PROTEINS: Structure, Function, and Genetics 6:306–315 (1989)

Ras Interaction with the GTPase-Activating Protein (GAP)

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ABSTRACT **Biologically active forms of Ras complexed to GTP can bind to the GTPase-activating protein (GAP), which has been implicated as a possible target of Ras in mammalian cells. In order to study the structural features of Ras required for this interaction, we have evaluated a series of mutant *ras* proteins for the ability to bind GAP and a series of Ras peptides for the ability to interfere with this interaction. Point mutations in the putative effector region of Ras (residues 32–40) that inhibit biological activity also impair Ras binding to GAP. An apparent exception is the Thr to Ser substitution at residue 35; [Ser-35]Ras binds to GAP as effectively as wild-type Ras even though this mutant is biologically weak in both mammalian and *S. cerevisiae* cells. In vitro, [Ser-35]Ras can also efficiently stimulate the *S. cerevisiae* target of Ras, adenylyl cyclase, indicating that other factors may influence Ras/protein interactions in vivo. Peptides having Ras residues 17–44 and 17–32 competed with the binding of Ras to *E. coli*-expressed GAP with IC_{50} values of 2.4 and 0.9 μM, respectively, whereas Ras peptide 17–26 was without effect up to 400 μM. A related peptide from the yeast GTP-binding protein YPT1 analogous to Ras peptide 17–32 competed with an IC_{50} value of 19 μM even though the YPT1 protein itself is unable to bind to GAP. These results suggest that determinants within Ras peptide 17–32 may be important for Ras binding to GAP.**

Key words: oncogene, GTP-binding protein, cancer, *S. cerevisiae* adenylyl cyclase

INTRODUCTION

The product of the Harvey (Ha) *ras* oncogene is a 21 kDa membrane-bound protein which binds GTP and GDP, and has an intrinsic GTPase activity.[1,2] The *ras* oncogene product is referred to as p21 or Ras. Ras has structural similarities with other regulatory G proteins, and is biochemically and biologically active only when complexed to GTP. Specific amino acid changes in Ras at positions 12, 13, 59, 61, and 63 impair the intrinsic GTPase activity, thereby stabilizing the protein in the active GTP form.[1,2] These oncogenic mutations of *ras* have been reported to occur in a variety of neoplasia, including human colorectal tumors, carcinomas from human exocrine pancreas, and myeloid leukemias.[1–4]

In the yeast *Saccharomyces cerevisiae, RAS* proteins regulate the levels of intracellular cAMP by stimulating adenylyl cyclase activity.[2] This interaction occurs only with RAS-GTP, and not with RAS-GDP.[5] Biochemical evidence has indicated that adenylyl cyclase is not the Ras target in mammalian cells.

Critical to our understanding of Ras function has been the analysis of the structure of this 189-amino acid oncogene product. Prior to the solution of the crystal structure of Ras, mutagenesis studies of Ras identified regions of the protein involved with membrane localization,[10] guanine nucleotide binding,[6–9] binding of a neutralizing antibody,[11] and interaction with target proteins.[11–14] Some of these regions are highlighted on the model of Ras shown in Figure 1 that is based upon crystallographic data.[15,16] Membrane localization is essential for Ras function in mammalian cells and involves Cys-186. The purine ring of GDP interacts with Asn-116 and Asp-119, and the phosphates lie near residues 12–16. The residues that comprise the Y13-259 antibody epitope are Glu-63, Ser-65, Ala-66, Met-67, Gln-70, and Arg-73. Antibody Y13-259 upon microinjection into mammalian cells or *Xenopus* oocytes inhibits DNA synthesis and morphological changes induced by Ras.[17,18] Although antibody Y13-259 neutralizes Ras function, mutations within the epitope reacting with Y13-259 do not impair Ras action indicating that Y13-259 action is indirect. The putative effector region is a surface domain of Ras spanning amino acids 32–40 that is critical for the interaction be-

Abbreviations: EF-Tu, elongation factor-Tu; GAP, GTPase-activating protein; Ha-*ras* p21, the 21 kDa protein product of the Harvey *ras* oncogene; Ras, the gene product of mammalian *ras;* RAS1, the gene product of *S. cerevisiae RAS1.*

Received May 8, 1989; revision accepted July 17, 1989.

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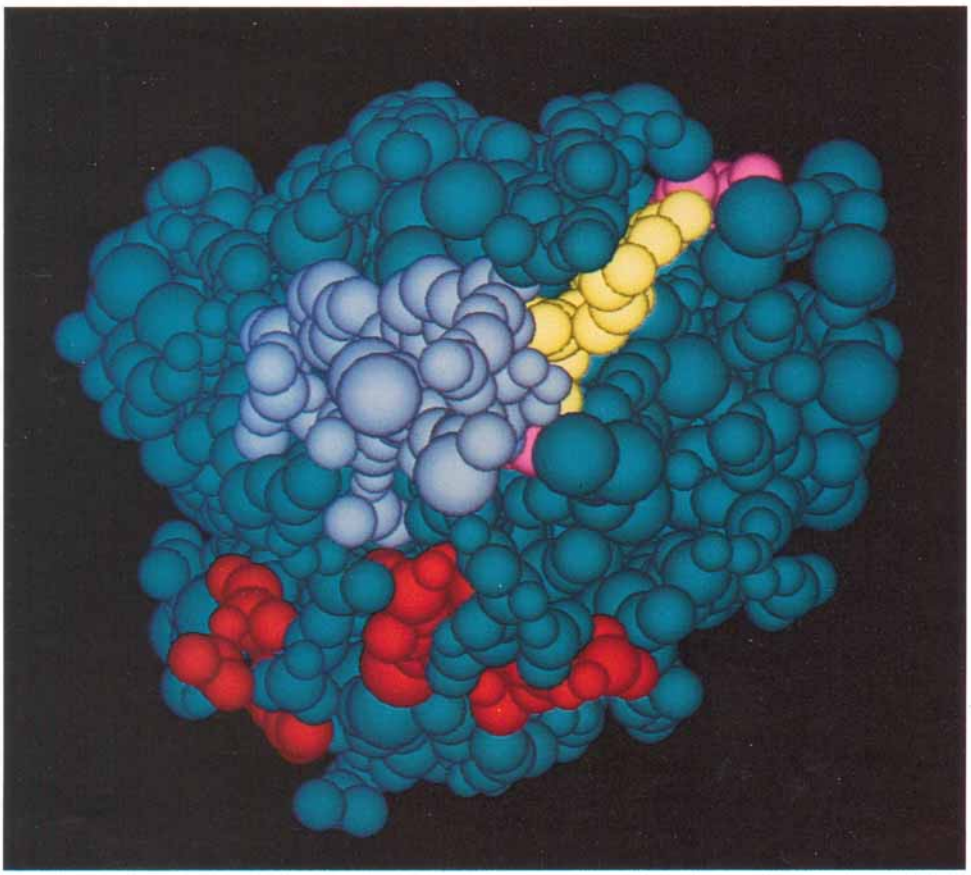

Fig. 1. Critical regions of Ras. On the structure of [Val-12]Ras complexed to GDP (yellow) are highlighted residues required for nucleotide binding (pink, amino acids 16 and 119), effector function (purple, amino acids 32–40), and the binding of the neutralizing antibody Y13-259 (red, amino acids 63, 65–67, 70, and 73).

tween Ras and its target.[11–14] Mutations in this region impair the cell transforming activity of Ras but are without effect on membrane localization or GDP binding. The region 32–40 could either be a direct point of interaction between Ras and its target or might influence the adoption of an active conformation.

Studies of the *ras* oncogene are directed toward identifying the Ras target in mammalian cells and understanding the mechanisms that regulate Ras activity. Recently, the GTPase-activating protein (GAP) was identified by Trahey and McCormick[19] as an activity in the cytosolic fraction of *Xenopus* oocytes which was able to stimulate the intrinsic GTPase activity of normal Ras 100-fold but was without effect on the impaired GTPase activity of oncogenic Ras. Through its interaction with Ras, GAP may be acting as an upstream negative regulator of Ras, by affecting the biochemical activity which determines the biological potency of the protein. Alternatively, GAP may act as a downstream target of Ras, in a manner analogous to the interaction between EF-Tu and the ribosome.[20,21,35]

In order to evaluate GAP function in the Ras pathway, GAP was purified as a monomeric 125 kDa polypeptide and the cDNA was cloned.[22–24] The GAP gene encodes a protein of 1044 amino acids. Although purified GAP does not stimulate the GTPase activity of oncogenic Ras mutants, it is able to physically bind to the GTP forms of both normal and oncogenic Ras.[23,25] Mutations in the Ras region 32–40 that impair biological function can also inhibit binding to GAP raising the possibility that GAP might be the immediate target of Ras.[23,25–27]

The sequence of GAP showed a significant amino acid similarity between regions in the N-terminal half of GAP and the sequences of the B and C boxes conserved among the noncatalytic domains of nonreceptor tyrosine kinases, the *crk* oncogene product and phospholipase C-148.[23,24] By deletion mutagenesis, a 343-amino acid C-terminal fragment of GAP was identified which has the same catalytic activity and Ras binding affinity as full length GAP purified from bovine brain.[28] Together, these observations suggest that GAP may have two structural domains, with the N-terminus having a functional role distinct from the C-terminal Ras binding domain.

In this paper, we have extended our initial obser-

vations on the interactions between Ras and GAP. The Ras structural requirements for binding to GAP in vitro are similar to those needed for stimulation of *S. cerevisiae* adenylyl cyclase activity, an effector enzyme of Ras. In addition, a peptide having Ras residues 17–32 competed for Ras/GAP binding suggesting that a point of contact between Ras and GAP is present in this region.

MATERIALS AND METHODS

Protein Purification

Mammalian GAP was purified from the cytosolic fraction of a bovine brain cerebral homogenate as described previously.[22] Full length recombinant GAP and [702–1044]GAP were expressed in *E. coli* using the *lac* Z promoter,[28] and were purified from the soluble fraction of an *E. coli* lysate.[28] Ha *ras* p21 mutants were expressed in *E. coli* and purified as previously described.[23,28]

Guanine Nucleotide Exchange of Ras

To prepare a substrate for the GAP assay, normal Ha-*ras* p21 was equilibrated with [γ-^{32}P]GTP (3000 Ci/mmol, ICN Radiochemicals), in the following reaction: 0.1 nmol of Ha-*ras* p21 and 0.2 nmol [γ-^{32}P]GTP were added to 1 ml of buffer containing 2 mM EDTA, 2 mM DTT, 100 μg/ml BSA (Sigma: fatty acid free), 25 mM Tris-HCl, pH 7.5. Following incubation for 15 minutes at 30°C, the reaction was chilled on ice and applied to a PD-10 column (Pharmacia) to remove free guanine nucleotide. The column was eluted at 4°C by adding successive 1 ml aliquots of buffer A (1 mM $MgCl_2$, 20 mM sodium Hepes, pH 7.5), and collecting 1 ml column fractions. Aliquots of each fraction were analyzed by a filter binding assay[22] to determine the efficiency of the guanine nucleotide exchange reaction. Only fractions having greater than 80% of the [γ-^{32}P]GTP incorporated into *ras* p21 were used in the GAP assays.

To prepare *ras* mutants as competitors for the kinetic binding assay, a similar exchange reaction was performed, except nonradioactive GTP (Sigma: Cat. #G-5631), GDP (Sigma: Cat. #G-6506), Gpp(NH)p, GTP[γS] or GDP[βS] (Boehringer Mannheim: Cat. #106402, 220647, and 528536, respectively), were added in a 10-fold molar excess over Ras. Following the exchange reaction, *ras* protein was concentrated to one-tenth volume by centrifugation at 4°C in a Centricon-10 microconcentrator (Amicon) at 5000*g*. This concentrated *ras* protein solution was diluted with 10 volumes of buffer A, and concentrated again to one-tenth volume. By repeating this procedure three more times, the free guanine nucleotide was removed, and the buffer was equilibrated with buffer A. The final concentration of the *ras* proteins was 2–4 mM.

GAP Assays

Ras GTPase activity was measured by the charcoal adsorption procedure.[23,28] Each 50 μl reaction contained 10 fmol purified GAP, 240 fmol Ha *ras* p21-[γ-^{32}P]GTP, and 1 mg/ml BSA in buffer A. Varying amounts of the competing *ras* mutants or peptides were added as indicated. Prewarmed reactions (24°C) were initiated by the addition of GAP, then incubated for 4 minutes at 24°C. The reactions were terminated by the addition of 5 volumes of ice cold 5% activated charcoal (Sigma: HCl washed) in 50 mM NaH_2PO_4. GTPase activity was quantitated by measuring the free [^{32}P]orthophosphate in the cleared supernatant after centrifugation of the charcoal mix. GAP-stimulated Ras GTPase activity in the absence of competitors was 40–60 fmol Ras-GTP hydrolyzed, the 100% control value. In the absence of GAP, GTP hydrolysis was not detectable during the incubation period of the assay.

Binding of Ras Peptides to GAP

Ras peptides were dissolved in either 100% dimethyl formamide (DMF, American Burdick and Jackson) or 100% dimethyl sulfoxide (DMSO, Fluka UV-spectroscopy grade) at a calculated peptide concentration of 1–2 mM. Following 1 minute in a Sonicor SC-200 bath sonicator at room temperature, the peptide solutions were vortexed, and then centrifuged for 5 minutes at room temperature in an Eppendorf microfuge to precipitate any undissolved material. The cleared peptide solutions (20 μl) were then added to 80 μl of buffer A in a dilution series covering a range of peptide concentrations from 0.02 μM up to the maximum solubility limit for each peptide. In each dilution, the solvent concentration was maintained at 20%. These peptides solutions were then centrifuged for 5 minutes at room temperature at 10,000*g*, and the cleared supernatants were divided into two parts. One part of each cleared peptide dilution was used in a GAP competition assay: 40 μl was added to a 5 μl solution containing 0.5 mg/ml BSA plus 240 fmol Ha-*ras* p21-[γ-^{32}P]GTP. Reactions were initiated upon addition of 5 μl of GAP (10 fmol), and analyzed as described above.

The other portion of the cleared peptide solution was measured spectrophotometrically to determine solubility and concentration of the peptides. Extinction coefficients were determined by measuring the absorbance at 277 nm of two peptides whose exact concentrations were quantitated by amino acid analysis. The extinction coefficients for peptide 17–37 dissolved in buffer A/20% DMF and peptide 91–103 dissolved in water/20% DMSO were 2580 and 2230 cm^{-1} M^{-1}, respectively. These values were used to determine the exact concentration for each peptide used at each point in the competition assay. Peptides lacking tyrosine were quantitated by amino acid analysis. In addition, peptide solubility

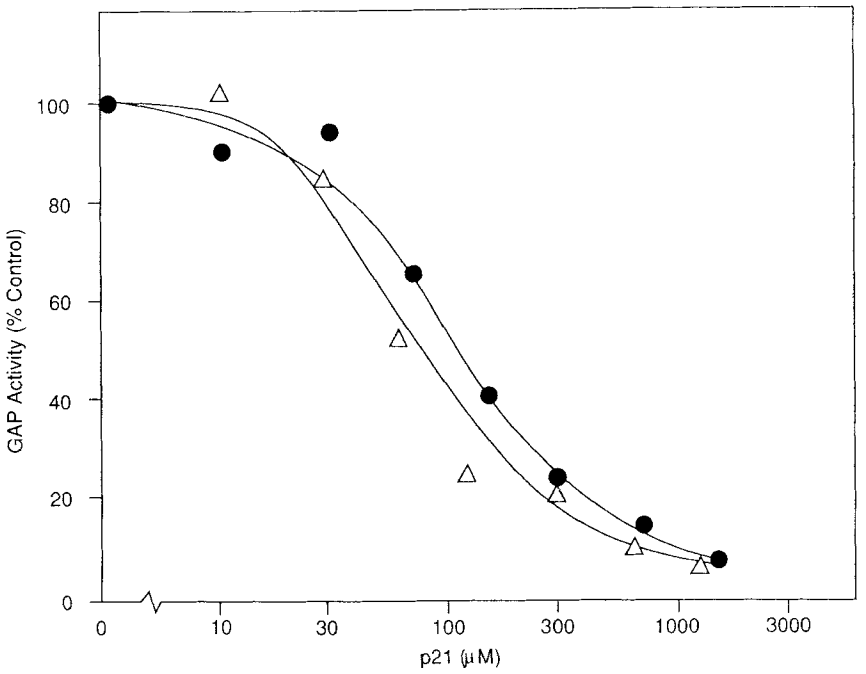

Fig. 2. GAP binding to the GTP and Gpp(NH)p complexes of Ras. Normal Ha *ras* p21 was complexed with GTP (●) or Gpp(NH)p (△) and added in increasing amounts to GAP reactions containing 10 fmol pure bovine brain GAP and 240 fmol of normal Ha *ras* p21-[γ-^{32}P]GTP. GAPactivity was quantitated by measuring the amount of [^{32}P]orthophosphate liberated from the GTP hydrolysis reaction. Activity is expressed as a percentage of the activity of GAP measured in the absence of competitors.

was confirmed by comparing the light scattering at 600 nm for each peptide solution measured before and after centrifugation. Only concentrations reflecting 100% solubility were used in the dose–response curves, and the maximum concentration used for each peptide competitor indicates the limit of solubility for that peptide.

Peptide Synthesis

The peptides were prepared by solid phase synthesis[29] using a double-coupling protocol for the introduction of all amino acids on the ABI automated peptide synthesizer (Model 430A). Deprotection and removal of the peptide from the resin support was effected by treatment with ligand HF.[30] Purification was achieved by preparative HPLC on a reverse-phase C_{18} silica column (25 cm × 2.5 in.) using a 0–100% gradient of acetonitrile containing 0.1% TFA.[31] Homogeneity was demonstrated by analytical HPLC and identity was confirmed by amino acid analysis.

Adenylyl Cyclase Assay

Yeast strain F1D[32] was grown in YEHD medium at 30°C, and membranes were prepared as described.[13] Since these membranes contain adenylyl cyclase, but are lacking *RAS* protein, stimulation of adenylyl cyclase activity may be measured following reconstitution with purified Ras complexed with GTP. Reactions containing 83 μg of F1D membranes and *ras* proteins at a final concentration of 100 μM were carried out in a 100 μl volume according to the procedure described by Casperson et al.[33] as modified.[13]

RESULTS

Guanine Nucleotide Dependence of Ras Binding to GAP

We have shown previously that both normal Ras and [Val-12]Ras are able to bind to purified bovine brain GAP in a GTP-dependent manner.[23] Since Ras is sensitive to GAP-stimulated GTP hydrolysis, the ability of Ras complexed to Gpp(NH)p, a nonhydrolyzable GTP analog, to bind to GAP was tested in a kinetic competition assay. As shown in Figure 2, the binding isotherms for Ras complexed to either GTP or Gpp(NH)p were similar, with IC_{50} values of 110 and 80 μM, respectively. Ras complexed to GTP[γS], another nonhydrolyzable GTP analog, is also able to bind GAP (data not shown), although GAP did not stimulate hydrolysis of Ras-GTP[γ-^{35}S] (as tested by incubation at 24°C for 30 minutes with 30 fmol of GAP).

The GDP complexes of normal Ras and [Val-12]Ras had little if any interaction with GAP up to 1 mM, the limit of our ability to concentrate Ras in active form.[23] [Leu-61]Ras-GTP has a binding affinity for GAP of 1.5 μM, which is 50- to 100-fold stronger than observed with normal Ras or [Val-12]Ras[23]; this observation enabled us to test for a binding in-

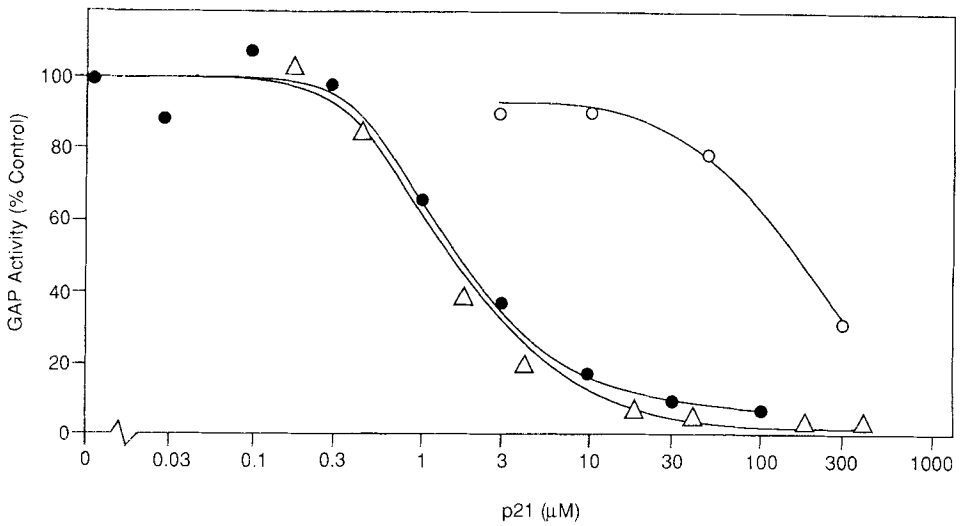

Fig. 3. Binding of GAP to either mammalian or yeast *ras* proteins. [Leu-61]Ha *ras* p21 was complexed to either GTP (●) or GDP (○), and *S. cerevisiae* [Leu-68]RAS1 (term. 185) was complexed to GTP (△) as described in Methods. GAP activity was measured with increasing amounts of these proteins.

teraction between GAP and the GDP complex of [Leu-61]Ras. As shown in Figure 3, [Leu-61]Ras-GDP (IC_{50} = 150 μM) was able to compete in the GAP assay with a potency 100-fold poorer than observed for the biologically active GTP complex (IC_{50} = 1.5 μM). An identical binding affinity (IC_{50} = 150 μM) was obtained using [Leu-61]Ras-GDP[βS], a GDP analog resistant to conversion to GTP (not shown).

Structural Determinants Essential for Ras Binding to GAP

S. cerevisiae and mammalian Ras share 82% amino acid similarity between residues 1 and 81 and 45% similarity between residues 82 and 164 beyond which there is no homology until the membrane localization consensus site.[2] Previously we observed that the N-terminal domain of *S. cerevisiae* RAS1 having residues 1–185 was sensitive to GAP in the GTPase assay.[22] To assess binding affinities of the evolutionarily diverse mammalian and yeast Ras for GAP, we compared mammalian [Leu-61]Ras p21 with the analogous *S. cerevisiae* RAS1 mutant, [Leu-68]RAS1(term. 185). As shown in Figure 3, the binding affinities for the GTP complexes of both [Leu-61]Ras p21 and [Leu-68]RAS1(term. 185) were the same with IC_{50} values of 1.5 and 1.1 μM, respectively. This observation demonstrates that all the determinants required for Ras/GAP interactions are conserved between mammalian and *S. cerevisiae ras* proteins.

Mutations in the putative effector region of Ras, residues at 32–40, can inhibit cell transforming activity and GAP binding.[11–14,21,23,25–27] Results in Table I summarize some of the GAP binding data for these mutants. The mutations were verified by DNA sequencing and by immunoblot analysis of the purified proteins using antisera that detect amino acid changes at Ras residues 33 and 35 or at residues 32 and 38 (not shown). In general, if a mutation in the effector region reduces cell-transforming activity it also reduces GAP binding. The Thr to Ser substitution at residue 35 is an apparent exception to this rule. Forms of mammalian Ras or *S. cerevisiae* RAS2 having this mutation are transformation defective and are biologically impaired in yeast cells.[13,14] However, [Ser-35]Ras binds effectively to GAP in a GTP-dependent manner (Table I) with an IC_{50} value of 200 μM (not shown) and is weakly responsive to GAP in a GTPase assay.[21,25] A poorer binding interaction was detected between GAP and [Val-12, Ser-35]Ras p21.

The apparent discrepancy between the weak biological activity and the efficient GAP binding activity of [Ser-35]Ras might indicate that GAP either is not the target of Ras or that Ras/protein interactions in vitro are not identical to those in vivo. To extend these observations, the various *ras* proteins were tested for the ability to reconstitute *S. cerevisiae* adenylyl cyclase activity in isolated membranes (Table I). This assay enables evaluation of the interaction of proteins with a known target system of Ras. [Ser-35]Ras was able to stimulate adenylyl cyclase activity in vitro even though this form of Ras is unable to complement RAS2 function in vivo as assayed by yeast growth on a nonfermentable carbon source.[13] [Val-12, Ser-35]Ras was weaker in its ability to stimulate adenylyl cyclase activity. The small stimulation of adenylyl cyclase activity observed with other mutant *ras* proteins was GTP dependent, indicating that a weak interaction was occurring. YPT1, which is a Ras-related 20 kDa GTP-binding

TABLE I. Interaction of Ras Mutants with GAP and *S. cerevisiae* Adenylyl Cyclase*

Ras mutant	GAP binding (% inhibition)	Yeast adenylyl cyclase reconstitution (pmol cAMP/minute/mg)
Normal Ras	100	12
[Asn-33]Ras	35	1
[Ala-35]Ras	35	0
[Ser-35]Ras	100	9
[Ala-38]Ras	0	0
[Asn-38]Ras	10	2
[Val-12]Ras	100	31
[Val-12, Asn-33]Ras	10	2
[Val-12, Ser-35]Ras	45	4
[Val-12, Asn-38]Ras	nd†	2
[Leu-61]Ras	100	17
YPT1	0	0

*Purified Ras proteins complexed to GTP were tested in GAP competition assays or in the yeast adenylyl cyclase reconstitution assay. The point mutations in the region 32–40 have been shown to reduce Ras biological potency.[11–14,21,25–27] In the GAP assay, *E. coli*-expressed GAP was used, and the % inhibition is indicated for assays having 1 mM competing protein. In the adenylyl cyclase assay, *ras* proteins were tested at 100 μM. Basal adenylyl cyclase activity in the presence of Mg^{2+} alone (no Ras) was 2 pmol/minute/mg, and this value was subtracted from the activity values obtained in the presence of *ras* proteins. The GDP complexes of the *ras* proteins were inactive (not shown).

†nd, not determined.

protein but which is functionally distinct,[34] did not bind to GAP and did not stimulate adenylyl cyclase activity. Forms of Ras with effector mutations did not compete with the ability of wild-type Ras to stimulate adenylyl cyclase activity (not shown).

Competition of Ras/GAP Interactions by Ras Peptides

Ras peptides were tested for the ability to compete with the binding between Ras and *E. coli*-expressed GAP, and the results are summarized in Table II. To ensure that the experimental results would not reflect any potential peptide insolubility, concentrated solutions of peptide in solvent were diluted into the aqueous assay buffer, centrifuged to remove insoluble material, and then the clarified supernatant was added to the protein mixture (see Methods).

A peptide having Ras residues 17–44 effectively competed for Ras/GAP binding with an IC_{50} value of 2.4 μM. This affinity is similar to that observed with [Leu-61] Ras-GTP. Effective competition was also observed for Ras peptides 17–37 and 17–32 which lack at least some of the residues identified by the Ras mutagenesis studies as being critical for Ras biological activity. Peptides 17–26, 23–37, and 31–43 were ineffective up to 400 μM. Mixtures of these inactive peptides were also without effect. Peptides from other regions of Ras such as 60–76 (the epitope for the neutralizing antibody Y13-259), 91–103, and 126–137 were also inactive; these peptides encompass residues which can be deleted from Ras without impairing cell transforming activity.[12] A peptide having YPT1 residues 22–37, analogous to Ras region 17–32, competed with an IC_{50} value of 19 μM, 21-fold poorer than that observed with Ras peptide 17–32. None of the peptides inhibited Ras intrinsic GTPase activity assayed in the absence of GAP or kirromycin-stimulated *E. coli* EF-Tu GTPase activity[35,36] (data not shown). In *S. cerevisiae* adenylyl cyclase reconstitution assays having 5 μM normal mammalian Ras-GTP and 10 μM competing peptide, adenylyl cyclase activity was inhibited 24 and 0% by Ras peptides 17–32 and 60–76, respectively.

A deletion analysis of GAP has identified a 343-amino acid C-terminal domain ([702–1044]GAP), which is sufficient to bind Ras and stimulate Ras GTPase activity.[28] As shown in Figure 4, Ras peptide 17–44 competed for binding to bovine brain GAP and *E. coli*-expressed [702–1044]GAP with IC_{50} values of 7.5 and 90 μM, respectively, a 12-fold difference. By comparison, [Val-12]Ras-GTP binds to both full length GAP and [702–1044]GAP with the same affinity (IC_{50} = 200 μM) as we have observed previously [28] for [Leu-61] Ras-GTP (IC_{50} = 2 μM).

DISCUSSION

In this paper, we have extended in greater detail our initial observations on the binding interaction between Ras and GAP. GAP is the only mammalian protein identified so far that physically interacts with the *ras* oncogene protein, and as such is an important link toward understanding the role of Ras in oncogenesis. By characterizing how these two proteins associate, we can begin to determine how Ras functions in the cell. The binding of Ras to GAP is clearly guanine-nucleotide dependent. The results in Figure 2 indicate that adoption of the active Ras conformation can be equally conferred by either GTP or Gpp(NH)p. Furthermore, there is at least a 100-fold preference for binding to GAP by this conformation over that adopted by Ras when complexed to GDP, which is biologically inactive.

Analyses of the *ras* protein have suggested that the domain having amino acid residues 32–40 is involved in the formation of the biologically active conformation of Ras.[11–14] Ras effector function appears to have been conserved between *S. cerevisiae* and mammalian cells.[2] If mammalian GAP is a downstream target protein of Ras it would be predicted that mutant *ras* proteins impaired in GAP binding in vitro would also be unable to stimulate the yeast adenylyl cyclase in vitro. As shown in Table I, *ras* proteins having mutations that weaken GAP binding were also less effective in stimulation of adenylyl cyclase. Of the forms of Ras having mutations in the region 32–40, only [Ser-35]Ras was fully active in these assays. [Ser-35]Ras expressed in

TABLE II. Effect of Peptides on Ras/GAP Interaction*

Peptide			Competition IC_{50} value (μM)
Ras	17–44	SALTIQLIQNHFVDEYDPTIEDSYRKQV	2.4
Ras	17–37	SALTIQLIQNHFVDEYDPTIE	3.6
Ras	17–32	SALTIQLIQNHFVDEY	0.9
Ras	17–26	SALTIQLIQN	>400
Ras	23–37	LIQNHFVDEYDPTIE	>400
Ras	31–43	EYDPTIEDSYRKQ	>400
Ras	60–76	GQEEYSAMRDQYMRTGE	>400
Ras	91–103	EDIHQYREQIKRV	>400
Ras	126–137	ESRQAQDLARSY	>400
YPTI	22–37	SCLLLRFSDDTYTNDY	19

*Peptides were prepared in DMF as described in Methods to ensure complete solubility and then tested in the GAP assay at various concentrations in order to determine an IC_{50} value. An IC_{50} value of >400 indicates no observable inhibition at the highest soluble peptide concentration tested (400 μM).

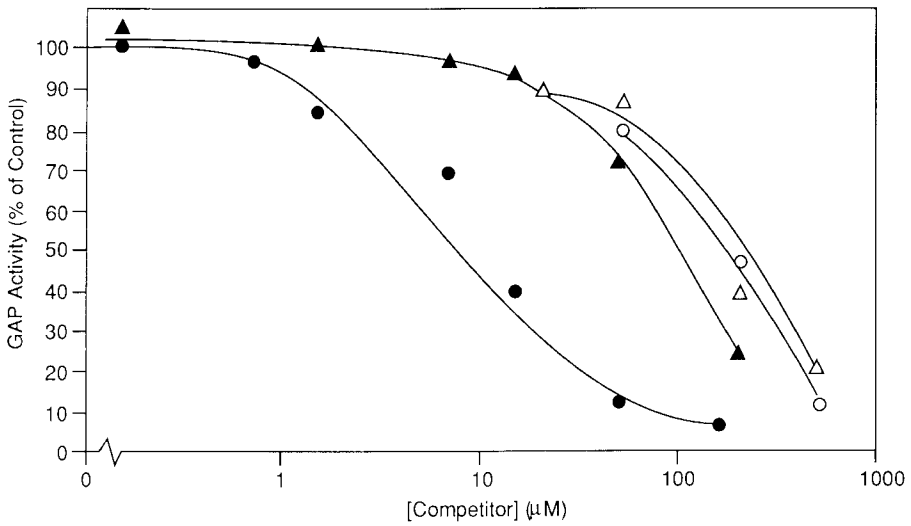

Fig. 4. Interaction of Ras protein and peptide with GAP and [702–1044]GAP. Reactions contained either bovine brain GAP (●,○) or *E. coli*-expressed [702–1044]GAP (▲,△). Competition of Ha *ras* p21-[γ-^{32}P]GTP hydrolysis was measured in the presence of increasing concentrations of [Val-12]Ha *ras* p21-GTP (○,△) or Ras peptide 17–44 (●,▲).

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                  *     * *   *   * *
Ras 17-32   [S] A [L] T I Q L I Q N H F V D E [Y]
YPT1 22-37  [S] C [L] L L R F S D D T Y T N D [Y]
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Fig. 5. Comparison of Ras and YPT1 sequences. Similarity between Ras residues 17–32 and the analogous region of YPT1, residues 22–37, is indicated for identical (boxed) and conserved (*) amino acids. Conservative amino acid replacements are defined as previously described,[23] with the following groupings: A, G, P, S, T; L, I, V, M; D, E, N, Q; K, R, H; F, Y, W; and C.

S. cerevisiae also stimulates yeast adenylyl cyclase in partially purified yeast membranes.[13]

The biochemical activity of [Ser-35]Ras in vitro apparently conflicts with the impaired biological function of this protein in vivo. Other investigators have reported that transformation-defective Ras mutants such as [Ile-40]Ras and [Ser-40]Ras are able to bind to GAP in vitro.[21] The results indicate that the in vitro binding of Ras to either GAP or *S. cerevisiae* adenylyl cyclase does not mimic perfectly the in vivo interaction, possibly suggesting that other factors might be involved. Although we do not know yet whether GAP is the target of Ras in mammalian cells, all biochemical and genetic evidence indicates that adenylyl cyclase is a target of Ras in *S. cerevisiae*.[2] Our results suggest that GAP and yeast adenylyl cyclase interact with *ras* proteins in a similar, although not necessarily identical, manner. For example, [Asn-33]Ras and [Ala-35]Ras appeared to interact with GAP better than with adenylyl cyclase (Table I). Thus, a comparison of Ras binding to GAP and yeast adenylyl cyclase cannot prove whether GAP functions as a regulator or the immediate target of Ras.

The results with Ras peptides identify another region of Ras, residues 17–32, important for its inter-

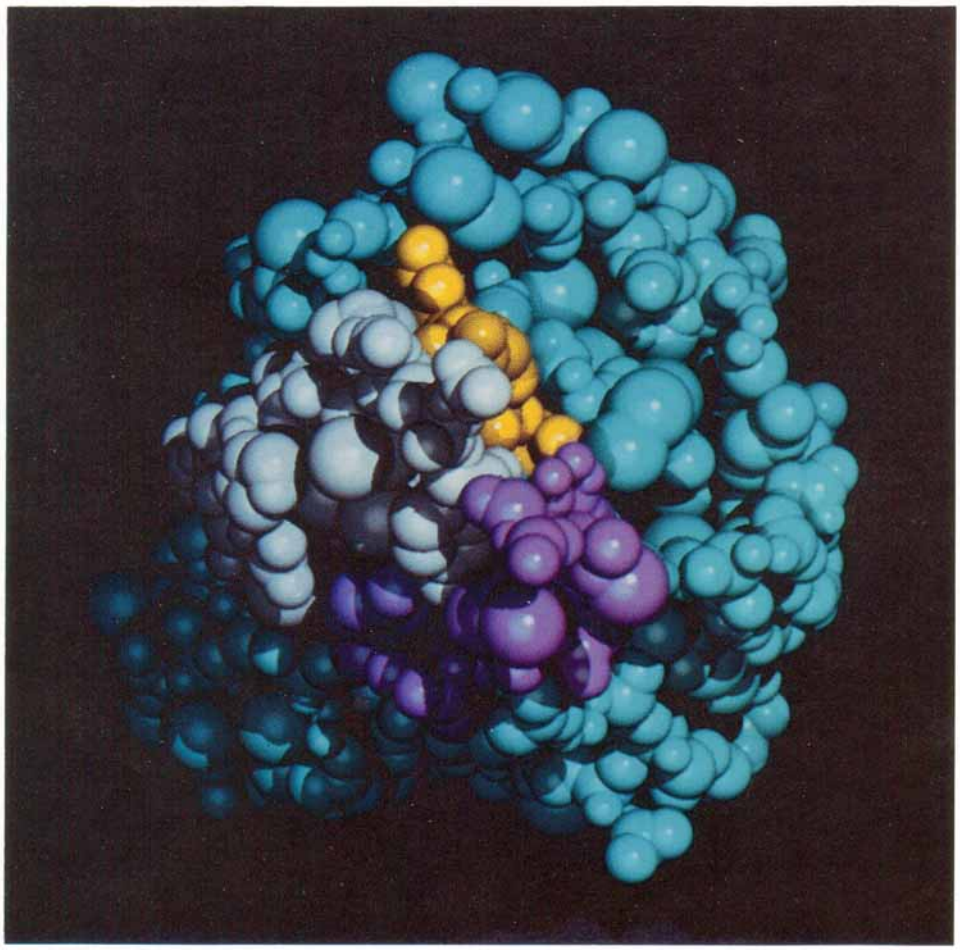

Fig. 6. Model of [Val-12]Ras complexed to GDP. GDP, yellow. Residues 17–32, gray. Residues 33–40, purple.

action with GAP. This region might influence the cell transforming activity of Ras and the tumor suppressing activity of Rap1A/K-rev; both proteins have identical sequences in the region 32-40 but are divergent in the region 21-31.[2] Previously, Willumsen et al.[12] observed that deletion of Ras Phe-28/Val-29 along with an insertion of Ser-Asp-Gln rendered Ras inactive for its ability to transform cells; the mutation was without effect on GDP binding. Interestingly, YPT1 peptide 22–37, analogous to Ras peptide 17–32, was also able to compete although with a 21-fold poorer affinity than observed with Ras peptide 17–32. The sequences of Ras 17–32 and YPT1 22–37, compared in Figure 5, reveal amino acid similarity throughout this region, and these two peptides might have similar secondary structure in solution. The inability of YPT1 protein to bind GAP indicates that other structural determinants might be involved.

At present, we do not know whether the peptides that inhibit Ras/GAP interactions are binding to Ras or binding to GAP. If the peptides were binding to Ras, we would predict an equally potent inhibition of Ras GTPase activity stimulated by either full-length GAP or [702–1044]GAP just as is observed when *ras* protein is used as the competitor. As shown in Figure 4, Ras peptide 17–44 was approximately 12-fold less potent in assays having [702–1044]GAP compared to assays having full-length GAP. In contrast, GAP peptide 888-910 from the presumed Ras binding site inhibited both GAP and [702-1044] GAP with similar affinities (approximately 100 μM, unpublished observations).[2,23] These results may indicate that the Ras peptide is binding to GAP. Deletion of the N-terminal domain of GAP might influence the structure or flexibility of the C-terminal half. The intact *ras* protein might be able to induce a tight-binding GAP conformation whereas the more flexible peptide would be less effective in this interaction.

In the crystal structure of Ras, an α-helix extends from residues 16–25 followed by a turn (residues 26–28), an extended coil (residues 29–36), and a β-sheet (residues 37–46). A model of Ras complexed to GDP is shown in Figure 6 which highlights residues in the regions 17–32 (gray) and 33–40 (purple). Both of these regions are well exposed over the region 24–40. The phosphates of GDP lie beneath this

region with Phe-28 interacting with the purine ring and Asp-30 near the ribose hydroxyl groups.[15,16] One can speculate that the binding of GTP might alter the conformation of this surface domain. It is possible that residues in both regions 17–32 (identified by the peptide studies) and 32–40 (identified by previous single amino acid substitution studies) are involved with Ras recognition and binding to other proteins. Lack of a competitive effect by Ras peptide 31–43 in the GAP assay does not rule out this region as being important for Ras/GAP interaction. It is possible that there is a minimal peptide size required for activity or a solution conformation that peptide 31–43 was unable to adopt. The hypotheses of Ras/GAP interactions generated by the peptide studies and protein modeling can now be tested directly by introducing single amino acid substitutions into the *ras* protein.

ACKNOWLEDGMENTS

We thank the following people: A. Ng for purified [702–1044]GAP; K. Stauffer for peptide synthesis; L. Wassel for amino acid analyses; O. Fasano for his gift of *S. cerevisiae* strain F1D; A. Parmeggiani for his gifts of kirromycin and purified *E. coli* EF-Tu; S.-H. Kim for the models of Ras and for helpful discussions; P. Anderson, R. Dixon, R. Freidinger, M. Rieman, and U. Vogel for helpful discussions.

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