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Biochemistry

Identification of effector residues and a neutralizing epitope of Ha-*ras*-encoded p21

(mammalian transformation/yeast *RAS* proteins/antibody-binding site/adenylate cyclase/guanine nucleotide-binding protein)

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ABSTRACT **To identify the amino acid residues of the Harvey (Ha) *ras*-encoded protein that are involved in protein–protein interactions, we have created a series of mutant Ha-*ras* proteins. In particular, amino acid substitutions have been introduced within two regions, residues 32–42 and 61–80, that are conserved among *ras* proteins from different species. We observed that amino acid substitutions at positions 35, 36, 38, 40, and, to a lesser extent, 39 and 78 reduce the biological potency of Ha-*ras* protein in both mammalian and *Saccharomyces cerevisiae* cells, without noticeably affecting the known intrinsic biochemistry of these proteins. The reduction of *in vivo* activity for these mutant *ras* proteins correlates with their reduced ability to stimulate yeast adenylate cyclase. The *ras*-protein-neutralizing antibody Y13-259 binds to six residues: Glu-63, Ser-65, Ala-66, Met-67, Gln-70, and Arg-73. Single substitutions for these residues reduce Y13-259 antibody binding by at least a factor of 1000 but do not significantly affect biological activity. These data are discussed in terms of the model for Ha-*ras* protein based on the structure of the elongation factor EF-Tu–GDP complex.**

The proteins encoded by the *ras* oncogenes are members of an evolutionarily conserved family of proteins that are present in mammalian and lower eukaryotic cells (1). These proteins, which are localized to the inner plasma membrane through the covalent attachment of lipid to a carboxyl-terminal cysteine residue, have intrinsic biochemical properties that are similar to those of the known guanine nucleotide-binding regulatory proteins (G proteins), such as the elongation factor EF-Tu, α-transducin, and the various G proteins that regulate mammalian adenylate cyclase (2, 3). Like these other G proteins, *ras* proteins specifically bind GDP and GTP and have a low GTP-hydrolytic activity (4–7). Oncogenic forms of the mammalian *ras* proteins—Harvey (Ha), Kirsten (Ki), and N-ras—arise from amino acid substitutions at specific positions: 12, 13, 59, 61, and 63 (see ref. 1 for review). The enhanced transforming potency of these mutant proteins correlates with their decreased GTPase activities, which presumably results in a higher intracellular concentration of the more active GTP complex.

Whereas the function of the *ras* proteins in mammalian cells remains unknown, normal cellular growth of *Saccharomyces cerevisiae* depends on the stimulation of membrane-bound adenylate cyclase by either of the yeast *ras* proteins, RAS1 or RAS2 (8–12). The mammalian *ras* protein will substitute for the yeast *RAS* protein in yeast cells, and a significantly altered form of the yeast *RAS1* protein will transform mammalian cells (13, 14). In addition to proteins that are highly homologous to Ha, proteins have been discovered—such as the yeast *YP2* protein and the *rho* proteins of human and *Aplysia*—that are distantly related to Ha, showing 30–40% homology (15, 16).

To identify the residues of Ha that are involved in protein–protein interaction, the effector sites, we have systematically made amino acid substitutions in Ha. Of the residues conserved among the *ras* proteins, we have identified residues, most notably those at positions 32–41, that appear to be invariable with respect to effector action. In addition, we have identified all of the residues that interact with the neutralizing monoclonal antibody Y13-259 (17, 18). The results of these studies suggest a specific structural basis for *ras* action.

MATERIALS AND METHODS

Mutagenesis and Expression. Mutations were introduced by oligonucleotide-directed mutagenesis, as described (6), and by the use of synthetic-gene fragment replacements. Ha mutants were expressed in *Escherichia coli* HB101 and purified to >90% homogeneity in the absence of denaturing steps, using ion-exchange chromatography followed by a high-performance liquid chromatography (HPLC) sizing step with a Pharmacia Superose 12 column (19).

Yeast Membranes. Cultures (50 ml) were grown in leucine-selective minimal medium (36) to OD_{600} 0.5–1.5. Spheroplasts were prepared by glusulase digestion (20), and a crude particulate fraction was prepared by homogenization [10–20 strokes with Dounce homogenizer pestle A (Thomas)] in 50 mM sodium 2-(*N*-morpholino)ethanesulfonate (Mes), pH 6.0/0.4 mM sodium EDTA/0.4 mM sodium EGTA/2 mM phenylmethylsulfonyl fluoride/2 mM dithiothreitol followed by centrifugation (100,000 × *g* for 30 min). Adenylate cyclase assays were performed at 30°C for 30 min with 20 μg of membrane protein and 10 mM $MgCl_2$ as described (20).

Immunology. For immunoblot analysis, purified proteins (2 μg) were electrophoresed in 12% polyacrylamide gels containing $NaDodSO_4$. Proteins were then transferred at 4°C onto nitrocellulose (Schleicher & Schuell BA83, 0.2 μm) for 1 hr at 350 mA. Filters were incubated with *ras*-protein-specific rat monoclonal antibody Y13-259 (1:500 dilution) in buffer A (25 mM Tris Cl, pH 7.5/1 mM sodium EDTA/150 mM NaCl/1% bovine serum albumin/1% powdered milk) and then washed with 0.9% NaCl/0.04% Nonidet P-40 (Sigma). Immunoreactive proteins were detected by autoradiography after incubation of filters with ^{125}I-labeled sheep anti-rat IgG (10 μCi, Amersham; 1 Ci = 37 GBq) in buffer A.

Peptides for Ha residues 60–76 (Ac-Gly-Gln-Glu-Glu-Tyr-Ser-Ala-Met-Arg-Asp-Gln-Tyr-Met-Arg-Thr-Gly-Glu-NH_2) and 31–43 (Ac-Glu-Tyr-Asp-Pro-Thr-Ile-Glu-Asp-Ser-Tyr-Arg-Lys-Gln-NH_2) were prepared on an Applied Biosystems 430A peptide synthesizer. [Ala^{65}]Peptide 60–76 (Ac-Gly-Gln-Glu-Glu-Tyr-Ala-Ala-Met-Arg-Asp-Glu-Tyr-Met-Arg-

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Abbreviations: Ha, Harvey *ras*-encoded protein; G protein, guanine nucleotide-binding regulatory protein; EF-Tu, elongation factor EF-Tu.

	10	20	30	40	50	60
Ha	M T E Y K L V V V G A G G V G K S A L T I Q L I Q N H F V D E Y D P T I E D S Y R K Q V V I D G E T C L L D I L D T A G					
Mras. SCRAS	E Y K V V V G G G V G K S A L T I Q Q F V D Y D P T I D S Y R K Q V V I D L D I L D T A G					
Dd. SP	E Y K V V G G G V G K S A L T I Q Q F V D Y D P T I D S Y R K I D L D L D T A G					
YP2	K G G V G K S L Y T I D L D T A G					

	70	80	90	100	110	120
Ha	Q E E Y S A M R D Q Y M R T G E G F L C V F A I N N T K S F E D I H Q Y R E Q I K R V K D S D D V P M V L V G N K C D L					
Mras. SCRAS	Q E E Y S A M R Q Y M R G E G F L V S Y Q I R V K D P V V G N K D L					
Dd. SP	Q E E Y S A M R Q Y M R G G F L V S Q I R V K D P V N K D L					
YP2	Q E Y R G V S I R V N K D L					

	130	140	150	160	170	180
Ha	A A R T V E S R Q A Q D L A R S Y G I P Y I E T S A K T R Q G V E D A F Y T L V R E I R Q H K L R K L N P P D E S G P G					
Mras. SCRAS	V A P E T S A K V A F Y L R R					
Dd. SP	V A E S A K V A F Y L R R					
YP2	V A E S A V A F R					

FIG. 1. Sequence comparisons among *ras* and *YP2* proteins. Ha sequence is listed in row 1. In row 2 are listed those residues of Ha that are conserved among the mammalian *ras* (Mras) proteins Ha, Ki, and N-ras and RAS1 and RAS2 of *Saccharomyces cerevisiae* (SCRAS). Row 3 has the residues of row 2 that are conserved in the proteins encoded by the *ras* genes of *Schizosaccharomyces pombe* (SP) (21) and *Dictyostelium* (Dd) (22). In row 4 are listed the residues of row 3 that are present in the YP2 sequence. The sequence of YP2 was aligned with that of Ha by aligning residue 6 of YP2 with residue 1 of Ha and by deleting residue 47 of YP2. Standard one-letter amino acid abbreviations are used. Positions where amino acid substitutions were made are designated (●).

Thr-Gly-Glu-NH_2) was purchased from Bachem Fine Chemicals (Torrance, CA). Peptide 60–76 was purified to homogeneity by HPLC on a C18 reversed-phase column (1 × 30 cm, Vydac) in an isocratic solvent system, containing 87% solvent A (0.1% trifluoroacetic acid in H_2O) and 13% solvent B (0.1% trifluoroacetic acid in acetonitrile). [Ala^{65}]Peptide 60–76 and peptide 31–43 were 98% and 80% pure, respectively. For competition experiments, [Val^{12},Thr^{59}]Ha was ^{32}P-labeled by autophosphorylation (6) for 2.5 hr at 37°C. An antibody complex for 20 reactions was formed by preincubating 10 μl of antibody with 1.2 ml of a 50% slurry of protein A-Sepharose [previously coated with rabbit anti-rat IgG (Cappel Laboratories, Cochranville, PA)] and 4 ml of buffer B (25 mM Tris Cl, pH 7.5/1% Triton X-100/0.5% sodium cholate/0.1% $NaDodSO_4$). This complex was washed three times with buffer B and four times with buffer C (50 mM Tris Cl, pH 7.5/150 mM NaCl). Competition reactions took place in buffer C (final volume 200 μl) for 16 hr at 4°C, after which the Sepharose beads were washed four times in buffer C prior to $NaDodSO_4$/PAGE. Peptide concentrations (pmol) in the competition reaction mixtures were quantitated by amino acid analysis.

RESULTS

Mutagenesis of Ha. We have found that bacterially expressed YP2 binds GTP and GDP specifically and has a low GTPase activity (unpublished data). Though homologous to ras, YP2 does not complement *ras* function in yeast. In Fig. 1, we have indicated those residues that are conserved among the *ras*-related proteins but do not occur in the YP2 sequence. Regions containing a cluster of these residues include the Ha sequences 3–9, 18–30, 33–42, 63–79, and 99–105. Amino acid substitutions were introduced at the positions indicated in Fig. 1. Assuming that YP2 and Ha have similar structures, we chose YP2 residues that represented nonconservative substitutions. Where comparison of sequences indicated that a residue might be important for structural integrity, conservative substitutions were chosen; e.g., Leu for Met.

Most of the amino acid substitutions were made in the gene for [Val^{12},Thr^{59}]Ha. This oncogenic variant functions in yeast cells and has a strong response in mammalian cells as assayed either by focus formation in NIH 3T3 cells or by the microinjection assay (19, 23). In addition, the autophosphorylation of the *ras* proteins at the Thr-59 residue is an intrinsic biochemical activity that can be assayed conveniently. The mutations that produced altered activities were also introduced into variants having the normal Ala-59 substitution. Biochemical integrity of the mutant proteins was monitored by the ability to be expressed in *E. coli* as soluble proteins that copurified with GDP and by the ability to autophosphorylate (Thr-59 derivatives) in the presence of 2 μM GTP. In this study, [Val^{12},Thr^{59},Glu^{104},Asn^{105}]Ha and [Glu^{104},Asn^{105}]Ha were the only proteins that were not obtained in a soluble form. All of the remaining mutants purified with endogenous nucleotide and possessed autophosphorylating activities similar to that of [Val^{12},Thr^{59}]Ha.

Binding Site of Monoclonal Antibody Y13-259. Monoclonal antibody Y13-259 broadly reacts with *ras* proteins, including all three mammalian *ras* proteins, RAS1 and RAS2 of *S. cerevisiae*, and the ras of *Dictyostelium* (24–26). The reactivity of Y13-259 toward all of the purified mutant proteins was explored by immunoblot analysis. As apparent from the autoradiogram of the immunoblot shown in Fig. 2, Y13-259

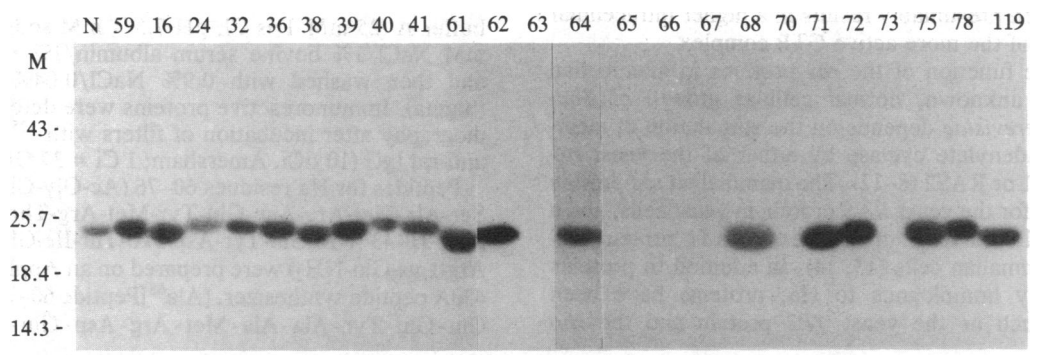

FIG. 2. Immunoblot of Ha mutants with *ras*-protein-specific monoclonal antibody Y13-259. Purified *ras* proteins (2 μg) were subjected to $NaDodSO_4$/PAGE and transferred to nitrocellulose. The residue at which a mutation was introduced is indicated above each lane. The actual amino acid changes are listed in Table 1. Lane N: normal Ha. M, molecular mass in kilodaltons.

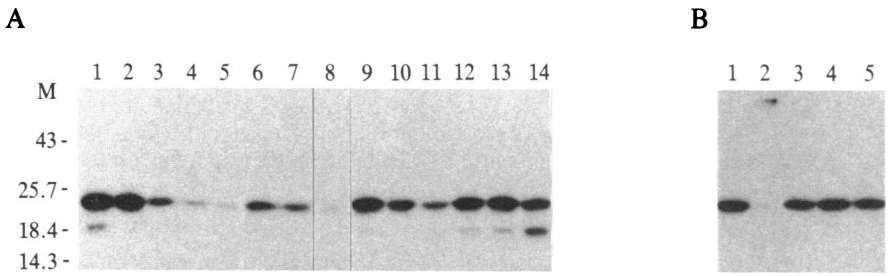

FIG. 3. Peptide competition for the ras antibody Y13-259 binding site. ^{32}P-labeled [Val^{12},Thr^{59}]Ha (8 pmol) was incubated with protein A-Sepharose-bound antibody Y13-259 (*A*) or antibody Y13-4 (*B*) in the presence of various unlabeled proteins and peptides and analyzed by NaDodSO$_4$/PAGE and autoradiography. M, molecular mass in kilodaltons. See *Materials and Methods* for peptide sequences and purity. Reactions were performed under limiting antibody conditions as determined by a second immunoprecipitation reaction (data not shown). (*A*) Lane 1: no competitor. Lane 2: 80 pmol of [Val^{12},Thr^{59},Thr^{66}]Ha. Lanes 3–5: [Val^{12},Thr^{59}]Ha at 16, 40, and 80 pmol. Lanes 6–8: peptide 60–76 at 11, 28, and 56 pmol. Lanes 9–11: [Ala^{65}]peptide 60–76 at 56, 560, and 5600 pmol. Lanes 12–14: peptide 31–43 at 75, 750, and 7500 pmol. (*B*) Lane 1: no competitor. Lane 2: 800 pmol of [Val^{12},Thr^{59}]Ha. Lane 3: 560 pmol of peptide 60–76. Lane 4: 560 pmol of [Ala^{65}]peptide 60–76. Lane 5: 750 pmol of peptide 31–43.

did not react with Ha mutants having substitutions at positions 63, 65, 66, 67, 70, or 73. However, reactivity with Y13-259 was observed for the mutants having substitutions at positions 61, 62, 64, 68, 71, 72, 75, or 78, as well as for the mutants having substitutions outside of the 60–80 sequence. Poorer reactivity compared to control was observed with the substitution of Leu for Arg-68. To explicitly define the binding site of Y13-259, a peptide having the sequence 60–76 was synthesized (peptide 60–76) as well as a peptide with the same sequence except that the residue corresponding to Ser-65 was replaced with Ala ([Ala^{65}]peptide 60–76). The ability of these peptides to compete with native Ha for binding to Y13-259 was measured in competition experiments with ^{32}P-labeled [Val^{12},Thr^{59}]Ha. As evident from the autoradiogram shown in Fig. 3*A*, peptide 60–76 demonstrated equivalent binding affinity to Y13-259 as did Ha protein. A 1000-fold equivalent of [Ala^{65}]peptide 60–76 was required to reduce the amount of bound Ha by 50%. No competition was observed with a control peptide having sequence 31–43 or with [Val^{12},Thr^{59},Thr^{66}]Ha. The ability of the peptides to compete was specific for Y13-259, as they were without effect against Y13-4 (Fig. 3*B*).

Residues at positions 69, 74, and 76 are not critically involved in binding Y13-259, as substitutions at these positions occur among the known *ras* proteins that bind Y13-259 (24–26). Since Gly-60 lacks a side chain, our antibody-binding experiments, with the mutant proteins and with the peptides, determine that the key side chains involved in Y13-259 binding are precisely those of Glu-63, Ser-65, Ala-66, Met-67, Gln-70, and Arg-73.

Biological Activity in Yeast and Mammalian Cells. Haploid yeast cells with *RAS1 ras2* genotype are unable to grow on nonfermentable carbon sources (27, 28). Constitutive expression of either *RAS1*, *RAS2*, or the normal and oncogenic variants of Ha complement this defect (14). *RAS1 ras2* strains, expressing these mutant Ha proteins, were assayed for their ability to grow on glycerol plates at 37°C (Table 1). Substitutions at positions 35, 36, 38, 40, and, to a lesser extent, 78 impaired the ability of the Ha protein to complement the *ras2* defect in yeast. Substitutions that resulted in diminished activity were at positions 32, 39, 67, and 72 and the double substitution at positions 104 and 105.

The ability of the mutant *ras* proteins to function in mammalian cells was determined by microinjection of purified proteins into NIH 3T3 cells. Proteins were microinjected at a concentration of 0.5 or 2.0 mg/ml, and their ability to produce morphological changes and to induce DNA synthesis was compared to those of [Val^{12},Thr^{59}]Ha, normal Ha, and the negative control [Val^{12},Thr^{59}]Ha-(1–174) (terminated at residue 174) (Table 1). All of the mutant proteins tested appeared as active as [Val^{12},Thr^{59}]Ha, except for those with

Table 1. Biological activity of Ha mutants

Substitution			Assay	
Residue(s)	From	To	Yeast	NIH 3T3
22	Gln	Arg	+	ND
25	Gln	Leu	+	ND
32	Tyr	His	± (+)*	ND (+)*
35,36	Thr,Ile	Ala,Leu	− (−)*	− (−)*
35†	Thr	Ala	−	ND
36†	Ile	Ala	−	ND
37	Glu	Ala	+	ND
38	Asp	Ala	− (−)*	−
39	Ser	Ala	±	+
40	Tyr	Lys	− (−)*	−
41	Arg	Leu	+	±
54	Asp	Ala	+	ND
61†	Gln	Leu	ND	+
62†	Glu	Gln	+	ND
63†	Glu	Gln	+	ND
64†	Phe	Leu	+	ND
65	Ser	Arg	+	+
66	Ala	Thr	+	+
67	Met	Ile	+	+
68	Arg	Leu	+	+
70	Gln	Ser	+	+
71	Tyr	Phe	+	+
72	Met	Ile	+	+
73	Arg	Gln	+	+
75	Gly	Ser	+	+
78	Phe	Ile	− (−)*	±
104,105	Lys,Asp	Glu,Asn	±	ND
117	Lys	Asn	+	+
(Terminated at 174)			−	−

Substitutions were introduced into [Val^{12},Thr^{59}]Ha, except where indicated for [Val^{12},Ala^{59}]Ha (*) or [Gly^{12},Ala^{59}]Ha (†). *Yeast assay.* Ha mutants were expressed in yeast cells *S. cerevisiae* 112-699 [*α leu2, ura3, his3, ras2-699(HIS3)*], using the yeast shuttle vector AAH5, which contains the yeast alcohol dehydrogenase-gene promoter and terminator and carries the auxotrophic marker for leucine (*LEU2*) (14). Colonies were restreaked from plates containing glucose but lacking leucine onto plates containing 2% peptone, 1% yeast extract, and 2% glycerol (YEPglycerol) and incubated at 37°C. Growth was scored after 2 days. *NIH 3T3 assay.* Purified proteins were microinjected into serum-starved, confluent NIH 3T3 cells (29, 30). Mutant proteins that, when injected at a concentration ≤0.5 mg/ml, gave distinct morphological changes and induced DNA synthesis similar to those produced by [Val^{12},Thr^{59}]Ha injected under the same conditions were scored +. Those proteins that were without any effect, even when injected at 2.0–3.0 mg/ml, were scored −. Those proteins that were active only at high concentrations (2.0–3.0 mg/ml) were scored ±. ND, not done.

substitutions at 35–36, 38, 40, or 78. Whereas only background activities were observed for substitutions at 35–36, 38, and 40, the Ile-78 variant was functional but had diminished potency.

Differential Stimulation of Yeast Adenylate Cyclase Activities. To quantitate the effector activity of the Ha mutants at the biochemical level, we measured the extent to which the yeast adenylate cyclase activity was stimulated by the *ras* mutant proteins expressed in yeast (Table 2). The expression of [Val^{12},Thr^{59}]Ha in haploid yeast cells (*RAS1 ras2*) gave a significant increase in membrane-bound adenylate cyclase activities. Mutants with substitutions at positions 35–36, 38, 39, or 40 and the double mutant, 104–105, showed activities <30% that of the control strain. The remaining mutants examined had >60% activities, which is consistent with the ability of the corresponding strains to grow on glycerol. Addition of the antibody Y13-259 reduced the adenylate cyclase activities by >80% for all the active mutants, except for those that we had shown to be Y13-259-binding residues (Table 2). We have observed that the addition of monoclonal antibodies Y13-4 or Y13-128 but not YA6-172 or Y13-238 also resulted in 80% decrease in the [Val^{12},Thr^{59}]Ha stimulation of adenylate cyclase activity (data not shown). The inhibitory effect of Y13-259 was not observed when an equivalent amount of peptide 60–76 was added to the assay sample (data not shown).

To determine whether the different adenylate cyclase activities were due to different potency or to different levels of expression, the levels of the Ha mutant proteins present in these membrane preparations were quantitated by immunoblot analysis or by autophosphorylation and immunoprecipitation. Levels detected were similar for the different variants, except for the Glu-104,Asn-105 variant, for which negligible expression was observed (data not shown).

Table 2. Adenylate cyclase activity in yeast membranes from strains expressing Ha mutants

Protein expressed	Relative activity	% inhibition by Y13-259
Control	1.0	88
Arg-22	0.6	97
His-32	0.6	95
Ala-35,Leu-36	0.03	—
Ala-38	0.08	—
Ala-39	0.3	86
Lys-40	0.2	—
Leu-41	1.2	83
Arg-65	1.0	4
Thr-66	2.8	32
Ile-67	0.6	20
Ser-70	1.8	36
Phe-71	2.6	94
Leu-72	1.2	90
Gln-73	1.5	1
Ser-75	0.8	95
Ile-78	0.6	87
Glu-104,Asn-105	0.2	—

[Val^{12},Thr^{59}]Ha mutants were expressed in yeast strain 112.699 (*RAS1 ras2*), using the vector AAH5 (14). Adenylate cyclase activity in membranes from yeast strains transformed with AAH5 or [Val^{12},Thr^{59}]Ha-AAH5 was 2.4 and 21.4 pmol/(min/mg), respectively. Activity in the presence of antibody Y13-259 was 0.8 and 2.5 pmol/(min/mg) for the AAH5 and [Val^{12},Thr^{59}]Ha-AAH5 strains, respectively. To directly compare the effects of mutations in this assay, activities are expressed relative to [Val^{12},Thr^{59}]Ha (control) corrected for basal activity with AAH5 alone. Antibody-containing assays were performed by incubating 20 μg of membranes with 2.5 μl of antibody Y13-259 for 15 min at 4°C before the assay was initiated at 30°C. Adenylate cyclase activity in the presence of antibody Y13-259 is expressed as % inhibition of activity in the absence of antibody. In control experiments, it was determined that the amount of antibody could be reduced by a factor of 15 without loss of inhibition and that the inhibitory effect was retained in antibody-treated membranes that were washed to remove free antibody (data not shown). The effect of antibody Y13-259 is not reported for those mutants with relative activities ≤0.2.

DISCUSSION

The effector activities for the Ha mutants, as measured by the mammalian microinjection assay, the growth of yeast transformants on glycerol, and the stimulation of yeast adenylate cyclase are overall consistent. The mutations that affected the biological activity were substitutions within the region 32–42 and at position 78. The double substitution at positions 104 and 105 appeared to have its major effect on the structural integrity of the protein. The small *ras* activity measured by the yeast glycerol-growth assay for this mutant presumably resulted from the low expression of active protein.

The determination of the structure of the EF-Tu–GDP complex offers a model for the structure of the *ras* protein (31–33). As reported elsewhere (19), our observations of altered nucleotide binding for Ha mutants having substitutions at positions 16 and 119 suggest a mode of binding GDP/GTP similar to that of EF-Tu for Ha, involving residues Lys-16, Asp-57, Asn-116, and Asp-119. Though the overall sequence homology between the *ras* proteins and EF-Tu is

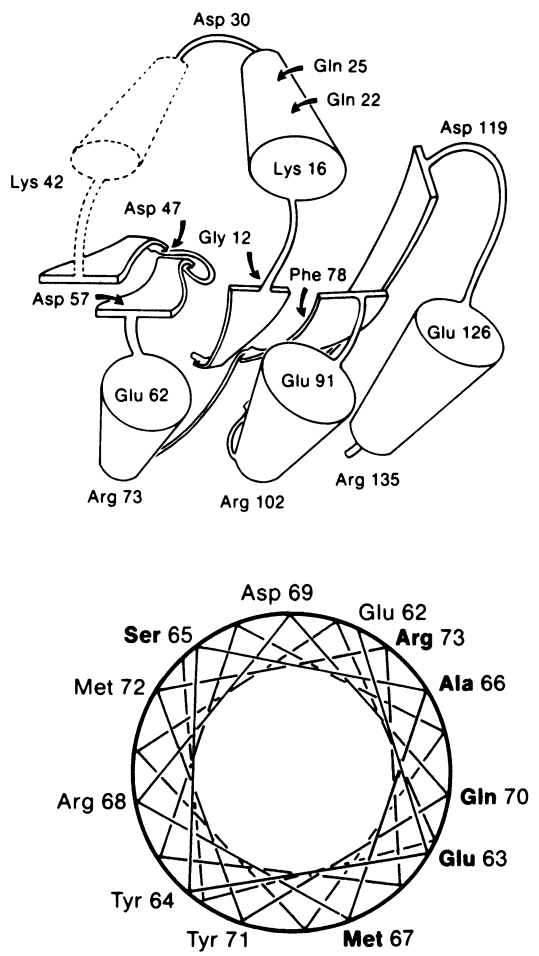

FIG. 4. (*Upper*) Model of Ha 1–135 based on the structure of EF-Tu–GDP. (*Lower*) The Edmundson wheel-helix projection (37) is shown for helix 62–73. Residues highlighted by bold print (Ser-65, Arg-73, Ala-66, Gln-70, Glu-63, and Met-67) are critical for antibody Y13-259 binding.

low, the conservation of the nucleotide-binding region indicates that the folding of the proteins is conserved. Analysis by amphiphilic-helix determinants (34) predicts amphiphilic helices for regions 16–30, 62–73, 91–102, and 126–135, which would align with the EF-Tu helices 24–40, 84–93, 114–125, and 144–158. The alignment of the ras sequence with that of the EF-Tu structure is shown schematically in Fig. 4.

The results from our mutagenesis study can be discussed in terms of the model of Ha based on the known structure of EF-Tu. Unfortunately, because the structure of EF-Tu was determined using trypsinized preparations, the exact structure of the region analogous to Ha 32–41 is not known for the EF-Tu protein. If this region exists as an amphiphilic helix, then the critical residues 32–41 would lie on the more hydrophobic side. Substitutions at positions that would lie on the more hydrophilic sides of proposed helices 16–30 (Gln-22 and Gln-25) and 35–40 (Glu-37 and Arg-41) had little influence on the effector activity of Ha.

In the model of Ha based on the EF-Tu structure, the region 62–73 would exist as an α-helix. The residues, which bind the antibody Y13-259, are spaced so they lie predominantly on the exposed, hydrophilic side of this helix (Fig. 4). The distances between the residues for this structure would be $\approx$20 Å, which is consistent with the dimensions of an antibody binding site (35). The binding to the three consecutive residues Ser-65, Ala-66, and Met-67 might require a distortion of the proposed helix.

It has been reported (18) that antibody Y13-259 neutralizes the biological activity of Ha when microinjected into mammalian cells. We have identified a neutralizing action of Y13-259 at the biochemical level in its inhibition of ras stimulation of yeast adenylate cyclase. However, our failure to detect a determinant in this region that is important for biological activity shows that this neutralization is indirect and results from either distortion of the protein or from steric factors.

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