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Aberrant Ras Regulation and Reduced p190 Tyrosine Phosphorylation in Cells Lacking p120-Gap

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The Ras guanine nucleotide-binding protein functions as a molecular switch in signalling downstream of protein-tyrosine kinases. Ras is activated by exchange of GDP for GTP and is turned off by hydrolysis of bound GTP to GDP. Ras itself has a low intrinsic GTPase activity that can be stimulated by GTPase-activating proteins (GAPs), including p120-Gap and neurofibromin. These GAPs possess a common catalytic domain but contain distinct regulatory elements that may couple different external signals to control of the Ras pathway. p120-Gap, for example, has two N-terminal SH2 domains that directly recognize phosphotyrosine motifs on activated growth factor receptors and cytoplasmic phosphoproteins. To analyze the role of p120-Gap in Ras regulation in vivo, we have used fibroblasts derived from mouse embryos with a null mutation in the gene for p120-Gap (*Gap*). Platelet-derived growth factor stimulation of $Gap^{-/-}$ cells led to an abnormally large increase in the level of Ras-GTP and in the duration of mitogen-activated protein (MAP) kinase activation compared with wild-type cells, suggesting that p120-Gap is specifically activated following growth factor stimulation. Induction of DNA synthesis in response to platelet-derived growth factor and morphological transformation by the v-*src* and EJ-*ras* oncogenes were not significantly affected by the absence of p120-Gap. However, we found that normal tyrosine phosphorylation of p190-rhoGap, a cytoplasmic protein that associates with the p120-Gap SH2 domains, was dependent on the presence of p120-Gap. Our results suggest that p120-Gap has specific functions in downregulating the Ras/MAP kinase pathway following growth factor stimulation, and in modulating the phosphorylation of p190-rhoGap, but is not required for mitogenic signalling.

Ras proteins are small guanine nucleotide-binding polypeptides that associate with the plasma membrane and cycle between an inactive GDP-bound form and a biologically active GTP-bound state (5, 6). Ras activation is of particular importance in coupling receptor tyrosine kinases to downstream signalling pathways involved in the control of cell proliferation and differentiation (19, 61, 73). Tyrosine kinases induce the exchange of GDP for GTP on Ras through SH2/SH3 adapter proteins such as Grb2, which binds through its SH2 domain to specific tyrosine phosphorylation sites on activated receptors, or on intermediate proteins such as Shc (4, 59, 60, 66). Grb2, in turn, binds through its SH3 domains to the Ras guanine nucleotide exchange factors mSos1 and mSos2 (11, 20, 22, 41, 65). GTP-bound Ras undergoes a conformational change such that it can associate with downstream targets, notably the Raf protein-serine/threonine kinase which activates the mitogen-activated protein (MAP) kinase protein kinase cascade (45, 72, 75). Ras-GTP also interacts specifically with GTPase-activating proteins (GAPs), which act to accelerate the weak intrinsic Ras GTPase activity by up to 5 orders of magnitude and thereby greatly accelerate the rate at which Ras is inactivated by hydrolysis of bound GTP to GDP (1, 5, 69).

Several GAPs have been identified in mammalian cells, including p120-Gap (70, 74), neurofibromin (the product of the *Nf1* tumor suppressor gene) (48, 77), and two forms of GAP1 (15, 23, 43, 76), one of which is regulated by binding to IP4. These of GAPs all share a GAP-related catalytic domain (GRD) which is both necessary and sufficient for stimulating Ras GTPase activity (47, 48, 77). They also possess a variety of additional sequences that distinguish the different GAP proteins from one another and are potentially involved in regulating their abilities to negatively regulate Ras in response to external signals (5). These noncatalytic regions of mammalian GAPs may also have additional biological functions, for example, by binding to effectors downstream from Ras (49). In addition, they may have functions that are independent of Ras. Such considerations have led to the notion that GAPs, which bind to the active signalling form of Ras, might potentially serve both as negative regulators and effectors of Ras.

p120-Gap is of particular interest in tyrosine kinase signalling, since the N-terminal region of p120-Gap possesses two SH2 domains, which flank a single SH3 domain (3, 54). The SH2 domains of p120-Gap are functional phosphotyrosine-binding modules that recognize phosphorylated motifs in activated growth factor receptors and specific cytoplasmic phosphoproteins (3, 54). Thus, p120-Gap binds to the activated β receptor for platelet-derived growth factor (βPDGF receptor), through recognition of an autophosphorylation site within the receptor kinase insert (Tyr 771) by the C-terminal p120-Gap SH2 domain (14, 36, 37). Such interactions can potentially recruit p120-Gap to the membrane (53, 55) and may therefore be involved in controlling Ras activity following growth factor stimulation. Consistent with a role in tyrosine kinase signalling, p120-Gap is itself phosphorylated at Tyr 460 by a variety of activated tyrosine kinases (9, 21, 29, 34, 42, 52, 53, 55). However, it remains unclear whether p120-Gap activity is inhibited or enhanced following growth factor receptor activation.

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The p120-Gap SH2 domains also bind two intracellular phosphotyrosine-containing proteins of 62 and 190 kDa in cells stimulated with a variety of growth factors or transformed by oncogenic tyrosine kinase variants (7, 21, 55, 64). p62 is a highly tyrosine phosphorylated but relatively minor protein that possesses a pleckstrin homology domain and multiple YXXP motifs (11a, 77a). p190 has a C-terminal catalytic domain that possesses GAP activity toward members of the Rho family of small GTPases (including Rho, Rac, and Cdc42), which are involved in control of the actin cytoskeleton, and an N-terminal guanine nucleotide-binding region (67, 68). The interactions of p62 and p190 with the p120-Gap SH2 domains require tyrosine phosphorylation but appear independent of Ras activation (44, 54, 55, 63, 67, 68). The central region of p120-Gap has a pleckstrin homology domain and a calcium/lipid-binding region (24, 25, 56), both of which could potentially regulate interactions of p120-Gap with phospholipids and hence with the membrane. At its C terminus, p120-Gap has a GRD that stimulates Ras GTPase activity (47) in a fashion that may be modulated by N-terminal p120-Gap elements (28). p120-Gap therefore participates in multiple protein-protein interactions through its SH2, SH3, and GRD domains and may therefore have several biological functions.

p120-Gap was originally identified as a polypeptide that negatively regulates wild-type Ras but not oncogenically activated Ras variants (27, 69). A variety of biological experiments, mostly based on overexpression of full-length p120-Gap, support the notion that p120-Gap inhibits signalling by Ras-GTP (2, 13, 18, 26, 58, 78). However, it remains possible that p120-Gap also has an effector function downstream of Ras. Indeed, the N-terminal region of p120-Gap has been suggested to synergize with a nonmyristylated v-Src mutant in inducing cellular transformation (17), to induce gene expression (51), to modulate coupling of muscarinic receptors with G_k (49), and to affect the actin cytoskeleton (50).

We have recently introduced a null mutation into the mouse gene for p120-Gap (*Gap*) (30). Mouse embryos that are homozygous for the mutant *Gap* allele die at approximately day 10.5 of embryogenesis and display a variety of defects, including aberrant formation of vascular structures and increased apoptosis in the nervous system. We have used these mutant embryos to obtain cells lacking p120-Gap, which we have employed to address the cellular functions of p120-Gap.

MATERIALS AND METHODS

Generation and characterization of wild-type, *Gap*$^{-/-}$, and *Nf1*$^{-/-}$ cell lines. Embryos were extracted at day 9.5 of gestation, the yolk sacs were removed, and the remaining tissue was incubated with trypsin for 5 min at 37°C. Cells were seeded in 24-well tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS). Early-passage cells were expanded and used for further experiments. All cell lines described in this study were derived from separate embryos. Genomic DNA isolated from yolk sacs and later from cell lines was prepared and probed for the presence of wild-type or mutant *Gap* and *Nf1* loci by PCR as described previously (30, 33). For protein analysis, cells were lysed in 125 mM Tris-Cl (pH 6.8)–20% glycerol–10% β-mercaptoethanol–10 mM dithiothreitol (DTT)–4.6% sodium dodecyl sulfate (SDS)–0.05% bromophenol blue (2× SDS-sample buffer) (39), DNA was sheared by using a syringe, and proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 7.0% gel and transferred to a polyvinylidene difluoride membrane by using a Bio-Rad (Hercules, Calif.) semidry blotting apparatus at 50 mA per gel for 60 min at room temperature. Membranes were blocked for 1 h at room temperature in 10 mM Tris-Cl (pH 7.4)–150 mM NaCl–0.2% Tween 20 (TBST) containing 5% dried milk, incubated with a polyclonal antiserum raised against p120-Gap, neurofibromin (16), or a fusion protein containing the βPDGF receptor kinase insert domain in TBST–5% dried milk for 1 h at room temperature, and washed twice for 10 min each time with TBST and twice for 5 min each time with 10 mM Tris-Cl (pH 7.4)–150 mM NaCl. Membranes were then incubated for 1 h with horseradish peroxidase-protein A (Bio-Rad) diluted 1:10,000 in TBST and washed as before. Reactive proteins were visualized by enhance chemiluminescence (Amersham Canada, Oakville, Ontario, Canada).

Determination of percentages of Ras-bound GTP. Cells were labeled for 16 h at 37°C in DMEM without phosphate and containing 20 mM HEPES (pH 7.2), 0.5% dialyzed CS, and 0.5 mCi of [^{32}P]orthophosphate per ml. Control cells or cell stimulated for 5 min at 37°C with 30 ng of PDGF BB per ml were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of lysis buffer (50 mM Tris-Cl [pH 7.5], 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Nonidet P-40, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]), and Ras was immunoprecipitated by incubation with 1 μg of anti-Ras monoclonal antibody Y13-259 on ice, followed by incubation with 100 μl of 10% (vol/vol) anti-rat immunoglobulin (Ig)-Sepharose. Immunoprecipitates were washed several times with lysis buffer; guanine nucleotides were eluted in 30 μl of elution buffer (0.2% SDS, 2 mM DTT 2 mM EDTA) and resolved by chromatography in 1 M KH_2PO_4 on 0.1-mm polyethyleneimine-impregnated cellulose. Results were quantified with a PhosphorImager.

Characterization of MAP kinase activation. For MAP kinase blots, cells were starved for 16 h in DMEM containing 20 mM HEPES (pH 7.2). Control cells or cell stimulated with 30 ng of PDGF BB per ml at 37°C for 5 min or for the time indicated were rinsed twice with ice-cold PBS lysed in 2× SDS-sample buffer and boiled, and DNA was sheared by using a syringe. Approximately equal amounts of proteins were resolved by SDS-PAGE and analyzed by immunoblotting with the anti-MAP kinase monoclonal antibody B3B9 as described above for other antisera. For analysis of MAP kinase activity, cells were lysed in HO lysis buffer (50 mM HEPES [pH 7.5], 100 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 100 μM Na_3VO_4, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml, 1 mM PMSF) and immunoprecipitated by incubation with polyclonal anti-MAP kinase serum prebound to protein A-Sepharose. Immunoprecipitates were washed twice with HO lysis buffer and twice with 10 mM HEPES (pH 7.5) containing 10 mM magnesium acetate and subsequently incubated for 15 min at 37°C with 30 μl of kinase mix (20 mM HEPES [pH 7.4], 10 mM magnesium acetate, 1 mM DTT, 25 μM ATP, 0.5 mg of myelin basic protein (MBP) per ml, and 1 μl of [γ-^{32}P]ATP per reaction); 5-μl aliquots were spotted onto phosphocellulose paper and washed several times in 75 mM phosphoric acid, and the radioactivity incorporated in MBP was quantified by Cerenkov counting.

Quantitation of PDGF-induced DNA synthesis. DNA synthesis was quantified by [^{3}H]thymidine incorporation. Cells were grown to confluence, starved for 16 h in DMEM containing 20 mM HEPES (pH 7.4) and 0.5% CS, and subsequently grown another 16 h in the presence or absence of 30 ng of PDGF BB per ml. [^{3}H]thymidine was added to a concentration of 5 μCi/ml; cells were incubated for another 5 h, washed twice with PBS, and fixed in methanol-acetic acid (3:1). DNA was precipitated with 10% trichloroacetic acid at 4°C and dissolved in 0.2 N NaOH. Radioactivity incorporated was quantified by scintillation counting.

Focus-forming assay. Cells were seeded at 5×10^5 per 10-cm-diameter dish and transfected by BES calcium phosphate precipitation with 5 μg of v-*src* cDNA cloned into pECE or 5 μg of pEJ-*ras* and pBabe-hygro; pBSKS$^+$ was used as the carrier. Cells were split 1:10 24 h after transfection in DMEM containing 5% CS. Cells were fixed in 10% methanol–10% acetic acid, stained with 0.4% crystal violet in 20% ethanol, and photographed 21 days after transfection. To determine transfection efficiencies, cells were grown in the presence of 50 μg of hygromycin per ml.

Analysis of protein phosphorylation. Cells were starved overnight in DMEM containing 20 mM HEPES (pH 7.5). Control cells or cells stimulated with 30 ng of PDGF BB per ml for 5 min at 37°C were rinsed two times with cold PBS and lysed in 1 ml of PLC-lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 10 mM sodium pyrophosphate, 100 μM sodium vanadate, 1 mM PMSF, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml) per 10-cm-diameter tissue culture dish. Lysates were cleared by centrifugation at 10,000 rpm in a microcentrifuge at 4°C, incubated with 5 μl of polyclonal antiserum raised against p190 (68) for 1 h on ice, and subsequently incubated for 1 h with 100 μl of 10% protein A-Sepharose or anti-mouse IgG-Sepharose at 4°C on an agitator. Sepharose beads were collected by centrifugation and washed four times with PLC-lysis buffer. Immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting with the monoclonal antiphosphotyrosine (anti-P.Tyr) antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, N.Y.). Membranes were blocked with TBST containing 5% bovine serum albumin and then incubated with the antibody diluted in TBST–5% bovine serum albumin. Washes and detection were done exactly as described for other antisera, using horseradish peroxidase–goat anti-mouse Ig (Bio-Rad) as a secondary antiserum. Immunoblotting with the anti-p190 serum was done as described above for other antisera.

For analysis of p62, the cells were labeled for 14 h in DMEM without methionine and containing 20 mM HEPES and 100 mCi of [^{35}S]methionine per ml. p62 was immunoprecipitated with 5 μl of monoclonal anti-p62, a gift of R. Roth (31). Immunoprecipitations were resolved by SDS-PAGE, transferred to Immobilon, and analyzed by autoradiography followed by immunoblotting with anti-P.Tyr monoclonal 4G10.

RESULTS

Isolation of cells deficient for p120-Gap. To study the role of p120-Gap in Ras regulation, and to compare the functions of p120-Gap and neurofibromin, we used cells isolated from *Gap*

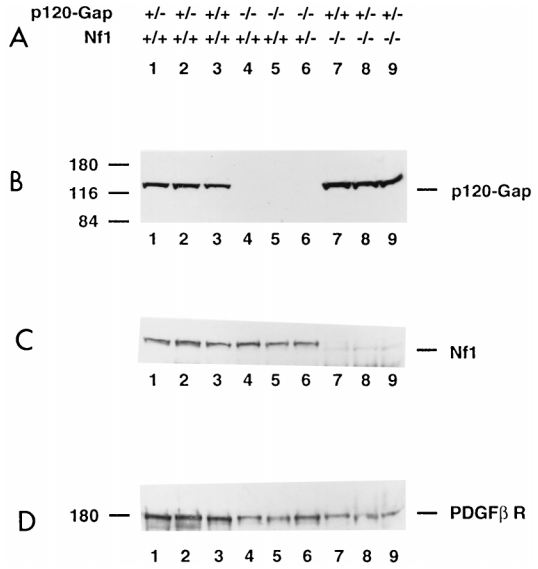

FIG. 1. p120-Gap, neurofibromin, and βPDGF receptor expression in fibroblast cell lines derived from wild-type, $Gap^{-/-}$, and $Nf1^{-/-}$ embryos. (A) Following mating of $Gap^{+/-}$ $Nf1^{+/-}$ compound heterozygous mice, resulting embryos were collected at day 9.5 of gestation. Cell cultures were established and genotyped by PCR analysis. (B) To test for p120-Gap expression, whole-cell lysates were analyzed by immunoblotting with a polyclonal anti-p120-Gap serum. (C) To test for neurofibromin expression, whole-cell lysates were analyzed by immunoblotting with a polyclonal antineurofibromin (Nf1) serum. (D) To test for βPDGF receptor expression, whole-cell lysates were analyzed by immunoblotting with a polyclonal anti-PDGF receptor serum. Sizes are indicated in kilodaltons.

and *Nf1* mutant mouse embryos. Mice homozygous for a loss-of-function mutation in *Gap* die at day 10.5 of embryonic development (30), while *Nf1*-deficient mouse embryos die at day 13 of development (8, 33). To allow the simultaneous isolation of embryos and cells defective in either p120-Gap or neurofibromin from the same cross, compound heterozygous mice with mutations in both the *Gap* and the *Nf1* genes were mated, and embryos were taken at day 9.5 of development. Individual embryos were trypsinized, and cultures from separate embryos were established. Genotypes were established by PCR analysis. Following genotyping of the *Gap* and *Nf1* loci, nine cultures were maintained, of which three were wild type or heterozygous for the *Gap* and *Nf1* genes, three were homozygous for the *Gap* mutation, and three were homozygous for the *Nf1* mutation.

To validate the genotypic analysis of these cells, each culture was examined by Western blotting for the presence of p120-Gap or neurofibromin (Fig. 1). Cells that were homozygous for the *Gap* mutation failed to express detectable levels of p120-Gap, in contrast to heterozygous or wild-type cells (Fig. 1). Similarly, cells that were homozygous for the mutant *Nf1* allele did not express neurofibromin (Fig. 1). These cells were also analyzed by immunoblotting with antibodies to the βPDGF receptor and were found to contain similar levels of the receptor (Fig. 1).

Aberrant Ras-GTP loading in PDGF-stimulated $Gap^{-/-}$ cells. To analyze Ras-GTP levels in the various cultures, cells were starved, labeled with [^{32}P]orthophosphate, and stimulated with PDGF. Each experiment was performed in triplicate, using cultures established from different embryos; the result shown is representative of several independent experiments. The percentage of Ras in the GTP-bound state was determined in PDGF-stimulated cells in comparison with control unstimulated cells. In the early-passage wild-type cells, Ras-GTP levels showed only a marginal detectable increase after 5 min of PDGF stimulation (Fig. 2). In serum-deprived $Gap^{-/-}$ cells, the basal level of Ras-GTP was similar to that observed in wild-type cells (approximately 13%). However, following stimulation of $Gap^{-/-}$ cells with PDGF for 5 min, the Ras-GTP levels rose about twofold to approximately 25% in each of the three $Gap^{-/-}$ cultures (Fig. 2). These results indicate that in these early-passage embryonic cells, the absence of p120-Gap does not affect the basal level of Ras-GTP but that PDGF-stimulated Ras-GTP levels are unusually high. The implication of these data is that p120-Gap does not play a significant role in maintaining the basal level of Ras-GTP but has a major part in the downregulation of Ras after growth factor stimulation. In contrast to the results obtained with $Gap^{-/-}$ cells, $Nf1^{-/-}$ cells did not show a significantly enhanced level of Ras activation in response to PDGF; instead, we have observed a somewhat variable increase in both the basal and PDGF-stimulated levels of Ras-GTP compared to wild-type cells (Fig. 2).

Activation of the MAP kinase pathway in $Gap^{-/-}$ cells. Ras-GTP interacts through its effector loop with Raf, which in turn stimulates the MAP kinase pathway (45, 72, 75). To investigate whether p120-Gap is absolutely required for the induction of MAP kinase activity, or alternatively whether it plays an indirect role in regulating MAP kinase activation through its effect on Ras-GTP levels, we assayed the activation state of MAP kinase in wild-type and $Gap^{-/-}$ cells. Wild-type or $Gap^{-/-}$ mutant cells were stimulated with PDGF and subjected to Western blot analysis with anti-MAP kinase antibodies (Fig. 3A). In wild-type cells, PDGF stimulation induces the conversion of MAP kinase to a form that shows decreased electrophoretic mobility during SDS-PAGE, due to coordinate phosphorylation on both tyrosine and threonine by MAP kinase kinase (Mek) (62). A very similar pattern of MAP kinase electrophoretic mobility was seen in two independent cultures of $Gap^{-/-}$ cells (Fig. 3A). In unstimulated $Gap^{-/-}$ mutant cells, MAP kinase migrated at the position typical of the inactive nonphosphorylated form but upon PDGF stimulation was completely converted to the more slowly migrating phosphorylated species, very similar to the activation of MAP kinase observed in wild-type and $Nf1^{-/-}$ cell lines (Fig. 3A).

It is perhaps surprising that in wild-type cells we observed such a small increase in Ras-GTP levels following stimulation with PDGF (Fig. 2) while at the same time MAP kinase became fully activated, as judged by a stoichiometric shift in mobility during SDS-PAGE (Fig. 3A). This may reflect a strong amplification of the Ras-GTP signal. It is also possible

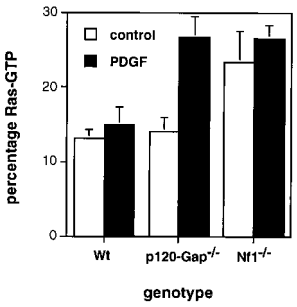

FIG. 2. Ras activation in cell lines that lack p120-Gap or neurofibromin. The percentages of Ras in the GTP-bound state were determined in control and PDGF-stimulated cells (5 min, 37°C) after separation of Ras-bound ^{32}P-labeled nucleotides by chromatography on cellulose-coated glass plates. The averages of three individual determinations on separate cell cultures, each derived from a separate embryo, are shown with standard deviations as error bars. Wt, wild type.

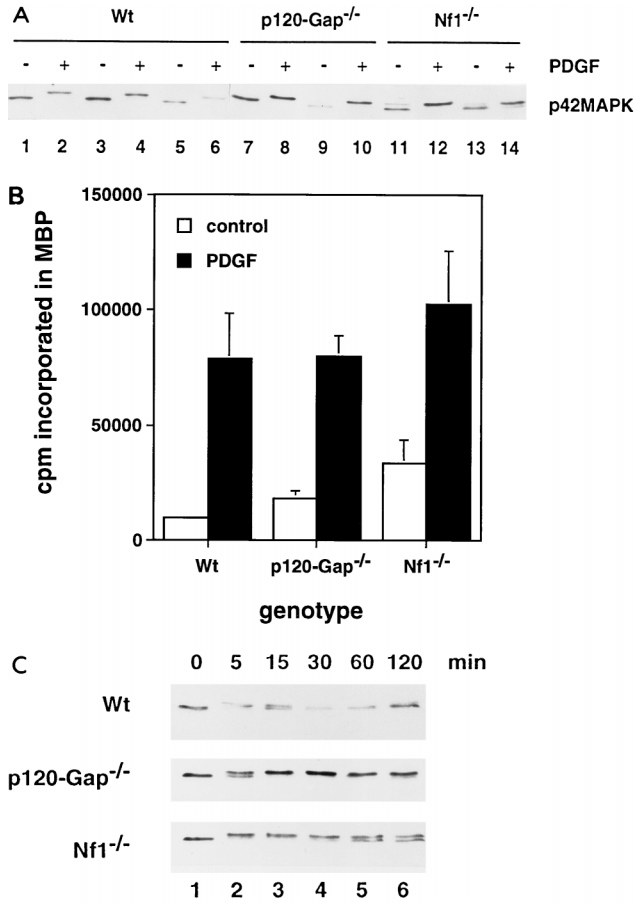

FIG. 3. Prolonged MAP kinase activation in response to PDGF in fibroblast cell lines that lack p120-Gap or neurofibromin. (A) Whole-cell lysates from serum-starved cells (lanes 1, 3, 5, 7, 9, 11, and 13) and cells stimulated with PDGF for 5 min at 37°C (lanes 2, 4, 6, 8, 10, 12, and 14) were analyzed for MAP kinase (MAPK) activation by immunoblotting with an anti-MAP kinase monoclonal antibody. Shown are results for wild-type (Wt) cells (lanes 1 to 6), $Gap^{-/-}$ cells (lanes 7 to 10), and $Nf1^{-/-}$ cells (lanes 11 to 14). (B) MAP kinase immunoprecipitates from control and cells stimulated with PDGF for 5 min at 37°C were analyzed for kinase activity, using MBP as a substrate. The averages of three determinations on cell cultures derived from independent embryos are shown with standard deviations as error bars. (C) Time course of MAP kinase activation, in response to stimulation with PDGF in wild-type, $Gap^{-/-}$, and $Nf1^{-/-}$ cells. Whole-cell lysates from serum-starved control cells or cells stimulated with PDGF for the times indicated were analyzed by anti-MAP kinase immunoblotting.

that there is a Ras-independent pathway that contributes to MAP kinase activation in these cells. The fact that an increase in Ras-GTP levels can contribute to MAP kinase activation is evident from the analysis of the $Gap^{-/-}$ mutant cells, where we observed a large increase in Ras-GTP levels in response to PDGF coupled to an extended duration of MAP kinase activation (see below).

As an alternative measure of MAP kinase activation, we assayed phosphorylation of MBP in vitro following immunoprecipitation of cell lysates with anti-MAP kinase antibodies. MAP kinase isolated from both wild-type and $Gap^{-/-}$ cells showed similar basal levels of MBP kinase activity; in both cases, PDGF stimulation resulted in a 5- to 10-fold increase in MBP kinase activity present in MAP kinase immunoprecipitates (Fig. 3B). Taken together, these results show that p120-Gap is not required for either MAP kinase phosphorylation or stimulation of MAP kinase activity in response to PDGF, indicating that p120-Gap is not an essential component of the signalling pathway between Ras and MAP kinase. In very early passage $Nf1^{-/-}$ cells, MAP kinase remained partially activated in serum-starved cells, but this constitutive activation of MAP kinase was lost upon passaging of the cells. MAP kinase became fully activated in $Nf1^{-/-}$ cells following PDGF stimulation (Fig. 3A and B). These data indicate that neurofibromin is dispensable for signalling from Ras to MAP kinase and indeed can exert an inhibitory effect on MAP kinase activation in unstimulated cells.

It is possible that p120-Gap, through its ability to downregulate Ras-GTP, influences the duration of MAP kinase activation. To test this notion, MAP kinase phosphorylation in wild-type and $Gap^{-/-}$ cells was examined at various times after PDGF stimulation. MAP kinase remained in the phosphorylated, active state for significantly longer in cells lacking p120-Gap (approximately 1.5 h) than in wild-type cells (15 min) (Fig. 3C). This finding is consistent with the hypothesis that a primary function of p120-Gap is in downregulating Ras-GTP, and therefore the MAP kinase pathway, following growth factor stimulation. In the absence of p120-Gap, MAP kinase apparently remains in the active state for a more prolonged period of time, presumably due to an extension in the lifetime of Ras-GTP. Similarly, MAP kinase activation was prolonged in the $Nf1^{-/-}$ cells, although not to the same extent as observed in $Gap^{-/-}$ cells, consistent with the fact that neurofibromin is also a negative regulator of Ras and the Ras/MAP kinase pathway.

DNA synthesis and oncogene-induced transformation in $Gap^{-/-}$ cells. Although $Gap^{-/-}$ cells apparently had normal growth rates, it remained possible that p120-Gap is important for a signalling pathway distinct from that involving MAP kinase but important for PDGF-induced DNA synthesis. As a measure of the mitogenic response to growth factors in $Gap^{-/-}$ cells, DNA synthesis was monitored in mutant cells following stimulation with PDGF. PDGF induced DNA synthesis to comparable extents in wild-type, $Gap^{-/-}$, and $Nf1^{-/-}$ cells (Fig. 4A). These results suggest that neither p120-Gap nor neurofibromin is essential for the induction of DNA synthesis by the PDGF receptor.

The involvement of p120-Gap in signalling pathways downstream of activated tyrosine kinases and Ras proteins was further examined by investigating the susceptibility of $Gap^{-/-}$ cells to transformation with the v-*src* and H-*ras* oncogenes. Established lines of wild-type and $Gap^{-/-}$ cells were transfected with expression vectors encoding the v-Src tyrosine kinase or transforming variant of H-Ras. Both v-*src* and H-*ras* induced the formation of transformed foci in monolayers of wild-type or $Gap^{-/-}$ cells (Fig. 4A), with similar efficiencies (data not shown). The morphologies of $Gap^{-/-}$ cells transformed by v-*src* or v-*ras* were indistinguishable from those of the corresponding transformed wild-type cells. Taken together, these results indicate that p120-Gap is not essential for the deregulation of cell growth and morphological transformation induced by v-*src*, nor is it necessary for cellular transformation by oncogenic *ras*.

Phosphorylation of p190 and p62 in $Gap^{-/-}$ cells. p120-Gap is normally associated with two phosphotyrosine-containing proteins, p190 and p62. Complex formation appears to be mediated primarily by recognition of phosphorylated tyrosine residues on p190 or p62 by the p120-Gap SH2 domains (54, 55). p62 is inducibly phosphorylated by a wide range of activated tyrosine kinases. p190-rhoGap is basally phosphorylated on tyrosine and associated with p120-Gap in some cell lines and can also undergo an increase in phosphorylation following

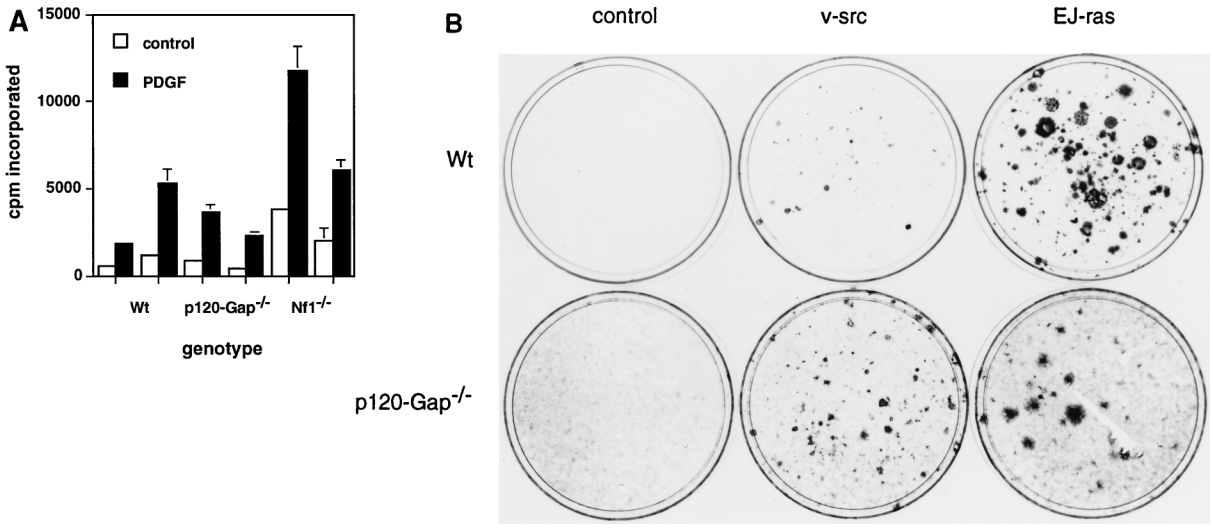

FIG. 4. p120-Gap is not required for induction of DNA synthesis or morphological transformation of fibroblasts by the v-*src* or EJ-*ras* oncogene. (A) Control serum-deprived cells and cells incubated in the presence of PDGF for 16 h were tested for their rates of DNA synthesis by [^{3}H]thymidine incorporation. The averages of four independent determinations are shown with standard deviations as error bars. Wt, wild type. (B) Wild-type and *Gap*$^{-/-}$ cells were transfected with eukaryotic expression plasmids containing the v-*src* cDNA or the EJ-*ras* gene. Both oncogenes transformed wild-type and *Gap*$^{-/-}$ cells with similar efficiencies.

growth factor stimulation or cellular transformation by oncogenic tyrosine kinases (10, 12, 21, 67).

In wild-type mouse embryonic cells, p190 was tyrosine phosphorylated even in the absence of growth factor stimulation and could be coprecipitated with p120-Gap (Fig. 5 and results not shown). In *Gap*$^{-/-}$ cells, the basal tyrosine phosphorylation of p190 was much lower than in wild-type cells, and only very low levels of tyrosine phosphorylation were attained following stimulation with PDGF (Fig. 5). Hence, p120-Gap serves to promote the tyrosine phosphorylation of p190-rhoGap. One possible consequence of p190-rhoGap tyrosine phosphorylation and resulting association with p120-Gap might to be relocalize p190 to the plasma membrane. However, p190 localization appeared similar in wild-type and *Gap*$^{-/-}$ cells (results not shown), regardless of growth factor stimulation. This result does not exclude the possibility that association with p120-Gap has a more subtle effect on p190 localization at the plasma membrane.

Tyrosine phosphorylation of the second p120-Gap associated protein p62 was also investigated. To quantitate tyrosine phosphorylation of p62, immunoprecipitations from [^{35}S]methionine-labeled cells were resolved by SDS-PAGE and transferred to Immobilon. The blot was first analyzed by autoradiography to quantitate p62 levels (Fig. 6A) and subsequently probed for the presence of phosphotyrosine (Fig. 6B). p62 was tyrosine phosphorylated in unstimulated cells, and we did not observe an increase in phosphorylation in response to PDGF in these cells (Fig. 6). In contrast, stimulation with epidermal growth factor resulted in an increase in p62 tyrosine phosphorylation in both wild-type and *Gap*$^{-/-}$ cells (results not shown). These data suggest that in contrast to p190, the binding of phosphorylated p62 to the p120-Gap SH2 domains is not essential to maintain normal p62 phosphorylation.

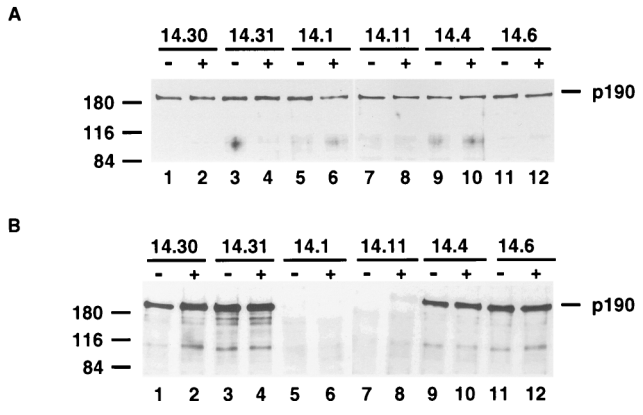

FIG. 5. Reduced p190 phosphorylation in *Gap*$^{-/-}$ but not in *Nf1*$^{-/-}$ cells. p190 immunoprecipitates from serum-starved control cells (lanes 1, 3, 5, 7, 9, and 11) and PDGF-stimulated cells (lanes 2, 4, 6, 8, 10, and 12) were split into two equal parts and analyzed by immunoblotting with anti-p190 serum (A) and an anti-P.Tyr antibody (B). Immunoprecipitates were from wild-type cells (lanes 1 to 4), *Gap*$^{-/-}$ cells (lanes 5 to 8), or *Nf1*$^{-/-}$ cells (lanes 9 to 12). Sizes are indicated in kilodaltons.

DISCUSSION

p120-Gap acts as a negative regulator of Ras in vivo. We have isolated cells from *Gap*$^{-/-}$ embryos and have used these cultures to examine the role of p120-Gap in controlling Ras

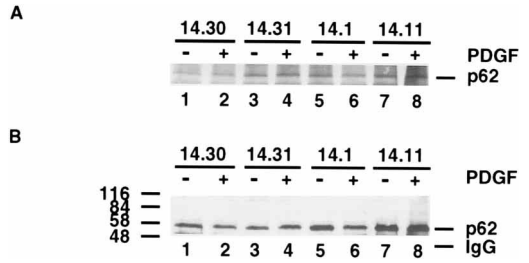

FIG. 6. Normal p62 phosphorylation in *Gap*$^{-/-}$ fibroblasts. p62 immunoprecipitates from [^{35}S]methionine-labeled serum-starved cells (lanes 1, 3, 5, and 7) and PDGF-stimulated cells (lanes 2, 4, 6, and 8) were analyzed by autoradiography (A) and anti-P.Tyr immunoblotting (B). Shown are results for wild-type cells (lanes 1 to 4) and *Gap*$^{-/-}$ cells (lanes 5 to 8). Sizes are indicated in kilodaltons.

activation, in mitogenic signalling and cellular transformation, and in the fate of the p120-Gap-associated proteins p190 and p62. In the absence of p120-Gap there is little change in the basal level of Ras-GTP, but the fraction of GTP-bound Ras following growth factor stimulation is markedly higher than in wild-type cells. Furthermore, MAP kinase activation is more prolonged in the $Gap^{-/-}$ cells. These data indicate that p120-Gap is specifically activated in response to PDGF stimulation and therefore serves to moderate both the extent and the duration of Ras activation in PDGF-stimulated cells. This finding is in agreement with the original purification of p120-Gap as a negative regulator of Ras (27, 69) and with the overexpression studies that indicated that p120-Gap acts to inhibit signalling by Ras-GTP (2, 13, 18, 26, 58, 78).

The observation that p120-Gap is activated by growth factor stimulation is consistent with the fact that p120-Gap possesses SH2 domains that bind inducibly to the activated growth factor receptors and other phosphotyrosine-containing proteins. The association of p120-Gap with activated receptors may be a way of recruiting this cytosolic protein to the plasma membrane, as observed in growth factor-stimulated cells, and thereby localizing p120-Gap in proximity with its target, Ras. This would be consistent with the observation that the inhibitory effects of p120-Gap on the proliferation of NIH 3T3 cells are enhanced by the incorporation of membrane-targeting signals, which result in the constitutive association of p120-Gap with the plasma membrane (32). Indeed, recent data indicate that a βPDGF receptor mutant that is specifically impaired in its ability to associate with p120-Gap is more active in stimulating the MAP kinase pathway than the wild-type receptor (35). In the absence of direct association with a receptor, p120-Gap may become localized to the membrane through association with p62 (57), potentially assisted by its pleckstrin homology and calcium/lipid-binding domains. There are also data to suggest that the amino-terminal region of p120-Gap influences the catalytic activity of the GRD C-terminal region (28). It therefore appears that growth factor receptors are coupled to Ras activation through SH2/PTB-containing adapter proteins such as Shc and Grb2 (20, 41, 65, 66), which are complexed with the mSos guanine nucleotide exchange factors, and to Ras downregulation through p120-Gap. This suggests that receptor tyrosine kinases precisely regulate Ras-GTP levels through SH2-containing proteins with opposing functions.

Both the intensity and duration of Ras/MAP kinase signalling may have a critical effect on cellular responses to growth factor signals (46, 71). The stimulatory proteins that elevate levels of Ras-GTP, and inhibitory proteins such as p120-Gap that inactivate Ras, apparently work in concert to set both the level of Ras-GTP attained after growth factor stimulation and the length of time for which the Ras signal persists. Thus, the activities of both exchange factors and GAPs are likely crucial in determining the biological effects of a particular growth factor. This may explain some of the consequences of mutating the *Gap* gene in mouse embryos. In particular, embryos homozygous for the *Gap* mutation show a greatly elevated level of apoptosis in the developing nervous system (30), which might potentially be attributed to aberrant activation of Ras signalling by neurotrophic factors.

The results obtained from an analysis of $Gap^{-/-}$ and $Nf1^{-/-}$ cells indicate that neurofibromin is not regulated by PDGF stimulation. Schwann cells from *Nf1*-deficient mice show an aberrantly high basal level of Ras-GTP but no unusually large increase in Ras-GTP after growth factor stimulation (38). Higher basal Ras-GTP levels and increased and prolonged Ras activation have also been observed in hemopoietic cells derived from $Nf1^{-/-}$ mice (40). In the early embryonic cells studied here, the absence of neurofibromin also seems to lead to an increase in the baseline of Ras-GTP, although this was somewhat variable. At a minimum, these data suggest that p120-Gap and neurofibromin regulate distinct aspects or Ras guanine nucleotide loading and respond to different extracellular cues. Consistent with this finding, $Gap^{-/-}$ and $Nf1^{-/-}$ mouse embryos have quite distinct phenotypes, while embryos homozygous for both mutations have a phenotype very much more severe than that elicited by either mutation alone (8, 30, 33). These data indicate that p120-Gap and neurofibromin have independent biological functions but likely act in synergy to control Ras activation.

Cellular functions of p120-Gap. A wealth of data suggest that the Raf serine/threonine kinase is a key Ras-GTP effector that activates the MAP kinase pathway (5, 45). Results from $Gap^{-/-}$ cell lines show that p120-Gap is not required for MAP kinase activation or stimulation of DNA synthesis in response to PDGF. As a more stringent test of the requirement for p120-Gap in signalling downstream from tyrosine kinases or Ras, other than as a regulator of Ras itself, we tested that ability of $Gap^{-/-}$ cells to be transformed by the *src* or *ras* oncogene. In neither case was there any striking difference between the phenotype of wild-type and $Gap^{-/-}$ transformed cells. Thus p120-Gap is not absolutely required to mediate the more obvious mitogenic and morphological changes induced by activated tyrosine kinases or Ras GTPases.

These results do not by any means exclude the possibility that p120-Gap has cellular functions in addition to its ability to downregulate Ras in response to tyrosine kinase signals. Indeed, this possibility is suggested by the finding that p120-Gap binds to p190-rhoGap, which through its capacity to inactivate Rho GTPases may control the actin cytoskeleton, cell shape, and cell movement (21, 67, 68). A biochemical difference can be discerned between p190 from wild-type and $Gap^{-/-}$ cells, in the sense that both the basal and growth factor-induced tyrosine phosphorylation of p190 is substantially reduced in the absence of p120-Gap. Hence, p120-Gap might potentially play a role in regulating Rho family GTPases and the cellular cytoskeleton through its effects on p190. This might be explained if the association of tyrosine-phosphorylated p190 with the p120-Gap SH2 domains protects p190 phosphotyrosine sites from the actions of tyrosine phosphatases. p62 phosphorylation appeared to be unaffected by the absence of p120-Gap, consistent with the notion that phosphorylation of p62 and that of p190 are regulated independently. A principal phenotype in $Gap^{-/-}$ mice involves a defect in the formation of vascular structures in the yolk sac and the dorsal aorta of the embryo, apparently due to defective movement or remodelling of endothelial cells (30). We are presently investigating the possibility that p120-Gap plays a role in directed cell movement.

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