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Platelet-Derived Growth Factor-Dependent Activation of Phosphatidylinositol 3-Kinase Is Regulated by Receptor Binding of SH2-Domain-Containing Proteins Which Influence Ras Activity

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Upon binding of platelet-derived growth factor (PDGF), the PDGF β receptor (PDGFR) undergoes autophosphorylation on distinct tyrosine residues and binds several SH2-domain-containing signal relay enzymes, including phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ), the GTPase-activating protein of Ras (RasGAP), and the tyrosine phosphatase SHP-2. In this study, we have investigated whether PDGF-dependent PI3K activation is affected by the other proteins that associate with the PDGFR. We constructed and characterized a series of PDGFR mutants which contain binding sites for PI3K as well as one additional protein, either RasGAP, SHP-2, or PLCγ. While all of the receptors had wild-type levels of PDGF-stimulated tyrosine kinase activity and associated with comparable amounts of PI3K activity, their abilities to trigger accumulation of PI3K products in vivo differed dramatically. The wild-type receptor, as well as receptors that recruited PI3K or PI3K and SHP-2, were all capable of fully activating PI3K. In contrast, receptors that associated with PI3K and RasGAP or PI3K and PLCγ displayed a greatly reduced ability to stimulate production of PI3K products. When this series of receptors was tested for their ability to activate Ras, we observed a strong positive correlation between Ras activation and PI3K activation. Further investigation of the relationship between Ras and PI3K indicated that Ras was upstream of PI3K. Thus, activation of PI3K requires not only binding of PI3K to the tyrosine-phosphorylated PDGFR but accumulation of GTP-bound Ras as well. Furthermore, PLCγ and RasGAP negatively modulate PDGF-dependent PI3K activation. Finally, PDGF-stimulated signal relay can be regulated by altering the ratio of SH2-domain-containing enzymes that are recruited to the PDGFR.

Activation of the platelet-derived growth factor (PDGF) β receptor (PDGFR) by ligand binding leads to autophosphorylation of the receptor at several distinct tyrosine residues, which triggers the recruitment of SH2-domain-containing molecules. One class of these receptor-associated proteins consists of enzymes, including phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ1 (PLCγ), the GTPase-activating protein of $p21^{ras}$ (RasGAP), Src family members Src, Fyn, and Yes, and the protein tyrosine phosphatase SHP-2 (20). A second class of proteins that associate with the phosphorylated PDGFR consists of SH2-domain-containing adaptor proteins, such as Shc, Nck, and Grb2 (1, 28, 46). These two classes of SH2-domain-containing proteins associate with the activated PDGFR in distinct ways. The enzymes bind only when the receptor is phosphorylated at certain tyrosine residues (20). In contrast, the adaptors bind in response to phosphorylation of the receptor at multiple tyrosine residues (23a, 46). Grb2 can also associate with the PDGFR in an indirect manner by binding to either tyrosine-phosphorylated SHP-2 or p85 (4, 25, 44).

Numerous groups have investigated the role of receptor-associated proteins in mediating biological responses. One strategy has been to use PDGFR phosphorylation site mutants, and these studies have determined that PI3K or other proteins which bind to the PI3K binding site(s) are required for PDGF-dependent initiation of DNA synthesis and protection from apoptosis (12, 23, 42, 45). Similarly, PLCγ appears to be required for PDGF-dependent DNA synthesis in certain cell types (42). The use of a microinjection strategy has identified a requirement for the Src family members for cell cycle progression (32, 33, 39). In contrast, the precise role for the other PDGFR-associated enzymes, RasGAP and SHP-2, has remained elusive. RasGAP is known to regulate the GTPase activity of $p21^{ras}$, which is required for numerous biological responses, including cell proliferation (6). Ras becomes activated via the activity of a guanine nucleotide exchange factor, such as SOS, which converts GDP-bound, inactive Ras to GTP-bound, active Ras (5). Conversely, RasGAP promotes Ras inactivation by stimulating the intrinsic GTPase activity of Ras, thus returning Ras from its active GTP-bound form back to the inactive GDP-bound form (6, 27). A second function for RasGAP in PDGFR signaling appears to be the inhibition of PLCγ tyrosine phosphorylation and thus PLCγ activation (43). Whether the mechanism involves Ras activation is currently being investigated. Conflicting lines of evidence exist pertaining to the importance of SHP-2 function in the regulation of PDGFR signal transduction, with different assays suggesting a variety of possibilities (3, 24, 31, 41). In light of these obser-

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vations, it is possible that RasGAP and/or SHP-2 plays either a negative or a weakly positive modulatory role in PDGFR-mediated signaling.

The participation of PI3K in numerous signaling pathways and biological responses has generated interest in understanding the mechanism by which PI3K activity is regulated. It appears that activation of PI3K requires at least three events: (i) translocation from the cytoplasm to the membrane, (ii) binding of the p85 subunit to tyrosine-phosphorylated proteins, and (iii) binding of active Ras to the p110 catalytic subunit (2, 17, 29, 34, 37). The relative contribution of each of these events to the activation occurring in PDGF-stimulated cells has not been carefully addressed, and there remains some controversy regarding the importance of Ras in activating PI3K.

Several lines of investigation have indicated that RasGAP and SHP-2 may be regulating PDGF-dependent PI3K activation. SHP-2 is capable of dephosphorylating PDGFR autophosphorylation sites, including one required for p85 binding, and thus SHP-2 binding may limit the amount of time that PI3K stays associated with the receptor (24). Provided that Ras is required for PDGF-mediated PI3K activation and that RasGAP functions to decrease the level of active Ras in a PDGF-stimulated cell, the recruiting of RasGAP could blunt PI3K activation by acting on Ras. Thus, it is possible that RasGAP and/or SHP-2 modulates PDGF-dependent activation of PI3K. To test this hypothesis, we constructed a series of mutant PDGFRs that contained the binding sites for PI3K and one of the other receptor-associated proteins: PI3K and SHP-2, PI3K and RasGAP, or PI3K and PLCγ. Characterization of these mutants revealed that all receptors were kinase active and that they associated with comparable amounts of PI3K in a PDGF-dependent manner. Despite their uniform ability to recruit PI3K, not all the receptors were able to activate PI3K, as measured by the accumulation of PI3K products in cells. Inhibition of PI3K activation directly correlated with the inability of the mutant receptors to activate Ras, suggesting that activation of Ras is a prerequisite for PDGF-mediated PI3K activation. Indeed, N17 Ras blocked Ras-mediated events and accumulation of PI3K products. Furthermore, PI3K inhibitors completely prevented PDGF-mediated PI3K activation but only modestly reduced the accumulation of GTP-Ras. These results strongly suggest that PDGF-dependent activation of PI3K requires not only association of PI3K with the activated PDGFR but accumulation of active Ras as well. Furthermore, in HepG2 and NIH 3T3 cells, Ras functions upstream of PI3K in the PDGFR signaling cascade. Finally, several of the signaling enzymes that associate with the PDGFR act to suppress PI3K activation, and in the case of RasGAP, the mechanism appears to involve decreasing the levels of active Ras.

MATERIALS AND METHODS

Cell lines. HepG2 cells were cultured as previously described (42). NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. NIH 3T3 transfectants were cultured in the above medium supplemented with 1 mg of G418 and 400 μg of hygromycin B per ml.

Construction of PDGFR mutants. Construction of the F5 and Y40/51 (42), Y40/51/71 (43), and the Y40/51/21 (called Y21/40/51 in reference 43) mutants has been previously described. To make the Y40/51/09 receptor, the 2.8-kb *Eco*RI-*Bam*HI fragment from F771 (23) was subcloned into Y1009/pVZ (42). The resulting PDGFR construct was subcloned into the pLXSN vector; virus was generated and used to infect cells as previously described (42). Mass populations of cells were selected on the basis of their ability to grow in the presence of G418 at a concentration of 2 mg/ml. Cells expressing uniform numbers of receptor were obtained by fluorescence-activated cell sorting, utilizing a human-specific anti-PDGFR monoclonal antibody, 7212 (15), which recognizes an epitope in the extracellular domain of the PDGFR, followed by staining with an anti-mouse secondary antibody coupled to fluorescein isothiocyanate fluorescent dye. The sorted cells were then grown out as mass populations. Periodic retesting of receptor levels indicated that receptor expression in all cell lines was comparable and that the level of expression was stable for at least several months.

Transfection and expression of Ras mutants. The inducible eukaryotic expression vector pOPRSVI CAT (Stratagene) containing N17 Ras was a generous gift from Anne Gardner. Ras inserts were subcloned as *Not*I-*Xho*I fragments into *Not*I-*Xho*I-cut pOPRSVI. NIH 3T3 cells were cotransfected with the Ras-containing constructs and a plasmid encoding the *lac* repressor p3′SS by using a calcium phosphate mammalian cell transfection kit (5 Prime/3 Prime, Inc.). Clones were selected on the basis of their ability to grow in the presence of 1 mg of G418 and 400 μg of hygromycin B per ml. Drug-resistant colonies were isolated and were induced to express N17 Ras by addition of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the culture medium for 16 to 20 h. Expression was analyzed by Western blotting (immunoblotting) with an anti-rat monoclonal antibody against Ras, AB-1 (1 mg/ml) (Oncogene Science). All 10 of the drug-resistant colonies tested expressed N17 Ras in an inducible manner. Levels of exogenously expressed Ras were approximately equal to the endogenous Ras levels in the clones used in these studies.

Western blot analysis. Western blotting was performed exactly as previously described (23) with the exception that following probing with the primary antibody, blots were probed with a horseradish peroxidase-conjugated secondary antibody. The blots were then developed with Western blot detection reagents (enhanced chemiluminescence; Amersham). To blot for SHP-2, a combination of anti-SHP-2 antisera was employed (34.2 and anti-Syp). The 34.2 antibody was a crude polyclonal rabbit serum raised against a glutathione *S*-transferase (GST) fusion protein that included the last 44 amino acids of the carboxyl terminus of human SHP-2. The anti-Syp antibody utilized has been previously described (14). Both of these antisera were diluted 1:1,000 for Western analysis.

In vitro kinase assay. Intrinsic tyrosine kinase activity of the PDGFR mutants was analyzed exactly as previously described (22). PDGFR was immunoprecipitated from HepG2 cells with an anti-PDGFR antibody, 30A (23). Twenty percent of the immunoprecipitate, representing approximately 2×10^5 cells, was used in a kinase assay containing 1 μg of GST-PLCγ (amino acids 550 to 850 of rat PLCγ) (43).

In vivo PI3K assay. Cells at 80 to 95% confluence were starved for 24 h in phosphate-free medium supplemented with 2 mg of bovine serum albumin (BSA) per ml. Following starvation, $^{32}P_i$ (ICN) was added to a final concentration of 0.2 to 1.0 mCi/ml; the cells were labeled for 3 h and then exposed to buffer or 50 ng of PDGF-BB per ml for 4 min. Cells were then placed on ice, washed three times with ice-cold H/S (20 mM HEPES [*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], 150 mM NaCl, pH 7.4) supplemented with 1 mM sodium orthovanadate, fixed by adding 750 μl of methanol–1 M HCl (1:1) containing 10 mg of crude brain phosphoinositide (Sigma) per ml, and collected into an Eppendorf tube containing 380 μl of chloroform. The samples were vortexed vigorously and then placed on a rotator at room temperature for 15 min. The samples were spun briefly to separate the phases, the aqueous phase was removed, and the chloroform phase was washed with 750 μl of methanol–0.1 M EDTA, pH 8.0 (1:1). The chloroform phase was then transferred to a 20-ml glass scintillation vial and dried under a stream of N_2. The lipids were deacylated and resolved by high-performance liquid chromatography (HPLC) with a SAX column, and the radioactivity in the various fractions was quantitated with a Radiomatic A500 on-line radioactivity counter (Packard).

In vitro PI3K activity assay. The PI3K activity in anti-PDGFR immunoprecipitates representing 2×10^5 cells was determined as previously described (23).

Ras activity assay. Activation of Ras was determined exactly as previously described (42) with the following modifications. Cells were stimulated with 50 to 100 ng of PDGF per ml for 4 min and lysed in BLB (25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1% Triton X-114, 10 mM benzamidine, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 10 μg of soybean trypsin inhibitor per ml, 1 mM ATP, 0.1 mM GTP, 0.1 mM GDP, and 1 mM Na_2HPO_4-NaH_2PO_4 [pH 7.4]) without antibody, and the insoluble material was pelleted by centrifugation. The detergent phase of the supernatant was separated from the aqueous phase, resuspended, and cleared as described previously (42), before incubation with protein G-Sepharose beads coupled to the anti-Ras rat monoclonal antibody Y13-259 at 4°C for 90 min.

MAPK gel shift assay. Cells were starved in Dulbecco's modified Eagle's medium plus 0.1% calf serum for 20 h before being exposed to buffer or 50 ng of PDGF per ml for 4 min at 37°C. Cells were then lysed in EB^{++} (21) without BSA, protein concentrations were determined by the Bio-Rad assay, and the amount of protein in each sample was equalized by dilution in EB^{++}. Samples were run on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and transferred to Immobilon. Blots were probed with a mouse monoclonal antibody against p42 and p44 mitogen-activated protein kinase (MAPK) (Zymed; 1:2,500). The faster/slower p42 band ratio was quantitated by densitometry and used as a measure of the extent of MAPK activation.

RESULTS

Previous studies have indirectly suggested a role for RasGAP and SHP-2 in PDGF-dependent PI3K signaling. We have directly investigated this possibility by constructing a series of PDGFR mutants which contained binding sites for the p85

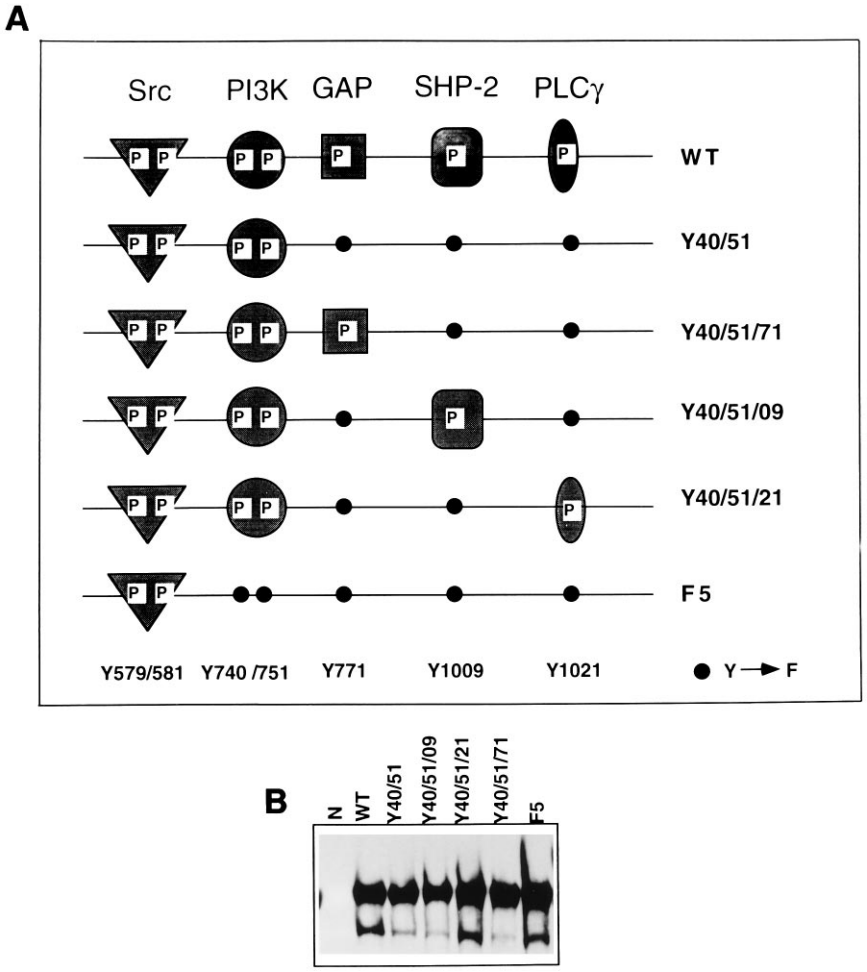

FIG. 1. Schematic of the PDGFR mutants. (A) The gray objects represent the receptor-associated proteins, and they are labeled at the top of the figure. P, phosphorylated tyrosine residue; ●, tyrosine-to-phenylalanine substitution. WT, wild-type PDGFR. F5 contains tyrosine-to-phenylalanine mutations at positions 740, 751, 771, 1009, and 1021. The names of the other mutants indicate which of the tyrosine residues have been restored. Only the last two numbers of the restored phosphorylation site are used in the name of the receptor. The cartoon indicates which of the receptor-associated proteins are expected to bind to each of the receptor mutants. (B) PDGFR Western blots of HepG2 cell lysates (representing approximately 3×10^4 cells) expressing the various PDGFRs. N indicates cells expressing the empty pLXSN vector.

subunit of PI3K and one other protein, either RasGAP, SHP-2, or PLCγ. Figure 1 illustrates this series of PDGFR mutants and the signaling proteins predicted to bind to them. Note that all of the mutants contain the Src binding site and that the F5 construct fails to associate with all four of the signaling molecules highlighted in this study.

The mutant cDNA constructs were prepared as described in Materials and Methods, subcloned into the retroviral expression vector pLXSN, and introduced to GP+E and PA317 virus-producing cells. The resulting virus was used to infect HepG2 cells (42), mass populations of cells were selected on the basis of G418 resistance, and selected cells were sorted by using an extracellular PDGFR antibody (7212) to obtain equivalent levels of receptor expression for each of the cell lines. Figure 1B is a Western blot of total cell lysates probed with an anti-PDGFR antibody (30A); it shows that all cell lines expressed similar amounts of receptor.

Characterization of the PDGFR mutants. We first tested the ability of each PDGFR mutant to stably interact with PLCγ, RasGAP, p85, and SHP-2 by Western blot analysis of receptor immunoprecipitates. Confluent, quiescent HepG2 cells expressing the various mutants either were left resting or were stimulated with 50 ng of PDGF per ml for 5 min at 37°C. Cells were lysed, and the PDGFR was immunoprecipitated with 30A and assayed by Western blot analysis. Binding of the receptor-associated proteins to the receptor mutants was very similar to what had been predicted (compare Fig. 1A with Fig. 2A). All receptors containing Y740 and Y751, tyrosines required for binding of PI3K, were capable of binding to the p85 regulatory subunit of PI3K in a PDGF-dependent manner. Probing receptor immunoprecipitates with antibodies specific to the α isoform of p85 detected only one of the two bands seen in Fig. 2A (data not shown), suggesting that multiple p85 isoforms associate with the activated PDGFR in HepG2 cells. Only receptors containing the sites required for RasGAP or SHP-2 binding (Y771 and Y1009, respectively) bound to either of these two proteins above background levels when stimulated by PDGF. Association of PLCγ with the activated PDGFR was also dependent on the presence of its binding site (Y1021) on the receptor. However, low amounts of PLCγ were consistently observed to bind to the Y40/51/09 mutant. This was not completely unexpected since it has been previously shown that site Y1009 can act as a low-affinity binding site for PLCγ (36).

Receptor tyrosine kinase activity was assessed by subjecting

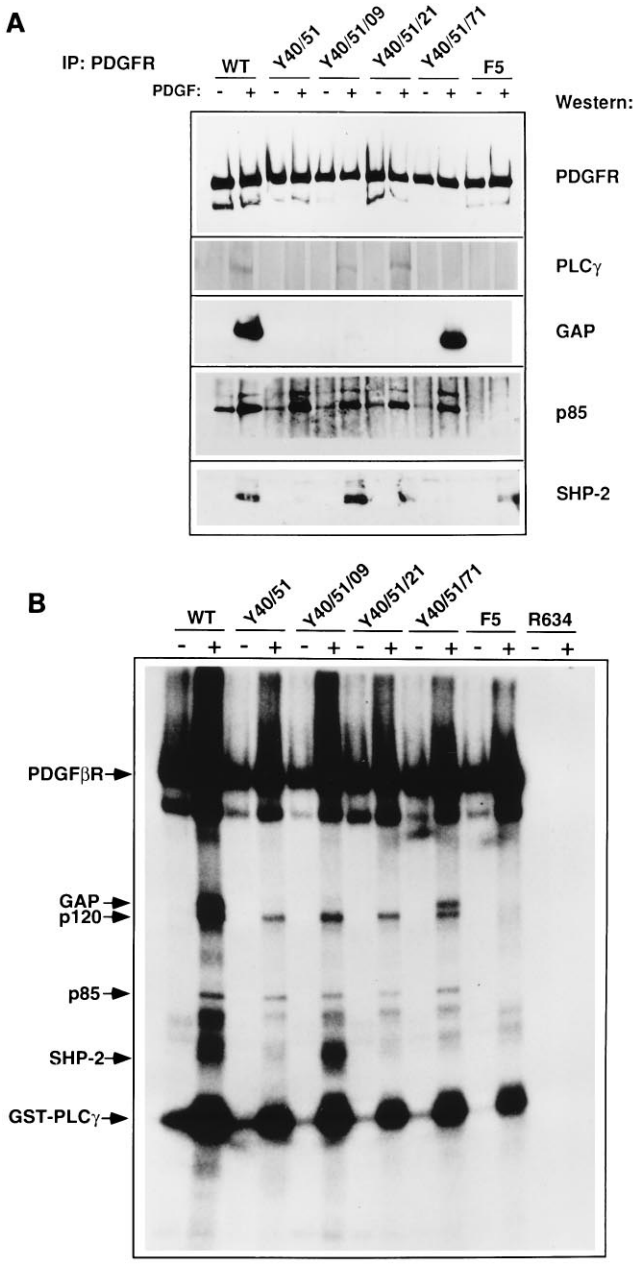

FIG. 2. Characterization of the proteins that associate with the PDGFR mutants. (A) Confluent, quiescent cultures of HepG2 cells expressing the various PDGFRs were left resting (−) or were stimulated for 5 min at 37°C with 50 ng of PDGF-BB per ml (+). The cells were lysed, the lysates were immunoprecipitated with an anti-βPDGFR antibody (30A), immunoprecipitates representing approximately 4.2×10^6 cells were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon, and the appropriate portion of the membrane was subjected to Western blot analysis with the antibodies indicated in the right-hand margin. p85, antiserum used to recognize the 85-kDa subunit of PI3K. (B) PDGFR immunoprecipitates, representing approximately 2×10^5 cells, were subjected to an in vitro kinase assay in the presence of an exogenous substrate (GST-PLCγ). The samples were resolved by SDS-PAGE, and then the gel was treated with KOH and subjected to autoradiography. R634 is the kinase-inactive PDGFR mutant.

the PDGFR immunoprecipitates to an in vitro kinase assay in the presence of [γ-^{32}P]ATP and an exogenous substrate, GST-PLCγ. All of the PDGFRs analyzed were able to phosphorylate the exogenous substrate and were activated by PDGF to a comparable extent (Fig. 2B). RasGAP, p85, and SHP-2, but not PLCγ, can be readily detected in an in vitro kinase assay if they are complexed with the PDGFR. The pattern of binding of the receptor-associated proteins to the activated PDGFRs observed in the in vitro kinase assay, shown in Fig. 2B, is very similar to the pattern observed with Western blot analysis, shown in Fig. 2A, further confirming the integrity of our panel of receptor mutants.

PI3K activity in vivo. To further characterize the receptor mutants, we tested their ability to activate PI3K by examining PDGF-dependent accumulation of PI3K products: phosphatidylinositol 3,4-bisphosphate (PI3,4P2) and phosphatidylinositol 3,4,5-triphosphate (PIP3). Confluent, quiescent cells were labeled with $^{32}P_i$ and were either left resting or stimulated with 50 ng of PDGF per ml at 37°C for 5 min. Lipid products were isolated by extracting the cells with chloroform-methanol; the samples were dried, deacylated, and then analyzed by HPLC. As expected, PDGF stimulation of cells expressing the wild-type (WT) or Y40/51 receptor resulted in the accumulation of PIP3, whereas stimulation of the F5 receptor, which binds poorly to p85 (Fig. 2A), did not trigger significant accumulation of PIP3 (Fig. 3A). Add-back of the tyrosine required for SHP-2 binding site (Y1009) to the Y40/51 receptor did not affect PDGF-dependent accumulation of PI3K products. Interestingly, restoration of either site 771 or 1021, required for binding of RasGAP or PLCγ, respectively, resulted in significant inhibition of PDGF-stimulated activation of PI3K (Fig. 3A). A similar trend was observed for PDGF-dependent production of PI3,4P2 (data not shown). These experiments indicate that not all receptors that recruit p85 are able to activate PI3K. Furthermore, receptors that associate with PI3K and PLCγ or PI3K and RasGAP fail to mediate significant accumulation of PI3K products, suggesting that PLCγ and RasGAP are able to inhibit activation of PI3K.

One explanation for why PI3K does not become activated in cells expressing the Y40/51/71 or Y40/51/21 receptor is that while p85 does bind to the receptor (Fig. 2A), the PI3K holoenzyme does not. In order to test this hypothesis, we determined whether PI3K activity is present in the PDGFR immunoprecipitates. The PDGFR was immunoprecipitated from resting or PDGF-stimulated cells expressing the various PDGFRs and subjected to an in vitro PI3K assay. This assay measures the amount of PI3K present in a PDGFR immunoprecipitate. The WT PDGFR immunoprecipitates contained robust PI3K activity provided that the cells had been stimulated with PDGF, whereas the F5 receptor immunoprecipitates contained only trace amounts of PI3K activity. In contrast to the in vivo data (Fig. 3A), all of the PDGFR mutants associated with WT levels of PI3K activity (Fig. 3B). These data suggest that binding of the PI3K holoenzyme to the tyrosine-phosphorylated PDGFR, which probably involves translocation of PI3K to a juxtamembrane location, is not sufficient for PDGF-dependent activation of PI3K.

Activation of Ras. The receptors that were unable to activate PI3K were those that associated with PI3K as well as RasGAP or PLCγ. This suggested that PLCγ and RasGAP are able to prevent PDGF-dependent accumulation of PI3K products. PLCγ hydrolyzes PI4,5P2, a PI3K substrate, so it is possible that when PI3K and PLCγ bind to the same receptor, these two enzymes compete for substrate. Consistent with this idea is the observation that the Y40/51/21 receptor poorly activates PLCγ (43).

A comparable explanation for how RasGAP interferes with activation of PI3K does not seem likely. Instead, the observations that PI3K activity is stimulated by active Ras and that RasGAP suppresses the accumulation of active Ras suggested

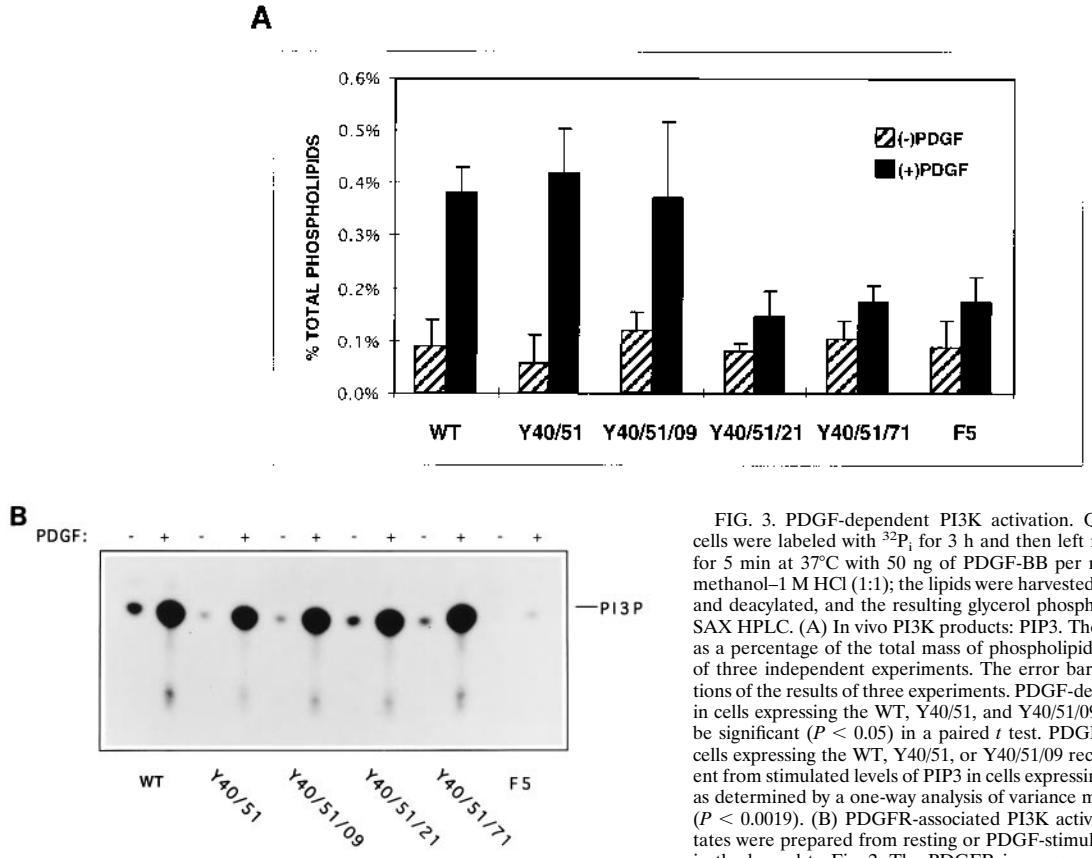

FIG. 3. PDGF-dependent PI3K activation. Quiescent cultures of HepG2 cells were labeled with $^{32}P_i$ for 3 h and then left resting (striped) or stimulated for 5 min at 37°C with 50 ng of PDGF-BB per ml (solid). Cells were fixed in methanol–1 M HCl (1:1); the lipids were harvested by extraction with chloroform and deacylated, and the resulting glycerol phosphoinositides were separated by SAX HPLC. (A) In vivo PI3K products: PIP3. The amount of PIP3 is presented as a percentage of the total mass of phospholipids extracted and is the average of three independent experiments. The error bars indicate the standard deviations of the results of three experiments. PDGF-dependent accumulation of PIP3 in cells expressing the WT, Y40/51, and Y40/51/09 receptors was determined to be significant ($P < 0.05$) in a paired t test. PDGF-stimulated levels of PIP3 in cells expressing the WT, Y40/51, or Y40/51/09 receptor were significantly different from stimulated levels of PIP3 in cells expressing Y40/51/21, Y40/51/71, or F5 as determined by a one-way analysis of variance multiple comparison procedure ($P < 0.0019$). (B) PDGFR-associated PI3K activity. Receptor immunoprecipitates were prepared from resting or PDGF-stimulated cells exactly as described in the legend to Fig. 2. The PDGFR immunoprecipitates were incubated with phosphatidylinositol (PI) and [γ-^{32}P]ATP, the phospholipids were resolved by thin-layer chromatography, and the plate was analyzed by autoradiography. The position of phosphatidylinositol phosphate (PI3P) is indicated in the right-hand margin. The autoradiogram shown is representative of three experiments.

that RasGAP may be inhibiting PI3K by a mechanism which involves Ras (6, 34). To determine whether there was a correlation between PDGF-dependent activation of PI3K and activation of Ras, we measured the ability of the PDGFR mutants to activate Ras. Confluent quiescent cells were labeled with $^{32}P_i$ and stimulated with 50 ng of PDGF per ml; then Ras was immunoprecipitated and Ras-associated nucleotides were separated by thin-layer chromatography. Figure 4A shows an autoradiogram of a representative experiment. The GTP/(GTP + GDP) ratio was quantitated by PhosphorImager analysis, and the composite of three independent experiments is shown in Fig. 4B. Ras was activated in cells expressing either the WT or the Y40/51 PDGFR but not in cells expressing the F5 receptor (Fig. 4). Restoring Y1009 to the Y40/51 receptor had no effect on PDGF-dependent Ras activation. However, addition of either the RasGAP binding site or the PLCγ binding site to the Y40/51 receptor prevented Ras activation (Fig. 4). We consistently observed a higher basal level of Ras-GTP in the mass population of cells expressing the Y40/51/71 receptor. While we do not understand the basis of this observation, the data clearly show that PDGF stimulation of cells expressing the Y40/51/71 receptor did not trigger an accumulation of active Ras but instead decreased the basal levels of Ras-GTP. The lack of Ras activation downstream of the Y40/51/71 receptor is consistent with RasGAP binding and RasGAP function; however, the inability of the Y40/51/21 receptor to mediate Ras activation was unexpected, since mutant receptors containing either sites Y40/51 or Y1021 alone can activate Ras. It is possible that the combination of these autophosphorylation sites on the same receptor molecule prevents the activation of an important intermediary protein, such as SOS. While the precise mechanism by which Ras is activated and suppressed has not yet been resolved, these data establish a firm correlation between a receptor's ability to activate Ras and its ability to drive the accumulation of PI3K products (Fig. 3 and 4).

Wortmannin treatment of cells expressing Y40/51. The correlation observed between PI3K and Ras activation begs the question of whether Ras lies upstream or downstream of PI3K in PDGFR-mediated signaling cascades. If PI3K catalytic function is required for Ras activation, then PI3K inhibitors should block Ras activation. Since there are several PDGF-dependent pathways that can lead to Ras activation, we utilized cells expressing the Y40/51 mutant, in which the only pathway(s) to activate Ras depends on the presence of tyrosines 740 and 751 (Fig. 4; compare F5 with Y40/51). Using this mutant instead of the WT PDGFR reduces the possibility that wortmannin-dependent changes in Ras activation would be masked by activation of Ras through a PI3K-independent pathway, such as those downstream of either Y1009 or Y1021 (42). Cells expressing the Y40/51 mutant were pretreated with the PI3K inhibitor wortmannin before addition of PDGF, and then Ras activation was measured either directly or via gel shift of MAPK in response to PDGF. At 100 nM wortmannin, PDGF-dependent accumulation of PI3K products was completely inhibited and GTP loading of Ras was reduced by 22.0% ± 2.9% compared with cells which were treated with vehicle (dimethyl sulfoxide) alone (Fig. 5). In contrast to its modest effect on Ras

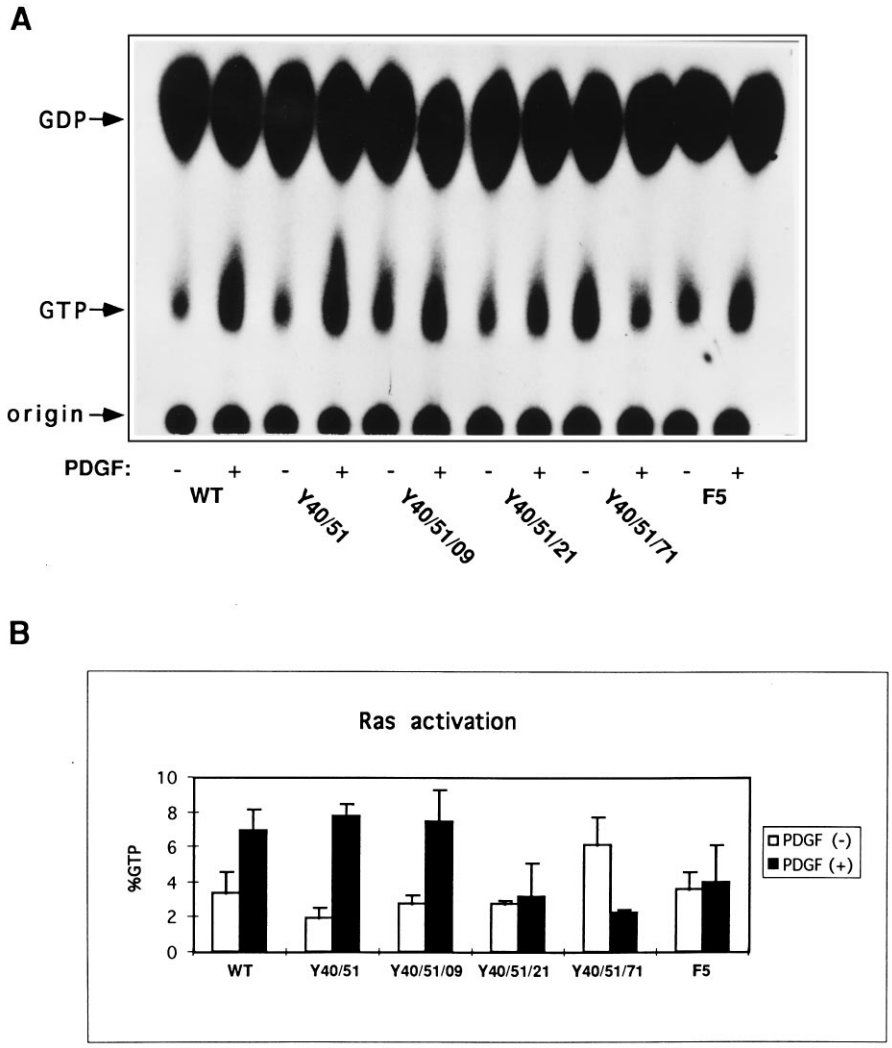

FIG. 4. PDGF-dependent Ras activation by the PI3K-positive double-add-back mutants. Confluent, quiescent HepG2 cells expressing the indicated PDGFR mutants were labeled with $^{32}P_i$, stimulated with 100 ng of PDGF-BB per ml for 5 min at 37°C, and lysed; Ras was immunoprecipitated with the Y13-259 anti-Ras antibody, and the nucleotides were resolved on a polyethyleneimine cellulose plate. (A) An autoradiogram of a representative experiment is presented. The origin as well as the positions of GDP and GTP are indicated. (B) The radioactivity in the GTP and GDP was quantitated with a Molecular Dynamics PhosphorImager, and the average ± the standard deviation of three independent experiments is presented. The data are expressed as percentages of GTP, which was calculated by dividing the counts per minute present in GTP by the counts per minute present in GTP plus GDP.

loading, wortmannin did not inhibit MAPK activation at any of the concentrations tested (10 nM to 1 μM) (Fig. 5C). Our inability to detect an effect on MAPK is likely due to the fact that MAPK lies several steps downstream of Ras activation, and therefore amplification of a signal initiated by Ras may render the slight inhibitory effect of wortmannin undetectable. The data suggest that PI3K activity appears to augment but is not required for PDGF-dependent activation of Ras in HepG2 cells.

Inhibition of in vivo PI3K activity in NIH 3T3 cells expressing dominant negative (N17) Ras. To complement the above experiments using a PI3K inhibitor, we used NIH 3T3 cells stably transfected with an IPTG-inducible expression vector encoding a dominant negative (N17) version of Ras as a second approach to determine whether Ras is required for activation of PI3K. If PDGF-dependent activation of PI3K requires active Ras, expression of dominant negative Ras should prevent activation of PI3K in cells stimulated with PDGF. Treating N17 cells with IPTG for 16 to 20 h induced the expression of the variant Ras protein to a level at least comparable to the amount of endogenous Ras, whereas IPTG had no effect on the NIH 3T3 parental cells (Fig. 6A and data not shown). Expression of N17 Ras did not affect PDGF-dependent events, including autophosphorylation of the PDGFR, PDGFR kinase activity, PDGFR association with p85 and PI3K activity, and PDGFR association with PLCγ, RasGAP, or SHP-2 (data not shown). As expected, PDGF failed to induce activation of Ras in cells which were induced to express N17 Ras, whereas IPTG had no effect on the parental NIH 3T3 cells (Fig. 6A). To further determine the extent of inhibition of Ras-mediated events, we measured activation of MAPK by a gel shift assay. Expression of N17 Ras severely reduced PDGF-dependent activation of MAPK but did not completely inhibit this event (Fig. 6B). Therefore, Ras may be modestly activated in the N17-expressing cells, which cannot be detected in our Ras loading assay.

Finally, we did studies to determine whether the expression of the N17 Ras mutant affected PDGF-dependent activation of

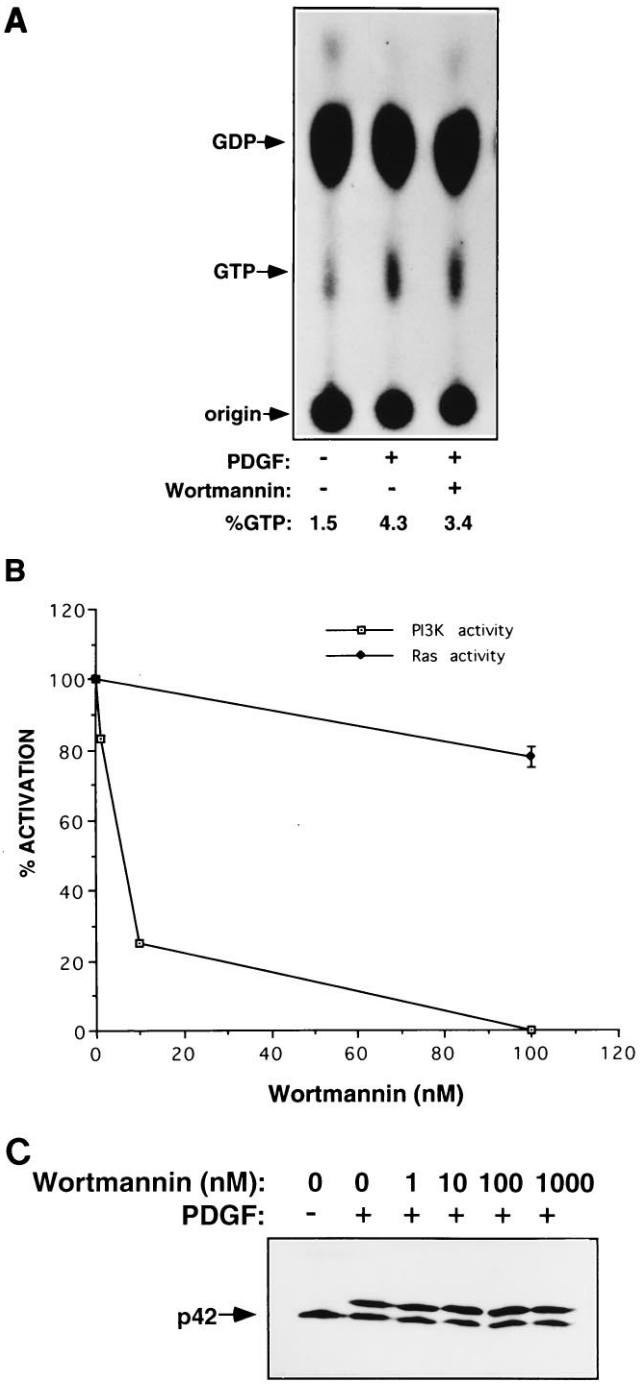

FIG. 5. Effect of wortmannin on PDGF-stimulated activation of Ras-mediated events in Y40/51 cells. HepG2 cells expressing the Y40/51 PDGFR were pretreated with either 100 nM wortmannin or vehicle (dimethyl sulfoxide) and were either left resting or stimulated with 100 ng of PDGF per ml for 5 min at 37°C. Activation of Ras was determined as described in the legend to Fig. 4. (A) An autoradiogram of a representative experiment is presented. (B) Effect of wortmannin on PDGF-stimulated PI3,4P2 (squares), as measured in Fig. 3, and Ras activation (diamonds), as measured in Fig. 4. The data are presented as a percentage of the activity of cells pretreated with vehicle (dimethyl sulfoxide) alone. The error bars on Ras activation from cells pretreated with 100 nM wortmannin represent the standard deviations of four independent experiments. (C) PDGF stimulation of MAPK in HepG2 Y40/51 cells: effect of wortmannin. Y40/51-expressing cells were preincubated for 20 min with the indicated concentrations of wortmannin before addition of 50 ng of PDGF per ml for an additional 5 min; cells were lysed, and cell lysate, representing 7.5×10^4 cells, was analyzed by MAPK Western blotting. The arrow points to the rapidly migrating (unphosphorylated) form of p42.

PI3K in vivo. The parental or N17-expressing NIH 3T3 cells were incubated with IPTG, labeled with ^{32}P, and stimulated for 4 min with 50 ng of PDGF per ml at 37°C; the cells were lysed, and phospholipids were extracted and subjected to HPLC analysis. PDGF-stimulated PI3,4P2 accumulation was consistently decreased by 50 to 60% in cells expressing N17 Ras, compared with the parental cells (Fig. 6C). The low level of Ras activation in the N17-expressing cells may account for the modest PDGF-stimulated increase in PI3,4P2 observed in the NIH 3T3 N17 cells. In addition, we found that the degree of inhibition of Ras activation (as measured by inhibition of MAPK gel shift) correlated with the extent of PI3K inhibition. MAPK activation was always inhibited by at least 50%, and in one of these experiments, the inhibition was greater than 90%; in this experiment we could not detect any PDGF stimulation of PI3K products. These studies reveal a strong correlation between Ras activation and accumulation of PI3K products and are consistent with our findings in the HepG2 cell system. Together our data indicate that Ras is required for maximal activation of PI3K in PDGF-stimulated cells; therefore, Ras appears to lie upstream of PI3K.

DISCUSSION

We have constructed and characterized a panel of PDGFR mutants in which we assessed the contributions of PLCγ, RasGAP, and SHP-2 to PDGF-dependent PI3K activation. These studies have shed light on the mechanism of PI3K activation and the role of PLCγ and RasGAP in this event. In addition, the experiments have revealed that PDGF-mediated signal relay can be regulated by varying the ratio of signal relay enzymes which are recruited to the PDGFR.

Mechanism of PI3K activation. As PI3K is more thoroughly investigated, it appears that activation of PI3K requires multiple events. Engagement of p85's SH2 domains with tyrosine-phosphorylated proteins or peptides changes the conformation of p85 and thereby activates the p110 catalytic subunit (29, 37). A second event involves translocation of PI3K from the cytoplasm to the membrane, where it can gain access to its substrate. In a growth factor-stimulated cell, this step appears to occur when PI3K associates with tyrosine-phosphorylated transmembrane growth factor receptors (17). PI3K is also phosphorylated on tyrosine and serine residues following growth factor stimulation, and serine phosphorylation of p85 inhibits PI3K's lipid kinase activity (7, 9, 19). Finally, Ras has been shown to activate PI3K, presumably via its ability to associate with the p110 catalytic subunit of PI3K in a GTP-dependent manner (34). Our data indicate that recruitment of PI3K to the PDGFR is not sufficient for activation of PI3K and that activation of Ras appears to be necessary for the PDGF-driven increase in PI3K products (Fig. 3 to 6).

The subject of whether Ras acts upstream or downstream of PI3K in growth factor-driven pathways remains controversial, as there is evidence supporting both possibilities. In support of the idea that PI3K is upstream of Ras, Hu et al. reported that in NIH 3T3 cells, expression of a constitutively active version of the p110 subunit of PI3K (p110*) induces transcription from the c-*fos* promoter and that this event can be blocked by co-expression of N17 Ras (16). Also, expression of p110* in *Xenopus* oocytes increased the level of GTP-bound Ras, caused activation of Raf-1, and induced Ras-dependent oocyte maturation (16). Furthermore, in CHO cells, expression of a mouse PDGFR lacking PI3K binding sites failed to mediate Ras activation; however, this same mutant was able to stimulate Ras activation in Baf-3 cells (38). Finally, PI3K inhibitors prevent activation of MAPK in normal fibroblasts and middle-T-trans-

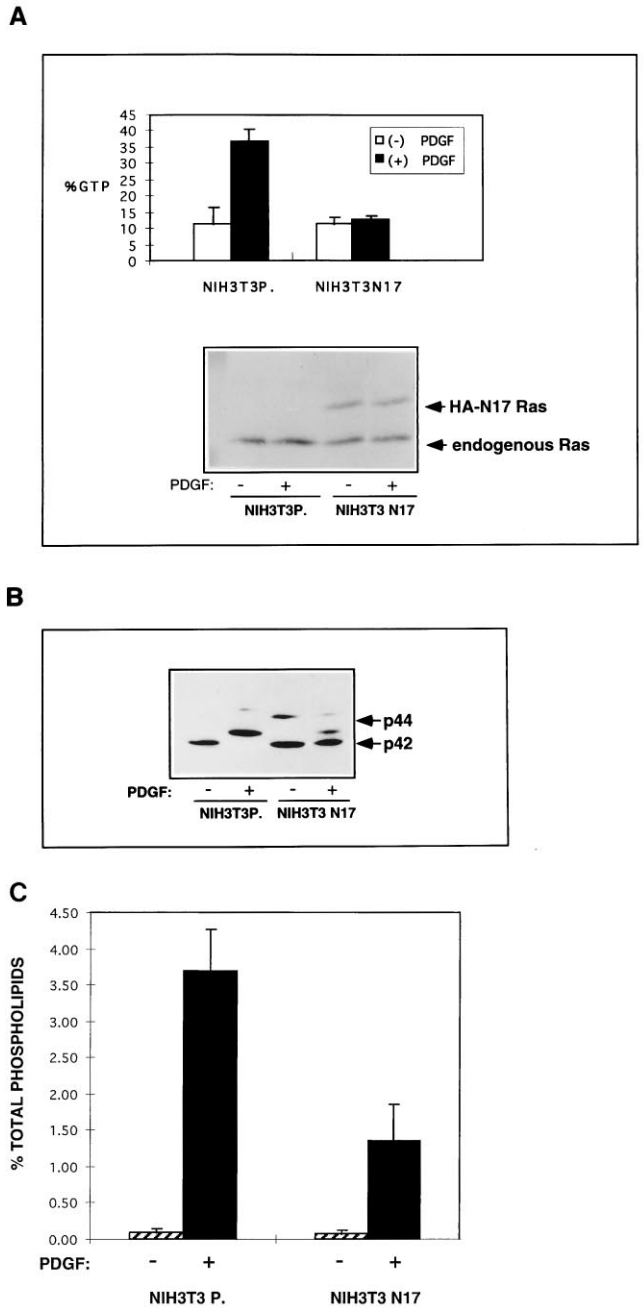

FIG. 6. Expression of N17 Ras in NIH 3T3 cells inhibits full activation of PI3K in vivo. (A) Ras activation. NIH 3T3 cells were transfected with an inducible hemagglutinin-tagged version of N17 Ras (HA-N17 Ras). Cells were induced by incubation with 5 mM IPTG for 16 to 20 h. HA-N17 Ras was observed as a band which migrated more slowly than endogenous Ras in an anti-Ras Western blot. Ras activation was measured as described in the legend to Fig. 4. The results represent three independent experiments, and the error bars indicate the standard deviations. (B) MAPK activation: PDGF-dependent MAPK gel shifts in parental (NIH 3T3P) and N17-expressing (NIH 3T3 N17) NIH 3T3 cells. Resting cells were left quiescent or stimulated with 50 ng of PDGF per ml, and then cells were lysed and total cell lysates were subjected to MAPK Western blotting. The arrows point to the unphosphorylated, rapidly migrating p42 and p44 MAPK isoforms. (C) PI3K activity: accumulation of PI3,4P2 in parental and N17-expressing cells. Resting or PDGF-stimulated cells were harvested, and PI3,4P2 was purified and quantitated. The data are expressed as a percentage of total phospholipids. The graph represents three independent experiments; the error bars indicate the standard deviations.

formed cells (40) or partially inhibit insulin-stimulated Ras activation (8).

Evidence supporting the concept that Ras is upstream of PI3K includes the findings that Ras-GTP stably associates with p110 and that it activates the enzyme in vitro. N17 Ras partially blocks growth factor-stimulated PI3K activation in PC12 and NIH 3T3 cells (34) (Fig. 6). In HepG2 cells, our data generally support the idea that PI3K is downstream of Ras (Fig. 3 to 5). Finally, mutant PDGFRs which do not bind or activate PI3K (for example, the Y1009 and Y1021 receptors) maximally activate Ras (reference 42 and unpublished observations). Thus, Ras activation appears to be a prerequisite for maximal PDGF-stimulated activation of PI3K in both HepG2 and NIH 3T3 cells.

We have found that inhibiting PI3K inhibits PDGF-stimulated Ras activation by 22% (Fig. 5), indicating that PI3K products and/or PI3K itself contributes to the accumulation of active Ras. A possible explanation is that PI3K competes with RasGAP for binding to the effector region of Ras (13). In this case, PI3K would prevent interaction of GTP-bound Ras with RasGAP and thereby contribute to the accumulation of activated Ras. It is also possible that PI3K products are required to suppress RasGAP activity (8). The recent realization that PI3K products bind certain SH2 domains offers another plausible explanation of how PI3K products could contribute to Ras activation (30). Provided that the SH2 domains of Grb2 and Nck interact with PIP3, then an increased concentration of PIP3 could result in the recruitment of SOS to the membrane via Grb2 or Nck. In summary, the relationship between PI3K and Ras may be multifaceted and flexible and thus may contribute to the nonuniform importance of PI3K to Ras activation seen in different cell types.

How does Ras contribute to PI3K activation? In vitro studies indicate that Ras-GTP binds to the p110 subunit and increases its catalytic activity (34, 35). This effect is synergistic with binding of tyrosine-phosphorylated proteins or peptides to the p85 subunit (35). In the in vivo setting, Ras may function to help recruit PI3K to the cell membrane in a mechanism similar to its role in Raf activation. However, unlike the Raf analogy, PI3K binds to the tyrosine-phosphorylated PDGFR and thereby attains a juxtamembrane location (17). This appears to be a Ras-independent event since we have not detected any difference in the ability of PI3K to stably associate with the PDGFR in cells expressing N17 Ras compared with control cells (23a). However, binding to the receptor, and thereby gaining access to the membrane, is not sufficient for activation of PI3K (Fig. 3). Ras-GTP may adjust PI3K's orientation relative to its substrate. The recent resolution of PLCδ's crystal structure predicts that the catalytic domain of PLCδ is inserted into the cell membrane (11). Since PLCδ and PI3K act on the same substrate, it is possible that PI3K also inserts into the membrane and that Ras-GTP facilitates this event.

Contribution of PLCγ, RasGAP, and SHP-2 to PDGFR signaling. Many distinct signaling complexes with nonidentical signaling capacities are likely to exist within a PDGF-stimulated cell. By constructing and expressing the PI3K double-add-back PDGFR mutants, we effectively increased the population of receptors that bind to PI3K and only one of the other major receptor-associated proteins, PLCγ, RasGAP, and SHP-2. Thus, the effect(s) of one of these enzymes on PDGFR-mediated PI3K signaling should be amplified. Utilizing these mutants, we have found that association of PDGFR with specific SH2-domain-containing proteins does have a dramatic effect on the PI3K pathway.

The impaired PI3K and Ras responses observed downstream of the Y40/51/21 mutant defy the logical prediction that

a receptor capable of binding to both PI3K and PLCγ would be capable of signaling. PI3K and PLCγ have both been identified as independent, positive downstream mediators of PDGFR signal transduction in this HepG2 system (42). Therefore, it was surprising that accumulation of PI3K products in cells expressing the Y40/51/21 mutant was attenuated. Since both PI3K and PLCγ utilize PI4,5P2 as a substrate, when PI3K and PLCγ are recruited to the same receptor molecule and thus to a similar pool of lipid substrate, competition for PI4,5P2 could result in a diminished signaling capability for both enzymes. Consistent with this hypothesis is the observation that PLCγ is poorly activated by the Y40/51/21 receptor (43). Alternatively, PI3K may be poorly activated by receptors that bind both PI3K and PLCγ because Ser-608 becomes extensively phosphorylated, which shuts down lipid kinase activity (7, 9). Serine kinase activity has been shown to copurify with PLCγ (30a); consequently, it is possible that this kinase phosphorylates and inactivates PI3K. Finally, given that Ras activation is a prerequisite for PI3K activation, it is possible that the defect in PI3K activation by the Y40/51/21 receptor is due to its inability to activate Ras. Ongoing experiments are aimed at distinguishing between these possibilities.

The Y40/51/71 receptor, which binds to both PI3K and RasGAP, was also crippled in its ability to activate Ras and PI3K in response to PDGF stimulation. Since the function of RasGAP is to enhance the hydrolysis of GTP bound to Ras, it is not surprising that the addition of the RasGAP binding site to the Y40/51 receptor results in the prevention of Ras activation. Therefore, the most likely explanation for the inability of the Y40/51/71 mutant to drive the accumulation of PI3K products is that RasGAP prevents accumulation of active Ras, which is a requirement for PDGF-dependent activation of PI3K. Indeed, we have recently observed that the F771 PDGFR, which does not bind RasGAP, is more efficient at mediating certain Ras-mediated events (7a). This is reminiscent of a nontransforming mutant of middle T antigen, which was fully capable of binding to PI3K but was unable to increase the levels of PI3K products in cells (26). This mutant was found to be deficient in the activation of Grb2 and Shc, suggesting that it was also deficient in activation of Ras (10). While it is likely that RasGAP is responsible for the impaired ability of the Y40/51/71 PDGFR to activate PI3K, we cannot rule out the possibility that an as yet unidentified inhibitor of PI3K is recruited to the receptor via site Y771 directly or by associating with RasGAP. This possibility, however, is unlikely since immunoprecipitates of the Y40/51/71 receptor coprecipitate with WT levels of PI3K activity, as determined by an in vitro PI3K assay (Fig. 3C).

While PLCγ and RasGAP appear to negatively regulate PDGFR signaling through PI3K, no apparent role for SHP-2 was observed. Tyrosine-phosphorylated SHP-2 recruits Grb2 and SOS to the PDGFR, and this is thought to contribute to PDGF-stimulated Ras activation (4, 25). Thus, by enhancing Ras activation, SHP-2 may contribute to PI3K activation. On the other hand, SHP-2 is capable of selectively dephosphorylating Y751 and Y771 on the PDGFR, decreasing the length of time that PI3K and RasGAP stay associated with the receptor (24). While this could contribute to regulation of PI3K activation, SHP-2's effects on PI3K and RasGAP binding are maximal at 25 min post-PDGF stimulation, a time at which we no longer detect PI3K products in PDGF-stimulated cells (unpublished observations). It appears that additional studies will be required to elucidate SHP-2's contribution to PDGFR signal relay.

Regulation of PDGFR signal relay. If recruiting RasGAP or PLCγ to receptors that have associated with PI3K prevents signaling by PI3K, then how does the WT receptor, which contains the binding sites for all these associated proteins, manage to activate PI3K? One explanation is that each WT receptor is not binding all of the receptor-associated proteins. Indeed, the PDGFR is not phosphorylated at all of the phosphorylation sites to the same extent; rather, at least a 16-fold difference exists in the stoichiometry of phosphorylation of the various tyrosine residues (18). Therefore, it is highly unlikely that all stimulated PDGFRs will bind to all of the possible SH2-domain-containing proteins at the same time. In the case of the WT receptor, many receptors may bind only to PI3K, or PI3K and a neutral protein like SHP-2, so that PI3K can become activated and its products can accrue. In the case of the mutant receptors, which have normal kinase activity but a reduced set of tyrosines that can be phosphorylated, the probability of PI3K and RasGAP or PI3K and PLCγ binding to the same receptor is increased, and consequently PI3K's signal is extinguished.

Association of the PDGFR with different combinations of SH2-domain-containing proteins may be a mechanism which provides the receptor with the flexibility necessary to respond to stimuli of varying intensity and duration. Binding of a neutralizing combination of SH2-domain-containing enzymes to a greater population of PDGFRs following an acute period of receptor stimulation may be important for proper temporal regulation of PDGFR signaling. Consistent with this possibility, we have observed that PI3K binds to the receptor very early following PDGF stimulation (~30 s), but the peak of RasGAP binding does not occur until approximately 5 min post-PDGF stimulation (23a). PDGFR binding of different combinations of associated proteins provides a mechanism by which PI3K responses can be fine-tuned to optimize signaling for specific biological events, such as mitogenesis, differentiation, and apoptosis. Such flexibility would be of paramount importance during stages of organismal development when the timing and integrity of PDGFR-mediated biological events, such as mitogenesis and cell migration, are critical to the proper formation of the developing embryo.

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