rac or Cdc42 proteins was analyzed by probing with a mixture of anti-Ras and anti-Myc antibodies (lower panel). The weaker band above the 97-kDa marker in lanes 1, 7, 8, and 9 represents endogenous p110 protein detected by the anti-p110 antibody; this signal is obscured in samples with strong overexpression of recombinant p110 molecules. (B) Akt activation in anti-HA precipitates was analyzed and quantitated as described for Fig. 4. Black bars show results obtained with p110*; shaded bars show data obtained with p110* · H.

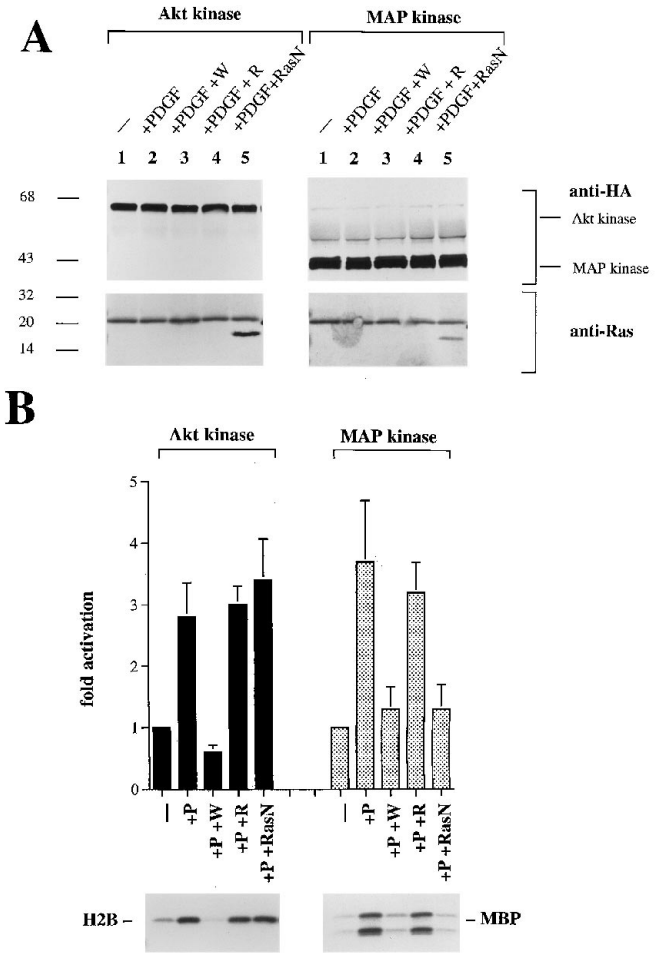

FIG. 6. Effects of wortmannin, rapamycin, and dominant negative Ras on PDGF-mediated activation of Akt or MAP kinase. Expression vectors for Akt or MAP kinase were cotransfected in parallel with either empty vector DNA or a vector that directs the expression of RasN17 (RasN). Cells were pretreated with or without wortmannin (W) or rapamycin (R) before stimulation with PDGF. (A) Expression of HA-tagged Akt, HA-tagged MAP kinase, and RasN17 was monitored by blotting with the indicated antibodies. (B) Akt and MAP kinase were precipitated from lysates and subjected to Akt and MAP kinase activity assays, respectively. Phosphorylation of either substrate histone H2B or myelin basic protein (MBP) was detected by autoradiography and quantitated with a Molecular Imager. The increase in Akt or MAP kinase activities is given relative to the levels of unstimulated kinases coexpressed with empty vector DNA. Each bar represents the mean of two transfections (upper panel). A representative autoradiograph is shown in the lower panel.

required for the activation of Akt. Using activated forms of PI 3-kinase, we can determine directly whether PI 3-kinase can activate Akt. Modified p110 molecules were transiently coexpressed with Akt. Akt was precipitated from lysates, and its activity was assayed with one-third of each precipitate (22) (Fig. 4B). Akt protein levels were analyzed by blotting of the remaining two-thirds of the precipitates (Fig. 4A). p110 · H and M · p110 stimulated the kinase activity of Akt between 5- and 10-fold, and p110* caused on average a 4-fold activity increase. The highest increases in Akt kinase activity were obtained in the presence of p110* · H or M · p110*, which stimulated Akt activity from 7- to more than 20-fold. No substantial increases in activity were observed with p110 or kinase-deficient derivatives. These results strengthen our previous finding that a low amount of PI 3-kinase activity can be more effective in triggering signaling responses when directed to the membrane than a predominantly cytoplasmic PI 3-kinase with a high specific activity. The data furthermore demonstrate that Akt is indeed a downstream effector of PI 3-kinase and that PI 3-kinase activation is sufficient to induce the Akt pathway.

PI 3-kinase-induced activation of Akt is independent of Ras, Rac, or Cdc42. Small G proteins have been implicated in PI 3-kinase signaling by a number of studies (18, 19, 29, 33, 39, 45, 54, 64, 65). To assess whether PI 3-kinase-induced stimulation of Akt is mediated by small GTPases, we have determined whether the expression of dominant negative forms of Ras, Rac, or Cdc42 can affect activation of Akt by PI 3-kinase. The expression of the dominant negative mutants did not interfere with the stimulation of Akt by activated PI 3-kinase molecules (Fig. 5B). We do not consider the small reduction observed with RacN17 significant, since a similar result was obtained when wt Rac was coexpressed (not shown). RasN17 did not interfere with PDGF-mediated Akt activation but did efficiently block PDGF-induced MAP kinase activation (Fig. 6)

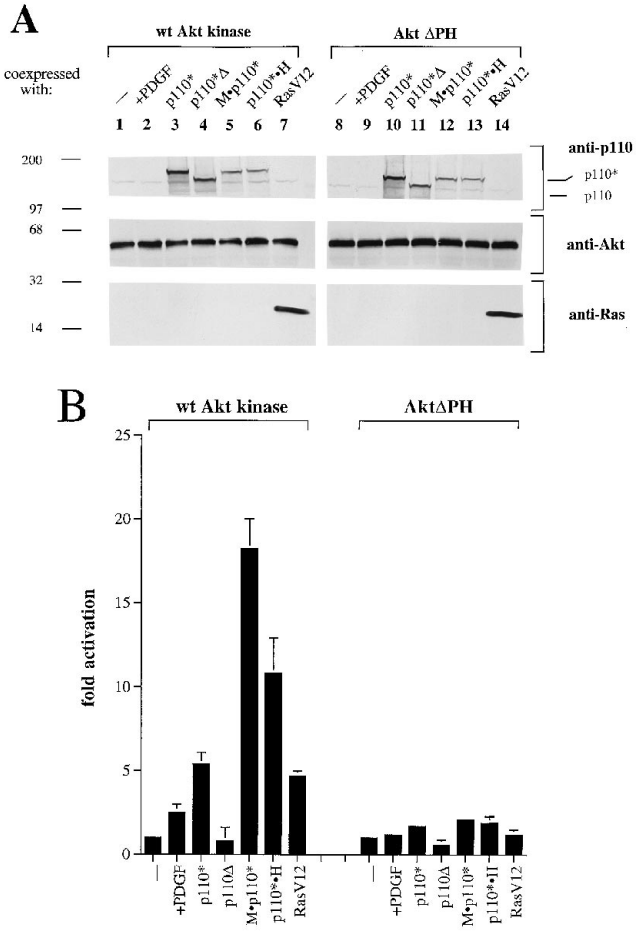

FIG. 7. A functional PH domain in Akt is required for PI 3-kinase mediated activation. wt Akt or a mutant Akt carrying a point mutation in the PH domain (AktΔPH) was coexpressed with the indicated p110* derivatives or RasV12. (A) Expression levels were monitored by immunoblotting. p110*, M · p110*, p110* · H, and p110Δkin were detected by an anti-p110 antibody U3A (upper panel). The weaker band above the 97-kDa marker represents endogenous p110 protein detected by the anti-p110 antibody. Recombinant Akt molecules were detected after precipitation with anti-HA antibody and blotting with an anti-Akt antibody (middle panel). The anti-HA immune complexes were analyzed for Akt activity in parallel (B) as described for Fig. 4. Each bar represents the mean of two independent experiments. The expression of activated Ras was detected by an anti-Ras antibody (lower panel).

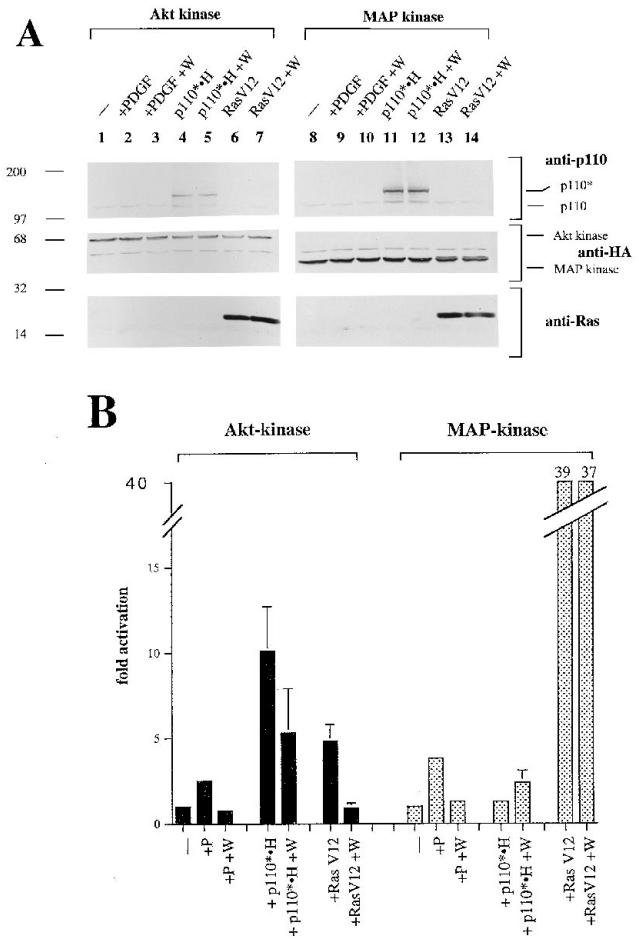

FIG. 8. Differential regulation of Akt and MAP kinases by PI 3-kinase and Ras. Recombinant Akt or MAP kinase were coexpressed with or without p110* · H and RasV12. Cells were treated with wortmannin (W) and stimulated with PDGF as indicated. (A) Expression levels were monitored by blotting. p110* · H was detected by an anti-p110 antibody U3A (upper panel). The weaker band above the 97-kDa marker represents endogenous p110 protein detected by the anti-p110 antibody. Recombinant Akt or MAP kinase were detected with anti-HA antibody (middle panel). The expression of RasV12 was detected by anti-Ras antibody (lower panel). (B) Anti-HA precipitates of Akt and MAP kinase were subjected to Akt and MAP kinase activity assays, respectively, and quantitated as described for Fig. 6. Each bar represents the mean of three experiments ± standard deviation. The wortmannin concentration used in this experiment was not sufficient to block the activation of Akt by the highly expressed p110* · H.

(2). RacN17 did interfere with p110*-induced JNK activation (see Fig. 9). These data indicate that none of the small G proteins tested is involved in PI 3-kinase-mediated activation of Akt.

We also tested the abilities of constitutively active forms of the small GTP-binding proteins to stimulate the kinase activity of Akt. RacV12 and Cdc42 V12 were not able to activate Akt (Fig. 5B). However, RasV12 activated Akt substantially above the background level. When RasV12 was coexpressed with forms of p110*, the stimulation was additive rather than synergistic. Because the activation of Akt either by activated forms of PI 3-kinase or by PDGF stimulation was not affected by dominant negative Ras (Fig. 5 and 6) (2), the data suggest that RasV12 might activate Akt in an independent pathway.

Akt stimulation by PI 3-kinase requires a functional PH domain in Akt. It was recently reported that PDGF-induced stimulation of Akt requires the pleckstrin homology (PH) domain present at its N terminus. To investigate whether the PH domain in Akt is essential for PI 3-kinase-mediated activation of the pathway, we introduced into the Akt PH domain a point mutation that renders it unresponsive to PDGF stimulation (13). When coexpressed with the indicated signaling molecules (Fig. 7A), AktΔPH was not significantly stimulated by either p110* variants or RasV12 (Fig. 7B). In contrast, wt Akt exhibited high levels of kinase activity when coexpressed with either p110*, M · p110*, p110* · H, or RasV12. These data show that the PH domain of Akt regulates its general responsiveness to stimuli like PDGF, activated PI 3-kinase, or RasV12 and suggest that they use similar mechanisms or intermediates for signaling to Akt.

Akt activation and MAP kinase activation are differentially regulated by PI 3-kinase and Ras. PDGF-mediated stimulation of both Akt and MAP kinase was reduced by treatment with 50 nM wortmannin (Fig. 6 and 8), which suggests that PI 3-kinase is involved in the regulation of both cellular responses. Surprisingly, membrane-localized p110* · H was not able to activate MAP kinase, although it efficiently activated Akt (Fig. 8). Therefore, in contrast to Akt activation, PI 3-ki-

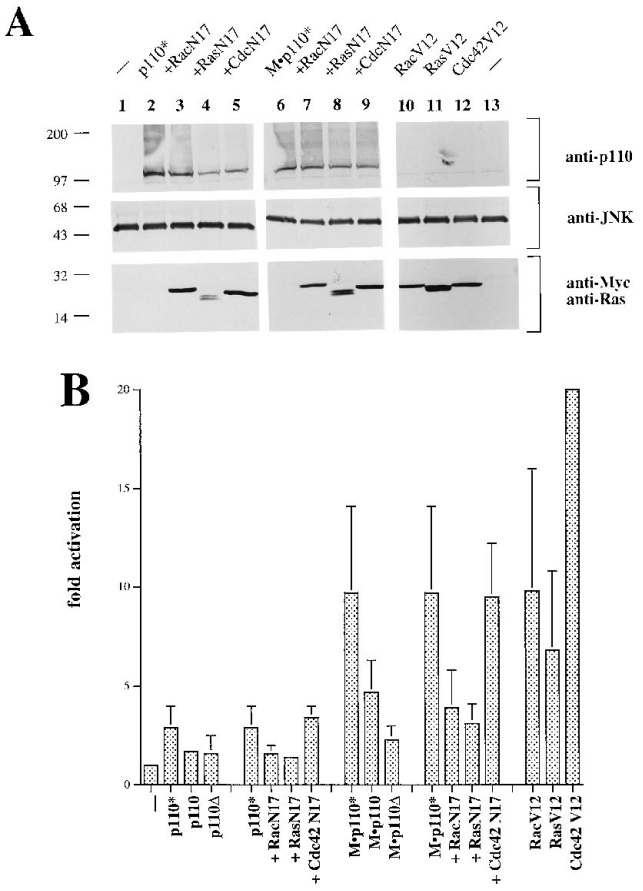

FIG. 9. Activated forms of p110 induce JNK. (A) HA-tagged JNK was coexpressed with the indicated p110 derivatives or activated forms of small G proteins. In addition, dominant negative forms of small G proteins were cotransfected to test their abilities to interfere with p110*- or M · p110*-induced activation of JNK. Expression levels were monitored as described above. (B) Anti-HA precipitates of JNK were subjected to a JNK activity assay using GST-Jun(1-89) as the substrate. Phosphorylation of the substrate protein was analyzed by SDS-PAGE and quantitated with a Molecular Imager. The increase in JNK kinase activity is given relative to the level of unstimulated kinase coexpressed with empty vector DNA. Each bar represents the mean of at least three transfections ± standard deviation.

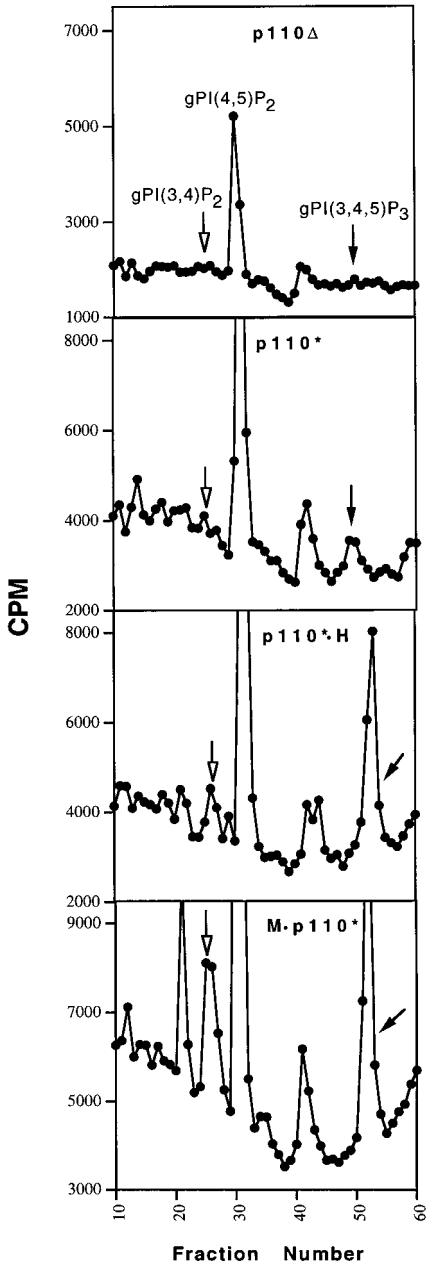

FIG. 10. Expression of constitutively active forms of PI 3-kinase leads to increased intracellular levels of 3′-phosphorylated phosphoinositides. COS-7 cells transiently expressing the indicated forms of p110 were radiolabeled with $^{32}P_i$. ^{32}P-labeled phospholipids were extracted, deacylated, and analyzed by anion-exchange HPLC. The results of a representative experiment are shown. One one-hundredth of each extract from one 10-cm-diameter plate was analyzed (3.1×10^6 cpm for p110Δ, 2.3×10^6 cpm for p110*, 3.2×10^6 cpm for p110* · H, and 3.0×10^6 cpm for M · p110*). The positions of the deacylated glycerophosphoinositides gPI 3,4-P_2 and gPI 3,4,5-P_3 in the elution profile (determined by cochromatography of the respective standards) are represented by open and filled arrows, respectively. The position of gPI 4,5-P_2 is also indicated. The identity of the peak observed to the left of gPI 3,4-P_2 in the M · p110* profile is unknown but has been speculated to be gPI 3,5-P_2 (47).

nase seems to be required but not sufficient for MAP kinase activation.

Expression of RasV12 caused a 5-fold increase in kinase activity of Akt and a 40-fold increase in MAP kinase activity (Fig. 8). Ras-induced activation of Akt was blocked by wortmannin, while Ras-induced activation of MAP kinase was unaffected by wortmannin. This result implies that with respect to MAP kinase, PI 3-kinase cannot act as a downstream effector of Ras. In contrast, Ras-induced Akt stimulation seems to require PI 3-kinase, since wortmannin abrogated this Ras-induced response. Our data suggest that the two signaling pathways are clearly distinct with respect to requirements for signaling molecules and their order of activation.

Membrane-localized PI 3-kinase activates JNK. It has been previously suggested that PI 3-kinase activation subsequently activates the GTP-binding protein Rac (18, 30, 59). Rac was shown to regulate JNK (6, 35). Activated JNK phosphorylates the ternary complex factor proteins Elk-1 and SAP-1, which in turn lead to the induction of c-*fos* and c-*jun* expression (14, 61). To investigate whether stimulation of the PI 3-kinase pathway can activate JNK, the indicated p110 derivatives were coexpressed with JNK in COS-7 cells (Fig. 9A). Activated forms of Rac, Ras, or Cdc42 were tested as positive controls. M · p110 stimulated JNK activity approximately fourfold compared with basal JNK activity (Fig. 9B). M · p110* caused a 10-fold increase in activation, which was as high as the activation achieved with RacV12. Dominant negative forms of Rac or Ras, but not Cdc42, reduced JNK stimulation induced by M · p110*, suggesting that Rac and Ras may act as downstream effectors of PI 3-kinase in this pathway, whereas Cdc42 appears to activate JNK via an independent pathway. Similar results on the activation of JNK were obtained with p110*, although the overall increase in JNK activity was less pronounced (Fig. 9B). These data show that activation of PI 3-kinase is sufficient for the induction of JNK activity, although it was not sufficient to induce MAP kinase (Fig. 8).

Expression of activated forms of PI 3-kinase results in increased intracellular levels of PI 3-phosphoinositides. The experiments described above demonstrate that recruitment of p110 to the membrane has a stimulatory effect on PI 3-kinase-mediated responses. One possible explanation for this finding is that either the lipid or protein substrates for PI 3-kinase are located at the membrane. To investigate how the expression of activated p110 derivatives affects the intracellular levels of 3′-phosphorylated phosphoinositides, transiently transfected COS-7 cells were metabolically labeled with $^{32}P_i$. Phospholipids were extracted, deacylated, and subjected to HPLC analysis (47). Expression of the membrane-localized forms of p110*, M · p110* and p110* · H, resulted in a substantial increase in the intracellular levels of PI 3,4-P_2 and PI 3,4,5-P_3 (Fig. 10) compared with control cells expressing the kinase-defective p110Δ. Further, the levels of PI 3-phosphoinositides that were generated correlated well with the relative efficiency of each p110 molecule in activating signaling pathways. M · p110* generated the highest levels of 3′-phosphorylated phospholipids and was the most effective in activating pp70 S6 kinase, Akt, and JNK (Fig. 10). p110* · H exhibited a slightly lower efficiency in generating phospholipids as well as in stimulating downstream events; the primarily cytoplasmic p110* was the least efficient in both. M · p110 and p110 · H generated intermediate levels of PI 3-phosphoinositides (not shown). These results suggest that the amount of phospholipids produced in vivo regulates PI 3-kinase dependent processes and support the idea that 3′-phosphorylated phosphoinositides function as second messengers.

DISCUSSION

In this study, we demonstrate that membrane localization of PI 3-kinase can induce signaling responses characteristic of growth factor induction. In particular, we show that activation

of the PI 3-kinase pathway by targeting p110 to the membrane is sufficient to activate the downstream signaling kinase pp70 S6 kinase, Akt/Rac, or JNK (Fig. 3, 4, and 9) but not MAP kinase (Fig. 8). The activation of S6 kinase and Akt appears to be Ras independent (36) (Fig. 5), while the activation of JNK by PI 3-kinase appears to be Ras dependent (Fig. 9). The attachment of heterologous membrane targeting signals to the catalytic subunit of PI 3-kinase, p110, represents an alternative way to generate constitutively active PI 3-kinase molecules. We previously described a constitutively active form of PI 3-kinase, p110*, which caused the induction of cellular responses without prior growth factor induction as a result of its high specific PI 3-kinase activity (19) (Fig. 2B and 3C). Recombinant p110 exhibited relatively low specific activity (28) (Fig. 2 and 3C), and even high levels of expression of p110 did not lead to substantial effects on signaling pathways (reference 19 and this study). However, when p110 was directed to the membrane, either by N-terminal myristoylation or by C-terminal farnesylation, even low amounts of PI 3-kinase activity were sufficient to trigger the induction of a subset of intracellular kinases more effectively than the highly active but cytoplasmic p110* (Fig. 3, 4, and 9). Consequently, the combination of the high PI 3-kinase activity of p110* with membrane-targeting signals provided for maximal activation of downstream responses. The intracellular levels of 3′-phosphorylated phosphoinositides that were generated in response to expression of the activated p110 derivatives correlated with the efficiency of each form of p110 in inducing downstream events (Fig. 10). Our results suggest a dual role for p85 with respect to PI 3-kinase activation. First, the iSH2 domain of p85 binds to p110 and modulates its enzymatic activity. In addition, p85 localizes p110 to the membrane by binding to activated growth factor receptors. Our data suggest that recruiting p110 to the membrane is important for the regulation of PI 3-kinase-dependent responses, probably because its lipid substrates are at the membrane.

We found previously that expression of p110* was sufficient to induce Ras-dependent *fos* promoter activation in fibroblasts and to activate the Ras/MAP kinase pathway in *Xenopus* oocytes (19). However, in this study, expression of p110* in mammalian fibroblasts was not sufficient to induce MAP kinase activation (Fig. 8), although low concentrations of wortmannin did inhibit PDGF-mediated stimulation of MAP kinase. These data indicate that the activation of MAP kinase in oocytes and fibroblasts may be regulated by different mechanisms. For example, MAP kinase induction during meiotic maturation in oocytes is dependent on expression of the Ser/Thr kinase Mos (11, 43). In contrast, there is no evidence for an involvement of Mos in regulating MAP kinase in somatic cells like fibroblasts, in which little if any Mos is expressed (38, 42). It is possible that the *fos* promoter induction that we previously observed in NIH 3T3 cells (19) is mediated by the JNK pathway, because expression of activated forms of PI 3-kinase is sufficient to activate JNK (Fig. 9). JNK activation leads to stimulation of c-*jun* and c-*fos* expression by phosphorylating the ternary complex factor component Elk-1 (14, 61).

Akt activation by p110* requires its PH domain (Fig. 7). PH domains have been implicated in the binding of phospholipids of the PI-phosphatide type (12). The Akt PH domain has been suggested to mediate regulatory protein-protein interactions (7) as well as to bind PI 3-phosphatides generated by PI 3-kinase (13). In a similar fashion, the products of PI 3-kinase could regulate small G proteins by binding to the PH domains in guanine nucleotide exchange factors or guanine nucleotide dissociation inhibitors.

In COS-7 cells, an activated form of Ras was able to induce Akt by more than fivefold over the background level (Fig. 5, 7, and 8). Our finding that wortmannin inhibited this Ras-induced Akt activation (Fig. 8) could be explained by a model that places Ras upstream of PI 3-kinase (29, 45). However, dominant negative Ras, RasN17, did not interfere with the PI 3-kinase-dependent activation of Akt by PDGF (Fig. 6) (2), which suggests that Ras mediates the activation of Akt by an independent pathway. In the same experiment, RasN17 efficiently blocked PDGF-induced activation of MAP kinase (Fig. 6), indicating that the Ras pathway was indeed inhibited. An alternative explanation for the observation that activation of Akt by RasV12 is wortmannin sensitive could be that expression of the oncogenic RasV12 leads to the production of autocrine factors. These autocrine factors could subsequently activate growth factor receptors, which in turn lead to the induction of signaling pathways, including the activation of PI 3-kinase. We have found that under our experimental conditions, RasV12 expression causes the secretion of autocrine factors into the cell culture medium which result in the activation of MAP kinase (data not shown). Unfortunately, because of the lack of a suitable anti-Akt antibody, we were not able to test whether these autocrine factors could also stimulate Akt kinase. We did not observe the induction of autocrine factors by p110* expression. In contrast to RasV12-induced Akt activation, wortmannin did not inhibit RasV12-induced MAP kinase activation (Fig. 8), which argues against a role of PI 3-kinase as a downstream effector of Ras in this pathway. Dominant negative forms of Ras or Rac, but not Cdc42, inhibited p110*-mediated JNK activation in COS-7 cells (Fig. 9), while they all had no effect on Akt kinase induction (Fig. 5). These results suggest that small G proteins mediate PI 3-kinase induced signaling in some but not all pathways.

Our data demonstrate that activation of the PI 3-kinase pathway by either membrane targeting of p110, overexpression of p110 molecules with high enzymatic activity, or a combination thereof can trigger the stimulation of downstream responses. Activation of PI 3-kinase can influence multiple pathways by the regulation of kinases known to be involved in mitogenic or stress responses. Our data do not unequivocally place PI 3-kinase upstream of Ras; however, they show that PI 3-kinase can stimulate a diverse group of signaling molecules, including some that are probably not in the Ras pathway.

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