The EMBO Journal vol.11 no.8 pp.2855–2862, 1992

RAS residues that are distant from the GDP binding site play a critical role in dissociation factor-stimulated release of GDP

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Communicated by G.Cesareni

We have previously shown that a conserved glycine at position 82 of the yeast RAS2 protein is involved in the conversion of RAS proteins from the GDP- to the GTP-bound form. We have now investigated the role of glycine 82 and neighbouring amino acids of the distal switch II region in the physiological mechanism of activation of RAS. We have introduced single and double amino acid substitutions at positions 80–83 of the *RAS2* gene, and we have investigated the interaction of the corresponding proteins with a yeast GDP dissociation stimulator (SDC25 C-domain). Using purified RAS proteins, we have found that the SDC25-stimulated conversion of RAS from the GDP-bound inactive state to the GTP-bound active state was severely impaired by amino acid substitutions at positions 80–81. However, the rate and the extent of conversion from the GDP- to the GTP-bound form in the absence of dissociation factor was unaffected. The insensitivity of the mutated proteins to the dissociation factor *in vitro* was paralleled by an inhibitory effect on growth *in vivo*. The mutations did not significantly affect the interaction of RAS with adenylyl cyclase. These findings point to residues 80–82 as important determinants of the response of RAS to GDP dissociation factors. This suggests a molecular model for the enhancement of nucleotide release from RAS by such factors.

Key words: GDP dissociation factor/gene replacement/*RAS*/*SDC25*/yeast

Introduction

RAS proteins can modulate growth in evolutionarily distant systems such as vertebrates and yeast (for reviews, see Tamanoi, 1988; Gibbs and Marshall, 1989; Broach and Deschenes; 1990; Lowy *et al.*, 1991). In these and other systems, the function of RAS is controlled by positive and negative effectors. Several regions of the RAS molecule have been shown to be involved in the interaction with the effectors (reviewed by Bourne *et al.*, 1990, 1991; Kaziro *et al.*, 1991; Pai, 1991). The 'switch I' region that includes residues 32–40 (human Ha-*ras* p21 coordinates) appears to be critical for biological activity as well as for interaction with GTPase activating proteins (GAPs). A second region, called 'switch II', extends from residue 60 to residue 75, and it includes tyrosine 64 which is important for interaction with GAP (Antonny *et al.*, 1991). It is also well established that C-terminal residues of RAS play a crucial role in determining its membrane association (Lowy *et al.*, 1991).

Mutagenesis studies have indicated that the distal part of the switch II region is required for the biological function of RAS. This region includes the conserved Gly82 of the yeast RAS2 protein, corresponding to Gly75 of the human p21 (Fasano *et al.*, 1988) and Gly75 of the *Caenorhabditis elegans let-60* RAS (Beitel *et al.*, 1990). However, data from Sigal *et al.* (1986) and Willumsen *et al.* (1986) indicated that

Table I. Replacement vectors used to introduce mutations into the chromosomal *RAS2* gene and predicted amino acid changes within the corresponding protein

Plasmid vector[a]	Amino acid and nucleotide sequence at position					
	79 ATG Met	80 CGC Arg	81 AAC Asn	82 GGC Gly	83 GAA Glu	84 GGA (wild-type) Gly
pR21[b]	ATG	GAC Asp	AAC			
pR22[b]	ATG	CGC	GAC Asp			
pR23				GAC Asp		
pR10[c]				AGC Ser		
pR26[d]				GGC	CAA Gln	
pR11[c]						AGA Arg
pR4[c]				AGC Ser		AGA Arg
pR24[b]	ATG	GAC Asp	GAC Asp			
pR25[b]	ATG	CGC	GAC Asp	GAC Asp		

[a] The indicated replacement vectors encode Gly at position 19. For a detailed structure, see Fasano *et al.* (1988). Chimeric vectors with a Val codon at position 19 were constructed as indicated in Materials and methods, and named as follows: pR221 (Val19Asp81), pR241 (Val19Asp80Asp81) and pR251 (Val19Asp81Asp82).
[b] Note the loss of an *Fsp*I site as a consequence of the mutation.
[c] See Fasano *et al.* (1988).
[d] Note the formation of a *Hae*III site as a consequence of the mutation.

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distal residues of the switch II region were dispensable for the interaction of RAS proteins with their target. Therefore, the functional role of distal switch II residues has remained obscure.

Using a biochemical approach, we previously found that Gly82 constitutes a pivot point during the conversion of RAS from the GDP- to the GTP-bound form (Kavounis *et al.*, 1991). By analysing the *in vitro* and *in vivo* properties of mutated RAS2 proteins harbouring single amino acid substitutions in this region, we now show that residues 80–81 play a critical role in the response of RAS to a GDP dissociation factor. These findings together support a mechanism for the function of dissociation factors in which the latter increase the rate of GDP dissociation from RAS via long-distance interactions.

Results

Impaired growth of yeast cells with a disrupted RAS1 gene and expressing RAS2 proteins carrying single and double amino acid substitutions in the region 80–82

We have previously described methods for the construction of yeast strains with a disrupted *RAS1* gene *(ras1⁻)*, and with the chromosomal *RAS2* gene replaced by a mutated allele encoding a RAS2 protein with randomly induced single or double amino acid substitutions (Fasano *et al.*, 1988). We found that residues 40, 82 and 84 of the RAS2 protein were critical for its physiological function. Subsequent biochemical analysis of a mutated $RAS2^{S82}$ protein indicated that the corresponding wild-type residue was important for proper nucleotide binding via long range interactions (Kavounis *et al.*, 1991). To explore further the role of the region that includes residue 82, we used site-directed mutagenesis techniques to construct isogenic yeast strains carrying single and double amino acid substitutions within positions 80–84 of the *RAS2* gene product (in a *ras1⁻* background, see Materials and methods). The phenotype of the yeast transformants was scored by replica plating on media containing different carbon sources, and by observing growth at different temperatures. The mutated strains that we constructed, the corresponding amino acid changes in the RAS2 protein, and their growth properties, are listed in Tables I and II. The genotype of the strains was verified as shown in Figure 1.

The results confirmed our previous observations that a single amino acid substitution at position 84 had no or marginal growth phenotype (Fasano *et al.*, 1988). Likewise, no evident growth phenotype was associated with the replacement of Gly82 and Glu83 by Ser and Gln, respectively (Fasano *et al.*, 1988 and Table II), while a double mutant Ser82 Arg84 showed a temperature-sensitive growth on glycerol plates. The introduction of Asp at position 82 resulted in inability to grow in glycerol plates at 30 and 37°C (Table II). From Table II it also appears that the growth defect was increasingly evident for amino acid substitutions from positions 82 to 80. In fact, a single amino acid change at position 81 resulted in a selective growth defect only on glycerol, while an amino acid change at position 80 affected growth both on glycerol and on glucose. Appropriate genetic tests showed that the growth defect on glycerol was not due to a ϱ^- genotype (see Fasano *et al.*, 1988), but was rather linked to the mutated *RAS2* gene. Mutations leading to a double amino acid substitution at positions 81–82 resulted in temperature-sensitive growth on glucose. A double amino acid substitution at positions 80–81 led to lethality. In fact, sporulation of a *RAS1/ras1⁻ RAS2/ras2-D80D81* diploid strain did not produce viable spores with a *ras1⁻ ras2-D80D81* genotype (for details, see Materials and methods).

Table II. Effect of the expression of mutated RAS2 proteins on growth[a]

Strain	Relevant genotype[b]	Amino acid at position						Growth			
		19	80	81	82	83	84	YPD		YPGLY	
								30°C	37°C	30°C	37°C
19D-1	*RAS2* (wt)	Gly	Arg	Asn	Gly	Glu	Gly	+	+	+	+
19DRV19[c]	*RAS2-V19*	**Val**						+	+	+	+
19DRD80	*ras2-D80*		**Asp**					±	±	−	−
19DRD81	*ras2-D81*			**Asp**				+	+	−	−
19DRV19D81	*ras2-V19D81*	**Val**		**Asp**				+	+	+	−
19DRD82	*ras2-D82*				**Asp**			+	+	−	−
[d]	ras2-S82				**Ser**			+	+	+	+
19DRQ83	*ras2-Q83*					**Gln**		+	+	+	+
[d]	*ras2-R84*						**Arg**	+	+	+	+
TS1-6[d]	*ras2-ts1*				**Ser**		**Arg**	+	+	+	−
[e]	*ras2-D80D81*		**Asp**	**Asp**				−	−	−	−
19DRV19D80D81	*ras2-V19D80D81*	**Val**	**Asp**	**Asp**				+	+	−	−
19DRD81D82	*ras2-D81D82*			**Asp**	**Asp**			+	−	−	−
19DRV19D81D82	*ras2-V19D81D82*	**Val**		**Asp**	**Asp**			+	+	−	−

[a]Note the absence of wild-type RAS1 and RAS2 proteins in the isogenic strains listed (19D-1 excepted).
[b]The strains were derived by transformation from a single *ras1⁻* parental strain. For details, see Materials and methods.
[c]Constructed as described by Feger *et al.* (1991).
[d]From Fasano *et al.* (1988).
[e]Lethal genotype, as established by the lack of viable haploid segregants from a sporulated diploid that was heterozygous for the mutations (see Materials and methods).

The lethality caused by a double amino acid substitution at positions 80–81 of RAS2 can be rescued by an additional activating amino acid change at position 19

The growth inhibition caused by single amino acid substitutions at positions 80–82 of the RAS2 protein (corresponding to residues 73–75 of the human H-*ras* p21) was in apparent contrast with previous observations by Sigal *et al.* (1986) and by Willumsen *et al.* (1986). In fact, using an oncogenically activated human p21, these authors had observed that single amino acid changes and deletions including positions 73 and 75 of p21 did not affect the transforming potential of the protein and its interaction with downstream elements. Therefore we tested the possibility that the defective phenotype resulting from a double amino acid substitution at position 80–81 could be bypassed by the presence of an additional, activating mutation. Indeed, we observed that the introduction of an activating amino acid substitution at position 19 (position 19 corresponds to 12 in

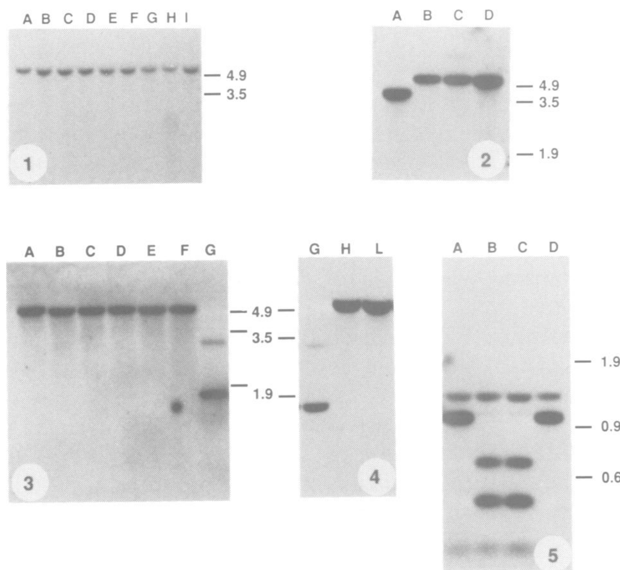

Fig. 1. Structure of genomic *RAS2* sequences in yeast transformants. The strain JR26-19D (relevant genotype *ras1⁻ RAS2 lys1-1*) was transformed with the purified *Eco*RI–*Hin*dIII fragment of the replacement vectors listed in Table I. The vectors carried various alleles of the *RAS2* gene and the selectable marker *SUP16* inserted into a non-essential 5′ flanking region (for details, see Materials and methods and Fasano *et al.*, 1988). Total yeast DNA from single transformant colonies was cleaved with either *Eco*RI (panels 1 and 2), *Eco*RI–*Fsp*I (panels 3 and 4), or *Hae*III (panel 5) and fractionated on a 1% agarose gel. Cleavage with *Eco*RI was used to determine the proper *RAS2::SUP16* integration pattern (Fasano *et al.*, 1988). *Fsp*I and *Hae*III were diagnostic for the presence or absence of mutations within the *RAS2* coding region (see Table I). After transfer to nitrocellulose, the filter was hybridized under stringent conditions with a radioactively labelled *Hpa*I fragment of the cloned *RAS2* gene (Powers *et al.*, 1984). Replacement vectors for the following *RAS2* alleles (Table I) were used. Panels 1, 3 and 4: lanes A and L, pR21, *ras2-D80::SUP16;* lane B, pR22, *ras2-D81::SUP16;* lane C, pR221, *ras2-V19D81::SUP16;* lane D, pR241, *ras2-V19D80D81::SUP16;* lane E, pR25, *ras2-D81D82::SUP16;* lane F, pR24, *ras2-D80D81::SUP16,* since a diploid recipient strain was used (see Materials and methods) the loaded DNA is from a selected haploid segregant (ABL2C, relevant genotype *RAS1 ras2-D80D81::SUP16);* lane G, pR2S, wild-type *RAS2::SUP16;* lane H, pR251, *ras2-V19D81D82::SUP16;* lane I, pR23, *ras2-D82::SUP16.* Panels 2 and 5: lane A, original strain JR26-19D, untransformed; lanes B and C, pR26, *ras2-Q83::SUP16;* lane D, pR2S, wild-type *RAS2::SUP16.* For the denomination of the corresponding yeast strains, see Table II.

the human p21, Powers *et al.*, 1984) in a RAS2 protein with a double 80–81 mutation restored viability, even though growth was not as good as for strains expressing a wild-type RAS2 protein (Table II). From the same table a clear, albeit incomplete suppression of other deleterious amino acid substitutions in the region 80–82 by the additional presence of Val at position 19 is also evident.

A RAS2 protein with a double amino acid substitution at positions 80–81 is capable of interacting with adenylyl cyclase

We investigated the biochemical properties of yeast membranes expressing a RAS2 protein with a double 80–81 amino acid substitution. The lethality of the double mutant alone (Table II) prevented its direct biochemical characterization in yeast. Therefore, we measured the adenylyl cyclase activity of membranes from strains expressing either a $RAS2^{V19}$ protein (genotype *ras1⁻ RAS2-V19*) or a protein with a triple amino acid substitution (genotype *ras1⁻ RAS2-V19D80D81*). In agreement with previous findings (Broek *et al.*, 1985) membranes from the strain expressing an activated $RAS2^{V19}$ protein showed an *in vitro* Mg^{2+}-dependent adenylyl cyclase activity that was not further stimulated by the non-hydrolysable GTP analogue Gpp(NH)p (Figure 2). The additional presence of a double amino acid substitution at positions 80–81 did not abolish the ability of the activated protein to stimulate adenylyl cyclase in the presence of Mg^{2+} ions. Interestingly, the Mg^{2+}-dependent activity of the triple mutant was stimulated by preincubation with Gpp(NH)p (Figure 2), thus suggesting that at least part of the protein was present in the membranes in a GDP-bound form. The higher activity of membranes expressing the $RAS2^{V19D80D81}$ protein was possibly related to the better expression of this protein *in vivo*, compared

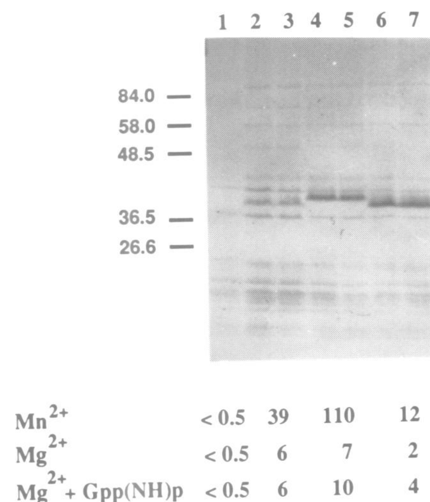

Fig. 2. Expression and adenylyl cyclase activities of wild-type RAS2, $RAS2^{V19}$ and $RAS2^{V19D80D81}$ proteins in yeast membranes. Total yeast membrane preparations (50 μg) were fractionated by SDS–PAGE and immunoblotted with RAS-specific polyclonal antibodies. With the exception of the sample in lane 1, membranes were loaded in duplicate. Strains used for the preparation of membranes were: ABE2A (*ras1⁻ ras2⁻ cyr1⁻*), lane 1; 19DRV19 (*ras1⁻ RAS2-V19*), (lanes 2 and 3); 19DRV19D80D81 (*ras1⁻ RAS2-V19D80D81*), lanes 4 and 5; 19D-1 (*ras1⁻ RAS2*), lanes 6 and 7. Numbers at the bottom of the figure represent average adenylyl cyclase activities (pmol cyclic AMP produced/mg membrane protein/min) in the presence of either 1 mM $MnCl_2$ or 5 mM $MgCl_2$. Where indicated, Gpp(NH)p was added at the final concentration of 100 μM. For details about the strains and the assay, see Table II and Materials and methods.

with RAS2^{V19} (Figure 2). We observed a lower electrophoretic mobility of the RAS2^{V19D80D81} protein compared with the wild-type (Figure 2), that was also detectable upon expression of the protein in bacteria (not shown).

The partial rescue of the biological activity of a mutated RAS2^{D80D81} protein by an activating amino acid substitution, and the ability of the triple mutated RAS2^{V19D80D81} protein to stimulate adenylyl cyclase in a Gpp(NH)p-dependent manner, suggested that the D80D81 substitution led to a protein that was defective in its physiological activation mechanism, but not in the interaction with adenylyl cyclase. To investigate directly the biochemical consequences of the replacement of residues 80–81, in the absence of the additional activating amino acid substitution at position 19, we used wild-type and mutated RAS2^{D80D81} proteins overexpressed and purified from *E.coli*. The availability of purified proteins (for the construction of expression vectors and for the purification procedure, see Materials and methods and Kavounis *et al.*, 1991) also facilitated a more quantitative analysis of their biochemical characteristics. Using an *in vitro* adenylyl cyclase reconstitution assay (De Vendittis *et al.*, 1986a; Feger *et al.*, 1991), we found that purified proteins (RAS2^{D80D81}, RAS2^{D80}, RAS2^{Q83}, RAS2^{S82}) in their Gpp(NH)p-bound form were almost as active as the wild-type protein in the stimulation of the yeast adenylyl cyclase activity of membranes from strains overexpressing the yeast adenylyl cyclase gene, and lacking active endogenous RAS proteins. A comparison between the wild-type and the RAS2^{D80D81} protein using this assay is shown in Table III. In addition, we found that protein concentrations required for half-maximal stimulation were nearly identical (Table III).

Purified RAS2 proteins carrying single and double amino acid substitutions at positions 80–82 show an impaired response to a nucleotide dissociation factor

The results described in the previous paragraphs suggested that residues 80–81 of the RAS2 protein were important for its physiological activation mechanism. GDP dissociation factors have been shown to be critical for the activation of RAS by stimulating the conversion from the inactive GDP-bound to the active GTP-bound form (Créchet *et al.*, 1990). Therefore, we tested the effect of the mutations on RAS–dissociation factor interaction. As a source of GDP dissociation factor, we used a bacterial extract enriched for the yeast SDC25 C-domain, a protein that has been shown to be very efficient in the stimulation of the conversion of RAS from the GDP to the GTP-bound form (Crechet *et al.*, 1990). We incubated purified RAS2 proteins in their GDP-bound form with a 20-fold molar excess of [^{3}H]Gpp(NH)p, and we followed the time course of formation of the radioactive RAS2–[^{3}H]Gpp(NH)p complex, in the absence and in the presence of the factor. Figure 3 shows that, while the rate of nucleotide exchange of the wild-type protein was strongly stimulated by the SDC25 C-domain, the RAS2^{D80} and RAS2^{D80D81} proteins were insensitive to stimulation. The RAS2^{S82} protein showed an intermediate sensitivity. It is important to note that the defect was of a kinetic nature. In fact, both the wild-type and the mutated proteins retained the capability to exchange nearly equimolar amounts of [^{3}H]Gpp(NH)p after incubation with excess nucleotide in the presence of low Mg^{2+} (not shown). Since it has been shown that the role of the SDC25 C-domain in the nucleotide exchange reaction is to accelerate the dissociation of the RAS-bound GDP (Crechet *et al.*, 1990), we determined the GDP off rates from preformed RAS2–[^{3}H]GDP complexes, in the absence and in the presence of the SDC25 C-domain. We found that the RAS2^{D80D81} and RAS2^{D80} proteins were almost completely insensitive to SDC25 stimulation (Figure 4 and Table IV). In fact, even in the presence of an amount of dissociation factor that strongly stimulated the GDP off rate from the wild-type protein, the RAS2^{D80D81} protein was marginally if at all stimulated by SDC25 (Table IV).

The rate of dissociation of GDP from the RAS2–GDP

Table III. *In vitro* adenylyl cyclase reconstitution assay with purified RAS2 proteins[a]

Purified protein	pmol	Adenylyl cyclase activity (units)[b]	
		Experiment 1	Experiment 2
RAS2 (wt)	0	13	13
	300	228	249
	600	372	404
	900	475	567
	1200	835	661
RAS2^{D80D81}	0	13	12
	300	155	156
	600	266	255
	900	374	359
	1200	461	428

[a] The RAS2 proteins were used in their Gpp(NH)p-bound form. Membrane preparations of *ras1$^-$ ras2$^-$* cells overexpressing the yeast adenylyl cyclase gene were used as a source of adenylyl cyclase. The final concentrations of $MgCl_2$ and Gpp(NH)p (including bound and free) in the assay were 5 mM and 100 μM, respectively. For details, see Materials and methods.
[b] Units of adenylyl cyclase activity correspond to pmol of cyclic AMP produced per mg of membrane proteins per min at 30°C.

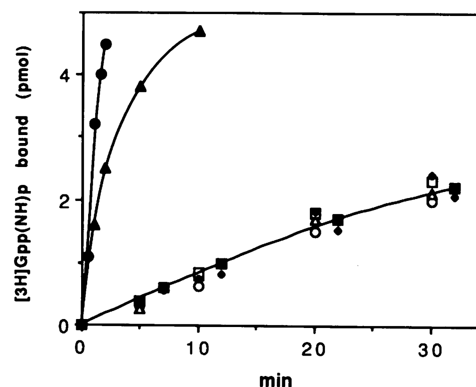

Fig. 3. Effect of amino acid substitutions in the region 80–82 of RAS on the spontaneous and SDC25-stimulated rate of formation of the RAS2–Gpp(NH)p complex. The reaction mixture contained, in a final vol of 170 μl of buffer E (see Materials and methods), 40 pmol of RAS2–GDP complex and either 6 pmol equivalents of partially purified SDC25 C-domain (filled symbols) or no SDC25 (empty symbols). Incubation was at 30°C. The reaction was started by the addition of 800 pmol of [^{3}H]Gpp(NH)p. At the indicated time intervals, 40 μl aliquots were withdrawn from the reaction mixture and the radioactive RAS2–nucleotide complex was determined by the nitrocellulose filter procedure. Symbols are: circles, wild-type RAS2 protein; triangles, RAS2^{S82}; squares, RAS2^{D80D81}; diamonds, RAS2^{D80}.

complex in the absence of the GDP dissociation factor was unaffected by the mutations (Table IV). This confirmed that residues 80–81 were important for a proper response of RAS to the SDC25 C-domain. We also investigated the effect of the mutations on the Gpp(NH)p off rates. Previously, an increased Gpp(NH)p off rate in the $RAS2^{S82}$ protein had been attributed to the introduction of mobility contraints in the hinge region corresponding to residue 82 (discussed by Kavounis *et al.*, 1991). The results, shown in Figure 5, confirmed that the Gpp(NH)p off rate of the $RAS2^{S82}$ protein was >2-fold faster than that of the wild-type protein (0.055 versus 0.025 min^{-1}), while a <2-fold increase was observed for the $RAS2^{D80D81}$ and $RAS2^{D80}$ proteins (calculated values 0.046 and 0.040 min^{-1}, respectively). Therefore, amino acid substitutions in the region 80–81 displayed a less pronounced effect than a substitution at position 82 on the Gpp(NH)p off rate. On the opposite side, the effect of mutations on dissociation factor-induced GDP release was much more dramatic for positions 80–81 than for position 82 (Table IV).

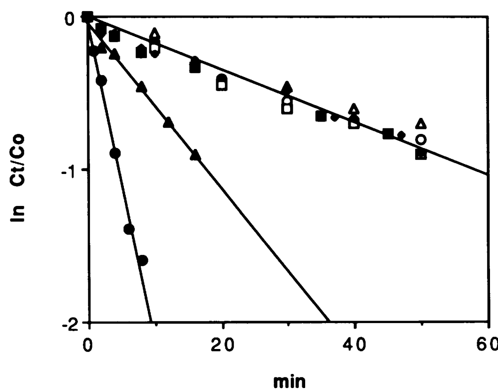

Fig. 4. Effect of amino acid substitutions in the region 80–82 of RAS on the spontaneous and SDC25-stimulated rate of dissociation of the RAS2–GDP complex. The reaction mixture contained, in a final vol of 300 μl of buffer E (see Materials and methods), 60 pmol of preformed RAS2–[^{3}H]GDP complex and either 2 pmol equivalents of partially purified SDC25 C-domain (filled symbols) or no addition (empty symbols). Incubation was at 30°C. The reaction was started by the addition of a 100-fold molar excess of unlabelled GDP. At the indicated time intervals, 45 μl aliquots were withdrawn from the reaction mixture and the radioactive RAS2–nucleotide complex was determined by the nitrocellulose filter procedure. Co is the initial amount of RAS–radioactive nucleotide complex, while Ct is the corresponding amount at the indicated time. Symbols are: circles, wild-type RAS2 protein; triangles, $RAS2^{S82}$; squares, $RAS2^{D80D81}$; diamonds, $RAS2^{D80}$.

Table IV. Effect of the SDC25 C-domain on the rate of dissociation of GDP from purified wild-type and mutated RAS2 proteins[a]

Protein	Amino acid at position				10^2k_{off} GDP (min^{-1})			
	80	81	82	83	–SCD25	+SCD25 (nM) 7	28	78
RAS2 (wild-type)	Arg	Asn	Gly	Glu	2	21	63	n.d.
$RAS2^{S82}$			**Ser**		2	6	29	n.d.
$RAS2^{Q83}$				**Gln**	2	21	n.d.	n.d.
$RAS2^{D80}$	**Asp**				2	2	4	5
$RAS2^{D80D81}$	**Asp**	**Asp**			2	2	3	3

Nucleotide off rates were calculated as described in Figure 4.

Discussion

Residues 80–81 are involved in the activation of the RAS2 protein by a GDP dissociation factor

The defective growth of yeast strains expressing a RAS2 protein with single and double amino acid substitutions in the region 80–82 (73–75 using Ha-*ras* p21 coordinates) indicates that this region is critical for the physiological function of the protein. Previous observations pointed to the importance of this region both in yeast and *C.elegans*. In fact, we reported that a Gly to Ser substitution at position 82 of the yeast RAS2 protein in a *ras1*$^-$ *ras2-ts1* yeast strain was necessary, but not sufficient to cause defective growth *in vivo* (Fasano *et al.*, 1988). Moreover, Beitel *et al.* (1990) showed that a recessive mutation at position 75 of the *C.elegans let-60* RAS led to a developmental defect.

The finding that residues 80–82 of the yeast RAS2 protein are important for a productive interaction with a GDP dissociation factor could provide a molecular explanation for the defective growth phenotypes of the corresponding mutants. We have observed a direct correlation between *in vitro* and *in vivo* data. The increasing severity of the biochemical defect going from amino acid substitutions at positions 82 to 80 was in line with the progressive impairment of growth associated with the expression of the corresponding proteins *in vivo*. However, we consider unlikely that a defective interaction with SDC25 is sufficient to explain the growth phenotype. In fact, a double amino acid substitution at positions 80–81 led to lethality, while the deletion of the *SDC25* gene in yeast had no apparent effect (Damak *et al.*, 1991). This can be reconciled with our observations if we assume that the mutated RAS2 proteins are unable to interact not only with SDC25, but also with the product of the essential *CDC25* gene, which shares extensive structural and functional similarity with *SDC25* (Damak *et al.*, 1991; Jones *et al.*, 1991). The observation that the overexpression of the 3′ terminal region of the

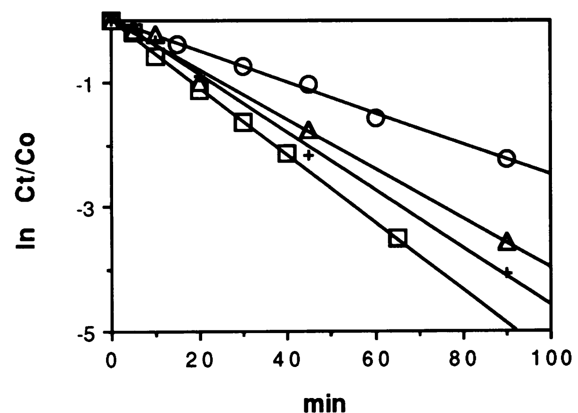

Fig. 5. Effect of amino acid substitutions in the region 80–82 of RAS on the rate of dissociation of the RAS2–Gpp(NH)p complex. The reaction mixture contained, in a final vol of 400 μl of buffer E (see Materials and methods), 25 pmol of preformed RAS2–[^{3}H]Gpp(NH)p complex. Incubation was at 30°C. The reaction was started by the addition of a 1000-fold molar excess of unlabelled Gpp(NH)p. At the indicated time intervals, 60 μl aliquots were withdrawn from the reaction mixture and the radioactive RAS2–nucleotide complex was determined by the nitrocellulose filter procedure. Co is the initial amount of Ras–radioactive nucleotide complex, while Ct is the corresponding amount at the indicated time. Symbols are: circles, wild-type RAS2 protein; triangles, $RAS2^{D80}$; crosses, $RAS2^{D80D81}$; squares, $RAS2^{S82}$.

CDC25 gene was able to suppress the partial growth defect of a yeast strain expressing a $RAS2^{S82R84}$ mutated protein is in line with this hypothesis (Frascotti *et al.*, 1991). Additional support is provided by the ability of an activating amino acid substitution at position 19 both to restore the viability of double mutants with amino acid substitutions at positions 80–81 (this paper) and to bypass $cdc25^-$ lethality (Broek *et al.*, 1987; Robinson *et al.*, 1987).

Other groups have described dominant negative mutations of the *RAS2* gene which possibly led to the formation of an inactive complex with a nucleotide dissociation factor (Powers *et al.*, 1989). The corresponding amino acid substitutions were at the level of residues 22 and 25, distant from the region 80–82 on a molecular scale. This suggests that the region 80–81 is necessary, but not sufficient to determine a productive interaction with GDP dissociation factors. This is also in line with the results of Hiroyoshi *et al.* (1991), who recently reported that post-translational modifications are required for the effect of a *smg* p21-specific dissociation factor. Other regions that might be involved in RAS–dissociation factor interaction include residues 66, 103–108 and 131–136 of the *C.elegans let-60 RAS* gene product (Beitel *et al.*, 1990). The involvement of the region 103–108 is also suggested by the work of Willumsen *et al.* (1991). Very likely, several regions of RAS play a role in the interaction with dissociation factors.

Recently, it has been shown that the *dbl* oncogene product is a dissociation factor that specifically catalyses the release of GDP from the human homologue of the RAS-related yeast CDC42 protein (Hart *et al.*, 1991; Ron *et al.*, 1991). The same authors reported that the *dbl* oncogene product did not act on H-*ras* p21. Since the CDC42 and the RAS2 proteins show no structural similarity at the level of distal switch II residues (reviewed by Valencia *et al.*, 1991a), this region could be a likely candidate for determining the specificity of RAS–dissociation factor interaction.

Long-range interactions play a critical role in dissociation factor-stimulated release of the nucleotide from RAS

We have previously shown that the replacement of Gly82 by Ser altered the kinetic properties of the nucleotide binding site (Kavounis *et al.*, 1991), even though Gly82 is distant from this site in the three dimensional structure of the highly homologous *ras* p21 (Pai *et al.*, 1989; Schlichting *et al.*, 1990). This suggested that the binding of macromolecular effectors to this region of the polypeptide chain, by inducing structural alterations analogous to those caused by the replacement of Gly by Ser, could affect nucleotide binding via long distance interactions. The results reported in this paper, indicating that residues 80–82 are critical for a productive interaction with the SDC25 C-domain, suggest that such a mechanism could be involved in the SDC25-induced dissociation of GDP from RAS. We envisage that, upon binding of SDC25 to RAS, either direct or indirect interactions involving residues 80–82 of RAS and SDC25 could lead to structural perturbations, that ultimately increase the off rate of the bound GDP. This hypothetical molecular mechanism for the stimulation of the GDP off rate is also in agreement with the three dimensional structure of RAS (Pai *et al.*, 1989; Schlichting *et al.*, 1990) and with recent biochemical studies on the interaction between this protein and the SDC25 C-domain (Mistou *et al.*, 1992). In fact, a physical link for the transmission of the effect between residues that are in close contact with the nucleotide and residues 80–82 could be provided by loop L4 and helix $\alpha 2$ in the RAS molecule (residues 67–80 of the yeast RAS2 protein). In this schematic model, residues 80–81 could be one of the major determinants for a proper response to the exchange factor, while Gly82 could have a prevalent role as a pivot point. Interestingly, Arg80 of the yeast RAS2 protein is conserved within the evolutionary tree of *RAS* genes involved in growth control (reviewed by Valencia *et al.*, 1991a,b). Like the corresponding Arg73 of the human *ras* p21, it might participate in the formation of a salt bridge with neighbouring residues (Tong *et al.*, 1991), and it is very likely surface-exposed. In fact, a mutated $RAS2^{D80}$ protein was unable to bind the RAS-specific monoclonal antibody Y13–259 (O.Fasano and A.C.Verrotti, unpublished). Earlier studies, indicating that the same monoclonal antibody (Furth *et al.*, 1982) was capable both of binding to the loop L4–helix $\alpha 2$ region (Sigal *et al.*, 1986) and of inhibiting GDP dissociation (Hattori *et al.*, 1987), also pointed to a crucial role of the corresponding region in determining the kinetic parameters of RAS–nucleotide interaction.

It should be noted that the high thermal mobility of loop L4 and helix $\alpha 2$ (Schlichting *et al.*, 1990) is in apparent contrast with a role for this region as a linker between nucleotide-proximal residues and distal positions 80–81. To reconcile structural data with functional observations, we should take into account the possibility that the high thermal mobility of the region including loop L4 and helix $\alpha 2$ could reflect a small number of distinct isomeric states of the polypeptide chain, rather than a loose structure. The observation by Antonny *et al.* (1991) that the replacement of Tyr71 by Trp (yeast RAS2 coordinates) did not modify the response of RAS to the SDC25 C-domain also confirms the possibility that intermediate residues on the 67–80 chain might act as linkers involved in the transmission to the nucleotide of dynamic perturbations that originate in the distal part of the switch II region.

Materials and methods

Media

The standard rich medium used was YPD (2% bacto-peptone, 1% yeast extract and 2% glucose). Media with carbon sources other than glucose contained either 3% glycerol (YPGLY) or 2% galactose (YPGAL). Complete synthetic medium contained 0.67% yeast nitrogen base without amino acids (Difco). Amino acids and nucleic acid bases were added as indicated by the yeast manual (Sherman *et al.*, 1983).

Yeast strains

The construction of haploid yeast strains with a disrupted *RAS1* gene ($ras1^-$), and with the chromosomal *RAS2* gene replaced by a mutated allele flanked by the selectable marker *SUP16* in a 5′ non-essential region, has been described previously (Fasano *et al.*, 1988). The strains were derived from JR26-19D (*MAT*a ade2-1 can*1-100 his3 leu2-3,112 lys1-1 ura3-52 ras1::URA3*, Fasano *et al.*, 1988) by transformation with appropriate yeast integration vectors in which point mutations leading to single or double amino acid changes had been introduced by site-directed mutagenesis (see next paragraph). The vectors were completely sequenced in order to exclude the presence of additional mutations. Table I shows a list of mutations introduced at the *RAS2* locus. The list of corresponding strains is shown in Table II. To eliminate any possible interference of the marker *SUP16* during the evaluation of the phenotypic effect of the mutations, we always compared the mutated yeasts with the JR26-19D-derived strain 19D-1 (genotype *ras1*$^-$ *RAS2::SUP16*), carrying a wild-type *RAS2* gene flanked by the marker *SUP16*. The construction of this strain has been described

by De Venditties *et al.* (1986a). To score mutations with a lethal phenotype (*ras2-D80D81*), we constructed the diploid strain FBC114 (relevant genotype *RAS1/ras1::URA3 RAS2/RAS2 ura3-52/ura3-52 lys1-1/lys1-1*) in which one *RAS1* allele was inactivated by the insertion of the marker *URA3* (Kataoka *et al.*, 1984). Upon transformation of the diploid with the *Eco*RI–*Hin*dIII fragment of the plasmid pR24 (Table I) and selection for Lys^+, *RAS1/ras1::URA3 RAS2/ras2-D80D81::SUP16* transformants were sporulated and the genotype of the germinated spores was assigned on the basis of the indicated markers. The construction of the strain ABE2A (relevant genotype *ras1-Δ ras2-Δ cyr1::HIS3 bcy1-11*), with the *CYR1* gene disrupted by the marker *HIS3* (Feger *et al.*, 1991) and with deletions within the coding region of the chromosomal *RAS1* and *RAS2* genes will be described elsewhere.

Yeast integration and expression vectors

Integration vectors for the replacement of the chromosomal *RAS2* gene with mutated alleles were derived from the plasmid pR2S (Fasano *et al.*, 1988). The *RAS2* gene (*Pst*I–*Hin*dIII fragment of pR2S) was first subcloned in M13mp18. Mutants were generated by site-directed mutagenesis, using the Amersham kit and appropriate oligonucleotides which differed from the wild-type sequence at the positions indicated in Table I. Wild-type *RAS2* sequences in pR2S were then replaced by mutated sequences to generate chimeric integration vectors. We also used the plasmid pR15, carrying a *RAS2* gene encoding valine at position 19 (*RAS2-V19*), to construct chimeric integration vectors in which mutations within the *Pst*I–*Hin*dIII region were combined with the activating mutation at codon 19. A list of the vectors is shown in Table I. For the overexpression of wild-type and mutated forms of the RAS2 protein in yeast we used the high copy number vector YEp51. A 1.2 kbp *Hpa*I–*Hin*dIII fragment of the *RAS2* gene including the complete coding region (Powers *et al.*, 1984) was cloned in the proper orientation using the *Sal*I and *Hin*dIII sites of the vector (Broach *et al.*, 1983). *Sal*I was rendered blunt-ended by filling-in before ligation.

Plasmids for the overproduction of mutated RAS2 proteins in E.coli

The vectors had the general structure of the previously described plasmid pAV1, that efficiently directs the synthesis in *E.coli* of the wild-type RAS2 protein under the control of an inducible λP_L promoter (Kavounis *et al.*, 1991). The following vectors for the expression of wild-type and mutated variants of the RAS2 protein were used in this study: pAV1 (wild-type RAS2), pAVS82 ($RAS2^{Ser82}$), pAVD80 ($RAS2^{Asp80}$), pAVQ83 ($RAS2^{Gln83}$), pAVD80D81 ($RAS2^{Asp80Asp81}$).

Purification of wild-type and mutated RAS2 proteins

Plasmid vectors for the synthesis of the yeast RAS2 protein in *E.coli* cells were constructed as described in the preceding paragraph. Pure, biologically active wild-type and mutated RAS2 proteins were obtained as described by Kavounis *et al.* (1991). Bacterial strains and induction conditions were also as described by the same authors.

Yeast transformations

Yeast transformations with either plasmid DNA or purified DNA fragments (10–20 μg) were carried out after treatment of yeast cells with lithium acetate, as described previously (Fasano *et al.*, 1988). Selection of transformants was carried out on synthetic medium, supplemented with appropriate amino acids and nucleic acid bases, using glucose as a carbon source.

Yeast membrane preparations and adenylyl cyclase assays

Yeast membranes were prepared as described by Fasano *et al.* (1988), from cells that were grown at 30°C in synthetic medium with appropriate amino acid supplements. The cells were harvested at an optical density of 0.6 at 595 nm. The adenylyl cyclase assay was as described by Broek *et al.* (1985), using the procedure of Salomon *et al.* (1974) for the separation of cyclic AMP from ATP. The assay was done in a final vol of 100 μl, using 60 μg of membrane proteins. One unit of adenylyl cyclase activity corresponds to 1 pmol of cyclic AMP produced per mg of membrane proteins in 1 min at 30°C. When indicated, the assay was carried out in the presence of Gpp(NH)p 100 μM. Either $MgCl_2$ or $MnCl_2$ was used as a divalent cation, at final concentrations of 5 mM and 1 mM, respectively. As a source of adenylyl cyclase for *in vitro* complementation assays with purified RAS2 proteins, we used membranes from the strain ABE2A (*ras1-Δ ras2-Δ cyr1::HIS3 bcy1-11*, see preceding paragraphs) transformed with the high copy number plasmid pYACE1 encoding the complete yeast adenylyl cyclase gene (Feger *et al.*, 1991). Membranes from this strain displayed a low RAS-independent activity that could be strongly stimulated by the addition of the purified RAS2 protein in its GTP- or Gpp(NH)p-bound form (Table III).

Immunoblotting procedures

Separation of crude extracts and membrane preparations by SDS–PAGE, transfer to nitrocellulose membrane filters and immunostaining with specific sera (immunoblots) was carried out as described by Feger *et al.* (1991). Polyclonal antibodies against the yeast RAS proteins have been described previously (De Vendittis *et al.*, 1986b).

Interaction of purified Ras proteins with guanosine nucleotides and with the SDC25 C-domain

Radioactive RAS2–nucleotide complexes were prepared by preincubating purified RAS2 proteins (complexed with GDP) with an excess of either $[^3H]$GDP or $[^3H]$Gpp(NH)p for 30 min at 30°C in 40 mM Tris–HCl, pH 7.5, 0.5 mM dithiothreitol, 0.1 mM $MgCl_2$. $(NH_4)_2SO_4$ at the final concentration of 100 mM was added to facilitate the exchange of the bound with the free radioactive nucleotide. The final concentration of Mg^{2+} in the experiments illustrated in this paper was 1 mM. In addition to Mg^{2+}, the standard reaction buffer (buffer E) contained 40 mM Tris–HCl pH 7.5, 100 mM NH_4Cl, 1 mM dithiothreitol, 3 mM $(NH_4)_2SO_4$, 0.1 mg/ml BSA and other components as indicated. The rate of dissociation of nucleotides from the corresponding RAS2–nucleotide complexes was measured by adding at zero time an excess of unlabelled GDP. The displacement of the radioactive nucleotide was followed at appropriate time intervals, either by the nitrocellulose filter procedure, or by loading aliquots of the reaction mixture on Sephadex G-50 columns, as described by De Vendittis *et al.* (1986b). Isolation and partial purification of the SDC25 C-domain was carried out by the method described in Crechet *et al.* (1990). The relative amount of SDC25 C-domain was determined by densitometric scanning.

Acknowledgements

We wish to thank Dr Chris Sander for helpful discussions, and Mrs Hilary Davies for reviewing the manuscript. C.K. was supported by a short-term EMBO fellowship. Work done in Italy was partially supported by a grant from the Associazione Italiana per la Ricerca sul Cancro.

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Received on February 10, 1992; revised on April 13, 1992