Differential antagonism of Ras biological activity by catalytic and Src homology domains of Ras GTPase activation protein

(CAAX motif/transformation/transactivation)

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ABSTRACT Ras p120 GTPase activation protein (GAP), a cytosolic protein, is a negative mediator and potential downstream effector of Ras function. Since membrane association is critical for Ras function, we introduced the Ras membrane-targeting signal (a 19-residue peptide ending in CAAX, where C = cysteine, A = aliphatic amino acid, and X = any amino acid) onto the GAP N-terminal Src homology 2 and 3 and the C-terminal catalytic domains (designated nGAP/CAAX and cGAP/CAAX, respectively) to determine the role of membrane association in GAP function. cGAP/CAAX and full-length GAP/CAAX, but not GAP or nGAP/CAAX, exhibited potent growth inhibitory activity. Whereas both oncogenic and normal Ras activity were inhibited by cGAP/CAAX, nGAP/CAAX, despite lacking the Ras binding domain, inhibited the activity of oncogenic Ras without affecting the action of normal Ras. Altogether, these results demonstrate that membrane association potentiates GAP catalytic activity, support an effector function for GAP, and suggest that normal and oncogenic Ras possess different downstream interactions.

Ras proteins function as molecular switches, via a regulated GDP/GTP cycle, to modulate signal-transduction pathways that control cell growth and differentiation (1). The Ras GDP/GTP cycle is controlled by proteins that stimulate guanine nucleotide dissociation (e.g., CDC25 and SDC25) to form the active, GDP-complexed protein (2–6) and by proteins that stimulate Ras GTPase activity [Ras p120 GTPase activation protein (GAP) and neurofibromin] to form the inactive, GTP-complexed protein (7, 8). Oncogenic Ras proteins are growth factor- and p120 GAP- and neurofibromin-stimulated GDP hydrolysis and consequently persist in the active, GTP-complexed form. However, since mutations in the Ras effector domain that block GAP binding also abolish transforming activity (9, 10), GAP may also serve as an essential downstream effector for mediating a Ras signal-transduction pathway (7, 8).

GAP can be divided into two functional domains. The N-terminal region, which contains the Src homology (SH) 2 and 3 sequences shared among nonreceptor tyrosine kinases and other proteins (11), may serve a regulatory function and promote interaction with specific phosphorytrosine-containing proteins such as the GAP-associated p62 and p190 phosphoproteins (12). The C-terminal catalytic domain contains the Ras binding domain and is sufficient for stimulating Ras GTPase activity (13). Thus the two domains of GAP may serve to promote a functional linkage between Ras and other signaling components to facilitate Ras-triggered cellular proliferation.

Whereas GAP is a predominantly cytosolic protein (14, 15), Ras proteins display a tight plasma membrane association, which is essential for transforming activity (16–18). Ras membrane association is triggered by three tightly linked posttranslational modifications (farnesylation, proteolysis, and carboxyl methylation) that are signaled by a consensus C-terminal CAAX (C = cysteine, A = aliphatic amino acid, and X = any amino acid) sequence present in all Ras proteins (16–18). These C-terminal modifications, together with sequences containing lysine residues or palmitoylated cysteine residues immediately upstream of the CAAX sequence, target Ras proteins specifically to the plasma membrane (19, 20). Since membrane association is critical for Ras function, and since translocation of GAP to the plasma membrane has been observed upon mitogenic stimulation of cells (21), GAP membrane association is presumably important in modulating its interaction with Ras. However, whether membrane association is important for GAP function and how membrane association influences the Ras–GAP interaction have not been determined. To address these questions, we have generated membrane-targeted forms of full-length GAP and of the individual N-terminal regulatory and C-terminal catalytic domains of GAP and determined their biological activities in NIH 3T3 cells. We observed that membrane association potentiated the negative regulatory function associated with the GAP C terminus, whereas the N terminus was found to specifically antagonize oncogenic, but not normal, Ras activity in a membrane-independent fashion.

MATERIALS AND METHODS

Molecular Constructs of Ras GAP. Taq polymerase chain reaction DNA amplification using synthetic oligonucleotides was performed to introduce BamHI restriction sites and 5′ ATG initiation or 3′ termination codons into the human p120 Ras GAP cDNA (14) to generate sequences encoding either the N-terminal (residues 1–666; designated nGAP) or C-terminal (residues 705–1047; designated cGAP) domains of Ras GAP. Additionally, constructs encoding membrane-targeted variants of GAP were generated by introducing the sequence encoding the 19 C-terminal residues of K-Ras4B into the C termini of nGAP (designated nGAP/CAAX), cGAP (cGAP/CAAX), and full-length GAP (GAP/CAAX). All mutated sequences were confirmed by dideoxy sequencing and introduced into the pZIP-NeoSV(X)1 retrovirus vector for expression in mammalian cells (22).

Cell Culture and Transfection Analysis. NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum and transfected by the calcium phosphate method as described (23). Transfected cultures were either subcultured in growth medium containing G418 (Geneticin; Gibco/BRL) at 400 μg/ml to isolate stably transfected cells

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Abbreviations: GAP, GTPase activation protein; SH, Src homology; CAT, chloramphenicol acetyltransferase; nGAP, N-terminal domain (residues 1–666) of GAP; cGAP, C-terminal domain (residues 705–1047) of GAP; neoφ, neomycin resistant; neoφ, neomycin sensitive. To whom reprint requests should be addressed.
expressing each GAP mutant or, alternatively, maintained in growth medium for 14–20 days for quantitation of foci of morphologically transformed cells. The drug-resistant colonies either were visualized by staining with crystal violet or were subcultured for analysis of protein expression. To determine the ability of different oncogenes to rescue cGAP/CAAX growth inhibition, we attempted to isolate G418-resistant colonies from cultures that were cotransfected with 50 ng of pZIP-cGAP/CAAX DNA [neomycin resistant (neo)] and a 40-fold molar excess of neomycin-sensitive (neo) expression plasmid constructs encoding transforming v-Src (psrc11), human Raf (p22W-raf), or Fos (pSM3-fos). To determine if each GAP variant was able to inhibit oncogenic Ras focus-forming activity, the focus-forming activity was determined for cells that were either transfected with 10 ng of a plasmid construct that encodes oncogenic H-Ras(12V) [pUC-ras(12V)] or cotransfected with pUC-ras(12V) and 2 μg of pZIP-GAP DNA encoding each GAP mutant sequence.

Subcellular Localization of GAP Proteins. G418-selected cells expressing the appropriate protein were metabolically labeled overnight with 200 μCi of [35S]methionine/cysteine (Tran35S-label; ICN). Fractionation of cells into crude membrane- (P100) and cytosol- (S100) containing fractions by centrifugation at 100,000 × g was done as described (23). Each fraction was then immunoprecipitated with anti-GAP antibodies that recognize either N- or C-terminal (RH6-2A) domains of Ras GAP, resolved by SDS/PAGE, and subjected to fluorography.

Transcriptional Activation Chloramphenicol Acetyltransferase (CAT) Assays. Transcriptional activation of expression from a Ras-responsive element promoter was done as described (24). Briefly, NIH 3T3 cells were transfected with 1 μg of the pBuX4-CAT reporter plasmid (25), together with either 150 ng of pZIP-ras(61L) for analysis of oncogenic Ras or 1000 ng of SDC25/C DNA (pRG1; provided by B. Tocque, Rhone-Poulenc Sante) for analysis of normal Ras, and 2 μg of each pZIP-GAP mutant. All transfections were performed in duplicate in 60-mm dishes, and the cells were harvested after 48 hr. A 30-μl aliquot of each supernatant was then assayed for CAT activity by incubation with 0.1 μCi (1 Ci = 37 GBq) of [14C]chloramphenicol (NEI) and 0.57 mM acetyl-CoA in 250 mM Tris-HCl (pH 7.8) in a final reaction volume of 138 μl. After 45 min, the reaction was halted by extraction with 500 μl of ethyl acetate and evaporated under vacuum, and the resulting pellet was dissolved in 10 μl of ethyl acetate and subjected to thin layer chromatography in a buffer of 5% (vol/vol) methanol/95% (vol/vol) chloroform. Assays were quantitated using an AMBIS beta scanner.

RESULTS

A Membrane-Targeted Form of the C Terminus of GAP (cGAP/CAAX) Exhibits Potent Inhibition of Cell Proliferation. To evaluate the role of membrane association in mediating GAP function, the 19-residue C terminus of K-Ras4B was added to the GAP N-terminal SH2/SH3-containing (residues 1–666; designated nGAP/CAAX) or the C-terminal catalytic (residues 705–1047; designated cGAP/CAAX) domains (Fig. 1). This Ras sequence contains a lysine-rich domain and a CAAX prenylation signal sequence and is sufficient to target heterologous, cytosolic proteins to the plasma membrane (19). Retrovirus expression constructs encoding the different GAP and chimeric GAP–Ras proteins were then used to determine their effects on cellular proliferation and Ras function in NIH 3T3 cells.

Initial analysis was done by transfecting retrovirus constructs encoding each GAP sequence into NIH 3T3 cells, followed by selection in G418-containing growth medium, to isolate cells stably expressing each GAP variant. Whereas colonies were readily observed (>200 colonies per dish) with

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**Fig. 1.** Structures of mutant GAP constructs. The 19-residue C terminus of K-Ras4B was added to full-length GAP, nGAP, and cGAP.

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**Fig. 2.** Growth inhibitory activity of GAP mutants. (A) NIH 3T3 cells were transfected with pZIP-GAP plasmid DNA constructs encoding GAP or cGAP/CAAX at 2 μg per 60-mm dish. Three days after transfection, cells were subcultured (1:10) into 100-mm dishes and maintained in growth medium containing G418 at 400 μg/ml to isolate transfected cells expressing each mutant GAP protein. After 14–20 days, the cells were fixed and stained with crystal violet. (B) Rescue of cGAP/CAAX growth inhibition by oncogenic Ras. NIH 3T3 cells were transfected with 50 ng of pZIP-cGAP/CAAX plasmid DNA alone (cGAP/CAAX–ras) or were cotransfected with 50 ng of pZIP-cGAP/CAAX and 2 μg of pMuMT-1 (encoding Ras61L) plasmid DNA (cGAP/CAAX + ras). Transfected cultures were then treated as described in A. (C) Growth inhibitory activity of GAP/CAAX and cGAP. Cells were transfected with 2 μg pZIP-GAP/CAAX or pZIP-cGAP and processed as described in A.
with this GAP mutant suggested that cGAP/CAAX expression was inhibitory for the growth of NIH 3T3 cells.

**cGAP/CAAX Growth Inhibition Is Reversed by Oncogenic Ras and Raf but not Src.** One possible basis for the inhibitory activity of cGAP/CAAX may be that this mutant GAP is constitutively activated in its catalytic function and, consequently, inhibits endogenous Ras function by promoting formation of the inactive, GDP-complexed protein. Since previous studies have shown that the loss of endogenous Ras function can be overcome by oncogenic Ras (26, 27), we determined whether coexpression of oncogenic Ras would alleviate cGAP/CAAX-mediated growth inhibition. Whereas no G418-resistant colonies were observed in the cultures transfected with 50 ng of pZIP-cGAP/CAAX DNA alone, drug-resistant colonies (>100 colonies per dish) were readily observed in cultures cotransfected with 2 μg of a neo<sup>+</sup> plasmid DNA expression construct (pMUT-1) that expresses oncogenic H-Ras(61L) (Fig. 2B). Cotransfection with a plasmid encoding a second oncogenic Ras mutant (12V) also reversed the growth inhibitory activity, while cotransfection with normal ras did not result in the isolation of G418-resistant colonies (Table 1). Interestingly, the G418-resistant cells displayed normal to only partially transformed morphologies, suggesting that cGAP/CAAX also antagonized Ras transforming activity (data not shown).

To further characterize the ability of oncogenic Ras(61L) to rescue cGAP/CAAX growth inhibition, pZIP-cGAP/CAAX was also cotransfected with expression constructs (neo<sup>+</sup>) encoding Ras(61L) mutants that are defective in either membrane association [pcDNA-ras(61L, 186S)] or GAP binding [pcDNA-ras(61L, 35A)] (24). Neither mutant reversed cGAP/CAAX growth inhibition (Table 1), suggesting that both Ras membrane association and GAP binding are required for reversion of cGAP/CAAX growth inhibition. Finally, while the Ras-independent transforming c-Raf protein could reverse cGAP/CAAX growth inhibition, neither the Ras-dependent v-Src protein nor two oncoproteins that function independently of the Ras pathway (v-Fos and mutant p53) displayed rescue of cGAP/CAAX inhibition (Table 1). This pattern of oncogenic rescue suggests that cGAP/CAAX growth inhibition is a consequence of blocking endogenous Ras function.

**Membrane Association and N-Terminal Truncation Both Potentiate GAP Catalytic Activity.** To further evaluate the basis for cGAP/CAAX growth inhibition, we characterized the growth inhibitory activities of a full-length GAP sequence that terminates in the Ras membrane-targeting sequence (GAP/CAAX) and a catalytic domain mutant that lacks this targeting signal (cGAP) (Fig. 1). No G418-resistant colonies were observed in cultures that were transfected with pZIP-cGAP/CAAX, whereas a very low frequency of drug-resistant colonies was observed with cultures transfected with pZIP-cGAP plasmid DNA (Fig. 2C). Thus, while the addition of a plasma membrane-targeting signal to GAP alone is sufficient to activate potent growth inhibitory activity, removal of the N-terminal domain also contributes significantly to the growth inhibitory activity of cGAP/CAAX.

Stably transfected cells that were established by cotransfection with pUC-ras(12V) and the different GAP constructs were metabolically labeled with [35S]methionine/cysteine and used for immunoprecipitation analysis with anti-GAP antibodies. Cells transfected with cGAP/CAAX or cGAP, but not GAP, expressed a 42-kDa species, which is consistent with the expected size of the GAP C-terminal domain (Fig. 3). Fractionation analysis showed that cGAP/CAAX was present exclusively in the P100 membrane fraction, whereas cGAP, which lacks the membrane-targeting sequence, was detected predominantly in the S100 cytosolic fraction (Fig. 3). Finally, a predominantly membrane-associated GAP/CAAX protein versus the predominantly cytosolic wild-type GAP was observed (Fig. 3). Thus, the Ras C terminus promotes the membrane association of both cGAP and GAP.

**nGAP/CAAX and cGAP/CAAX Exhibit Differential Inhibition of Normal and Oncogenic Ras Activity.** To evaluate the specific effects of the different GAP mutants on either normal or oncogenic Ras activities, transcriptional activation assays were performed. While oncogenic Ras proteins activate transcription from promoters that contain Ras-responsive elements (24, 25), normal Ras displays limited, or no, activation of this function. However, exogenous expression of the yeast Ras guanine nucleotide-dissociation stimulatory protein SDC25/C in mammalian cells can activate endogenous Ras activity (28) and induce transcriptional transactivation from Ras-responsive element-containing promoters via activation of endogenous Ras (3). Therefore, we were able to determine the effects of each GAP construct on both oncogenic (Ras(61L)) and normal (SDC25/C-mediated) Ras transactivation activity.

For these assays, we utilized a CAT reporter gene under the control of a β-globin promoter that contains four tandem Ras-responsive elements (PEA-1) derived from the polyoma virus enhancer (pBX4-CAT) (25). While all GAP variants inhibited the ability of oncogenic Ras to transactivate CAT activity (ranging from 46% to 89% inhibition), the strongest inhibition (>80%) was observed with the membrane-targeted forms of GAP and cGAP (Fig. 4A). A significant, but lower, inhibitory activity was also observed with the non-membrane-targeted forms of GAP and cGAP (46–64%). By comparison, equivalent and strong inhibition was observed

### Table 1. Rescue of cGAP/CAAX growth inhibition by coexpression of oncogenic proteins

<table>
<thead>
<tr>
<th>Oncogenic protein coexpressed&lt;sup&gt;a&lt;/sup&gt;</th>
<th>G418-resistant colonies&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>H-Ras (normal)</td>
<td>–</td>
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<tr>
<td>H-Ras(12V)</td>
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<td>H-Ras(61L)</td>
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<td>H-Ras(61L, 35A)</td>
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<td>H-Ras(61L, 186S)</td>
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<tr>
<td>c-Raf (p22W-raf)</td>
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<tr>
<td>v-Src (pscr11)</td>
<td>+</td>
</tr>
<tr>
<td>v-Fos (pSM3-fos)</td>
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<td>p53(135V)</td>
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<sup>a</sup>NIH 3T3 cells were cotransfected with 50 ng of pZIP-cGAP/CAAX DNA (neo<sup>+</sup>) and 2 μg of the oncogene-containing plasmid DNA (neo<sup>+</sup>) and then selected in G418-containing medium.

<sup>b</sup>The appearance of G418-resistant colonies was quantitated after 3 weeks; +++, >100 colonies per 100-mm dish; +++, 20–100 colonies per dish; –, no colonies were detected.
with either the cytosolic or membrane-targeted forms of nGAP (68–78%). Thus, membrane association potentiated only the activity associated with cGAP or full-length GAP.

In contrast to the inhibition of oncogenic Ras by either N- or C-terminal GAP constructs, only GAP variants containing the catalytic domain (cGAP, cGAP/CAAX, GAP/CAAX, and GAP) inhibited SDC25/C-induced transactivation via endogenous normal Ras function (Fig. 4B). Both cGAP and GAP inhibited SDC25/C-induced transactivation (32–43%), while their membrane-targeted counterparts displayed 2-fold greater (66–80%) inhibitory activities. In contrast, neither nGAP nor nGAP/CAAX exhibited any significant inhibitory activity. Thus, only GAP mutants containing the C-terminal catalytic domain were inhibitory for normal Ras-induced transcriptional activation, and this activity was enhanced by membrane association.

The N Terminus of GAP Inhibits Oncogenic Ras Transforming Activity. NIH 3T3 cotransfection assays were done to determine if nGAP could also inhibit oncogenic Ras focus-forming activity. While transfection of 10 ng per dish of pZIP-ras(61L) DNA alone resulted in >40 foci per dish, cotransfection with excess (2 μg) pZIP-nGAP/CAAX plasmid DNA resulted in a significant (>50%) reduction in transformed foci (Fig. 5). Since a comparable reduction in focus-forming activity was also observed with nGAP, membrane association does not additionally enhance this activity. Since full-length GAP exhibited a significantly lower inhibition (17%) of focus-forming activity than that seen with the nGAP constructs, removal of the catalytic domain potentiates GAP inhibition of oncogenic Ras transformation.

DISCUSSION

The results described here support the model that Ras GAP is both a negative regulator and a downstream effector target for Ras function (7, 8). The growth inhibitory activity associated with the C-terminal GAP constructs suggests that membrane association and, to a lesser degree, removal of the N-terminal regulatory domain constitutively activate GAP catalytic activity. In contrast, the nGAP constructs lacked any growth inhibitory activity and, instead, preferentially inhibited oncogenic, but not normal, Ras activity. The utility of the N-terminal GAP variants to inhibit transcriptional activation and transformation induced by oncogenic Ras suggests that this region of GAP interacts with downstream components essential for the Ras transformation pathway. However, the inability of nGAP to inhibit normal Ras function suggests that the downstream interactions involved in normal cell proliferation are distinct from those that mediate Ras transformation.

One possible basis for cGAP/CAAX growth inhibition is that this protein competes with Ras binding sites at the plasma membrane. However, we have stably expressed a number of defective Ras proteins (22, 24) that possess the same plasma membrane targeting signal yet do not display such a phenotype. Instead, the potent growth inhibitory activity of cGAP/CAAX is likely to be a consequence of constitutively activated catalytic activity. This possibility is supported by two observations. First, while neither nGAP/CAAX nor nGAP was growth inhibitory, the full-length GAP/CAAX variant did exhibit potent growth inhibitory activity. Second, cGAP/CAAX growth inhibitory activity is reversed by coexpression with oncogenic Ras or Raf but not Src or normal Ras. This pattern of oncogene rescue is the same as that observed for the Ras(17N) dominant inhibitory mutant, which also possesses a similar growth inhibitory phenotype (26).
The growth inhibitory activities of cGAP/CAAX and GAP/CAAX suggest that membrane association potentiates the negative regulatory function of GAP. However, since removal of the N-terminal domain to form cGAP also activates GAP inhibition of growth, the N-terminal domain may serve as a negative regulator of GAP catalytic activity. Similarly, Schweighoffer et al. (3) observed that the C terminus alone, but not full-length GAP, suppresses oncogenic Ras transactivation activity. Therefore, the binding of phosphoproteins to the SH2/SH3 domains may be analogous to removal of the N terminus and may serve to modulate the catalytic activity of full-length GAP (8).

In contrast to cGAP/CAAX, nGAP/CAAX displayed no growth inhibitory activity and can be stably expressed in untransformed NIH 3T3 cells. Instead, both nGAP and nGAP/CAAX were found to be potent inhibitors of oncogenic Ras activity. The preferential inhibition of oncogenic versus normal Ras activity is analogous to a similar selective inhibition observed with cytosolic GAP binding mutants of Ras [e.g., H-ras(61L,186S)] (29, 30). Like nGAP, H-ras(61L,186S) is believed to preferentially block oncogenic Ras activity by preventing Ras stimulation of a downstream target (27). Finally, the comparable activities of the cytosolic and membrane-targeted versions of nGAP suggest that membrane association does not regulate this GAP function and therefore that this domain probably interacts with a cytosolic downstream target.

Previous studies have determined that full-length GAP inhibits transformation by normal Ras, V-Src, and c-Fms but not oncogenic Ras (31-34). Our observation that the N-terminal domain alone can inhibit oncogenic Ras activity suggests that removal of the catalytic domain can activate a latent activity negatively regulated by the C terminus. A similar unmasking of an N-terminal GAP activity via removal of the C-terminal domain has also been observed in two other recent studies. First, while GAP inhibition of the muscarinic atrial K+ channel is dependent on Ras, deletion mutants of GAP that lack the catalytic domain are inhibitory in a Ras-independent manner (35). Second, the GAP N-terminal domain alone, but not full-length GAP, was observed to be capable of activating transcription of a fos promoter reporter construct (36). The results from these two studies suggest a model in which Ras binds to the GAP catalytic domain and induces a conformational change that exposes the N-terminal SH2/SH3 domain, thereby allowing GAP to interact with a putative downstream target protein "X". Removal of the C-terminal sequences may eliminate the requirement for Ras binding and result in a constitutively activated N-terminal domain that then complexes with protein X.

Two different biological consequences may be envisioned to occur as a result of a constitutive nGAP association with the downstream protein X. This association may complete the pathway in a Ras-independent fashion and result in growth stimulation. Alternatively, completion of the Ras pathway may require simultaneous association of protein X with the N terminus and Ras with the C terminus of GAP. Thus, nGAP may serve as a competitive antagonist for protein X with the endogenous GAP–Ras complex and prevent formation of the Ras signaling complex. While the results of Medema et al. (36) support the first possibility, our observations are consistent with the second scenario. Since the GAP SH2/SH3 domain appears to interact with various phosphotyrosine-containing proteins, it is possible that different consequences of expressing GAP variants lacking the catalytic domain may reflect their interaction with different protein Xs in the different cell systems and assays used. Establishing the identity of the putative downstream protein X(s) will be critical for identifying the Ras signal transduction pathway(s) and for determining whether normal versus oncogenic Ras promote their activities via distinct pathways.