The Function of the p190 Rho GTPase-activating Protein Is Controlled by Its N-terminal GTP Binding Domain*

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p190 is a GTPase-activating protein (GAP) for the Rho family of GTPases. The GAP domain of p190 is at the C terminus of the protein. At its N terminus, p190 contains a GTP binding domain of unknown significance. We have introduced a mutation (Ser
→ Asn) into this domain of p190 that decreased its ability to bind guanine nucleotide when expressed as a hemagglutinin (HA)-tagged protein in COS cells. In vitro, both the wild type and S36N mutant HA-p190 proteins showed similar GAP activities toward RhoA, but when expressed in NIH 3T3 fibroblasts only wild type p190 appeared able to function as a RhoGAP. Wild type HA-p190 induced a phenotype of rounded cells with long, beaded extensions similar to that seen when Rho function is disrupted by ADP-ribosylation. HA-p190(S36N), although expressed at a similar level to the wild type protein, had no discernible effect on the cells. The beaded extension phenotype induced by wild type HA-p190 required GAP function. A GAP-defective mutant, p190(R128A), had no effect on cell morphology. Moreover, the beaded extension phenotype could be suppressed by co-expression of a gain-of-function Rho mutant, RhoA(G14V), or Rac mutant, Rac1(G12V). Activation of the Jun kinase (JNK) via muscarinic receptors was inhibited by wild type HA-p190, but JNK activity was enhanced by the S36N mutant. Co-expression of HA-p190 with a fragment containing only the mutated GTP binding domain partially inhibited the beaded extension phenotype, suggesting that it may sequester a factor required for p190 function. Taken together these data demonstrate that within the cell, the Rho/Rac GAP activity of p190 can be regulated by the N-terminal GTP binding domain.

Within cells there are numerous proteins that bind to and hydrolyze GTP and that function as molecular switches. One class of GTPases, which has been called the Ras superfamily, can be subdivided into families according both to sequence similarity and function. The Ras family of GTPases includes Rho, Rac, Cdc42, and TC10 is involved in various aspects of cytoskeletal organization (1). Additionally, Rho GTPases can activate the Jun N-terminal kinase (JNK) and can regulate the transcriptional activity of certain genes (2).

Actions of the Ras-like GTPases are tightly controlled and are dictated by the state of nucleotide binding, which alters the conformation of discrete “switch regions” in the proteins (3). The change in conformation allows the GTPases to interact with their appropriate targets (4). Regulation of the Ras-like GTPases is accomplished by GEFs (guanine nucleotide exchange factors), which catalyze the exchange of GDP for GTP on the GTPases, and GAPs (GTPase-activating proteins), the function of which is to catalyze the hydrolysis of bound GTP to GDP (5).

GAPs have been identified that act specifically either on Ras or Rho GTPases. RasGAPs include p120 (6) and neurofibromin (7), whereas p190, Bcr, and RhoGAP act on the Rho subfamily of GTPases (8). Many of these GAPs possess, in addition to the sequences responsible for enhancing GTP hydrolysis, domains that are found in diverse signal transduction components. For example, the p190 RhoGAP contains a C-terminal catalytic domain and at its extreme N terminus a region with significant sequence similarity to the guanine nucleotide binding domain of the Ras superfamily of GTPases (9). This region is most similar to the yeast Rab GTPase, Ypt1, but it contains unusual amino acid residue substitutions within key motifs. Through this N-terminal region, p190 has been shown to bind GTP, and mutations within the GTP binding domain of p190 exhibit altered nucleotide binding affinities (10). However, the significance of this GTP binding domain is unknown.

In addition to their ability to enhance hydrolysis of GTP, some GAPs may be downstream effectors of the GTPases with which they interact. The p120 RasGAP is believed to be involved in signal transduction events (11). It recognizes tyrosine-phosphorylated p190 RhoGAP (12, 13), and in cells transformed by tyrosine kinases, most of the p120 RasGAP is found complexed to p190 (14). The formation of a p120-p190 complex may provide a means for the Ras and Rho GTPase pathways to coordinate their signals. The p190 RhoGAP is predominantly cytoplasmic, although changes in localization have been reported to occur when cells are stimulated with certain growth factors. In murine fibroblasts that overexpress c-Src, for example, stimulation with epidermal growth factor induces a rapid and transient condensation of p190 RhoGAP (15). Cheng et al. (16) have shown that in chronic myelogenous leukemia cells the association of p190 with p120 parallels an increase in actin polymerization and cell adhesion induced by phorbol esters. However, these treatments have not been shown directly to alter the GAP activity of p190.

A new p190 RhoGAP isoform has been discovered, called p190-B. This protein is expressed in a number of cell types and is clustered at adherence sites where the cell membrane at-
taches to fibronectin (17). This interaction could establish a transmembrane link between integrins and the Rho family of GTPases, but there is no evidence to date that integrins modulate p190 GAP activity.

The existence of an unusual GTP binding domain (GBD) in p190 suggests the interesting possibility that it functions to control the RhoGAP domain. To investigate this hypothesis, we created a mutant p190 protein in which Ser36 was changed to Asn. This mutation is homologous to the Ras(S17N) mutation that generates a protein with decreased affinity for nucleotide binding (18). By using this mutant protein we showed that binding of GTP is required for p190 RhoGAP function within the cell, even though in vitro RhoGAP activity is not affected. We speculate that regulatory factors tightly control the GTP/GDP ratio of p190 which in turn regulates its GAP activity and hence modulates the Rho GTPases.

**EXPERIMENTAL PROCEDURES**

**Production of Epitope-tagged p190 and GFP-fused p190—**A HindIII-EcoRI fragment was isolated from p190 cDNA clone 391 (9) provided by R. Weinberg and J. Settleman (Massachusetts Institute of Technology). This fragment, which lacks the 5’ 670 base pairs of the p190 open reading frame, was subcloned into pBS. The first 670-base pair fragment was amplified by polymerase chain reaction (PCR), to include a 5’ BamHI site. The PCR product was cut with BamHI and HindIII and subcloned into pBS-p190 plasmid. The reconstructed p190 open cloning site in the same frame as pKH3, 3’ was inserted into a vector (K7-GFP) that contains a subcloned into pKH3, which attaches, in frame, a triple HA1 tag to the BamHI reading frame, was subcloned into pBS. The first 670-base pair fragment was amplified by polymerase chain reaction (PCR), to include a 5’ BamHI site. The PCR product was cut with BamHI and HindIII and subcloned into pBS-p190 plasmid. The reconstructed p190 open reading frame was excised from pBS using BamHI and EcoRI and subcloned into pKH3, which attaches, in frame, a triple HA1 tag to the N terminus (19). To make GFP-p190, the BamHI-EcoRI p190 fragment was inserted into a vector (K7-GFP) that contains a BamHI-EcoRI cloning site in the same frame as pKH3, 3’ to the GFP open reading frame (20).

**Creation of p190 Mutants—**The mutation Ser17 → Asn in Ras creates a mutant GTPase that cannot stably bind guanine nucleotides. The equivalent residue in p190 is Ser26. We mutated this residue to Asn to produce p190(S26N) by overlap polymerase chain reaction (PCR) (21) using pKH3-p190 as a template, a mismatched 5’ primer and a 3’ primer about 800 base pairs downstream. The PCR product, containing the mutation, was digested with BamHI and HindIII and then inserted in place of the wild type p190 fragment in pKH3. To create a GAP-defective p190, a point mutation was introduced so as to alter Arg1283 → Ala as GST-RhoA and cleaved with thrombin to remove the erichia coli

**RhoGAP Assay—**5 μg of RhoA protein, which was produced in Escherichia coli in GST-RhoA and cleaved with thrombin to remove the GST, was loaded with 20 μCi of [32P]GTP (3000 Ci/mmol); the labeled protein was then incubated with immunoprecipitated wild type or mutant p190 proteins for the specified time points. RhoGAP activity was detected as the loss of [γ-32P]GTP bound to RhoA. In control experiments (data not shown), RhoA loaded with [γ-32P]GTP showed no significant reduction in [32P] counts when in the presence of either wild type or mutant p190 proteins. The amount of [32P] bound to protein was determined through a filter binding assay (24).

**JNK Assay—**INI 3T3 cells, which stably express the human m1 type (hm1) muscarinic receptor (19), were transfected with plasmids as indicated in Fig. 8. Transfected cells were stimulated with either 100 μM of distilled water (control) for 15 min, 100 μM of carbachol for 15 min, or 100 μM of anisomycin/10-cm plate of cells for 20 min. The cells were lysed in the following buffer: 25 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM MgCl2, 0.5 μM Triton X-100, 0.1% Triton X-100, 20 μM of aprotinin/ml, 10 μM of leupeptin/ml, 1 μM phenylmethylsulfonyl fluoride, and 1 μM o-phenylphenylhydrazine. The lysates were clarified by centrifugation at 14,000 × g. Aliquots of detergent-soluble protein each extract were removed from each sample tube and were fractionated on SDS-gel electrophoresis. The purified proteins were then transferred onto nitrocellulose membrane and immuno-oblotted as above. HA-tagged proteins were immunoprecipitated with the remaining detergent-soluble extracts and washed 3 times in Buffer A (2 mM Na2VO4 and 0.1% Nonidet P-40 in phosphate-buffered saline), washed 1 time in Buffer B (100 mM MOPS (pH 7.5), 0.5 μM LiCl), and washed 1 time in Kinase Buffer (12.5 mM MOPS (pH 7.5), 12.5 mM

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Function of p190 Rho/RacGAP GTP binding domain

34362
β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM EGTA, plus 0.5 mM NaF and 0.5 mM Na3VO4). Immunoprecipitated proteins were resuspended in 30 μl of Kinase Buffer, to each reaction tube was added 2 μg of purified GST-cJun-(1–79) and 2 μCi of [γ-32P]ATP. The reaction tubes were incubated at 30 °C for 20 min at which time the reactions were halted by the addition of 10 μl of 4× Laemmli sample buffer. Immunocomplex kinase reactions were fractionated by SDS-gel electrophoresis on 12% polyacrylamide gels and autoradiographed.

RESULTS

Creation and Characterization of Mutant p190 Proteins—Recombinant p190 protein expressed in SF9 insect cells is bound primarily to GTP (10). To investigate the function of nucleotide binding to p190, it was necessary to create a mutation that eliminated binding of GTP. We therefore created a mutation within the p190 guanine nucleotide binding domain (GBD) that is homologous to the Ser17 Asn mutation in Ras (25). When expressed in bacteria, the Ser36Asn p190 mutant did not specifically bind GTP, which is consistent with the notion that the S36N mutation does not detectably affect GTP binding. These results strongly support the conclusion that, when expressed in mammalian cells, the N-terminal GBD of p190 binds to both GDP and GTP and that the S36N mutant, like the analogous S17N mutant of Ras, possesses a greatly reduced affinity for guanine nucleotides.

Overexpression of Wild Type but Not Mutant p190 Proteins Induces Beaded Extensions in NIH 3T3 Fibroblasts—To determine the effect of the S36N mutation on p190 function, both wild type and S36N p190 were expressed by transient transfection as HA-tagged proteins in NIH 3T3 fibroblasts. The cells were fixed and stained for HA. Overexpression of wild type HA-p190 produced a distinct phenotype in which the cells rounded up and extended very long, beaded, dendritic-like processes, as observed by indirect immunofluorescence (Fig. 2). This phenotype is similar to that seen when either C3 botulinum toxin or RhoGDI is microinjected into Swiss 3T3 cells (26, 27). A similar phenotype was produced when HA-p190 was expressed in COS and BHK cells (data not shown). Fig. 2 shows representative cells that had been transfected with p190, p190(S36N), or with a GAP-defective mutant, p190(R1283A) (23), and stained with an anti-HA antibody. Remarkably, only those cells transfected with wild type p190 exhibited the beaded extension phenotype. Cells transfected with either the S36N or R1283A mutant looked normal, even though expression levels of the mutants and wild type p190 were similar, as determined from the fluorescence intensities.

The Phenotypic Changes Induced by p190 Overexpression Result from Cell Body Retraction Accompanied by Formation of Long, Beaded Extensions—To investigate how the p190 overexpression phenotype was produced, NIH 3T3 cells were transfected with a vector that encodes a GFP-p190 fusion protein and monitored by time lapse imaging, beginning 36 h post-
Function of p190 Rho/RacGAP GTP binding domain

The Phenotype Produced by p190 Is Dose-dependent—To confirm that the lack of effect of p190(S36N) on cell morphology was not a result of expression differences, we transfected both COS and NIH 3T3 cells with a range of plasmid doses. In these experiments 5, 10, or 25 μg of pRK7GFP-p190 plasmid. GFP-p190 images were captured by fluorescence microscopy with a 20× objective lens at the indicated times using a Hamamatsu camera and Open Lab software. Selected images were processed using Adobe Photoshop 4.0, and pixel intensities were inverted to make the thin dendrite-like processes more clearly visible.

The similarity between the beaded extension phenotype induced by overexpression of p190 in NIH 3T3 cells, and that induced by botulinum C3 toxin or RhoGDI (26, 27), argues that this phenotype is a consequence of the p190 RhoGAP activity, which depletes the cell of Rho:GTP (and/or Rac:GTP). To test this hypothesis, we used three independent approaches. First, we ascertained that the GBD domain was not sufficient for induction of the beaded extension phenotype. NIH 3T3 cells were transfected with plasmids expressing the isolated HA-tagged GBD domain, residues 1–266, of wild type p190. Transfected cells were fixed and stained for HA. Immunofluorescence showed that cells expressing HA-GBD appeared normal. Therefore, the GBD of p190 is not responsible for the induction of the beaded extension phenotype.

In the second approach, we created a GAP-defective p190. The GAPs of many small GTPases contain a conserved Arg residue that is essential for catalyzing nucleotide hydrolysis (22, 28). This residue is located at position 1283 in p190 (Fig. 4). As a third, independent approach, we asked whether the co-expression of Rho or Rac could reverse the phenotype induced by p190. We reasoned that a gain-of-function Rho mutant, which is constitutively GTP-bound and is resistant to GAP activity (29, 30), would likely counteract the effects of

p190 RhoGAP Activity Is Essential for Induction of Morphological Changes—The similarity between the beaded extension phenotype induced by the overexpression of p190 in NIH 3T3 cells, and that induced by botulinum C3 toxin or RhoGDI (26, 27), argues that this phenotype is a consequence of the p190 RhoGAP activity, which depletes the cell of Rho:GTP (and/or Rac:GTP). To test this hypothesis, we used three independent approaches. First, we ascertained that the GBD domain was not sufficient for induction of the beaded extension phenotype. NIH 3T3 cells were transfected with plasmids expressing the isolated HA-tagged GBD domain, residues 1–266, of wild type p190. Transfected cells were fixed and stained for HA. Immunofluorescence showed that cells expressing HA-GBD appeared normal. Therefore, the GBD of p190 is not responsible for the induction of the beaded extension phenotype.

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p190 most effectively, but increased levels of wild type Rho might partially reverse the effects also. NIH 3T3 cells were therefore co-transfected with HA-p190 and HA-RhoA, the gain-of-function mutant HA-RhoA(G14V) and, as a negative control, with the dominant-interfering mutant HA-RhoA(S19N).

As can be seen from Fig. 6A and Table I, RhoA(G14V) completely inhibited the induction of beaded extensions by p190. Wild type RhoA caused a partial inhibition, reducing the incidence of beaded extensions from about 25 to 8% of the transfected cells. The RhoA(S19N) mutant produced cells of abnormal phenotype that were difficult to score consistently. The values given are, however, minimum estimates, and the S19N mutant did not block expression of the beaded extension phenotype. Importantly, the co-expression of the RhoA constructs did not perturb the expression of p190 (Fig. 6B). Therefore, inhibition of the beaded extension phenotype was specific to RhoA-GTP function and was not a result of the inhibition of p190 expression. RhoA(S19N) is believed to act by sequestering upstream Rho exchange factors, thereby reducing production of endogenous Rho:GTP. One might therefore predict that...
The GTP Binding Domain (GBD) of p190 Regulates GAP Activity in Vivo but Not in Vitro—Although the GBD is located at the extreme N terminus of p190, and the GAP domain is at the C terminus, one could imagine an allosteric interaction that could allow the GBD to regulate GAP activity directly. To test this possibility, we expressed HA-tagged p190 and the S36N mutant in COS cells, and then immunoprecipitated the proteins from cell lysates and assayed them for GAP activity using recombinant RhoA loaded with [γ-32P]GTP. As shown in Fig. 5, both the wild type and the S36N mutant p190s displayed similar in vitro RhoGAP activities. Immunoblotting of the same lysates revealed similar levels of expression of these two proteins (Fig. 5). Therefore, despite the inability of p190(S36N) to induce the beaded extension phenotype, the mutant is not defective in GAP activity in vitro. We interpret this result to mean that the GBD of p190 modulates p190 GAP activity indirectly, perhaps by interaction with a regulatory factor.

To investigate this hypothesis further, we asked whether the co-expression of the isolated GBD could interfere with p190 function. Cells were therefore transfected with HA-p190 plus wild type HA-p190(1–266) or with HA-p190(1–266)S36N. Both of these isolated GBD fragments expressed efficiently, at molar levels about 10-fold better than that of the full-length HA-p190 (Fig. 1). The unmutated GBD had no effect on the incidence of the beaded extension phenotype induced by p190 (Table II). However, expression of the S36N GBD significantly lowered the percent of cells displaying this phenotype, without reducing the level of expression of p190 (Fig. 7). Our interpretation of these data, which is consistent with the results described previously, is that the GBD of p190 must be in the GTP-bound state to allow GAP function within the cell. The S36N mutation in the GBD, analogous to the S17N mutation of Ras, reduces its affinity for guanine nucleotides but enhances its affinity for a guanine nucleotide exchange factor (GEF). Overexpression of the isolated S36N GBD would sequester this exchange factor, thereby reducing the fraction of full-length p190 in the functional, GTP-bound state.

p190 Inhibits Carbachol-mediated JNK Activation—We wished to test the model described above in an assay that was independent of cell morphology. Gain-of-function mutants of the Rac/Rho family of GTPases can stimulate activity of the Jun N-terminal protein kinase (JNK) (2). Activation of JNK by G-protein-coupled receptors, such as the muscarinic receptors, is also mediated by Rac/Rho GTPases (34). We therefore predicted that the overexpression of HA-p190 would block this activation, by suppressing the formation of Rac/Rho:GTP. A similar block to JNK activation has been demonstrated previously, using the isolated catalytic domain of p190 (31).

To test this prediction, cells expressing the human muscarinic receptor, subtype 1 (hmr1), were transfected with HA-tagged JNK and wild type or S36N mutant p190 and were stimulated with the muscarinic agonist carbachol. JNK was immunoprecipitated from cell lysates and assayed using GST-c-Jun as a substrate. As shown in Fig. 8, carbachol stimulated JNK activity, by suppressing the formation of Rac/Rho:GTP. A similar block to JNK activation has been demonstrated previously, using the isolated catalytic domain of p190 (31).
Function of p190 Rho/RacGAP GTP binding domain

**DISCUSSION**

This study presents the first evidence of a role for guanine nucleotide binding to the N-terminal GBD of the p190 Rho/RacGAP. The isolated C-terminal GAP domain has been shown previously to stimulate the GTPase activity of Rho, Rac, and Cdc42 in vitro and to cause both a loss of stress fibers and cell shape changes consistent with an inhibition of Rho function (8). No linkage between the N-terminal GBD and the C-terminal GAP domains has previously been reported, and in vitro studies have shown that point mutants in the GBD that are defective in GTP binding possess a similar GAP activity to wild type p190 (10). We now demonstrate that, in transfected NIH 3T3 cells, the nucleotide bound state of the p190 Rho/RacGAP GBD influences cellular morphology and JNK activity, effects that are likely to be mediated by the GAP activity of p190.

NIH 3T3 cells transfected with wild type, HA-tagged, p190 Rho/RacGAP exhibited a pronounced rounding of the cell body and beaded, dendritic-like extensions similar to those seen when Rho function is impaired by C3 botulinum toxin or by injection of RhoGDI (26, 27). Remarkably, the expression at similar levels of a mutant p190 defective in GTP binding (S36N) did not induce this phenotype. Wild type p190, but not the S36N mutant, also inhibited the activation of JNK by carbachol.

a nucleotide exchange factor for the p190 GBD. In the absence of a functional exchange factor, the GTP/GDP ratio of the p190 GBD falls, which (by an unknown mechanism) then disables a functional exchange factor, the GTP/GDP ratio of the p190 GAP. The isolated C-terminal GAP domain has been shown previously to stimulate the GTPase activity of Rho, Rac, and Cdc42 in vitro and to cause both a loss of stress fibers and cell shape changes consistent with an inhibition of Rho function (8). No linkage between the N-terminal GBD and the C-terminal GAP domains has previously been reported, and in vitro studies have shown that point mutants in the GBD that are defective in GTP binding possess a similar GAP activity to wild type p190 (10). We now demonstrate that, in transfected NIH 3T3 cells, the nucleotide bound state of the p190 Rho/RacGAP GBD influences cellular morphology and JNK activity, effects that are likely to be mediated by the GAP activity of p190.

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The data therefore argue that the N-terminal GTP binding domain can regulate the GTPase activity toward RhoA either by altering the accessibility of RhoGTP to the GAP domain of p190 due to mislocalization of p190, or via interactions with factors that regulate GAP activity. We have considered each of these hypotheses. To test whether the loss of GTP binding to p190 causes the protein to mislocalize within the cell, such that it is inaccessible to Rho and Rac, we used confocal laser microscopy to visualize cells co-expressing HA-tagged and GFP fusion p190 proteins. However, we were unable to detect any obvious difference in distribution of the two proteins (data not shown).

Another possibility is that p190 wild type and S36N associate differentially with cellular factors. One obvious candidate is the p120 RasGAP which has been shown to be capable of high affinity association with tyrosine-phosphorylated p190 (12–14, 37). We have not been able to detect any regulatory effect of p120 binding on p190 RhoA GAP activity. For example, the co-expression of p120GAP in COS cells with p190 did not significantly alter the specific activity of p190 RhoGAP under conditions in which both proteins were co-precipitated (data not shown). Furthermore, under the conditions used for our in vitro GAP assays, neither wild type nor S36N were associated with detectable amounts of endogenous p120 RasGAP (data not shown). Both the wild type and S36N p190 were tyrosine-phosphorylated to the same level. We conclude that neither the state of p190 tyrosine phosphorylation nor association with p120 GAP are responsible for the effect of ectopic p190 expression or the inability of the mutant p190(S36N) to induce that effect.
In the GDP-bound state, p190 may associate with an inhibitor factor. However, this model predicts that the expression of the S36N mutant GBD would sequester the inhibitory factor and thereby enhance the beaded extension phenotype induced by co-transfecting wild type p190. This was not the case. Rather, the S36N GBD partially suppressed the induction of the beaded extension phenotype which we discuss in further detail below. We cannot, however, discount this model entirely because the factor may be abundant and was not fully sequestered by the mutant GBD.

An alternative model is that p190-GTP associates with an effector or target protein that is a co-activator of the GAP domain. Precedent exists for this model in the Ran GTPase system. For instance, although Ran GAP is a highly efficient catalyst of GTP hydrolysis in vitro, RanBPH1 can stimulate its activity severalfold and appears to be required in vivo (38, 39). In our model the mutant p190(S36N) would not bind the co-activator and so could not function as an efficient GAP within the intact cell. One might argue that co-expression of the isolated GBD would sequester the co-activator and thereby suppress the effect of the wild type p190. However, we find that neither the full-length p190 nor the isolated GBD are exclusively GTP-bound when expressed in COS cells, and it may not be technically feasible to express a high enough concentration of GTP-bound GBD within the cells to sequester a significant fraction of co-activator.

Both models suggest that the p190 GAP activity is controlled by the guanine nucleotide-bound state of its GBD. Guanine nucleotide binding would then be regulated by other factors. A p190 GBD-GAP activity has already been described in lysates of GTP-bound GBD within the cells to sequester a significant fraction of co-activator.

function p190 Rho/RacGAP GTP binding domain

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