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A Human Protein Selected for Interference with Ras Function Interacts Directly with Ras and Competes with Raf1

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The overexpression of some human proteins can cause interference with the Ras signal transduction pathway in the yeast *Saccharomyces cerevisiae*. The functional block is located at the level of the effector itself, since these proteins do not suppress activating mutations further downstream in the same pathway. We now demonstrate, with in vivo and in vitro experiments, that the protein encoded by one human cDNA (clone 99) can interact directly with yeast Ras2p and with human H-Ras protein, and we have named this gene *rin1* (Ras interaction/interference). The interaction between Ras and Rin1 is enhanced when Ras is bound to GTP. Rin1 is not able to interact with either an effector mutant or a dominant negative mutant of H-Ras. Thus, Rin1 displays a human H-Ras interaction profile that is the same as that seen for Raf1 and yeast adenylyl cyclase, two known effectors of Ras. Moreover, Raf1 directly competes with Rin1 for binding to H-Ras in vitro. Unlike Raf1, however, the Rin1 protein resides primarily at the plasma membrane, where H-Ras is localized. These data are consistent with Rin1 functioning in mammalian cells as an effector or regulator of H-Ras.

The *ras* genes encode signal-transducing guanine nucleotide-binding proteins that are involved in mitogenic response and differentiation in eucaryotes (10, 11, 20, 27). To carry out these functions, Ras proteins make physical contact with a variety of proteins that are classified as either regulators of Ras activity or signalling effectors. The regulators of Ras include the GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors. These proteins are able to directly stimulate the intrinsic properties of Ras that control its guanine nucleotide-bound state and hence its signalling activity (2, 6). In some cases, these proteins may also serve in an effector capacity to propagate signals from Ras. In the yeast *Saccharomyces cerevisiae*, adenylyl cyclase has been shown to act as the primary effector of Ras (29, 30). In mammalian cells, Raf1 was identified as a common downstream component of Ras signalling pathways. Mammalian Raf1 and yeast adenylyl cyclase were shown to physically interact with Ras in both two-hybrid and direct binding experiments (13, 18, 19, 31, 33, 34, 36). Recently, direct interaction of phosphatidylinositol 3-kinase with Ras has also been demonstrated (23). These interactions display the predicted hallmarks of Ras-effector binding; they are enhanced by GTP-bound Ras and they are blocked by Ras effector domain mutations (26, 35). Interestingly, although these Ras effectors (yeast adenylyl cyclase, Raf1, and phosphatidylinositol 3-kinase) have the same Ras interaction characteristics, they do not have any significant sequence similarity.

We have previously described the isolation of human cDNAs that, when expressed in *S. cerevisiae*, are able to suppress an activated *RAS2* allele ($RAS2^{V19}$). Yeast cells carrying this mutation have elevated cyclic AMP (cAMP) levels as a result of the overstimulation of adenylyl cyclase by Ras. These cells are exquisitely sensitive to heat shock because of an inability to exit the cell cycle (24). Three human cDNAs including Rin1 (clone 99) were able to suppress the $RAS2^{V19}$ mutant but not other heat shock-sensitive yeast mutants with elevated cAMP levels (5). This result is consistent with a direct interference with Ras function. Sequence analysis of Rin1 indicated a limited sequence similarity with GAPs (5), suggesting a possible physical interaction with Ras. We now demonstrate direct and specific binding of Rin1 to both yeast and human Ras.

MATERIALS AND METHODS

Strains, growth conditions, and yeast assays. *S. cerevisiae* CTY10-5d (*MAT***a** *ade2 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3*::*lexAop-lacZ* [3a]) was used for Ras2p interaction experiments. Strain L40 is *MAT***a** *trp1 leu2 his3 LYS2*::*lexAop-HIS3 URA3*::*lexAop-lacZ* (11a, 33) and was used for H-Ras and Rap1A experiments (both *lacZ* and *HIS3* reporter gene assays). For heat shock experiments, strain TK161-R2V (*MAT***a** *leu2 his3 ura3 trp1 ade8 can1 RAS2*^{V19}) (30) was used. Yeast colonies were patched in duplicate and replica plated onto normal growth medium at 30°C or on medium that was incubated at 55°C for 4 min. They were then allowed to recover at 30°C for 2 to 3 days.

For two-hybrid interaction assays, cells harboring the two indicated plasmids were patched on selective medium (synthetic complete-leucine-tryptophan), to ensure uptake of both hybrid plasmids, and assayed as described previously (3) for LacZ activity (blue color) indicating a productive interaction. For *HIS3* induction assays, transformants were selected on medium without tryptophan or leucine, and single colonies were then streaked on medium without histidine and grown for 2 days.

Plasmid constructions. The yeast *RAS2* (wild-type and activated mutant) genes were PCR amplified and ligated into the GAL4 activation domain (GAD) vector pGAD424 (1) that was modified to accept a *Sal*I-*Not*I insert (this construct was renamed pGAD425). This was done by digestion with *Eco*RI and *Pst*I and then ligation to the oligonucleotides 5′-AATTCATGTCGACGGTACCG CGGCCGCTGCA-3′ and 5′-GCGGCCGCGGTACCGTCGACATG-3′. Each yeast *RAS2* sequence was constructed with a Cys-to-Ser mutation at position 318 which blocks palmitoylation and reduces membrane localization but does not alter Ras2p function (15). This was done by first performing a PCR using the primers 5′-CGATGTCGACCATGCCTTTGAACAAGTCG-3′ and 5′-GATAG GATCCACCCGATCCGCTCTTG-3′. The first primer introduced a *Sal*I site, and the second primer introduced a *Bam*HI site just prior to codon 318. This product was digested with *Sal*I and *Bam*HI and ligated into pBluescriptKS (Stratagene) to yield pKS-RAS2. This plasmid was digested with *Bam*HI and *Not*I and ligated with the oligonucleotides 5′-GATCGTGTATTATAAGTTA GC-3′ and 5′-GGCCGCTAACTTATAATACAC-3′, which reconstructed the carboxy-terminal end of *RAS2* but incorporated the Cys-to-Ser mutation at residue 318. The resulting complete *RAS2* sequence was then removed by using *Sal*I and *Not*I and ligated into pGAD425 to create pGAD-RAS2. The same procedure was followed for both wild-type *RAS2* and $RAS2^{V19}$.

The LexA DNA binding domain fusions of human H-*ras* wild-type and mutant constructs were generously provided by Anne Vojtek and Jonathan Cooper, Fred Hutchinson Cancer Research Center, Seattle, Wash. The yeast adenylyl cyclase (Cyr1p[606-1608]) used represents a truncated form of the *CYR1* gene (4) cloned as a *Sal*I-*Not*I fragment into a LexA activation domain vector, pBTM116 (1a),

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that was modified to permit *Sal*I-*Not*I cloning (this construct was renamed pBTM117). This modification was carried out in the same manner (with the same oligonucleotides) as the modification of pGAD424. The same *CYR1* fragment was ligated into the pGAD425 vector. The Rin1 used includes the entire coding region of cDNA 99 (5) and was cloned into both the modified LexA DNA binding domain vector, pBTM117 (for yeast Ras2p interactions), and the GAD vector, pGAD425 (for H-Ras interactions). This was done by first moving the 1-kb *Sal*I-to-*Bam*HI fragment of cDNA 99 and then adding the 0.7-kb *Bam*HI-to-*Not*I fragment (this was necessary because two *Not*I sites exist in the clone). Cdc25p[907-1589] and Raf1[48-178] are VP16 fusion constructs in a pVP16 vector (33) and were generously provided by A. Vojtek and J. Cooper. Rap1A was PCR amplified from human brain cDNA (Clontech), using the primers 5′-CATAGTCGACCTAGAGCAGCAGACATGA-3′ and 5′-GCTGGATCCT GATGCGTGAGTACAAGCTA-3′, and cloned into pBTM116 by using *Bam*HI-*Sal*I.

Wild-type and mutant yeast *RAS2* genes were amplified by PCR and cloned into the glutathione *S*-transferase (GST) vector pGEX-2T (Pharmacia) that was modified by oligonucleotide insertion to accept a *Sal*I-*Not*I insert. To do this, the *Sal*I site was inserted at the existing *Bam*HI site, using the oligonucleotide 5′-GATCGTCGAC-3, and the *Not*I site was introduced at the existing *Eco*RI site, using the oligonucleotide 5′-AATTGCGGCCGC-3′. The human H-Ras (wild type and activated mutant)–GST fusion constructs were the generous gift of A. Vojtek and J. Cooper (33). The effector domain mutant (GST–H-Ras^{L35R37}) was constructed in the GST fusion vector pGEX-3X (AmRad). This vector was cut with *Eco*RI, treated with Klenow enzyme, then cut with *Bam*HI. The insert was prepared from LexA–H-Ras^{L35R37} (see above) by digestion with *Pst*I, treatment with Klenow enzyme, and then digestion with *Bam*HI. The H-Ras^{L35R37} fragment was purified and ligated into the prepared pGEX-3X vector.

Rin1 (cDNA 99) was cloned into the in vitro transcription vector pSP64-XβM (14), previously modified to contain an *Eco*RV site (25), which was further modified to accept a *Sal*I-*Not*I fragment (pSP64-XβSN). This was done by first destroying the existing *Sal*I site by digestion with *Sal*I, treatment with Klenow enzyme, and ligation of the blunt ends. The resulting plasmid was then digested with *Nco*I and *Eco*RV and ligated with the oligonucleotides 5′-CATGTCGACT TGCGGCCGC-3′ and 5′-GCGGCCGCAAGTCGA-3′ to create unique *Sal*I and *Not*I sites. Rin1 was cloned into this vector with the same strategy used for cloning it into pBTM117 and pGAD425.

In vitro binding and competition experiments. GST-Ras (yeast and human) fusion proteins were purified from bacteria by established methods, using glutathione-Sepharose (Pharmacia). These proteins were then loaded with GTPγS or GDP as described previously (33). In the case of yeast Ras2^{V19}, this resulted in incomplete exchange with the guanine nucleotide present on the purified protein (see Results and Discussion), and so a modified protocol was also used. Immobilized protein was rinsed with loading buffer (50 mM Tris [pH 7.5], 20 mM KCl, 7.5 mM EDTA, 1 mM dithiothreitol) at room temperature. Guanine nucleotide (GTPγS or GDP) was added to a concentration of 1.2 mM, the mixture was incubated at 37°C for 20 min and then chilled to 4°C, and $MgCl_2$ was added to a concentration of 6 mM. The Rin1 sequence (cDNA 99) was expressed from an in vitro transcription vector (see above). Rin1 RNA was synthesized and translated in vitro, using reticulocyte lysate (Promega) in the presence of [^{35}S]methionine. For binding experiments, 6 μl of ^{35}S-Rin1 lysate was incubated with resin-bound GST-Ras protein in buffer (20 mM Tris [pH 7.4], 25 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.01% Nonidet P-40, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 1% dry milk, 0.6 mM guanine nucleotide) for 1 h at 4°C. The resin was washed (four times for 15 min each time at 4°C) with washing buffer (binding buffer with 80 mM NaCl, 0.5% Nonidet P-40, no dry milk, and no guanine nucleotide). After being washed, the resin was boiled in sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For competition experiments, binding was carried out as described above but in the presence of Raf1 protein as a maltose-binding protein (MBP) fusion. MBP-Raf1 proteins were purified as described previously (7), using constructs generously provided by A. Vojtek and J. Cooper. Equal amounts of MBP-Raf1[51-131] and MBP-Raf1[51-131]^{L89} were determined by the Bradford assay and SDS-PAGE analysis. For competition experiments, ^{35}S-Rin1 signals were quantitated with an Ambis radioimager.

Immunofluorescence. Rin1 was expressed from the modified pGEX-2T vector to yield a GST-Rin1 fusion that was purified on a glutathione column and injected into rabbits with Titermax. The resulting Rin1 and preimmune sera were purified by using protein A-Sepharose (Pharmacia) and used at a 1:100 dilution. HeLa cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum and treated as described previously (32) for immunofluorescence and nuclear staining with propidium iodide. GST or GST-Rin1 was included at 60 μg/ml during incubation with Rin1 antiserum in order to demonstrate signal specificity. The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma).

Nucleotide sequence accession number. The corrected Rin1 sequence has been submitted to GenBank (accession number L36463).

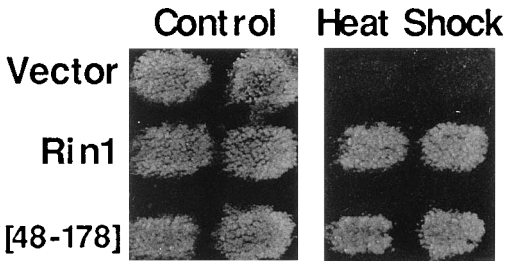

FIG. 1. Heat shock assay. *S. cerevisiae* TK161-R2V (30) carries an activated *RAS2*^{V19} allele. This strain was transformed with pBTM117 (vector), pLexA-Rin1, or VP16-Raf1[48-178] (pRIP51 [33]). Expression of hybrid protein versions of Rin1 and Raf1 is clearly able to interfere with Ras2p^{V19} function and relieve heat shock sensitivity. pGAD-Rin1 was also tested and gave the same result (data not shown). Vector sequences do not show this activity.

RESULTS AND DISCUSSION

To ascertain whether Rin1, the protein encoded by cDNA 99 (5), functions through a direct interaction with the Ras2 protein, we created transcription factor fusion protein constructs (8) for use in two-hybrid interaction experiments. Each of the constructs was first tested for its ability to suppress an endogenous activated *RAS2* allele. As can be seen in Fig. 1, Rin1 (clone 99) is still able to confer heat shock resistance, even when expressed as a hybrid fusion protein, indicating that the fusion protein retains its Ras interference properties. Also shown is a Raf1 fusion construct originally isolated from a library by two-hybrid screening (33). As shown, the Ras effector Raf1 is also capable of suppressing activated yeast *RAS2*^{V19}. The positive correlation between interference and two-hybrid transcriptional activation suggests that the two assays reflect related binding functions. That is, binding of Rin1 (or Raf1) to Ras is directly responsible for suppression of Ras function and hence heat shock sensitivity. In each case, this presumably reflects competition with yeast adenylyl cyclase for Ras binding. This result also implies that significant quantities of the fusion proteins are available in the cytoplasm as well as the nucleus.

We then proceeded to test for direct binding. As shown in Table 1, the Rin1 fusion protein is capable of interacting with a yeast Ras2 fusion protein, as judged by LacZ activity. Binding is seen with either wild-type Ras2p or an activated mutant, Ras2p^{V19}. Also shown is the interaction of yeast Ras2p with adenylyl cyclase, a known effector of Ras.

To determine if the human Rin1 gene product could interact with a human Ras protein, we used H-Ras fusion proteins that include a DNA binding domain. As shown in Table 1, the Rin1 protein, expressed as a GAD fusion, is clearly able to interact with both wild-type and activated H-Ras fusion proteins. No signal is detected when either partner is replaced by a vector alone. In addition, the interaction is equally strong when Rin1 is expressed as a binding domain fusion and H-Ras is expressed as a GAD fusion (data not shown). No binding signal is seen with an effector domain mutant, H-Ras^{L35R37} (35). There is also no binding to a dominant negative mutant (H-Ras^{A15}) (22) which exists primarily in a GDP-bound or nucleotide-free form (16). These results indicate that the H-Ras–Rin1 interaction requires an intact Ras effector domain and that Ras in its GTP-bound state is the preferred partner. Also demonstrated are the known interactions of H-Ras with Cyr1p (yeast adenylyl cyclase) and Cdc25p (a guanine nucleotide exchange factor and positive regulator of Ras2p) and with Raf1 (33). The results show that Rin1 has a Ras interaction profile that is indistinguishable from that of Raf1 and yeast adenylyl cyclase.

TABLE 1. Two-hybrid interaction analysis using a *lacZ* reporter[a]

Interacting protein[b]	Ras protein[b]						
	pGAD		pLexA				
	Ras2p	Ras2p^{V19}	H-Ras	H-Ras^{V12}	H-Ras^{L35R37}	H-Ras^{A15}	Rap1A
Cyr1p[606-1608]	Blue	Blue	Blue	Blue	White	White	White
Cdc25p[907-1589]	ND	ND	Blue	Blue	Blue	Blue	White
Raf1[48-178]	ND	ND	Blue	Blue	White	White	Blue
Rin1	Blue	Blue	Blue	Blue	White	White	White

[a] Blue, a positive signal following incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactoside; white, no signal; ND, not determined.
[b] Ras2p indicates the protein product of yeast *RAS2*; H-Ras is the product of the human Ha-*ras* gene. Superscript notation indicates the identities and positions of altered residues of mutant alleles. Numbers in brackets indicate the first and last codons of the fragment being expressed. Yeast Ras proteins were expressed as activation domain fusions, and human Ras proteins were expressed as DNA binding domain fusions. LexA-Cyr1p was used with GAD-Ras2p constructs. GAD-Cyr1p was used with LexA–H-Ras constructs. LexA-Rin1 also interacts with a pGAD-H-Ras construct (data not shown). Other fusions were as described in Materials and Methods.

These results were confirmed by using the *HIS3* reporter construct present in the same yeast strain and analyzing growth on histidine-deficient medium. As with the *lacZ* expression experiments, H-Ras was shown to interact with Rin1 (Fig. 2, sector 6) as well as the control Ras partners Cdc25p (Fig. 2, sector 2) and yeast adenylyl cyclase (Fig. 2, sector 4). The allele specificity of Ras-Rin1 interactions clearly parallels that of Ras-Raf1 (33, 31, 36); interactions take place with wild-type H-Ras or H-Ras^{V12} but not with the effector mutant H-Ras^{L35R37} or with the dominant negative mutant H-Ras^{A15}.

To examine direct binding of Rin1 to yeast and human Ras proteins, we produced ^{35}S-labeled Rin1 protein in an in vitro expression system and tested its ability to bind to GST-Ras fusion proteins attached to a resin. The Rin1 protein specifically binds to both yeast and human Ras, with no binding to GST alone (Fig. 3). Binding occurs with either wild-type or activated mutant Ras (yeast Ras2p^{V19} or human H-Ras^{V12}) but does not take place with a Ras mutant carrying effector domain mutations. In addition, the binding of Rin1 to Ras is enhanced by GTP. For wild-type yeast and human Ras and for activated human Ras, the difference between GTPγS-loaded and GDP-loaded Ras is quite large. For the activated yeast Ras (Ras2p^{V19}), the difference appears much smaller and may simply reflect poor loading with GDP. In a subsequent experiment using a modified guanine nucleotide loading procedure (see Materials and Methods), yeast Ras2p, both wild type and activated, showed essentially complete dependence on GTP binding for Rin1 interaction (data not shown).

Therefore, using two-hybrid reporter gene activation, direct in vitro binding, or heat shock sensitivity as a determinant of Ras interference, we found that Rin1 shows the same pattern as Raf1. In addition, the guanine nucleotide and effector residue requirements for Ras partnership are completely shared by Rin1 and Raf1. We therefore examined whether Raf1 protein can compete with Rin1 for binding to Ras protein. Figure 4 shows that Rin1 binding to wild-type human H-Ras is greatly reduced with increasing amounts of Raf1[51-131], a minimum Ras-interacting domain fragment of Raf1 (7). With amounts of Raf1 that are roughly equal to that of the H-Ras bound to the column, Rin1 binding is reduced by 48% (Fig. 4, lane 3). With 10-fold more Raf1, Rin1 binding is decreased by 93% (Fig. 4, lane 4). A parallel experiment with Raf1[51-131]^{L89}, a mutant form of Raf1 that binds poorly to Ras (7), showed no competition (Fig. 4, lanes 5 to 7). This result confirms that Rin1 and Raf1 interact with Ras through overlapping determinants. Recent experiments have demonstrated that yeast adenylyl cyclase and Raf1 can also compete for binding to Ras (either yeast or mammalian), although with different affinities (18).

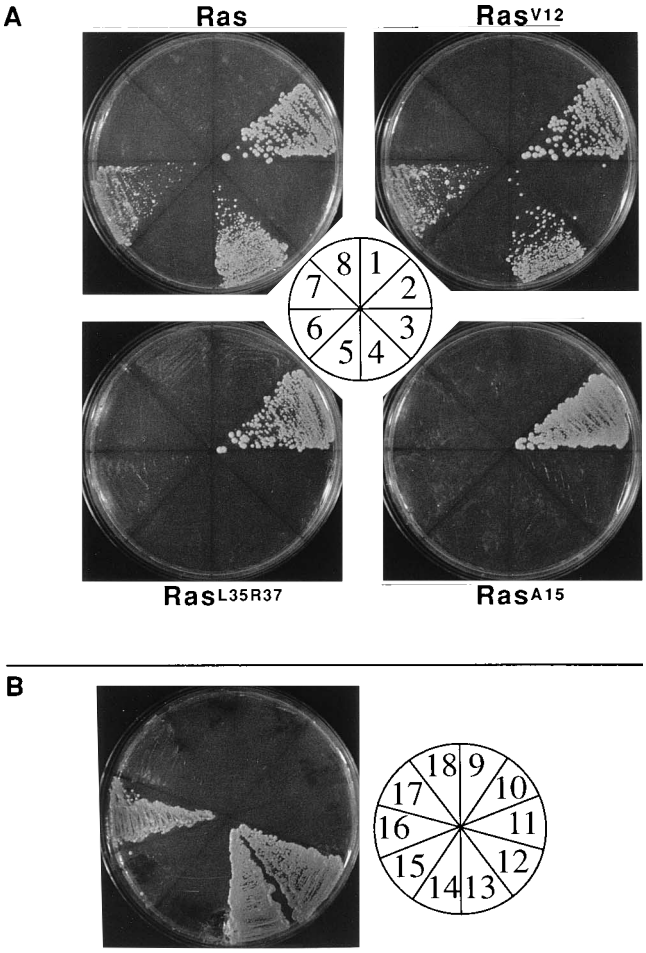

FIG. 2. Two-hybrid interactions by histidine selection. *S. cerevisiae* L40 (see Materials and Methods) carries the *his3* mutation and the *lexAop-HIS3* allele that permits growth on histidine-deficient media if a two-hybrid transcription complex is formed. These cells were transformed with the combination of two hybrid plasmids indicated. In each case, multiple transformants were tested, and all gave the same result. (A) Allele specificity of interactions with human H-Ras. The allele of H-Ras used is shown for each plate. The effector mutant Ras^{L35R37} also carries an activation mutation (33). Sectors: 1, pVP16 plus LexA-Ras; 2, VP16-Cdc25p[907-1589] plus LexA-Ras; 3, VP16-Cdc25p[907-1589] plus pBTM117; 4, GAD-Cyr1p[606-1608] plus LexA-Ras; 5, GAD-Cyr1p[606-1608] plus pBTM117; 6, GAD-Rin1 plus LexA-Ras; 7, GAD-Rin1 plus pBTM117; 8, pGAD425 plus LexA-Ras. (B) Interactions with Rap1A. The yeast strains in each sector harbor the following plasmids (in this panel, Ras indicates wild-type H-Ras only): 9, LexA-Ras plus pGAD425; 10, LexA-Rap1A plus pVP16; 11, LexA-Ras plus pVP16; 12, LexA-Rap1A plus VP16-Raf1[48-178]; 13, LexA-Ras plus VP16-Raf1[48-178]; 14, pBTM117 plus VP16-Raf1[48-178]; 15, LexA-Rap1A plus GAD-Rin1; 16, LexA-Ras plus GAD-Rin1; 17, pBTM117 plus GAD-Rin1; 18, LexA-Rap1A plus pGAD425.

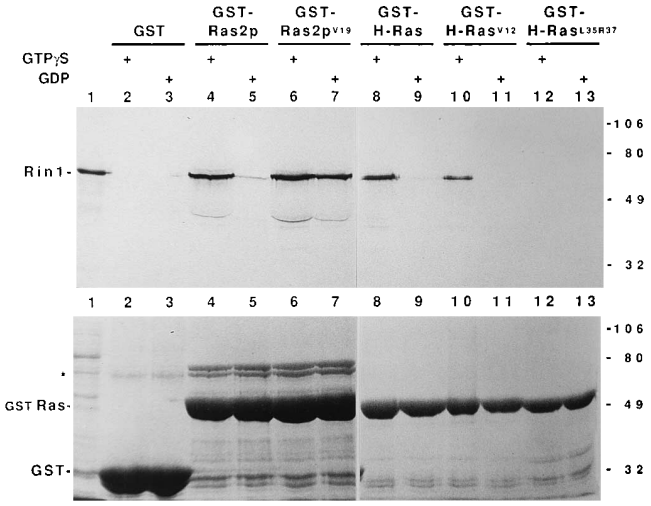

FIG. 3. In vitro binding. In vitro-translated Rin1 was incubated with immobilized GST or various GST-Ras fusion proteins. After washing, resin-bound proteins were analyzed by gel electrophoresis. The top panel shows the resulting autoradiogram, and the bottom panel shows the Coomassie blue-stained gel. The guanine nucleotide bound is indicated at the top, and positions of molecular mass markers (in kilodaltons) are shown at the right. Lane 1, ^{35}S-Rin1 lysate alone; lanes 2 to 13, binding assays using GST or the indicated GST-Ras fusion protein. The asterisk indicates the presence of two high-molecular-weight forms of the GST-yeast Ras2p fusion. The molecular weight of the upper band is the predicted molecular weight for the complete fusion protein. The lower band and the intense band presumably represent breakdown products. It is not clear which species of Ras2p are primarily responsible for Rin1 binding. The GST–H-Ras and GST proteins migrate approximately according to their predicted molecular weights. The in vitro-produced Rin1 is predicted to have a molecular mass of 53 kDa but is seen to migrate at a rate corresponding to a slightly higher mass.

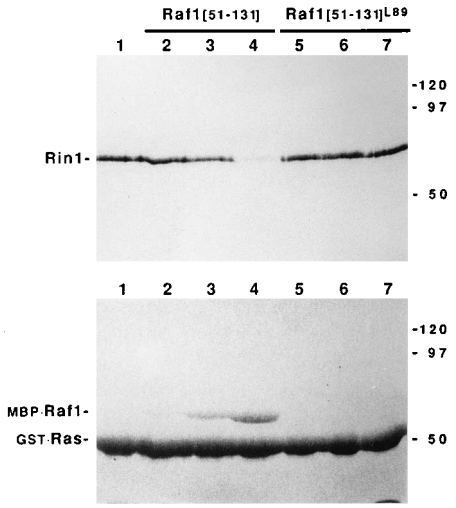

FIG. 4. Competition between Raf1 and Rin1. Resin-bound GST–H-Ras (GTPγS loaded) was incubated with ^{35}S-Rin1 alone (lane 1) or in the presence of increasing amounts of MBP-Raf1[51-131] (lanes 2 to 4) or MBP-Raf1[51-131]^{L89} (lanes 5 to 7). The top panel is an autoradiogram showing the Rin1 protein bound to Ras. The bottom panel is the same gel (Coomassie blue stained) indicating the GST-Ras and MBP-Raf1 released from the resin. Lane 4 represents a saturating amount of Raf1[51-131], since the same amount of bound Raf1[51-131] is seen when one-third as much starting protein is used (data not shown). The ratio of bound Raf1[51-131] to Ras indicates that some GST-Ras cannot be bound, as has been reported by others (33). Positions of molecular mass markers (in kilodaltons) are indicated on the right.

A careful reanalysis of the primary sequence of Rin1, and comparison with a recently isolated rat Rin1 cDNA, revealed an error in the published sequence (5). Figure 5 shows the corrected nucleotide sequence and the affected amino acid sequence. This correction alters the last 25 predicted residues and extends the reading frame by 71 amino acids. Interestingly, a comparison of the full, corrected primary sequence of Rin1 with the Raf1 sequence showed no significant alignment. Other proteins that interact with Ras in an effector domain-dependent manner (yeast adenylyl cyclase, phosphatidylinositol 3-kinase, and a variety of GAPs) also do not align with Raf1. This may indicate that the effector domain residues are only a small (though critical) portion of a large binding surface or that the binding determinants of these Ras-binding proteins are widely scattered throughout their primary structure.

It is known that Raf1 can also bind to at least one Ras-related protein, Rap1 (36). Rap1A and mammalian Ras are 53% identical overall and 100% identical in their effector domains (21). The binding of Rap1A to Raf1 and to GAP (9) may play a critical role in the ability of Rap1A to suppress Ras function in mammalian cells (12). To determine whether the similarity of interaction profiles for Rin1 and Raf1 extends beyond their ability to bind to Ras, we examined whether Rin1 could interact with Rap1. Table 1 demonstrates the interaction of Raf1 with Rap1A. Unlike Raf1, however, Rin1 shows no detectable Rap1A binding activity. The same result was observed when growth on histidine-deficient medium was used as a measure of expression of a *HIS3* reporter gene (Fig. 2). Interestingly, yeast adenylyl cyclase, another known effector of Ras, also does not interact with Rap1A (Table 1). Overall, Rin1 has an interaction profile that is indistinguishable from that of yeast adenylyl cyclase and may represent a new class of mammalian Ras-interacting proteins that do not associate with Rap1.

To address the opportunity for Rin1-Ras interactions in mammalian cells in vivo, subcellular localization of the Rin1 protein was examined. Antibody raised against a GST-Rin1 fusion protein shows Rin1 protein to be largely confined to the plasma membrane. It is dispersed throughout the membrane, with areas of higher local concentration giving rise to a punctate pattern (Fig. 6). This distribution puts Rin1 in the proximity of Ras, which is primarily membrane associated, and seems to provide an opportunity for the two proteins to interact. Residence at the plasma membrane is a property shared with yeast adenylyl cyclase, while Raf1 is primarily cytoplasmic prior to membrane translocation and activation by Ras. Indeed, recruitment of Raf1 to the plasma membrane is a primary event in its activation (17, 28).

Local clustering of Rin1 on the membrane (Fig. 6) is not

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1171-CGCCTGCCCCCTGGGGCCCTGGCCCACAGGCTGCCCACCACTGGCTACCTCGTCTACCGCCGG
 391-ArgLeuProProGlyAlaLeuAlaHisArgLeuProThrThrGlyTyrLeuValTyrArgArg

     GCAGAGTGGCCTGAGACCCAGGGGGCTGTGACAGAGGAGGAGGGCAGTGGGCAGTCAGAGGCA
     AlaGluTrpProGluThrGlnGlyAlaValThrGluGluGluGlySerGlyGlnSerGluAla

     AGAAGCAGAGGGGAGGAGCAAGGGTGCCAGGGAGATGGGGATGCTGGGGTCAAAGCCAGCCCC
     ArgSerArgGlyGluGluGlnGlyCysGlnGlyAspGlyAspALaGlyValLysAlaSerPro

     AGGGACATTCGGGAACAGTCTGAGACAACTGCTGAAGGGGGCCAGGGTCAAGCCCAGGAAGGC
     ArgAspIleArgGluGlnSerGluThrThrAlaGluGlyGlyGlnGlyGluAlaGlnGluGly

     CCTGCTCAGCCAGGGGAACCAGAGGCAGAGGGAAGCCGGGCAGCAGAGGAGTAG
     ProAlaGlnProGlyGluProGluAlaGluGlySerArgAlaAlaGluGlu---
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FIG. 5. Corrected sequence of Rin1 (cDNA 99). The location of an additional nucleotide (compared with the original published sequence) is indicated with an asterisk. The nucleotide and amino acid numbering is from the original sequence (5). The corrected amino acid sequence for the extended open reading frame is given and differs only beyond the sequence correction. The stop codon is marked with three dashes. Sequencing was carried out with Sequenase (U.S. Biochemical).

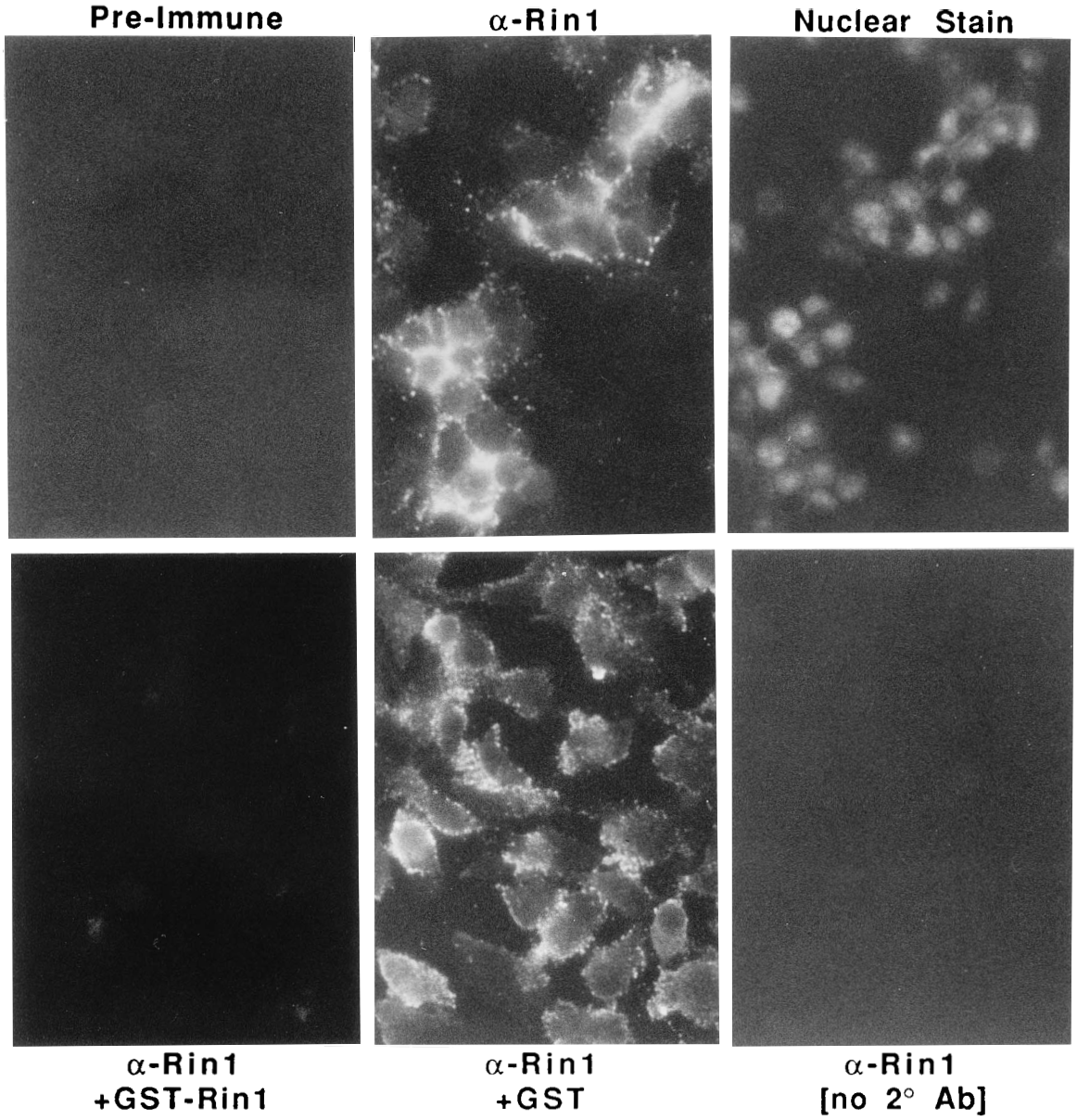

FIG. 6. Immunofluorescence localization of Rin1 in HeLa cells. Cells were fixed and treated with preimmune or Rin1 (α-Rin1) antiserum. No signal is seen with preimmune serum. Incubation with GST or GST-Rin1 protein demonstrates Rin1 specificity of the antibody that was raised against GST-Rin1. The experiment done in the absence of secondary antibody (no 2° Ab) demonstrates that the Rin1 antiserum and other reagents do not contribute to the fluorescence signal.

seen with Ras, which suggests a possible association with other functional structures, such as focal adhesions; this possibility is being investigated further. In addition, the tissue type expression pattern of Rin1 message indicates high levels in the brain (10a) and may reflect a specialized role for this protein in Ras signaling.

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