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p190 RhoGAP, the Major RasGAP-Associated Protein, Binds GTP Directly

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In mitogenically stimulated cells, a specific complex forms between the Ras GTPase-activating protein (RasGAP) and the cellular protein p190. We have previously reported that p190 contains a carboxy-terminal domain that functions as a GAP for the Rho family GTPases. Thus, the RasGAP-p190 complex may serve to couple Ras- and Rho-mediated signalling pathways. In addition to its RhoGAP domain, p190 contains an amino-terminal domain that contains sequence motifs found in all known GTPases. Here, we report that p190 binds GTP and GDP through this conserved domain and that the structural requirements for binding are similar to those seen with other GTPases. While the purified protein is unable to hydrolyze GTP, we detect an activity in cell lysates that can promote GTP hydrolysis by p190. A mutated form of p190 that fails to bind nucleotide retains its RasGAP binding and RhoGAP activities, indicating that GTP binding by p190 is not required for these functions. The sequence of p190 in the GTP-binding domain, which shares structural features with both the Ras-like small GTPases and the larger G proteins, suggests that this protein defines a novel class of guanine nucleotide-binding proteins.

The ability of numerous proteins to bind and hydrolyze GTP has been maintained throughout evolution as a switching mechanism for a variety of cellular functions (16). Processes as diverse as translational elongation, protein sorting, and signal transduction are mediated by the various classes of GTPases that have now been identified (4, 5). The tightly regulated transition of these proteins between the GTP-bound active state and the GDP-bound inactive state appears to accompany a significant conformational change (24) that determines their ability to interact with appropriate targets (5). In the case of the small Ras-like GTPases, the transition between these states is partly regulated by a group of specific GTPase-activating proteins (GAPs) that directly promote the hydrolysis of GTP when bound to these GTPases (3). Although the nature of the enzymatic activity of GAPs suggests that they function as down-regulators of GTPases, there is also evidence that supports an effector function for these proteins (1, 6, 9, 10, 15, 23, 37, 38). Thus, their role in GTPase-mediated cellular signalling pathways has not yet been clarified.

Our studies of the RasGAP have led to the identification of the major RasGAP-associated protein, p190, which forms a specific complex with RasGAP in growth-stimulated cells (11, 26, 33). We have previously reported that p190 itself functions biochemically as a specific GAP for members of the Rho GTPase family and that this activity is encoded in a carboxy-terminal domain of the protein that is related to other RhoGAPs (32). The Rho GTPases, which are about 30% identical to the Ras proteins (16), have been implicated in cytoskeletal organization and cell morphology (29, 30). Thus, formation of the RasGAP-p190 complex brings into close proximity two GAPs with distinct substrate specificity and may serve to coordinate signalling pathways mediated by Ras and Rho GTPases.

In addition to the carboxy-terminal RhoGAP domain, p190 contains within its amino-terminal region several sequence motifs that are shared by all known GTPases (33). These conserved sequences include the GXXXXGK(S/T) phosphate-binding motif, the DXXG Mg^{2+}-binding motif, and the N/TKXD guanine-binding motif (5), all of which are present in p190 with the proper spacing. These sequence motifs, which are also referred to as G-1, G-3, and G-4, respectively, have been found to be important for guanine nucleotide-binding and GTPase activities in a variety of cellular proteins, including the low-molecular-weight Ras-like GTPases, the α subunits of heterotrimeric G proteins, the translation elongation factors, ADP ribosylation factors, and subunits of the signal recognition particle (SRP) (4, 5). The presence of such sequences in p190 suggests that p190 should similarly exhibit guanine nucleotide-binding activity. Here, we report that the purified p190 protein binds directly to GTP and GDP through the amino-terminal GTPase-like domain. While purified p190 does not appear to have an intrinsic GTP-hydrolyzing activity under the conditions tested, we have detected an activity in fibroblast lysates that can promote GTP hydrolysis by p190.

MATERIALS AND METHODS

Cell culture and metabolic labeling. Rat-1 and *src*-transformed Rat-1 (rat-*src*) fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cells were metabolically labeled by incubation for 12 h in methionine-free medium that was supplemented with [^{35}S]methionine (NEN Express label) at 0.2 mCi/ml. *Spodoptera frugiperda* Sf9 insect cells were cultured in Grace's complete medium, and infection with high-titer baculovirus stocks was carried out according to standard protocols. Metabolic labeling of Sf9 cells with [^{35}S]methionine was carried out as previously described (32) except that labeling was for 2 h. For pulse-chase analysis, baculovirus-infected Sf9 cells were incubated for 1 h in methionine-free medium supplemented with [^{35}S]methionine (NEN Express label) at 0.1 mCi/ml. Following the 1-h pulse, the cells were rinsed with Tris-buffered saline and incubated in Grace's complete medium for the indicated

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chase times. ^{32}P labeling of baculovirus-infected Sf9 cells was done 48 h postinfection by incubation for 12 h in low-phosphate medium (Gibco) supplemented with 10% fetal calf serum that had been dialyzed against 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9)–0.9% NaCl. $^{32}P_i$ was added to a final concentration of 0.5 mCi/ml.

Immunoprecipitation and Western blotting (immunoblotting). Cells were lysed by scraping in 50 mM HEPES (pH 7.4)–150 mM NaCl–1.5 mM $MgCl_2$–5 mM EGTA–10% glycerol–1% Triton X-100–5 μg of aprotinin per ml–5 μg of leupeptin per ml–1 mM phenylmethylsulfonyl fluoride. The lysates were clarified by centrifugation at 13,000 × *g* and precleared by incubation with normal mouse serum and protein A-Sepharose beads (50% slurry) at 4°C for 1 h. The samples were then incubated with monoclonal antibodies directed against either p190 (32) or RasGAP (33) and with protein A-Sepharose beads at 4°C for a total of 2 h. The recovered antigen-antibody complexes were washed five times in 20 mM HEPES (pH 7.0)–150 mM NaCl–1 mM EDTA–1% Nonidet P-40–1% sodium deoxycholate–0.1% sodium dodecyl sulfate (SDS) and one time in 20 mM HEPES (pH 7.5)–0.5 M LiCl–0.5% Nonidet P-40. For Western blotting, the immunoprecipitated material was released from the protein A-Sepharose beads by boiling in SDS sample buffer for 3 min and then electrophoresed through an SDS–7.5% polyacrylamide gel. Transfer to nitrocellulose, washing, and antibody incubation were carried out as described previously (33) except that a 1:5,000 dilution of goat anti-mouse serum conjugated to horseradish peroxidase (Bio-Rad) was used as the secondary antibody. The blots were developed by using the Renaissance (DuPont NEN) enhanced chemiluminescence system. Samples immunoprecipitated from ^{35}S-labeled lysates were electrophoresed similarly and then treated with Autofluor (National Diagnostics), dried, and subjected to fluorography.

Construction of p190 mutants. Site-directed mutagenesis of p190 was carried out by using the Mut-a-gene kit (Bio-Rad) on single-stranded template derived from a plasmid carrying the 703-bp *Hin*dIII-*Acc*I fragment from the p190 coding sequence (33). The resulting construct was sequenced to confirm that only the targeted nucleotides were altered during the mutagenesis procedure. The corresponding region in a full-length wild-type p190 clone was replaced by the mutated fragment, and the complete mutant p190 coding sequence was cloned into the baculovirus expression vector pEV55 for generation of high-titer baculovirus stocks as described previously (32).

NDP kinase assay. Samples for analysis were added to a reaction mixture containing 25 mM HEPES (pH 7.9), 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, and [γ-^{32}P]ATP (0.2 μCi, 3,000 Ci/mmol) in a 20-μl volume. Nucleoside diphosphate (NDP) kinase (0.04 U; Boehringer Mannheim) was then added, and each sample was incubated at 30°C for 10 min. The reactions were stopped by the addition of EDTA to 10 mM, and an aliquot of each sample was spotted on a polyethyleneimine-cellulose plate. The reaction products were resolved by thin-layer chromatography (TLC) in 0.75 M KH_2PO_4 (pH 3.4) and visualized by autoradiography. In some cases, products were quantified by scintillation counting of spots excised from the filter.

Guanine nucleotide release assay. p190 was isolated from baculovirus-infected Sf9 cells by immunoprecipitation, and the washed protein A-Sepharose/p190 beads were resuspended in 20 mM HEPES (pH 7.0)–100 mM NaCl–2 mM EDTA and incubated at 37°C. At the indicated time points, an aliquot of the supernatant was removed for the NDP kinase assay and an equivalent amount of the incubation buffer was added to the beads to replace the volume. A duplicate immunoprecipitate was boiled for 5 min in the buffer described above, and the eluted nucleotide was subjected to NDP kinase assay to determine the total amount of nucleotide bound to p190. The amount of radioactivity in the ^{32}P-labeled GTP spot corresponding to each sample was quantified by scintillation counting, and the amount of nucleotide released at each time point was expressed as a percentage of the total nucleotide bound to p190.

GTP hydrolysis. p190 was immunoprecipitated from ^{32}P-labeled p190-baculovirus-infected Sf9 cells, and the washed protein A-Sepharose/p190 beads were divided into four equal aliquots. The beads were resuspended in 20 mM HEPES (pH 7.5)–100 mM NaCl–5 mM $MgCl_2$ and incubated at 37°C for the indicated times. Nucleotide associated with p190 at each time point was released from the protein by boiling the beads in 20 mM HEPES (pH 7.0)–100 mM NaCl–2 mM EDTA for 5 min and then resolved by TLC as described for the NDP kinase assay. Fibroblast extracts used to detect promotion of GTPase activity by p190 were prepared from rat-*src* cells by homogenization in 25 mM HEPES (pH 7.5)–100 mM NaCl–1 mM $MgCl_2$–protease inhibitors.

RhoGAP assay. RhoA protein produced in *Escherichia coli* was loaded with [γ-^{32}P]GTP, the labeled protein was then incubated with lysate prepared from uninfected or infected Sf9 cells for 15 min at 30°C, and RhoGAP activity was measured in a filter binding assay as described previously (32).

RESULTS

The p190 protein contains sequence motifs found in all GTPases. We have previously reported the identification of the complete coding sequence of the major RasGAP-associated protein, p190 (33). Near its amino terminus, the predicted p190 protein sequence contains motifs that have been found to be important for guanine nucleotide-binding and GTPase activity in a variety of cellular proteins (5). The consensus sequences for these motifs and the corresponding amino acids in p190 are shown in Fig. 1A. In addition to the GXXXX GK(S/T) phosphate-binding motif, the DXXG Mg^{2+}-binding motif, and the N/TKXD guanine-binding motif, p190 contains two additional motifs, ETSA and NVXXAF, which have been previously found only in the smaller Ras-like GTPases (5). Figure 1B shows a sequence alignment between the amino-terminal domain of p190 and one of the more closely related small GTPases, Ypt2 (18). The spacing between these motifs is also conserved in p190.

Detection of guanine nucleotide associated with p190. The presence of these amino acid sequence motifs suggests that p190 is likely to exhibit guanine nucleotide-binding activity as well as GTP-hydrolyzing activity. We have previously described the expression and purification of the full-length p190 protein in the baculovirus system (32). Therefore, we were able to use purified recombinant p190 to test directly for guanine nucleotide-binding activity. We first attempted to incorporate radiolabeled GTP onto purified p190 in an EDTA-facilitated exchange reaction. Using conditions in which we could readily detect exchange of GTP onto a recombinant RhoA GTPase (50 nM) in a filter binding assay, no binding of GTP to p190 was detected, even at protein concentrations as high as 100 nM (20 μg/ml). Further attempts to achieve exchange under a variety of magnesium and salt concentrations and at several temperatures were also unsuccessful (data not shown). A requirement for specific nucleotide exchange-promoting fac-

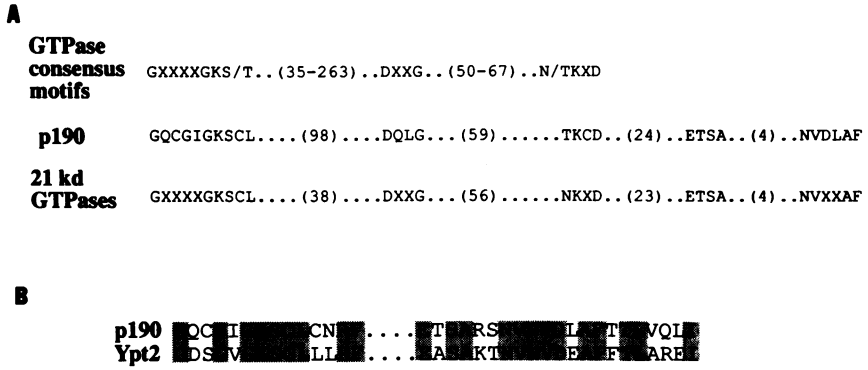

FIG. 1. Sequence alignment of the amino-terminal region of p190 with GTPase consensus motifs. (A) The consensus sequences of the conserved amino acid motifs found in all guanine nucleotide-binding proteins and in the small Ras-like GTPases and the corresponding sequences present in p190 are shown. The numbers in parentheses indicate the spacing between each motif. (B) Alignment of p190 with one of the more closely related small GTPases, Ypt2 (18). Amino acids that are identical in both are highlighted.

tors has been reported for other G proteins, such as EF-Tu (19, 31).

Since it was not possible to achieve efficient nucleotide exchange with recombinant p190 protein in vitro, we attempted to determine whether p190 isolated from cells is associated with guanine nucleotide. One standard method of guanine nucleotide detection is spectrophotometry at 253 nm, the peak wavelength of absorbance for GDP and GTP. However, we estimated that a minimum of 2 mg of a purified protein of the size of p190 (10 nmol/ml) would be necessary to detect protein-associated guanine nucleotide, assuming 1:1 binding stoichiometry. Therefore, we developed a more sensitive assay for guanine nucleotide detection that relies on the enzyme NDP kinase to detect guanine nucleotide that is released from purified protein following denaturation. NDP kinase can transfer the labeled phosphate from [γ-^{32}P]ATP to any NDP present in the reaction mixture (2). Our strategy was to heat denature p190 to release associated nucleotide and to subject the released nucleotide to NDP kinase analysis. Any p190-associated GDP should be converted to radiolabeled GTP in the assay. The reaction products, which are predicted to consist of radiolabeled ATP and GTP, can then be resolved by TLC and detected by autoradiography. While the role of NDP kinase as a potential regulator of GTPases in vivo is controversial, these experiments rely only on the well-documented in vitro catalytic activity of the enzyme.

Control NDP kinase reactions with [γ-^{32}P]ATP and NDP kinase with or without added unlabeled GDP were first performed to demonstrate that the GDP is converted to radiolabeled GTP under the conditions used (Fig. 2A). Moreover, the yield of radiolabeled GTP in these assays was found to be linearly dependent on the amount of input GDP over a wide range (data not shown). We then assayed association of guanine nucleotide with baculovirus-produced p190 which was purified by immunoprecipitation of lysates from infected Sf9 insect cells. The immunoprecipitates each contain approximately 1 μg (5 pmol) of p190. Immunopurification, as opposed to column purification, allowed us to perform several informative negative controls in parallel. Following immunoprecipitation with a p190-specific antibody, protein A-Sepharose beads

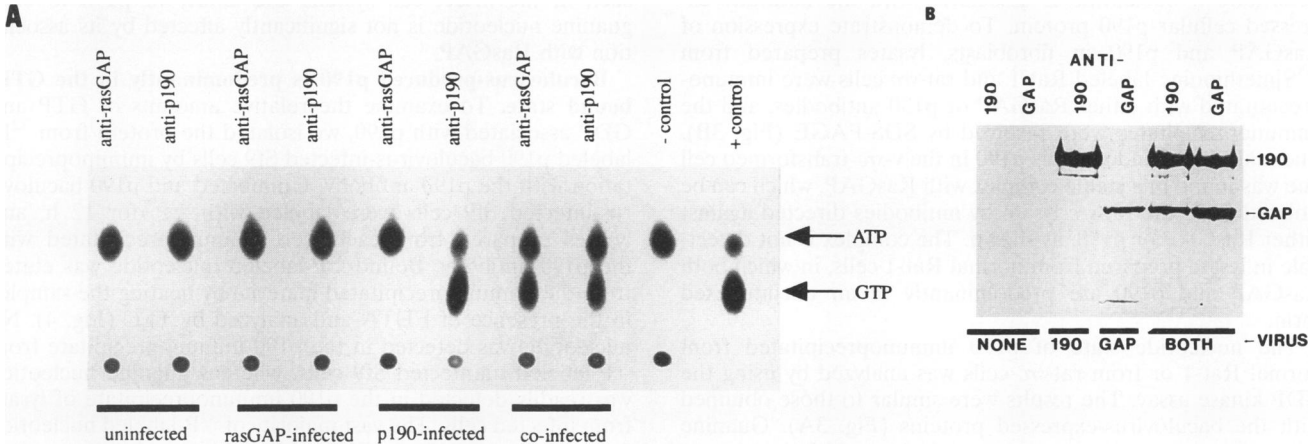

FIG. 2. NDP kinase assay of baculovirus-produced p190. (A) Anti-RasGAP and p190 immunoprecipitates of lysates from baculovirus-infected cells were boiled, and the eluted samples were subjected to NDP kinase assay and TLC. A thin-layer chromatograph of the reaction products is shown, with arrows indicating the relative migrations of ATP and GTP. Negative and positive controls for the NDP kinase assay are indicated. (B) Lysates prepared from uninfected or baculovirus-infected Sf9 cells were incubated with the indicated viruses and metabolically labeled with [^{35}S]methionine 48 h postinfection, and prepared lysates were subjected to immunoprecipitation with the indicated monoclonal antibodies. Samples were then resolved by SDS-PAGE and fluorographed.

were extensively washed in radioimmunoprecipitation assay (RIPA) buffer and then heated briefly in the presence of EDTA to promote release of any associated nucleotide. Released material was then subjected to the NDP kinase assay. As expected, labeled GTP was produced in the NDP kinase reaction after assay of material released from p190 protein immunoprecipitated from p190-infected Sf9 cells, indicating the association of guanine nucleotide with p190. No labeled GTP was generated from several negative controls, which included immunoprecipitations with anti-p190 or anti-RasGAP antibodies of lysate from uninfected cells, an anti-RasGAP immunoprecipitate of p190-infected cells, or an anti-p190 immunoprecipitate of RasGAP-infected cells, indicating that the guanine nucleotide detected in immunoprecipitates from the p190-infected cells is specifically associated with p190.

We also used this assay to address a possible effect of association of p190 with RasGAP on guanine nucleotide binding to p190. To demonstrate that RasGAP-p190 association can occur in the insect cell system, Sf9 cells coinfected with the RasGAP and p190 baculoviruses were metabolically labeled with [^{35}S]methionine, the labeled lysate was incubated with either RasGAP or p190 antibody, and the immunoprecipitated material was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2B). Both RasGAP and p190 antibodies were able to immunoprecipitate efficiently a complex containing the two proteins, as shown. To detect associated guanine nucleotide, similar immunoprecipitations were performed from unlabeled cells, and the material that was eluted by EDTA treatment of the washed beads was subjected to an NDP kinase assay. p190 isolated from coinfected cells by immunoprecipitation with either the RasGAP or p190 antibody was associated with guanine nucleotide (Fig. 2A). No nucleotide was detected in either RasGAP or p190 immunoprecipitates prepared from cells infected with RasGAP baculovirus alone.

These results suggest that the p190 protein complexed with RasGAP is also associated with guanine nucleotide. We have also confirmed that only guanine nucleotide, as opposed to other types of nucleotide, is associated with p190 by two-dimensional TLC analysis of nucleotide produced in the NDP kinase assay (data not shown).

We carried out a similar experiment in Rat-1 fibroblasts to determine if nucleotide is associated with the naturally expressed cellular p190 protein. To demonstrate expression of RasGAP and p190 in fibroblasts, lysates prepared from [^{35}S]methionine-labeled Rat-1 and rat-*src* cells were immunoprecipitated with either RasGAP or p190 antibodies, and the immunoprecipitates were resolved by SDS-PAGE (Fig. 3B). The majority of endogenous p190 in the v-*src*-transformed cell line was found in a stable complex with RasGAP, which can be isolated from the rat-*src* lysate by antibodies directed against either RasGAP or p190, as shown. The complex is not detectable in lysate prepared from normal Rat-1 cells, in which both RasGAP and p190 are predominantly in an uncomplexed form.

The nucleotide state of p190 immunoprecipitated from normal Rat-1 or from rat-*src* cells was analyzed by using the NDP kinase assay. The results were similar to those obtained with the baculovirus-expressed proteins (Fig. 3A). Guanine nucleotide was found to be associated with p190 isolated from both Rat-1 and rat-*src* cells with the p190 antibody. No nucleotide was detected in the RasGAP immunoprecipitate from the Rat-1 cell line but was readily detectable in the analogous immunoprecipitate from the rat-*src* cell line, in which endogenous p190 is complexed with RasGAP. As was

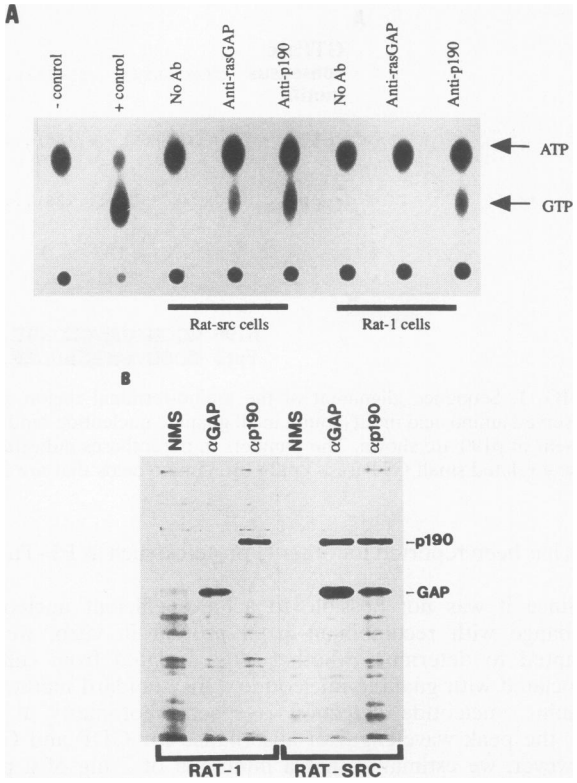

FIG. 3. NDP kinase assays of immunopurified p190 from fibroblasts. (A) Guanine nucleotide associated with p190 isolated from Rat-1 and rat-*src* cells was assayed with NDP kinase following immunoprecipitation of lysates with RasGAP or p190 antibodies (Ab), as indicated. A thin-layer chromatograph of the reaction products is shown, with arrows indicating the relative migrations of ATP and GTP. Negative and positive controls for the NDP kinase assay are also included. (B) Rat-1 and rat1-*src* cells were metabolically labeled with [^{35}S]methionine, and lysates prepared from each were incubated with the indicated antibody. The immunoprecipitates were electrophoresed through an SDS–7.5% polyacrylamide gel. NMS, normal mouse serum; αGAP, monoclonal GAP antibody B4F8 (33); αp190, monoclonal p190 antibody D2D6 (32).

seen in the insect cell system, the ability of p190 to bind guanine nucleotide is not significantly affected by its association with RasGAP.

Baculovirus-produced p190 is predominantly in the GTP-bound state. To examine the relative amounts of GTP and GDP associated with p190, we isolated the protein from ^{32}P-labeled p190 baculovirus-infected Sf9 cells by immunoprecipitation with the p190 antibody. Uninfected and p190 baculovirus-infected Sf9 cells were labeled with $^{32}P_i$ for 12 h, and lysates prepared from each were immunoprecipitated with the p190 antibody. Bound ^{32}P-labeled nucleotide was eluted from the immunoprecipitated material by heating the samples in the presence of EDTA and analyzed by TLC (Fig. 4). No nucleotide was detected in the p190 immunoprecipitate from ^{32}P-labeled uninfected Sf9 cells, whereas guanine nucleotide was readily detected in the p190 immunoprecipitate of lysate from infected cells. The vast majority of ^{32}P-labeled nucleotide associated with p190 is GTP, although a small amount (approximately 5%) of labeled GDP was detected. It is not possible, however, to determine precisely the ratio of GTP to GDP associated with p190 because of potential differences in the proportions of GTP and GDP that become radiolabeled in the intracellular pools of nucleotide.

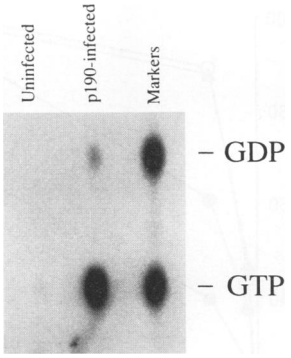

FIG. 4. Detection of p190-associated guanine nucleotide by P_i labeling of infected Sf9 cells. Lysates prepared from ^{32}P-labeled uninfected Sf9 cells and from ^{32}P-labeled p190 baculovirus-infected Sf9 cells were subjected to immunoprecipitation with the p190 antibody. ^{32}P-labeled guanine nucleotide associated with each immunoprecipitate was eluted, and GTP and GDP were resolved by TLC. The migration of ^{32}P-labeled GDP and GTP standards is also shown.

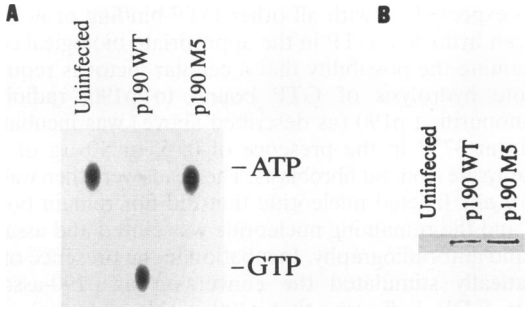

FIG. 5. p190 mutant M5 does not bind guanine nucleotide. (A) Uninfected, wild-type (WT) p190-infected, and mutant M5-infected insect cells were lysed and subjected to immunoprecipitation with the p190 monoclonal antibody. Associated guanine nucleotide was eluted and detected by NDP kinase assay followed by TLC. (B) Western blot analysis of material immunoprecipitated from lysates of uninfected, wild-type p190-infected, and mutant M5-infected Sf9 cells with the p190 antibody to demonstrate that equivalent amounts of wild-type and mutant protein were analyzed in the NDP kinase assay.

Mutation of the TKCD motif in p190 eliminates guanine nucleotide binding. The results of the NDP kinase and phosphate labeling experiments indicate that p190 is associated with guanine nucleotide. However, we have not ruled out the possibility that the detected nucleotide is derived from an unidentified p190-associated protein that is present in the immunoprecipitates. If p190 binds nucleotide directly, we predict that the conserved guanine nucleotide-binding motifs in the amino-terminal domain of p190 are required for this interaction. To test this directly, we constructed a mutant form of p190 (mutant M5) in which the conserved TKCD motif was mutated to TDCV. Structural studies of the Ras GTPase have shown that the conserved lysine in the $p21^{ras}$ N/TKXD motif stabilizes the guanine nucleotide-binding loop while the conserved aspartic acid residue binds directly to the guanine base (27, 28). Mutation of the aspartic acid residue in $p21^{ras}$ has been shown to reduce significantly guanine nucleotide binding in vitro and results in an increased dissociation rate of bound GTP (7, 8, 13). Substitution of both amino acids in p190 was predicted to eliminate its ability to bind guanine nucleotide. p190 was immunoprecipitated from Sf9 cells which were infected with either wild-type p190 baculovirus or mutant M5 baculovirus. Nucleotide released from each immunoprecipitate following incubation in EDTA was assayed with NDP kinase. While labeled GTP was readily detectable in the NDP kinase assay of material released from immunoprecipitated wild-type p190, no nucleotide was found to be associated with the immunoprecipitated p190 M5 protein, indicating that p190 containing the double substitution in the TKCD motif does not bind guanine nucleotide detectably (Fig. 5A). Western blot analysis of the immunoprecipitated material analyzed in the NDP kinase assay showed that equivalent amounts of p190 protein were present in the wild-type and mutant M5 samples (Fig. 5B). These data suggest that the conserved sequence motifs present in the amino-terminal domain of p190 function as they do in the well-characterized guanine nucleotide-binding proteins and confirm that the detected guanine nucleotide is directly associated with the p190 protein.

GTP hydrolysis by p190 requires a cellular GAP-like activity. Guanine nucleotide-binding proteins cycle between inactive GDP-bound and active GTP-bound states, and this balance is regulated in part by the rate of GTP hydrolysis (3). To detect potential p190 GTPase activity, we examined immunoprecipitates of p190 from ^{32}P-labeled p190 baculovirus-infected Sf9 cells. As described above, most of the ^{32}P-labeled nucleotide associated with p190 in these cells is GTP. GTPase activity was assayed by incubating the immunoprecipitated material (bound to beads) at 37°C in the presence of magnesium. Samples were taken after 0, 10, 30, and 120 min of incubation, and the nucleotide bound to p190 at each time point was eluted by heating in the presence of EDTA and subjected to TLC (Fig. 6A). No detectable hydrolysis of GTP to GDP occurred over the 2-h time course of this experiment, indicating that p190 does not possess intrinsic GTP-hydrolyzing activity under the conditions used. Because we are measuring hydrolysis of radiolabeled GTP that is prebound to p190, the GTPase reaction obeys first-order kinetics and, as such, is independent of protein concentration.

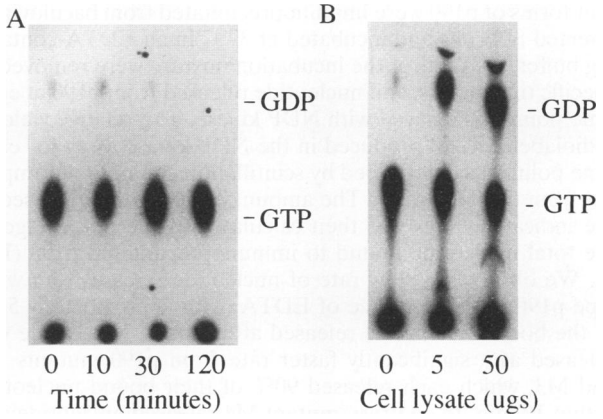

FIG. 6. GTPase assays of p190. (A) p190 was immunoprecipitated from ^{32}P-labeled baculovirus-infected Sf9 cells and assayed for GTPase activity by incubation at 37°C in the presence of magnesium for the indicated amounts of time. The ^{32}P-labeled guanine nucleotide associated with p190 at the indicated time points was released from the protein by boiling in EDTA and resolved by TLC. The arrows show the migration of ^{32}P-labeled GDP and GTP standards. (B) The GTPase activity of p190 immunoprecipitated from ^{32}P-labeled baculovirus-infected Sf9 cells was assayed at 37°C for 30 min in the presence of 0, 5, or 50 μg of rat fibroblast lysate, as indicated. The ^{32}P-labeled guanine nucleotide associated with p190 was detected as in panel A.

It is expected, as with all other GTP-binding proteins, that p190 can hydrolyze GTP in the appropriate biological context. To examine the possibility that a cellular factor is required to promote hydrolysis of GTP bound to p190, radiolabeled immunopurified p190 (as described above) was incubated for 30 min at 37°C in the presence of 0, 5, or 50 μg of a total cellular lysate from rat fibroblasts. The beads were then washed to remove any labeled nucleotide that did not remain bound to p190, and the remaining nucleotide was eluted and assayed by TLC and autoradiography. Incubation in the presence of lysate dramatically stimulated the conversion of p190-associated GTP to GDP, indicating that p190 is able to hydrolyze GTP (Fig. 6B). Although cell lysates contain GTPase activity, in these experiments all of the observed conversion of GTP to GDP must be mediated by p190, since the p190-nucleotide complex is immobilized on beads throughout the reaction, and the GTP bound to p190 is therefore unavailable as substrate for other GTPases.

Mutant forms of p190 exhibit altered nucleotide affinities. Structural features of GTPases are important determinants of their affinity for guanine nucleotides (5). To determine whether p190 binds nucleotide in a manner similar to that of other GTPases, we constructed three amino acid substitution mutants of p190 that are predicted to exhibit altered nucleotide-binding properties. Mutant M2 is a Thr-to-Ile substitution in the TKCD motif, mutant M3 is a Thr-to-Leu substitution in the ETSA motif, and mutant M4 is an Asp-to-Ala substitution in the DQLG motif. In the context of the Ras protein, mutations analogous to M2 and M3 give rise to Ras proteins that exhibit a reduced affinity for guanine nucleotide and a higher nucleotide exchange rate (7, 13, 34). A mutation analogous to M4 in Ras exhibits an increased relative affinity for GDP over GTP (20). p190 baculoviruses were prepared for each mutant, and expression of mutant p190 protein was confirmed (data not shown). The relative affinities of each protein for GDP were determined by measuring the rate at which nucleotide is released from the protein in the presence of EDTA, which facilitates the release of guanine nucleotide from GTPases as a result of the requirement for magnesium for the protein-nucleotide association (17). Wild-type or mutant forms of p190 were immunoprecipitated from baculovirus-infected Sf9 cells and incubated at 37°C in an EDTA-containing buffer. Aliquots of the incubation mixture were removed at specific time points, and nucleotide released from p190 at each time point was assayed with NDP kinase. The relative yield of radiolabeled GTP produced in the NDP kinase assay for each time point was determined by scintillation counting of samples cut from the TLC plate. The amount of nucleotide released at the indicated times was then calculated as the percentage of the total nucleotide bound to immunoprecipitated p190 (Fig. 7). We observed a slow rate of nucleotide release from wild-type p190 in the presence of EDTA, with approximately 50% of the bound nucleotide released after 8 min. Nucleotide was released at a significantly faster rate from p190 mutants M2 and M3, which each released 90% of their bound nucleotide within 10 min. In contrast, mutant M4 released nucleotide at a rate that was about six times slower than the rate for the wild-type protein. These results suggest that p190 mutants M2, M3, and M4 exhibit alterations in nucleotide affinity that are similar to those seen with analogous substitutions in Ras.

GTP binding to p190 is not required for RasGAP-p190 complex formation or RhoGAP activity. By analogy with other GTPases, the nucleotide state of p190 is likely to influence the conformation of the protein. Thus, it is possible that guanine nucleotide binding regulates one of its other biochemical properties, such as RhoGAP activity or association with RasGAP. We used the p190 mutant M5 to determine if the absence of associated guanine nucleotide affects these other properties of the protein. To analyze p190 association with RasGAP, lysates were prepared from [^{35}S]methionine-labeled Sf9 cells infected with RasGAP virus alone or coinfected with RasGAP plus wild-type p190 viruses or RasGAP plus p190 mutant M5 viruses. Following immunoprecipitation with RasGAP antibodies, RasGAP-p190 complex formation was analyzed by SDS-PAGE and fluorography (Fig. 8). In this assay, a readily detectable complex was observed between RasGAP and wild-type or mutant M5 forms of p190, indicating that the

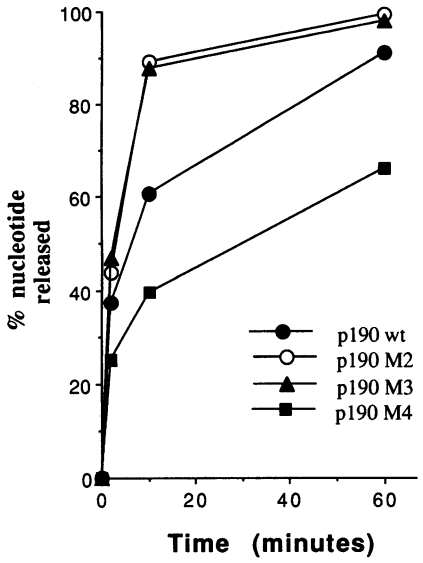

FIG. 7. Nucleotide release rates of wild-type and GTPase domain mutants of p190. The rate of EDTA-facilitated nucleotide release from immunopurified baculovirus-produced p190 was determined as described in Materials and Methods. The amount of nucleotide released at 37°C is expressed as a percentage of the total amount of nucleotide bound to p190 at each time point and is plotted against time of incubation. The rate of nucleotide release from wild-type (WT) p190 and each of the mutants was determined in at least four independent experiments.

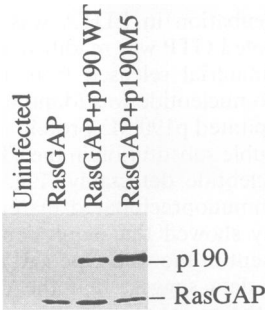

FIG. 8. Nucleotide-free p190 retains RasGAP-binding ability. Sf9 insect cells were either uninfected, infected with RasGAP virus alone, or coinfected with RasGAP plus wild-type (WT) p190 viruses or RasGAP plus p190 mutant M5 viruses, as indicated. Cells were labeled metabolically with [^{35}S]methionine 48 h postinfection, and lysates were subjected to immunoprecipitation with RasGAP antibodies. The immunoprecipitated material was electrophoresed through an SDS–7.5% polyacrylamide gel, treated with Autofluor, and fluorographed. The migration of RasGAP and p190 proteins is indicated.

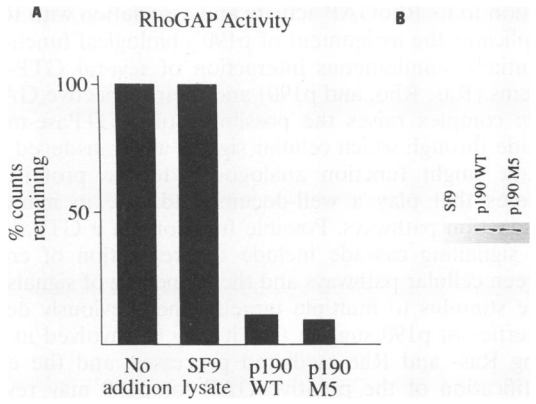

FIG. 9. Nucleotide-free p190 retains RhoGAP activity. (A) RhoGAP assays were performed with uninfected or infected insect cell lysates as described in Materials and Methods. Results of the filter binding assay are displayed as the percentage of counts remaining on the filter. WT, wild type. (B) Western blot analysis of uninfected, wild-type (WT) p190-infected, and mutant M5-infected insect cells with a p190 antibody to demonstrate that equivalent amounts of wild-type and mutant proteins were analyzed.

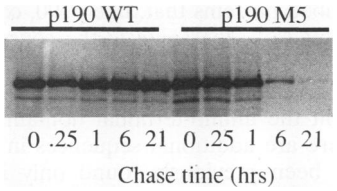

FIG. 10. Nucleotide-free p190 is unstable. The relative stabilities of wild-type (WT) p190 and mutant M5 p190 proteins were determined by pulse-chase analysis as described in Materials and Methods. At the indicated chase times, ^{35}S-labeled lysates were subjected to immunoprecipitation with p190 antibodies. The immunoprecipitated material was electrophoresed through an SDS–7.5% polyacrylamide gel, treated with Autofluor, and fluorographed.

ability of p190 to bind guanine nucleotide is not required for the formation of the RasGAP-p190 complex. We have also determined that purified p190 protein which has been stripped of associated guanine nucleotide with EDTA can still bind RasGAP in vitro (data not shown).

We have previously reported that the p190 protein exhibits a specific GAP activity toward the Rho family GTPases. To determine if guanine nucleotide binding is required for the RhoGAP activity of p190, we analyzed the RhoGAP activity of mutant M5 p190 protein. Lysates prepared from uninfected Sf9 cells and from Sf9 cells infected with either wild-type p190 baculovirus or mutant M5 baculovirus were tested for RhoGAP activity on purified bacterially produced RhoA protein, using a previously described filter binding assay (32). The mutant p190 protein was able to stimulate the GTPase activity of RhoA in a manner identical to that of the wild-type protein, indicating that the association of p190 with guanine nucleotide is not required for its RhoGAP activity (Fig. 9A). Western blot analysis of the lysates used in the experiment indicated that equivalent amounts of wild-type and mutant M5 p190 protein were analyzed in the RhoGAP assay (Fig. 9B).

Nucleotide-free p190 exhibits reduced stability. GTP-binding proteins are often unstable in the absence of associated guanine nucleotide (12, 14). Therefore, we examined the stability of wild-type and mutant M5 p190 produced in insect cells, using a pulse-chase assay. Cells infected with wild-type or mutant M5 p190 virus were metabolically labeled with [^{35}S]methionine for 1 h, then chased for various times with unlabeled methionine, and lysed. Cell lysates were subjected to p190 immunoprecipitation followed by SDS-PAGE and fluorography (Fig. 10). Relative protein amounts were approximated by densitometry. The wild-type p190 protein is extremely stable, exhibiting no detectable decay even after 21 h of chase. In contrast, mutant M5 protein, although synthesized at normal levels, was rapidly degraded, exhibiting an apparent half-life of about 2 h. Thus, the wild-type p190 protein is at least 10-fold more stable than the mutant that fails to bind nucleotide.

DISCUSSION

We have demonstrated that the major RasGAP-binding protein, p190, is associated with guanine nucleotide both in the recombinant insect cell system and in cultured rodent fibroblasts. Moreover, nucleotide association appears to be independent of p190's interaction with RasGAP. The absence of detectable guanine nucleotide associated with p190 mutant M5, in which the highly conserved N/TKxD motif has been mutated, confirms that the binding of GTP to p190 is direct and is mediated through the amino-terminal GTPase-like domain of the protein. The altered nucleotide affinities seen with three single amino acid substitution mutants of the GTPase-like domain of p190 indicate that the association of guanine nucleotide with p190 involves structural requirements similar to those of other GTPases.

As is true with other GTPases, the nucleotide-free form of p190 exhibits significantly reduced stability. The dramatic difference in protein stability between wild-type and mutant M5 p190 (greater than 10-fold) suggests that a large proportion of the wild-type protein in our preparations is associated with nucleotide. While it is formally possible that the two amino acid substitutions in mutant M5 result in protein instability by a nucleotide-independent effect, we believe this to be unlikely since the other amino acid substitutions that we have generated in this region of the protein all give rise to stable proteins that bind nucleotide.

The nucleotide associated with baculovirus-produced p190 is largely in the form of GTP, suggesting that the protein lacks a significant intrinsic GTP-hydrolyzing activity. The failure of immunopurified p190 to hydrolyze GTP detectably during a 2-h incubation is consistent with this possibility. Purified ADP ribosylation factor, which binds GTP and GDP, also lacks a detectable intrinsic GTP-hydrolyzing activity (21), and it has been reported that membrane preparations can promote hydrolysis of GTP by ADP ribosylation factor (35). Our observation that an activity in fibroblast lysates can promote GTP hydrolysis by p190 suggests that a cellular GAP may play a role in regulating the nucleotide state of p190. The eventual identification of such a GAP is likely to reveal clues about the biological function of p190.

The release rate of nucleotide from p190 under low magnesium conditions ($t_{1/2}$ = 8 min) is slower than that of the smaller Ras-like GTPases ($t_{1/2}$ < 30 s) (17), raising the possibility that a cellular factor is required to promote nucleotide dissociation from p190. Our inability to load p190 with radiolabeled GTP in vitro is consistent with this possibility. It is also possible that some cellular factor is required to promote association of p190 with nucleotide. The SRP 54K subunit, a GTP-binding protein which also lacks a significant intrinsic GTPase activity, requires the SRP receptor to promote GTP binding by increasing the affinity of SRP 54K for GTP (25). An analogous mechanism may be required to promote GTP binding to p190. Notably, the SRPs and a small number of additional GTPases are the only

previously described proteins that, like p190, contain a threonine residue instead of asparagine in the conserved N/TKxD motif (5).

While the consensus motifs seen in all known GTPases are clearly present in the amino-terminal domain of p190, it is notable that there are additional sequences in this region of p190 that have been previously found only in the smaller Ras-like GTPases. These motifs include the ETSA sequence and the NVxxAF sequence, which are present in p190 with the same spacing that they have in some Ras and Rab family proteins (5). The ETSA sequence of the 21-kDa GTPases, which is situated near the guanine ring of GTP according to the Ras crystal structure and interacts indirectly with guanine nucleotide (27, 28), is not seen in the larger G proteins. The function of the NVxxAF motif of p190, which is also well conserved in the small GTPases, is unknown.

Although the GTPase sequence motifs of p190 most closely resemble those of the smaller Ras-like GTPases, the substantially longer spacing between the G-1 and G-3 loops of p190 (98 amino acids) is closer to that seen in the larger G-protein α subunits. Recently, it has been reported that this domain in G_α functions to promote GTPase activity even when isolated from the core GTP-binding domain. It was suggested that this insert domain, which is considerably larger than that of the smaller GTPases, functions biochemically as a GAP and that this accounts for the relatively high intrinsic GTP hydrolysis rate of G-protein α subunits (22, 36). Interestingly, p190 contains a relatively large insert domain (98 amino acids) and yet appears to lack intrinsic GTPase activity. Thus, the function of this large insert domain in p190 is unclear. Notably, p190 also exhibits a structural feature that is not found in any other GTPases. While nearly all previously described GTPases contain three or four hydrophobic residues directly preceding the first glycine of the G-1 loop (5), p190 has three charged residues in that region. As the function of the hydrophobic residues in this region has not been elucidated, it is difficult to predict how their absence might influence GTP binding and hydrolysis by p190. Thus, the structural features of p190 suggest that it may define a novel class of GTP-binding proteins, having properties of both the smaller Ras-like GTPases and the larger G proteins.

The role of GTP binding in p190 function is difficult to assess until the role of p190 in cellular signalling is more clearly established. The guanine nucleotide state of GTPases is generally associated with distinct conformational changes in the protein (24) that determine its ability to interact with one or more additional cellular proteins. For example, Ras-GTP, but not Ras-GDP, binds specifically to an amino-terminal domain of c-Raf (39). We have previously demonstrated that p190 contains a carboxy-terminal domain that functions as a GAP for Rho family GTPases (32) and that p190 is tightly associated with RasGAP in growth-stimulated cells (11, 26, 33). Therefore, we hypothesized that the nucleotide state of p190 might affect its interaction with one of these proteins. However, the ability of p190 mutant M5 to complex with RasGAP and to function normally as a RhoGAP suggests that the nucleotide binding to p190 is not required for these interactions. Thus, the nucleotide state of p190 is likely to affect some as yet unidentified property of the protein. It may be that the putative p190-specific GAP functions as an effector target of p190 or, alternatively, that interaction of p190 with this putative GAP regulates some aspect of p190 function. It is also possible that the nucleotide state of p190 affects its ability to interact with one or more additional cellular proteins that have not yet been identified.

The observation that p190 exhibits GTP-binding activity in addition to its RhoGAP activity and association with RasGAP complicates the assignment of p190's biological function. The potentially simultaneous interaction of several GTP-binding proteins (Ras, Rho, and p190) and their respective GAPs in a single complex raises the possibility of a GTPase-mediated cascade through which cellular signals are transduced. Such a cascade might function analogously to the protein kinase cascades that play a well-documented role in many signal transduction pathways. Possible functions of a GTPase-mediated signalling cascade include the regulation of cross-talk between cellular pathways and the branching of signals from a single stimulus to multiple targets. The previously described properties of p190 suggest that it may be involved in coordinating Ras- and Rho-mediated processes, and the eventual identification of the putative GAP for p190 may reveal yet another signalling pathway that is integrated with Ras and Rho.

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