Yeast *RAS2* mutations modulating the ras-guanine exchange factor interaction

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Abstract We have used a two-hybrid approach to test various forms of *Saccharomyces cerevisiae* Ras2p for their ability to interact with the human guanine nucleotide exchange factor HGRF55. We have previously shown that a strong two-hybrid interaction is found between the HGRF55p and the dominant negative Ras2p(G22A) form of ras [Camus et al. (1995) Oncogene 11, 951–959]. We show here that the substitution N123I which weakens the guanine nucleotide binding also promotes ras-GEF interaction. We demonstrate that the R80D substitution alone completely abolishes the interaction of Ras2p(G22A) with GEF, whereas substitutions at positions 81, 82 and 73 have only small effects. Since residue 73 is involved in the response of ras to GEF, we propose that it plays a role in the conformational change induced by the GEF rather than in its binding. Those results emphasize the role of the α2 helix of the switch II region in the recognition of the GEF family.

Key words: Ras protein; Guanine exchange factor (GEF); Two-hybrid system; Cellular signalling; CAAX box; *Saccharomyces cerevisiae*

1. Introduction

Ras proteins act as molecular switches in several different signalling pathways. They are active when bound to GTP and switch their conformation to the inactive state when GTP is hydrolyzed to GDP [1,2]. The active form is recognized by different proteins in different organisms such as raf, ralGDS and phosphatidyl inositol-3 kinase (PI-3 kinase) in mammalian cells or adenylyl cyclase in *Saccharomyces cerevisiae*. Inactivation and activation of ras are controlled by two proteins, a GTPase activating protein (GAP) and a guanine nucleotide exchange factor (GEF), respectively. Although the target of ras differs, the catalytic domains of its regulatory elements are conserved throughout evolution to the same extent as ras proteins. This conservation reflects the need for specific interactions between ras and its regulators. Indeed ras and the active domain of GEF have been functionally exchanged between yeast and mammalian cells [3–7].

In the past decade extensive work has been devoted to the analysis of the structure-function relationship of the ras proteins. As a result, the effector domain of ras (switch I region) promoting the interaction with downstream targets and GAP has been well delimited [8–11]. In contrast, the portions of ras involved in the interaction with the GEF are much less characterized. Although genetic and biochemical analyses have identified several positions which are important for the response to the GEF [12–17], those which are more directly involved in the interaction and those which relay the conformational change remain to be defined.

The best candidates for a specific ras-GEF interaction lie in the α2-helix of the switch II region (amino acids 61–77 or 68–84, for mammalian rasp21 and *S. cerevisiae* Ras2p respectively). Substitutions at position 66 in the ras homologue *let-60* of *Caenorhabditis elegans*, affects its normal function in vulval development [18]. If this mutation acts by uncoupling the GEF and ras, it is expected both to relieve a dominant negative ras mutation sequestering the GEF and not to interfere with the dominant activation due to a ras oncogenic mutation. Indeed substitutions in p21ras at the same position lead to these phenotypes when assessed in NIH3T3 cells [12]. In contrast to wild type cells, the ratio of bound GTP on this ras mutant was not modified by overexpression of the yeast GEF Sdc25p, known to be active towards mammalian ras. Therefore the replacement of alanine by threonine or valine at position 66 (73 in Ras2p from *S. cerevisiae*) prevents, in vivo, the action of ras-GEF. The substitution G75S (in p21ras) was also shown to give similar phenotypes in vivo [12]. The replacement of the corresponding conserved glycine in *S. cerevisiae* Ras2p at position 82 by larger residues was shown to hinder the transition from inactive to active form [19]. In addition, amino acid substitutions in nearby residues at positions 80 and 81 were shown to impair the response to GEF in vitro on purified proteins [20]. Therefore the α2 helix, which is mobile and known to switch during the conformational change, appears to play a critical role in the response to GEF.

In order to assess the involvement of these ras residues in the interaction of the ras protein with a GEF we have used the two-hybrid system that we have recently developed to test the interaction between mutant forms of the human exchange factor HGRF55 and the yeast Ras2p(G22A) protein [21]. The Ras2p was fused to the activation domain of Gal4p and we analyze here the effect of selected mutations of Ras2p on interaction with the GEF in a two-hybrid system.

2. Materials and methods

2.1. Strains

The strains Y526 (*MATa Δgal4 Δgal80 URA3::GAL1-lacZ ura3 his3 ade2 lys2 leu2 trp1*; kindly provided by M. Werner, CEA, France) and HF7c (*MATa ura3-52, his3-200 lys2-801 ade2-101 trp1-901 leu2-3 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17-mers)$_3$-CYC1-lacZ* from the Clontech matchmaker kit) were used for the two-hybrid analysis with respectively the *lacZ* reporter gene and his-

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tidine prototrophy selection. The activity of all the Gal4-Ras2p and Gal4-Hgrf55p fusion proteins was tested respectively in the strains OL514 (*Δras1 Δras2 rca1*; Georges Renault, personal communication) and OL97.1-11B (*MATa cdc25-5 ura3 leu2 his3 his7*; [22]) by complementation assays.

2.2. Plasmid constructions

The details for the constructions of the pGBT9-*RAS2*, pGBT9-*RAS2*^{ala22} and pGAD424-*C-Hgrf* plasmids are described in [21]. Site directed mutagenesis by double-primer method ([25], see also modifications in [21]) was used to introduce appropriate mutations in the *RAS2* wild type or *RAS2*^{ala22} mutant context. The mutagenic primers designed to overlap the codon to be mutagenized are listed in Table 1. In most cases, two to three independent mutagenized clones were thus recovered and tested in the two-hybrid system. All mutations were checked by sequence analysis with automated DNA sequencing based on the dideoxy chain-termination method [23], using the ABI 373A sequencer. The double-strand DNAs were sequenced using the prism kit (Applied biosystem). During site mutagenesis of codon 82 (G to S substitution), a clone harboring an extra mutation (TTG→TCG; L30→S) along with the desired one was obtained.

2.3. β-Galactosidase assays

β-galactosidase activity was assayed for Y526 transformants either on filters or in liquid as previously described [1,3–5,12,19,21,24–32]. All assays were performed at least twice with several independent yeast transformants.

2.4. Protein analysis

Whole cell protein extracts were prepared as previously described [3,21,24–28,33]. Extracts were loaded on SDS-polyacrylamide gel [30] and immunoblotting was performed as previously described [34]. Ras2p protein antibodies were previously described [26].

3. Results and discussion

3.1. Requirements for ras to interact with GEF

In order to monitor the interaction between ras and its GEF we have used the system previously described [21]. Briefly, we have chosen to fuse the 281 carboxy-terminal residues of the catalytic domain of the human exchange factor HGRF55p to the activation domain of Gal4p since this region is sufficient to suppress *cdc25* mutations [6]. The entire open reading frame of the yeast *RAS2* gene was fused to the DNA-binding domain of the *GAL4* gene. The interaction of ras with GEF using the two-hybrid system has been reported previously by other groups using either a Ace1p system [35] or a lexA based system [36]. As in other reports, the stronger interaction was observed with a dominant negative mutation (Table 2). The Ras2p(G22A) is an altered form of ras which leads to a dominant negative phenotype in yeast [37]. This phenotype can be suppressed by overexpression of Cdc25p and has been interpreted as a sequestration of the major yeast GEF, the Cdc25p factor, by the Ras2p(G22A) protein. The two-hybrid results shown in Tables 2 and 3 confirm the much stronger interaction of this substituted form with a GEF. With the classical Gal4p-based two-hybrid system described here we get at least two orders of magnitude difference between the mutated and the wild type form of Ras2p (Table 2) as compared to the five-fold factor found in the Ace1p system [35].

In contrast to others reports, we did not, however, detect the interaction with the wild type ras protein in our system although the two-hybrid interaction has also been tested with the more sensitive reporter gene *HIS3* presents in the HF7c strain (Table 2 and data not shown). This result cannot be explained by the lack of either Ras2pwt or Hgrf55p proteins since the complementation assays performed in the appropriate strains work (see Sections 2 and 3). This also cannot be assigned to the heterologous system that we use because the same results have been obtained using yeast Cdc25p and Ras2p proteins (Marco Geymonat, personal communication).

Moreover, because of competition between the membrane localization signal of ras protein and the two-hybrid nuclear localization signal, the detection of the Ras-GEF interaction has required the inactivation of the carboxy-terminal CAAX box by a substitution of the conserved cysteine to arginine [21]. On the other hand, this result reveals that neither isoprenylation nor endoplasmic membrane association is strictly required for the Ras-GEF recognition, but we cannot exclude that membrane association enhances the interaction.

3.2. Preferential binding of GEF to dominant negative alleles of ras

As mentioned above, a preferential binding of the GEF to the dominant negative form Ras2p(G22A) has been observed in all three independent two-hybrid assays ([2,6,10,14–17,23,34–39], and this work). In addition, we extend this observation to another substitution, Ras2p(N123I), which also leads to a dominant negative form of ras (Table 2). It has been reported that two substitutions in the guanine nucleotide pocket NKXD of v-H-ras [27], N116I and K117E, allow the formation of a stable complex with the GEF domain of Sdc25p [29]. These modifications result in a reduced affinity for guanine nucleotide and confirm that empty forms of ras display a stronger affinity for the GEF [31]. In our experiments we find that only the N123I (N116I) substitution promotes a strong two-hybrid interaction (Table 2) whereas the Ras2p(K124E) (K117E in v-H-ras) behaves as the wild type and is even able to complement the deletion of *RAS* genes (Fig. 1 and see Section 3.5).

This difference can be explained by the differential requirement for GTP to promote the dissociation of the complex ras-GEF which was measured to be 0.08 mM and 10 mM for the p21(K117E) and p21(N116I) respectively [29]. With a cellular concentration in the millimolar range, only the Ras2p(K124E)-GEF complex is expected to be displaced by GTP. Thus in vivo, the Ras2p(K124E) protein no longer

Table 1
Oligonucleotides used for the site-directed mutagenesis of *RAS2* or *RAS2*^{ala22}

RAS2/oli16	(A73→T)	^{208}GAA TAC TCT aCT ATG AGG GAA C^{229}
RAS2/oli17	(A73→V)	^{208}GAA TAC TCT gtT ATG AGG GAA C^{229}
RAS2/oli18	(R80→D)	^{226}GAA CAA TAC ATG gaC AAC GGC GA^{248}
RAS2/oli19z	(N81→D)	^{229}CAA TAC ATG CGC gAC GGC GAA G^{250}
RAS2/oli20	(G82→S)	^{234}C ATG CGC AAC tcC GAA GGA TTC C^{256}
RAS2/oli21	(N123→I)	^{358}GTT GTT GGT AtC AAA TCT GAT TTA G^{382}
RAS2/oli22	(K124→E)	^{358}GTT GTT GGT AAC gAA TCT GAT TTA G^{382}

The nucleotides modified by mutagenesis are in lower case. The corresponding codons are underlined and the amino acid substitutions are indicated in parentheses. The positions of the oligonucleotides on the *RAS2* sequence are indicated using the first ATG as position one.

forms a stable complex with GEF as observed in the two-hybrid assay (Table 2). Moreover, the different behavior observed here between these N116I and K117E substitutions argues in favor of the selectivity of this assay to follow the Ras-GEF interaction.

3.3. Substitutions in the α2 helix of ras differentially affect the interaction with the GEF

Second mutations have been introduced in Ras2p(G22A) to test their effect in the two-hybrid interaction assay. We have chosen to change positions 73, 80, 81 and 82 which are known to be involved in the response to GEF [12,13,17,20].

3.3.1. Position 73 in Ras2p is not essential for the two-hybrid interaction. We have chosen to analyze substitutions at position 66 (73 in yeast Ras2p), which was considered the best candidate for ras-GEF interaction since its substitution leads to a stable protein that affects the mammalian cells response to GEF, abolishes the sequestering effect of dominant negative mutation at position 17 in p21ras but does not alter the activated conformation of an oncogenic ras protein [12]. As shown in Table 3, neither A to T nor A to V substitution at position 73 in yeast Ras2p eliminates the two-hybrid interaction. The A73T substitution has no detectable effect on the GEF interaction and the A73V substitution only reduces the level of β-galactosidase by a factor of two. This result shows that this position is not a key determinant for the establishment of an interaction with the GEF in the two-hybrid system used here. The greater effect observed with the A73V substitution as compared to the A73T substitution could still indicate some interference with the GEF interaction due to steric hindrance. However, the two-fold effect measured in the two-hybrid assay appears too small to account for the biological effects reported for these substitutions in vivo. Therefore it seems more likely that the alanine at position 73 (66 in p21ras) is required for the proper change in ras conformation induced by the binding of the GEF rather than for the binding itself. Indeed, the introduction of a larger residue in the switch II region might block the conformational change required for ras activation in response to GEF.

3.3.2. Position 80 in Ras2p has an essential role for ras GEF interaction. The R80D substitution has a dramatic effect in preventing any activation of the *GAL1* promoter (Table 3). This result is not due to the absence or to the degradation of the corresponding protein in yeast, since for all ras mutant tested here, equal levels of protein were detected by Western blotting (not shown). This result confirms the importance of position 80 in ras for the action of a GEF. It could suggest that this arginine residue is directly involved in the interaction by its positive charge at the surface of the molecule. It is interesting to note that with the same two-hybrid system, the only substitution found in the GEF to be able to disrupt the interaction with ras was the change of an arginine to an alanine [21]. Moreover, it has been found that with a reverse ion pair change at the positively charged residue R1374 in yeast Cdc25p and the negatively charged residue E63 in H-ras, it was possible to partially suppress the non-functional state of each mutant protein [38]. Taking together, these results reinforce the possibility that ras-GEF interaction is controlled by a salt bridge between the two proteins.

Table 2
Two-hybrid interaction of various single ras mutant forms with guanine exchange factor Hgrf55p

Tested interaction	β-Galactosidase activity
HGRF55p/Ras2p(wild type)	0.1
HGRF55p/Ras2p(G22A)	9.7
HGRF55p/Ras2p(N123I)	10
HGRF55p/Ras2p(K124E)	0.1
HGRF55p/Ras2p(R80D)	0.1
HGRF55p/Ras2p(N81D)	0.1
HGRF55p/Ras2p(G82S)	0.1

The β-galactosidase activity is expressed in nmol of *o*-nitrophenyl-β-D-galactoside hydrolysed per minute and per mg of protein. The value of 0.1 corresponds to the background level. A value of 1000 is obtained for the entire Gal4p activator itself.

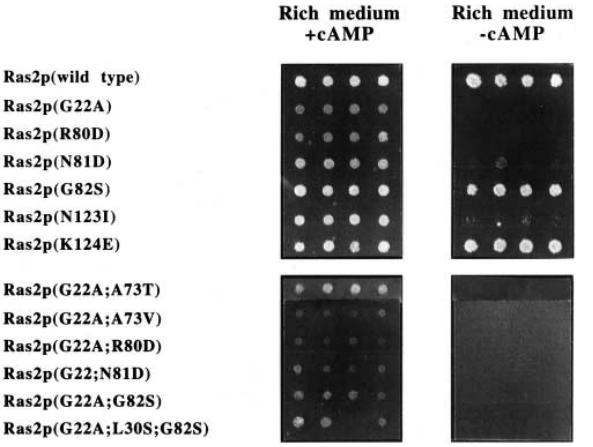

Fig. 1. Complementation assays in strain OL514 (Δ*ras1* Δ*ras2 rca*). The plasmids used in the two-hybrid system harboring a Ras2p wild type or mutant forms were tested for complementation in strain OL514. Four independent yeast transformants first grown on selective medium containing 5 mM cAMP were further tested on rich medium with or without cAMP. Growth was carried out at 30°C for 3 days.

In contrast to the effect of the substitution at position 80, substitutions at non-charged positions N81 or G82 show only mild effects on the ras-GEF interaction (Table 3). In previous results obtained [17], it was difficult to appreciate the contribution of a substitution at position 81 as compared to position 80 since they were both associated. We demonstrate here that the single substitution at position 81 does not significantly change the ras-GEF interaction. However, this mutation affects the activity of ras as shown in the complementation assay (Fig. 1). Position 82, which is important for the transition from the inactive to the active state of ras [19], does not appear to be directly involved in the interaction with the GEF nor in the biological activity of ras (Fig. 1).

3.4. Substitution at position 30 might alter ras nucleotide bound state

In the course of G82S mutagenesis we have obtained a clone harboring an additional mutation which leads to the substitution L30S (see Section 2). This Ras2p(G22A; L30S; G82S) mutant form has been introduced in the two-hybrid assay. The β-galactosidase activity detected is 10-fold smaller than that measured for the Ras2p(G22A; G82S) form (Table 3). This L30S substitution might be expected to reduce the GTPase activity leading to an accumulation of the ras.GTP bound form that no longer interacts with GEF. Recently, L23 in rasp21 (equivalent to L30 in Ras2p) has been shown to be involved in strong contact with the strictly conserved F156

Table 3
Two-hybrid interaction of Ras2p(G22A) associated mutant forms with guanine exchange factor HGRF55p

Tested interaction	β-Galactosidase activity[a]	Ratio (%)[b]
HGRF55p/Ras2p(G22A)	9.7	100
HGRF55p/Ras2p(G22A;A73T)	10.3	106
HGRF55p/Ras2p(G22A;A73V)	4.7	48
HGRF55p/Ras2p(G22A;R80D)	0.1	<1
HGRF55p/Ras2p(G22A;N81D)	11	113
HGRF55p/Ras2p(G22A;G82S)	7.8	80
HGRF55p/Ras2p(G22A;L30S;G82S)	0.85	8.7

[a]β-Galactosidase activity is expressed in nmol of *o*-nitrophenyl-β-D-galactoside hydrolysed per minute and per mg of protein.
[b]β-Galactosidase activity is expressed as the percentage of the remaining activity detected for a given Ras2p double or triple mutant form relative to the activity measured for Ras2p(G22A).

residue (F149 in yeast Ras2 protein) [39]. This conserved phenylalanine residue also participates in the GDP/GTP switch since a F149L substitution leads to increased levels of ras-GTP in vivo [39]. According to these results, it could be suggested that the L30S mutation may alter the ras nucleotide binding state and thus modify the ras-GEF interaction as observed with the two-hybrid assay. However, we cannot exclude that the folding of the Ras2p(G22A; L30S; G82S) mutant protein is so altered that the ras-GEF interaction is greatly affected but not abolished.

3.5. Complementation assay of the ras constructs

We have checked the ability of all ras constructions to complement a yeast strain deleted from the *RAS1* and *RAS2* genes (Fig. 1). Strain OL514 (*Δras1 Δras2 rca1*) contains the *rca1/pde2* mutation which affects the phosphodiesterase involved in the cyclic adenosine 3′,5′-monophosphate (cAMP) degradation [40]. The lethality caused by *ras1* and *ras2* deletions can thus be rescued by the addition of cAMP in the growth medium. When a given *RAS* gene is introduced, the transformed strain will grow without addition of exogenous cAMP only if the protein is functional. All ras mutants were tested in that strain. We have found that the wild type *RAS2* and the two mutants $ras2^{G82S}$ and $ras2^{K124E}$ were able to complement the defect of *RAS* (Fig. 1). This result indicates that the fusion of the Ras2p protein to the activation domain of Gal4p does not impair its ability to interact with its target and activator. In addition it shows that the CAAX box is not essential for suppression of the growth defect in yeast, at least when the corresponding ras protein is overexpressed.

The lack of complementation exhibited by the other constructs suggests that they are not or much less functional. As previously mentioned, it is not due to the degradation of the fusion proteins since they are all found in similar amounts in Western blotting (not shown). This absence of growth is expected for the dominant negative forms Ras2p(K123E) and Ras2p(G22A). Moreover, the dominant negative phenotype associated with the single G22A substitution cannot be rescued by any of the combined second mutation (Fig. 1), not even by the R80D one which strongly inhibited ras-GEF interaction measured by the two-hybrid assay and thus relieves the sequestration effect of the G22A substitution (Table 3). The complementation assay also confirms that substitutions at positions 80 and 81 lead to inactive forms as already observed [20].

4. Conclusion

Our results emphasize the importance of the α2 helix in the response of ras to the GEF. The orientation of this helix has been proposed to change between the GDP and the GTP form [1,2,6,9,10,13–16,20,23,32,34,35,37–39]. Therefore its involvement in the interaction with GEF could be the basis for a specificity in the recognition of the ras.GDP bound form by a guanine exchange factor. The fact that in vitro, GEF are also able to release GTP suggests the existence of other sites of interaction with ras [24]. The region 103–108 of ras, which does not change during the transition from the GDP to the GTP state, could be one of these since it has been shown that substitutions in that region abolished the response to Cdc25p [15,16].

Among the various positions required for the GEF effect on ras, only one was found to be critical in the two-hybrid interaction. Although we cannot exclude that the change in conformation induced by substitution G22A slightly modifies the ras-GEF interaction, we can postulate that residue 80 in ras2p is the most directly involved amino acid in this contact. The substitutions in position 73 themselves may just prevent the change in conformation induced by the interaction and required to form the active state of ras.

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