Distal Switch II Region of Ras2p Is Required for Interaction with Guanine Nucleotide Exchange Factor*

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The interaction of Saccharomyces cerevisiae Ras2p with the catalytic domain of the GDP/GTP exchange factors (GEFs) mouse CDC25m, yeast Cdc25p, and Sdc25p was analyzed by introducing the substitution R80D/N81D into Ras2p S24N, a mutant that is shown to interfere with the Ras2p wild type (wt)-GEF interaction by forming a stable complex. The triple mutant, like Ras2p R80D/N81D, did not interfere with the action of GEF on Ras2p wt (or H-Ras p21) and was unable to form a stable complex with GEF. The GEF stimulation of the nucleotide dissociation of the triple mutant was virtually abolished and strongly decreased with the double mutant.

The affinities of Ras2p S24N/R80D/N81D for GDP and GTP was decreased 3 and 4 orders of magnitude, respectively, like that of Ras2p S24N, whereas the double mutant behaved as Ras2p wt. Like Ras2p S24N and unlike Ras2p R80D/N81D, the GTP-bound triple mutant did not activate adenylyl cyclase. Thus, the triple mutant and Ras2p S24N have opposite properties toward the binding to GEF but similarly modified behaviors toward GDP, GTP, and adenylyl cyclase. This work emphasizes the determinant role of the distal switch II region of Ras2p for the interaction with GEF and the different structural background of the interaction with adenylyl cyclase.

Ras proteins are GTPases that regulate cell growth and differentiation by cycling between the active GTP-bound and the inactive GDP-bound states. The level of these two forms is determined by a number of proteins that interact with Ras proteins and constitute the first element of a cascade of kinases influencing the activity of transcription factors. The interaction of Ras proteins with GEFs is a major target of the GTP-bound form of Ras proteins and constitutes the first element of a cascade of kinases influencing the activity of transcription factors. The interaction of Ras proteins with GEFs is a major target of the GTP-bound form of Ras proteins and constitutes the first element of a cascade of kinases influencing the activity of transcription factors.

1 The abbreviations used are: GEF, GDP/GTP exchange factor of Ras proteins; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; wt, wild type.

2 Some of the designations used are: Ras proteins, the products of RAS genes in general including human H-Ras p21 and yeast S. cerevisiae Ras2p; Ras2p, the product of S. cerevisiae RAS2 gene; Ras2p S24N, the product of the RAS2 S24N dominant allele; Ras2p R80D/N81D, the product of the ras2 R80D/N81D recessive allele; Ras2p S24N/R80D/N81D, a construct derived from Ras2p S24N and Ras2p R80D/N81D; CDC25m, the catalytic domain of a mouse brain-specific GEF; Cdc25p-C, the catalytic domain of the S. cerevisiae GEF Cdc25p; Sdc25p-C, the catalytic domain of the S. cerevisiae GEF Sdc25p; CDC25m, a general term designating Cdc25p, Sdc25p, and CDC25m.

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Ras2p for the action of GEF action and the specific properties of the mechanism of interference of Ras2p mutants.

MATERIALS AND METHODS
Preparation of Ras Proteins—Ras2p wt and mutants were produced in Escherichia coli strain SC51 as recombinant protein fused with glutathione S-transferase using the pGEX vector. Ras2p S24N was constructed as reported (Poullet et al., 1995). Ras2p R80D/N81D was obtained from the Ndd-Sal fragment from pAVBD09811 (Varrotti et al., 1992) into the Smal site of pGEX2T (pGEX2T R80D/N81D). Ras2p S24N/R80D/N81D was engineered by inserting the Ndd-Pst fragment of pAV1 carrying the S24N mutation (Poullet et al., 1995) as replacement for the homologous wild type segment in pGEX2T R80D/N81D. The Ndd-Sal fragment from p5Kc-Hras (Grass et al., 1985), containing the full-length reading frame of human c-H-ras p21, was cloned into the Smal site of pGEX2T to express the glutathione S-transferase–H-Ras p21 fusion. The transformed E. coli strains were grown at 28 °C in 2 liters of LB-rich medium containing 50 μg/ml ampicillin. Cell cultures were induced at a density of 0.3 A600 units with 0.1 mM isoprpyl-β-D-thiogalactospyranoside. After 12–15 h of growth, the cells were collected by centrifugation, washed, and sonicated thrice at 4 °C, at which temperature all the subsequent steps were carried out (Poullet et al., 1995). The affinity chromatography on glutathione-agarose and the thrombin treatment to remove the fused glutathione S-transferase were carried out as reported (Poullet et al., 1995). The faster the purification procedure, the higher was the content of the intact Ras2p products. The purified preparations contained −65% of full-length form (plus −30 and −5%) of the 29- and 37-kDa proteolytic forms, respectively.

Mono-Q HR 5/5 chromatography (fast protein liquid chromatography, Pharmacia Biotech) using a linear 20–30 mM KCl gradient (50 ml in 25 mM Tris-HCl, pH 7.8, 2 mM MgCl2, 7 mM 2-mercaptoethanol, and 10 μg/ml DTT) allowed the separation of nearly homogeneous full-length Ras2p from the 29-kDa form. The p21 was purified on Mono-Q HR 5/5 using the same gradient. After concentration in an Amicon ultrafiltration apparatus (Dialo membrane PM10), the Ras proteins were stored at −20 °C in 25 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 30 mM KCl, 50% glycerol, 1 mM DTT, and 10 μg/ml DTT.

Purification of Sdc25p-C, Cdc25p-C, and CDC25-CMm—Cdc25p-C (509 amino acids), Sdc25p-C (550 amino acids), and CDC25-CMm (250 amino acids) expressed in E. coli strain SC51 as glutathione S-transferase fusions were purified after removal of glutathione S-transferase by thrombin treatment (J. Acquet et al., 1994; Poullet et al., 1995).

Ras2p Nucleotide Interaction—The dissociation constants (Kd) and the dissociation rate constants (koff) of Ras2p nucleotide complexes were determined by the nitrocellulose binding assay using 0.45-μm nitrocellulose discs that were washed twice with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.8, 1 mM MgCl2, 150 mM NaCl, and 7 mM 2-mercaptoethanol. The Koff was determined by the nitrocellulose binding assay using 0.45-μm nitrocellulose discs that were washed twice with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl2, and 0.05 mM DTT.

RESULTS
Properties of Ras2p S24N—In mammalian cells, the mutation S17N induces a dominant negative phenotype (Stacey et al., 1991). This was also the case with the S. cerevisiae homologous substitution S24N, as was shown by genetic analysis of the RAS2 S24N phenotype.3 To characterize the interfering properties of Ras2p S24N in vitro in a quantitative manner, we examined its effect on the dissociation rate of Ras2p wt [3H]GDP induced by Sdc25p-C (Fig. 1A), Cdc25p-C (Fig. 1B), and CDC25-CMm (Fig. 1C) or on p21 [3H]GDP in the presence of CDC25-CMm-C (Fig. 1D). In all cases the response of Ras wt to GDP and Cdc25p-depletion was the same when the primary GEFs were used for Ras2p wt, as compared with the Bradford method). The Koff was determined by the nitrocellulose binding assay using 0.45-μm nitrocellulose discs that were washed twice with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl2, and 7 mM 2-mercaptoethanol. The Koff was determined by the nitrocellulose binding assay using 0.45-μm nitrocellulose discs that were washed twice with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl2, and 7 mM 2-mercaptoethanol. The Koff of Ras2p GDP and Ras2p GDP complexes were calculated by Scatchard plots according to the equation:

\[ r = \frac{N_{\text{Nucleotide free}}}{1 - \frac{r}{r_{\text{max}}} (n - r)} \]  

(Eq. 1)

where is the average mol of GDP or GTP bound per mol of Ras2p and is the number of binding sites. To eliminate traces of GDP from concentrations, the concentrated solution of the radiolabeled solution of GDP or GTP was equilibrated with buffer B at 4 °C. The flow rate controlled by the fast protein liquid chromatography system was 0.04 ml/min, and 200-μl fractions were collected 250 min after loading. Ras2p and CDC25-CMm were detected by determining the bound [3H]GDP and the [3H]GDP release activity, respectively. In the former case, 20-μl aliquots were incubated for 10 min at 30 °C with 2 μM [3H]GDP (25–180 GBq/mmol) in a 50-μl reaction mixture (25 mM Tris-HCl, pH 7.5, 2 mM EDTA, 20 mM (NH4)2SO4 and 1 mM DTT). In the latter case, the reaction was started by adding 3 pmol of preformed Ras2p [3H]GDP in 30 μl of buffer B to a 20-μl aliquot, to which 3 μl containing 9 nmol of cold GDP were added. The decrease in radioactivity was measured after 15 min at 30 °C. Immunostained Western blot using 140-μl lophosphates was performed according to Poullet et al. (1995) with Ras2p-specific antibodies and CDC25-CMm antibodies obtained from rabbit immunized against purified CDC25-CMm.

Assay of Adenylyl Cyclase Activity—The Ras2p GDP- and Cdc25p-dependent adenylyl cyclase activity was measured at 30 °C (Créchet et al., 1993) using 30 μg/ml of membranes (as a source of adenylyl cyclase) from S. cerevisiae strain AAT7B-31 (genotype: ade2 cdc25-1 LEU2 CR14 hi5A leu2–3, 112 lys 1–1 ras1–A ras2:URA3 ura3–52; Mirisola et al., 1994). The membranes were prepared according to Mirisola et al. (1994) with the following modifications. The cells, grown at 30 °C in YEPD (2% bacto-peptone, 1% yeast extract, and 2% glucose) medium, were collected at 1.2 A550 units, disrupted in 2 volumes of 50 mM Tris-HCl, pH 6.2, 0.05 mM EDTA, 0.1 mM DTT containing a mixture of protease inhibitors (Boehringer Mannheim): 2 mM Pefablock-Sc (4-[2-Aminomethyl]-benzenesulfonylfluoride hydrochloride), 2 μg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 60 μg/ml antipain. The membranes were stored in the same buffer with 10% glycerol.

Other Methods—The purity of proteins was estimated by Coomassie Blue staining on 12% SDS-polyacrylamide gel electrophoresis, by the Bradford method (Bradford, 1976), and for Ras proteins their concentration was estimated by their ability to bind [3H]GDP in the presence of a saturating concentration of the labeled substrate (0.7–1 mol/mol of Ras as compared with the Bradford method).

3) J.-B. Créchet and O. Fasano, unpublished observations.
Sdc25p without affecting the intrinsic GDP/GTP exchange (Verrotti et al., 1992). The introduction of these two substitutions in Ras2p S24N (Fig. 2) caused the loss of the ability to interfere with the Cdc25p-C-mediated Ras2p wt \([3H]GDP\) dissociation rate even at concentrations as high as 1.5 \(\mu M\), whereas in the same conditions 0.5 \(\mu M\) Ras2p S24N reduced twice the dissociation rate of Ras2p wt \([3H]GDP\). This shows that the nature of residues Arg 80 and Asn 81 is essential for the interfering properties of Ras2p S24N.

The ability of the various Ras2p species to form stable complexes with Cdc25p on gel filtration was also analyzed. For these experiments, we used the truncated form of Ras2p to improve the resolution of the eluted products. CDC25Mm-C was preferred to Cdc25p-C or Sdc25p-C, because unlike the purified forms of these two yeast GEFs (Poullet et al., 1995), it does not display hydrophobic interactions with the acrylamide-agarose matrix, leading to a retention on filtration chromatography. CDC25Mm-C was added in a 2 to 1 molar excess over Ras2p. In these conditions, as deduced from the ability of the eluted fractions to bind \([3H]GDP\) and stimulate the dissociation of Ras2p \([3H]GDP\), more than 80% of the GDP free-Ras2p wt was engaged in a stable complex with CDC25Mm-C (Fig. 3A). In contrast to Ras2p wt, the triple mutant did not form a complex with CDC25Mm-C, even in the absence of nucleotides (Fig. 3B), like Ras2p R80D/N81D (Fig. 3C). As was previously shown with Sdc25p-C (Poullet et al., 1995), the complex Ras2p S24N-CDC25Mm-C (Fig. 3D) required for dissociation much higher concentrations of nucleotide (0.1 mM GDP (Fig. 3E) or GTP (Fig. 3F)) than Ras2p wt (1 \(\mu M\), not shown). The effect of the R80D/N81D to relieve the S24N effect stresses the importance of Arg 80 and Asn 81 for the binding to GEF. The existence of a 1:1 stoichiometry between Ras2p and GEF in the stable complex was proved by immunotransfer Western blot analysis on SDS-polyacrylamide gel electrophoresis (not shown).

The Action of GEF on the Nucleotide Interaction of the Various Ras2p Species—The analysis of the interaction between the various mutants and GTP or GDP was carried out in the presence of 10 mM MgCl2. The stabilization by this cation of the Ras-nucleotide complex (De Vendittis et al., 1986a; Hall & Self, 1986) was required to obtain dissociation rates measurable by the nitrocellulose binding assay in the case of high concentrations of GEF or fast intrinsic dissociation rates as found for Ras2p S24N-GDP (see below).

Table I shows that the intrinsic dissociation rate of the GDP complex of the triple mutant was 300 times faster than that of the Ras2p wt complex, corresponding to the fast intrinsic dissociation of Ras2p S24N-GDP, whose rate could, however, still be enhanced by CDC25Mm-C, to a 10-fold smaller extent than...
Fig. 2. Ras2p S24N/R80D/N81D does not interfere with the Cdc25p-C-dependent dissociation of Ras2p wt[3H]GDP. Ras2p wt[3H]GDP dissociation was measured in buffer A with 1.5 mM MgCl₂ in the absence (○) or the presence (●) of 2.2 nm Cdc25p-C and after preincubation of GEF with 0.5 μM Ras2p S24N (●), 0.5 μM (○), or 1.5 μM Ras2p R80D/N81D (●) and 0.5 μM (○) or 1.5 μM (▲) Ras2p S24N/R80D/N81D. The intrinsic Ras2p wt[3H]GDP dissociation rate (○) was not modified by adding 0.5 μM Ras2p S24N (▲).

Ras2p wt, whereas the rate of the triple mutant was virtually insensitive to GEF. Because even in the presence of 10 MgCl₂, the dissociation rates of the GTP complexes of the triple and S24N mutants were too fast to be measured by the nitrocellulose binding assay, the analysis of these complexes was limited to the calculation of the dissociation constants (K_d) from Scatchard plots, obtained with nucleotide-free Ras2p and GTP concentrations varying between 4 and 80 μM (Table II). The K_d values of the GTP complex of Ras2p S24N (14.4 μM) and the triple mutant (97 μM) lay in the same range and were much higher than that of Ras2p wt (3 nm, Créchet et al., 1990a). The K_d values of their GDP complexes, as determined for concentrations of GDP between 0.2 and 6 μM, were close (1.6 μM for Ras2p S24N and 2.5 μM for the triple mutant) and 1 order of magnitude smaller than those of the GTP complexes.

In agreement with our previous observations (Verrotti et al., 1992), the double mutant displayed virtually the same intrinsic GDP “off” rate as Ras2p wt. As shown in Table I, its response to GEF was not totally abolished, unlike that of the triple mutant. A concentration of 0.83 μM CDC25⁴⁴⁻C, stimulating 250 times the dissociation rate of Ras2p wt-GDP, was found to increase six times the dissociation rate of the GDP complex of the double mutant. Therefore, the strength of its interaction was 40 times weaker than that of Ras2p wt.

Effect of S80/81 and 24 Substitutions on the Cdc25p-mediated Activation of Ras2p wt on Adenylyl Cyclase—To study the effect of the various mutants on the Ras2p wt-dependent adenylyl cyclase activity, cell membranes were prepared from the mutated S. cerevisiae strain AAT3B-1Δ, carrying deletions in RAS1, RAS2, and CDC25 genes (Mirisola et al., 1994). The viability of this strain is ensured by the adenylyl cyclase CRI4 mutation that bypasses the requirement for RAS and CDC25 due to a higher intrinsic activity. Because the CR14-encoded adenylyl cyclase activity is enhanced by the Ras protein much more than the wild type adenylyl cyclase (De Vendittis et al., 1986b), the sensitivity of the assay is increased. It is known that the addition of Ras2p-GDP to yeast membranes from this strain strain as a source of adenylyl cyclase is ineffective in stimulating the cAMP production (Créchet et al., 1993). The presence of GEF restores the adenylyl cyclase activity via the conversion of Ras2p-GDP to Ras2p-GTP. Fig. 4 illustrates the capacity of the various Ras2p mutants to interfere with the action of Ras2p wt in a reconstituted system for adenylyl cyclase activity. Ras2p S24N hinders in a concentration-dependent manner the reconstitution of adenylyl cyclase activity dependent on the regeneration of Ras2p wt-GTP by Cdc25p-C. No difference in the inhibitory effect was observed between the 42-kDa native form and the 29-kDa C-terminal truncated form of Ras2p S24N, further confirming that the C-terminal region of Ras2p, at least if not post-translationally processed, is irrelevant for the interaction with the catalytic domain of GEF. Increasing amounts of the triple mutant did not reveal any interference with the activation of adenylyl cyclase dependent on the conversion of Ras2p wt-GDP to Ras2p wt-GTP by Cdc25p-C. As in a way expected, no interfering effect could be detected with Ras2p R80D/N81D. The moderate increase in cAMP production was due to the partial regeneration of the Ras2p R80D/N81D-GTP complex, because the GTP- and Gpp(NH)p-bound forms of this mutant can activate membrane-bound adenylyl cyclase (Verrotti et al., 1992; Mirisola et al., 1994). The observation that a five to six times higher concentration of its GDP complex was needed to induce half the Cdc25p-dependent adenylyl cyclase activity obtained with Ras2p wt-GDP is in agreement with the marked decrease in the affinity of the double mutant for GEF (cf. Table I).

From Fig. 4, one can derive that neither Ras2p S24N nor the triple mutant was able to stimulate the adenylyl cyclase activity. The lack of activity cannot be explained by a reduced affinity for GTP. Because the K_d values of their GTP complexes lie in the 10–90 μM range (Table II), more than 50% of the bound GDP should be exchanged with GTP within a few seconds due to the high intrinsic GDP dissociation rates of these two mutants and the high concentration of GTP present in the assay (0.5 mM). In agreement with this, no adenylyl cyclase activity was detected with the preformed GTP complexes of these two Ras2p mutants.

To analyze whether the double and triple Ras2p mutants were still capable of interacting with adenylyl cyclase in an unproductive manner, we have carried out competition experiments between these mutants and Ras2p wt with respect to the activation of adenylyl cyclase. Neither Ras2p S24N nor Ras2p S24N/R80D/N81D in their GTP bound-form using a concentration excess up to 10 times over Ras2p wt-GTP could compete for the adenylyl cyclase activation. These results (not illustrated) show that the inability of these two Ras2p mutants to activate adenylyl cyclase is related to a defective binding to their target.

**DISCUSSION**

This work shows that the property of substitutions R80D/N81D to abolish the interference on the Ras2p wt-GEF interaction induced by mutation S24N is associated with the loss of the ability to form a stable complex with GEF. The relationships between these two effects emphasize the importance of the distal switch II region for the binding to GEF. R80D/N81D and S24N are substitutions that induce opposite effects on the properties of Ras proteins. The former mutation has been reported to eliminate the response to GEF, affecting neither the intrinsic GTP/GTP exchange reaction of Ras2p nor its affinity for GDP and GTP (Verrotti et al., 1992; Pouillet et al., 1995). In vivo, it has a lethal effect that can be rescued by a constitutively activating mutation that bypasses the need for GEF (Verrotti et al., 1992). Substitution S24N induces dominant negative properties similar to those described for the homologous p21S17N (Johnson et al., 1993) that are based on the formation of a stable complex with GEF, leading to the sequestration of the exchange factor. In this work, the characterization of
Ras2p R80D/N81D has been extended by demonstrating that its insensitivity to GEF is caused by a strong decrease in the affinity between these two proteins. As an important feature, the introduction of R80D/N81D into Ras2p S24N does not influence two selective properties induced by the latter mutation: the strongly decreased affinity for GTP and GDP and the inability to activate adenylyl cyclase. Therefore, the suppression of the tight interaction with GEF virtually constitutes the only major difference between the triple mutant and Ras2p S24N.

The switch II region is known to be the structure of Ras undergoing the most extensive changes depending on whether GDP or GTP is bound, as shown by x-ray diffraction and NMR studies (Pai et al., 1990; Miller et al., 1992). The pivotal role of glycine 82 (Gly75 in Ras p21), located at the boundary of the C-terminal end of helix α2 and loop L5, is crucial for the transitions between the GDP- and GTP-bound forms of Ras proteins (Stouten et al., 1993; Kavounis et al., 1991), strongly suggesting that the physiological action of GEF requires a specific conformation of loop L4/helix α2/loop L5 for inducing a productive interaction. The competition phenomena described between nucleotide and GEF for binding to Ras2p (Poullet et al., 1995; this work) could arise from the coordination existing between the helix α2 region and the nucleotide binding network (Stouten et al., 1993). In the dominant negative mutants of Ras, the binding to GEF, which in the case of the wild type Ras protein represents a transient intermediate state, becomes a stabilized specific state of the Ras molecule (Feig & Cooper, 1988; Farnsworth & Feig, 1991). Interfering properties are induced by mutations of specific residues of the nucleotide binding site, particularly of those involved in a direct perturbation of the magnesium ion-nucleotide coordination (Farnsworth & Feig, 1991; Jung et al., 1994). As a common feature, these Ras mutants display a decrease in the affinity for GDP and GTP by several orders of magnitude. Because the nucleotide binding site is strictly correlated with the structural elements playing a key role in the interaction with ligands (effector loop) or in the

![Fraction number graph](image)

**Figure 3.** Ras2p S24N/R80D/N81D does not form a stable complex with CDC25<sup>MM</sup>. The complex formation was determined on AcA44 Ultrogel columns equilibrated or not with the indicated concentrations of guanine nucleotide. The Ras2p- and CDC25<sup>MM</sup>-C-containing fractions were detected by measuring the [3H]GDP binding (●) and the [3H]GDP releasing activity (○), respectively.

**Table I**

The dissociation rate constants (k<sub>-1</sub>) of the GDP complexes of Ras2p wt, Ras2p R80D/N81D, Ras2p S24N, or Ras2p S24N/R80D/N81D as a function of increasing concentrations of CDC25<sup>MM</sup>

The dissociation rate constants of the various Ras2p [3H]GDP complexes were determined as described under "Materials and Methods" in buffer A containing 10 mM MgCl₂, with or without the indicated concentrations of CDC25<sup>MM</sup>. NM, nonmeasurable with the nitrocellulose binding assay due to a dissociation rate that is too fast.

|                     | 10<sup>-1</sup>k<sub>-1</sub> | 10<sup>-1</sup>k<sub>-1</sub> | 10<sup>-1</sup>k<sub>-1</sub> | 10<sup>-1</sup>k<sub>-1</sub> |
|---------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                     | Ras2p wt | Ras2p R80D/N81D | Ras2p S24N | Ras2p S24N/R80D/N81D |
| − GEF               | 0.55 ± 0.03 | 0.79 ± 0.03 | 161 ± 5 | 160 ± 4.2 |
| + CDC25<sup>MM</sup>-C (100 nm) | 10.3 ± 0.2 | 0.98 ± 0.04 | 336 ± 11 | 160 ± 4.8 |
| + CDC25<sup>MM</sup>-C (280 nm) | 31.1 ± 1.4 | 2.39 ± 0.08 | NM | 191 ± 6.3 |
| + CDC25<sup>MM</sup>-C (830 nm) | 135 ± 3.3 | 4.86 ± 0.06 | NM | 237 ± 3.6 |
the interaction with regulators and effectors. Point substitutions in this area can modify in a drastic manner states (switch I and II regions). It is not surprising that a single specific conformation determining the active and inactive interaction with GEF, likely by a decrease in the affinity between these two proteins. These observations are in agreement with the determinant role of the helix α2 region in the interaction with GEF. The finding that substitutions situated on the boundary between helix α3 and loop L7 in p21 also hinders the GEF signal (Segal et al., 1995) does not contrast with this conclusion. In fact, in the three-dimensional model of p21, the helix α2 region and the helix α3/loop L7 boundary are located on the nearly same exposed surface of the Ras molecule and are in contact (Stouten et al., 1993). Consequently, conformational changes in helix α2/loop L5 also influences the state of the helix α3/loop L7 region. This situation, together with the negative effect of their mutation on the GEF action, suggests that both regions are involved in the interaction with GEF, either directly as part of the Ras2p binding site for GEF or indirectly as key elements for inducing the active conformation of this binding site that is very probably located nearby. Unlike this, experimental evidence indicates that the phosphoryl binding loop 4, flanking the N-terminal end of helix α2, only participates in the transmission of the GEF signal, because point substitutions in this loop impair the GEF-dependent dissociation rate of the nucleotide without substantially decreasing the affinity for GEF (Mistou et al., 1992).

Despite the relevance of the conclusions derived from these studies, the precise nature of the structural elements delimiting the GEF binding surface between Ras and GEF remains as yet unclear. In fact, helix α2/loop L5 and helix α3/loop L7, the two most probable regions of Ras proteins involved in a direct interaction with GEF, have only been characterized by mutagenic analysis. More direct functional methods, such as competition experiments using oligopeptides corresponding to the Ras2p or p21 regions including the various α-helices and flanking sequences, have failed so far to locate a specific structure or structures of Ras proteins directly involved in the interaction with GEF. Consequently, the functional findings still need to be confirmed by structural studies directed to unveil the three-dimensional relationships of the Ras-GEF complex.

Unlike Ras2p S24N, whose GTP complex is incapable of activating adenylyl cyclase as already reported for the corresponding p21 mutant (Farnsworth et al., 1991), Ras2p R80D/N81D-Gpp(NH)p was found to display the same affinity for adenylyl cyclase as Ras2p wt, only the Vmax of the adenylyl cyclase activation was somewhat decreased (Verrotti et al., 1992; Mirisola et al., 1994). These results, which in part differ with the conclusions derived from experiments using p21 mutants and yeast adenylyl cyclase (Segal et al., 1995), clearly indicate that the binding site for GEF is distinct from the site activating the adenylyl cyclase. The selectivity of the additional presence of R80D/N81D on the specific conformation induced by S24N concerning the activation of adenylyl cyclase, further confirms that the GEF signal originates from regions different from the binding site for the nucleotide and the coordinated magnesium ion. In this work we have demonstrated that the interaction of the triple and Ras2p S24N mutants with respect to adenylyl cyclase is based on a truly defective binding between these two proteins and not on the formation of an inactive complex.

In conclusion, the observations that substitution of serine 24, a residue of the nucleotide/magnesium ion binding site, can induce a stable interaction with GEF and that additional mutations in helix α2 abolish this interaction put emphasis on the close relationships of the helix α2/loop L5 region with the specific binding site for GEF.

4 E. Jacquet and J. B. Créchet, unpublished observations.
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