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Protein-Tyrosine Kinases Regulate the Phosphorylation, Protein Interactions, Subcellular Distribution, and Activity of p21^{ras} GTPase-Activating Protein

MICHAEL F. MORAN,[1]† * PAUL POLAKIS,[2] FRANK McCORMICK,[2] TONY PAWSON,[1,3] AND CHRISTINE ELLIS[1]

Department of Molecular and Medical Genetics, University of Toronto,[3] and Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital,[1] Toronto, Ontario M5G 1X5, Canada, and Department of Molecular Biology, Cetus Corporation, Emeryville, California 94608[2]

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The p21^{ras} GTPase-activating protein (GAP) down-regulates p21^{ras} by stimulating its intrinsic GTPase activity. GAP is found predominantly as a monomer in the cytosol of normal cells. However, in cells expressing an activated cytoplasmic protein-tyrosine kinase, p60$^{v\text{-}src}$, or stimulated with epidermal growth factor, GAP becomes phosphorylated on tyrosine and serine and forms distinct complexes with two phosphoproteins of 62 and 190 kDa (p62 and p190). In v-*src*-transformed Rat-2 cells, a minor fraction of GAP associates with the highly tyrosine phosphorylated p62 to form a complex that is localized at the plasma membrane and in the cytosol. In contrast, the majority of GAP enters a distinct complex with p190 that is exclusively cytosolic and contains predominantly phosphoserine. Epidermal growth factor stimulation also induces a marked conversion of monomeric GAP to higher-molecular-weight species in rat fibroblasts. The GAP-p190 complex is dependent on phosphorylation and shows reduced GAP activity. These results indicate that protein-tyrosine kinases induce GAP to form multiple heteromeric complexes, which are strong candidates for regulators or targets of p21^{ras}.

Tyrosine kinases require functional *ras* proteins to generate mitogenic and oncogenic responses (21, 29). This fact suggests that p21^{ras} might act downstream of tyrosine kinases in a mitogenic signaling pathway and implies that tyrosine kinases can stimulate p21^{ras} activity. The membrane-associated p21^{ras} proteins are biologically inactive when bound to GDP and are activated by the exchange of GDP for GTP, which induces a marked conformational change in p21^{ras} (22, 31). The normal mechanism by which this activation occurs is unknown, although p21^{ras} guanine nucleotide release/exchange activities have recently been described (6, 34, 35).

One protein known to interact with p21^{ras} is the Ras GTPase-activating protein (GAP) (31). GAP stimulates the otherwise weak intrinsic GTPase activity of p21^{ras} and thereby promotes the return of p21^{ras} to an inactive GDP-bound state. Thus, GAP is a negative regulator of p21^{ras}. However, since GAP interacts with the effector domain of p21^{ras}, spanning at least residues 32 to 40, it has been suggested that GAP might also serve as a target of the p21^{ras}-GTP complex (1, 4). Hence, the transient interaction of GAP with p21^{ras}-GTP at the plasma membrane might simultaneously induce GTP hydrolysis and the generation of a downstream signal involving a GAP-associated activity or protein.

The C-terminal half of GAP, which is structurally related to the *IRA*1 and *IRA*2 gene products of *Saccharomyces cerevisiae* (30) and the human NF-1 protein (36), is sufficient to accelerate p21^{ras} GTPase activity (17). The amino-terminal region of GAP contains two Src homology 2 (SH2) domains and an intervening SH3 domain, which are conserved in phospholipase C-γ, Src-like cytoplasmic tyrosine kinases, and the v-Crk oncoprotein and are implicated in the interactions of tyrosine kinases with their targets (2, 12, 19, 23). This finding suggests that GAP might serve as a link between tyrosine kinases and p21^{ras}. Recently we and others have shown that GAP is indeed phosphorylated by a variety of oncogenic tyrosine kinases, including the v-*src*, v-*abl*, and v-*fps* gene products, as well as the ligand-stimulated receptors specific for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony-stimulating factor 1 (7, 18, 25). GAP also forms a complex with autophosphorylated PDGF receptors (10, 11), which can be mimicked in vitro by using GAP SH2 domains expressed in bacteria (2, 19).

GAP coimmunoprecipitates with two tyrosine-phosphorylated proteins of 62 and 190 kDa (p62 and p190) from lysates of cells expressing activated tyrosine kinases (7). In v-*src*-transformed cells, p62 is a highly tyrosine phosphorylated protein whose phosphorylation by p60$^{v\text{-}src}$ and association with GAP are both consequences of its affinity for SH2 domains (19). The activation of tyrosine kinases may therefore induce the formation of signaling complexes involving GAP, p62, and p190, which could in turn regulate the transduction of mitogenic signals through p21^{ras}. Here we investigate the formation of these GAP complexes.

MATERIALS AND METHODS

Materials. Polyclonal rabbit antibodies against the entire human GAP protein (used in experiments shown in Fig. 6 only) or residues 171 to 448 were used as described previously (7, 10). Mouse monoclonal antibody PY-69 (ICN Immunobiologicals, Costa Mesa, Calif.; used in experiments

* Corresponding author.
† Present address: Banting and Best Department of Medical Research, University of Toronto, 112 College Street, Toronto, Ontario, M5G 1L6, Canada.

shown in Fig. 6 only) or affinity-purified polyclonal rabbit phosphotyrosine (P.Tyr)-specific antibodies were prepared as described previously (9, 14). Rabbit anti-mouse immunoglobulin (RamIg) was from Organon Teknika. EGF was a gift of Jeff Kudlow. Potato acid phosphatase (PAP) was obtained from Sigma and Boehringer Mannheim. Protein A-Sepharose beads, PVP-360 (polyvinylpyrrolidone; average molecular weight, 360,000), and the various protease and phosphatase inhibitors were from Sigma. Enzyme-linked antibodies were from Bio-Rad, the enhanced chemiluminescence substrates and ^{125}I-protein A were from Amersham, and chromatographic media were from Pharmacia.

Cell culture. Rat-1 cells that express approximately 2.5 × 10^5 human EGF receptors per cell (R1hER; 32a) and Rat-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Rat-2 cells (3) or NIH 3T3 cells transformed by v-*src* were grown in DMEM containing 5% calf serum. R1hER cells were maintained in DMEM with 0.5% calf serum for 48 h prior to stimulation with 80 nM EGF.

Cell labeling, lysis, and immunoprecipitation. For metabolic labeling with [^{35}S]methionine, subconfluent cells in 10-cm-diameter dishes were incubated for 2 h with 2 ml of methionine-free DMEM containing 5% dialyzed FBS and [^{35}S]methionine (150 μCi/ml; 1,000 Ci/mmol; Amersham). Serum-starved R1hER cells (see above) were similarly labeled but for 4 h with [^{35}S]methionine and with 2% dialyzed FBS prior to EGF stimulation. Alternatively, proteins were metabolically labeled by incubating cells with 500 μCi of an equal mixture (by activity) of [^{3}H]Leu, -Lys, -Phe, -Pro, and -Tyr (approximately 70 to 190 Ci/mmol; Amersham) for 3 h in 3 ml of DMEM lacking the above five amino acids and supplemented with 5% dialyzed FBS. For phosphate labeling, cells were incubated for 2 h in 3 ml of phosphate-free DMEM with 2% dialyzed FBS and 5 mCi of $^{32}P_i$ (ICN).

After labeling, cells were rinsed with cold phosphate-buffered saline (PBS), lysed with 2 ml of PLCLB (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM $NaPP_i$, 1 mM Na_3VO_4) containing 10 μg each of aprotinin and leupeptin per ml and 1 mM phenylmethylsulfonyl fluoride (PMSF), and clarified by centrifugation as described previously (7). Prior to immunoprecipitation with affinity-purified antibodies, clarified lysates from metabolically labeled cells were precleared by incubating the cells for 1 h with 50 μl of nonimmune rabbit serum preadsorbed to 50 μl of protein A-Sepharose, removing the complex by centrifugation, and repeating the procedure with 10 μg of RamIg preadsorbed to 5 μl of protein A-Sepharose. For anti-GAP immunoprecipitations, 10 μl of crude antiserum or 1 μg of affinity-purified antibody was preadsorbed to 10 or 5 μl, respectively, of protein A-Sepharose beads, washed twice with PLCLB, and then incubated with the lysate for 90 min at 4°C. As controls, equivalent amounts of nonimmune rabbit serum or RamIg were used. Immune complexes were washed three times with HNTG (20 mM HEPES [pH 7.0], 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4), resuspended in sodium dodecyl sulfate (SDS) sample buffer, heated to 100°C for 3 min, and then electrophoretically resolved through a 7.5% polyacrylamide gel containing SDS. ^{32}P samples were electrophoretically transferred from polyacrylamide gels to nitrocellulose or Immobilon membranes and then subjected to autoradiography or fluorography. SDS-polyacrylamide gels containing ^{35}S samples were either subjected to autoradiography or impregnated with En^{3}Hance (NEN) and then subjected to fluorography.

To disrupt protein-protein complexes prior to immunoprecipitation (see Fig. 1), cells were lysed in buffer containing 10 mM sodium phosphate (pH 7.0), 0.5% SDS, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, and 10 μg each of aprotinin and leupeptin. Lysates were heated at 100°C for 3 min, cooled to 4°C, forced through a 25-gauge needle, and diluted with RIPA buffer (33) prior to immunoprecipitation.

Immunoblotting. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose (Schleicher & Schuell) or Immobilon (Millipore) membranes. Blots were blocked and subsequently probed with anti-P.Tyr antibodies and ^{125}I-protein A as described previously (12, 19). The blocking solution for anti-GAP antibodies contained 5% Carnation skim milk powder in Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5], 150 mM NaCl). Anti-GAP immunoblots were incubated with 0.2 μg of affinity-purified anti-GAP antibodies per ml for 60 min at room temperature and then washed four times for 10 min each time with TBS containing 0.05% Tween 20 (TBST). Blots were probed for 60 min with 5 μCi of ^{125}I-protein A (35 μCi/μg), washed as described above, and exposed to Kodak XAR X-ray film at −75°C with an intensifying screen. For the experiment shown in Fig. 6, the secondary incubation was with the appropriate goat anti-rabbit or anti-mouse horseradish peroxidase conjugate diluted 1:20,000 in TBST for 60 min at room temperature, followed by four 5-min washes in TBST. After a 1-min incubation in enhanced chemiluminescence substrate solution, the wet blots were covered with plastic wrap and exposed to Kodak RP X-ray film for 1 to 10 min. Exposure times were such that the intensities of bands were within the range of linear response of the film.

Phosphatase treatment. Anti-GAP immunoprecipitates were prepared from [^{35}S]methionine-labeled Rat-2 v-*src*-transformed cells as described above. After two washes in HNTG, the immune complexes were washed twice in phosphatase buffer (PAPB; (20 mM HEPES [pH 7.0], 5% glycerol, 0.05% Triton X-100, 2.5 mM $MgCl_2$, 50 μg of aprotinin per ml, 10 μg of leupeptin per ml). The immune complexes were divided into two equal aliquots for incubation with the phosphatase or buffer only. PAP was prepared by dissolving 150 μg of the enzyme (recovered by centrifugation of 150 μl of the 1-mg/ml ammonium sulfate suspension) in 1 ml of PAPB. This was desalted by being passed over a Sephadex G-25 column previously equilibrated with PAPB. The enzyme was recovered in a volume of 1.5 ml at a concentration of 0.1 mg/ml. The immune complex was then resuspended in either 200 μl of desalted PAP (20 μg; 1 U) or buffer alone and incubated at 30°C for 60 min. The immune complexes were then washed three times with HNTG and resuspended in SDS sample buffer.

Subcellular fractionation. The following procedures were performed at 4°C. Cells were scraped into PBS (10 mM sodium phosphate [pH 7.4], 150 mM NaCl) and then collected by centrifugation. Cell pellets (0.1 g [wet weight] of cells) were resuspended with 1 ml of hypotonic buffer (10 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 50 μg of leupeptin per ml, 1 mM PMSF, 1 mM Na_3VO_4); 10 min later, the cells were transferred to a Dounce homogenizer and further disrupted by 25 strokes with a tight-fitting pestle. The homogenate was adjusted to the indicated NaCl concentration from a 5 M stock solution, and nuclei were removed by centrifugation at 800 × *g* for 5 min. The postnuclear supernatant was centrifuged at 100,000 × *g* for 40 min. The supernatant fraction (S100) was adjusted to 1% Nonidet P-40 (NP-40) from a 10% stock. The pellet (P100) was gently

rinsed with 1 ml of PBS and then resuspended in 1 ml of hyptonic buffer containing 1% NP-40. For detection of P.Tyr-containing GAP and GAP-associated proteins, the S100 and P100 fractions were immunoprecipitated with affinity-purified anti-GAP or control RamIg antibodies and then subjected to immunoblotting with anti-GAP or anti-P.Tyr antibodies and ^{125}I-protein A.

Tryptic peptide mapping. Phosphopeptide mapping of nitrocellulose-bound proteins was performed essentially as described by Luo et al. (15). ^{32}P-labeled protein bands were excised from membranes, washed five times with 1 ml of distilled water each time, coated with 0.5% PVP-360 in 0.1 M acetic acid for 30 min at 37°C, and then washed five more times with water. The immobilized protein was then digested with 10 μg of tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington) in 200 μl of 50 mM NH_4HCO_3 for 2 h at 37°C, at which time 10 μg of fresh trypsin was added and the incubation was continued for another 2 h. The membrane was removed, and the soluble tryptic peptides were lyophilized, oxidized with performic acid, and washed by three cycles consisting of the addition of 1 ml of water and then lyophilization. The resulting oxidized tryptic peptides were mapped in two dimensions on thin-layer cellulose plates by electrophoresis at pH 2.1 and ascending chromatography as described previously (33).

Gel filtration and ion-exchange chromatography. The following procedures were performed at 4°C. Cells were gently scraped from 10-cm-diameter plates into 0.75 ml of Tris-saline containing 100 μM Na_3VO_4 and centrifuged at 1,000 × *g* for 15 s. Cells were resuspended with 300 μl of PLCLB per 5×10^6 cells and clarified by centrifugation at 15,000 × *g* for 15 min. Then 200 μl of the lysate was injected onto a Superose-12 column (HR 10/30; Pharmacia) that was equilibrated with PLCLB. The column was run at a flow rate of 0.3 ml/min, and fractions of 0.60 or 0.75 ml were collected and immunoprecipitated with 10 μl of anti-GAP serum preadsorbed to 10 μl of protein A beads. The immune complexes were resolved by SDS-PAGE followed by Western blotting. The column was calibrated by using catalase (232,000) and ferritin (440,000) as molecular weight standards and by using blue dextran to determine the void volume.

For resolution of GAP complexes by S-Sepharose, 2×10^9 v-*src*-transformed NIH 3T3 cells were lysed in 20 mM Tris-HCl [pH 8.0]–1 mM EDTA–0.5 mM DTT–0.5 mM PMSF–0.2 mM Na_3VO_4–10 μg each of pepstatin and leupeptin per ml and then centrifuged at 200,000 × *g* for 60 min. The supernatant was adjusted to pH 6.5 with monobasic sodium phosphate and then applied to a 5-ml column of S-Sepharose equilibrated in 50 mM sodium phosphate [pH 6.5]–1 mM EDTA–0.5 mM DTT–0.2 mM Na_3VO_4–10 μg each of pepstatin and leupeptin per ml. The column was washed and eluted in the same buffer at a flow rate of 12 ml/h with an 80-ml linear gradient of 0 to 0.5 M NaCl, and fractions of 2 ml were collected. Aliquots of even-numbered fractions were assayed for GAP activity and subjected to immunoprecipitation and immunoblotting for the determination of GAP and GAP-associated proteins. Two fractions that contained approximately equal amounts of GAP immunoreactivity, but only one of which contained GAP-associated p190, were selected for the experiment presented in Fig. 6.

GAP activity. GAP and the GAP-p190 complex were resolved by cation-exchange chromatography (see above) and assayed for their ability to stimulate GTP hydrolysis by $p21^{ras}$. The GAP assay was performed as described in Polakis et al. (24): $p21^{N\text{-}ras}$ was prebound to [γ-^{32}P]GTP by combining 1 μg of purified protein (100 μg/ml in 20 mM Tris-HCl [pH 7.5]–0.1% NP-40) with 20 μl of 15 mM Tris-HCl (pH 7.5)–7.5 mM EDTA–5 μCi of [γ-^{32}P]GTP (6,000 Ci/mmol; NEN) and incubating the mixture for 10 min at 30°C. Then 2-μl aliquots of column fractions were mixed with 16 μl of assay buffer (25 mM Tris-HCl [pH 7.5], 6.25 mM $MgCl_2$, 1 mM DTT, 625 μM GTP, 1.25 mg of bovine serum albumin per ml, 0.06% NP-40), and the reaction was started by the addition of 2 μl of $p21^{ras}$-[γ-^{32}P]GTP. After incubation at 23°C for 10 min, the reaction was stopped by the addition of 4 ml of ice-cold 25 mM Tris-HCl (pH 7.5)–0.1 mM NaCl–5 mM $MgCl_2$ and rapid vacuum filtration through nitrocellulose. Filters were washed twice with 4 ml of the same buffer, dried, and quantified by liquid scintillation counting.

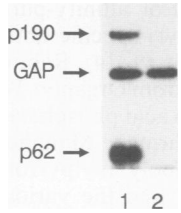

FIG. 1. Evidence that p62 and p190 are complexed to GAP. Rat-2 v-*src* cells were lysed either in buffer containing 1% Triton X-100 (lane 1) or in buffer containing 0.5% SDS (lane 2). The lysate containing SDS was denatured by heating to 100°C prior to dilution and immunoprecipitation. Nondenatured (lane 1) and denatured (lane 2) lysates were immunoprecipitated with anti-GAP antiserum and analyzed by Western blotting with anti-P.Tyr antibodies and ^{125}I-protein A.

RESULTS

GAP complex formation is induced and maintained by phosphorylation. GAP coimmunoprecipitates with the phosphoproteins p62 and p190 from lysates of v-*src*-transformed or EGF-stimulated rat fibroblasts. To investigate whether p62 and p190 are precipitated as a consequence of a physical interaction with GAP, lysates of v-*src*-transformed Rat-2 cells were heated for 3 min at 100°C in 0.5% SDS prior to dilution and immunoprecipitation with anti-GAP antibodies and subsequent Western blot analysis with anti-P.Tyr antibodies. Denaturation abolished the precipitation of both p62 and p190 (Fig. 1), although recognition of GAP was unaffected. These results show that p62 and p190 are not immunoprecipitated directly by anti-GAP antibodies but rather are complexed with GAP in Rat-2 v-*src* lysates. To assess the relative amounts of p190 and p62 bound to GAP and the dependence of these interactions on tyrosine kinase activity, Rat-2 v-*src* cells were metabolically labeled with [^{35}S]methionine or a mixture of five tritiated amino acids (Leu, Lys, Phe, Pro, and Tyr) and immunoprecipitated with affinity-purified anti-GAP antibodies. Similar amounts of p190 and GAP could be identified in these immunoprecipitates (Fig. 2A). In Fig. 2B, lysates from [^{35}S]methionine-labeled Rat-2 v-*src* and parental Rat-2 cells were immunoprecipitated with anti-GAP serum (lanes 1 to 4). Lane 5 of Fig. 2B shows an anti-GAP immunoprecipitation from $^{32}P_i$-labeled Rat-2 v-*src* cells and is included for comparison to demonstrate the comigration of ^{35}S- and ^{32}P-labeled p190

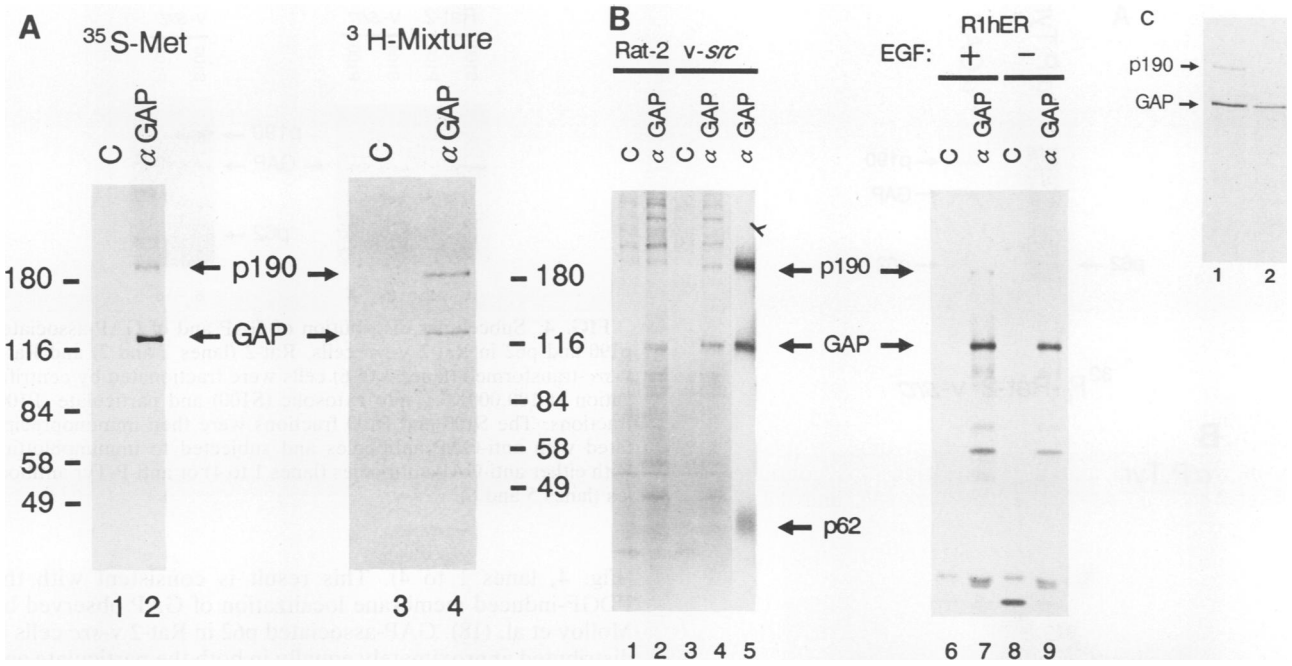

FIG. 2. Induction by the v-*src* and EGF receptor tyrosine kinases of phosphorylation-dependent association of GAP and p190. (A) Rat-2 v-*src* cells were metabolically labeled with [^{35}S]methionine or a mixture of ^{3}H-amino acids (Leu, Lys, Phe, Pro, and Tyr), lysed, and immunoprecipitated with control antibodies (C; lanes 1 and 3) or affinity-purified anti-GAP antibodies (αGAP; lanes 2 and 4). Sizes (in kilodaltons) are marked at the left and right. (B) [^{35}S]methionine-labeled Rat-2 (lanes 1 and 2) and Rat-2 v-*src* (lanes 3 and 4) cells were immunoprecipitated with control (C) or anti-GAP (αGAP) antiserum. For comparison, an anti-GAP immunoprecipitate from ^{32}P-labeled Rat-2 v-*src* cells was resolved on the same gel to show the migration of phosphorylated p190, GAP, and p62 (lane 5). R1hER cells (lanes 6 to 9) were radiolabeled with [^{35}S]methionine and then incubated for 30 min at 37°C in the presence (+) or absence (−) of 80 nM EGF. Cell lysates were then immunoprecipitated with control or anti-GAP antiserum. (C) Anti-GAP (affinity-purified antibody) immunoprecipitates from [^{35}S]methionine-labeled Rat-2 v-*src* cells were incubated with buffer alone (lane 1) or with PAP (lane 2). The treated and untreated samples were then analyzed by gel electrophoresis.

and GAP and the absence of a ^{35}S-labeled band corresponding to p62. In contrast to the v-*src*-transformed cells, the normal Rat-2 cells contained little or no GAP-associated p190 (Fig. 2B, lanes 2 and 4). A similar induction of p190 binding was observed following EGF stimulation of Rat-1 cells that overexpress the human EGF receptor (R1hER) (Fig. 2B, lanes 6 to 9). This result indicates that a substantial fraction of GAP becomes physically associated with p190 as a consequence of p60$^{v\text{-}src}$ or EGF receptor kinase activity.

p62, in contrast to p190, could not be readily identified in anti-GAP immunoprecipitates from Rat-2 v-*src* cells radiolabeled with [^{35}S]methionine or ^{3}H-amino acids, although it is easily detected following metabolic labeling with $^{32}P_i$ (7). p62 therefore appears to be a minor but highly phosphorylated protein. The faint band at ~52 kDa (Fig. 2A, lane 4) does not comigrate with p62, and its identity is unknown. Additional nonspecific bands are evident in the anti-GAP immunoprecipitates derived by using whole serum (Fig. 2B) that are absent in the immunoprecipitates performed with affinity-purified anti-GAP antibodies (Fig. 2A).

Phosphorylation appears essential for the continued interaction of GAP and p190. Incubation of the anti-GAP immunoprecipitates from [^{35}S]methionine-labeled Rat-2 v-*src*-transformed cells with potato acid phosphatase (PAP) released p190, but not GAP, from the immune complex (Fig. 2C, lane 2). Anti-P.Tyr immunoblotting confirmed that the phosphatase treatment completely removed phosphotyrosine from the GAP immunoprecipitates (data not shown). This experiment was repeated several times, and similar results were obtained by using PAP from different sources. However, contamination of the various phosphatase preparations with a p190-specific protease activity would also account for the loss of p190 from the GAP immunoprecipitates. Since PAP removes phosphate groups indiscriminately, we cannot determine from these experiments whether p190-GAP complex formation requires phosphorylation of tyrosine or serine residues or both. However, the majority of protein-bound phosphate in p190 and GAP was found to be phosphoserine (7), and we have been able to purify the GAP-p190 complex from Rat-2 v-*src* lysates completely depleted of tyrosine phosphorylated proteins with anti-P.Tyr antibodies (18a). We cannot assess the role of phosphorylation in the GAP-p62 interaction by these experiments, since p62 was not detected by [^{35}S]methionine labeling (Fig. 2A).

p62 is a major substrate of p60$^{v\text{-}src}$. To investigate whether p62 is a prominent substrate for p60$^{v\text{-}src}$, Rat-2 v-*src*-transformed cells were metabolically labeled with $^{32}P_i$, lysed, and immunoprecipitated with anti-P.Tyr antibodies or anti-GAP antibodies. Among the spectrum of ^{32}P-labeled proteins immunoprecipitated with anti-P.Tyr antibodies, a major phosphoprotein comigrated with the GAP-associated p62 (Fig. 3A). The 62-kDa protein immunoprecipitated with anti-P.Tyr antibodies and p62 isolated with anti-GAP antibodies were found to have identical tryptic phosphopeptide maps (Fig. 3B), indicating that they correspond to the same polypeptide(s). By comparing the amount of ^{32}P-labeled GAP-associated p62 with the total p62 immunoprecipitated

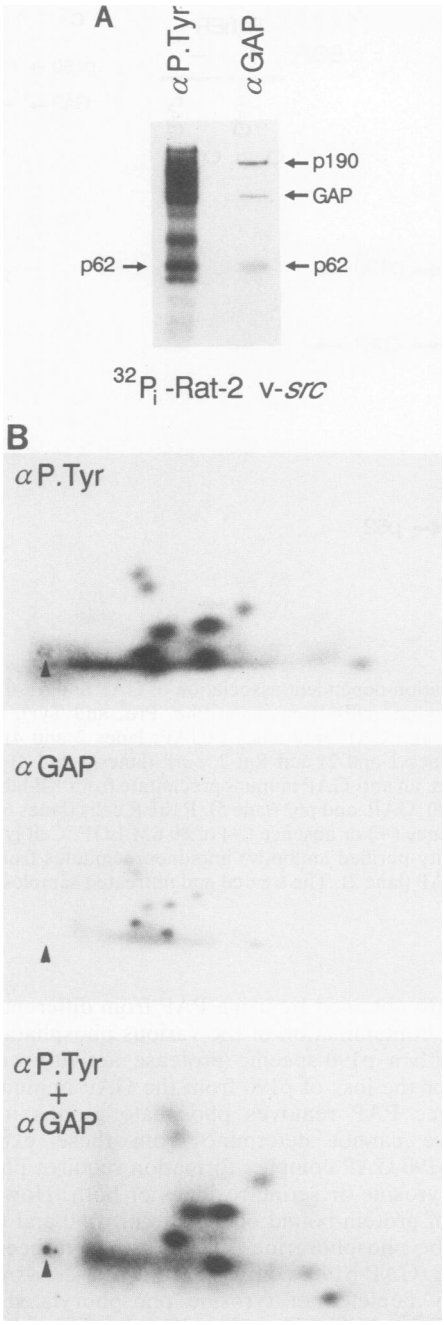

FIG. 3. (A) Comparison of anti-P.Tyr (αP.Tyr) and anti-GAP (αGAP) immunoprecipitates from $^{32}P_i$-labeled Rat-2 v-*src* cells. (B) Tryptic phosphopeptide maps. The indicated p62 bands from the blot in panel A were excised, digested with trypsin, and then resolved either alone or together, as indicated, in two dimensions by thin-layer electrophoresis and chromatography (see Materials and Methods). Arrowheads indicate the origin of sample loading.

with anti-P.Tyr antibodies, we estimate that at least 10% of tyrosine phosphorylated p62 is stably complexed with GAP.

Location of GAP complexes. In normal Rat-2 cells, GAP appeared to be entirely soluble, as judged by subcellular fractionation. However, transformation by $p60^{v\text{-}src}$ elicited a redistribution such that ~8% of GAP in Rat-2 v-*src* cells became associated with the particulate fraction of the cell

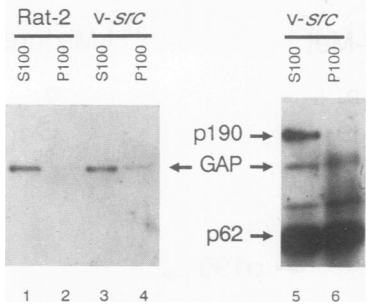

FIG. 4. Subcellular distribution of GAP and of GAP-associated p190 and p62 in Rat-2 v-*src* cells. Rat-2 (lanes 1 and 2) and Rat-2 v-*src*-transformed (lanes 3 to 6) cells were fractionated by centrifugation at 100,000 × *g* into cytosolic (S100) and particulate (P100) fractions. The S100 and P100 fractions were then immunoprecipitated with anti-GAP antibodies and subjected to immunoblotting with either anti-GAP antibodies (lanes 1 to 4) or anti-P.Tyr antibodies (lanes 5 and 6).

(Fig. 4, lanes 1 to 4). This result is consistent with the PDGF-induced membrane localization of GAP observed by Molloy et al. (18). GAP-associated p62 in Rat-2 v-*src* cells is distributed approximately equally in both the particulate and soluble fractions, whereas the major GAP-p190 complex appears to be completely cytosolic (Fig. 4, lanes 5 and 6).

The interactions of GAP with the particulate fraction of Rat-2 v-*src* cells is at least partially ionic in nature. Increasing the salt concentration of whole-cell lysates reduced the yield of GAP in the P100 fractions following ultracentrifugation (data not shown). Without the addition of salt, ~18% of GAP was particulate, and this value decreased to ~5% when the lysate was adjusted to 500 mM with NaCl. At a physiological salt concentration of 150 mM NaCl, ~8% (7.5 ± 1.1 standard deviation; $n = 3$) of GAP appeared to be membrane associated (Fig. 4). The amount of particulate p62 also decreased with increasing ionic strength, but the ratio of p62 to GAP remained constant (data not shown). Thus, there was no preferential loss of either monomeric GAP or GAP-p62 from the P100 fraction when the salt concentration was increased. GAP contains a hydrophobic N-terminal region as well as SH2 and SH3 domains, all of which would have the potential to promote membrane or cytoskeletal localization (32).

Tyrosine kinases shift monomeric GAP into distinct heteromeric complexes. The hypothesis that tyrosine kinases induce a significant proportion of GAP to complex with p190 predicts that the apparent size of native GAP should shift upon growth factor stimulation or transformation by v-*src* and that the majority of GAP should cofractionate with p190. To test this possibility, detergent lysates of EGF-stimulated or v-*src*-transformed rat fibroblasts were applied to a Superose-12 gel filtration column, and the fractions were immunoprecipitated with GAP antibodies.

Anti-GAP immunoblotting revealed that with serum-starved R1hER cells, GAP eluted from the sizing column at approximately 200 kDa, as does GAP purified from baculovirus-infected insect cells, which likely corresponds to the GAP monomer. At 30 min after EGF stimulation there was an almost complete conversion of GAP to larger 300 to 500-kDa species (Fig. 5a). In proliferating asynchronous Rat-2 cells and Rat-2 v-*src* cells, a broader pattern of GAP elution was apparent. The majority of GAP from the v-*src*-transformed rat cells eluted at 300 to 500 kDa, whereas in normal Rat-2 cells a majority of GAP eluted at a lower

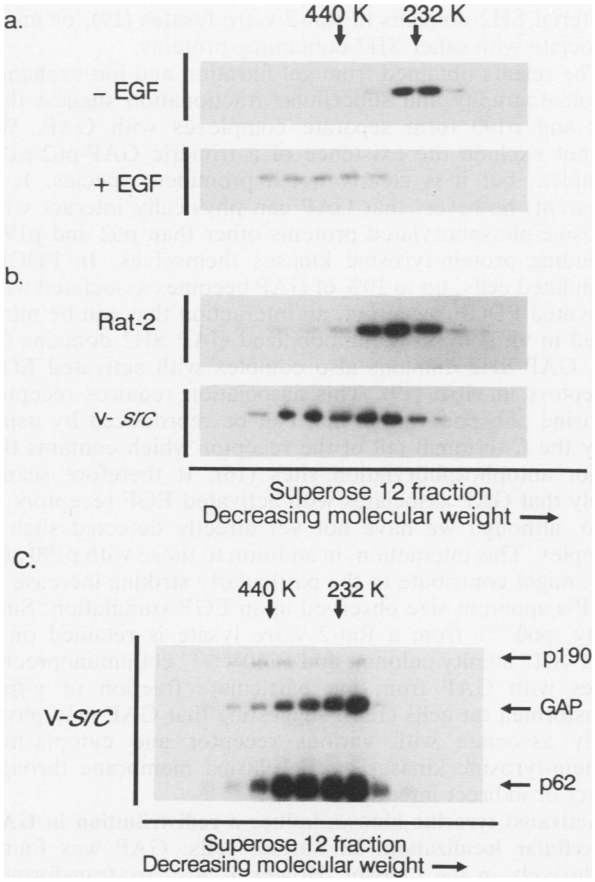

FIG. 5. Evidence that GAP forms independent complexes with p62 and p190. (a) R1hER cells were serum starved for 48 h, incubated for 30 min with (+EGF) or without (−EGF) 80 nM EGF, lysed, and fractionated by gel filtration on a Superose-12 column. Fractions (high molecular weight to the left) were then immunoprecipitated and immunoblotted with anti-GAP antibodies. (b) Normal Rat-2 cells and Rat-2 v-*src* cells were lysed and fractionated by gel filtration chromatography. The distribution of GAP was determined by immunoprecipitation and immunoblotting as described above. (c) Rat-2 v-*src* cells were lysed, fractionated by gel filtration chromatography, and subjected to anti-GAP immunoprecipitation and anti-P.Tyr immunoblotting. The elution of molecular weight standards is indicated in each case. K, Kilodaltons.

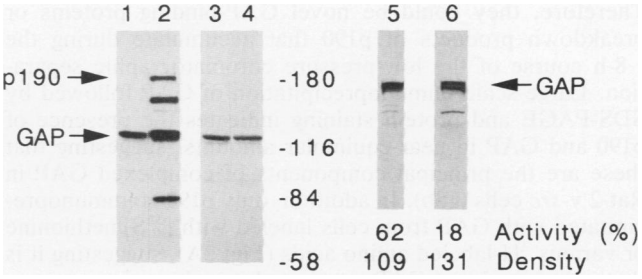

FIG. 6. Evidence that GAP-p190 has decreased GAP activity. Monomeric GAP (lanes 1, 3, and 5) was chromatographically resolved from GAP complexed to p190 (lanes 2, 4, and 6) as described in Materials and Methods. Anti-GAP immunoprecipitates were subjected to SDS-PAGE and immunoblotted with either anti-P.Tyr (lanes 1 and 2) or anti-GAP (lanes 3 and 4) antibodies. Additionally, GAP fractions subjected to SDS-PAGE without prior immunoprecipitation were immunoblotted with anti-GAP antibodies (lanes 5 and 6). GAP immunoreactivity in these fractions was quantified densitometrically and is expressed in arbitrary units (Density). GAP activity toward $p21^{ras}$-GTP was determined in these same fractions as described in Materials and Methods and is expressed as the percentage of $p21^{ras}$-bound GTP hydrolyzed (Activity). Sizes (in kilodaltons) are indicated to the right of lane 4.

molecular size (Fig. 5b). This finding suggests that the $p60^{v\text{-}src}$ tyrosine kinase activity in Rat-2 v-*src* cells promotes an increase in the fraction of GAP protein engaged in multiprotein complexes in comparison with normal cells. To examine the tyrosine phosphorylation of proteins in the GAP complexes, a Rat-2 v-*src* lysate was resolved through the sizing column. Fractions were immunoprecipitated with anti-GAP antibodies, and the immunoprecipitates were blotted with anti-P.Tyr antibodies (Fig. 5c). The majority of the total GAP, containing relatively little P.Tyr, eluted at a high molecular size (>440 kDa) coincident with, and presumably as a consequence of, its interaction with p190. A similar coelution through Superose of GAP-p190 from the cytosolic (S100) fraction of EGF-stimulated R1hER cells was observed (6a). In *src*-transformed cells, the more highly tyrosine phosphorylated GAP species, which involves a minor fraction of total GAP, fractionated at a lower molecular size either by itself or with p62 (Fig. 5c). Since GAP-associated p62 eluted at a lower size than most of p190, it is likely that GAP forms independent complexes with either p62 or p190. Indeed, GAP-p190, GAP-p62, and GAP monomer have been completely resolved from each other by chromatography on S-Sepharose (23a).

These results suggest that GAP exists in multiple forms in v-*src*-transformed or EGF-stimulated cells. Activation of the $p60^{src}$ tyrosine kinase induces the majority of GAP to form a cytosolic complex containing p190. A minor, more highly tyrosine phosphorylated subpopulation of GAP is particulate and presumably localized at the plasma membrane, as is approximately one half of a GAP-p62 complex. Since we can identify p62 only in its phosphorylated form, we cannot determine whether its association with GAP is affected by tyrosine kinase activation.

GAP-p190 has decreased GTPase-activating activity. If mitogenic signals emanating from tyrosine kinases require $p21^{ras}$ proteins, then it seems logical that the tyrosine phosphorylation of GAP or the binding of phosphoproteins to GAP in response to tyrosine kinase activation might affect its ability to down-regulate $p21^{ras}$-GTP. Tyrosine phosphorylation of partially purified GAP has no obvious effects on its activity (data not shown). GAP and p190-associated GAP were resolved with a cation-exchange column (Fig. 6, lanes 1 and 2). The amounts of GAP protein in these fractions were shown by anti-GAP immunoblotting to be approximately equivalent (Fig. 6, lanes 5 and 6). However, comparison of the Ras GTPase-activating activities of these fractions showed that the fraction containing monomeric GAP had specific activity 4.1-fold greater than that of the fraction containing complexed GAP. Anti-GAP immunoprecipitation (lanes 3 and 4) removed all of the Ras GTPase-activating activity and GAP protein from these fractions, indicating that all of the activity measured in these fractions is due to GAP (data not shown).

Lane 2 of Fig. 6 shows the coimmunoprecipitation of p190 as well as two additional P.Tyr-containing proteins of 150 and 80 kDa with GAP. These latter two proteins are not recognized by polyclonal anti-GAP antibodies (Fig. 6, lane 4) and are not present in anti-GAP immunoprecipitates of the starting material prior to chromatography (data not shown).

Therefore, they could be novel GAP-binding proteins or breakdown products of p190 that accumulate during the ~8-h course of the low-pressure chromatographic separation. Large-scale immunoprecipitation of GAP followed by SDS-PAGE and protein staining indicates the presence of p190 and GAP in near-equimolar amounts, suggesting that these are the principal components of complexed GAP in Rat-2 v-*src* cells (18b). In addition, only p190 coimmunoprecipitated with GAP from cells labeled with [^{35}S]methionine or various ^{3}H-labeled amino acids (Fig. 2A), suggesting it is the most prominent GAP-associated protein.

DISCUSSION

Activated protein-tyrosine kinases induce GAP to form multiple heteromeric protein-protein complexes. Stimulation with growth factors such as EGF or expression of an activated cytoplasmic tyrosine kinase, p60$^{v\text{-}src}$, induces marked changes in GAP phosphorylation, in its interactions with other proteins, and in its distribution within the cell. In v-*src*-transformed and EGF-stimulated cells, approximately 40 to 70% of cytoplasmic GAP complexes with p190 (as determined from densitometric analysis of the autoradiographs shown in Fig. 5a and b), whereas this complex is generally low or undetectable in normally proliferating Rat-2 cells. Interestingly, although the formation of this complex is greatly stimulated by p60$^{v\text{-}src}$ or EGF receptor tyrosine kinase activity, GAP associated with p190 in v-*src*-transformed cells contains relatively little P.Tyr. Indeed, only 3 to 4% of GAP is tyrosine phosphorylated in Rat-2 v-*src*-transformed cells (18a), and this P.Tyr-GAP subpopulation migrates at a lower molecular weight through a sizing column than does the presumptive GAP-p190 complex. In addition, P.Tyr-GAP is at least partially membrane associated, whereas GAP-p190 is completely cytosolic. In v-*src*-transformed cells, both GAP and p190 are phosphorylated principally on serine residues (7). Thus, the apparent sensitivity of the GAP-p190 interaction to phosphatase treatment suggests that serine phosphorylation may be required for the maintenance of the GAP-p190 complex. It is known that activated tyrosine kinases stimulate protein-serine/threonine kinases, such as p74^{raf} and MAP (mitogen-activating protein) kinase (20, 26). The GAP-p190 complex forms relatively slowly following tyrosine kinase activation (7), suggesting that their association might require the intermediary activation of a protein-serine/threonine kinase.

In contrast to p190, GAP-associated p62 is apparently a minor protein that becomes very rapidly and highly tyrosine phosphorylated upon activation of p60^{src} or the EGF receptor. Indeed p62 is one of the most prominent substrates of p60$^{v\text{-}src}$ identified in a lysate of v-*src*-transformed cells (Fig. 3A), and its phosphorylation in cells expressing wild-type and mutant v-*src* or v-*fps* genes has been shown to correlate with transforming activity (19). In vitro binding experiments using GAP SH2 domains generated in *Escherichia coli* have indicated that tyrosine-phosphorylated p62 binds with high affinity to the GAP SH2 region (19). We speculate that phosphorylation of p62 by p60$^{v\text{-}src}$ enhances its affinity for GAP SH2 domains and thereby directly induces the GAP-p62 complex. However, since antibodies to p62 are not yet available, we cannot rule out the possibility that p62 is associated with GAP prior to its phosphorylation. Only 10% of total P.Tyr-containing p62 appears to be stably associated with GAP. The remainder of phosphorylated p62 might be monomeric, consistent with the ease with which p62 binds bacterial SH2 domains in Rat-2 v-*src* lysates (19), or might associate with other SH2-containing proteins.

The results obtained from gel filtration and ion-exchange chromatography and subcellular fractionation suggest that p62 and p190 form separate complexes with GAP. We cannot exclude the existence of a trimeric GAP-p62-p190 complex, but it is clearly not a prominent species. It is apparent, however, that GAP can physically interact with tyrosine-phosphorylated proteins other than p62 and p190, including protein-tyrosine kinases themselves. In PDGF-stimulated cells, up to 10% of GAP becomes associated with activated PDGF receptors, an interaction that can be mimicked in vitro by using immobilized GAP SH2 domains (2, 11). GAP SH2 domains also complex with activated EGF receptors in vitro (19). This association requires receptor tyrosine phosphorylation and can be reproduced by using only the C-terminal tail of the receptor which contains the major autophosphorylation sites (16). It therefore seems likely that GAP associates with activated EGF receptors in vivo, although we have not yet directly detected such a complex. This interaction, in addition to those with p190 and p62, might contribute to the particularly striking increase in GAP's apparent size observed upon EGF stimulation. Similarly, p60$^{v\text{-}src}$ from a Rat-2 v-*src* lysate is retained on a GAP-SH2 affinity column, and P130$^{gag\text{-}fps}$ coimmunoprecipitates with GAP from the particulate fraction of v-*fps*-transformed rat cells (18a), suggesting that GAP may physically associate with various receptor and cytoplasmic protein-tyrosine kinases at the plasma membrane through direct or indirect interactions.

Activated tyrosine kinases induce a redistribution in GAP subcellular localization. In normal cells, GAP was found exclusively in the cytosol. However, in v-*src*-transformed cells, approximately 8% of GAP becomes associated with the particulate fraction and contains at least threefold more P.Tyr per mole than does cytosolic GAP (a minimal estimate based on several repetitions of the experiment shown in Fig. 4). These results are consistent with those obtained by Molloy et al. (18) using PDGF-stimulated cells. A subpopulation of this membrane-associated GAP is complexed with p62.

These results indicate that GAP undergoes a complex series of protein-protein interactions following tyrosine kinase activation. The initial migration of GAP to the membrane may occur as a consequence of the increased affinity of its SH2 domains for autophosphorylated sites on activated growth factor receptors or p60$^{v\text{-}src}$ (19). Once at the membrane, GAP may be phosphorylated on tyrosine and associate with the prominent tyrosine kinase substrate p62. It is possible that tyrosine phosphorylation or association with p62 displaces GAP from the tyrosine kinase. While only a small fraction of GAP is phosphorylated on tyrosine, this transient modification may subsequently affect GAP subcellular localization, serine phosphorylation, and/or protein-protein interactions. In v-*src*-transformed Rat-2 cells, the majority of GAP is located in the cytosol, where it is phosphorylated on serine and complexed with p190. It will be interesting to determine whether this cytosolic GAP species has been transiently phosphorylated on tyrosine and plasma membrane associated prior to its serine phosphorylation and interaction with p190.

Protein-tyrosine kinases and the Ras signaling pathway. Since GAP may play a critical role in downstream p21^{ras} signaling, proteins associated with GAP are candidates for p21^{ras} targets. For example, a p21^{ras} mutant with an Asp-38 → Glu substitution in the effector region still associates with

GAP but remains GTP bound and is biologically inactive. This suggests that GAP is not the only component of the $p21^{ras}$ target and further implicates GAP-associated proteins in downstream signaling from $p21^{ras}$ (13).

A logical outcome of the assembly of GAP complexes that accompanies tyrosine kinase activation would be an increased proportion of $p21^{ras}$ in the GTP-bound state, and such an increase in $p21^{ras}$-GTP has been reported in mouse fibroblasts following activation of a variety of oncogenic or growth factor receptor tyrosine kinases (8, 27, 28). One possible mechanism to accomplish this would be the inhibition of GAP's GTPase-activating effect on $p21^{ras}$. Our results show that the activity of GAP complexed to p190 is about fourfold lower than that of monomeric GAP. Thus, tyrosine kinase activation might inhibit GAP by promoting its interaction with p190. Our results suggest that this effect may be indirect, occurring as a consequence of the activation of a protein-serine kinase. In this context, it is intriguing that in appropriate CHO and mouse 3T3 cells, the c-*raf* protein serine/threonine kinase becomes activated following PDGF stimulation and, like Src, GAP, and other SH2-containing proteins in Rat-2 cells, becomes transiently associated with PDGF receptors (10, 20). Furthermore, Downward et al. (5) observed decreased GAP activity and increased GTP-bound Ras following activation of protein kinase C in T cells. This finding raises the possibility that protein kinase C promotes GAP-p190 formation, resulting in GAP inhibition and increased GTP-Ras. Clearly there are several candidate serine kinases that may be responsible for the intense serine phosphorylation of GAP and p190 observed following tyrosine kinase activation (7). It should be noted that we have not compared the kinetics of GAP-p190 formation and $p21^{ras}$-GTP accumulation following tyrosine kinase activation to establish a causal relationship between these phenomena. In addition, tyrosine kinases might also increase GTP-bound $p21^{ras}$ by stimulating a $p21^{ras}$ guanine nucleotide exchange factor.

Our present data indicate that GAP undergoes a complex series of interactions following tyrosine kinase activation, which we suggest are important in stimulating the Ras signaling pathway. Binding to GAP may allow p62 and p190 to access $p21^{ras}$, and hence these proteins are compelling candidates to provide the upstream activator and downstream effector functions required by Ras. The requirements for serine and tyrosine phosphorylation for the assembly or maintenance of these complexes may ensure the proper integration and coordination of signals from different protein kinase pathways. The emerging model is that activated tyrosine and serine kinases phosphorylate GAP, p62, and p190 to promote their assembly into functional signaling complexes. The propagation of mitogenic signals via phosphorylation-mediated protein-protein interactions involving SH2 domains may be an efficient mechanism to maintain tight coupling between growth factor binding to receptors and cell division.

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