Role of Phosphate–Magnesium-Binding Regions in the High GTPase Activity of rac1 Protein†

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ABSTRACT: rac1, a member of the low molecular mass GTP-binding protein family, has a 20-fold higher GTPase activity than H-ras, but the structural motifs responsible for this property do not appear to reside within the conserved amino acids of the consensus GTP-binding domains [Ménard, L., Tomhave, E., Casey, P. J., Uhing, R. J., Snyderman, R., & Disbury, J. R. (1992) *Eur. J. Biochem 206,* 537–546]. In this study, we determined the contribution, to the GTPase activity, of additional amino acids found in the phosphate–magnesium-binding (PM) regions of rac1. rac1 has three different amino acids from H-ras in each of the first two PM regions, while the third PM region is identical to that of H-ras. Mutation of the amino acids in the first PM region (aa 10–17) to the corresponding amino acids found in H-ras showed that modification of one of them, Asp11, resulted in a 50% decrease of the GTPase in rac1, whereas Gly13 and Ser17 had no effect. In the second PM region (aa 29–35), modification of the Pro29-Gly30 pair also reduced GTPase activity by 50% in rac1. rac1 mutated at positions 11 and 29 as well as 30 (P1–P2 mutant) had a 3–4-fold reduced GTPase activity compared to native rac1 (190 vs 552 pmol of GTP hydrolyzed/nmol of GTPγS-binding protein/min at 37 °C), suggesting a cooperative (but nonadditive) interaction between both domains. All mutants with reduced GTPase activity also had reduced affinity for GDP, shown by both equilibrium binding and GDP dissociation measurements. The most striking effect was found in the P1–P2 mutant where the k_d for GDP was reduced by more than 2 orders of magnitude (12.8 vs 0.04 μM for rac1). This change resulted in a dramatic increase in the dissociation rate of GDP for the P1–P2 mutant, producing a protein in which k_{cat} was the rate-limiting step in the hydrolysis of GTP. In contrast to the effects observed for interaction with GDP, dissociation of GTPγS was much less affected in all the mutants. Our data suggest that Asp11 and Pro29-Gly30 profoundly influence the hydrolysis of GTP by rac1 and account for about 50% of the increased activity of rac1 compared to H-ras. Additional structural motifs responsible for the remaining increased GTPase activity found in rac1 probably lie outside the conserved nucleotide-binding domains.

Low molecular mass GTP-binding proteins (LGBPs[1]) are a family of G proteins (guanine nucleotide-binding regulatory protein) distinct from the heterotrimeric G proteins (Bourne et al., 1991; Hall, 1990). LGBPs typically have a molecular mass of 20–30 kDa, are monomeric, and have a low GTP hydrolytic activity as compared to heterotrimeric G proteins (Bourne et al., 1991; Gilman et al., 1987). Recent evidence suggests that LGBPs are involved in such diverse processes as signal transduction, exocytosis, and cytoskeletal organization (Hall, 1990, 1992; Satoh et al., 1991). rac1, one member of the family of LGBPs, has a wide tissue distribution, yet is enriched in terminally differentiated myeloid cells (Didsbury et al., 1989; Polakis et al., 1989). Recent evidence has demonstrated rac1 and a closely related LGBP (rac2) are involved in the activation of the NADPH oxidase of phagocytic leukocytes (Abo et al., 1991; Knaus et al., 1991, 1992; Mizuno et al., 1992, Rotrosen et al., 1992; Heyworth et al., 1992) and cytoskeleton organization (Ridley et al., 1992).

With one exception, rac1 protein contains all of the conserved amino acids present in the consensus domains of H-ras involved in guanine nucleotide binding and GTP hydrolysis. In the second guanine nucleotide-binding (G) region, the consensus sequence NKXD is replaced by TKXD in rac1. We previously demonstrated that mutation of Thr115 to Asn115 in rac1 (to form the NKXD motif) had little effect on the GTP hydrolytic properties of the protein. On the basis of their primary structure and their high degree of homology, low molecular mass GTP-binding proteins are believed to share the overall three-dimensional structure of the H-ras protein, especially in the guanine nucleotide-binding domains. In contrast to the other LGBPs, rac1 has a much higher GTPase activity under low free Mg^{2+} concentrations (Ménard et al., 1992). The GTPase activity of rac1 was found to be ≈20-fold higher than that of H-ras (Ménard et al., 1992), and the amino acid(s) responsible for the increased GTPase activity was(were) shown to lie outside the conserved amino acids of the consensus GTP-binding domains (Ménard et al., 1992). The guanine nucleotide-binding domain is composed of the phosphate–magnesium-binding (PM) regions and guanine base-binding

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[1] Abbreviations: aa, amino acids; EDTA, ethylenediaminetetraacetic acid; G protein, guanine nucleotide-binding regulatory protein; G region, guanine nucleotide-binding region; GAP, GTPase-activating protein; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; GTPase activity, guanosine triphosphate hydrolytic activity; GTPγS, guanosine 5′-γ-thiotriphosphate; LGBPs, low molecular mass GTP-binding proteins; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; PM region, phosphate–magnesium-binding region; PPACK, D-phenylalanylprolylarginyl chloromethyl ketone; [α-^{32}P]GDP, guanosine 5′-[α-^{32}P]diphosphate; [α-^{32}P]-GTP, guanosine 5′-[α-^{32}P]triphosphate; [γ-^{35}S]GTP, guanosine 5′-[γ-^{35}S]triphosphate.

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(G) regions. On the basis of our previous results, the G regions are not responsible for the higher GTPase found in rac1 (Ménard et al., 1992). The PM regions are likely candidates to account for some, or all, of the difference in GTPase activity between rac1 and H-ras. The third PM region (aa 56–60) is identical in rac1 and H-ras, but some differences are present in the first two PM regions. A series of mutants was generated by polymerase chain reaction to replace the nonconserved amino acids in rac1 with the corresponding amino acids in H-ras in order to assess their contribution to the higher GTPase activity found in rac1.

MATERIALS AND METHODS

Materials

Dithiothreitol was from Boerhinger-Mannheim (Indianapolis, IN). Phenylmethanesulfonyl fluoride was from Sigma (St. Louis, MO). The sources for the other chemicals have been described previously (Ménard et al., 1992). All other chemicals were reagent grade.

Methods

rac1 Expression and Purification. rac1 protein was expressed as a fusion protein linked to glutathione transferase using the pGEX-2T expression system as described previously (Ménard et al., 1992). Cells (1 L) were grown to an A_{600} of 0.6–0.8, and protein expression was induced with 0.25 mM isopropyl β-D-galactoside for 2 h. Bacteria were collected by cenrifugation (3000*g*, 10 min) and washed once with 150 mM Tris-HCl (pH 8.0 at 20 °C), 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride (buffer A). The cell pellet was flash-frozen in liquid nitrogen and stored at −70 °C until use. On the day of the purification, the pellet was rapidly thawed, resuspended in 20 mL of buffer A containing 0.1 mM GDP and 4 mg/mL lysozyme, and incubated on ice for 30 min. $MgCl_2$ and Triton X-100 were added to final concentrations of 10 mM and 0.1% (w/v), respectively. Deoxyribonuclease and ribonuclease were added to final concentrations of 50 μg/mL, and the incubation was continued for an additional 30 min on ice. The Triton concentration was increased to 1%, and the suspension was centrifuged at 40000*g* for 15 min at 4 °C. The supernatant was mixed for 10 min at room temperature with 1.0 mL of glutathione–Sepharose-4B resin equilibrated in buffer A containing 1% Triton X-100, 0.1 mM GDP, and 10 mM $MgCl_2$. The resin was washed five times with 10 mL of buffer A containing 1% Triton X-100, 0.1 mM GDP, and 10 mM $MgCl_2$, twice with 10 mL of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1 mM GDP, 0.1 mM DTT, and 10 mM $MgCl_2$, and twice with 25 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 2.5 mM $CaCl_2$ (buffer B). Thrombin (30 units) in 2.0 mL of buffer B was added to the resin and incubated for 1 h at room temperature. rac1 protein was eluted from the resin with two 1.0-mL aliquots of buffer B without $CaCl_2$. Nonidet P-40 and D-phenylalanylprolylarginyl chloromethyl ketone (PPACK, a specific thrombin inhibitor) were added to the eluate to final concentrations of 0.01% and 1 μM, respectively. The purified protein was aliquoted and frozen at −70 °C, where it was stable for at least 6 months. Four different preparations of purified protein were used in this study without noticeable differences between each preparation.

Purification of H-ras Protein. Bacterially expressed H-ras was purified as described by Reiss et al. (1990). The protein preparation was greater than 90% pure, as judged by scanning Coomassie blue-stained polyacrylamide gels.

Site-Directed Mutagenesis. Point mutations in rac1 cDNA were generated by the polymerase chain reaction, as described previously (Ménard et al., 1992). The DNAs were sequenced to confirm the mutations and the integrity of the coding region using dideoxyDNA sequencing. The mutated proteins were expressed and purified exactly as described above for rac1. Three different preparations for each different mutant were used in this study without noticeable differences between each preparation.

Guanine Nucleotide Hydrolysis Assay. GTP hydrolysis was determined by quantifying the production of GDP using thin-layer chromatography on PEI-F cellulose, as described previously (Ménard et al., 1992). Reactions were typically carried out at 37 °C in a final volume of 50 μL of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.428 mM $MgCl_2$, 10 μg/mL bovine serum albumin, 10 μM GTP, 1 μCi/mL [α-^{32}P]-GTP (3000 Ci/mmol), and 1 μM PPACK (0.3 μM free Mg^{2+}, standard incubation buffer). Samples were incubated for 10 min at 37 °C and then the reaction was started by the addition of protein. The reaction was terminated after 15 min by the addition of 10 mM GTP, 10 mM GDP, 10 mM GMP, 0.5% SDS, and 2 mM EDTA (final concentrations). Aliquots were spotted on PEI-F cellulose sheets (20 × 20 cm), which were developed in a solution containing 1 M LiCl and 1 M formic acid. R_f values for GTP, GDP, and GMP were 0.26, 0.49, and 0.69, respectively. The guanine nucleotides were visualized under ultraviolet light, the spot was excised, and radioactivity was measured by liquid scintillation counting. GTP hydrolysis was calculated as the radioactivity in GDP/(GMP + GDP + GTP). In all of the experiments, the production of GDP in the control (no protein) was subtracted from the samples; this value was always less than 2% of the total GTP.

Guanine Nucleotide-Binding Assay. The preparation of [α-^{32}P]GDP has been described previously (Ménard et al., 1992). The rates of binding of [γ-^{35}S]GTP or [α-^{32}P]GDP were measured as described previously (Ménard et al., 1992). Briefly, proteins were incubated in standard incubation buffer (0.5 mL, see above) at 100 pmol/mL. The reactions were carried out with either [γ-^{35}S]GTP (1 μM, 20 Ci/mmol) or [α-^{32}P]GDP (2 μM, 30 Ci/mmol). After a 15-min incubation, 200 μM cold nucleotide was added, and 25-μL aliquots were removed at 10-s intervals and added to 2 mL of ice-cold 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 25 mM $MgCl_2$, and 0.01% bovine serum albumin. The samples were filtered through nitrocellulose membranes (0.45 μm, Millipore), and the filters were washed six times with 3 mL each of the same buffer. Filters were dried and radioactivity was measured by liquid scintillation counting.

Equilibrium Binding Experiments. Proteins were incubated at 50 pmol/mL in 100 μL (final volume) of standard incubation buffer for 15 min with [α-^{32}P]GDP. Nucleotide concentrations were varied between 5 nM and 100 μM. Binding was determined as described above.

Other Methods. Protein concentrations were measured using a Bio-Rad protein determination kit and bovine serum albumin as the standard.

RESULTS AND DISCUSSION

We have shown rac1 protein to have a 20-fold higher GTPase activity than H-ras under conditions of low Mg^{2+} concentrations (Ménard et al., 1992), conditions known to increase the rate of nucleotide exchange in LGBPs (Hall & Self, 1986; John et al., 1988; Ménard et al., 1992). Of all the amino acids found in the conserved nucleotide-binding regions of

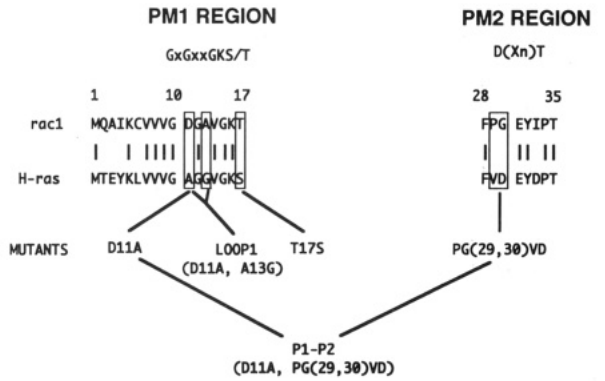

FIGURE 1: Sequence alignment of rac1 and H-ras in the phosphate–magnesium-binding (PM) regions. Identical amino acids are linked by a vertical line. Mutated amino acids are boxed. The consensus sequences are shown for each loop (Valencia et al., 1991; Bourne et al., 1991) . The single letter amino acid codes are used, X is any amino acid.

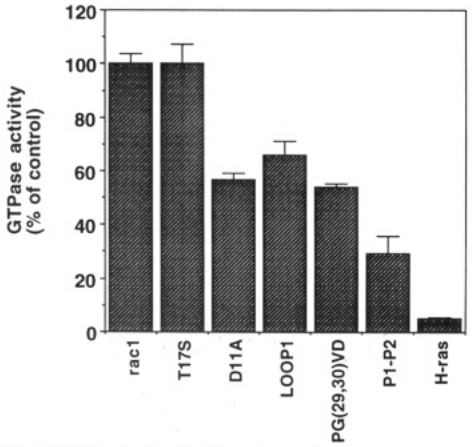

FIGURE 2: GTP hydrolysis of rac1, various mutants, and H-ras. Proteins were incubated in the standard incubation buffer (containing 0.3 μM free Mg^{2+}) at 10 μg/mL for 15 min. GTP hydrolysis was measured by thin-layer chromatography as described in Materials and Methods. The activity of the control (rac1) was 552.6 pmol of GTP hydrolyzed/nmol of GTPγS-binding protein. All hydrolysis values were corrected for the amount of GTPγS-binding protein for each protein preparation. The values represent the mean of two or three experiments performed in duplicate.

LGBPs, rac1 contains different amino acids in the first two PM regions and the second G region (NKXD). We previously showed that the second G region did not contribute significantly to the higher GTPase activity, thus suggesting that differences in PM regions might be responsible for some, or all, of the higher GTPase activity found in rac1. The primary structure of rac1 and H-ras in the first two PM regions is shown in Figure 1. We converted rac1 primary structure to H-ras using the polymerase chain reaction. Three mutants were made in the PM1 region (D11A, T17S, and loop 1) and one in the PM2 region [PG(29,30)VD], as well as a "double" mutant in both regions (i.e., positions 11 and 29–30, P1–P2 mutant). rac1 and the various mutants were expressed as fusion proteins using the pGEX-2T expression vector. The fusion proteins were purified by affinity chromatography on glutathione–Sepharose. rac1 and the different mutants were eluted from the column by specific cleavage with thrombin. The purity of the different protein preparations was >95% as judged by Coomassie blue staining of polyacrylamide gels (data not shown).

The effects of single or multiple amino acid substitutions in the first two PM regions on the GTPase activity of rac1 are shown in Figure 2. Of the three different amino acids found

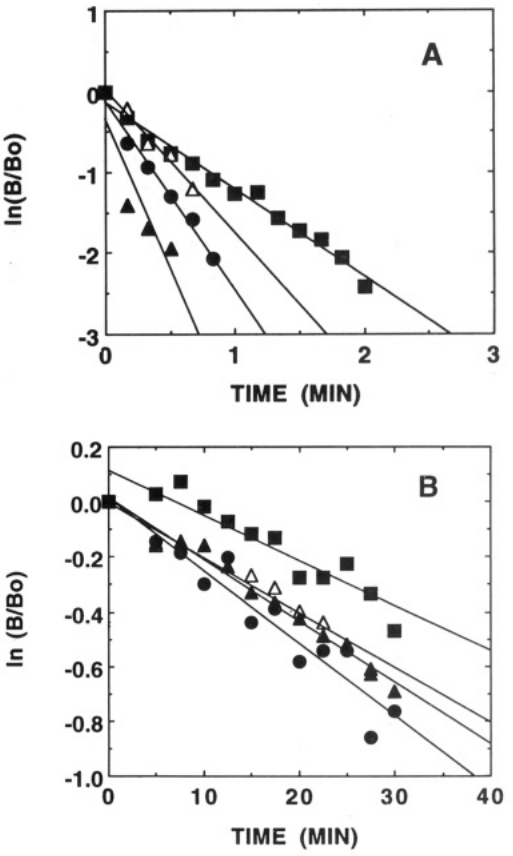

FIGURE 3: GDP and GTPγS dissociation from rac1 and various mutants. 100 pmol/mL each of the four proteins was incubated in the standard buffer (0.3 μM free Mg^{2+}) at 20 °C. (A) GDP and (B) GTPγS dissociation rates. The proteins were allowed to associate with the nucleotide for 15 min, and unlabeled GDP or GTPγS was then added at 200 μM final concentration. The level of nucleotide bound to rac1 (■), D11A (●), PGVD (△) and P1–P2 (▲) was determined at the indicated times by rapid filtration on nitrocellulose membranes, as described in Materials and Methods. B, amount of bound nucleotide; B_0, total amount of bound nucleotide at time 0. The values are representative of two or three separate sets of experiments.

in the PM1 region between rac1 and H-ras, mutation at position 11 (D11A) reduced the GTPase activity 2-fold, whereas mutation at position 17 (T17S) had no effect. As shown by the double mutant loop 1, Ala13 does not seem to contribute to the higher GTPase activity in rac1, suggesting that Asp11 is the amino acid responsible for the increased activity found in the PM1 region.

In the PM2 region of H-ras, amino acids 29–30 are involved in the binding of the ribose moiety of guanine nucleotides (Pai et al., 1990; Milburn et al., 1990), whereas position 33 is part of the effector region which interacts with GTPase-activating protein (GAP) (McCormick, 1989; Milburn, 1990; Gibbs et al., 1990; Bourne et al., 1991). rac1 possesses Pro-Gly compared to Val-Asp in H-ras, at positions 29–30, suggesting a potential role for these amino acids in the difference in activity of rac1. Conversion of these two amino acids to those present in H-ras reduced the GTPase activity of rac1 2-fold [PG(29,30)VD mutant, Figure 2]. Mutation of the three amino acids of both PM regions in rac1 (positions 11 and 29–30) to those present in H-ras resulted in a protein with one-third to one-fourth of the GTPase activity of the native protein (Figure 2).

The nucleotide-binding properties of the mutants with altered GTPase activity were determined. As shown in Figure 3, the release of GDP was increased in all of the mutants,

Table I: Guanine Nucleotide Off-Rates of Various rac1 Mutants[a]

protein	GDP_{off} (min^{-1})	$GTP\gamma S_{off}$ (min^{-1})
rac1	1.10 ± 0.04	0.013 ± 0.003
D11A	2.14 ± 0.13	0.025 ± 0.002
PG(29,30)VD	1.87 ± 0.13	0.018 ± 0.003
P1–P2	>6.0	0.017 ± 0.006

[a] Guanine nucleotide-binding assays were carried out using [α-^{32}P]GDP or [γ-^{35}S]GTP in 0.3 μM free Mg^{2+}, as described in Materials and Methods. After 15 min of incubation, 200 μM unlabeled nucleotide was added, and the amount of bound nucleotide was measured by rapid filtration. The k_{off} values were determined from the slopes of the binding curves, such as those shown in Figure 3. The values represent the mean ± sd of two or three sets of independent experiments.

Table II: Equilibrium Dissociation Constants of Various rac1 Mutants for GDP[a]

protein	$k_{d,GDP}$ (nM)
rac1	43 ± 1
D11A	609 ± 37
PG(29,30)VD	113 ± 18
P1–P2	12750 ± 3889

[a] Guanine nucleotide-binding assays were carried out as described in Table I. GDP concentrations were varied between 5 nM and 100 μM. k_d's were determined from linear curve fitting of Scatchard plots. The values represent the mean ± sd of two or three independent sets of experiments carried out in duplicate.

whereas the GTPγS off-rate was less affected. The most noticeable effect was the increase in $k_{off,GDP}$ of the P1–P2 mutant (Table I). The increased GDP off-rates translated into reduced affinity for GDP as measured by equilibrium binding (Table II). The D11A mutant had a much lower $k_{d,GDP}$ (1 order of magnitude) than rac1, suggesting that this amino acid is interacting, directly or indirectly, with GDP. The effect was even more pronounced in the P1–P2 mutant, where the $k_{d,GDP}$ was reduced by almost 3 orders of magnitude compared to rac1.

Mutations at positions 12 and 13 are oncogenic in H-ras (Barbacid, 1987; Bourne et al., 1991). These amino acids are part of the first loop, which is localized in the PM1 region (Milburn et al., 1990; Pai et al., 1990). Although mutations at position 12 do not change the structure of the loop significantly (Milburn et al., 1990; Privé et al., 1992), mutations at position 13 are expected to induce significant structural changes in the PM1 region due to the unusual structure of the backbone angles of Gly13 (Pai et al., 1990; Valencia et al., 1991). Our data showed that Asp11 was crucial for the interaction of rac1 with GDP and its GTPase activity, whereas Ala13 was not important. These results suggest that rac1 has a different loop 1 structure and could probably hydrolyze GTP through a different mechanism than H-ras, a hypothesis reinforced by the lack of an effect of the D63E mutation on rac1 GTPase activity (data not shown).

The PM2 region in H-ras has been studied mostly for its involvement in the interaction with GAP (McCormick, 1990; Bourne et al., 1991; Gibbs et al., 1990). In the crystal structure of H-ras, the side-chain carbonyl of Asp30 and the main-chain carbonyl of Val29 interact with the hydroxyl residues of the ribose moiety of the guanine nucleotide (Pai et al., 1991). The corresponding amino acids in rac1 are Pro-Gly, which may interact with the ribose in a quite different manner than Val-Asp due to their different structure. Changing Pro29 and Gly30 in rac1 to Val29 and Asp30 reduces the affinity of the protein for GDP, suggesting that Pro-Gly in rac1 forms a more stable interaction with GDP than Val-Asp or that the mutation affected the overall structure of the loop, altering the interaction of other amino acids with GDP. The D11A mutant had a much more reduced k_d for GDP than the PGVD mutant (Table II), suggesting that PM1 is a more important region for the interaction with GDP than PM2, a situation similar to H-ras (Pai et al., 1990; Milburn et al., 1990).

GTP hydrolysis in G proteins is controlled by the release of bound GDP, whereas in LGBPs, the rate of hydrolysis per se (i.e., k_{cat}) is usually the rate-limiting step (Ménard et al., 1992; Gilman, 1987). A reduction in either rate or both could lead to a reduction in GTP hydrolysis. The P1–P2 mutant had a 3–4-fold decreased GTPase activity coupled with a dramatically increased GDP off-rate. This indicates that the reduction in GTPase activity in the P1–P2 mutant most probably results from a reduction in k_{cat}. The results suggest that Asp11, Pro29, and Gly30 are involved in the GTP hydrolysis process in rac1. However, the present data do not enable us to determine whether the amino acids are directly or indirectly (through propagation of structural changes) involved in GTP hydrolysis. These questions will be better answered after the elucidation of the crystal structure of rac1.

Our results demonstrate that the PM1 and PM2 regions account for a large portion of the increased GTPase activity in rac1 compared to H-ras. Since rac1 has a ≈20-fold higher GTPase activity than H-ras, the results suggest that the amino acids responsible for the remaining 5-fold higher GTPase activity lie outside of the conserved GTP-binding domains and that these amino acids could be present over a large area of the protein. We have tried to localize the other amino acids involved in GTP hydrolysis in rac1 by generating chimeras between rac1 and H-ras. Unfortunately, in contrast with the mutants analyzed here, all chimera constructs produced insoluble proteins when expressed in *Escherichia coli*. This suggests that the structure of rac1 is probably quite different from that of H-ras in several domains. Determination of the crystal structure of the protein will provide a definitive answer to that question.

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