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Identification of Residues Critical for Ras(17N) Growth-Inhibitory Phenotype and for Ras Interaction with Guanine Nucleotide Exchange Factors

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The Ras(17N) dominant negative antagonizes endogenous Ras function by forming stable, inactive complexes with Ras guanine nucleotide exchange factors (GEFs; e.g., SOS1). We have used the growth-inhibitory phenotype of Ras(17N) to characterize two aspects of Ras interaction with GEFs. First, we used a nonprenylated version of Ras(17N), designated Ras(17N/186S), which no longer associates with the plasma membrane and lacks the growth-inhibitory phenotype, to address the importance of Ras subcellular location and posttranslational modification for its interaction with GEFs. We observed that addition of an N-terminal myristylation signal to Ras(17N/186S) restored the growth-inhibitory activity of nonprenylated Ras(17N). Thus, membrane association, rather than prenylation, is critical for Ras interaction with Ras GEFs. Second, we used a biological selection approach to identify Ras residues which are critical for Ras(17N) growth inhibition and hence for interaction with Ras GEFs. We identified mutations at residues 75, 76, and 78 that abolished the growth-inhibitory activity of Ras(17N). Since GEF interaction is dispensable for oncogenic but not normal Ras function, our demonstration that single-amino-acid substitutions at these three positions impaired the transforming activity of normal but not oncogenic Ras provides further support for the role of these residues in Ras-GEF interactions. Finally, Ras(WT) proteins with mutations at these residues were no longer activated by mammalian SOS1. Altogether, these results suggest that the Ras intracellular location and Ras residues 75 to 78 are critical for Ras-GEF interaction.

The four human Ras proteins (H-, N-, K4A-, and K4B-Ras) are members of a large superfamily of small guanine nucleotide-binding proteins that function as molecular switches to regulate signal transduction pathways important for modulating cell growth and differentiation (1, 3). Ras protein function is controlled by a GDP-GTP cycle that is regulated by at least two distinct classes of regulatory proteins. First, GTPase-activating proteins (p120 GAP and neurofibromin/NF1 GAP) recognize the active, GTP-bound protein and stimulate the intrinsic GTPase activity of Ras proteins to form the inactive, GDP-bound protein (2). Second, guanine nucleotide exchange factors (GEFs) promote the dissociation of bound nucleotide to promote the formation of the active, GTP-bound state (13). Several Ras GEFs have recently been molecularly cloned and characterized. These include mammalian homologs of the yeast CDC25 and SDC25 (designated GNRF, CDC25^{Mm}, or mCDC25) (24, 35, 41) and the *Drosophila* SOS (SOS1 and SOS2) (4) proteins. smg GDS represents a fourth Ras GEF (15), whereas the Vav protein, which shares homology with the Dbl family of putative GEFs for the Rho family of Ras-related proteins, has also been shown to stimulate Ras GDP-GTP exchange (17).

Although the precise role of each GEF regulatory protein remains to be established, the observation that a putative GEF-complexing Ras mutant, Ras(17N), is a potent inhibitor of cell proliferation supports a critical role for GEF in Ras function (14, 37). Present evidence suggests that the growth-inhibitory activity of the constitutively GDP-bound Ras(17N) protein is a consequence of its formation of an inactive complex with Ras GEFs (30), thus preventing the activation of endogenous Ras. Presently, there is no direct evidence that Ras(17N) growth inhibition is due to this mechanism. However, support for this mechanism is provided by the demonstration that overexpression of the yeast SDC25 protein, which functions as a Ras GEF (31), reverses Ras(17N) growth inhibition in NIH 3T3 cells (33).

Although guanine nucleotide binding is essential for Ras function, Ras biological activity is also critically dependent on its localization to the inner face of the plasma membrane (11, 22). Ras membrane association is triggered by a series of three closely linked posttranslational modifications (prenylation, proteolysis, and carboxylmethylation) that are signaled by the C-terminal CXXX (where C is cysteine and X is any amino acid) sequence, which is present on all Ras proteins. Mutation of the cysteine residue of the CXXX motif prevents prenylation, and such mutant proteins are no longer associated with the plasma membrane, are cytosolic, and are completely nontransforming. However, although it is clear that prenylation and membrane association are essential for Ras function, their precise contributions to Ras function remain to be established.

Ras function may be dependent on prenylation to promote the interaction of Ras proteins with regulatory proteins such

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as GAPs and GEFs that control its GDP-GTP cycle or with upstream (e.g., receptor tyrosine kinases) or downstream (e.g., serine/threonine kinases) components required for completion of the Ras signaling pathway (1). This requirement may reflect the possibility that such proteins recognize only the prenylated form of Ras. For example, smg GDS stimulates GDP-GTP exchange on only the prenylated forms of K-Ras4B and RhoA (26). Additionally, Ras prenylation was found to be required for Ras activation of mitogen-activated protein kinase in a cell-free assay (19).

Alternatively, the membrane location produced upon prenylation may be required for Ras interaction with other proteins. This possibility is supported by the observation that the fatty acid myristate can replace prenylation to promote Ras membrane association and transforming activity (7). In addition, although Ras GAPs can readily stimulate the GTPase activity of nonprenylated Ras proteins, plasma membrane targeting greatly potentiates the negative regulatory activity of both p120 GAP and NF1 GAP (10, 18). Thus, it may be the subcellular location, rather than prenylation itself, that is required for Ras interaction with other proteins.

In the studies presented here, we have used the Ras(17N) dominant inhibitory mutant to address the role of Ras GEFs in Ras function. Our results suggest that membrane association, rather than prenylation per se, is critical for Ras-GEF interactions and that the Ras domain containing residues 75 to 78 may be essential for GEF activation of normal Ras biological activity but is dispensable for the transforming activity of oncogenic Ras.

MATERIALS AND METHODS

Molecular constructs. The Ras(17N/186S) mutant contains a cysteine-to-serine substitution of the cysteine residue in the C-terminal CXXX prenylation signal sequence present in all Ras proteins (11, 22). This Ras(17N) variant no longer undergoes prenylation, is not membrane associated, and lacks the growth-inhibitory activity of Ras(17N) (14). We have previously shown that addition of an N-terminal myristylation signal sequence to the N terminus of Ras proteins can substitute for prenylation to restore membrane association and transforming activity (7). To generate a myristylated form of Ras(17N/186S), a 0.7-kb *Hin*dIII-*Bam*HI fragment that encodes residues 6 to 189 of the Ras(17N/186S) mutant protein (provided by Larry Feig) was ligated with a 0.4-kb *Bgl*II-*Hin*dIII fragment that encodes an 11-residue N-terminal myristylation signal sequence (derived from the rat leukemia virus Gag protein) and the first five residues of human H-Ras and then introduced into the *Bam*HI site of the pZIP-NeoSV(x)1 retrovirus vector plasmid [designated Myr-*ras*(17N/186S)] (7). pZIP retrovirus constructs of *ras*(61L), *ras*(17N), *ras*(17N/186S), and Myr-*ras*(186S) were constructed as described previously (7).

An expression construct encoding the catalytic domains of mouse SOS1 (provided by D. Broek) was generated by PCR amplification of cDNA sequences of mouse SOS1 with a 5′ synthetic oligonucleotide that introduced an *Eco*RI site and initiating ATG codon and a 3′ oligonucleotide that introduced a stop codon followed by an *Eco*RI restriction site. The PCR-generated fragment encoding human mouse mSOS1 residues 559 to 1071 was then introduced into the unique *Eco*RI site of a modified version of pZIP-NeoSV(x)1 (pZBR) and was designated pZIP-SOS(c) (30a).

In vitro random mutagenesis. In vitro random mutagenesis of pZIP-Myr-*ras*(17N/186S) plasmid DNA was done as described previously (14). Briefly, 30 μg of plasmid DNA in 30 μl of H_2O was mixed with 150 μl of ethylene glycol and heated to 70°C for 5 min. After a 27-μl aliquot was removed for the nonmutated control, 16 μl of hydroxylamine solution (0.5 M in 0.2 M sodium PP_i) was added to the remaining DNA solution. Incubation was continued at 70°C for 60 min, with 30-μl aliquots being removed at 10-min intervals, and the reaction was stopped by the addition of 80 μl of ice-cold stop solution (0.6 M Tris-HCl [pH 8.0], 1.0 M NaCl, 20% acetone). Each DNA fraction was then centrifuged through 1.0-ml Sephadex G-100 columns to remove the residual hydroxylamine. The degree of mutagenesis was quantitated by determining the reduction of ampicillin resistance of the treated DNA after transformation into *Escherichia coli* HB101. DNA aliquots representing 30 to 40% reduction in ampicillin resistance (corresponding to approximately one mutation per molecule) were then used for transfection into NIH 3T3 cells as described below.

Cell culture and transfection assays. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, and DNA transfections by the calcium phosphate precipitation technique were done as described previously (7), with each dish containing 100 to 1,000 ng of pZIP-*ras*H DNA encoding each mutant Ras protein. Transfected cells were maintained in growth medium, and transformed foci were quantitated after 14 days. Transfected NIH 3T3 cells were also selected in growth medium containing 400 μg of G418 (Geneticin; GIBCO BRL) per ml to establish cell lines expressing each mutant Ras protein or to quantitate cell growth inhibition.

Subcellular localization and analysis of Ras expression. G418-selected NIH 3T3 cells expressing each mutant protein were labeled overnight in growth medium supplemented with 200 μCi of [^{35}S]methionine/cysteine (Tran^{35}S-label; ICN) per ml. For fractionation analysis, labeled cells were separated into crude membrane (P100)- and cytosol (S100)-containing fractions by centrifugation (100,000 × *g* for 30 min) as described previously (7). Ras proteins were immunoprecipitated from each fraction with the rat Y13-259 and mouse 146-3E4 (specific for H-Ras; Quality Biotech, Camden, N.J.) anti-Ras monoclonal antibodies resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subjected to fluorographic analysis as described previously (7). In vivo guanine nucleotide association analysis was done essentially as described previously (7). Briefly, transfected NIH 3T3 cells expressing each of the different mutant proteins were metabolically labeled overnight with 200 μCi of $^{32}P_i$ (Dupont NEN) per ml. The labeled cells were first lysed with 1% Nonidet P-40 detergent buffer and then subjected to immunoprecipitation with Y13-259. The bound guanine nucleotides were released from the immunoprecipitated Ras proteins and separated by thin-layer chromatography on PEI cellulose plates, and the percentage of GTP bound was quantitated by AMBIS beta scanning.

Transcriptional activation CAT assays. Transcriptional activation of expression from a promoter which contains Ras-responsive elements (Ets-1 and AP-1 binding motifs) was done essentially as described previously (10). Briefly, NIH 3T3 cells were transfected with 1 μg of the pB4X-CAT chloramphenicol acetyltransferase (CAT) reporter plasmid (provided by B. Wasylyk) (8) together with 2 μg of each pZIP-*ras* mutant. The ability of Ras GEFs to stimulate the ability of normal Ras to activate transcription from pB4X-CAT was determined by cotransfection with 100 ng of pZIP-SOS(c). To evaluate the ability of Ras(17N) to compete with mouse SOS1, cultures were cotransfected with 2 μg of pZIP-*ras*H(WT) and 0.1 μg of pZIP-*SOS*(c) plasmid DNAs

together with 1.0 μg of pZIP-Ras(17N). All transfections were performed in duplicate in 60-mm dishes. The cells were harvested after 48 h and lysed in 100 μl of 250 mM Tris-HCl (pH 7.8) by three cycles of freeze-thawing. Lysates were clarified by microcentrifugation, with the resulting supernatant heated to 62°C to inactivate any endogenous acyltransferase activity, and then subjected to a second clarification step. A 30-μl aliquot of each supernatant was then assayed for CAT activity by incubation with 0.1 μCi of [^{14}C]chloramphenicol (NEN) and 0.34 mM acetyl coenzyme A 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 5% methanol–95% chloroform. Assays were quantitated by using an AMBIS beta scanner.

RESULTS

Membrane association, rather than prenylation, is required for 17N growth inhibition. Previous studies have established that isoprenylation and membrane association are required for the growth-inhibitory activity of Ras(17N) (14). To determine whether Ras(17N) function is dependent on isoprenylation or membrane association, we generated a nonprenylated form of Ras(17N) that was targeted to membranes via myristylation. We have previously shown that addition of an 11-amino-acid myristylation signal sequence to oncogenic Ras will substitute for isoprenylation and restore Ras transforming activity (7). Therefore, we introduced this N-terminal myristylation signal onto the N terminus of a cytosolic form of Ras(17N) [H-Ras(17N/186S)] to determine whether myristate addition could replace isoprenylation to restore the growth-inhibitory phenotype.

The growth-inhibitory activity of myristylated, nonprenylated Ras(17N) [designated Myr-Ras(17N/186S)] was then compared with that of both prenylated and nonprenylated forms of Ras(17N). Although cultures transfected with 500 ng of pZIP-*ras*(WT) plasmid DNA and subjected to selection in G418-containing growth medium exhibited the appearance of a near-confluent dish (>500 colonies) of drug-resistant colonies, cultures transfected with pZIP-*ras*(17N) exhibited a much reduced concentration of G418-resistant colonies (Fig. 1). As described previously (14), the nonprenylated, cytosolic version of Ras(17N) [Ras(17N/186S)] lacked growth-inhibitory activity and displayed a greatly enhanced concentration of G418-resistant colonies. However, the addition of an N-terminal myristylation signal sequence to this mutant [Myr-Ras(17N/186S)] restored potent growth-inhibitory activity (Fig. 1). Furthermore, since we observed fewer G418-resistant colonies with the myristylated than the prenylated form of 17N, the growth-inhibitory activity of Myr-Ras(17N/186S) is apparently greater than that of the prenylated Ras(17N) protein. Thus, the membrane association triggered by myristate can promote a sufficiently proper location of Ras(17N) to allow expression of its growth-inhibitory phenotype. These results suggest that membrane association, rather than prenylation, is required for GEF recognition of Ras proteins.

Isolation of Ras(17N) mutants which lack the growth-inhibitory phenotype. Although it is believed that Ras(17N) growth inhibition is a consequence of its formation of an inactive complex with Ras GEFs, thereby preventing GEF activation of endogenous Ras activity (14, 30), there is no direct evidence for this model. To provide a better understanding of the biochemical mechanism of Ras(17N) growth inhibition and to identify domains of Ras important for Ras-GEF interaction, we have used a random-mutagenesis method to identify second-site mutations that abolish the growth-inhibitory phenotype of Ras(17N). Since we observed that the myristylated form of Ras(17N) [Myr-Ras(17N/186S)] displayed stronger growth-inhibitory activity than did the prenylated version [Ras(17N)], we used a retrovirus construct [pZIP-Myr-*ras*(17N/186S)] encoding this protein for the random-mutagenesis studies.

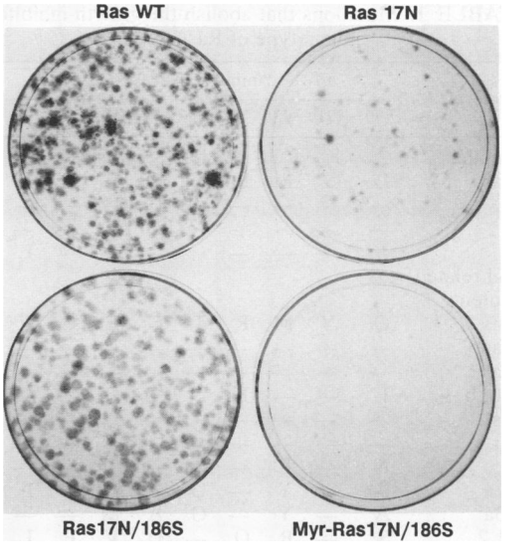

FIG. 1. Myristylation restores the growth-inhibitory activity of a nonprenylated, cytosolic version of Ras(17N) [Ras(17N/186S)]. NIH 3T3 cells were transfected with 500 ng of pZIP-*ras* constructs encoding Ras(WT), Ras(17N), Ras(17N/186S), and Myr-Ras(17N/186S) and selected in G418-containing growth medium. After 14 days, the drug-resistant colonies were visualized by staining with crystal violet.

pZIP-Myr-*ras*(17N/186S) plasmid DNA was treated with hydroxylamine to introduce random base pair substitutions (G-to-A missense mutations) by using previously described methods (14). The hydroxylamine-treated DNA was then transfected into NIH 3T3 cells, and G418-resistant colonies were isolated. Since stable expression of Ras(17N) is not tolerated, the transfection of control, untreated pZIP-Myr-*ras*(17N/186S) plasmid DNA typically resulted in the isolation of only rare G418-resistant colonies (less than 1 colony per 100-mm dish) (Fig. 1). In contrast, occasional G418-resistant colonies were observed with the hydroxylamine-treated DNA. Altogether, 16 G418-resistant colonies of proliferating cells were observed in three 100-mm dishes. The 16 individual colonies that arose from the hydroxylamine-treated DNAs were then isolated to establish clonal cell lines for further analysis.

We anticipated that a variety of mutations in Myr-Ras(17N/186S) may account for the outgrowth of G418-resistant cells. For example, in addition to mutations that may prevent Ras-GEF interaction, the loss of the growth-inhibitory activity of Ras(17N) may be a consequence of mutations that introduce premature stop codons that then encode truncated, nonfunctional proteins. Alternatively, missense mutations that cause protein instability may have been introduced. To determine whether these two classes of mutations were present, the cells representing the 16 indi-

TABLE 1. Mutations that abolish the growth-inhibitory phenotype of Ras(17N)

Protein	Amino acid at Ras residue:										
	70	71	72	73	74	75	76	77	78	79	80
Ras mutants											
H-Ras	Q	Y	M	R	T	G	E	G	F	L	C
75E	—[a]	—	—	—	—	E	—	—	—	—	—
75K	—	—	—	—	—	—	K	—	—	—	—
78L	—	—	—	—	—	—	—	—	L	—	—
Ras and related proteins											
H-Ras	Q	Y	M	R	T	G	E	G	F	L	C
N-Ras	—	—	—	—	—	—	—	—	—	—	—
K-Ras	—	—	—	—	—	—	—	—	—	—	—
Rap1a,b	L	—	—	K	N	—	Q	—	—	A	L
R-ras	—	—	—	—	A	—	H	—	—	—	L
TC21	—	—	—	—	—	—	—	—	—	—	L
Ral	N	—	F	—	S	—	—	—	—	—	—
Rab1a	S	—	Y	—	G	A	H	—	I	I	V
Rab3a	A	—	Y	—	G	A	M	—	—	I	L
Rac1,2	S	—	P	Q	—	D	V	F	L	I	—
RhoA,B,C	S	—	P	D	—	D	V	I	L	M	—

[a] — indicates identity with H-Ras at the indicated residue.

vidual G418-resistant colonies were used for Western blot analysis to assay for expression of the 22-kDa Myr-Ras protein. Of the 16 cell lines, only 2 (designated clones 4-5 and 6-6) expressed a detectable 22-kDa Myr-Ras protein (data not shown). Thus, the remaining 14 drug-resistant cell lines may express either truncated or unstable Myr-Ras proteins.

Another possible mutation that would abolish Myr-Ras(17N/186S) growth inhibition would be the loss of the glycine residue, which is the site of myristate addition, in the N-terminal myristylation sequence. However, fractionation analysis of the 4-5 and 6-6 cells indicated that both of these cell lines expressed a membrane-associated form of p22 Myr-Ras (data not shown), suggesting that these two Myr-Ras variants still undergo myristylation. Therefore, the apparent loss of growth-inhibitory activity of these two Myr-Ras variants may be the consequence of mutations that either removed the 17N mutation or contained mutations at sequences that prevented their interaction with Ras GEFs.

Cell lines 4-5 and 6-6 were used for further analysis to determine the nature of the mutation(s) which allowed stable expression of Myr-Ras(17N). To address this directly, genomic DNA was isolated from cell lines 4-5 and 6-6, subjected to PCR amplification with primers corresponding to the 5′ and 3′ sequences of the Myr-*ras*(17N/186S) cDNA, subcloned into the M13mp18 phage construct, and analyzed by dideoxy sequencing. Sequences derived from both 4-5 and 6-6 retained the coding sequence for asparagine at codon 17. Instead, the sequence derived from 4-5 cells contained a single-base substitution at codon 78 of the H-Ras coding sequence which results in a Phe (TTC)-to-Leu (CTC) substitution, whereas the sequence derived from 6-6 cells contained two base substitutions at codons 75 and 76 which result in a double substitution of Gly (GGG) to Glu (GAG) at position 75 and of Glu (GAG) to Lys (AAA) at position 76 (Table 1). These results suggest that the Ras sequence corresponding to residues 75 to 78 is essential for Ras(17N) growth inhibition and may therefore be important for interaction with Ras GEFs.

Mutations at residues 75 to 78 abolish 17N growth inhibition and reduce normal, but not oncogenic, Ras transforming potential. To characterize the role of residues 75 to 78 in Ras function, we first introduced the same single-amino-acid substitutions into this region of the normal, prenylated version of the Ras(17N) mutant protein. As we had observed with the Myr-Ras(17N/186S) protein, these substitutions also abolished the growth-inhibitory activity of the prenylated version of Ras(17N) (Fig. 2A). Whereas cultures transfected with 100 ng of pZIP-*ras*(17N) displayed very few G418-resistant colonies [which typically express no, or very low levels of, Ras(17N) (14)], cultures transfected with versions of Ras(17N) containing amino acid substitutions at residues 75, 76, and 78 all showed the appearance of drug-resistant colonies that were comparable in number to those observed with cells transfected with Ras(WT). Interestingly, although the 6-6 Myr-Ras(17N) revertant contained two amino acid substitutions, either substitution alone was sufficient to reverse Ras(17N) growth inhibition. Finally, the expression of Ras(17N) proteins with mutations at residues 75 to 78 were readily detectable in stably transfected NIH 3T3 cells, indicating that these mutations did not reverse Ras(17N) growth inhibition by merely causing protein instability (Fig. 2B). Thus, the ability of mutations at residues 75 to 78 to abolish the growth-inhibitory phenotype of prenylated Ras(17N) defines a Ras domain which is critical for the dominant negative nature of this mutant and suggests that this region may be important for interaction with Ras GEF.

Since oncogenic forms of Ras are no longer responsive to GAP-stimulated GTP hydrolysis, these proteins are constitutively GTP bound and are presumably independent of GEF stimulation for their transforming activity. Consistent with this possibility, genetic studies with *Saccharomyces cerevisiae* have shown that activated forms of Ras can overcome the loss of yeast RAS GEF (CDC25) function (5) and that Ras(17N) growth inhibition can be reversed by coexpression of oncogenic Ras (14). Consequently, if residues 75 to 78 are important for interaction with a Ras GEF, we would expect this domain to be critical for the activity of normal, but not oncogenic, Ras proteins. To address this possibility, we introduced either single or double mutations at residues 75 to 78 into both normal (WT) and oncogenic (12R) Ras sequences and determined their transforming activities in transfected NIH 3T3 cells. The potent focus-forming activity of Ras(12R) was not abolished by the individual substitutions at residues 75 to 78 (Table 2), and cells expressing these mutants also exhibited the same transformed morphology as cells expressing nonmutated Ras(12R) (Fig. 3). In contrast, when the individual mutations were introduced into residue 75, 76, or 78 of normal Ras(WT) protein, transforming activity was found to be significantly reduced (Table 2). Thus, this demonstration that Ras residues 75 to 78 are dispensable for oncogenic but not normal Ras-transforming activity is consistent with their possible involvement in GEF interactions. Furthermore, since oncogenic Ras transforming activity and preferential binding to GTP (Fig. 2C) are retained, mutations at residues 75 to 78 do not merely reverse Ras(17N) activity by abolishing Ras guanine nucleotide binding. The reversal of Ras(17N) growth inhibition by mutations at residues 75, 76, and 78 was not due to these mutants producing transforming proteins since cells overexpressing these proteins had normal morphology and did not generate transforming foci. Finally, since the double 75E,76K mutant reduced the transforming activity of Ras(12R) without significantly reducing the level of protein expression (Fig. 2B) or Ras(12R) GTP/GDP ratio (Fig. 2C), it is possible that this mutant has a more drastic effect on Ras conformation that also perturbs effector function.

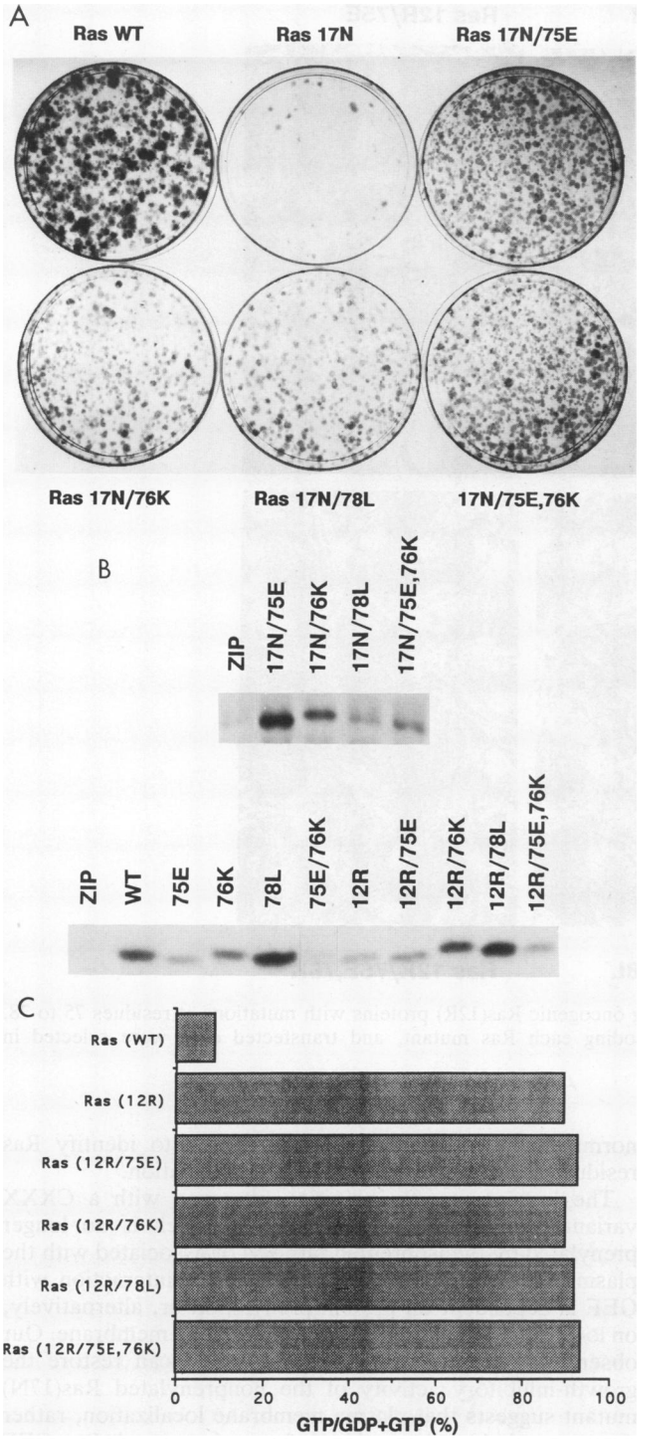

FIG. 2. Mutations at residues 75 to 78 reverse the Ras(17N) growth-inhibitory phenotype in NIH 3T3 cells. (A) Growth of G418-resistant cells. NIH 3T3 cells were transfected with 500 ng of pZIP-*ras* constructs encoding Ras(WT), Ras(17N), Ras(17N/75E), Ras(17N/76K), Ras(17N/78L), and Ras(17N/75E,76K). G418-resistant colonies were visualized by staining with crystal violet. (B) Expression of Ras proteins with mutations at residues 75 to 78 following isolation of stably transfected cells. Transfected cells were selected in G418-containing growth medium, metabolically labeled with [^{35}S]methionine-[^{35}S]cysteine, and immunoprecipitated with 146-3E4 anti-H-Ras monoclonal antibody. Ras proteins were detected by SDS-PAGE and fluorography. All proteins were readily detected, indicating that mutating residues 75 to 78 did not cause significant protein instability. (C) In vivo guanine nucleotide association analysis. NIH 3T3 cells expressing each of the different mutant proteins were metabolically labeled overnight with $^{32}P_i$ and then solubilized in detergent buffer. Labeled guanine nucleotides bound to Ras proteins were immunoprecipitated and analyzed by thin-layer chromatography.

TABLE 2. Transforming activity of mutants of Ras(WT) and Ras(12R) with mutations at residues 75 to 78[a]

Mutant	No. of foci/μg of DNA in:		
	Expt 1	Expt 2	Expt 3
WT	78	52	29
75E	<1	7	6
76K	ND[b]	12	<1
78L	8	16	11
75E,76K	<1	6	<1
12R	4,410	3,115	3,100
12R/75E	1,565	2,400	3,060
12R/76K	ND	2,775	3,450
12R/78L	3,440	3,310	3,150
12R/75E,76K	65	590	665

[a] NIH 3T3 cells were transfected with 1 μg (experiments 1 and 2) or 2 μg (experiment 3) of pZIP-*ras*(WT) or with 20 ng of pZIP-*ras*(12R) constructs per 60-mm dish and incubated in growth medium for 14 days prior to quantitation of transformed foci. Each experiment was performed in quadruplicate.
[b] ND, not done.

Mutations at residues 75 to 78 perturb Ras interaction with GEF. The consequences of substitutions at residues 75 to 78 on the biological activities of Ras(WT), Ras(12R), and Ras(17N) suggest that this domain is important for interaction with a Ras GEF. To address this possibility directly, we used transient-transfection transcription assays to determine whether the catalytic domain of mouse SOS1 could still stimulate the activities of Ras(WT) mutant proteins with mutations at residues 75 to 78. For these assays, we used the ability of Ras GEFs and normal Ras to synergistically stimulate transcriptional activation from a CAT reporter construct (pB4X-CAT) which contains Ras-responsive sequence elements (the Ets-1 and AP-1 DNA-binding motifs from the polyoma virus enhancer) in its promoter sequence (10, 33). Although oncogenic Ras proteins can efficiently (10- to 20-fold) activate transcription from pB4X-CAT (10), normal Ras proteins display only weak stimulation of transcription. However, cotransfection with pZIP-SOS1(c) can stimulate (approximately threefold) the activity of normal Ras(WT) protein in this assay (Fig. 4). In contrast, Ras(WT) proteins with mutations at residue 75, 76, or 78 were found to be significantly impaired in their ability to be transcriptionally activated by SOS1. A similar loss of responsiveness was also observed when these mutant proteins were coexpressed with constructs encoding the catalytic domains of mammalian CDC25 or yeast SDC25 Ras GEFs (data not shown). Thus, residues 75, 76, and 78 are important for proper interaction with these Ras GEFs.

Mammalian SOS1 can overcome the inhibitory activity of Ras(17N). Although recent studies have shown that yeast SDC25 is a Ras GEF and that overexpression of SDC25 can overcome the inhibitory activity of Ras(17N) (33), it is presently not known which mammalian GEF(s) is the target for Ras(17N) inhibition. Therefore, we used SOS1-induced transcription activation from the pB4X-CAT reporter plas-

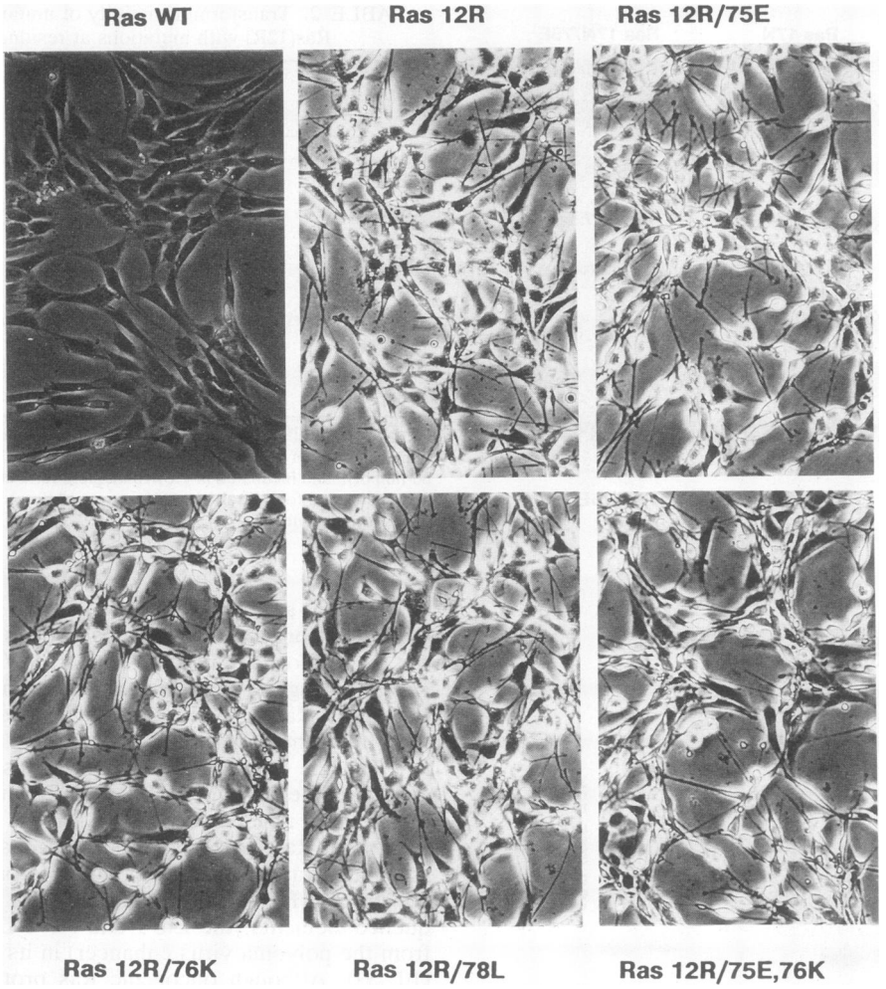

FIG. 3. Transformed morphology of NIH 3T3 cells stably expressing oncogenic Ras(12R) proteins with mutations at residues 75 to 78. NIH 3T3 cells were transfected with 20 ng of pZIP constructs encoding each Ras mutant, and transfected cells were selected in G418-containing growth medium.

mid to address the functional relationship between Ras(17N) and the catalytic domain of mouse SOS1. We observed that Ras(17N) completely blocked the ability of the catalytic domain of SOS1 and Ras(WT) to stimulate transcription activation from this Ras-responsive reporter (Fig. 5). However, cotransfection with an additional 25-fold excess of pZIP-*SOS1*(c) plasmid DNA was found to reverse this inhibition. Consequently, these results suggest that SOS1 is at least one of the GEFs which are inactivated by Ras(17N).

DISCUSSION

The Ras(17N) mutant protein is a potent inhibitor of cell proliferation and has been used widely as a dominant negative inhibitor of endogenous Ras function (14, 37). Ras(17N) preferentially binds to GDP and consequently is believed to form an inactive complex with Ras GEFs (14). Consistent with this proposal, overexpression of a yeast Ras GEF (SDC25) reverses Ras(17N) growth inhibition (33). Similarly, overexpression of yeast CDC25 overcomes the inhibitory activity of an analogous dominant negative mutant of yeast RAS2(15A) (30). In this study, we have used Ras(17N) to evaluate the role of Ras GEFs in regulating the activities of normal and oncogenic Ras proteins and to identify Ras residues which are important for this regulation.

The loss of growth inhibition observed with a CXXX variant of Ras(17N), Ras(17N/186S), which is no longer prenylated by the isoprenoid farnesyl or associated with the plasma membrane (14), suggests that Ras interaction with GEF is dependent on protein prenylation or, alternatively, on localization of Ras proteins to the plasma membrane. Our observation that the fatty acid myristate can restore the growth-inhibitory activity of the nonprenylated Ras(17N) mutant suggests that plasma membrane localization, rather than prenylation, is required for a functional Ras-GEF interaction. A membrane requirement for Ras interaction with GEFs is unexpected since the mammalian SOS and CDC25 homologs are active on unprocessed Ras proteins and are present in the cytosol at significant levels (6, 35). Thus, it would be expected that a cytosolic form of Ras(17N) should still form a complex with GEF and would still be growth inhibitory. However, since translocation of SOS to the plasma membrane via the Grb2 adaptor protein is required for activation of Ras GDP-GTP exchange (6, 9, 12, 16, 21, 28, 32, 36), it may be that only membrane-associated forms of GEFs are capable of efficiently interacting with

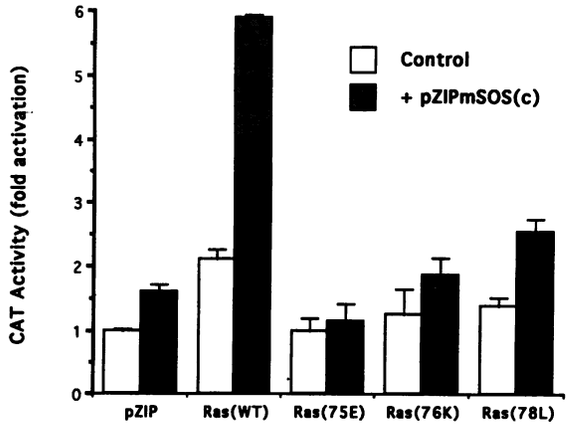

FIG. 4. Mutation of Ras residues 75, 76, and 78 blocks SOS1-stimulated transcriptional activation from Ras-responsive elements in the pB4X-CAT reporter construct. NIH 3T3 cells were cotransfected with 1 μg of the indicated pZIP-*ras* mutant alone or together with 100 ng of pZIP-SOS(c) (catalytic domain of mouse SOS1) plus 1 μg of the pB4X-CAT reporter construct. Whereas Ras(WT) showed a threefold stimulation when coexpressed with SOS(c), the 75E, 76K, and 78L mutants were poorly responsive to SOS1 stimulation, suggesting that this region is critical for GEF-mediated activation of Ras. Results are representative of at least three independent experiments, which were performed in duplicate.

Ras. This may reflect the possibility that the membrane-associated forms of GEFs are differentially modified (e.g., by phosphorylation) to facilitate their interaction with membrane-bound Ras proteins in vivo.

Although the Ras sequences responsible for Ras interaction with negative regulatory GTPase-activating proteins has been established (primarily residues 32 to 40), the Ras domains important for GEF interactions are presently poorly defined (23, 29). In this study, we have used a random-mutagenesis method to introduce second-site mutations that abolish the growth-inhibitory phenotype of Ras(17N) as one approach to identify Ras residues that may be important for Ras-GEF interaction. Additionally, since oncogenic but not

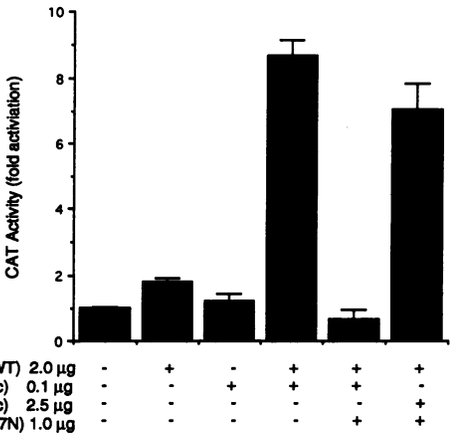

FIG. 5. Ras(17N) inhibition of SOS1 activity is overcome by overexpression of SOS1. Although neither Ras(WT) nor SOS1(c) alone showed significant stimulation of transcription from pB4X-CAT, cotransfection of Ras(WT) and SOS(c) resulted in eightfold stimulation. Ras(17N) efficiently blocked this activation. However, excess SOS(c) was able to reverse this inhibitory activity.

normal Ras proteins can overcome a CDC25 defect in *S. cerevisiae* (5), we also expected that such mutations would impair the transforming activity of normal but not oncogenic Ras proteins. We observed that single-amino-acid substitutions of residue 75, 76, or 78 completely abolished the growth-inhibitory activity of Ras(17N) and decreased the transforming activity of normal but not oncogenic Ras. Furthermore, Ras(WT) proteins with substitutions at these residues were impaired in their ability to be activated by the catalytic domain of SOS1. Taken together, these observations suggest that these mutations have impaired the ability of Ras to associate with GEFs and that residues 75 to 78 are important for a functional Ras-GEF interaction. Although it is possible that the mutations at residues 75 to 78 merely reversed the guanine nucleotide-binding defect caused by the S17N mutation, our observation of unchanged levels of GTP (~90%) complexed to Ras(12R) proteins containing mutations at these positions is inconsistent with this possibility.

Our observation that residues 75 to 78 are important for Ras-GEF interaction is similar to results described by Verrotti et al. (40), who determined that yeast RAS2 residues 80 and 81 (corresponding to human Ras residues 73 and 74) were important for stimulation of GDP-GTP exchange by SDC25. They observed that amino acid substitutions at these two residues impaired the function of wild-type RAS2 but not G19V mutants of RAS2. Consequently, taken together with our results, residues 73 to 78 may represent a Ras domain which is essential for GEF activation of Ras GDP-GTP exchange. Whether mutations at these residues perturb both stimulation and binding or merely perturb stimulation is presently not known. We are currently assessing the ability of Ras proteins with mutations at positions 75 to 78 to associate with Ras GEFs.

In contrast to our observations, two recent site-specific mutagenesis studies have implicated other Ras residues which may be important for Ras-GEF interaction. First, Mistou et al. (25) determined that single-amino-acid substitutions in loops L2 (T35A, D38A, and D38E) and L4 (Q61H, Q61L, E62H, and E63H) all strongly impaired stimulation, but not binding, by SDC25. These residues are in the two regions, designated switch I (residues 32 to 38) and II (residues 60 to 76), whose conformations differ in the GDP- and GTP-bound states of Ras proteins and which are also involved in GAP binding and stimulation. Second, Segal et al. observed that deletion of H-Ras residues 97 to 105, which are dispensable for Ras transforming activity, impaired Ras GEF (yeast and mouse CDC25, yeast SDC25) activation of the ability of Ras to stimulate yeast adenylate cyclase activity (34). The consequence of this deletion on GEF binding was not determined. Although it is possible that the different observations made in these studies merely reflect the use of different assays for Ras-GEF interaction, it is also likely that, whereas some Ras residues will be important for GEF binding, others will be required for GEF stimulation.

How might residues 75 to 78 affect the interaction of Ras with GEF? One logical possibility is that these residues directly associate with GEFs (Fig. 6). Alternatively, they may critically influence the conformational distinction between the GDP- and GTP-bound forms of Ras that allows preferential recognition of Ras-GDP by GEFs. This second possibility is supported by the observation that yeast CDC25 binds preferentially to the GDP-bound form of RAS2 and that a conformational change to the GTP-bound state abolishes CDC25 binding (27). As mentioned above, the switch I and switch II domains that distinguish the GDP- and GTP-

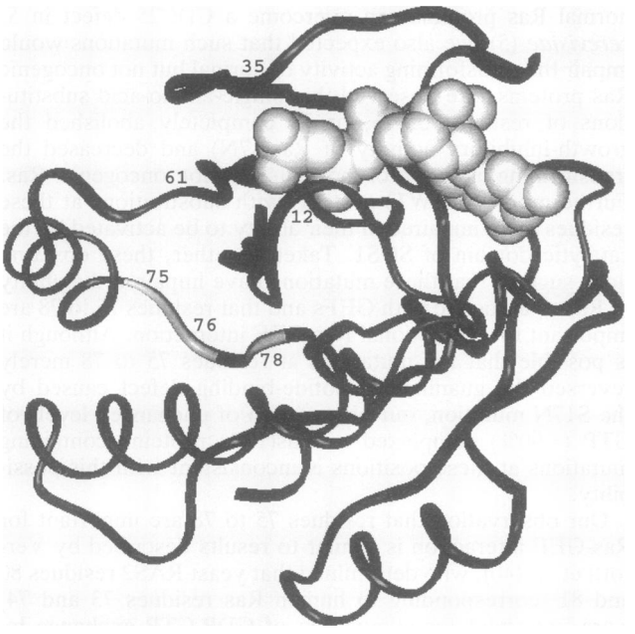

FIG. 6. Location of Ras residues 75, 76, and 78. Ras residue 75 may represent a critical position for the conformational switch between Ras-GDP and Ras-GTP. Residue 76 is located in a loop which is exposed at the surface of the Ras protein and may be involved in interaction with Ras GEFs. The effects of a substitution at residue 78 may be due to perturbation of the orientation or flexibility of residues 75 and 76.

complexed forms of Ras have been defined by x-ray structural analyses (42). The backbone flexibility of residues 75 and 77 has been speculated to be important for the conformational change between the biologically active Ras-GTP and inactive Ras-GDP states (20). Furthermore, Kavounis et al. (20) reported that a G82S (residue 75 in human Ras) mutation in yeast RAS2 protein weakened interactions with the nonhydrolyzable GTP analog Gpp(NH)p (20). They speculated that this replacement might hinder attainment of the active conformation of the RAS2 protein without significantly affecting the GDP bound state. Thus, mutation of specific residues involved in binding to GEF or residues important for attainment of the GDP- versus GTP-bound form could perturb Ras-GEF interactions. Finally, it is also important to note that mutations in residues that facilitate the GEF-induced guanine nucleotide exchange may also be expected to impair Ras-GEF interactions.

A critical role for residues that distinguish the GDP and GTP versions of Ras may also explain why several domains have been implicated to be important for Ras-GEF interactions. For example, Stouten et al. (38) recently proposed a model, which is based on the crystal structures of Ras-GTP and EF-Tu-GDP, that defines specific residues in helix α2 and loop L5 (switch II) (changes in which are propagated to helix α3 and loop L7) which are important for conversion of Ras between its active and inactive forms. This may account for the observation that deletion of residues 103 to 108 in α3-L7 impairs Ras activation by CDC25 (34). Therefore it is not entirely surprising that residues identified by different investigators as being important for GEF interaction are either surface-accessible residues in helices α2 and α3 (residues 73, 74, 104, and 105) (Fig. 6), whose conformation differs between GTP- and GDP-bound forms, or residues (residues 75 to 77) whose flexibility is important for formation of the active, GTP-bound form. Consequently, GEFs may interact with several exposed residues present in both α2 and α3 whose spatial distribution is dependent on the guanine nucleotide state of Ras.

Biochemical analyses of the specificity of Ras GEF proteins such as CDC25 or SOS suggest that they are specific for Ras proteins and do not stimulate guanine nucleotide exchange on other members of the Ras superfamily (6, 35). As shown in Table 1, most members of the Ras superfamily show amino acid divergence in the Ras residues that have been identified in this study as important for GEF interaction (39). For example, Rho and Rab protein members show divergence at all three positions corresponding to Ras residues 75, 76, and 78. Interestingly, although Ral proteins are not responsive to Ras GEFs, they share the same residues at these three positions. Thus, residues flanking positions 75, 76, and 78 may also be important for determining the specificity of Ras GEFs for Ras proteins. Further mutational studies of this region in Ras and Ras-related proteins will help to establish the structural basis for specific stimulation of Ras by Ras GEFs. Additionally, biochemical and structural analysis of Ras proteins with mutations at these residues may help to elucidate the structural basis for the differential recognition of the GDP- versus GTP-complexed forms of Ras by GEFs and to determine how GEFs may trigger the formation of Ras-GTP.

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ADDENDUM IN PROOF

After submission of this article, mutations at residues 66 and 75 of Ras were reported to perturb its interaction with SDC25 (L. R. Lowe and C. J. Marshall, Oncogene **8**:2583–2590, 1993).

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