Ras proteins from \textit{Saccharomyces cerevisiae} differ from mammalian Ha-Ras in their extended C-terminal hypervariable region. We have analyzed the function of this region and the effect of its farnesylation with respect to the action of the GDP/GTP exchange factors (GEFs) Cdc25p and Sdc25p and the target adenyl cyclase. Whereas Ras2p farnesylation had no effect on the interaction with purified GEFs from the Cdc25 family, this modification became a strict requirement for stimulation of the nucleotide exchange on Ras using reconstituted cell-free systems with GEFs bound to the cell membrane. Determination of GEF effects showed that in cell membrane the Cdc25p dependent activity on Ras2p was predominant over that of Sdc25p. In contrast to full-length GEFs, a membrane-bound C-terminal region containing the catalytic domain of Cdc25p was still able to react productively with unfarnesylated Ras2p. These results indicate that in membrane-bound full-length GEF the N-terminal moiety regulates the interaction between catalytic domain and farnesylated Ras2p-GDP. Differently from GEF, full activation of adenyl cyclase by the interaction with both full-length exchange factors Cdc25p and Sdc25p, even if this step of maturation was found to facilitate the interaction. The use of Ha-Ras/Ras2p chimaeras of different length emphasized the key role of the hypervariable region of Ras2p in inducing maximum activation of adenyl cyclase and for a productive interaction with membrane-bound GEF.

Ras proteins are GTPases cycling between the active GTP-bound state and the inactive GDP-bound state. They transmit extracellular signals that regulate cell growth and differentiation (1). The level of activated Ras is controlled by the GTPase-activating protein and the GDP/GTP exchange factor (GEF)\(^1\) which in the case of \textit{Saccharomyces cerevisiae} are Ira1p/Ira2p (2, 3) and Cdc25p, respectively (4). This organism harbors a second RasGEF (Sdc25p, Ref. 5) of unclear functions, that can complement Cdc25p (6–8). Ras1p and Ras2p regulate the activity of adenyl cyclase and cAMP-dependent protein kinases (9). One major difference between yeast and mammalian Ras proteins lies in their C-terminal hypervariable region which in the case of Ras from the former organism is much more extended (~120 versus ~20 aa residues). The function of this overextended C-terminal region is as yet unclear. Association with the cell membrane is an essential condition for the function of Ras proteins. Translocation of Ras to the inner surface of the membrane is promoted by sequential post-translational modifications of the C-terminal CAAX consensus box (10). The first step, the farnesylation of cysteine, is followed by proteolytic cleavage of the AAAX peptide, methyl-esterification of the exposed isoprenylated cysteine and in the case of human N-Ras, Ha-Ras, and \textit{S. cerevisiae} Ras1p and Ras2p, palmitoylation of one or two cysteines located upstream to the CAAX motif (11). After farnesylation, AAAX proteolysis and methylation, Ras proteins are still mainly cytosolic; their tight association with the plasma membrane requires palmitoylation (12–14) or for K-Ras a signal composed of a polybasic domain (14). In mammals, farnesylation was reported to be essential for the action of the ubiquitary exchange factor SOS (15); it targets Raf to the cell membrane (16–19) and is necessary for transformation (20). In yeast farnesylation of Ras2p was found to be important for the interaction with the adenyl cyclase-CAP complex (21, 22). Information on the role of farnesylation in the activity of yeast Cdc25p and Sdc25p is so far limited to the observation that the isolated catalytic domain of Cdc25p promoted the nucleotide exchange on prenylated Ras and even more strongly on unprocessed Ras (15).

In this work we have analyzed the role of the C-terminal hypervariable region of Ras proteins and its farnesylation in the interaction with both full-length exchange factors Cdc25p and Sdc25p, and in the activation of adenyl cyclase. As methodological approach, a well defined reconstituted \textit{in vitro} system using membrane preparations from isogenic yeast strains was utilized in order to mimic \textit{in vivo} conditions and compensate for the fact that the isolated full-length Cdc25p and Sdc25p are not yet available despite considerable efforts. In this context the activities of these two GEFs as components bound to the cell membrane were characterized and compared with the activity of membrane-bound GEF C-terminal region. The obtained results have further enlightened the regulatory role of the N-terminal region of GEF on the C-terminal catalytic domain and demonstrated the absolute requirement of Ras2p farnesylation for a productive interaction. The construction of Ha-Ras/Ras2p chimaeras has selectively defined the impor-
tance of the hypervariable region of yeast Ras for the activation of adenyl cyclase.

**EXPERIMENTAL PROCEDURES**

**Media, Plasmids, and Yeast Methods**—The standard rich medium used was YEPD (2% bacto-peptone, 1% yeast extract, and 2% dextrose). Selective synthetic media contained 0.67% yeast nitrogen base without amino acids (Difco) supplemented with all the auxotrophic requirements as described (23) and 2% dextrose or 3% glycerol or 2% raffinose as carbon source to a cell density of 0.15 × 10^6/ml. Yeast strains transformed by these plasmids were grown at 30 °C in farnesylation buffer with 0.5 mM Fpp (Isotopchim) and in the presence of a protease inhibitors mixture (2 mM Pefablock S-C, Roche Molecular Biochemicals). Level of farnesylation of the various Ras proteins using [3H]Fpp as substrate was analyzed by electrophoresis on 1% agarose gels and radioautography. For yeast transformation with DNA fragments of the same method used for Ras1p. Mono-Q HR5/5 chromatography (FPLC system, Amersham Pharmacia Biotech) with a linear 20–230 mM KCl gradient (50 ml in 25 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 7 mM β-mercaptoethanol, and 10 μM GDP) allowed the separation of full-length protein products of the C-terminal truncated forms of Ras2p. The C-terminal catalytic domain of Sdc25p (Sdc25p, 550 aa), Cdc25p (C-Cdc25p, 509 aa), CDC25K (CDC25Km, 285 aa) were purified as described (36) and the full-length CDC25Km (1262 aa) was obtained as N-terminal fusion with the maltose-binding protein (38).

**Functions of the Hypervariable Region of Ras2p**

**Preparation of Farnesyl-Protein Transferase—**E. coli JM101 containing the plasmid pG14-2/12 (39) was used to express the coupled S. cerevisiae RAM1/RAM2 gene products encoding farnesyl transferase (FTase). The transformed strain was grown at 24 °C in 4 liters of LB-rich medium with 50 μg ml⁻¹ ampicillin and induced at a cell density of 0.5 A₅₅₀ with 0.1 mM isopropyl-β-D-thiogalactopyranoside. After 12–15 h, the cells were collected by centrifugation, washed, and sonicated four times for 30 s at 4 °C in 100 ml of buffer A (25 mM Tris-HCl, pH 7.8, 10 μM ZnCl₂, 1 mM MgCl₂, 1 mM dithioreitol) containing 80 mM NaCl, 0.25 mM CaCl₂, 5 mM dithiothreitol (Roche Molecular Biochemicals), and protease inhibitors (2 mM Pefablock S-C, 1.7 μg ml⁻¹ pepstatin, 2.5 μg ml⁻¹ aprotinin, 1 μg ml⁻¹ leupeptin; Roche Molecular Biochemicals). Supernatant from 2 h centrifugation at 140,000 × g was loaded on a HiPrep 16/10 Source 30Q column (Amersham Pharmacia Biotech). The most active fractions eluted between 180 and 250 mM NaCl in buffer A were applied to an immobilized chymotryptic gel chromatography system carrying the heparin-agarose TSK (corresponding to the C-terminal residues of Ras-B and purified (40). The collected active eluted fractions (~50% pure) were concentrated by ultrafiltration and stored in buffer A with 50% glycerol at −20 °C.

**Preparation of in Vitro Farnesylated Ras—**FTase activity was tested by measuring the amount of [3H]farnesyl moieties transferred from [3H]farnesyl pyrophosphate (Fpp, Isotopichrom) to intact purified Ras products. The standard reaction mixture contained in 50 mM Tris-HCl, pH 7.8, 10 μM ZnCl₂, 2.5 mM MgCl₂, 200 nM farnesylated or unfarnesylated preformed Ras proteins, and 70 μM [3H]Fpp (specific activity: 694 MBq mmol⁻¹). The reaction was started with 0.3–1 μl purified FTase. After 45 min at 30 °C, an aliquot was applied to glass fiber filter (MFS, Advace GASS) and the reaction stopped in 1 M HCl/ethanol at 0 °C. Filters were washed 4 times with cold ethanol, washed, and counted in a Wallac 1410 liquid scintillation spectrometer. The blank values obtained without Ras were subtracted.

**Preparation of Protein**—For FTase activity testing, the amount of [3H]farnesyl moieties transferred from [3H]farnesyl pyrophosphate (Fpp, Isotopichrom) to intact purified Ras products. The standard reaction mixture contained in 50 mM Tris-HCl, pH 7.8, 10 μM ZnCl₂, 2.5 mM MgCl₂, 0.25 mM CaCl₂, 5 mM dithiothreitol (farnesylation buffer), 10 μM GDP, 5 μM Ras proteins, and 70 μM [3H]Fpp (specific activity: 694 MBq mmol⁻¹). The reaction was started with 0.3–1 μl purified FTase. After 45 min at 30 °C, an aliquot was applied to glass fiber filter (MFS, Advace GASS) and the reaction stopped in 1 M HCl/ethanol at 0 °C. Filters were washed 4 times with cold ethanol, washed, and counted in a Wallac 1410 liquid scintillation spectrometer. The blank values obtained without Ras were subtracted.

**Preparation in Vitro Farnesylated Ras—**GDP, GTP, or GTP-S-bound Ras products (4 μM) were farnesylated by a 45-min incubation at 30 °C in farnesylase buffer with 0.5 μM purified FTase, 100 μM cold Fpp (Isotopichrom) and in the presence of a protease inhibitors mixture (2 μg ml⁻¹ aprotinin, 1 μg ml⁻¹ leupeptin, 60 μg ml⁻¹ antipain, 2 mM Pefablock S-C, Roche Molecular Biochemicals). Level of farnesylation of the various Ras products using [3H]Fpp as substrate was analyzed by electrophoresis on NuPAGE N membrane (Schleicher & Schuell) from a high sodium bisulfite PAGE gel (33). For yeast transformation with DNA fragments of the same method used for Ras1p. Mono-Q HR5/5 chromatography (FPLC system, Amersham Pharmacia Biotech) with a linear 20–230 mM KCl gradient (50 ml in 25 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 7 mM β-mercaptoethanol, and 10 μM GDP) allowed the separation of full-length protein products of the C-terminal truncated forms of Ras2p. The C-terminal catalytic domain of Sdc25p (Sdc25p, 550 aa), Cdc25p (C-Cdc25p, 509 aa), CDC25K (CDC25Km, 285 aa) were purified as described (36) and the full-length CDC25Km (1262 aa) was obtained as N-terminal fusion with the maltose-binding protein (38).

**Preparation of Farnesyl-Protein Transferase—**E. coli JM101 containing the plasmid pG14-2/12 (39) was used to express the coupled S. cerevisiae RAM1/RAM2 gene products encoding farnesyl transferase (FTase). The transformed strain was grown at 24 °C in 4 liters of LB-rich medium with 50 μg ml⁻¹ ampicillin and induced at a cell density of 0.5 A₅₅₀ with 0.1 mM isopropyl-β-D-thiogalactopyranoside. After 12–15 h, the cells were collected by centrifugation, washed, and sonicated four times for 30 s at 4 °C in 100 ml of buffer A (25 mM Tris-HCl, pH 7.8, 10 μM ZnCl₂, 1 mM MgCl₂, 1 mM dithioreitol) containing 80 mM NaCl, 0.25 mM CaCl₂, 5 mM dithiothreitol (Roche Molecular Biochemicals), and protease inhibitors (2 mM Pefablock S-C, 1.7 μg ml⁻¹ pepstatin, 2.5 μg ml⁻¹ aprotinin, 1 μg ml⁻¹ leupeptin; Roche Molecular Biochemicals). Supernatant from 2 h centrifugation at 140,000 × g was loaded on a HiPrep 16/10 Source 30Q column (Amersham Pharmacia Biotech). The most active fractions eluted between 180 and 250 mM NaCl in buffer A were applied to an immobilized chymotryptic gel chromatography system carrying the heparin-agarose TSK (corresponding to the C-terminal residues of Ras-B and purified (40). The collected active eluted fractions (~50% pure) were concentrated by ultrafiltration and stored in buffer A with 50% glycerol at −20 °C.

**Farnesyl-Protein Transferase Assay—**FTase activity was tested by measuring the amount of [3H]farnesyl moieties transferred from [3H]farnesyl pyrophosphate (Fpp, Isotopichrom) to intact purified Ras products. The standard reaction mixture contained in 50 mM Tris-HCl, pH 7.8, 10 μM ZnCl₂, 2.5 mM MgCl₂, 0.25 mM CaCl₂, 5 mM dithiothreitol (farnesylation buffer), 10 μM GDP, 5 μM Ras proteins, and 70 μM [3H]Fpp (specific activity: 694 MBq mmol⁻¹). The reaction was started with 0.3–1 μl purified FTase. After 45 min at 30 °C, an aliquot was applied to glass fiber filter (MFS, Advace GASS) and the reaction stopped in 1 M HCl/ethanol at 0 °C. Filters were washed 4 times with cold ethanol, washed, and counted in a Wallac 1410 liquid scintillation spectrometer. The blank values obtained without Ras were subtracted.
Functions of the Hypervariable Region of Ras2p

FIG. 1. Diagram of the various Ras constructs. The solid bars indicate regions originating from Ha-Ras and the open bars refer to those from Ras2p.

washed twice with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 100 mM NH₄Cl, 10 mM MgCl₂, 7 mM β-mercaptoethanol. The filters were then counted for radioactivity.

Adenyl Cyclase Assay—The adenyl cyclase assay was carried out as described (41). Yeast membranes were prepared from cells grown in their respective selective medium (36) and collected at a cell density of 1.5 × 10⁶ units/mL as used as source of membrane-associated adenyl cyclase and GEF (Cdc25p, Sdc25p, or both factors). The CAMP production was determined after 18 min at 30 °C at which time 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 GTPyS complex, contained either 30–40 μg of membrane preparation for the yeast strains expressing the CRI4-adenyl cyclase gene or 3.5 μg of membrane preparation for the yeast strain TS1-6 which harbors an overexpression vector for the wild-type adenyl cyclase gene (CYR1). These amounts of membranes in the assay gave similar levels of Ras-uncoupled adenyl cyclase activity as determined in the presence of 1.5 mM MnCl₂. Preformed Ras-GDP, GTP, or GTPyS complexes were obtained in the presence of 0.5 mM of the corresponding unlabelled guanine nucleotide and farnesylated or unfarnesylated Ras proteins in their preformed states. Unprenylated complexes were treated identically but omitting the FTase. The reaction was started with a mixture containing 50 mM MES, pH 6.2, 5 mM MgCl₂, GTP, or GTPyS (0.5 mM), cAMP (0.5 mM), [γ-³²P]ATP (0.3 mM, 5 GBq mmol⁻¹), theophylline, creatine phosphate, and creatine kinase.

Other Methods—Protein concentration were measured by the Bio-Rad (Bio-Rad) with the indicated molecular mass (kDa). SDS-PAGE was carried out using a 12.5% acrylamide separating gel. DNA probes were ³²P-labeled with the Megaprime DNA labeling system from Amersham Pharmacia Biotech.

RESULTS

Properties of the Biological Components—The properties of the biological components used in this work were carefully characterized. The experiments involving yeast membranes were carried out with different membrane preparations in order to assure reproducibility of the results. Fig. 1 illustrates the forms and constructs of Ras used for our experiments. They were at least 90% pure on Coomassie Blue-stained SDS-PAGE (Fig. 2A) and stable for at least several months when kept at 20 °C in storage buffer (36). It is important to emphasize that all purified Ras species displayed a molar stoichiometric GDP/GTP yS complex, contained either 30–40 μg of membrane preparation for the yeast strains expressing the CRI4-adenyl cyclase (CRI4-adenyl cyclase) gene or 3.5 μg of membrane preparation for the yeast strain TS1-6 which harbors an overexpression vector for the wild-type adenyl cyclase gene (CYR1). These amounts of membranes in the assay gave similar levels of Ras-uncoupled adenyl cyclase activity as determined in the presence of 1.5 mM MnCl₂. Preformed Ras-GDP, GTP, or GTPyS complexes were obtained in the presence of 0.5 mM of the corresponding unlabelled guanine nucleotide and farnesylated or unfarnesylated Ras proteins in their preformed states. Unprenylated complexes were treated identically but omitting the FTase. The reaction was started with a mixture containing 50 mM MES, pH 6.2, 5 mM MgCl₂, GTP, or GTPyS (0.5 mM), cAMP (0.5 mM), [γ-³²P]ATP (0.3 mM, 5 GBq mmol⁻¹), theophylline, creatine phosphate, and creatine kinase.

At first, we examined whether in vitro farnesylation of Ras2p affected the intrinsic interaction with GDP and the GDP dissociation rate mediated by Cdc25p and Sdc25p catalytic domains but no effect was found (Table II), differently from the observations of other authors using the catalytic domain of Cdc25p (15). Because the specific activities of these various GEF catalytic domains are different (43), their concentration in the assays was adjusted to give a comparable stimulation on the Ras2p-GDP dissociation rate. Full-length Cdc25p and Sdc25p could not be tested because their isolation has as yet to be achieved. However, differently from a report on full-length SOS (15) we were unable to see any enhancement by farnesylation of the activity of full-length, purified mouse CDC25M isoform of its catalytic domain C-CDC25M (Table II).

Farnesylation of Ras2p Is Strictly Required for the Nucleotide Exchange Activity Mediated by Membrane-bound Cdc25p or Sdc25p—The complete disruption of both RAS1 and RAS2 genes or of the CDC25 gene is lethal (6, 44, 45). However, introduction of the CRI4 mutation (T1651I, Ref. 46) into adenyl cyclase gene bypasses the requirement for both RAS and CDC25 genes via the constitutive production of low levels of CAMP. CRIH-adenyl cyclase activity is still strongly stimulated by Ras-GTP proteins in vivo and in vitro (26, 46). To take advantage of these properties, we have used a set of isogenic yeast strains in a CRIH and rasI, ras2 background. Starting from strain AAT3B, strains were constructed, in which CDC25 (AAT3B-Δ1), SDC25 (AAT3B-ΔS25) or both these genes (AAT3B-Δ2) were disrupted. It is essential to stress that measurement of Ras nucleotide exchange dependent on membrane-bound Cdc25p or Sdc25p cannot be directly determined by the classical methods on nitrocellulose or gel filtration, as a likely consequence of the inherent properties of the association between exchange factors and cell membrane. For this reason, in most our experiments the exchange activity on Ras was followed indirectly in a reconstituted adenyl cyclase assay. The validity of this method was proved in previous work (41). Membrane preparations from AAT3B, AAT3B-Δ1, AAT3B-ΔS25,
TABLE II

In vitro farnesylation of Ras2p does not influence Cdc25<sup>gef</sup>-dependent GDP dissociation rate

The reaction was performed as described under * Experimental Procedures* with 6 nM C-Cdc25p, 48 nM C-Sdc25p, 90 nM C-CDC25<sub>1877–1927</sub>, and 230 nM full-length CDC25<sub>1877–1927</sub>, and was started with 200 nM farnesylated or unprenylated Ras2p<sup>1927–2022</sup>/H[GDP] complex.

| Ras2p | Farnesylated Ras2p |
|-------|-------------------|
| GEF   | 1.83 ± 0.16        |
| GEF + C-Cdc25p (500 aa) | 12.8 ± 0.6       |
| GEF + C-Sdc25p (550 aa) | 18.5 ± 0.7       |
| GEF + C-CDC25<sub>1877–1927</sub> (285 aa) | 24.0 ± 0.8       |
| GEF + Full-length CDC25<sub>1877–1927</sub> | 4.6 ± 0.14       |

FIG. 3. Dependence on farnesylation of Ras2p for exchange activity promoted by membrane-associated GEFs (A) and its effect on adenylyl cyclase interaction (B). Adenylyl cyclase activity was measured as a function of increasing concentrations of purified full-length Ras2p-GDP (A) or of preformed Ras2p-GTP<sup>S</sup> complex (B) as unprenylated (filled symbols) or farnesylated form (open symbols) in the presence of membranes from strain AAT3B containing both Cdc25p and Sdc25p, AAT3B-ΔS25 with only Cdc25p, AAT3B-ΔC with only Sdc25p, AAT3B-ΔS and AAT3B-Δ2 lacking both GEFs, respectively. The background activity of membranes in the absence of Ras2p was subtracted. Data are mean ± S.E. of values from four independent experiments using different preparations for each yeast strain. Error bars smaller than the symbols are not shown. Standard deviations of panel B are expressed in Table III.

and AAT3B-Δ2 were used as a source of adenylyl cyclase in combination with either Cdc25p or Sdc25p, or both GEFs for *in vitro* assays in which purified intact Ras2p was added exogenously. This hybrid *in vitro* system reproduces in *vivo* conditions in which Cdc25p or Sdc25p are anchored to the membrane. We could so analyze selectively the effect of the membrane-associated GEF(s) on increasing concentrations of unfarnesylated or farnesylated Ras2p-GDP via the extent to which the generated Ras-GTP could activate adenylyl cyclase.

Fig. 3A confirms that unfarnesylated Ras2p-GDP was unable to activate adenylyl cyclase whatever membrane preparation was used and shows that restoration of adenylyl cyclase is strictly dependent on farnesylation of Ras2p-GDP. The stimulation was slightly reduced when only Cdc25p or Sdc25p, or both GEFs for *in vitro* assays in which Cdc25p or Sdc25p are anchored to the membrane. We could so analyze selectively the effect of the membrane-associated GEF(s) on increasing concentrations of unfarnesylated or farnesylated Ras2p-GDP via the extent to which the generated Ras-GTP could activate adenylyl cyclase.

Differently from Intact Yeast GEF, Farnesylation of Ras2p Is Not Required for the Exchange Activity Dependent on Membrane-coupled C-terminal Region of Cdc25p—For a more detailed investigation of the specific effects of Ras2p farnesylation on the response to GEF, we have transformed a yeast strain depleted of genomic CDC25 and SDC25 with pyEDP1/82, pFC1, or pNID25–1 overexpressing full-length Cdc25p and Sdc25p, and the C-terminal region of the former (C-Cdc25p 877–1589), respectively. Figs. 4, A and B, confirm the strict dependence on farnesylation of Ras2p-GDP for the regeneration of the active complex mediated by overexpressed membrane-associated Cdc25p or Sdc25p. In both conditions, saturation curves were similar showing that farnesylated Ras2p can react with the same efficiency with either exchange factor.

Differently from membrane-bound intact GEF, farnesylation is not required for the exchange activity if a membrane-associated GEF lacking the N-terminal moiety (C-Cdc25p 877–1589) is used (Fig. 4C). In this case, Ras2p-GDP can rapidly be converted to its activated form even if unprenylated. With prenylated Ras2p, the saturation curve was similar to that observed with membrane-bound full-length Cdc25p. In conclusion, the requirement of Ras2p farnesylation for activation by membrane-associated intact GEF appears not to be the consequence of membrane targeting of Ras2p, since the C-terminal region of Cdc25p can react to the same extent with unfarnesylated or farnesylated form of Ras2p. The dependence on farnesylation is a selective property inherent in the membrane-associated full-length Cdc25p, indicating that the N-terminal moiety specifically controls the interaction with farnesylated Ras2p. The same is probably valid also for Sdc25p.
functions of the hypervariable region of Ras2p

**Fig. 4.** Farnesylation of Ras2p is a specific requirement for full-length Cdc25p and Sdc25p but not for the catalytic domain of Cdc25p-dependent exchange activity. Adenylyl cyclase activity dependent on the regeneration of Ras2p-GTP complex mediated by yeast GEFs was analyzed as a function of increasing concentrations of farnesylated (□) and unprenylated (○) Ras2p-GDP complex in the presence of membranes from strain AAT3B-Δ2R2F1 expressing either full-length Cdc25p (A), full-length Sdc25p (B), or the catalytic domain of Cdc25p (C) under the conditions described under "Experimental Procedures." The background activity of membranes in the absence of Ras2p was subtracted. Data are mean ± S.E. of values from three independent experiments using different membrane preparations for each yeast strain. **Errors bars** smaller than symbols are not shown.

**Fig. 5.** Comparative responses of CR4 (A) and CYR1 (B) adenylyl cyclase gene products to Ha-Ras-GTP-S complexes. Activation of adenylyl cyclase CR4 (A) and CYR1 (B) gene product were compared as a function of increasing concentration of unprenylated (filled symbols) or prenylated (empty symbols) form of Ras2p (■, ○), Ha-Ras (Ha-Ras 1–173/Ras2p 307–322) (●, ○) complexed with GTP-S. The assays were performed as described in the legend to Fig. 3 using 30–35 μg of membranes from indifferently strain AAT3B or AAT3B-Δ2 (A) or 3.5 μg of membrane from yeast strain TS1–6 overexpressing wild-type adenylyl cyclase (B). The background activity of membranes in the absence of Ras2p was subtracted. The results shown are the average of three independent experiments. Standard errors are expressed in the derived Table III.

produced $K_a$ values were 26 and 100 nM, respectively (Table III). The ability of unprenylated Ras2p to interact with CYR1 gene product was very low ($K_a > 800$ nM, Table III), unprenylated Ha-Ras 1–173/Ras2p 307–322 being nearly unable to induce any cAMP production.

The Hypervariable Region of Ras2p Is Required for Full Adenylyl Cyclase Activation while Its Prenylation Increases the Efficiency of the Interaction—The primary structures of Ha-Ras and Ras2p display domains with differing homology. The N-terminal domains (residues: 1–51 in Ha-Ras and 8–88 in Ras2p) show a 90% homology, the middle domains (82–173 in Ha-Ras and 89–181 in Ras2p) a 50% homology, whereas no similarity exists in the C-terminal regions except for the CAAX box (47). Having observed that Ras2p is a better activator of adenylyl cyclase than Ha-Ras, we tried to determine what region of the C-terminal moiety of Ras2p was involved in this effect. For this, we constructed two chimaeras: Ha-Ras 1–81/Ras2p 89–322 containing in addition to the N-terminal domain of Ha-Ras the Ras2p middle domain (50% homology with Ha-Ras) and its hypervariable C-terminal region, and Ha-Ras 1–173/Ras2p 182–322 containing only the hypervariable region of Ras2p.

These two constructs, which binds stoichiometrically GDP and GTP and are fully farnesylated, showed the same intrinsic dissociation rate values for GDP (Table IV), i.e. slower than that of Ras2p ($1.1 \times 10^{-2}$ min$^{-1}$ versus $1.83 \times 10^{-2}$ min$^{-1}$) and close to that of Ha-Ras p21; also their response to C-Cdc25p (509 aa) being similar. Therefore, these two constructs conserved the inherent properties of Ha-Ras. Concerning the cAMP production as a function of unprenylated Ras-GTP-S concentrations, Fig. 6 shows that the presence of the two C-terminal Ras2p domains can both increase $V_{max}$ and affinity of Ha-Ras for CR4 adenylyl cyclase to the levels observed with Ras2p (Table III).

Experiments were carried out to compare the profiles of activation of CR4 (Fig. 7, panel A) and CYR1 (Fig. 7, panel B) gene products with increasing concentrations of the various prenylated Ras forms. With both farnesylated chimaerases, the saturation levels were comparable to that with farnesylated Ras2p. This result emphasizes the role of the extended C-terminal region of Ras2p for maximum activation of adenylyl cyclase. The extent of activation is particularly evident with the CYR1 product, since the two fused domains of Ras2p increased by 5–6 times the value of $V_{max}$ observed with farnesylated Ha-Ras. The affinity constants estimated from inverse plots of these experiments are summarized in Table III. Farnesylated Ha-Ras/Ras2p chimaeras show affinity constants identical to those of farnesylated intact Ras2p for the CR4 (7–8 nM) and CYR1 gene (26–38 nM) products. Comparison of the $V_{max}$ and $K_a$ values of prenylated and unprenylated Ras strongly suggests that the C-terminal region of Ras2p encompassing residues 173–307 includes specific structures important for the activation of adenylyl cyclase, whereas farnesylation is mainly involved in promoting the efficiency of the interaction.

Elements Determining the Sensitivity of Ras to the Membrane-associated Catalytic Domain and Full-length Cdc25p—We have also analyzed the sensitivity of Ha-Ras/Ras2p chimaeras to membrane-bound Cdc25p or C-Cdc25p 877–1589 using the adenylyl cyclase reconstitution assay. Increasing concentrations of the different Ras-GDP constructs were used in their unprenylated and prenylated form. Fig. 8A shows that full-length Cdc25p can fulfill its exchange activity...
not only on Ras2p but also on the various Ha-Ras/Ras2p chimaeras with high efficiency, provided that these products were farnesylated. Differently from unprenylated Ras2p, unprenylated Ha-Ras 1–173/Ras2p 307–322 could not be farnesylated. Membranes from either strain AAT3B or AAT3B-SDC25 were used to reconstitute adenylyl cyclase activity. For standard errors, see the derived Table III. The results show that the background activity of membranes in the absence of Ras2p was subtracted. The results shown are the average of three independent experiments. Standard errors are expressed in the derived Table III.

FIG. 6. Specific involvement of the C-terminal region of Ras2p for maximal adenylyl cyclase activation. Adenylyl cyclase activation was measured as a function of increasing concentrations of GTP·S complexes of farnesylated Ras2p (○), Ha-Ras (Ha-Ras 1–173/Ras2p 307–322) (●), Ha-Ras 1–81/Ras2p 89–322 ( ), and Ha-Ras 1–173/ Ras2p 182–322 (▲) as described in the legend to Fig. 3. The results represent three independent experiments performed in the presence of membranes from either strain AAT3B or AAT3B-SDC25 as source of the CHY gene product. For the first time, it was possible to define the extent of cell-membrane exchange activity dependent on Cdc25p or Sdc25p interaction by bypassing the lethality of the deletions. In this system the activity of membrane-associated full-length GEFs was strictly dependent on farnesylation of Ras2p and could be attributed to Sdc25p. For the first time, it was possible to define the extent of cell-membrane exchange activity dependent on Sdc25p acting as a second yeast GEF. Genetic analysis has shown that the SDC25 gene can functionally complement a cde25 mutation and that the SDC25 and CDC25 genes are differentially transcribed, the former being expressed late during growth (8). The same Km and Vmax of membranes with over-
puriﬁed C-Cdc25p can exert a GDP dissociating activity on Ras2p 89–322 (Ras2p). This demonstrates the essential role of this region after fusion with the hypervariable region of Ras2p (residues 182–322). This strategy was practically unable to stimulate adenylyl cyclase and only its farnesylation enabled some stimulation. Even with farnesylated Ha-Ras maximum activation of adenylyl cyclase was much lower than with Ras2p, an effect that was more pronounced with wild-type adenylyl cyclase than with its CRI4 mutant. However, farnesylated Ha-Ras showed a higher afﬁnity for both CRI4 and CYR1 gene products, almost as high as that of farnesylated Ras2p. To clarify the reasons for the functional differences, we extended the observations that an activated Ha-Ras Val-12/Ras2p chimaera containing the ﬁrst 73 amino acids of Ha-Ras Val-12 stimulates adenylyl cyclase more efﬁciently than Ha-Ras (53) for this purpose, we constructed two N-terminal Ha-Ras/C-ter Ras2p chimaeras including C-terminal regions of Ras2p of different length (Ha-Ras 1–81/Ras2p 89–322 and Ha-Ras 1–173/Ras2p 182–322) comprising the extended hypervariable region of Ras2p. Both constructs revealed the same proﬁle of adenylyl cyclase activation as Ras2p, thereby deﬁning the hypervariable domain as an additional important element for full reconstitution of the activity. Other major determinants for the interaction with adenylyl cyclase are the effector region (residues 32–40 in Ha-Ras and 39–47 in Ras2p) (31, 55–59), its flanking residues and the switch 2 region (31, 58). By means of the CRI4-encoded product, which is highly sensitive to Ras2p even if unprenylated, we showed that maximum stimulation of adenylyl cyclase by prenylated Ras2p or Ha-Ras/Ras2p chimaeras is identical to that obtained with prenylated Ras2p. This emphasizes the crucial role of the hypervariable region for maximum activation and shows that the major effect of farnesylation is to increase the afﬁnity between Ras2p and adenylyl cyclase rather than to stimulate adenylyl cyclase activity.

Even if there is still some uncertainty about a direct interaction between Ras proteins and adenylyl cyclase, due to the need of the adenylyl cyclase tightly associated protein CAP (60, 61) for a proper response to post-translationally modiﬁed Ha-Ras (22), it is possible that the basic nature of the C-terminal region of Ras2p could favor an efﬁcient interaction with the leucine repeat-rich region following the N-terminal region of adenylyl cyclase. This interaction could induce a suitable conformation for maximum activation of the catalytic domain. Leucine-rich repeat regions (61) are critical in mediating protein-protein interactions. In fact, it is known that farnesylation is not sufﬁcient for a stable anchoring of Ras to the plasma membrane, palmitoylation of the upstream cysteine being required (12–14). Our observations are suggestive for a function of the N-terminal domain regulating the activity of the catalytic domain as proposed in Ref. 49. Farnesylation of Ras could induce a topological orientation allowing the accessibility to the catalytic domain of Cdc25p, where the region 1374–1444 is essential for this interaction (50). Evidence in vitro and in vivo has indicated that the N-terminal moiety of mammalian Ras-GEFs CDC25Mm (38) and SOS (51) down-regulates the activity of the catalytic domain, despite a modular organization fully different from the N-terminal region of Cdc25p or Sdc25p.

Another relevant aspect of this work is the effect of Ha-Ras farnesylation on the response to GEF and adenylyl cyclase. As for Ras2p, farnesylation of Ha-Ras is required for a response to membrane-associated Cdc25p, but differently from Ras2p, unprenylated Ha-Ras is insensitive to membrane-associated C-Cdc25p 877–1589 and becomes as responsive as Ras2p only after fusion with the hypervariable region of Ras2p (residues 182–322). This demonstrates the essential role of this region for a productive interaction with the C-terminal domain of Cdc25p and disagrees with a suggested negative regulatory role of the C-terminal portion of Ras2p, which was proposed from the observation that Ras proteins lacking the C-terminal domain can bypass cdc25 mutations (52). We observed that puriﬁed C-Cdc25p can exert a GDP dissociating activity on Ha-Ras or Ha-Ras 1–173/Ras2p 307–322 even better than on Ras2p. These results show that with membrane-associated components protein interaction becomes subject to more selective constraints than with soluble components. Differently from region 8–181 (homologous to region 1–173 in Ha-ras) which is acidic, the hypervariable region 182–322 displays a highly positive net charge, that together with the presence of extensive hydrophobic stretches is likely essential for binding to membrane-associated GEF.

Ha-Ras p21 was reported to be able to substitute for yeast Ras proteins in sustaining growth and adenylyl cyclase activation, but complementation of the defect of yeast Ras genes was not efﬁcient (53, 54). In our hands unprenylated recombinant Ha-Ras was practically unable to stimulate adenylyl cyclase and only its farnesylation enabled some stimulation. Even with farnesylated Ha-Ras maximum activation of adenylyl cyclase was much lower than with Ras2p, an effect that was more pronounced with wild-type adenylyl cyclase than with its CRI4 mutant. However, farnesylated Ha-Ras showed a higher afﬁnity for both CRI4 and CYR1 gene products, almost as high as that of farnesylated Ras2p. In order to clarify the reasons for the functional differences, we extended the observations that an activated Ha-Ras Val-12/Ras2p chimaera containing the first 73 amino acids of Ha-Ras Val-12 stimulates adenylyl cyclase more efﬁciently than Ha-Ras (53) for this purpose, we constructed two N-terminal Ha-Ras/C-ter Ras2p chimaeras including C-terminal regions of Ras2p of different length (Ha-Ras 1–81/Ras2p 89–322 and Ha-Ras 1–173/Ras2p 182–322) comprising the extended hypervariable region of Ras2p. Both constructs revealed the same proﬁle of adenylyl cyclase activation as Ras2p, thereby deﬁning the hypervariable domain as an additional important element for full reconstitution of the activity. Other major determinants for the interaction with adenylyl cyclase are the effector region (residues 32–40 in Ha-Ras and 39–47 in Ras2p) (31, 55–59), its flanking residues and the switch 2 region (31, 58). By means of the CRI4-encoded product, which is highly sensitive to Ras2p even if unprenylated, we showed that maximum stimulation of adenylyl cyclase by prenylated Ras2p or Ha-Ras/Ras2p chimaeras is identical to that obtained with prenylated Ras2p. This emphasizes the crucial role of the hypervariable region for maximum activation and shows that the major effect of farnesylation is to increase the afﬁnity between Ras2p and adenylyl cyclase rather than to stimulate adenylyl cyclase activity.

Even if there is still some uncertainty about a direct interaction between Ras proteins and adenylyl cyclase, due to the need of the adenylyl cyclase tightly associated protein CAP (60, 61) for a proper response to post-translationally modiﬁed Ha-Ras (22), it is possible that the basic nature of the C-terminal region of Ras2p could favor an efﬁcient interaction with the leucine repeat-rich region following the N-terminal region of adenylyl cyclase. This interaction could induce a suitable conformation for maximum activation of the catalytic domain. Leucine-rich repeat regions (61) are critical in mediating protein-protein interaction (62), as has been recently shown in SUR-8, a conserved Ras-binding protein that contains this core consensus and positively regulates Ras-mediated signaling in Caenorhabditis elegans (63).

Farnesylation of Ras2p is not required for adenylyl cyclase activation, as has been tested with CRI4-adenyl cyclase. However, as already reported for the wild-type CYR1 product (21), farnesylation of Ras2p-GTP increases the afﬁnity for adenylyl cyclase. Compared with the wild-type one, CRI4-adenyl cyclase shows not only an increased sensitivity to Ras2p (26, 46) but also a higher afﬁnity for farnesylated Ras. One should stress that the differences between these two adenylyl cyclases are only quantitative.
Adenylyl cyclase is not as strongly associated with the plasma membrane as Cdc25p and Sdc25p, does not contain a hydrophobic region resembling a membrane-spanning domain (64) and in ras1ras2bvy1 cells it is located in the soluble fraction (65). Overexpression of Cdc25p has been reported to translocate adenylyl cyclase to the membrane fraction (65), while disruption of IRA1 gene dislocates it from the membrane (66), indicating that both Cdc25p and Ira1p are involved in anchoring adenylyl cyclase to the membrane. A complex between the Cdc25p SH3 domain and adenylyl cyclase not mediated by CAP has been demonstrated (67). Taken together these data suggest the existence of the cell in a large oligomer complex including Cdc25p, Ira1p, and adenylyl cyclase. The association of Ras2p, Cdc25p, and adenylyl cyclase (68) is further supported by this work highlighting the common function of the C-terminal hypervariable region of Ras2p and its farnesylation in promoting the interaction with and/or activation of membrane-bound Cdc25p and adenylyl cyclase. However, neither Cdc25p nor Sdc25p, even when overexpressed, are able to increase the affinity of farnesylated Ras2p for adenylyl cyclase.

In conclusion, this work shows that the cellular localization of Ras2p, its regulators Cdc25p and Sdc25p and target adenylyl cyclase requires structural modifications that are dispensable under conditions of soluble purified components. We have highlighted the essential role of Ras2p farnesylation for GEF responsiveness and the involvement of its C-terminal hypervariable region in the interaction with Cdc25p. Compared with Ha-Ras, this region has been found to contain structural elements essential for the activation of adenylyl cyclase, the farnesylation facilitating this interaction.