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Platelet-Derived Growth Factor Stimulation of GTPase-Activating Protein Tyrosine Phosphorylation in Control and c-H-*ras*-Expressing NIH 3T3 Cells Correlates with p21^{ras} Activation

CHRISTOPHER J. MOLLOY,[1]† TIMOTHY P. FLEMING, DONALD P. BOTTARO, ANTONIO CUADRADO, AND STUART A. AARONSON*

Laboratory of Cellular and Molecular Biology, National Cancer Institute, Building 37, Room 1E24, Bethesda, Maryland 20892

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Platelet-derived growth factor (PDGF) stimulation of NIH 3T3 cells leads to the rapid tyrosine phosphorylation of the GTPase-activating protein (GAP) and an associated 64- to 62-kDa tyrosine-phosphorylated protein (p64/62). To assess the functions of these proteins, we evaluated their phosphorylation state in normal NIH 3T3 cells as well as in cells transformed by oncogenically activated v-H-*ras* or overexpression of c-H-*ras* genes. No significant GAP tyrosine phosphorylation was observed in unstimulated cultures, while PDGF-BB induced rapid tyrosine phosphorylation of GAP in all cell lines analyzed. In NIH 3T3 cells, we found that PDGF stimulation led to the recovery of between 37 and 52% of GAP molecules by immunoprecipitation with monoclonal antiphosphotyrosine antibodies. Furthermore, PDGF exposure led to a rapid and sustained increase in the levels of p21^{ras} bound to GTP, with kinetics similar to those observed for GAP tyrosine phosphorylation. The PDGF-induced increases in GTP-bound p21^{ras} in NIH 3T3 cells were comparable to the steady-state level observed in serum-starved c-H-*ras*-overexpressing transformants, conditions in which these cells maintained high rates of DNA synthesis. These results imply that the level of p21^{ras} activation following PDGF stimulation of NIH 3T3 cells is sufficient to support mitogenic stimulation. Addition of PDGF to c-H-*ras*-overexpressing cells also resulted in a rapid and sustained increase in GTP-bound p21^{ras}. In these cells GAP, but not p64/62, showed increased tyrosine phosphorylation, with kinetics similar to those observed for increased GTP-bound p21^{ras}. All of these findings support a role for GAP tyrosine phosphorylation in p21^{ras} activation and mitogenic signaling.

Platelet-derived growth factor (PDGF) is one example of a class of mitogenic polypeptides that bind to specific cell surface receptors, leading to the activation of their intrinsic tyrosine kinase activity. Although the critical intracellular targets of the receptors remain largely undefined, the necessity of functional receptor tyrosine kinase activity supports the hypothesis that tyrosine phosphorylation of specific substrates is required for mitogenic signaling. Support for the role of protein tyrosine phosphorylation in the regulation of cell growth has derived from the identification and characterization of a number of molecules possessing this activity and the involvement of their constitutive activation in tumorigenesis. These molecules include non-membrane-spanning tyrosine kinases of the *src* family as well as membrane-spanning tyrosine kinases such as *erb*B/epidermal growth factor receptor, *erb*B-2, *trk*, and *met* (for reviews, see references 18 and 39).

Several recent studies have identified potential target proteins of receptor and nonreceptor tyrosine kinases. These proteins include enzymes involved in phospholipid metabolism such as phospholipase C-γ (23, 29, 41) and phosphatidylinositol-3-kinase (39, 42). Furthermore, interactions between activated receptors and intracellular tyrosine kinases have been described (16, 22). Many of these protein-protein interactions involving protein tyrosine kinases appear to be mediated by nonenzymatic domains of the molecules termed SH2 domains, which may represent an important regulatory mechanism in mitogenic signaling (21). In the case of phospholipase C-γ, the kinetics and extent of tyrosine phosphorylation correlate with in vivo enzymatic degradation of its substrate, phosphatidylinositol bisphosphate, yielding the second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (7, 24).

Functional Ras proteins (p21^{ras}) have been indirectly implicated in the signal transduction pathway used by certain growth factors, including PDGF (32). These small guanine nucleotide-binding molecules are active in their GTP-bound state and are inactive when bound to GDP (12, 25, 36). Transforming point mutations, which activate *ras* function, result in a retention of the GTP-bound form (for a review, see reference 3). Such molecules are potently mitogenic when microinjected into quiescent fibroblasts (37). However, the precise functional role of p21^{ras} in mitogenic signal transduction remains to be elucidated.

Ras proteins are regulated in vivo by a GTPase-activating protein (GAP), which greatly stimulates the conversion of GTP-bound p21^{ras} to the inactive, GDP-bound form (38). However, GAP has no attenuating effect on oncogenic p21^{ras}, allowing these molecules to remain in their active GTP-bound states. There is also some evidence that GAP-p21^{ras} interactions may be important in the signal-transduc-

* Corresponding author.

† Present address: Department of Cardiovascular Biochemistry, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543.

ing function of $p21^{ras}$, since several laboratories have shown that the GAP-binding domain of $p21^{ras}$ corresponds to the known Ras effector domain (1, 6, 17, 27, 28).

A potentially important connection between growth factors and the $p21^{ras}$-GAP system has derived from the demonstration that GAP is rapidly phosphorylated on tyrosine residues in response to certain growth factors, including PDGF and epidermal growth factor (10, 11, 19, 20, 30). Cellular transformation by nonmembrane tyrosine kinases, including v-*src* and v-*fps*, has also been associated with tyrosine phosphorylation of GAP in vivo (5, 10). An associated 64- to 62-kDa protein (p64/62), which is tyrosine phosphorylated and coimmunoprecipitates with GAP, has also been observed in v-*src* and v-*fps* transformants as well as in response to epidermal growth factor triggering (10, 32).

The extent and functional consequences of tyrosine phosphorylation of GAP and GAP-associated proteins in $p21^{ras}$ signaling functions have not been determined. One possibility is that tyrosine phosphorylation transiently impairs the attenuating activity of GAP on $p21^{ras}$, causing an increase in the levels of activated, GTP-bound $p21^{ras}$. This hypothesis is consistent with recent evidence that PDGF stimulation can increase levels of GTP-bound $p21^{ras}$ by two- to threefold (14, 35). However, the biological significance of these modest increases in $p21^{ras}$ activation is not known. Alternatively, tyrosine phosphorylation of GAP and/or p64/62 may be required for $p21^{ras}$ coupling to downstream biochemical signaling pathways. Thus, this study was undertaken to investigate the role of PDGF-stimulated tyrosine phosphorylation of GAP and GAP-associated proteins in mitogenic signaling pathways.

MATERIALS AND METHODS

Cells and reagents. NIH 3T3 mouse embryonic fibroblasts and transformants expressing v-H-*ras* or high levels of cellular H-*ras* (c-H-*ras*) were established in this laboratory. Experiments were performed with both mass cultures and specific clones of the *ras*-expressing transformants. At least three selected clones of cells that overexpressed c-H-*ras* were used. Biochemical results reported for these cell lines were reproducible in all clones tested. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, Md.) containing 10% calf serum and penicillin-streptomycin. PDGF-BB homodimer was obtained from Amgen Biologicals (Thousand Oaks, Calif.). Monoclonal antiphosphotyrosine (anti-P-Tyr) antibodies (immunoglobulin G2bk) were purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). Monoclonal anti-$p21^{ras}$ antibodies (Y13-259) were purchased from Oncogene Sciences Inc. (Manhasset, N.Y.). Antipeptide antisera specific for the α and β PDGF receptors were prepared as previously described (26). Anti-GAP peptide antisera were generously provided by Mark Marshall (Merck, Sharp & Dohme Research Laboratories) or prepared as described previously (40).

Immunoprecipitation, immunoblotting, and DNA synthesis assays. Cultures were incubated overnight in DMEM without serum, supplemented with 10 nM selenium (GIBCO-BRL) and 10 μg of transferrin (Collaborative Research, Bedford, Mass.) per ml. The cells were then either left untreated or stimulated with PDGF-BB (100 ng/ml) for the indicated times. DNA synthesis as measured by [^{3}H]thymidine incorporation (DuPont, NEN, Boston, Mass.) was performed as previously described (13). For biochemical analyses, cultures were rinsed twice in ice-cold phosphate-buffered saline (PBS) containing 1 mM sodium orthovanadate (Na_3VO_4) and lysed on ice in 0.5 ml of P-Tyr lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg each of aprotinin, leupeptin, and pepstatin A per ml). Lysates were collected in 1.5-ml microcentrifuge tubes and subjected to ultrasonic disruption for 10 s followed by centrifugation at 14,000 × *g* at 4°C for 10 min. Aliquots containing equal amounts of protein (2 to 3 mg) were subjected to immunoprecipitation, in which 2 μg of anti-P-Tyr was used per mg of cell lysate. To maximize the anti-P-Tyr recovery of substrates, in some experiments, supernatants remaining after the primary immunoprecipitation were reimunoprecipitated with fresh anti-P-Tyr. Antipeptide antisera were diluted to a final concentration of 1:100. For competition experiments, antisera were preincubated for 20 min in a small amount of P-Tyr lysis buffer containing 20 μg of the appropriate peptide. All immunoprecipitations were carried out for 2 h on ice, and immune complexes were then recovered by using protein G-agarose (Gammabind G; Genex Corp., Gaithersburg, Md.). Immunoprecipitates were washed five times in P-Tyr lysis buffer, solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer, and resolved by SDS-PAGE and immunoblotting as previously described (8). Aliquots of whole cell lysates (100 μg) removed prior to immunoprecipitation were also routinely analyzed by immunoblotting. Immunoreactive bands were visualized by using ^{125}I-protein A and autoradiography. In some experiments, radioactivities of the individual bands were quantitated and compared by using a model 400E Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.).

Detection of guanine nucleotides bound to $p21^{ras}$. Confluent cultures were starved in serum-free DMEM for 18 h and incubated in phosphate-free DMEM containing 0.5 mCi of $^{32}P_i$ (Dupont, NEN) for 3 h at 37°C. Quiescent cells were then treated with Na_3VO_4 (0.1 mM for 30 min at 37°C) followed by either PDGF-BB (100 ng/ml) or phorbol 12-myristate 13-acetate (PMA; 100 nM) for the indicated times. Pretreatment of the cells with Na_3VO_4 did not have any significant effect on the steady-state levels of GAP or GAP-associated protein tyrosine phosphorylation in these experiments. In some experiments, cells were pretreated for 24 h with 500 nM PMA to down-regulate protein kinase C (PKC). For $p21^{ras}$ extraction, after agonist stimulation, cells were washed with ice-cold PBS and lysed in 0.5 ml of ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, aprotinin and leupeptin (10 μg/ml each), arachidonic acid (100 μg/ml), and 50 μg of monoclonal anti-$p21^{ras}$ antibody (Y13-259) per ml. In control experiments, a competing peptide for anti-$p21^{ras}$ was also included. The lysates were sonicated and centrifuged at 14,000 × *g* for 10 min at 4°C. Soluble supernatants were precleared for 30 min by the addition of 0.1 ml of activated charcoal slurry (10%, wt/vol) which had been previously blocked with bovine serum albumin (10 mg/ml). Charcoal particles were removed by centrifugation, and radioactivity in the remaining lysates was quantitated by trichloroacetic acid precipitation of representative aliquots. Immunoprecipitations of lysates containing equivalent counts were then performed by addition of 40 μl of a 50% suspension of Gammabind G which had been precoated with rabbit anti-rat immunoglobulin G (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.). Immune complexes were washed four times

TABLE 1. Effects of PDGF-BB on [^{3}H]thymidine incorporation in control and *ras*-transformed NIH 3T3 cells[a]

Expt	Treatment	[^{3}H]thymidine incorporation[b]		
		NIH 3T3	c-H-*ras*	v-H-*ras*
1	No addition	1.00 ± 0.05	5.24 ± 0.55	8.61 ± 0.96
	PDGF-BB	8.42 ± 0.46	11.59 ± 1.21	9.73 ± 0.84
2	No addition	1.00 ± 0.12	17.86 ± 2.15	12.70 ± 0.76
	PDGF-BB	13.11 ± 1.55	50.59 ± 1.88	19.36 ± 2.63

[a] Cells (10^5 per well) were plated in 24-well tissue culture plates. After attachment, medium was changed to serum-free DMEM for 18 to 24 h as described in Materials and Methods. Cells were then left untreated or were challenged with saturating concentrations of PDGF-BB (10 ng/ml). After 15 h, [^{3}H]thymidine (2 μCi/ml) was added to each well, and labeling was continued for 6 h.

[b] Normalized to the value for [^{3}H]thymidine incorporation in control NIH 3T3 cells in each experiment and expressed as fold stimulation over control ± standard error (n = 3). The basal level of [^{3}H]thymidine incorporation (no addition) for experiment 1 was 766 ± 38 cpm; for experiment 2, basal [^{3}H]thymidine incorporation was 1,853 ± 227 cpm.

with 1 ml of lysis buffer and twice with 1 ml of PBS plus 20 mM $MgCl_2$, resuspended in 1 M KH_2PO_4 (pH 3.4) buffer (20 μl), and then heated for 3 min at 95°C. The supernatant (10 μl) was then spotted onto a sheet of polyethyleneimine-cellulose (J. T. Baker Corp., Phillipsburg, N.J.), and bound guanine nucleotides in the p21^{ras} immune complexes were resolved by thin-layer chromatography and detected by autoradiography as described previously (14, 15). In some experiments, radioactive content of the individual spots was quantitated by using a Molecular Dynamics model 400E Phosphorimager.

RESULTS

Tyrosine phosphorylation of GAP and GAP-associated proteins in serum-starved control and *ras*-transformed NIH 3T3 cells. PDGF stimulation of quiescent NIH 3T3 cells has previously been shown to induce rapid tyrosine phosphorylation of GAP (30). Since several studies have indicated that GAP may have a role as part of an effector complex with p21^{ras} (1, 6, 17, 28), it is possible that downstream signaling, involving GAP-Ras interactions, is facilitated by GAP tyrosine phosphorylation. To examine whether such a modification is required for *ras* function, we analyzed NIH 3T3 cells transformed by an oncogenically activated *ras* mutant, v-H-*ras*, and cells transformed by overexpression of the normal c-H-*ras* gene. Because cells transformed by activated *ras* genes have been reported to be defective in certain responses to PDGF (2, 4, 34), including the ability to induce tyrosine phosphorylation of GAP (19), we also sought to evaluate the effects of PDGF in the same transformants.

When subjected to serum starvation for different time periods (16 to 24 h), NIH 3T3 cells became quiescent, exhibiting low levels of [^{3}H]thymidine incorporation (Table 1). However, despite serum deprivation, significant levels of DNA synthesis were observed in the *ras* transformants, implying that transformation induced by either v-H-*ras* or c-H-*ras* overexpression resulted in autonomy from continued growth factor stimulation. In the quiescent NIH 3T3 cells, PDGF stimulation led to an 8- to 13-fold increase in [^{3}H]thymidine incorporation, roughly comparable to the basal levels of DNA synthesis observed in the c-H-*ras* cells (5- to 18-fold over levels for NIH 3T3 controls) or v-H-*ras* cells (8- to 13-fold over levels for NIH 3T3 controls). PDGF stimulation of the c-H-*ras* cells further increased [^{3}H]thymidine incorporation two- to threefold over the level for unstimulated c-H-*ras* cells, but PDGF had less of an effect on the v-H-*ras* transformant cell line (Table 1).

In the absence of PDGF, no significant tyrosine phosphorylation of GAP was detectable in either of the *ras* transformants, similar to results observed in quiescent NIH 3T3 cells (Fig. 1). In contrast, PDGF-BB stimulation for 10 min resulted in a marked stimulation of GAP tyrosine phosphorylation in each of the cell lines analyzed (Fig. 1). The overall extent of PDGF-stimulated GAP tyrosine phosphorylation varied according to the particular *ras* transformant tested. However, the respective levels of PDGF-stimulated GAP tyrosine phosphorylation correlated with the relative levels of PDGF receptor autophosphorylation observed as well as levels of immunodetectable PDGF receptors present in each cell line (data not shown). All of these findings indicated that continued DNA synthesis stimulation by v-H-*ras* or overexpressed c-H-*ras* did not require detectable GAP tyrosine phosphorylation. Thus, tyrosine phosphorylation is unlikely

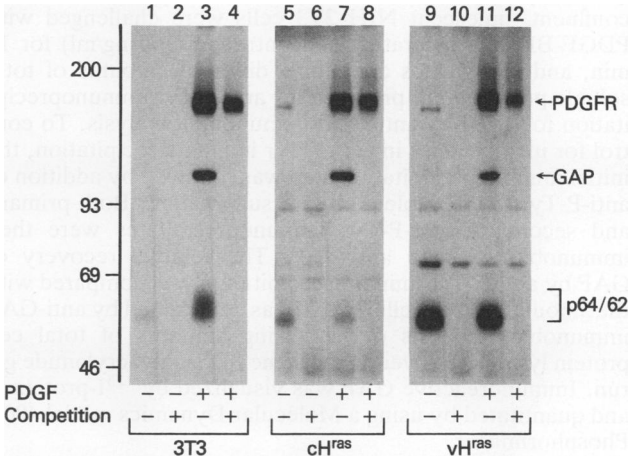

FIG. 1. Tyrosine phosphorylation of GAP and GAP-associated proteins in control and *ras*-transformed NIH 3T3 cells. Immunoprecipitates obtained by using an anti-GAP peptide antiserum were resolved on SDS-polyacrylamide gels and then subjected to immunoblot analysis with anti-P-Tyr antibodies. Cells were serum starved overnight and then were left untreated or were stimulated with PDGF-BB for 10 min prior to lysis as indicated. Lanes: 1 to 4, anti-GAP immunoprecipitates from wild-type NIH 3T3 cells; 5 to 8, anti-GAP immunoprecipitates from cells transformed by overexpression of c-H-*ras*; 9 to 12, anti-GAP immunoprecipitates from cells transformed by v-H-*ras*. Where noted, GAP peptide was included during the immunoprecipitation step as a competition control. Note that tyrosine phosphorylation of GAP was observed only after PDGF stimulation. In the c-H-*ras* cells, p64/62 tyrosine phosphorylation was not increased following PDGF addition (lane 7). Data are representative of independent experiments that were performed at least three times.

to be required for a GAP effector function with respect to p21^{ras}.

We next compared the effects of serum starvation or PDGF stimulation on tyrosine phosphorylation of a GAP-associated protein, p64/62. As shown in Fig. 1, anti-GAP immunoprecipitates from PDGF-stimulated (lane 3) but not serum-starved (lane 1) NIH 3T3 cells contained significant amounts of a 62- to 64-kDa tyrosine-phosphorylated protein (p64/62) as well as a pronounced tyrosine-phosphorylated band of 185 kDa corresponding to activated PDGF receptors. GAP and p64/62 coimmunoprecipitation could be completely blocked by GAP peptide competition (lane 4), indicating the specificity of their coimmunoprecipitation.

Anti-GAP immunoprecipitates from v-H-*ras* transformants maintained in serum-free medium contained significant amounts of tyrosine-phosphorylated p64/62 (Fig. 1, lane 9). Despite marked PDGF stimulation of GAP tyrosine phosphorylation in these cells, they showed no further increase in p64/62 tyrosine phosphorylation (lane 11). Analysis of several independent clones of c-H-*ras* overexpressor transformants revealed only a low level of tyrosine-phosphorylated p64/62 under serum-free conditions. In these cells, PDGF stimulation also resulted increased GAP tyrosine phosphorylation, but we observed no additional increase in the amounts of tyrosine-phosphorylated p64/62 associated with GAP (lane 7). Taken together, these results indicate that PDGF-stimulated GAP tyrosine phosphorylation does not always correlate with increased phosphorylation or association with p64/62.

PDGF-induced GAP tyrosine phosphorylation can involve a significant fraction of total cellular GAP. To estimate the percentage of total cellular GAP molecules which undergo tyrosine phosphorylation in response to PDGF stimulation, confluent, quiescent NIH 3T3 cells were challenged with PDGF-BB at a saturating concentration (100 ng/ml) for 10 min, and cell lysates containing different amounts of total soluble protein were prepared for anti-P-Tyr immunoprecipitation followed by anti-GAP immunoblot analysis. To control for inefficiencies in anti-P-Tyr immunoprecipitation, the initial immunoprecipitation step was followed by addition of anti-P-Tyr to the depleted lysate supernatant. Both primary and secondary anti-P-Tyr immunoprecipitates were then immunoblotted with anti-GAP. The relative recovery of GAP by anti-P-Tyr immunoprecipitation was compared with the amount of total cellular GAP, as determined by anti-GAP immunoblot analysis of increasing amounts of total cell protein lysates resolved in the same SDS-polyacrylamide gel run. Immunoreactive GAP was visualized by ^{125}I-protein A and quantitated by using a Molecular Dynamics model 400E Phosphorimager.

The intensity of the GAP signal increased as a function of the amount of cell lysate tested (Fig. 2A). Assuming that immunoprecipitation with anti-P-Tyr resulted in complete recovery of tyrosine-phosphorylated proteins, phosphorimage comparison of the intensity of the anti-P-Tyr-recovered GAP bands with those observed for the total cell lysates revealed that between 37 and 52% of the total cellular GAP was recovered by anti-P-Tyr in the PDGF-stimulated cells (Fig. 2B). Thus, these results strongly suggest that in NIH 3T3 fibroblasts, GAP is an excellent physiologic substrate for activated PDGF receptor tyrosine kinases. It is also possible that some of the anti-P-Tyr-recovered GAP could be the result of coimmunoprecipitation with other tyrosine-phosphorylated molecules. However, anti-P-Tyr recovery of GAP from serum-starved v-H-*ras* cells, which contain significant levels of the tyrosine-phosphorylated p64/62 in the absence of GAP tyrosine phosphorylation, was less than 1% (data not shown). Furthermore, we have observed efficient anti-P-Tyr recovery of GAP from PDGF-stimulated NIH 3T3 cells lysed under conditions that disrupt noncovalent protein interactions (cells lysed in 1% SDS at 95°C) (data not shown). Thus, we conclude that PDGF stimulates tyrosine phosphorylation of a significant portion of cellular GAP in NIH 3T3 cells.

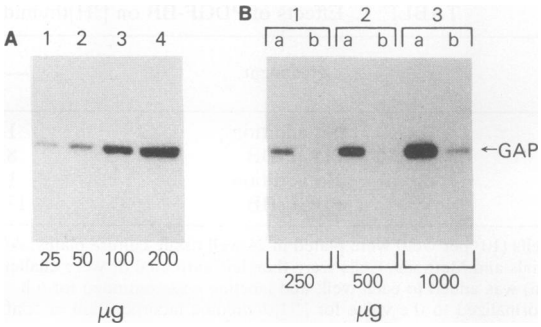

FIG. 2. Induction by PDGF of the tyrosine phosphorylation of a large percentage of cellular GAP. (A) Anti-GAP immunoblot analysis of PDGF-stimulated whole cell lysates. The amount of protein used per lane is noted at the bottom. (B) Anti-GAP immunoblot of anti-P-Tyr-recovered GAP from PDGF-stimulated cells. Aliquots of cell lysates containing the indicated amounts of protein were immunoprecipitated with an excess amount of anti-P-Tyr (lanes 1a, 2a, and 3a). Following the first immunoprecipitation, fresh anti-P-Tyr was added to supernatants and the immunoprecipitation was repeated (lanes 1b, 2b, and 3b). It should be noted that >90% of the proteins recognized by anti-P-Tyr were recovered in the first immunoprecipitation step. Comparison of the intensity of GAP in the anti-P-Tyr-recovered samples (a plus b lanes) with that in whole cell lysate blot was used to estimate the percentage of cellular GAP which was tyrosine phosphorylated. Anti-P-Tyr-recoverable GAP was calculated to be between 37 and 52% of total cellular GAP following quantitation of bound radioactivity by using a Molecular Dynamics model 400E Phosphorimager.

PDGF stimulation of GAP tyrosine phosphorylation correlates with functional activation of p21^{ras}. Since one function of GAP is to attenuate p21^{ras} activity by stimulating hydrolysis of p21^{ras}-GTP, tyrosine phosphorylation of GAP has been hypothesized to reduce this effect, allowing increased p21^{ras} signaling (17, 28, 30). In support of this concept, recent studies have demonstrated that certain growth factors and activated protein tyrosine kinases can increase the ratio of endogenous p21^{ras} bound to GTP, the active form of the Ras protein (14, 35). Thus, GAP tyrosine phosphorylation directly, or its association with tyrosine-phosphorylated p64/62, might play an important role in achieving such an effect. To evaluate p21^{ras} activation, we analyzed guanine nucleotides bound to p21^{ras} in serum-starved or PDGF-stimulated NIH 3T3 cells. Any increase in p21^{ras} bound to GTP could then be correlated to changes in tyrosine-phosphorylated GAP or p64/62. For these purposes, we also evaluated p21^{ras} activation in cells transformed by v-H-*ras* or the overexpression of c-H-*ras*.

The level of GTP-bound p21^{ras} was barely detectable in comparison with the level of p21^{ras}-GDP in intact quiescent NIH 3T3 cells (Fig. 3A, lane 1). Upon PDGF-BB stimulation for 10 min, we observed significant three- to fivefold increases in GTP-bound p21^{ras} (lane 2). These results were reproducible in three independent experiments. The level of GTP-bound p21^{ras} achieved was modest compared with that

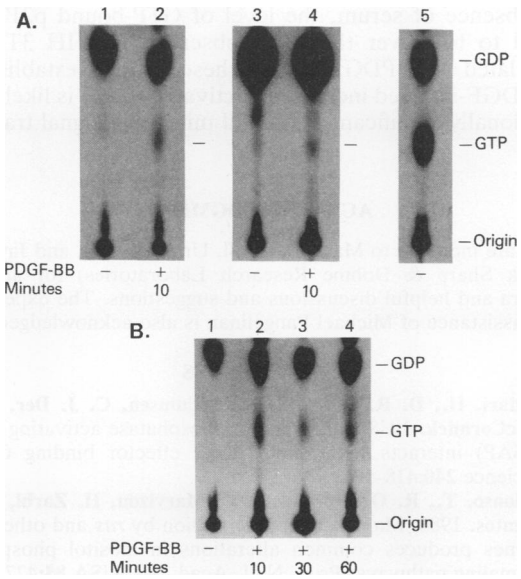

FIG. 3. Induction of rapid activation of p21^{ras} by PDGF stimulation of intact NIH 3T3 cells. (A) Thin-layer chromatographic analysis of p21-bound guanine nucleotides following anti-p21 immunoprecipitation from wild-type NIH 3T3 cells (lanes 1 and 2) and cells transformed by overexpression of c-H-*ras* (lanes 3 and 4) or v-H-*ras* (lane 5). Cells were serum starved overnight and then were either left untreated or stimulated with PDGF-BB for 10 min as indicated. Note that levels of active p21^{ras} (GTP bound) are increased by PDGF stimulation in both wild-type and c-H-*ras*-transformed cells following addition of PDGF (lanes 2 and 4). (B) Time course of p21^{ras} activation following PDGF stimulation of NIH 3T3 cells. Data are representative of independent experiments performed at least three times.

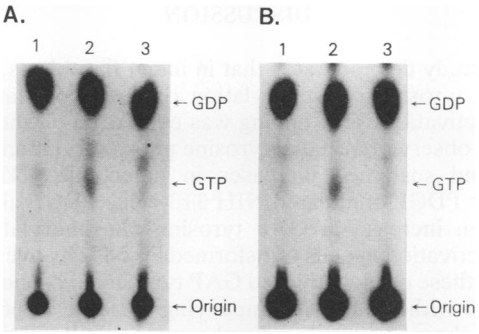

FIG. 4. Evidence that PDGF-stimulated p21^{ras} activation is independent of PKC activation, as determined by thin-layer chromatographic analysis of p21^{ras}-bound guanine nucleotides following anti-p21^{ras} immunoprecipitation from wild-type NIH 3T3 cells. (A) Cells were serum starved overnight and then left untreated (lane 1) or stimulated with either PDGF-BB (lane 2) or PMA (lane 3) for 30 min. Note that levels of GTP-bound p21^{ras} are increased by PDGF treatment but not by PMA treatment. (B) Serum-starved NIH 3T3 cells were pretreated with PMA for 24 h to down-regulate PKC and then left untreated (lane 1) or stimulated with PDGF-BB (lane 2) or PMA (lane 3). As shown in lane 2, PDGF-BB stimulated an increase in the level of GTP-bound p21^{ras}. Under these conditions, protein levels of PKC were reduced to less than 10% of the level found in untreated cells (data not shown).

observed in unstimulated v-H-*ras*-transformed cells (lane 5). By phosphorimage analysis, we estimated that the percentage of GTP-bound p21^{ras} induced by PDGF stimulation of NIH 3T3 cells was between 5 and 15% of total bound nucleotide. In contrast, levels of GTP-bound p21^{ras} in v-H-*ras*-transformed cells was estimated to be approximately 40% of bound nucleotide (lane 5). We did not observe a detectable increase in GTP-bound *ras* following PDGF stimulation of the v-H-*ras* transformants (data not shown).

To evaluate the biologic significance of PDGF-stimulated p21^{ras} activation in NIH 3T3 cells, we also measured the extent of GTP-bound p21^{ras} in c-H-*ras*-overexpressing transformants. The increased level of GTP-bound p21^{ras} achieved in PDGF-stimulated NIH 3T3 cells was greater than the steady-state level of p21^{ras}-GTP in the c-H-*ras*-overexpressing transformants subjected to serum starvation (Fig. 3A; compare lanes 2 and 3). Since under these conditions the c-H-*ras* transformants maintained high levels of DNA synthesis (Table 1), only relatively low levels of GTP-bound p21^{ras} must be required for its mitogenic signaling functions. Thus, the level of PDGF-stimulated GTP-bound p21^{ras} in NIH 3T3 cells is likely to be biologically significant.

We also analyzed p21^{ras}-bound guanine nucleotides at different times following PDGF stimulation. As shown in Fig. 3B, increased GTP-bound p21^{ras} was sustained for at least 1 h following PDGF addition to NIH 3T3 cells. Similar results were also obtained in c-H-*ras* transformants following PDGF addition (data not shown). These data are consistent with the sustained time course observed for GAP tyrosine phosphorylation in response to PDGF (30). As noted above, PDGF-stimulated GAP tyrosine phosphorylation in the c-H-*ras* overexpressor cells was not accompanied by any significant increase in tyrosine phosphorylation of the GAP-associated protein p64/62. Nonetheless, PDGF-BB stimulated a significant increase in the ratio of p21^{ras} bound to GTP (Fig. 3A, lane 4). Thus, in these cells, PDGF-induced p21^{ras} activation cannot be attributable to increased tyrosine phosphorylation of p64/62.

PDGF stimulation of p21^{ras} activation is independent of PKC. Recently, Downward et al. (9) have shown that stimulation of the T-cell antigen receptor causes rapid increases in the level of GTP-bound p21^{ras}. In this system, p21^{ras} activation was reported to result from PKC-dependent inhibition of GAP, by a mechanism that remains to be determined. PDGF stimulation of fibroblasts results in PKC activation through phospholipase C-γ-mediated phosphoinositide breakdown (for a review, see reference 42). Thus, we sought to determine whether this mechanism could be responsible for p21^{ras} activation observed in PDGF-stimulated NIH 3T3 cells. For these experiments, we used the potent PKC agonist PMA. This compound stimulates PKC when acutely administered to cells. However, chronic treatment leads to catabolism of the activated enzyme, effectively depleting the cells of PKC (33).

Short-term (30-min) PMA treatment of quiescent NIH 3T3 cells did not stimulate any increase in the level of GTP-bound p21^{ras} (Fig. 4A, lane 3). Furthermore, when cells were pretreated with PMA for 24 h to down-regulate PKC, PDGF was still able to induce an increase in p21^{ras} bound to GTP (Fig. 3B, lane 2). Under these conditions, PKC levels in the same cells decreased by more than 90%, as monitored by immunoblotting with a peptide antiserum against PKC (data not shown). Taken together, our findings indicate that the PDGF-stimulated activation of p21^{ras} in fibroblasts is not dependent on PKC activation.

DISCUSSION

This study demonstrates that in intact fibroblasts, PDGF-induced tyrosine phosphorylation of GAP correlates with $p21^{ras}$ activation. This finding was evidenced by the similar kinetics observed for GAP tyrosine phosphorylation and the rapid and sustained increases in detectable GTP-bound $p21^{ras}$ in PDGF-stimulated NIH 3T3 cells. PDGF also stimulated an increase in GAP tyrosine phosphorylation and $p21^{ras}$ activation in cells transformed by c-H-*ras* overexpression. In these cells, increased GAP tyrosine phosphorylation occurred without any accompanying increase in p64/62 tyrosine phosphorylation. Furthermore, PDGF-stimulated $p21^{ras}$ activation did not require PKC activation.

GAP has been suggested to act as a downstream effector as well as an attenuator of $p21^{ras}$ function (1, 6, 17, 28, 43). Theoretically, phosphorylation of GAP and/or GAP-associated proteins could facilitate its effector role, perhaps by coupling the GAP-$p21^{ras}$ complex to other molecules. Our finding that *ras* transformants lacked detectable levels of tyrosine-phosphorylated GAP argues that GAP tyrosine phosphorylation is not required for a GAP effector function. Our results support the concept that tyrosine phosphorylation of GAP results in the inhibition of its GTPase-stimulating activity, leading to increases in GTP-bound $p21^{ras}$, which then effects mitogenic signaling through some as yet undetermined downstream components. Our demonstration that GAP is an excellent substrate for activated PDGF receptors and that up to 50% of cellular GAP molecules undergo tyrosine phosphorylation within 10 min of PDGF stimulation further supports this model.

Previous studies have failed to demonstrate that direct tyrosine phosphorylation of GAP in vitro has any effect on $p21^{ras}$ GTPase activity (14, 31). However, the conditions used in these experiments may have lacked critical endogenous cofactors and therefore may not have reflected physiologic conditions in intact cells. For example, a recent study by Moran et al. (32) showed that in certain cells containing activated tyrosine kinases, a subpopulation of phosphorylated GAP associated with a cytoplasmic phosphoprotein (p190). This GAP-p190 complex exhibited decreased GTPase activity (32). Furthermore, epidermal growth factor and PDGF have also been shown to stimulate GAP phosphorylation on serine as well as tyrosine residues (10, 29a). Thus, serine phosphorylation of GAP may also modulate its activity in vivo. It is therefore possible that GAP tyrosine phosphorylation is an initial priming event, required for phosphorylation of GAP by an appropriate serine kinase(s) that may be ultimately responsible for inhibition of GAP activity. In addition, the results presented here do not exclude the possibility that PDGF also stimulates a $p21^{ras}$ guanine nucleotide exchange factor, allowing for an increase in $p21^{ras}$ bound to GTP following displacement of GDP.

Satoh et al. (35) have reported that in fibroblasts overexpressing the c-H-*ras* gene, certain growth factors, including PDGF, induce an increase in the levels of $p21^{ras}$ bound to GTP. Gibbs et al. (14) also detected increased GTP-bound $p21^{ras}$ in NIH 3T3 cells stimulated by PDGF as well as in cells transformed by oncogenes encoding protein tyrosine kinases. However, neither of these studies addressed the biologic significance of the levels of GTP-bound $p21^{ras}$ observed. In this study, we measured the steady-state level of GTP-bound $p21^{ras}$ in NIH 3T3 cells overexpressing normal $p21^{ras}$. Under conditions in which $p21^{ras}$ overexpression was sufficient to maintain a high level of DNA synthesis in the absence of serum, the level of GTP-bound $p21^{ras}$ was found to be lower than that observed in NIH 3T3 cells stimulated with PDGF. All of these findings establish that the PDGF-induced increase in activated $p21^{ras}$ is likely to be functionally significant in normal mitogenic signal transduction.

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