Characterization of *Saccharomyces cerevisiae* Ras1p and Chimaeric Constructs of Ras Proteins Reveals the Hypervariable Region and Farnesylation as Critical Elements in the Adenylyl Cyclase Signaling Pathway

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ABSTRACT: Ras1p and Ras2p, from *Saccharomyces cerevisiae*, are GTP-binding proteins that are essential elements in the signaling cascade leading to the activation of adenylyl cyclase. To overcome proteolytic activities that have hampered biochemical studies of Ras1p so far, its gene was genetically modified after which full-length Ras1p could be obtained. The interaction of farnesylated and unprenylated Ras1p with guanine nucleotides, guanine nucleotide exchange factors, GTPase activating proteins, and adenylyl cyclase was compared to Ras2p and human Ha-Ras interactions. Farnesylation of Ras proteins was demonstrated to be a prerequisite for membrane-bound guanine nucleotide exchange factor dependent formation of Ras-GTP complexes, and for efficient Ras-mediated adenylyl cyclase activation. To relate observed functional deviations with sequence differences between Ras1p and Ras2p, which reside almost exclusively within the hypervariable region, truncated versions and chimaeras of the Ras proteins were made. The characteristics of these constructs point to the presence of the hypervariable region of yeast Ras proteins for an efficient activation of adenylyl cyclase. The importance of the latter was confirmed as inhibition of the activation of adenylyl cyclase by an isolated farnesylated hypervariable region of Ras2p could be shown. This strongly suggests that the hypervariable region of Ras proteins can interact directly with adenylyl cyclase.

Ras proteins are GTPases cycling between the active GTP-bound and the inactive GDP-bound states. Thus, they act as molecular switches in Ras-mediated signal transduction controlling cell proliferation, differentiation, and apoptosis (*1*). The ratio of GTP to GDP-bound form is regulated by two kinds of regulators, GTPase activating proteins (GAPs),[1] which enhance their low intrinsic GTP hydrolytic activity, and GDP-GTP exchange factors (GEFs), which promote the regeneration of the active GTP-bound form (*1*−*3*).

The budding yeast *Saccharomyces cerevisiae* has two *RAS* genes, *RAS1* and *RAS2*, encoding proteins homologous to mammalian Ras proto-oncogene products. Yeast Ras proteins are essential for growth; they are positive regulatory elements of adenylyl cyclase (for reviews, refs *4*−*6*) and thus regulate the cAMP-dependent protein kinase pathway. In *S. cerevisiae*, the *IRA1* and *IRA2* gene products were identified as GAP-like proteins (*7*), and the *CDC25* and *SDC25* genes products as GEFs (*8*−*11*). Ira1p and Ira2p contain a domain, located in the intermediary portion of the molecule, that shares similarities with the catalytic core of mammalian GAPs, p120-GAP and neurofibromin (*7*, *12*). Yeast Cdc25p and its homologue Sdc25p, the first protein for which Ras-specific nucleotide exchange activity was characterized (*10*), are members of the $Cdc25^{GEF}$ family and represent the prototypes of ras-specific GEFs.

All Ras proteins undergo a series of posttranslational modifications at the carboxy terminal sequence CAAX motif, where C is a cysteine, A is usually an aliphatic amino acid, and X is any amino acid. These modifications, which comprise a farnesylation, a cleavage, a methyl esterification, and a palmitoylation, respectively, are essential for their membrane localization and therefore for biological activity (*13*, *14*). Palmitoylation is essential for the tight association of Ras proteins with the plasma membrane, since after the first three modification steps (farnesylation, proteolysis, and methylation) Ras proteins remain largely cytosolic. For K-Ras, which is not palmitoylated, a stretch of a basic residues (lysine) located just upstream of the CAAX motif is required for plasma membrane localization (*15*, *16*). The single modification step, farnesylation, on Ha-Ras was reported to be important for the action of exchange factor SOS (*17*), and it was found in Ras2p from *S. cerevisiae* to be required for $Cdc25^{GEF}$ membrane-bound exchange activity (*18*) in addition to efficient interaction with the adenylyl cyclase-CAP complex (*19*).

Ras1p and Ras2p appear to be functionally interchangeable; deletion of either the *RAS1* or *RAS2* gene has no consequences for yeast cell growth on glucose, while deletion of both genes is lethal (*20*). They differ in their pattern of

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[1] Abbreviations: GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; $CDC25^{Mm}$, mouse RasGRF, a neuronal RasGEF; PAGE, polyacrylamide gel electrophoresis; aa, amino acid; GTPγS, guanosine 5′-*O*-(thiotriphosphate); MES, 4-morpholineethanesulfonic acid.

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gene expression and essentially by the selective repression of *RAS1* transcripts in media containing a nonfermentable carbon source (*21*). The primary structures of yeast Ras1p (309 aa), Ras2p (322 aa), and that of mammalian Ha-Ras display domains with different homologies. The N-terminal region including residues 8−181 of Ras1p and Ras2p can be almost perfectly aligned (90% homology), and is highly similar to the corresponding 1−173 residues of Ha-Ras. The Ras sequence, C-terminal to this domain, except for the C-terminal CAAX box, diverges between the various Ras proteins and is called the hypervariable region (*22*). The major difference between yeast and mammalian Ras proteins lies in the sequence and the size of the C-terminal hypervariable region, which in the case of Ras1p and Ras2p is much more extended. The function of this overextended C-terminal region is not fully understood, and our knowledge is limited to our previous observation defining the importance of the hypervariable domain of Ras2p for the activation of yeast adenylyl cyclase (*18*).

In contrast to Ras2p (*10*, *18*, *23*−*25*), Ras1 protein has never been purified to homogeneity in its full-length form and consequently has not yet been subjected to intense biochemical characterization. In the present work, the biochemical properties of Ras1p have been characterized in detail and compared to Ras2p and Ha-Ras. The interaction with GDP and GTP, GEFs, GAPs, and the effector adenylyl cyclase were characterized in particular. The role of the hypervariable domain and the specific effect of farnesylation on these different interactions were investigated. Among the most important results, we observed that, as compared to Ras2p, Ras1p displays an enhanced affinity for GDP and GTP, and a reduced sensitivity to the catalytic domains of members of $Cdc25^{GEF}$ family and Ira2pGAP. Similar to Ras2p, farnesylation of Ras1p increases affinity for adenylyl cyclase and is strictly required for nucleotide exchange activity mediated by membrane bound full-length Cdc25p and Sdc25p exchange factors. The important role of the hypervariable domain could be demonstrated by comparing the potency of various chimaeric constructs of Ras proteins in stimulation of adenylyl cyclase activity. Furthermore, the isolated hypervariable domain of Ras2p inhibited Ras-mediated adenylyl cyclase stimulation. Our results underline the determinant role of the hypervariable domain of the yeast Ras proteins, in particular that of Ras2p, in an efficient adenylyl cyclase activation. Structural elements of this domain appear to interact directly with adenylyl cyclase.

MATERIALS AND METHODS

Expression and Purification of Proteins. Ras proteins is a general term used in this work for designating human Ha-Ras p21, *S. cerevisiae* Ras1p, Ras2p, and all chimaeric products. For clarity, we have named the various constructs throughout this paper as follows: Ras1p (amino acids 1−293 of Ras1p followed by amino acids 307−322 of Ras2p); Ras1p Δ-C (amino acids 1−181 of Ras1p followed by amino acids 307−322 of Ras2p); Ras2p Δ-C (amino acids 1−181 of Ras2p followed by amino acids 307−322 of Ras2p); Ras1p/Ras2p (amino acids 1−181 of Ras1p followed by amino acids 182−322 of Ras2p); and Ha-Ras/Ras1p (amino acids 1−173 of Ha-Ras followed by amino acids 182−293 of Ras1p and amino acids 307−322 of Ras2p). cHa-Ras p21, full-length Ras2p and Ha-Ras/Ras2p were obtained as described (*18*, *25*). Ras1p, Ha-Ras/Ras1p, and Ras1/Ras2p were expressed as glutathione *S*-transferase fusion proteins using plasmid vectors purchased from Amersham Biosciences. All the following Ras expression vectors were constructed using Expand high fidelity PCR system (Roche). *Ras1* gene was amplified from yeast genomic DNA of A5C strain *(a can1−100 his3 leu2−3, 112 lys1−1 ura3−52 ras2::LEU2*; relevant genotype *RAS1*, *ras2*) (*26*) using the 5′ primer GCGGGATCCATGCAGGGAAATAAATCAAC and the 3′ primer GATGAATTCTCAACAAATTATACAACAAC. The amplified fragment was cloned into the *Bam*HI-*Eco*RI sites of the vector pGEX-2TK giving the pGEX-2TK(*RAS1*) expression vector. Chimaeric Ras1p 1−181/Ras2p 182−322 and Ras1p 1−181/Ras2p 307−322 were constructed from pGEX-2T(*RAS2*) (*24*), using the 5′ primers AACGTAGACGAGGCCTTTTATAGCCTTATTCGTTTGGTAAGGGACGACGGTGGGAAATACAATAGC/ATGTTGACGGAAAATGACAACTCC and AACGTAGACGAGGCCTTTTATAGCCTTATTCGTTTGGTAAGGGAGACGGTGGGAAATACAATAGCATG/ACCAGTGAAGCCTCCAAG, respectively, containing *Stu* I restriction site (underlined) in the *Ras1* sequence and the 3′ primer pG24 (GGGGTTCCGCGCACATTTCCCCG) corresponding to a pGEX region 300 bases downstream of the multiple cloning site. The *Stu*I-*Eco*RI amplified fragments were cloned into pGEX-2KT-(*RAS1*). Chimaeric Ras1p 1−293/Ras2p 307−322 was constructed from pGEX-2T(*RAS2*) using the 5′ primer CAGTCTGCTGAGCCACAAAAAAATTCA/ACCAGTGAAGCCTCCAAG containing *Esp*I restriction site (underlined) and the 3′ primer pG24. The *Esp*I-*Eco*RI amplified fragment was cloned into pGEX-2KT(*RAS1*) leading to pGEX-2KT(f-*RAS1*) expression vector. Chimaeric Ras2p 1−181/Ras2p 307−322 was constructed from pGEX-2T-(*RAS2*) using the 5′ primer TTTTGGATACTGCAGGGCAGG and the 3′ chimaeric primer CGGGAATTCTTAACTTATAATACAACAGCCACCCGATCCGCTCTTGGAGGCTTCACTGGT/AGTCTTGTTGTACTTGCCGCC containing a *Eco*RI restriction site (underlined). The *Esp*I-*Eco*RI amplified fragment (134-bp) was cloned into pGEX-2T(*RAS2*). Chimaeric Ha-ras 1−173/Ras1 182−309/Ras2 307−322 was constructed from pGEX-2T(*H-ras*) (*27*) using the 5′ primer GACGTGCCCATGGTGCTGGTGGGG and the 3′ primer CGTATTATCCAGTTGACGATT/AGGGTTCAGCTTCCGCAG containing *Nco*I and *Hind*II restriction sites respectively (underlined). The resulting 195-bp *Nco*I-*Hind*II amplified fragment and the 560-bp *Hind*II-*Tth*111−I fragment from pGEX-2KT(f-*RAS1*) were ligated and cloned into pGEX-2T(*H-ras*). The Ras2p 182−322 and Ras1p 182−293/Ras2p 307−322 variable domains were constructed from pGEX-2T(*RAS2*) and pGEX-2KT(f-*RAS1*) using the 5′ primers CGCGGATCCTTGACGGAAAATGACAACTCC and CGCGGATCCAATCGTCAACTGGATAATACG respectively containing the *Bam*HI restriction site (underlined) and the 3′primer pG24. The amplified fragments were cloned into the *Bam*HI-*Eco*RI sites of the vector pGEX-2T. Accuracy of the amplifications was confirmed by nucleotide sequencing of the cloned genes.

The different Ras proteins were expressed in *Escherichia coli* SCS1 and prepared as described in ref *25*. Affinity chromatography using glutathione-agarose (Clontech) and thrombin treatment removing the N-terminal fused glutathione *S*-transferase were followed by chromatography on

Mono-Q HR5/5 (FPLC system, Amersham Pharmacia Biotech) with a 20−230 mM KCl linear gradient (50 mL) in 25 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 10% glycerol, 7 mM β-mercaptoethanol and 10 μM GDP or using Resource-S in the case of Ras1p 1−181/Ras2p 182−322 and variable domain of Ras2p, with the same salt gradient, in 50 mM MES−KOH pH 6.2. These ion exchange chromatography steps allowed the separation of full-length Ras from their proteolytic C-terminal truncated forms.

The C-terminal catalytic domain of GEFs Sdc25p (C-Sdc25p, 550 amino acids), Cdc25p (C-Cdc25p, 509 amino acids), and CDC25Mm (C-CDC25Mm, 285 amino acids); farnesyl-protein transferase; and the GAPs GAP-334 (amino acids 714−1047), GST-fused NF1−414 (amino acids 1145−1558) and Ira2p-383 (amino acids 1644−2026) were obtained as previously described (*18*, *25*, *27*).

Protein concentrations were measured using the Bradford method (Bio-Rad) or in the case of yeast membranes using the Lowry method (*28*), using BSA as a standard. SDS−PAGE was carried out using a 10 or 12.5% acrylamide separating gel.

Farnesyl-Protein Transferase Assay and Preparation of in Vitro Farnesylated Ras. The amount of [^{3}H]farnesyl moiety transferred from [^{3}H]farnesyl pyrophosphate (Fpp, PerkinElmer life sciences) to intact purified Ras products was determined using purified *E. coli* recombinant *S. cerevisiae* farnesyl protein transferase (FTase) as described in ref *18*. Level of farnesylation of the various Ras products was analyzed by electro-transfer to Nytran-N membrane (Schleicher & Schuell) from a 12.5% SDS−PAGE followed by autoradiography of the membrane pretreated with intensifier EN^{3}HANCE (NEN).

GDP, GTP, or GTPγ-S-bound Ras products (4−6 μM) were farnesylated using a 60 min incubation at 30 °C in farnesylation buffer (50 mM Tris-HCl, pH 7.8, 10 μM $ZnCl_2$, 2.5 mM $MgCl_2$, 0.25 mM $CaCl_2$, 5 mM dithiotreitol) with 0.6 μM purified FTase, 100 μM cold Fpp (Isotopchim) and in the presence of a protease inhibitors cocktail (2 μg mL^{-1} aprotinin, 1 μg mL^{-1} leupeptin, 60 μg mL^{-1} antipain, 2 mM Pefablock S-C, Roche).

Ras·Nucleotide Interaction. Association rates of Ras proteins with GDP or GTP and dissociation rates of Ras·GDP and Ras·GTP complexes were determined at 30 °C in standard buffer (25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 60 mM NH_4Cl, 0.05 mg mL^{-1} BSA, 1 mM dithiothreitol) using the nitrocellulose filtration procedure (*29*, *25*).

For the determination of association rates, nucleotide-free Ras proteins were prepared after incubation of pure Ras·GDP proteins (200−300 pmol in 30 μL) for 20 min at 30 °C in 25 mM Tris-HCl, pH 7.8, 100 mM $(NH_4)_2SO_4$, 10 mM NaEDTA, 0.05 mg mL^{-1} BSA, 1 mM dithiothreitol. The solution was passed through a Sephadex G-25 fine column (18 × 0.4 cm) at 4 °C equilibrated with 25 mM Tris-HCl, pH 7.5, 500 mM $(NH_4)_2SO_4$, 0.5 mM NaEDTA, 0.05 mg mL^{-1} BSA, 7 mM β-mercaptoethanol. A final concentration of 6 mM $MgCl_2$ was added to the GDP-free Ras containing fraction.

Prior to each GTP-containing assay, 3 μM [^{3}H]GTP (PerkinElmer life sciences) was preincubated in standard buffer for 10 min at 30 °C with 10 μg of pyruvate kinase and a 150-fold excess of phospho*enol*pyruvate.

Association rates of Ras products with GDP or GTP were measured in standard buffer containing 2−30 nM nucleotide-free Ras and 7.5−45 nM [^{3}H]GDP (specific activity, 130 Bq pmol^{-1}, PerkinElmer life sciences) or 7.5−100 nM [^{3}H]-GTP (specific activity, 120 Bq pmol^{-1}). The reaction was started with nucleotide-free Ras proteins. Aliquots of the reaction mixture were withdrawn every 10 s for a 2-min period and filtered on nitrocellulose discs that were washed twice with 3 mL of ice-cold 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 60 mM NH_4Cl, 1 mM dithiotreitol. The nitrocellulose membranes were then counted for radioactivity in a Wallac 1410 (PerkinElmer life sciences) scintillation spectrometer. The apparent second-rate constants for association of Ras products and guanine nucleotides were calculate according to the equation $[1/(b - a)]\ \ln[a(b - x)/b(a - x)] = k'_{+1}t$ where a is the initial concentration of [^{3}H]-GDP or [^{3}H]GTP, b is the initial concentration of nucleotide-free Ras proteins and x is the concentration of Ras·[^{3}H]GDP or Ras·[^{3}H]GTP complexes formed at the different times, t (*29*). The initial concentration b was determined from the radioactivity bound to each GDP-free Ras species in the presence of a saturating amount of [^{3}H]GDP, after 20 min incubation in standard buffer at 30 °C. This represented 70−90% of the protein concentration as determined using the BioRad protein assay.

For the determination of dissociation rate constants (k_{-1}), preformed labeled Ras·[^{3}H]GDP or Ras·[^{3}H]GTP complexes were prepared by incubating for 15 min at 30 °C 6 μM Ras-GDP in 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM dithiotreitol and 0.05 mg mL^{-1} BSA with 25 μM [^{3}H]GDP or [^{3}H]GTP (200 GBq mmol^{-1}). The dissociation rates were measured in 70 μL of standard buffer with or without GEF, as indicated in the legends of tables, with 200 nM preformed Ras·[^{3}H]GDP or Ras·[^{3}H]GTP complex and a 1000-fold excess of the corresponding nonlabeled nucleotide. The reaction was started with labeled Ras·nucleotide complex. At time intervals, aliquots (9 μL) were filtered on nitrocellulose discs that were then washed and counted as described above.

Measurement of GTPase Activity. The various Ras·[γ-^{33}P]-GTP complexes were preformed by incubating Ras·GDP (6−30 μM) at 25 °C for 10 min with a 2−3-fold excess of [γ-^{33}P]GTP (60−100 Bq/pmol) in 25 mM Tris-HCl, pH 7.8, 5 mM NaEDTA, 0.05 mg mL^{-1} BSA, 1 mM dithiotreitol and then stabilized by the addition of 6 mM $MgCl_2$.

GTPase activity was determined in 70 μL reaction mixture containing in 25 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiotreitol, preformed Ras·[γ-^{33}P]GTP complex (500 nM). The hydrolysis of the [γ-^{33}P]GTP was measured at 25 °C by following the liberation of γ-^{33}Pi in time using the charcoal method: (9 μL aliquots were withdrawn and the reaction was stopped with 400 μL of a 4% suspension of activated charcoal in 20 mM H_3PO_4. After centrifugation, the radioactivity in 250 μL of supernatant was counted. Control experiments were performed using the same conditions and the amount of GTP hydrolysis obtained in the absence of Ras was subtracted.

The GAP-stimulated GTPase activities were determined in 15 μL reaction mixture after 5 min incubation time in the presence of increasing concentrations of Ras·[γ-^{33}P]GTP, (0.02−40 μM) and 30 nM Ira2p-383, 4 nM GAP-334 or 4 nM NF1-414. Intrinsic GTPase activities were subtracted

from the results. Double reciprocal plots allowed the calculation of the K_m and the corresponding maximal stimulated GTPase activities (k_{cat}).

Adenylyl Cyclase Assay. The adenylyl cyclase assay was carried out as described (*30*). Yeast membranes were prepared from yeast strains TS1−6 overexpressing wild-type adenylyl cyclase (*CYR1* gene product) or isogenic yeast strains expressing the *CRI4*-encoded adenylyl cyclase (CRI4-adenyl cyclase) gene, AAT3B depleted of *RAS1* and *RAS2* genes, AAT3B-Δ2R2H, depleted of *RAS1*, *RAS2*, and both *CDC25* and *SDC25* genes, and AAT3B-Δ2R2H transformed with either pYEDP1/8/2, pFC1, or pIND25−1 overexpressing full-length Cdc25p and Sdc25p, and the C-terminal region of the former (C-Cdc25p 877−1589), respectively (for the complete genotypes of yeast strains used see ref *18*). Membranes prepared from these strains were used as source of membrane-associated adenylyl cyclase and GEF (Cdc25p, Sdc25p, or both factors). The cAMP production was determined after 18 min at 30 °C in which time interval the reaction was linear. The 100 μL reaction mixture with the indicated concentrations of farnesylated or unfarnesylated Ras proteins in their preformed GDP, GTP, or GTPγS complexes contained either 30−40 μg of membrane preparation for the yeast strains expressing the CRI4-adenylyl cyclase or 3.5 μg of membrane preparation for the yeast strain TS1−6 producing wild-type adenylyl cyclase. These amounts of membranes in the assay gave similar levels of Ras-uncoupled adenylyl cyclase activity as determined in the presence of 1.5 mM $MnCl_2$. Preformed Ras-GDP, -GTP, or -GTPγS complexes were obtained in the presence of 0.5 mM of the corresponding unlabeled guanine nucleotide and farnesylated as described above. Unprenylated complexes were treated identically; however, the FTase was ommited. The reaction was started with a mixture containing 50 mM MES, pH 6.2, 5 mM $MgCl_2$, GTP or GTPγS (0.5 mM), cAMP (0.5 mM), [α^{33}-P]ATP (0.3 mM, 5 GBq $mmol^{-1}$), theophylline, creatine phosphate, and creatine kinase.

The illustrated data are means of values from at least three independent experiments using different membrane preparations for each yeast strain.

RESULTS

Purification of Ras1p and Various Ras Constructs. To overcome the sensitivity of Ras1p to proteolytic C-terminal degradation (*25*, *31*), and to obtain an intact Ras1p that could be fully farnesylated, we constructed a modified Ras1 protein in which the 16 C-terminal residues were replaced by the 16 C-terminal residues of Ras2p (Ras1p 1−293/Ras2p 307−322). Furthermore, the protein was produced as a fusion with gluthatione-*S*-transferase (GST) using pGEX vectors to allow rapid purification using affinity chromatography. In addition, to analyze the effect of the homologous and hypervariable domain of Ras1p and Ras2p on the interaction with GEFs, GAPs, and adenylyl cyclase, various constructs of Ras were produced and purified, as schematically illustrated in Figure 1. Similarly, all the constructs harboring a Ras1p domain were produced as chimaeric products with the C-terminal 307−322 fragment of Ras2p (see Figure 1). The GST moiety was removed after thrombin treatment. Under these conditions, as shown in Figure 2A, the produced and purified Ras proteins appeared essentially pure and 60−100% in their full-length size on Coomassie blue-stained SDS−PAGE, the residual bands with lower molecular mass corresponding to the respective C-terminal truncated forms.

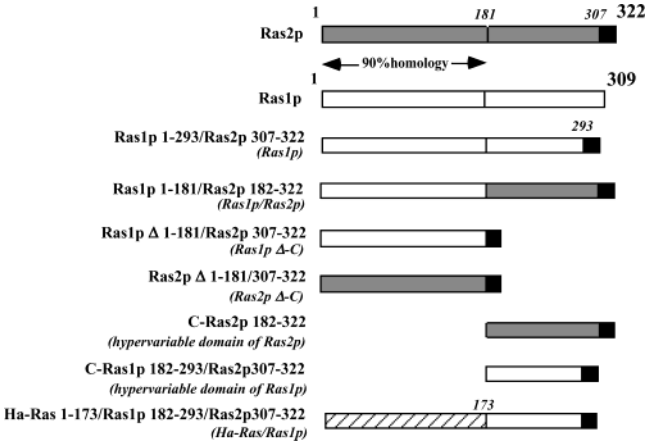

FIGURE 1: Diagram of the various Ras constructs. The gray bars indicate regions originating from Ras2p, the open bars refer to those of Ras1p the dashed bars to Ha-Ras regions, and the black bars refer to region encompassing residues 307−322 from Ras2p. The various Ras products were constructed as described in Materials and Methods.

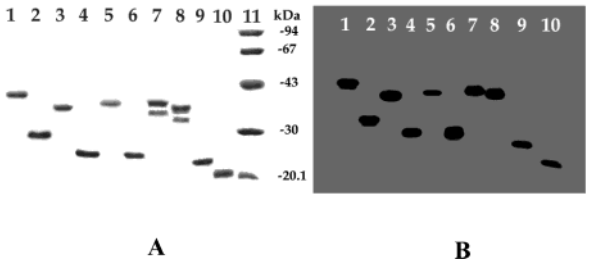

FIGURE 2: Coomassie Blue-stained SDS−PAGE of the various purified Ras constructs (A) and autoradiography of ^{3}H-farnesylated Ras products after electro-transfer (B). Coomassie blue-stained SDS−PAGE (A) of 1−1.5 μg of Ras2p (lane 1), Ras2p Δ-C (lane 2), Ras1p (lane 3), Ras1p Δ-C (lane 4), Ras1p/Ras2p (lane 5), Ha-Rasp21 (lane 6), Ha-Ras/Ras2p (lane 7), Ha-Ras/Ras1p (lane 8), hypervariable domain of Ras2p (lane 9), hypervariable domain of Ras1p (lane 10), and 4 μg of markers (Amersham Biosciences) with the indicated molecular mass expressed in kDa (lane 11). Autoradiography after electrotransfer (B) of 5−10 pmol of each Ras product after in vitro farnesylation with 25 pmol of [^{3}H]Fpp as donor of farnesyl group (832 GBq/mmol) (See Materials and Methods) following the same sequence as above.

All purified Ras species displayed an approximate 1:1 stoichiometric GDP or GTP binding and were stable for several months when kept at −20 °C in storage buffer (*25*). The FTase assay, using ^{3}HFpp as substrate shows that farnesylation was complete: 1 mol of farnesyl group bound per mole of intact Ras product. Figure 2B illustrates the farnesylation of the various Ras forms after electrotransfer on a nylon membrane.

Intrinsic Properties of Ras1p. Table 1 reports the apparent association and dissociation rate constants and the derived dissociation constant ($K'_d = k'_{-1}/k'_{+1}$) of the Ras-GDP and GTP complexes. The major difference between Ras1p and Ras2p is represented by a higher association rate constant and a modestly higher dissociation rate constant of Ras1p for both nucleotides. As a result, Ras1p has an affinity that is 4−6-fold higher for GDP and GTP than for Ras2p.

Remarkably, deletion of the hypervariable domain in Ras2p hardly affected the interaction with the nucleotides,

Table 1: Apparent Association and Dissociation Rate Constants and Derived Equilibrium Constant ($K'_d = k'_{-1}/k'_{+1}$) of the GDP and GTP Complexes of the Various Ras Constructs

complexes	association rate constant (k'_{+1}) 10^5 M^{-1} s^{-1}	dissociation rate constant (k'_{-1}) 10^{-4} s^{-1}	dissociation half-lives (min)	dissociation constant (K'_d) (nM)
Ras1p·GDP	31.18 ± 3.90	4.45 ± 0.04	26	0.14
Ras2p·GDP	2.53 ± 0.25	2.20 ± 0.03	53	0.87
Ha-Ras·GDP	50 ± 10	1.83 ± 0.09	63	0.04
Ras1p Δ-C·GDP	0.53 ± 0.06	7.17 ± 0.50	16	13.5
Ras2p Δ-C·GDP	6.55 ± 0.63	2.28 ± 0.08	51	0.35
Ras1p/Ras2p·GDP	19.0 ± 1.06	4.67 ± 0.12	25	0.25
Ras1p·GTP	7.80 ± 0.68	2.72 ± 0.10	42	0.35
Ras2p·GTP	1.09 ± 0.02	1.55 ± 0.05	75	1.42
Ha-Ras·GTP	20.0 ± 1.15	1.21 ± 0.03	96	0.06
Ras1p Δ-C·GTP	0.47 ± 0.07	3.0 ± 0.08	38	6.4
Ras2p Δ-C·GTP	1.54 ± 0.08	1.60 ± 0.02	72	1.03
Ras1p/Ras2p·GTP	7.80 ± 0.20	2.63 ± 0.21	44	0.34

Table 2: Comparison of the Effects of Various $cdc25^{GEF}$ on the Apparent dissociation rates of Ras1p·GDP and Ras2p·GDP Complexes

	10^2 k'_{-1} (min^{-1})			
complex	− GEF	+ C-Cdc25p	+ C-Sdc25p	+ C-$CDC25^{Mm}$
Ras2p·GDP	1.3 ± 0.02	13.4 ± 0.6 (10.3)	19.7 ± 0.9 (15.1)	9.1 ± 0.5 (7.0)
Ras1p·GDP	2.7± 0.03	9.6 ± 0.5 (3.6)	12.9 ± 0.7 (4.8)	11 ± 0.6 (4.0)

[a] The dissociation rates were measured as described in Materials and Methods with 200 nM Ras·[^{3}H]-GDP complexes in the presence of the C-terminal catalytic domain of Cdc25p (8 nM), or Sdc25p (70 nM) or mouse $CDC25^{Mm}$ (50 nM). Numbers in brackets indicate the stimulation fold.

Table 3: Comparison of the K_m and K_{cat} of Ras2p·GTP and Ras1p·GTP for Different GAP-like Catalytic Domains[a]

	Ras2p·GTP		Ras1p·GTP	
	k_m (μM)	k_{cat} (s^{-1})	k_m (μM)	k_{cat} (s^{-1})
Ira2p-383	11.5	0.34	7	0.08
GAP-334	6	1.2	11	2.5
NF1-414	0.4	0.16	0.18	0.08

[a] GTPase activity was carried out as described in Materials and methods after 5 min incubation at 25 °C in the presence of 30 nM Ira2p-383, or 4 nM GAP-334 or 4 nM NF1-414 in the presence of increasing concentrations of Rasp·[γ-^{33}P]GTP. K_m and K_{cat} were determined from double reciprocal plots. Error of measurements lies between 10 and 20%.

whereas this deletion had a drastic effect on Ras1p interaction, especially with respect to the association rate constants. This results in an affinity that is 2 orders of magnitude lower for GDP (13.5 vs 0.14 nM) and 18-fold lower for GTP (6.4 vs 0.35 nM). Fusion of the C-hypervariable domain of Ras2p to the Ras1p Δ-C domain, as in construct Ras1p/Ras2p, restores the kinetics of nucleotide interaction to the level of Ras1p. The substitution of the last 16 C-terminal residues of Ha-ras p21 by those of Ras2p appears not to affect the interaction with nucleotides since the determined association and dissociation rate constants are very similar to that previously obtained with cellular Ha-Ras encoded p21 (*32*, *33*).

In experiments not shown, we observed that in vitro farnesylation of the various Ras species did not affect the kinetics parameters of nucleotide interaction.

The intrinsic GTPase activity of Ras1p (1.5×10^{-4} s^{-1}) is comparable to Ras2p (1.1×10^{-4} s^{-1}) and identical to Ha-Ras (1.5×10^{-4} s^{-1}; ref *32*). Deletion of the hypervariable domain in Ras1p or Ras2p does not affect the GTPase activity (respectively 1.4×10^{-4} and 1.2×10^{-4} s^{-1}).

Stimulation of the Intrinsic Properties of Ras1p by GEFs and GAPs. Because the specific activities of the catalytic domains of the individual CDC25-family members (Cdc25p, Sdc25p, $CDC25^{Mm}$) are different (*34*), their concentration in the assay was adjusted to obtain a comparable stimulation of the intrinsic GDP-dissociation rates. Table 2 shows that the catalytic domain of all the three GEFs tested activate the dissociation of Ras1p·GDP. Although the order of GEF sensitivity for Ras1p and Ras2p seems comparable (C-Cdc25p being the strongest stimulator and C-$CDC25^{Mm}$ the weakest), the stimulating activity is 2−3 times lower on the Ras1p·GDP complex. As for Ras2p (*18*), we observed that in vitro farnesylation of Ras1p has no influence on the GEF catalytic domain-mediated GDP dissociation rate (not shown).

Table 3 shows that the K_m values of the GAP-stimulated GTPase activities of Ras1p and Ras2p are comparable for the various GAP catalytic domains. The K_m values of Ras1p·GTP for Ira2p and GAP catalytic domains are similar (7 and 11 μM, respectively), while that for NF1 catalytic domain is lower (0.18 μM). The K_m values of Ras1p·GTP for NF1 and GAP catalytic domains are similar to those obtained with Ha-Ras·GTP (*35*, *36*).

Similar to Ras2p·GTP, the k_{cat} value of GAP-334-stimulated Ras1p GTPase activity (2.5 s^{-1}) is higher than the values obtained in the presence of Ira2p-383 and NF1-414 (0.08 s^{-1}). Relative to the intrinsic GTPase rate constants of Ras1p (1.5×10^{-4} s^{-1}) and Ras2p (1.1×10^{-4} s^{-1}) under the same conditions, the catalytic domain of GAP can stimulate the GTPase of either Ras1p or Ras2p by 4 orders of magnitude, whereas the Ras1p GTPase and the Ras2p GTPase were stimulated 600 and 1400-fold respectively by NF1-414. Remarkably, the k_{cat} value of the stimulatory action of Ira2p-383 on Ras2p is 6-fold higher than that for Ras1p.

The GTPase of Ras1p Δ-C and Ras2p Δ-C were stimulated by Ira2p-383 to exactly the same extent as the respective full-length proteins. In vitro farnesylation of Ras2p or Ras1p has no effect on either intrinsic or GAP-catalytic domains-stimulated GTPase activities (not illustrated).

Importance of Ras1p Farnesylation for Adenylyl Cyclase Activation and for Guanine Nucleotide Exchange Activity

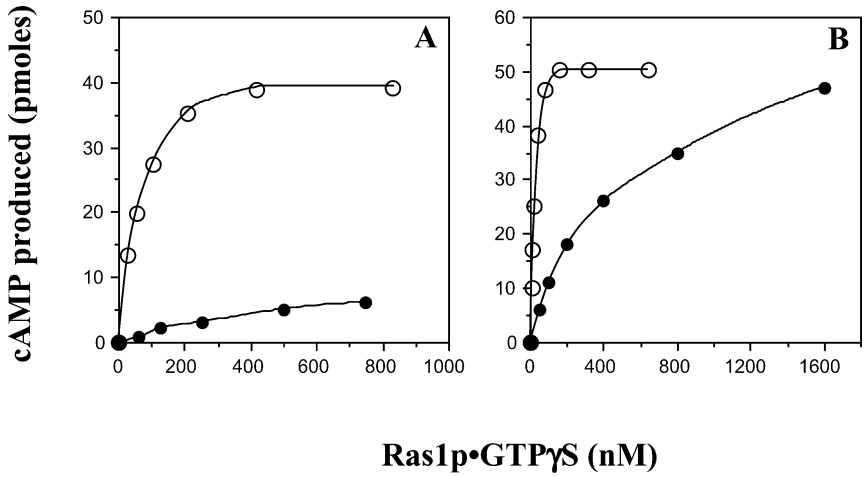

FIGURE 3: Importance of Ras1p farnesylation for activation of *CYR1* (A) or *CRI4* (B) adenylyl cyclase gene product. Activation of adenylyl cyclase *CYR1* (A) or *CRI4* (B) gene product were compared as function of increasing concentration of unprenylated (filled symbols) or prenylated (empty symbols) form of Ras1p complexed with GTPγS. Assay were performed as described in Materials and Methods using 3.5 μg of membranes from yeast strain TS1−6 overexpressing wild-type adenylyl cyclase (A) or 35 μg of membranes from yeast strain AAT3B (B). The background activity of membranes in the absence of Ras1p was subtracted. The results shown are the average of three independent experiments. Standard errors are expressed in the derived Table 4.

Table 4: K_a and V_{max} of *CRI4* and *CYR1* Gene Products for the Various Ras•GTP Species

	CRI4 gene product		*CYR1* gene product	
Ras•GTP species	K_a (nM)	V_{max} (pmol^{-1} min^{-1})	K_a (nM)	V_{max} (pmol^{-1} min^{-1})
Ras1p	400 ± 27	3 ± 0.28		
Ras2p	110 ± 7.5	4.4 ± 0.4		
farnesylated Ras1p	25.4 ± 2.3	3.13 ± 0.35	60 ± 1.0	2.42 ± 0.03
farnesylated Ras2p	6.5 ± 0.7	4.35 ± 0.50	26 ± 2.9	4.66 ± 0.47
farnesylated Ha-Ras	55 ± 6.2	1.85 ± 0.1	103.6 ± 8.5	1.16 ± 0.18
farnesylated Ras1p Δ-C	55.3 ± 6.0	1.8 ± 0.09	122 ± 10.5	1.27 ± 0.06
farnesylated Ras2p Δ-C	50.5 ± 5.0	1.94 ± 0.1	108 ± 9.6	1.5 ± 0.09
farnesylated Ras1p/Ras2p	19 ± 2.1	4.7 ± 0.6	32.5 ± 3.1	4.55 ± 0.4
farnesylated Ha-Ras/Ras1p	51 ± 5.0	2.9 ± 0.08	92 ± 8.0	2.37 ± 0.03

[a] Values were calculated from double-reciprocal plots. The K_a values represent the concentration of Ras•GTP product giving a half-maximal activity of adenylyl cyclase.

Mediated by Membrane-Bound Cdc25p and Sdc25p. Adenylyl cyclase activation by purified Ras1p•GTP was analyzed in a cell-free reconstituted system using membranes from AAT3B or TS1−6 yeast strains with disrupted *RAS1* and *RAS2* genes in the former (*37*) and with a disrupted *RAS1* and a thermo-sensitive *RAS2* gene in the latter (*38*). In the absence of functional *RAS* genes, adenylyl cyclase activity is undetectable and membrane preparations from these strains could be used as a convenient source of adenylyl cyclase for in vitro complementation assays with purified RAS products. Lethal phenotype caused by depletion of *RAS* genes is suppressed by overexpression of wild-type *CYR1* gene product using the high copy number vector pYACE1 (*39*) in the TS1−6 strain and by a *CRI4* mutation in the *CYR1* gene (T 1651 I) in the AAT3B strain. This mutation bypasses the requirement of *RAS1*, *RAS2*, and *CDC25* essential genes via the constitutive, low level production of cyclic AMP (*26*), and increases the RAS dependent adenylyl cyclase activity (*30*, *39*).

The Ras1p•GTPγS-dependent adenylyl cyclase activation was determined as a function of increasing concentrations of prenylated or unprenylated Ras1p•GTPγS complex. Figure 3A shows that prenylation of Ras1p markedly increased CYR1 adenylyl cyclase activation. Although less pronounced, the effect of farnesylation is also evident on CRI4 adenylyl cyclase activation (Figure 3B).

Taking the extent of activation as a measure for the productive interaction between Ras1p and adenylyl cyclase, we determined by double reciprocal plots the concentrations inducing half-maximum activation (K_a) and the corresponding V_{max} (See Table 4). We observed that farnesylation increases the affinity of Ras1p for CRI4 adenylyl cyclase 16 times (K_a values = 25 vs 400 nM for unprenylated Ras1p). This effect of prenylation is much more potent with the *CYR1* gene product. The K_a value of unprenylated Ras1p for CYR1 was too high to be determined, whereas that for farnesylated Ras1p was calculated to be 60 nM.

Taking advantage of the property of the *CRI4* mutation to bypass disruption of *RAS1*, *RAS2*, *CDC25* essential genes, we have used the AAT3B-Δ2R2H yeast strain (relevant genotype: *CRI4*, *ras1*$^-$, *ras2*$^-$, *cdc25*$^-$, *sdc25*$^-$, ref *18*) transformed with vectors overexpressing either full-length Cdc25p, Sdc25p, or a catalytic domain of Cdc25p encompassing residues 877−1589 (C-Cdc25p) to analyze the influence of prenylation of Ras1p on GEF activity.

Membranes preparations from these strains were used as a source of both adenylyl cyclase and GEF. Guanine nucleotide exchange activity was measured indirectly in a hybrid in vitro reconstituted system reproducing in vivo conditions in which the exchange factor is associated with the membranes. The activity of the membrane-bound GEF(s) on the Ras1p•GDP complex was determined indirectly using

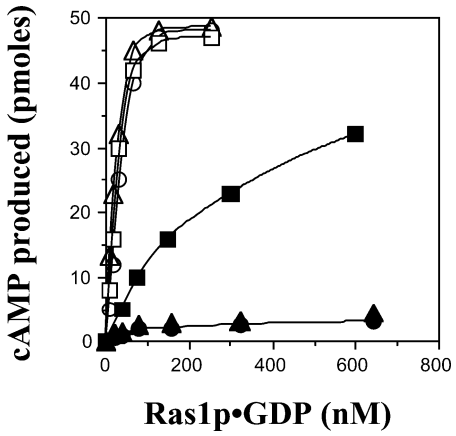

FIGURE 4: Requirement of Ras1p farnesylation for full-length membrane-bound Cdc25p and Sdc25p-dependent guanine nucleotide exchange activity. Adenylyl cyclase activity dependent on the regeneration of Ras1p·GTP complex mediated by yeast GEFs was analyzed as a function of increasing concentrations of farnesylated (empty symbols) and unfarnesylated (filled symbols) Ras1p·GDP complex in the presence of membrane from yeast strains AAT3BΔ2R2H overexpressing either full-length Cdc25p (▲, Δ), full-length Sdc25p (●, ○) or the catalytic domain of Cdc25p (residues 877−1589) (■, □) under the conditions described under Materials and Methods. The background activity of membranes in the absence of Ras1p was subtracted.

the extent to which the generated Ras1p·GTP complex could activate adenylyl cyclase as an indicator. This method has been successfully used in earlier works (*18*, *30*).

As observed in the saturation curves (Figure 4), farnesylated Ras1p can react with a similar efficiency with membrane-bound Cdc25p, Sdc25p or C-Cdc25p. Restoration of adenylyl cyclase activity mediated by membrane bound full-length Cdc25p or Sdc25p is strictly dependent on the farnesylation of Ras1p·GDP. In contrast, the membrane-associated C-Cdc25p lacking the N-terminal 876 residues stimulates GDP to GTP exchange activity on Ras1p even if unprenylated. These results, together with earlier data on Ras2p (*18*), indicate that farnesylation of yeast Ras is a common requirement for membrane-associated full-length GEF activation and suggests a role of the GEFs N-terminal moiety in the control of the interaction with farnesylated yeast Ras proteins.

Activation of Adenylyl Cyclase by Ras Proteins. The activation of wild-type *CYR1* and *CRI4* adenylyl cyclase was determined as a function of increasing concentrations of various Ras proteins. The kinetic parameters of Ras1p-mediated *CYR1* and *CRI4* activation show intermediate values when compared to Ras2p and Ha-Ras (Figure 5; Table 4).

Deletion of the hypervariable region in both yeast Ras proteins has a negative effect on the K_a and V_{max} values, the effect being more pronounced on Ras2p than on Ras1p (Table 4). Remarkably, the parameters of Ras1p Δ-C and Ras2p Δ-C are practically identical to those of Ha-Ras.

To evaluate the role of the hypervariable domain of Ras1p and Ras2p, we constructed the chimaeras Ras1p/Ras2p and Ha-Ras/Ras1p (Figure 1). As depicted in Figure 6, replacement of the hypervariable domain of Ras1p by that of Ras2p enhances the Ras1p activation potential to the level obtained with Ras2p. This replacement also leads to an increased affinity for both adenylyl cyclases (see Table 4 for K_a values). Similarly, fusion of the hypervariable domain of Ras1p to the N-terminal homologous moiety of Ha-Ras (1−173) leads to an increased V_{max} for the products of *CYR1* and *CRI4* to a level close to that of Ras1p. However, this fusion has little effect on the affinity for the two adenylyl cyclases (51−55 nM for CRI4 and 92−103 nM for CYR1).

Inhibition of Ras-Mediated Adenylyl Cyclase Activation by the Isolated Hypervariable Domain of Yeast Ras Proteins. To highlight the determinant role of this region, hypervariable domains of Ras1p and Ras2p were isolated, purified, and farnesylated in vitro (Figure 2). The effect of increasing concentrations of the hypervariable domain of Ras1p or Ras2p on adenylyl cyclase activation was determined using a fixed amount of prenylated Ras2p·GTPγS. Figure 7 shows that the hypervariable region of Ras2p can inhibit the Ras2p·GTPγS-dependent activation of adenylyl cyclase in a concentration-dependent manner, whereas no significant inhibition was obtained with hypervariable domain of Ras1p in the chosen concentration range.

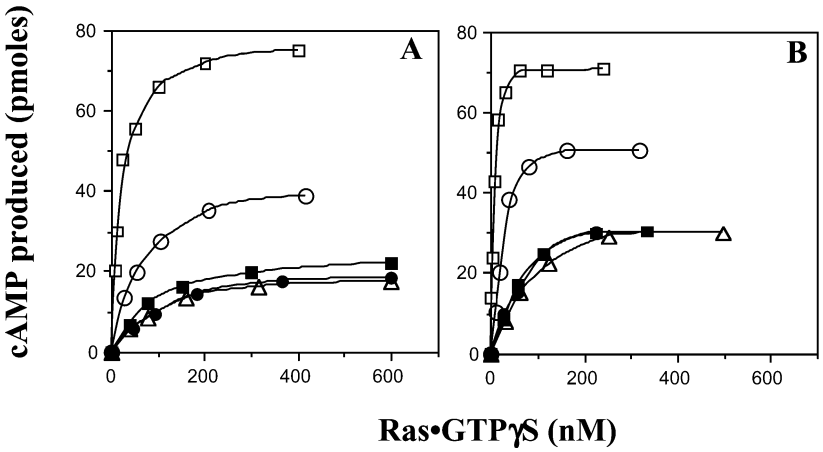

FIGURE 5: Comparative responses of *CYR1* (A) and *CRI4* (B) adenylyl cyclase to farnesylated Ras1p, Ras2p, and to their respective farnesylated C-terminal hypervariable truncated forms in complex with GTPγS. Activation of *CYR1* (A) and *CRI4* (B) gene product were compared as function of increasing concentrations of prenylated Ras1p (○), Ras2p (□), Ha-Ras (Δ), Ras1p Δ-C (●), Ras2p Δ-C (■), complexed with GTPγS. The assays were performed as described in legend to Figure 1 using 3.5 μg of membranes from yeast strain TS1−6 overexpressing wild-type adenylyl cyclase (A) or 35 μg of membranes from yeast strain AAT3B (B). Background activity of membranes in the absence of Ras was subtracted. The results shown are the average of three independent experiments; standard errors are expressed in the derived Table 4.

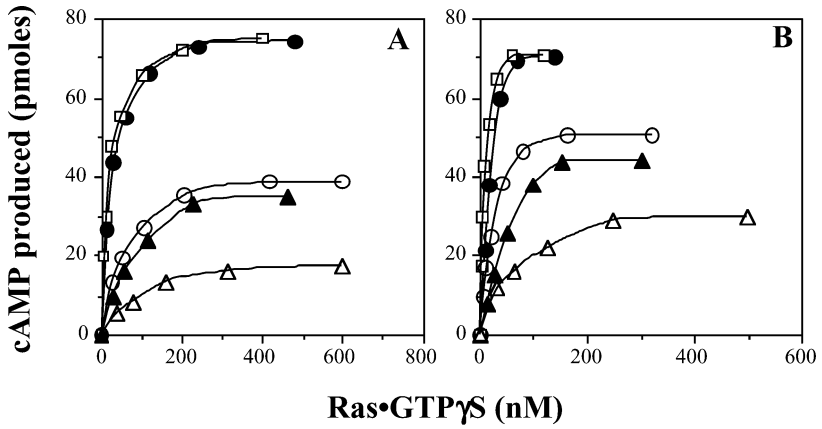

FIGURE 6: Role of hypervariable domain of Ras1p and Ras2p for activation of *CYR1* (A) and *CRI4* (B) adenylyl cyclase. Reconstituted adenylyl cyclase activity was determined as function of increasing concentrations of the various farnesylated Ras products in their active preformed GTPγS complex state: Ras2p (□), Ras1p (○), Ha-Ras (Δ), Ras1p/Ras2p (●), Ha-Ras/Ras1p(▲), in the presence 3.5 μg of membranes from yeast strain TS1−6 overexpressing wild-type adenylyl cyclase (A) or 35 μg of membranes from yeast strain AAT3B (B). Background activity of membranes in the absence of Ras was subtracted. The results shown are the average of three independent experiments; standard errors are expressed in the derived Table 4.

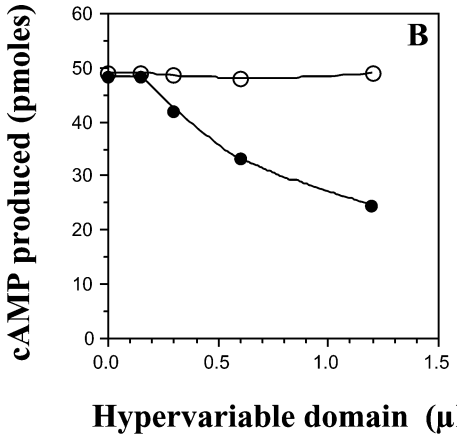

FIGURE 7: Specific inhibitory effect of Ras2p•GTPγS dependent adenylyl cyclase activation by increasing concentrations of isolated farnesylated hypervariable domain of RAS2p. cAMP production was determined in the presence or 35 μg of membranes from yeast strain AAT3B. Increasing concentrations of in vitro farnesylated hypervariable domain of Ras2p (●) or hypervariable domain of Ras1p (○) were preincubated for 10 min at 30 °C with membranes before adding a fixed-amount (3 pmol) of preformed farnesylated Ras2p•GTPγS. Reaction was started with nucleotides mixture as described in Materials and Methods. Background activity of membranes in the absence of Ras was subtracted.

DISCUSSION

Due to its instability and marked proteolytic activity phenomena during extraction and purification, Ras1p has not, to date, been obtained in a homogeneous, purified form. Consequently, in contrast to the well-characterized Ras2p (*18*, *23*−*25*, *31*), very little biochemical data concerning yeast Ras1p has been described. To overcome C-terminal proteolytic degradation and with the aim at obtaining full-length Ras1 protein, a Ras1 product was constructed in which the C-terminal 16 residues were replaced by those of Ras2p. This modification, together with the use of the pGEX expression system that allows fast protein purification by affinity chromatography, enabled the isolation of pure full-length Ras1 protein, allowing its biochemical characterization.

The interactions of Ras1p with the guanine nucleotides GDP and GTP showed 4−6-fold higher affinities as compared with those of Ras2p (Table 1), resulting primarily from increased association rate constants. This deviation was not expected since the nucleotide-binding site lies in the highly homologous N-terminal region of the Ras proteins. Even more surprisingly, deletion of the hypervariable region has a large effect on the nucleotide binding characteristics of Ras1p, but not of Ras2p. Changes in the association rate constants resulted also in 100- and 20-fold reductions in affinity of Ras1p for GDP and GTP, respectively. Remarkably, replacement of the hypervariable region of Ras1p by that of Ras2p did not lead to significant changes in nucleotide affinities of Ras1p. Apparently, the hypervariable region of either yeast Ras protein is crucial for a high nucleotide affinity of Ras1p, but not for Ras2p.

The intrinsic GTPase activity of Ras1p (9.2×10^{-3} min^{-1}) was very similar to that of Ras2p and Ha-ras. This is in agreement with the fact that the elements (Thr-35, Gln 61, Glu 63 in Ras, and Thr 42, Gln 68, Glu 70, respectively, in Ras1p and Ras2p) involved in the GTPase mechanism (*40*, *41*) are identical. In addition, the intrinsic hydrolysis rate of Ras1p and Ras2p was not affected by deletion of their C-terminal hypervariable regions.

Stimulation of the GTPase activity by catalytic domains of different RasGAPs resulted in deviating effects with Ras1p and Ras2p (Table 3). Whereas at most 2-fold differences could be observed in K_m and k_{cat} values for the human proteins GAP334 and NF1−414, the yeast protein Ira2p-383 appeared to stimulate the GTPase activity of Ras1p 6-fold less efficiently than that of Ras2p, but with similar K_m values. It is tempting to speculate about a putative specificity of the yeast Ira1 and Ira2 proteins for Ras1p and Ras2p, respectively. However, the isolation of Ira1p has yet to be achieved to continue this study.

In a standard GEF assay, Ras1p appears to be less sensitive than Ras2p to the in vitro action of the catalytic domains of three GEFs: yeast proteins C-Cdc25p, C-Sdc25p, and mammalian C-Cdc25^{Mm} (Table 2). Three elements of the Ras sequence are thought to be involved in the interaction with GEFs (*42*−*44*): switch I, switch II, and residues 108−112 in yeast Ras (corresponding to 101−105 in Ha-Ras). The switch I region, also called the effector loop, is identical in the yeast Ras proteins. In previous studies, we have shown the determinant role of distal switch II, in particular positions

81 and 82, of Ras2p for interaction with GEF (*24*, *25*). Interestingly, Ras1p and Ras2p differ solely at position 81 within this region (Thr and Asn, respectively) suggesting that this deviation is related to the difference in GEF-sensitivity. Alternatively, it may also be affected by position 108, which is a glutamine and leucine in Ras1p and Ras2p, respectively.

Farnesylation of Ras1p has no affect on the kinetics parameters of nucleotide interaction or the GDP dissociation rate mediated by the catalytic domains of yeast GEFs. However, in a reconstituted cell-free system with near-physiological conditions of interaction between Ras proteins and other membrane bound components, the activity of membrane associated full-length Cdc25p or Sdc25p was strictly dependent on farnesylation of Ras1p. In contrast to intact GEFs, farnesylation of Ras1p is not required for exchange activity dependence of membrane-associated C-Cdc25p. This observation is in agreement with the proposed function of the N-terminal domain of Cdc25p in controlling the interaction with farnesylated Ras2p (*18*). Farnesylation of Ras1p·GTP is critical for adenylyl cyclase activation (Figure 3), similar to Ras2p (*18*, *19*). The effect of farnesylation is more pronounced with wild-type CYR1 adenylyl cyclase than with CRI4 adenylyl cyclase whose mutation underlies a constitutive but low production of cAMP and induces an increased response to Ras·GTP (*26*, *39*). This intrinsic property of CRI4 adenylyl cyclase allowed us to demonstrate that farnesylation is not involved in increasing the Ras-dependent catalytic activity, but in increasing the affinity for adenylyl cyclase (Table 4). Prenylation enhances the affinity of Ras1p for CRI4 adenylyl cyclase almost 16 times, whereas the V_{max} values of the adenylyl cyclase activity mediated by farnesylated and unfarnesylated Ras1p are identical.

Comparison of the potential of activation of adenylyl cyclase by different farnesylated Ras products complexed with GTPγS shows that Ras1p-dependent activation is 2 times lower than that of Ras2p and 2 times higher than that of Ha-Ras. Differences in the efficiency of wild-type adenylyl cyclase activation between the various Ras proteins have already been observed in previous reports (*45*, *46*). The affinity of farnesylated Ras1p·GTPγS for both types of adenylyl cyclase is also intermediary between those of farnesylated Ras2p and farnesylated Ha-Ras·GTPγS complexes and is in the nanomolar range.

After deletion of their C-terminal hypervariable region, the GTPγS complexes of Ras2p and Ras1p display a comparable level of activation and a similar affinity for CRI4 or CYR1 adenylyl cyclase. Since these V_{max} and K_a values are also close to those of Ha-Ras, this strongly suggests that the extended hypervariable regions of Ras1p and Ras2p play a role in the activation of adenylyl cyclase.

To confirm the involvement of the hypervariable region of Ras1p and Ras 2p in the potency level of adenylyl cyclase activation, two chimeras were constructed: (i) a Ras1p/Ras2p construct composed of the homologous N-domain of Ras1p followed by the hypervariable domain of Ras2p, and (ii) a Ha-Ras/Ras1p construct containing in addition to the N-terminal homologous domain of Ha-Ras, the hypervariable domain of Ras1p. We observed that these farnesylated constructs in complex with GTPγS completely restored the maximal activation of Ras2p and Ras1p, respectively, for both wild type and CRI4 adenylyl cyclase. The substitution of the Ras1p hypervariable domain by that of Ras2p is also accompanied by an increased affinity for adenylyl cyclases.

The specific role of the hypervariable domain of Ras proteins and of Ras2p, in particular, as an element interacting with adenylyl cyclase was highlighted by the competitive inhibitory effect of the isolated and farnesylated hypervariable Ras2p domain on prenylated Ras2p·GTPγS-mediated adenylyl cyclase activation (Figure 7). Our results demonstrate thus that the extended hypervariable domain of yeast Ras, essentially the region encompassing residues 182−306 of Ras2p, is an element that, together with elements such as the effector region, its flanking residues, and the switch II region (*38*, *38*, *47*, *48*), is important for maximum activation of adenylyl cyclase.

In conclusion, the detailed biochemical characterization of yeast Ras1p protein in comparison to Ras2p and human Ha-Ras, has revealed many differences and a number of important features concerning the interaction with guanine nucleotides, positive and negative regulators and the effector adenylyl cyclase. Farnesylation of yeast Ras products was demonstrated to be essential for an increased interaction with adenylyl cyclase and for membrane-associated GEF responsiveness. Significantly, our results emphasize the involvement of the hypervariable region of yeast Ras proteins in inducing a suitable conformation for a productive interaction and activation of adenylyl cyclase.

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