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Role of glycine-82 as a pivot point during the transition from the inactive to the active form of the yeast Ras2 protein

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Ras proteins bind either GDP or GTP with high affinity. However, only the GTP-bound form of the yeast Ras2 protein is able to stimulate adenylyl cyclase. To identify amino acid residues that play a role in the conversion from the GDP-bound to the GTP-bound state of Ras proteins, we have searched for single amino acid substitutions that selectively affected the binding of one of the two nucleotides. We have found that the replacement of glycine-82 of the Ras2 protein by serine resulted in an increased rate of dissociation of Gpp(NH)p, a nonhydrolysable analog of GTP, while the GDP dissociation rate was not significantly modified. Glycine-82 resides in a region that is highly conserved between the yeast and human proteins. However, this residue is structurally distant from residues that participate in the binding of the nucleotide, as determined from the crystal structure of the human H-*ras* gene product. Therefore, the ability of the nucleotide binding site to discriminate between GDP and GTP is dependent not only on residues that are spatially close to the nucleotide, but also on distant amino acids. This is in agreement with the role of glycine-82 as a pivot point during the transition from the GDP- to the GTP-bound form of the Ras proteins.

S. cerevisiae; Ras; Point mutation; Guanosine diphosphate; Guanosine triphosphate

1. INTRODUCTION

The Ras proteins bind either GTP or GDP in an alternating fashion to assume 'on' and 'off' states, thus playing a regulatory role in diverse cellular processes such as growth regulation and secretion [1]. The conversion from the GTP-bound to the GDP-bound form is mediated by the GTPase activity of the proteins, while the regeneration of the GTP-bound from the GDP-bound form takes place through a nucleotide exchange reaction. Several amino acid side chains and backbone residues that are in close proximity to the nucleotide participate in bonding interactions with either GTP and GDP, and it has been shown that the replacement of these amino acids by others can affect the binding of the nucleotide [2]. Furthermore, comparitive crystallographic analysis of the GTP- and GDP-bound forms of the human H-ras p21 protein indicates that the regions spanning residues 30–37 and 60–76 of the polypeptide chain undergo a major conformational change after the conversion from one form to the other [3,4]. However, due to the high thermal mobility of the regions, crystallographic data alone do not allow one to predict the role of each amino acid in this conformational transition. To approach this problem by an alternative method, we have introduced single amino acid substitutions at different positions of the yeast Ras2 gene product, and we have analysed the nucleotide binding properties of the purified proteins. We started our analysis from the conserved glycine-82 of the yeast Ras2 protein, since previous data from random mutagenesis analysis indicated that this residue could be important for the attainment of the active, GTP-bound state [5]. The results indicate that a mutated Ras2 protein in which glycine-82 has been replaced by serine interacts with GDP like the wild-type Ras2. However, the interaction with the nonhydrolysable GTP analog Gpp(NH)p is weaker in the mutant than in the wild-type. This indicates that the replacement of glycine-82 by a larger residue hinders selectively the attainment of the active conformation of the Ras2 protein, while not affecting significantly the GDP-bound state.

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Abbreviations: PAGE, polyacrylamide gel electrophoresis; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; GDP-βS, guanosine-5′-*O*-(2-thiodiphosphate); EDTA, [ethylenedinitrilo]tetraacetic acid; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Purification of wild-type and mutated Ras2 proteins*

The expression vectors pAV1, that directs the synthesis of the wild-type yeast Ras2 protein under the control of the λP_L promoter, is

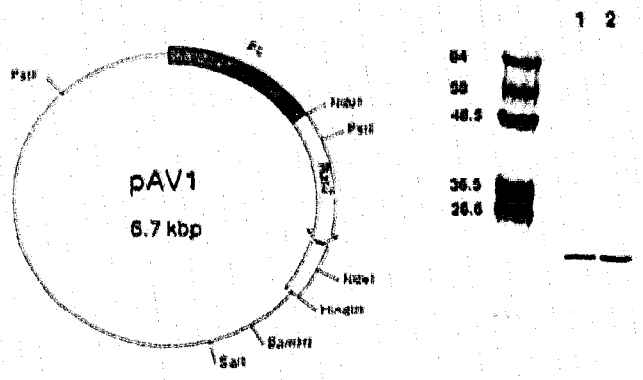

Fig. 1. Expression and purification of wild-type and mutated yeast Ras2 proteins from *E. coli*. The figure shows a schematic map of the expression vector pAV1, that directs the synthesis of the wild-type yeast Ras2 protein under the control of the λP_L promoter. pAV1 was constructed from an expression vector for the human H-*ras* protein [17] after replacing an *NdeI-SalI* insert encoding the human protein with an *NdeI-HindIII* fragment encoding the complete coding region (indicated by an arrow) and 3' untranslated sequences of the yeast *RAS2* gene [18], the *NdeI* site that includes the first ATG codon of the *RAS2* coding sequence was not present in the cloned gene [18], but it was engineered by standard site-directed mutagenesis techniques to ensure a correct initiation for the Ras2 protein. A 346 bp *HindIII-SalI* fragment from pBR322 was also included as a linker between the *HindIII* site of the insert [18] and the *SalI* site of the vector. The figure also shows a Coomassie staining of the purified wild-type (line 1) and mutated (Gly-82 → Ser, line 2) Ras2 protein, after fractionation by SDS-PAGE on a 12.5% gel. 1.5 μg of the purified proteins were loaded in each slot. Numbers on the left correspond to molecular mass standards in kDa.

described in Fig. 1. The vector pAVS82 encodes a Ras2 protein with serine replacing glycine in position 82. It was obtained from pAV1 after changing the codon 82 (GGC) to AGC by standard site-directed mutagenesis techniques. For expression of the corresponding protein, the vectors were introduced into the *E. coli* strain N4830 [6] and the synthesis of the Ras2 protein was induced as described by De Vendittis et al. [7]. About 75 g of cells were suspended at 0°C in 250 ml of 40 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 20 mM KCl, 0.5 mM dithiothreitol, 20 μM GDP (buffer TK), and sonicated (10 × 20 s, 100 W). The crude extract was centrifuged at 40 000 rpm for 1 h in a Beckman type 45 Ti rotor, and solid ammonium sulphate was added to the supernatant to 35% saturation. After 30 min at 0°C, the precipitated material was collected by centrifugation at 16 000 rpm in a Sorvall SS-34 rotor and discarded. Ammonium sulphate was then added to 65% saturation. After 2 h incubation at 0°C, the precipitated material (containing the Ras2 protein) was collected by centrifugation and resuspended in 100 ml of buffer TK. The solution was dialyzed for 12 h with three changes against 2 liters of TK buffer, and loaded on a DEAE-Sephacel column (5 × 30 cm) equilibrated with the same buffer. The column was washed with 500 ml of buffer, and eluted with a linear 0.02–0.5 KCl gradient in 250 ml of TK buffer. Aliquots from selected fractions were fractionated by SDS-PAGE on a 12.5% gel and immunoblotted with the Ras-specific monoclonal antibody Y13-259 [8]. A minor proportion of the Ras2 protein, with an apparent molecular mass of 38 kDa, was eluted at a KCl concentration of about 90 mM. Most of the immunoreactive Ras2 protein appeared as a 20 kDa band at a KCl concentration of about 250 mM. It should be noted that a much higher proportion of the 38 kDa form was found using a previously described purification procedure in which the ammonium sulphate precipitation step was omitted during the preparation of the crude extract [7]. The 20 kDa form of the Ras2 protein was further purified by AcA 44 gel filtration chromatography in TK buffer, and by a Mono-Q ion exchange column that was eluted with a linear 0.02–0.4 M KCl gradient in TK buffer. The protein was active in the stimulation of the adenylate cyclase activity of *ras1*$^-$ *ras2*$^-$ *CR14* strains in its Gpp(NH)p-bound, but not in its GDP-βS-bound form [9]. Both the purified wild-type and mutated protein were free of detectable contaminants, as shown by Coomassie blue staining after SDS-PAGE (Fig. 1).

2.2. *Interaction of Ras proteins with guanosine nucleotides*

To prepare specific Ras2/nucleotide complexes, we preincubated purified Ras2 proteins (complexed with GDP) with an excess of either [^{3}H]GDP or [^{3}H]Gpp(NH)p for 30 min at 30°C in 40 mM Tris-HCl, pH 7.5, 20 mM KCl, 0.5 mM dithiothreitol, 1 mM EDTA. The presence of EDTA was required to facilitate the exchange of the bound with the free radioactive nucleotide. The reaction was terminated by the addition of $MgCl_2$ at the indicated final concentration. Control experiments showed that no detectable loss of nucleotide binding activity was detected over three days incubation at 10°C. To measure the rate of dissociation of nucleotides from the corresponding Ras2/nucleotide complexes, we started the reaction by the addition of excess unlabelled GDP. The displacement of the radioactive nucleotide was followed at appropriate time intervals, by loading aliquots of the reaction mixture on Sephadex G-50 columns, as described by De Vendittis et al. [7]. This technique allowed a more precise measurement of the amount of protein/nucleotide complex than the commonly used nitrocellulose filter procedure.

3. RESULTS

3.1. *The introduction of serine at position 82 of the purified Ras2 protein affects selectively the affinity for Gpp(NH)p.*

Using the procedure described in section 2, we purified both the wild-type Ras2 protein and a mutated variant carrying a Gly-82 → Ser substitution as

Table 1

Titration of the GDP-bound form of purified wild-type and mutated (Gly-82 → Ser) Ras2 proteins with a small excess of [^{3}H]GDP or [^{3}H]Gpp(NH)p

Initial Ras2·GDP complex[a]	Radioactive nucleotide added	Yield of radioactive Ras2/nucleotide complex at equilibrium (%)
Ras2 (wt) 5.1 μM	[^{3}H]GDP (21 μM)	102.5[b]
Ras2 (Ser-82) 5.1 μM	[^{3}H]GDP (21 μM)	98.5[b]
Ras2 (wt) 5.1 μM	[^{3}H]Gpp(NH)p (21 μM)	63.5
Ras2 (Ser-82) 5.1 μM	[^{3}H]Gpp(NH)p (21 μM)	39.2

[a] The protein concentration was determined using the method of Lowry et al. [20], using bovine serum albumin as a standard. One μg of pure protein was considered to be equivalent to 50 pmol.

[b] The contribution of the GDP carried over with the protein was included in the calculation of the specific activity.

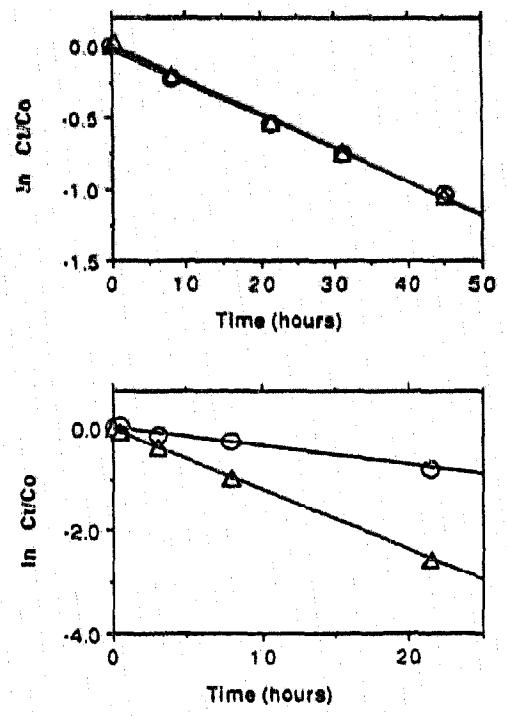

Fig. 2. Rate of dissociation of [^{3}H]GDP and [^{3}H]Gpp(NH)p from the corresponding Ras2/nucleotide complexes. The reaction mixture contained, in a final volume of 200 μl of 50 mM Tris-acetate, pH 8.0, 100 mM KCl, 0.5 mM dithiothreitol, 6.5 mM $MgSO_4$, and 1.5 mM EDTA, 1 nmol of either the wild-type (circles) or a mutated (Gly-82 → Ser, triangles) Ras2 protein complexed with either [^{3}H]GDP (upper panel) or [^{3}H]Gpp(NH)p (lower panel). The concentration of each nucleotide (including bound and free) was 25 μM, with a specific activity of about 600 cpm/pmol. The reaction was started by the addition of cold GDP to the final concentration of 1 mM. Following incubation at 10°C, aliquots of 30 μl were withdrawn from the reaction mixture at appropriate time intervals, and the radioactive Ras/nucleotide complex was determined by a gel filtration procedure (see section 2).

homogeneous species with an apparent molecular mass of 20 kDa (Fig. 1). Both proteins, purified as a complex with GDP, could exchange the bound nucleotide with excess exogenous [^{3}H]GDP or with the nonhydrolysable GTP analog [^{3}H]Gpp(NH)p (Table I). The time course of the exchange reaction was identical for both proteins (not shown), and the calculated stoichiometry of the protein/[^{3}H]GDP complex at equilibrium was close to 1:1 (Table I). When a small excess of [^{3}H]Gpp(NH)p was used for the exchange reaction, the amount of Ras/[^{3}H]Gpp(NH)p complex at equilibrium was apparently lower than 100%, due to the presence of competing unlabelled GDP carried over with the protein (Table I). Moreover, the relative yield of the Ras/[^{3}H]Gpp(NH)p complex was lower for the mutated protein harboring serine instead of glycine at position 82, than for the wild-type (Table I). This could be explained either by an increased affinity of the mutated protein for GDP, or by a decreased affinity for Gpp(NH)p. To discriminate between the two possibilities, we determined the values of the apparent dissociation rate constants of the two nucleotides from the wild-type and mutated proteins. The results, shown in Fig. 2, indicated that in all cases the dissociation of the protein/nucleotide complex followed first-order kinetics. However, while the dissociation rate constant for GDP was essentially the same for both proteins, the dissociation rate constant for Gpp(NH)p was 3.3-fold faster for the mutant than for the wild-type (Fig. 2 and Table II).

4. DISCUSSION

The conversion of the yeast Ras2 protein from the GTP-bound active to the GDP-bound inactive form is mediated by the GTPase activity of the protein, while the regeneration of the active form from the inactive one takes place by a nucleotide exchange reaction (for reviews, see [10–12]). Since the GTP-bound, but not the GDP-bound form of the Ras2 protein is able to stimulate adenylate cyclase [9,13], the activity of the latter enzyme can be regulated by physiological effectors that are capable of modulating either the GTPase or the nucleotide exchange reaction of Ras. The role of macromolecular effectors can be partially mimicked by single amino acid substitutions at critical positions on the Ras polypeptide. Indeed, it has been shown that amino acid substitutions at some positions can affect either the rate of the GTPase or of the nucleotide exchange reaction, or both [10–12]. As expected, crystallographic analysis has shown that all the amino acid residues that have so far been shown to be critical for the biological function of Ras are either involved in bonding interaction with, or are spatially very close to the nucleotide [14].

One exception to this rule is represented by amino acid residues that are important for the association of Ras proteins with the inner face of the plasma membrane [15]. We have previously shown that another ap-

Table II

Apparent dissociation rate constants of the Ras/GDP and Ras/Gpp(NH)p complexes at 10°C for the wild-type and a mutated Ras2 protein harboring a Gly-82 to Ser substitution[a]

Ras2 protein	k_{off}GDP (10^3 min^{-1})	k_{off}Gpp(NH)p (10^3 min^{-1})
Gly82 (wild type)	0.38	0.60
Ser82 (mutant)	0.39	2.00

[a] Dissociation rate constants were calculated from Fig. 2.

parent exception is provided by mutations at positions 82 and 84 of the yeast Ras2 protein (corresponding to the conserved glycines 75 and 77 of the human ras p21 gene products). These residues are physically very distant from the nucleotide (a schematic two-dimensional view of the relationship between glycine-82 and the nucleotide binding domain of Ras is shown in Fig. 3), and yet their replacement by larger residues resulted in inability of the mutated protein to stimulate adenylyl cyclase in vitro and in impairment of Ras-dependent phenotypes in vivo [5]. We speculated that this region was flexible and that glycines were required for the transition from the inactive (GDP-bound) to active (GTP-bound) state. This hypothesis was also in agreement with the suggestion by Jurnak et al. that glycines-75 and -77 of the human H-*ras*p21 serves as a pivot point (together with glycine-60) for the conformational change of the polypeptide segment 57–75 associated with the transition from the inactive to the active state [2]. The data reported in this paper suggest that an increase of the relative affinity for GDP of the mutated protein harboring Ser at position 82 might play a role in the expression of the defective phenotype of the double mutant 82–84 in vivo [5]. However, the observation that the replacement of both Gly-82 and Gly-84 by other amino acids was required for defective growth in vivo indicates that the two residues together are important for the physiological function of Ras2 [5]. The biochemical characterization of a protein with a double amino acid substitution and with Gly-84 alone replaced by Arg will possibly help to clarify this issue.

The increased off-rate for Gpp(NH)p observed after the replacement of Gly-82 by Ser raises the question of how a residue that is distant from the nucleotide binding site can affect the binding of the nucleotide to Ras. A possible explanation is provided by the model shown in Fig. 3. An important feature of this model is that the replacement of a glycine in a pivot point of the polypeptide chain (indicated by an arrow) by larger residues will hinder the conformational change that is associated with the exchange of GTP for GDP and vice versa. However, due to the high thermal mobility of the region encompassing residues 61–66 of the human protein (corresponding to residues 68–73 of the yeast Ras2 protein) [16], it has not been possible to predict from structural data whether or not small structural distortions induced by the replacement of one amino acid with another at position 82 could affect nucleotide binding. Our observations suggest that the polypeptide chain including residues 68–82 (yeast coordinates), even though very mobile in some points, could be able to transmit motions from residues that contact the nucleotide to more distant regions of the protein and vice versa (Fig. 3). Therefore, following the binding of Gpp(NH)p, the presence of Ser at position 82 could hinder the attainment of the proper conformation of the nucleotide binding site. This is also in line with the observation that binding of the Ras-specific antibody Y13-259 to this region interferes with nucleotide exchange [8,21,22]. A prediction of our model is that modulation of nucleotide binding, and of Ras function, could result from the binding of ligands (potential antibiotics and/or macromolecular effectors of Ras function) to the mobile region between the nucleotide binding site and glycine-82.

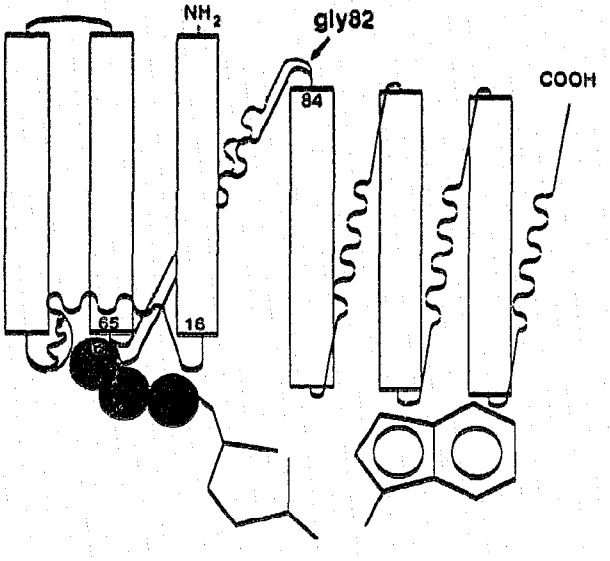

Fig. 3. A model illustrating how mobility constraints resulting from the replacement of glycine-82 by a larger residue could lead to a distorted Gpp(NH)p-bound state of the Ras2 protein. The hypothetical topological order of secondary structure of the nucleotide binding domain of the yeast Ras2 protein (strands of β-sheet are shown as rectangles and helices as wavy lines) has been drawn schematically, using the structure determined by Pai et al. for the human H-*ras* p21 gene product [16]. The yeast and the human protein are highly related over the amino-terminal 166 amino acids used for the determination of the structure. In fact, 65% of the amino acids are identical, while conservative substitutions are observed for most of the remaining ones [18,19]. The nucleotide is not in scale with the protein, and only relevant topological relationships are indicated. Circles indicate phosphate residues. Protein regions that are predicted to be affected following the replacement of GDP by Gpp(NH)p are schematically indicated in gray.

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REFERENCES

[1] Hall, A. (1990) Science 249, 635–640.
[2] Jurnak, F., Heffron, S. and Bergmann, E. (1990) Cell 60, 525–528.
[3] Schlichting, I., Almo, S.C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsh, W., Pai, E.F., Petsko, G.A. and Goody, R.S. (1990) Nature 345, 309–315.

[4] Brünger, A.T., Milburn, M.V., Tong, L., De Vos, A.M., Jancarik, J., Yamaizumi, Z., Nishimura, S., Ohtsuka, E. and Kim, S. (1990) Proc. Natl. Acad. Sci. USA 87, 4849–4853.
[5] Fasano, O., Crechet, J.B., De Venditiis, E., Zahn, R., Feger, G., Vitelli, A. and Parmeggiani, A. (1988) EMBO J. 7, 3375–3383.
[6] Gottesman, M.E., Adhya, S. and Das, A. (1980) J. Mol. Biol. 140, 57–75.
[7] De Venditiis, E., Zahn, R. and Fasano, O. (1986) Eur. J. Biochem. 161, 473–478.
[8] Furth, M.E., Davis, L.J., Fleurdelys, B. and Scolnick, E.M. (1982) J. Virol. 43, 294–304.
[9] De Venditiis, E., Vitelli, A., Zahn, R. and Fasano, O. (1986) EMBO J. 5, 3657–3363.
[10] Tamanoi, F. (1988) Biochim. Biophys. Acta 948, 1–15.
[11] Gibbs, J.B. and Marshall, M.S. (1989) Microbiol. Rev. 53, 171–185.
[12] Broach, J.R. and Deschenes, R.J. (1990) Adv. Cancer Res. 54, 79–139.
[13] Field, J., Brock, D., Kataoka, T. and Wigler, M. (1987) Mol. Cell. Biol. 7, 2128–2133.
[14] Pai, E.F., Kabsch, W., Krengel, U., Holmes, K., John, J. and Wittinghofer, A. (1989) Nature 341, 209–214.
[15] Hancock, J.H., Magee, A.I., Childs, J.E. and Marshall, C.J. (1989) Cell 57, 1167–1177.
[16] Pai, E.F., Krengel, U., Petske, G.A., Goody, R.S., Kabsch, W. and Wittinghofer, A. (1990) EMBO J. 9, 2351–2359.
[17] Gross, M., Sweet, R.W., Sathe, G., Yokoyama, S., Fasano, O., Goldfarb, M., Wigler, M. and Rosenberg, M. (1985) Mol. Cell. Biol. 5, 1015–1024.
[18] Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J. and Wigler, M. (1984) Cell 36, 607–612.
[19] Dhar, R., Nieto, A., Koller, R., DeFeo-Jones, D. and Scolnik, E.M. (1984) Nucleic Acids Res. 12, 3611–3618.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Sigal, I.S., Gibbs, J.B., D'Alonzo, J.S. and Scolnick, E.M. (1986) Proc. Natl. Acad. Sci. USA 83, 4725–4729.
[22] Hattori, S., Clanton, D.J., Satoh, T., Nakamura, S., Kaziro, Y., Kawakita, M. and Shih, T.Y. (1987) Mol. Cell. Biol. 7, 1999–2002.