The GTPase and Rho GAP Domains of p190, a Tumor Suppressor Protein That Binds the \( M_r 120,000 \) Ras GAP, Independently Function as Anti-Ras Tumor Suppressors

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ABSTRACT

p190 is a Tyr-phosphorylatable G protein of \( M_r 190,000 \) that binds NH\(_2\)-terminal SH2 domains of GAP, a Ras GAP of \( M_r 120,000 \). p190 contains at least two functional domains: a GTPase domain at the NH\(_2\) terminus and a GAP domain at the COOH terminus that can attenuate signal-transducing activity of three distinct G proteins (Rac, Rho, and CDC42). Here, we demonstrate that overexpression of either an antisense p190 RNA or a dominant negative mutant (Asn\(_{36}^+\)) of p190 GTPase domain (residues 1–251) but not the wild-type p190 GTPase domain is able to transform normal NIH/3T3 fibroblasts. Furthermore, overexpression of either the wild-type p190 GTPase domain or the COOH-terminal GAP domain can suppress v-Ha-Ras-induced malignant transformation. These results indicate that p190 contains at least two distinct anti-Ras tumor suppressor domains, the GTPase and GAP domains, and suggest that one of the mechanisms underlying the suppression of Ras-transformation by p190 is the attenuation by p190 GAP domain of Rac/Rho/CDC42 signalings, which are essential for Ras-transformation. In fact, the p190 GAP domain alone suppresses the expression of the c-Fos gene, which is mediated by Rac/Rho/CDC42 and is required for oncogenicity of Ras.

INTRODUCTION

Oncogenic Ras mutants, such as v-Ha-Ras, exert their transforming action by activating several distinct effectors (Ras/GTP-binding proteins), including Raf, GAP1,\(^3\) PI-3 kinase, PKC-\(\zeta\), and Ras-GDS (1–5). Interestingly, a Tyr-phosphorylated protein of \( M_r 190,000 \) called p190 binds the NH\(_2\)-terminal SH2 domains of GAP1 through the phospho-Tyr residues at positions 1087 and 1105 (6, 7), and therefore Ras and p190 are linked through GAP1. The GAP1 SH3 domain flanked by the two SH2 domains is required for Ras to promote the Xenopus oocyte maturation (8) and also for the oncogenicity of Ras (9), and the SH232 fragment of GAP1 alone is indeed oncogenic in mammalian fibroblasts, albeit its oncogenicity is rather weak (10). These findings have suggested the possibility that p190 is involved in the regulation of oncogenic Ras signaling in either positive or negative manners. However, thus far no experimental data have been reported to support or substantiate this notion. In addition to the NH\(_2\)-terminal GTPase domain, which binds GTP or GDP, p190 contains a GAP domain at the COOH terminus that stimulates the intrinsic GTPase activity of Rac, Rho, and CDC42, thereby attenuating their signal-transducing activity (11–14). The recent findings that both Rac and Rho are required for the oncogenicity of Ras and that at least Rac acts downstream of Ras (15, 16) have prompted us to examine whether p190 can interfere with oncogenic action of v-Ha-Ras by either attenuating the oncogenic Rac/Rho signalings, binding the SH2 domains of oncogenic GAP, or both.

Interestingly, a very low GTPase activity of p190 is highly stimulated by an as yet uncharacterized GAP(s) present in mammalian cell extracts (14). This indicates that p190, like other G proteins, requires a specific GEF that can convert the inactive GDP/p190 to the active GTP/p190 for its reactivation. The physiological role of Ras in mitogenic signaling has been extensively studied by overexpressing either an antisense RNA that blocks de novo synthesis of the corresponding Ras, or a dominant negative mutant (Asn\(_{17}^+\)) of Ras that preferentially forms a complex with GDP and can sequester the Ras activators [i.e., Ras GEFs, such as SOS (17, 18)]. Each G protein, regardless of its size, contains a single so-called “hallmark” three-amino acid motif (i.e., Gly-Lys-Ser/Thr) near its NH\(_2\) terminus, in which the third residue, Ser/Thr, controls its affinity for GDP/GTP (19). In small G proteins, such as Ras, Rac, and Rap1, this critical “third” residue corresponds to Ser\(_{17}^+\), whereas in p190 it corresponds to Ser\(_{36}^+\). Replacement of the “third” Ser/Thr by Asn in any G proteins thus far tested converts each molecule to its dominant negative mutant (15–17). Thus, in an attempt to identify the physiological role of p190 in the regulation of cell proliferation, we have overexpressed in normal NIH/3T3 fibroblasts either an antisense p190 RNA or a dominant negative mutant (Asn\(_{36}^+\)) of p190 GTPase domain. This mutant, like Asn\(_{17}^+\) mutants of small G proteins, such as Ras and Rac, is expected to bind predominantly GDP, instead of GTP, and therefore it can sequester the putative GEF for p190. Furthermore, we have overexpressed both the p190 GTPase and GAP domains in v-Ha-Ras-transformed NIH/3T3 cells to see whether each domain alone can interfere with the oncogenic action of Ras.

MATERIALS AND METHODS

Construction of the Plasmids Expressing p190-N, p190-NN, p190-NA, and p190-C (COOH-terminal GAP Domain). Using the rat p190 cDNA (11) as a template for PCR (PCR), we have amplified an EcoRI DNA fragment of 780 bp, which encodes the NH\(_2\)-terminal GTPase domain of the resultant plasmids are called p190-NS, p190-NA, pl9O-NN, and p190-C (COOH-terminal GAP Domain). Using the rat p190 cDNA (11) as a template for PCR (PCR), we have amplified an EcoRI DNA fragment of 780 bp, which encodes the NH\(_2\)-terminal GTPase domain of the resultant plasmids as described previously (20) in both sense (S) and antisense (A) orientations. The orientation of the EcoRI fragment insert nation codon (TGA) at the 3' end. This fragment was subcboned into the EcoRI site of the retroviral vector pMV7 as described previously (20) in both sense (S) and antisense (A) orientations. The orientation of the EcoRI fragment insert nation codon (TGA) at the 3' end. This fragment was subcboned into the EcoRI site of the retroviral vector pMV7 as described previously (20) in both sense (S) and antisense (A) orientations. The orientation of the EcoRI fragment insert nation codon (TGA) at the 3' end. This fragment was subcboned into the EcoRI site of the retroviral vector pMV7 as described previously (20) in both sense (S) and antisense (A) orientations. The orientation of the EcoRI fragment insert nation codon (TGA) at the 3' end. This fragment was subcboned into the EcoRI site of the retroviral vector pMV7 as described previously (20) in both sense (S) and antisense (A) orientations. The orientation of the EcoRI fragment insert.

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3 The abbreviations used are: GAP1, Ras GAP of \( M_r 120,000 \); GEF, GTP/GDP exchange factor; JNK, Jun kinase; MEK, MAP kinase kinase; PDBu, phosphoribulose isomerase; PIP2, phosphatidylinositol; PIP3, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; p190-C or p190-GAP or RHG, COOH-terminal GAP domain of p190; p190-NN, NH\(_2\)-terminal GTPase domain; p190-NA, antisense p190 NH\(_2\)-terminal domain; p190-NN, Asn\(_{36}^+\) mutant of p190 NH\(_2\)-terminal domain; p190-NS, sense p190 NH\(_2\)-terminal domain; RT, reverse transcription; SH232, the SH2-SH3-SH3 fragment of GAP1.
Fig. 1. A. Expression of antisense p190 DNA and p190-NN in NIH/3T3 transfected. The steady-state levels of either p190-NA, p190-NN, or p190-NS mRNAs in the corresponding transfecteds were estimated by a semiquantitative RT-PCR analysis using total RNAs extracted from each clone as described in “Materials and Methods.” 800, 450, and 450S, p190-NA, -NN, and -NS PCR products, respectively; S, 1-kb standard DNA ladder. Left, Lanes 1–5, p190-NSs 40, 41, 43, 49, and 51; Lanes 6–9, p190-NAs 40, 42, 45, and 46; Lane 10, no transfection; Lanes 11–14, pMV7 vectors a and b alone; Lane S, standard DNA marker. Right, Lanes 1–4, p190-NNs 1, 10, 4, and 12; Lane 5, no transfection; Lane 6, pMV7 vector alone; Lane S, standard DNA marker. To detect the exogenous rat p190-NS, -NA, or -NN mRNAs exclusively without amplifying the endogenous mouse full-length p190 mRNAs, we used the antisense PCR primer a specific oligonucleotide corresponding to the sequence approximately 50 bp downstream of the EcoRI insertion site of the vector pMV7. Furthermore, as the sense PCR primers to amplify both the p190-NS and -NN mRNAs, we used a p190 oligonucleotide called NG120, which starts at the 120th codon, and to amplify the 190-NA mRNA, we used a p190 oligonucleotide called NG251, which starts at the 251st codon. Thus, the RT-PCR products of p190-NS and -NN mRNAs are approximately 450 bp long, whereas that of p190-NA mRNA is approximately 800 bp long. B, the antisense p190 DNA transfection greatly reduces the accumulation of full-length p190. As described in “Materials and Methods,” the steady-state protein levels of full-length p190 in both p190-NA transfecteds and the parental normal 3T3 cells were estimated by an immunoblot analysis of each cell lysate (20 μg of protein) using an antibody specific for p190. Lanes 1 and 2, the parental normal NIH/3T3 cells without and with pMV7 vector-alone transfection, respectively; Lane 3, p190-NN transfected; Lane 4, p190-NS transfected; Lane 5–8, p190-NA transfecteds 40, 42, 45, and 46; Lane 10, the position of full-length p190 protein band. C, expression of p190-NS and v-Ha-Ras genes in the p190-NS transfecteds. The steady-state levels of both p190-NS (left) and v-Ha-Ras (right) mRNAs were estimated by a semiquantitative RT-PCR analysis as described in Fig. 2A. 450S, p190-NS PCR product; 600, v-Ha-Ras PCR product; S, 1-kb standard DNA ladder. Left (panel): Lanes 1–6, p190-NS transfecteds A1, A9, B1, B8, B10, and C1; Lane 7, no transfection; Lanes 8–9, pMV7 vector alone and b. Right, Lane 1, no transfection; Lane 2, pMV7 vector alone; Lanes 3–9, p190-NS transfecteds A1, A9, B1, B8, B110, C1, and D1; Lane 10, normal NIH/3T3 cells. Lane 11, v-Ha-Ras DNA template (1 μl). D, autoradiogram for v-Ha-Ras GTPase in the p190-NS transfecteds. As described in “Materials and Methods,” v-Ha-Ras in the cell lysate, each containing 100 μg of protein, derived from the parental v-Ha-Ras transfecteds or p190-NS transfecteds was immunoprecipitated and autophosphorylated, and the radioactive v-Ha-Ras band (arrow) was visualized. Lanes 1 and 2, parental v-Ha-Ras transfecteds without and with pMV7 vector alone transfection; Lanes 3–9, p190-NS transfecteds; Lane 10, normal NIH/3T3 cells (negative control).

(GAP or RHG). These plasmids were purified by CsCl density gradient centrifugation and used for transfection.

**Assay for Colony-forming Ability in Soft Agar of Normal and v-Ha-Ras-transformed NIH/3T3 Cells Transfected with the p190-NA and p190-C Plasmids.** The normal NIH/3T3 fibroblasts were transfected with either p190-NS, p190-NA, p190-NN, or the vector alone (1 μg each) as complexes with liposomes (35 μg) as described previously (20, 21). The v-Ha-Ras transformants were also transfected with either p190-NS, p190-NA, or the vector alone as described above. The resultant G418-resistant transfecteds were cloned in the presence of 400 μg/ml G418 (a neomycin analogue) as described previously (20). The colony-forming ability of the parental clones (normal and v-Ha-Ras-transformed) and each transfected was examined in soft agar by incubating 1000 cells/plate at 37°C for 2 or 3 weeks under the standard culture conditions (20) in the presence or absence of PDBu.

**RT-PCR Analysis of p190 and v-Ha-Ras mRNAs.** Total RNAs from each transfected clone were isolated as described previously (22, 23). Five μg of each RNA preparation were then used as a template to synthesize cDNA (23). The same aliquots of all cDNA preparations were used as templates for PCR to selectively amplify rat p190-NS, -NA, -NN, and -C and v-Ha-Ras DNA sequences using the corresponding unique pairs of sense (the exogenous Kozak sequences) and antisense primers (23). Under these PCR conditions, the amount of PCR products is linearly proportional to the initial amount of cDNA used as a template and is far below the saturation point (23).

**Autokinase Assay for v-Ha-Ras GTPase.** Unlike normal Ras GTPases, v-Ha-Ras GTPase, which uniquely contains Thr at position 59, is autophosphorylated at this position during the hydrolysis of GTP (24). According to the previously described autokinase assay procedures (25), the cell lysate containing 100 μg of protein prepared from either the parental v-Ha-Ras transformants or p190-NS transfecteds was subjected to immunoprecipitation with anti-Ras monoclonal antibody Y 1 3—259, and then each immunoprecipitate, resuspended in kinase buffer (50 mM Tris-HCI, pH 7.5, 5 mM MgCl2, and 1 mM DTT), was subjected to the autokinase assay with 10 nm [γ32P] GTP at 37°C for 30 min and to 15% SDS-PAGE. The autophosphorylated v-Ha-Ras band was visualized by using a PhosphorImager (Molecular Dynamics).

**Immunoblot Analysis of p190 Levels in Antisense p190 DNA Transfectants and c-Fos/v-Ha-Ras Levels in p190-C Transfectants.** Cells (106) of each transfected were suspended in 350 μl of 2 × SDS sample buffer and heated at 95°C for 15 min, and the total proteins (20 μg) in each sample were separated by 7% SDS-PAGE with a prestained protein marker (NOVEX-San Diego, San Diego, CA) as a molecular weight standard. The subsequent Western transfer was performed in a semi-dry blotter (Novex, Australia) by using polyvinylidene difluorde membrane (Millipore, Bedford, MA) at 120 mA for 2.5 h as described previously (26). The membrane was first blocked with PBS buffer (10% skim milk powder, 0.05% Tween 20 in PBS) at 4°C overnight, and then washed with PBSS buffer (0.1% BSA, 0.05% Tween 20 in PBS) three times for 7 min each.

For assaying the p190 levels, the washed membrane was incubated with 5 ml of 0.1% (1:1000) rabbit antiserum against p190 in PBS at 25°C for 90 min and washed with PBSS three times for 20 min each. The resultant membrane was further incubated with 5 ml of 0.033% (1:3000) goat antirabbit IgG.
conjugated with horseradish peroxidase (BioRad) at 25°C for 40 min and washed with PBSB three times for 20 min each. For assaying c-Fos or v-Ha-Ras levels, the washed membrane was incubated with a diluted (1:20) monoclonal antibody against c-Fos or v-Ha-Ras (OncoGene Science) in a similar manner. After being washed with PBSB, the membrane was further incubated with a goat antimouse IgG conjugated with horse radish peroxidase (DAKO Corp.) and washed with PBSB. These membrane were then incubated with ECL Western blotting detection reagent (Amersham Corp., United Kingdom) at 25°C for 1 min and exposed to XAR5 film (Kodak) for 12 min.

**Assay for Rho GAP Activity of p190 GAP Domain.** Cells (10^6) of each p190-C transfecant or parental v-Ha-Ras-transformants were disrupted in a RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP40, 0.5% deoxycholate, 100 μM sodium vanadate; 50 μM leupeptin, and 40 units/ml aprotinin). After a brief sonication, each homogenate was centrifuged at 10,000 × g for 10 min, and the resultant supernatant (extract) was collected. The protein concentrations were determined by the Bradford method (27). Rho GAP activity of each extract (20 μg of protein) was assayed according to the standard charcoal method (13), by incubating [γ-32P]GTPγRho complex with ECL Western blotting detection reagent (Amersham Corp., United Kingdom) at 25°C for 1 mm and exposed to XAR5 film (Kodak) for 12 min.

**RESULTS**

**Oncogenicity of an Antisense p190 RNA and a Dominant Negative Mutant of the p190 GAP Domain.** In an attempt to examine whether the NH2-terminal GTPase domain of p190 (p190-N) is oncogenic or is required for normal cell growth, or alternatively whether it acts as a tumor suppressor, using a retroviral vector called pMV7, we have transfected normal NIH/3T3 fibroblasts with the following three distinct DNAs derived from rat p190 cDNA (11): (a) p190-NS DNA encoding the wild-type p190 GTPase domain (residues 1–251) in the sense direction; (b) p190-NN DNA encoding a dominant negative mutant of p190 GTPase domain in which Ser→Asn is replaced by Asn; and (c) p190-NA DNA encoding the p190 GTPase domain but in the antisense direction (antisense p190 DNA). As negative controls, the cells were also transfected with the vector alone, which expresses only G418 resistance selectable marker. Among the resultant transfectants that are G418 resistant, more than 50 distinct clones were isolated in each category, and the levels of the corresponding mRNA in each transfectant was first scanned by RT-PCR as described under "Materials and Methods," and several high-expressors in each category were selected out for further detail analysis (Fig. 1A). The ability of these high-expressors to form colonies in soft agar (anchorage-independent growth) was then examined, because this in vitro phenotype is most tightly associated with the ability of malignant cells to develop tumors in animals. We found that the clones overexpressing either the p190-NA RNA or p190-NN are able to form colonies in soft agar, although both size and number of colonies are significantly smaller than those derived from v-Ha-Ras-transformed cells (Fig. 2A and Table 1, compared with Fig. 2B and Table 2). However, like the control (vector alone) transfectants, none of the clones overexpressing the sense wild-type p190 GTPase domain (p190-NS) formed any colony in soft agar (see Table 1). These observations suggest that the sense wild-type p190 GTPase domain alone is not oncogenic at all (or its expression level is still not high enough for malignant transformation); instead, both the antisense p190 and Asn mutant transfectants are potentially malignant. RT-PCR analysis has confirmed that both the antisense and sense RNAs corresponding to the NH2-terminal GTPase domain of p190, as well as its dominant negative mutant, are highly expressed in these selected transfectants (Fig. 1A). Furthermore, immunoblot using rabbit anti-p190 polyclonal antibody (13) has also confirmed that overexpression of the antisense p190 RNA greatly reduces the steady-state protein level of the endogenous mouse full-length p190 in each high antisense expression (Fig. 1B). Because no proper antibody detecting the p190 GTPase domain alone has been available as yet, we were unable to estimate the protein levels of the p190 GTPase domain (wild-type or mutant) in the corresponding high-RNA expressors.

Tumor-promoting agents, such as 12-O-tetradecanoylphorbol-13-acetate or other phorbol esters, can activate several PKC isotypes (except PKC-μ) that phosphorylate and activate the Ser/Thr kinase Raf, a downstream effector of Ras, and have been shown to potentiate the oncogenicity of v-Ha-Ras in mice (28), the NH2-terminal SH2-SH3-SH2 domain of GAP1 (10) and full-length Raf in fibroblasts such as NIH/3T3 cells (29). Thus, we have examined the effect of a tumor promoter called PDBu on the soft agar colony-forming ability of both p190-NA and p190-NN transfectants. PDBu (50 ng/ml) greatly increased both the size and the number of colonies derived from the antisense p190 transfectants to levels that are almost indistinguishable from those of v-Ha-Ras-transformed cells (Fig. 2A and Table 1, compared with Fig. 2B and Table 2). PDBu also significantly enhanced the malignant phenotype of the Asn mutant transfectants but showed no significant effect on either the normal NIH/3T3 fibroblasts or the wild-type p190 GTPase domain transfectants. These additional observations more convincingly indicate that not only full-length p190 but also its wild-type NH2-terminal GTPase domain alone must act as potent tumor suppressors. It is noteworthy that oncogenicity of the antisense p190 DNA appears to be not only higher than that of the Asn mutant, at least in the presence of PDBu, but also more sensitive to the tumor-promoting action of PDBu (Fig. 2A and Table 1), suggesting the possibility that an additional domain(s) other than the NH2-terminal GTPase domain, perhaps the COOH-terminal GAP domain, also contributes to the tumor suppressor activity of p190 in a phorbol ester-sensitive manner.

**Anti-Ras Action of the p190 GAP Domain (p190-C).** Because the p190 COOH-terminal domain (residues 1186–1513) is a GAP for Rac/Rho/CDCA42 GTPases (11–14), it can attenuate the transducing activity of Rac and Rho, which are required for the oncogenicity of Ras (15–16). Thus, it is conceivable that the p190 GAP domain alone
is sufficient to suppress malignant transformation caused by oncogenic Ras mutants such as v-Ha-Ras. Several pGAP-C transfectants derived from v-Ha-Ras transformants that express a high level of p190-GAP mRNA were screened by RT-PCR analysis (Fig. 3A), confirming that the p190-GAP fragment in these transfecants was found to be much higher than that in the parental v-Ha-Ras transformants (see Fig. 3B), as summarized in Table 2, overexpression of p190-GAP fragment strongly suppresses the anchorage-independent growth in soft agar under the conditions where the control vector alone has no effect on the growth of v-Ha-Ras transformants. Furthermore, an immunoblot analysis shows that these p190-GAP overexpressors still maintain the essentially same levels of v-Ha-Ras as the parental cells (see Fig. 4A). However, c-Fos protein levels are drastically reduced in these p190-GAP overexpressors compared with those in the parental v-Ha-Ras transformants (see Fig. 4B). These observations clearly indicate that (a) the p190 GAP domain alone is a tumor suppressor that blocks oncogenic action of v-Ha-Ras, and (b) this GAP domain exerts its anti-Ras action by blocking a downstream Rac/Rho/CDC42 signaling pathway(s) which leads at least to the activation of c-Fos gene.

Anti-Ras Action of the Wild-Type p190 GTPase Domain. We have shown recently that overexpression of the NH2-terminal SH232 domain of GAP1 also can convert the normal rat 3Y1 fibroblasts to weakly malignant cells that can form only small colonies in soft agar and that the malignancy is significantly enhanced by the phorbol ester PDBu (10). This SH232 effect is reminiscent of those of both the Asn36 mutant and antisense DNA of p190 GTPase. Because GAP1 is a downstream target of Ras and the SH2 domain of GAP1 binds Tyr-phosphorylated p190, it is conceivable that both Ras and the GAP1 SH232 domain can transform the normal cells in part by interfering with the tumor suppressor action of p190 GTPase.

In an attempt to examine this possible anti-Ras action of the p190 GTPase domain, we have transfected v-Ha-Ras-transformed NIH/3T3 cells with the sense wild-type GTPase domain of p190 (p190-NS) or just the vector pMV7 alone. Again, more than 50 distinct G418-resistant transfectants in each category were cloned, and several p190-NS high-expressors were selected out by RT-PCR analysis (Fig. 1C) for further characterization, such as their colony forming ability in soft agar. As shown in Fig. 2B and Table 3, the Ras-transfomers lost almost completely their anchorage-dependent growth upon overexpression of p190-NS, and their anchorage-independent growth was completely suppressed.

Table 1

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*Large, more than 100 cells/colony; medium, around 30 cells/colony; small, fewer than 10 cells/colony. Each value was the average of the data from three independent experiments, and the SD in each case was < 5%.

Fig. 3. A, expression of p190-GAP mRNA in p190-C transfectants derived from v-Ha-Ras-transformants. The steady-state levels of p190 mRNA in p190-C (RHG) transfectants (Lanes 3–7) are compared with those of the parental v-Ha-Ras-transformants transfected with the vector alone (Lane 2) by RT-PCR analysis as described in "Materials and Methods." Lane 1, RHG C without reverse transcriptase (control); Lane 3, clone RHG A; Lane 4, clone RHG B; Lane 5, clone RHG C; Lane 6, clone RHG D; Lane 7, clone RHG E. Lane M, DNA size marker (1 kb ladder). Arrow, the PCR product corresponding to p190-C cDNA of 1.0 kb. B, Rho GAP activity in the extracts from p190-GAP (RHG) overexpressors. Extracts were prepared from v-Ha-Ras-transfomers transfected with the vector alone (pMV7) or with p190-C (RHG A–E), and assayed for the Rho GAP activity as described in "Materials and Methods." Columns, average values obtained from three independent assays; SD < 5%.
expression of the wild-type p190 GTPase domain. The antisense p190 RNA expression (which is potentially oncogenic) showed no significant negative effect on the anchorage-independent growth of the parental Ras-transformants in the absence of PDBu (data not shown). Furthermore, both RT-PCR analysis and autokinase assay for v-Ha-Ras GTPase have confirmed that these p190-NS high-expressors still maintain both v-Ha-Ras mRNA and enzymatic activity levels as high as the parental transformants and vector-alone transfectants (Fig. 1, C and D), indicating that the observed reversion of transformation is due not to a loss of v-Ha-Ras expression but to overexpression of the GTPase domain of p190. The above findings provide the first direct evidence indicating that p190 GTPase domain alone can interfere with the oncogenic action of v-Ha-Ras at least, but the detailed mechanism underlying its anti-Ras or antioncogenic action and its target specificity still remains to be clarified in the future.

On a solid substratum, these p190-NS overexpressors are still able to grow as rapidly as the parental Ras-transformants in a liquid culture until they form a confluent monolayer. However, unlike the parental transformants, these p190-NS expressors form no detectable foci by piling up on top of each other (data not shown), indicating that overexpression of p190-NS is not cytotoxic, but simply restores the contact inhibition of cell growth (which is lost in the parental Ras-transformants), as does overexpression of several other anti-Ras tumor suppressor genes encoding NF2 (23), F-actin cappers such as gelsolin and tensin (9, 30), and Ras-binding fragments of NF1 and Raf (22, 31).

**DISCUSSION**

Here we have demonstrated that overexpression of either the wild-type p190 GTPase domain (p190-N, residues 1–251) or GAP domain (p190-C, residues 1186–1513) can suppress v-Ha-Ras-induced malignant transformation of NIH/3T3 fibroblasts. Furthermore, we have shown that (a) the normal NIH/3T3 cells can be transformed by overexpression of either p190-NA or a dominant negative mutant of the p190 GTPase domain (p190-NN), and (b) in the presence of a phorbol ester, the oncogenicity of p190-NA, which blocks the de novo synthesis of full-length p190, appears to be significantly higher and more PDBu-sensitive than that of p190-NN, which could block only the function of the GTPase domain. These findings clearly indicate that (a) the p190 is a novel tumor suppressor, and (b) it contains at least two distinct anti-Ras tumor suppressor domains, the NH2-terminal GAP and COOH-terminal DNA-binding domain alone, suppresses Ras-induced malignant transformation (33). Thus, it is quite conceivable that p190 GAP domain exerts its anti-Ras action by blocking the Rac/CDC42-mediated activation of JNK. Here, we have shown that p190 GAP domain suppresses both c-Fos gene expression and Ras-induced malignant transformation. This finding strongly supports the notion that c-Fos is also essential for oncogenicity of Ras. Interestingly, c-Fos and c-Jun form a heterodimer called AP1–1, which is required for the activation of various genes, including c-Jun gene (34). Furthermore, oncogenic Ras mutants activate the c-Fos gene through at least two

---

**Table 2** p190 GAP domain alone suppresses v-Ha-Ras-induced malignant phenotype

<table>
<thead>
<tr>
<th>Clone</th>
<th>No. of colonies</th>
<th>Suppression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental v-Ha-Ras transformants</td>
<td>960 (large)</td>
<td>0</td>
</tr>
<tr>
<td>Vector alone transfectants</td>
<td>930 (large)</td>
<td>3</td>
</tr>
<tr>
<td>p190-GAP transfectants:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone A</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Clone B</td>
<td>4 (small)</td>
<td>99.5</td>
</tr>
<tr>
<td>Clone C</td>
<td>40 (small)</td>
<td>96</td>
</tr>
<tr>
<td>Clone D</td>
<td>15 (small)</td>
<td>98.5</td>
</tr>
<tr>
<td>Clone E</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*a* Both the criteria for classification of the colony size and the SD of the data presented in this table were essentially the same as those described in Table 1.

---

**Fig. 4.** Expression of v-Ha-Ras and c-Fos in p190-GAP overexpressors derived from v-Ha-Ras-transformed NIH/3T3 cells. The steady-state protein levels of v-Ha-Ras (A) and c-Fos (B) were estimated by immunoblot analysis of each cell lysate (20 μg of protein) as described in “Materials and Methods.” V, v-Ha-Ras-transformants (parental); P, vector (pMV7) alone transfected; C, p190-C transfectant RHG C; E, p190-C transfectant RHG E.
distinct pathways: (a) Ras-GAP (SH2/SH3/SH2); and (b) Ras-Raf MEK-MAP kinase/Elk 1/serum responsive factor (35, 36). A third pathway involved in the activation of the c-Fos gene includes Rho/Rac/CDC42, which activates serum responsive factor (37). Thus, it is most likely that p190 GAP domain exerts its anti-Ras action by blocking a Rho/Rac/CDC42-mediated activation of the c-Fos gene. It was also demonstrated, however, that Rac activates PI-4 kinase, which producesPIP2 (38). The resultant PIP2 then binds profilin, dissociates the profilin/actin monomer complex, which caps the barbed end of actin filament (F-actin), and consequently uncaps this end (38). The Rac-induced uncapping allows the actin filament to elongate further by adding actin monomers at this end. Rac activates PI-3 kinase by binding its catalytic subunit p110 (2), and PI-3 kinase is required for the Rac activation (15). Furthermore, Ras induces the activation of Rac (15), and in turn, Rac induces membrane ruffling (39). Thus, it is reasonable to assume that Ras also can induce uncapping of F-actin barbed ends through the action of PI-3 kinase/Rac/PI-4 kinase/PIP2 signaling cascade. In other words, if the uncapping of F-actin is required for oncogenicity of Ras, the recapping of F-actin by either barbed end cappers, such as gelsolin, tensin, and cytochalasins, or PIP2-sequesters, such as gelsolin, cofilin, and profilin, could suppress Ras-induced malignant transformation. In fact, several F-actin cappers, such as a gelsolin mutant, tensin, and cytochalasins, as well as PIP2-binding mutants of cofilin, are capable of suppressing v-Ha-Ras-induced malignant transformation of NIH/3T3 cells (9, 30). These observations clearly indicate that the Rac-induced uncapping of F-actin is required for oncogenicity of Ras, and they further suggest the possibility that barbed-end-capping drugs could be potentially useful for the treatment of Ras-associated cancers, which represent more than 30% of total human carcinomas. We have shown here that the tumor suppressor p190 blocks Ras signaling, in part through the GAP domain (p190-C), which attenuates Rac, thereby blocking membrane ruffling. Interestingly, a novel anticancer drug called SCH51344, which suppresses Ras-induced malignant transformation (40), also blocks Ras/Rac-induced membrane ruffling.4

How can the tumor suppressor p190-N (GTPase domain) interfere with the oncogenic action of Ras? Because p190-N is a GTP-dependent signal transducer (G protein),5 it is most likely that the p190-N/GTP complex exerts its anticancerogenic effects by activating its downstream effectors (p190-N/GTP binding proteins) in the same manner that the Ras/GTP complex exerts its oncogenic effects by activating several distinct effectors of Ras such as Raf, GAP1, PI-3 kinase, PKC-ε, andRal-GDS (1–5). Thus far the only proteins that have been shown to bind the active form of this novel signal transducer (i.e., the p190-N/GTP complex) are as yet uncharacterized p190 GTPase-activating proteins called GAPXs, which are present in mammalian cell extracts (14). Although the GAPXs are considered attenuators of p190-N signal-transducing activity as they convert the GTP/p190-N to the inactive GDP/p190-N, it is also quite possible that GAPXs serve as downstream effectors of p190-N because GAP1 serves as both attenuator and effector of Ras (1). In an attempt to understand the molecular mechanism underlying the anti-Ras action of p190-N, we are currently purifying and characterizing GAPXs and a few other p190-N/GTP binding proteins, using the yeast two-hybrid system or the p190-N/GTP/GST fusion protein. Thus far, microinjection of p190-N has no effect on either Rac-dependent membrane ruffling (39) or activation of JNK kinase cascade (32, 41).6 Furthermore, p190-N has no effect on the best-characterized Ras/Raf/MEK/MAP kinase cascade, and therefore it is likely that p190-N regulates another as yet uncharacterized Ras-mediated pathway(s) by a novel mechanism. Because v-Src requires Ras for its oncogenicity (1), it is most likely that p190-N interferes with v-Src-induced malignant transformation, also.

In the past, tumor suppressor genes or candidates, such as the RB, NFI, NF2, APC, DCC, and p53 genes, were identified initially on the basis that either their loss or their mutations are found tightly associated with the development of tumors in specific tissues, such as eyes, brain, and colon, of cancer patients. However, in addition to p190, several other tumor suppressor gene products that bind actin filament, such as gelsolin, tensin, HS1, cofilin, α-actinin, and vinculin, were identified recently by their ability to suppress malignant transformation of NIH/3T3 cells caused by oncogenic Ras mutants or SV40 virus (9, 28, 42, 43). Most of these cytoskeletal tumor suppressors are either barbed end cappers or PIP2-sequesters. It would be of great interest to examine whether either loss, disruption or mutations of a human p190 gene and several other antioncogenes encoding F-actin binders are tightly associated with the tumor development in pancreas or colon of patients in whom the majority of Ras-associated carcinomas have been found.

Although we have shown that both the GTPase and GAP domains of p190 independently suppress Ras transformation and that full-length p190 is a tumor suppressor, whether overexpression of full-length p190 also can suppress Ras-transformation still remains to be clarified. The biological function of the remaining large domain (residues 252–1185) of p190 that lies between the GTPase and GAP domains is thus far entirely unknown. This third domain might affect the anti-Ras tumor suppressor activity of both the GTPase and GAP domains. The anti-Ras action of full-length p190 is under investigation. We realized recently that the GRF-1 cDNA cloned previously from a human breast cancer cell line (MCF-7) is a variant or mutant of the mammalian p190 (type A) cDNA (11, 44). Because the GRF-1 cDNA lacks the third base (G) of the 388th codon and the first base

\[ \text{Suppression (\%)} \]

<table>
<thead>
<tr>
<th>Clone</th>
<th>Small</th>
<th>Medium</th>
<th>Large</th>
<th>Total</th>
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<tr>
<td>No transfection</td>
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<td>112</td>
<td>51</td>
<td>810</td>
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<tr>
<td>+ Vector alone</td>
<td>597</td>
<td>121</td>
<td>42</td>
<td>760</td>
</tr>
<tr>
<td>p190-NS clones:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Clone B10   | 15    | 24     | 24    | 63    | 92   
| Clone A 1   | 32    | 23     | 21    | 76    | 90   
| Clone B 8   | 48    | 41     | 8     | 97    | 88   
| Clone A 9   | 65    | 25     | 12    | 102   | 87   
| Clone D 1   | 55    | 40     | 26    | 121   | 85   
| Clone C 1   | 76    | 38     | 27    | 141   | 83   
| Average     | 49    | 32     | 19    | 100   | 87   

a Both the criteria for classification of the colony size and the SD of the data presented in this table were essentially the same as those described in Table 1.

4 C. C. Kumar, personal communication.
5 R. Roof, personal communication.

\[ \text{Unpublished observation.} \]
(C) of codon 1167 of the full-length p190-A CDNA, the gene product contains only residues 389–1166 of p190-A and the extra 55 frame-shifted amino acids and lacks entirely both the GTPase and GAP domains that are tumor suppressors. Thus, it would be interesting to examine whether this tumor cell line expresses the full-length p190-A gene, in addition to its truncated variant/mutant (GRF-1).

ACKNOWLEDGMENTS

We are grateful to Sarah Parsons and Shintaro Iwashita for antibodies specific for p190. Richard Roof for unpublished information on GDP/GTP-binding of p190 GAP domain; Tony Burgess for consistent encouragement specific for p190; Richard Roof for unpublished information on GDP/GTP activation of v-Ha-Ras is modulated by amino acid residue 12. Proc. Natl. Acad. Sci. USA, 81: 2674–2678, 1984.


The GTPase and Rho GAP Domains of p190, a Tumor Suppressor Protein That Binds the \( M_\text{r} \) 120,000 Ras GAP, Independently Function as Anti-Ras Tumor Suppressors


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