The EMBO Journal vol.11 no.7 pp.2391 – 2397, 1992

Mutations of Ha-*ras* p21 that define important regions for the molecular mechanism of the SDC25 C-domain, a guanine nucleotide dissociation stimulator

Michel-Yves Mistou[1], Eric Jacquet[1], Patrick Poullet[1], Hans Rensland[2], Petra Gideon[2], Ilme Schlichting[2], Alfred Wittinghofer[2] and Andrea Parmeggiani[1]

[1]S.D.I. no. 61840 du C.N.R.S., Laboratoire de Biochimie, Ecole Polytechnique, F-91128 Palaiseau Cedex, France and [2]Max-Planck-Institut für Medizinische Forschung, Abteilung Biophysik, Jahnstrasse 29, D-6900 Heidelberg, FRG

Communicated by A.Bernardi

The SDC25 C-domain is a very active guanine nucleotide dissociation stimulator (GDS) isolated from *Saccharomyces cerevisiae* which acts equally well on Ha-*ras* p21 and yeast RAS2. These properties make the SDC25 C-domain a suitable tool to study the basic mechanism of a GDS. The action of the SDC25 C-domain was analysed by mutation of structurally important regions of p21. Substitutions that influence the coordination of Mg^{2+}·GDP or the interaction of the guanine ring were found to stimulate the intrinsic dissociation of GDP and suppress the action of the SDC25 C-domain. No relevant effects were observed with mutations in the phosphate binding loop L1 or by deleting the last 23 C-terminal residues of p21. Substitutions in the switch region 1 (loop L2) and 2 (loop L4) of p21 strongly impaired the action of this GDS; however, we show that this effect is not related to a decreased affinity of the SDC25 C-domain for the mutated p21. No functional competition could be found between this GDS and the catalytic domain of the human GTPase activating protein (GAP). This indicates that GDS and GAP bind to different sites of the p21·nucleotide complex, even though the same mutations in loops L2 and L4 regions affect the activity of both effectors. Since these two regions appear not to be involved directly in the interaction with GDS, we conclude that the negative effect induced by their mutation is related to their function as switches of selective conformations during the GDP to GTP exchange reaction catalysed by GDS.

Key words: GTPase activating protein/guanine nucleotide dissociation stimulator/oncogenesis/RAS proteins

Introduction

Ha-*ras* p21, a guanine nucleotide binding protein, is an essential element for growth control and differentiation of the human cell (Bourne *et al.*, 1990, 1991). As for all guanine nucleotide binding proteins, the GTP-bound form of p21 is the 'on' switch of the downstream pathway and the GDP-bound form is the 'off' switch. Therefore the GTPase and GDP release of p21 are pivotal activities probably regulated by external signals. The existence of a GTPase activating protein (GAP) has been well established (Trahey and McCormick, 1987; Gibbs *et al.*, 1988) and recently guanine nucleotide dissociation stimulators (GDSs) for ras proteins have been identified in mammals (Downward *et al.*, 1990; Huang *et al.*, 1990; Wolfman and Macara 1990). In *Saccharomyces cerevisiae*, the SDC25 C-domain, a product of the 3′ terminal region of *SDC25*, has been isolated on the basis of its capacity to suppress the lethality associated with mutation of *CDC25*, a gene encoding a membrane-bound GDS of RAS proteins (Boy-Marcotte *et al.*, 1989; Garreau *et al.*, 1990; Jones *et al.*, 1991). *SDC25* belongs to a rapidly growing family of genes displaying extensive homologies, isolated from various organisms such as *S.cerevisiae* (*CDC25*, *SDC25*), *Schizosaccharomyces pombe* (*Ste6*, Hughes *et al.*, 1990) and drosophila (*SOS*, Simon *et al.*, 1991), whose products appear to activate GTP-binding proteins regulating diverse important pathways. The properties of the SDC25 C-domain to be active also on p21 both *in vitro* (Créchet *et al.*, 1990a) and *in vivo* (Rey *et al.*, 1991) has allowed us to analyse the basic properties of a GDS by modifying important structural subsites of p21, such as those involved in the specific conformations of the GDP- and GTP-bound states or in the binding of the substrate, taking into account the three-dimensional structure at high resolution (Pai *et al.*, 1989, 1990; Tong *et al.*, 1989; Schlichting *et al.*, 1990). Our results characterize structural elements of p21 essential for the action of this GDS, shedding light on the molecular mechanisms involved in the stimulated p21·nucleotide dissociation.

Results

The GDS activity of the SDC25 C-domain on Ras proteins

The SDC25 C-domain is a very active stimulator of the nucleotide release from p21, at least as active as reported for the purified GDS from brain (Huang *et al.*, 1990) and the GDS of smg-p21A (Hiroyoshi *et al.*, 1991). The SDC25 C-domain can decrease the half-life of the p21·GDP complex by several hundred-fold (Figure 1); the response of p21 and *S.cerevisiae* RAS2 is approximately equal. The determination of the GDP to GTP exchange as a function of increasing concentrations of p21·GDP, under conditions in which the velocity is linear and proportional to the SDC25 C-domain concentration (Figure 2), allows the estimation of the k_{cat} of the reaction; the efficiency of the interaction between this GDS and p21 can be inferred from the $K_{m\ p21\cdot GDP}$. These properties justify the use of the SDC25 C-domain with a heterologous RAS protein to study the basic features of the GDS mechanism.

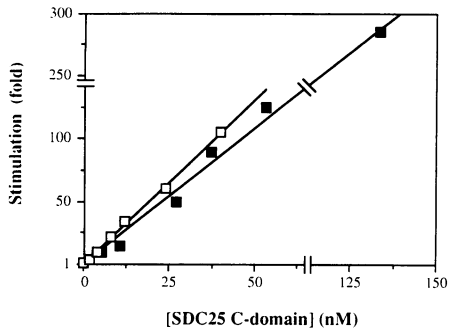

Fig. 1. Effect of the SDC25 C-domain concentration on the stimulation of the GDP dissociation rate. The dissociation rates of p21wt·GDP (■) and RAS2wt·GDP (□) complexes were determined for each point kinetically as described in Materials and methods. The effect (fold increase in stimulation) is expressed as compared with the intrinsic GDP dissociation rate.

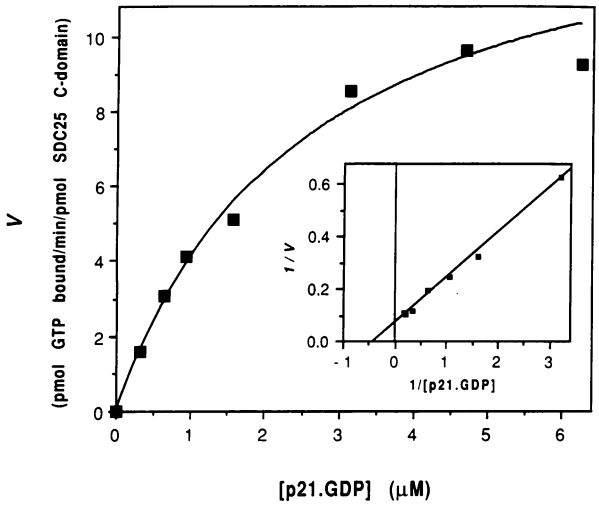

Fig. 2. Plot of the initial GDP−[γ-^{32}P]GTP exchange rate of p21wt in the presence of the SDC25 C-domain as a function of the p21·GDP concentration. The inset shows the double reciprocal plot.

Effect of mutations of p21 on the action of the SDC25 C-domain

For this study, we have utilized the GDP complexes of p21 mutants modified in important regions (Figure 3). An experiment using the fluorescence method is illustrated in Figure 4 and the results obtained with the diverse mutants are reported in Table I.

In the phosphate-binding L1 loop (residues 10−15), the substitution of two important residues, Gly12 and Gly13, has little effect on the activity of the SDC25 C-domain. Replacement of Gly12 with Valine does not alter the wild-type pattern at all. In the C-terminal truncated p21 Valine but not Proline, in the place of Gly12, slightly increases the SDC25 C-domain effect, while Aspartate induces a 50% inhibition. Substitution of Val12 is oncogenic (Barbacid, 1987), an effect associated with the inhibition of the p21 GTPase and the response to GAP (Trahey and McCormick, 1987; Gibbs *et al.*, 1988). Therefore mutation of this residue causes different effects on the response to GAP and GDS. Also the oncogenic mutation Gly13 to Valine does not change the response to GDS, while inhibiting the GAP effect. Unlike this, Gly13 to Serine moderately inhibits the SDC25 C-domain stimulation without essentially affecting the action

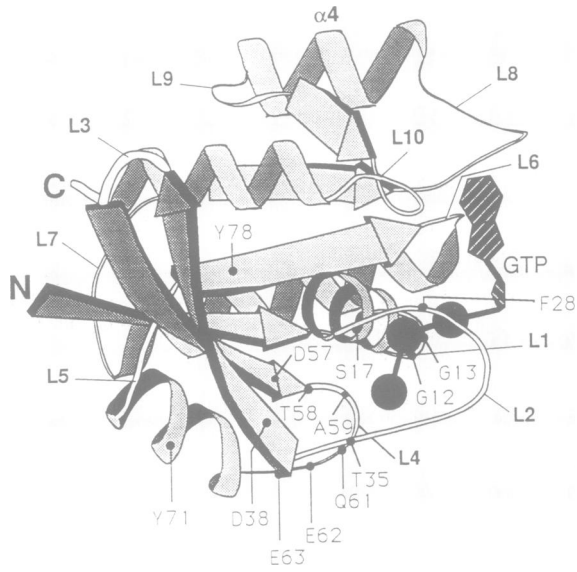

Fig. 3. The three-dimensional structure of the p21·GTP complex with the residues that have been substituted. Derived from Wittinghofer and Pai (1991).

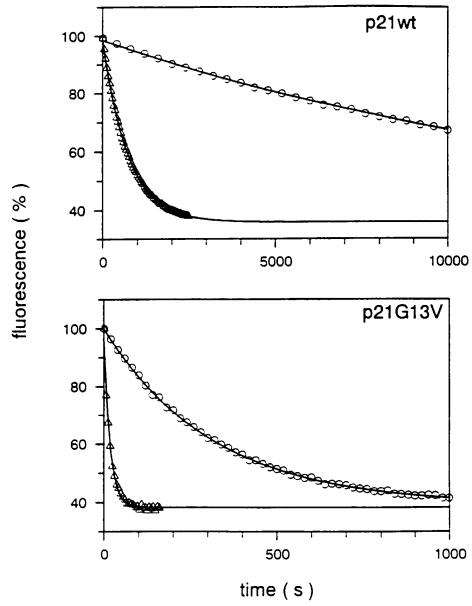

Fig. 4. Dissociation rates of p21·GDP complexes, as measured with the fluorescence assay. **Upper panel**, p21wt; **lower panel**, p21G13V; in the absence (○) and in the presence (△) of the SDC25 C-domain. The fluorescence signal decreases to the 35% fluorescence of unbound mantGDP.

of GAP. Interestingly, the intrinsic dissociation of the p21·GDP complex and the GDS effect can be affected in a different way by mutations. For instance, p21G13V has a dramatically increased intrinsic GDP dissociation, but the response to GDS is unchanged; the longer half-life of p21G12V (260 versus 104 min of p21wt) is not suppressed by GDS whose effect is the same as in p21wt.

The switch regions 1 and 2 include structures showing specific conformational changes upon binding of GDP or GTP (Milburn *et al.*, 1990; Schlichting *et al.*, 1990). The former contains the L2 loop and thus the largest part of the so-called effector region (residues 30−38); it is highly exposed to the solvent and is essential for the biological

Table I. Influence of the SDC25 C-domain on the half-life of GDP complexes of Ha-*ras* p21 mutants

Proteins	Half-life (min)[a]		Stimulation (fold)
	− SDC25 C domain	+ SDC25 C domain[b]	
p21wt	104	6.5	16
p21wt[c]	116	7.8	14.8
p21wt[d]	165	8.6	19.2
p21wt'[c]	85	4.2	20.2
p21G12V	260	17	15.3
p21G12V[c]	283	16.5	17.1
p21G12V'[c]	290	12.5	23.2
p21G12P'[c]	155	9	17.2
p21G12D'[c]	147	14.3	10.3
p21G13S	27.3	2.7	10.1
p21G13V[d]	3.7	0.2	18.5
p21S17A[d]	0.9	0.6	1.5
p21F28L[d]	5.6	2.4	2.3
p21T35A	87	18.5	4.7
p21D38E'[c]	146	27.5	5.3
p21D38A'[c]	250	140	1.8
p21D57A	20	8	2.5
p21T58V	100	5.7	17.5
p21A59T	12.5	2.1	6
p21Q61L	43	7.5	5.7
p21Q61H	70	35	2
p21E62H	99	64	1.5
p21E63H	127	40	3.1
p21Y71F	51	6.5	7.8
p21F78Y	85	5	17

[a]The half-life is calculated from the dissociation rate constant
[b]The final concentration of SDC25 C domain in all experiments is 8 nM
[c]Measured by filtration on Sephadex G25
[d]Measured by the fluorescence assay
All other nontruncated p21 proteins were tested by the nitrocellulose binding assay. The prime (') indicates truncated form of p21 (containing residues 1−166).

activity of ras and the stimulation by GAP (Adari *et al.*, 1988; Calés *et al.*, 1988). Substitutions in this area strongly reduce the stimulation by the SDC25 C-domain, as found by replacing residues 35 (p21T35A) and 38 (p21D38E'), two highly conserved residues that are part of the C1 cluster which forms a structural core around Mg^{2+} and phosphate binding L1 loop (Valencia *et al.*, 1991a). Asp38 to Glutamate has a stronger effect than Asp38 to Alanine, inducing a nearly total inhibition of the SDC25 C-domain activity.

The switch region 2 comprises the most mobile part of p21 (residues 61−64) situated on the L4 loop (Milburn *et al.*, 1990; Pai *et al.*, 1990; Tong *et al.*, 1991). In p21 mutants, whose three-dimensional structure is known, this is the most altered region as compared with the wild-type (Krengel *et al.*, 1990). Mutations in this region inhibit the activity of GAP (Maruta *et al.*, 1991; Gideon *et al.*, 1992). Gln61 is supposed to play an important role in the hydrolysis of GTP (Calès *et al.*, 1988; Pai *et al.*,1990), an activity in which also Glu63 may be somehow involved (Fasano *et al.*, 1984; Pai *et al.*, 1990; Gideon *et al.*, 1992), while the function of the highly conserved Glu62 is not yet defined. Concerning the SDC25 C-domain, substitution of any residue of the triad 61−63 causes a drastic inhibition of the GDS activity, while the intrinsic dissociation of the nucleotide is not drastically affected.

In GTP-binding proteins Mg^{2+} plays a crucial role in the binding of substrate and the catalytic activity. The replacement of residues involved in the coordination of Mg^{2+}, such as Ser17 and Asp57, is characterized by a fast intrinsic dissociation of GDP which suppresses the response to the SDC25 C-domain. This effect is particularly strong in the Ser17 to Alanine mutation, which induces a dissociation of the GDP complex independent of Mg^{2+} (J.John, H.Rensland, I.Schlichting, I.Vetter, G.D.Borasio, R.S.Goody and A.Wittinghofer, submitted) and inhibits the SDC25 C-domain response by 90%. In the Asp57 to Alanine mutation the inhibition is 70%. Ser17 and Asp57 bind to Mg^{2+} directly and via a water molecule, respectively, in both p21·GppNp and p21·GDP (Pai *et al.*, 1989, 1990; Tong *et al.*, 1991; Milburn *et al.*, 1990). Substitution of Phe28 (with Leucine, Reinstein *et al.*, 1991), a highly conserved residue, whose side-chain is involved in a aromatic−aromatic interaction with the guanine ring (Pai *et al.*, 1990; Tong *et al.*, 1991), accelerates the GDP dissociation rate and suppresses the GDS activity, thus displaying the same kind of effect as Ser17 and Asp57.

Of the other substitutions examined, Thr58 to Valine does not modify the effect of the SDC25 C-domain, as compared to p21wt, while Ala59 to Threonine, a well-known oncogenic mutation inducing autophosphorylating activity (Shih *et al.*, 1982; John *et al.*, 1988) considerably decreases the intrinsic half-life of p21·GDP and the GDS effect. These two residues are situated on the L4 loop, as part of the C1 cluster (Valencia *et al.*, 1991a) and are highly conserved. Substitution of Tyr71 with Phenylalanine, located on the

Table II. Influence of the SDC25 C-domain on the half-life of the various p21·[γ-^{35}S]GTP complexes

Proteins	GAP activity[b]	GAP affinity (μM)[b]	Half-life[a]		Stimulation (-fold)
			− SDC25 C domain (min)	+ SDC25 C domain (min)[c]	
p21wt	yes	4.8	151	14	10.8
p21T35A	no	–	28	8	3.5
p21D38A′	no	10	91	75	1.2
p21Q61L	no	0.1	57	9	6.3
p21Q61H	weak	2.0	79	39	2.0
p21E62H	yes	0.5	94	76	1.2
p21E63H	yes	1.0	105	54	1.9

[a]The half-life is calculated from the dissociation rate constant.
[b]From Krengel *et al.* (1990) for wild-type p21, other data are from Gideon *et al.* (1992). Affinity between GAP and p21 mutants corresponds to the concentration of the GppNp mutated complexes inhibiting by 50% the GAP activity on the wild-type GTP complex.
[c]The final concentration of SDC25 C-domain in all experiments is 8 nM.

surface of the molecule and conserved in ras and several ras-like proteins decreases by half the SDC25 C-domain stimulation, while replacement of Phe78 with Tyrosine has no apparent functional effect.

Deletion of the C-terminal part of p21

As shown in Table I, the intrinsic dissociation of p21wt′ (John *et al.*, 1989) and p21G12V′ is not decreased as compared with the corresponding full-length proteins, while the SDC25 C-domain is even somewhat more active on the C-terminal truncated protein. Thus the GDS activity of the SDC25 C-domain is independent of the interaction with the tail of p21.

The response of p21·GTP to GDS is inhibited by mutation of the switch regions 1 and 2, but not by the catalytic domain of GAP

The SDC25 C-domain is also active on the p21·GTP complex (Créchet *et al.*, 1990a). Mutations in the switch regions 1 and 2 that in the three-dimensional model are the two subsites most affected by the presence of the γ-phosphate, exert a marked inhibitory effect on GDS also with the p21·GTP complex. To avoid interference with the intrinsic p21 GTPase, [γ-^{35}S]GTP, a slowly hydrolysable analogue of GTP was used as substrate (Scherer *et al.*, 1989). In comparison with the corresponding GDP complex, the SDC25 C-domain stimulation is 40% lower in p21wt. A similar decrease (20 to 40%) in the effect of SDC25 C-domain is obtained with various p21 mutants (Table II). A notable exception is represented by mutants in position 61, in which the response to GDS is the same for the GTP and GDP complexes.

Mutations of the switch regions 1 and 2 inhibit the action of GAP (Adari *et al.*, 1988; Calés *et al.*, 1988; Gideon *et al.*, 1992; J.John, H.Rensland, I.Schlichting, I.Vetter, G.D.Borasio, R.S.Goody and A.Wittinghofer, submitted). However, we have been unable to detect any inhibition of the SDC25 C-domain activity on the [γ-^{35}S]GTP complex of p21wt and p21Q61L using increasing concentrations of GAP334 up to 250 times in molar excess, as tested by nitrocellulose binding and dynamic fluorescence measurements (not illustrated). In the case of p21Q61L to which GAP and its catalytic domain binds much more strongly than to p21wt (Shaber *et al.*, 1989), 84% of p21Q61L[γ-^{35}S]GTP was calculated to be complexed with GAP334, assuming a dissociation constant of 5 μM (A.Lautwein and A.Wittinghofer, unpublished).

Table III. $K_{m\ p21\cdot GDP}$ and k_{cat} of the SDC25 C-domain-dependent GDP−[γ-^{32}P]GTP exchange rate for p21 mutants[a]

Proteins	$K_{m\ p21\cdot GDP}$ (μM)	k_{cat} (min^{-1})
p21wt	2.3	14.5
p21T35A	1.6	5
p21D57A	1.2	0.8
p21Q61L	1.5	13.5
p21Q61H	0.8	1.5
p21E62H	0.7	0.4
p21E63H	1.6	1.7

[a]The final concentration of SDC25 C domain in all experiments is 16 nM

Low responsive p21 mutants still bind the SDC25 C-domain

To examine in more details the effect of mutations in the two regions which play a crucial role in the GTP- and GDP-bound conformations of p21, we have determined the enzymatic parameters (K_m and k_{cat}) of the exchange reaction catalysed by the SDC25 C-domain, following the method illustrated in Figure 2. To our surprise, the K_m values of the low responsive p21 mutants tested were not increased and have even become significantly smaller (Table III). This observation strongly suggests that the inhibited GDS activity is not related to a decreased binding of the SDC25 C-domain to the mutated proteins.

Discussion

It is well established that the requirement for *CDC25*, a gene encoding a GDS of yeast RAS proteins (Jones *et al.*, 1991), can be suppressed by the 3′ terminal region of *SDC25* (Boy-Marcotte *et al.*, 1989). The SDC25 C-domain induces a deregulated, permanently activated phenotype of the *S.cerevisiae* cell, as a consequence of an increased GDP to GTP exchange (Créchet *et al.*, 1990a). It is very active with and highly specific for both yeast RAS2 and Ha-*ras* p21; of the ras-like proteins tested (rap2, ral1A and rap1A from mammals; YPT1 from *S.cerevisiae* and ryh1 from *S.pombe*), none has been found to be sensitive to this GDS (Parmeggiani *et al.*, 1992). Therefore though the role of *SDC25* in

S.cerevisiae is not yet clarified, the SDC25 C-domain appears to be a model GDS for both yeast and human systems.

By modifying p21, we have shown that the switch regions 1 and 2 as well as the network of interactions involved in the coordination of the Mg^{2+}·nucleotide complex and the positioning of the guanine base, are crucial elements for GDS activity; whereas the phosphate binding L1 loop, a rather rigid structure important for the strength of the nucleotide binding and the C-terminal region are not relevant. Our results agree with the observations that mutations of p21 residues 35, 38 and 61 inhibit GDS activity from human placenta (Downward *et al.*, 1990). However, whereas this GDS has been reported to be inactive on urea-extracted p21 lacking the C-terminal part, the SDC25 C-domain is very active on our truncated p21. Differences inherent in the properties of the truncated p21 preparations may play a role in this discrepancy; however, it is possible that the SDC25 C-domain which was identified as a suppressor of a regulated yeast gene (*CDC25*), can express a constitutive activity independently of a specific activation mechanism which may be required for other GDS proteins. In fact, another GDS acting on smg·p21 (rap1B) has been reported to need the presence of the C-terminal domain of the latter (Hiroyoshi *et al.*, 1991).

The biological relevance of our data is emphasized by the recent isolation of conditional *Drosophila ras* mutants that show a decreased efficiency in the signal transmission of the tyrosine kinase pathway (Simon *et al.*, 1991). In this system the ras residues found to be mutated are Asp38 and Glu62, which in human p21 we have shown to be crucial for the SDC25 C-domain activity. Therefore the altered phenotype described in *Drosophila* could be caused by the inhibition of the regeneration of the GTP-bound complex catalysed by the SDC25-like protein encoded by *SOS*.

The modified response of a mutant to an effector may be caused either by the alteration of a structural determinant required for molecular recognition, resulting in lower affinity, or by an impaired mechanism of action. The former possibility does not apply to the p21 proteins mutated in the switch regions 1 and 2, since no decrease in the affinity for GDS has been detected. This observation together with the lack of competition with GAP, whose direct interaction with the effector loop has been postulated (Adari *et al.*, 1988; Schaber *et al.*, 1989), suggests that the binding site of the SDC25 C-domain is not located in these two regions. Differences in the p21 structures that recognize GAP and GDS are also suggested by the observation that the SDC25 C-domain does not act on the ras-like proteins Rap1Awt and Rap1AT61Q (E.Jacquet and M.Y.Mistou, unpublished results) which have the same effector region as Ha-*ras* p21 and can bind Ha-*ras* p21 GAP (Frech *et al.*, 1990).

How can mutations in the switch regions 1 and 2, which are two adjacent structures on the p21 surface, induce negative effects on the GDS independently from a direct interaction? A possible explanation can be derived from a similar system (EF-Tu and EF-Ts), where the GDP to GTP exchange reaction has been shown to follow a double substitution mechanism (Hwang and Miller, 1985) that implies a stable nucleotide-free EF-Tu·EF-Ts complex as intermediate between the formation of the GDP and GTP complexes. Such a stable complex, also reported for a ras-like protein (Bischoff and Ponstingl, 1991), has been

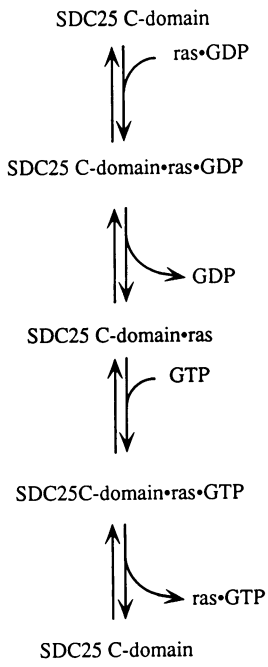

Fig. 5. Kinetic model of the GDP to GTP exchange reaction of p21 catalysed by GDS.

observed between RAS2 or p21 and SDC25 C-domain on gel-filtration (P.Poullet and H.Rensland, unpublished results). The occurrance of the reaction sequence (illustrated for p21 in Figure 5), alternating the nucleotide-free and -bound state of the RAS protein, implies that the GDS activity is associated with a succession of specific conformational changes. It is therefore not surprising that regulatory elements such as the switch regions, whose structure has been shown to be most affected by mutations (Krengel *et al.*, 1990), can influence the response to GDS even if the p21 binding site for GDS is located in other structures.

Substitution of p21 residues, such as Phe28, Ser17 and Asp57 involved in the substrate binding, markedly increase the intrinsic nucleotide dissociation activity, suppressing the effect of GDS. The same effect is obtained in RAS2, if Thr152 corresponding to Thr144 in p21 (a residue that interacts with the guanine base via Asn116) is substituted by Ile (Créchet *et al.*, 1990b; J.-B.Créchet and P.Poullet, unpublished). These results define a number of specific residues involved in the interaction with the substrate as potential targets of the GDS action. With other residues less directly involved in the binding of the substrate, the effects of mutations on the intrinsic nucleotide release can vary considerably compared with the response to the GDS, thus illustrating the complexity of the interaction network involved in the transmission of the signals for nucleotide release.

Our observation that strong inhibitory effects can arise from mutations of the switch regions 1 and 2 without correlation with a deficient interaction, emphasizes the possibility that other exposed structural elements of the molecule may be involved in the GDS recognition site of p21. Genetic studies have revealed several 'hot-spots' of residues involved in the biological activation that represent potential interaction sites with GDS. Point mutations in the α4 helix region of the *let-60 ras* gene product of

Caenorhabditis elegans give rise to recessive lethal phenotypes that could result from an altered activation by its specific GDS (Beitel *et al.*, 1990). From this study residues 75 or 103–108 may also take part in the activation by GDS. The involvement of the latter region has also been suggested by Willumsen *et al.* (1991), who have shown that mutations of residues 102 and 103 on the α3 helix abolish the biological functions of p21, an effect possibly associated with an altered guanine nucleotide exchange function. Mutations in the distal switch region 2 of RAS2 protein indicate that the conserved Gly82 and Gly84 (in p21, Gly75 and Gly77, respectively) are key elements for the activation of the RAS pathway (Fasano *et al.*, 1988; Kavounis *et al.*, 1991). Recently, it has been shown that substitution of Arg80 and Asn81 of RAS2 (in p21, Arg73 Thr74, respectively) gives rise to recessive lethal phenotypes and abolishes the response to the SDC25 C-domain (J.B.Créchet and O.Fasano, unpublished). Participation of the L8 and L10 loops, which are vicinal to the α4 helix, in the GDS interaction has also been derived from comparison of structural and functional properties of p21 and EF-Tu (Valencia *et al.*, 1991b). All these studies show how complex the mechanism of the GDS action is, suggesting great caution in the interpretation that functional modifications are a consequence of a direct alteration of the binding site of p21 for GDS. At the same time they indicate further potential areas for site directed mutagenesis and biochemical characterization to correlate the diverse biological observations.

Materials and methods

Biological components

The 3′ terminal part of the *S.cerevisiae SDC25* gene coding for the 550 C-terminal residues was expressed from the pTTQ19 vector in *Escherichia coli* strain 71/18. Isolation and partial purification of the SDC25 C-domain was carried out by modification of the method described in Créchet *et al.* (1990a; P.Poullet and A.Parmeggiani, unpublished). Pure p21 and the mutant p21 were prepared as described (Tucker *et al.*, 1986; John *et al.*, 1989). RAS2 was purified as described in Créchet *et al.* (1990b). GAP334, the catalytic domain of human GAP (residues 714–1047), was produced from ptrc99A (Amman *et al.*,1988) in *E.coli* CK600 wild-type and purified as described by Gideon *et al.* (1992).

GDS activity

The SDC25 C-domain guanine nucleotide release activity was determined either by nitrocellulose binding or filtration on short Sephadex G25 columns (Créchet and Parmeggiani, 1986) or fluorescence measurements (John *et al.*, 1990). In the first two methods, the labelled GDP and GTP complexes of p21 were prepared by incubating 1 μM p21 (or RAS2 in a few experiments of Figure 1) for 5 min at 30°C in 50 mM Tris–HCl pH 7.5, 2 mM EDTA, 100 mM NH_4Cl, 0.5 mg/ml BSA, 1 mM DTT and 5 μM [^{3}H]GDP (2000 c.p.m./pmol, Amersham) or [γ-^{35}S]GTP (1000 c.p.m./pmol, DuPont–NEN). $MgCl_2$ (3 mM) was then added. The dissociation reaction, in a final volume of 250 μl with or without 8 nM SDC25 C-domain, was started with the addition of 500-fold excess of unlabelled GDP or GTP. Aliquots (45 μl) were filtered through nitrocellulose discs (Sartorius 11306, 0.45 μm) which were washed twice with 3 ml ice-cold standard buffer (50 mM Tris–HCl pH 7.6, 100 mM NH_4Cl, 1 mM $MgCl_2$ and 7 mM 2-mercaptoethanol). The retained radioactivity on the discs was measured in a Wallac 1410 (LKB) liquid scintillation counter. For the truncated forms of p21 (p21′ represents p21Δ167–189) that are not quantitatively retained on nitrocellulose, the aliquots were filtered on 0.5×15 cm Sephadex G25 columns at 4°C (Créchet et Parmeggiani, 1986) in the presence of 10 mM $MgCl_2$ to inhibit the release of the nucleotide from p21, and then radioactivity in the protein containing fractions was counted. In the case of very fast GDP dissociation, dynamic fluorescence measurements were performed using a SLM 8000 spectrophotometer and 3′-*N*-methylantraniloyl 2′-deoxy derivatives of GDP (mantGDP, John *et al.*, 1990). The conditions are similar to those used in the filter binding method, except that 40 mM HEPES–NaOH pH 7.6 and 2 mM $MgCl_2$ replaced 50 mM Tris–HCl pH 7.5 and 1 mM $MgCl_2$ and no EDTA was used. The concentration of mantGDP and nucleotide-free p21 was 2 μM and 1 μM, respectively. The reaction was initiated by the addition of a 200 to 300-fold excess of GDP. Excitation and emission wavelengths were normally 370 and 440 nm, respectively. For the determination of the initial rate of GDP to GTP exchange, various concentrations of the p21·GDP complexes were incubated at 30°C in 200 μl of buffer A (50 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM DDT, 0.5 mg/ml BSA) in the presence of 16 nM SDC25 C-domain. The exchange reaction was started by the addition of 30 μM [γ-^{32}P]GTP (1000 c.p.m./pmol, Amersham). After 20, 40 and 60 s to ensure the linearity of the kinetics, the p21-bound radioactivity was determined on 60 μl aliquots by nitrocellulose binding. All the experiments described were repeated at least twice; the variations observed were within 10 to 20%.

Acknowledgements

We express our gratitude to Drs Anne-Lise Haenni, Roger S.Goody, Matthias Frech and Alfred Lautwein for valuable discussion and advice, and to Dr Ken Holmes for continuous interest and support. We are indebted to Drs Michel Jacquet for providing us the 3′-region of the *SDC25* sequence, D.Gallwitz for the gift of YPT1 and ryh1, P.Chardin for rap2 and ral1A, and V.Pizon and A.Tavitian for rap1AT61Q. This work was supported by the Association pour la Recherche sur le Cancer (grant no. 6377), Ligue Nationale Française contre le Cancer, and Institut National de la Santé et la Recherche Médicale (grant no. 900109). P.P. was recipient of a fellowship of the Ministère de la Recherche et de la Technologie.

References

Adari,H., Lowy,D.R., Willumsen,B.M., Der,C.J. and McCormick,F. (1988) *Science*, **240**, 518–521.
Amman,E., Ochs,B. and Abel,K.-J. (1988) *Gene*, **69**, 301–315.
Barbacid,M. (1987) *Annu. Rev. Biochem.*, **56**, 779–827.
Beitel,G.J., Clark,S.G. and Horvitz,H.R. (1990) *Nature*, **348**, 503–509.
Bourne,H.R., Sanders,D.A. and McCormick,F. (1990) *Nature*, **348**, 125–132.
Bourne,H.R., Sanders,D.A. and McCormick,F. (1991) *Nature*, **349**, 117–127.
Boy-Marcotte,E., Damak,F., Camonis,J., Garreau,H. and Jacquet,M. (1989) *Gene*, **77**, 21–30.
Bischoff,F.R. and Ponstingl,H. (1991) *Nature*, **354**, 80–82.
Calés,C., Hancock,J.F., Marshall,C.J. and Hall,A. (1988) *Nature*, **332**, 548–551.
Créchet,J.B. and Parmeggiani,A. (1986) *Eur. J. Biochem.*, **161**, 647–653.
Créchet,J.B., Poullet,P., Mistou,M.-Y., Parmeggiani,A., Camonis,J., Boy-Marcotte,E., Damak,F. and Jacquet,M. (1990a) *Science*, **248**, 866–868.
Créchet,J.B., Poullet,P., Camonis,J., Jacquet,M. and Parmeggiani,A. (1990b) *J. Biol. Chem.*, **265**, 1563–1568.
Downward,J., Riehl,R., Wu,L. and Weinberg,R.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5998–6002.
Fasano,O., Aldrich,T., Tamanoi,F., Taparowsky,E., Furth,M. and Wigler,M. (1984) *Proc. Natl. Acad. USA*, **81**, 4008–4012.
Fasano,O., Crechet,J.B., De Vendittis,D., Zahn,R., Feger,G., Vitelli,A. and Parmeggiani,A. (1988) *EMBO J.*, **7**, 3375–3383.
Frech,M., John,J., Pizon,V., Chardin,P., Tavitian,A., Clark,R., McCormick,F. and Wittinghofer, A. (1990) *Science*, **249**, 169–171.
Garreau,H., Camonis,J., Guitton,C. and Jacquet,M. (1990) *FEBS Lett.*, **269**, 53–59.
Gibbs,J.B., Schaber,M.D., Allard,J.W., Sigal,I.S. and Scolnick,E.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5026–5030.
Gideon,P., John,J., Frech,M., Lautwein,A., Clark,R., Scheffler,J.E. and Wittinghofer,A. (1992) *Mol. Cell. Biol.*, in press.
Hiroyoshi,M., Kaibuchi,K., Kawamura,S., Hata,Y. and Takai,Y. (1991) *J. Biol. Chem.*, **266**, 2962–2969.
Huang,Y.K., Kung,H.-F. and Kamata,T. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8008–8012.
Hughes,D.A., Fukui,Y. and Yamamoto,M. (1990) *Nature*, **344**, 355–357.
Hwang,Y.W. and Miller,D.L. (1985) *J. Biol. Chem.*, **260**, 11498–11502.
John,J., Frech,M. and Wittinghofer,A. (1988) *J. Biol. Chem.*, **263**, 11792–11799.
John,J., Schlichting,I., Schiltz,E., Rüsch,P. and Wittinghofer,A. (1989) *J. Biol. Chem.*, **264**, 13086–13092.
John,J., Sohmen,R., Feuerstein,J., Linke,R., Wittinghofer,A. and Goody,R.S. (1990) *Biochemistry*, **29**, 6058–6065.

Jones,S., Vignais,M.L. and Broach,J.R. (1991) *Mol. Cell. Biol.*, **11**, 2641–2646.
Kavounis,C. *et al.* (1991) *FEBS Lett.*, **281**, 235–239.
Krengel,U., Schlichting,I., Scherer,A., Schumann,R., Frech,M., John,J., Kabsch,W., Pai,E.F. and Wittinghofer,A. (1990) *Cell*, **62**, 539–548.
Maruta,H., Holden,J., Sizeland,A. and D'Abaco,G. (1991) *J. Biol. Chem.*, **266**, 11661–11668.
Milburn,M.V., Tong,L., de Vos,A.M., Brünger,A., Yamaizumi,Z., Nishimura,S. and Kim,S.H. (1990) *Science*, **247**, 939–945.
Pai,E.F., Kabsch,W., Krengel,U., Holmes,K.C., John,J. and Wittinghofer,A. (1989) *Nature*, **341**, 209–214.
Pai,E.F., Krengel,U., Petsko,G.A., Goody,R.S., Kabsch,W. and Wittinghofer,A. (1990) *EMBO J.*, **9**, 2351–2359.
Parmeggiani,A., Mistou,M.Y., Jacquet,E., Poullet,P. and Créchet J.B. (1992) in Lacal,J.C. and McCormick,F (eds) *The Ras Superfamilies of GTPases*. CRC Press, in press.
Reinstein,J., Schlichting,I., Frech,M., Goody,R.S. and Wittinghofer,A. (1991) *J. Biol. Chem.*, **206**, 17700–17706.
Rey,I., Schweighoffer,F., Barlat,I., Camonis,J., Boy-Marcotte,E., Guilbaud,R., Jacquet,M. and Tocqué,B. (1991) *Oncogene*, **6**, 347–349.
Schaber,M.D., Garsky,V.M., Boylan,D., Hill,W.S., Scolnick,E.M., Marshall,M.S., Sigal,I.S. and Gibbs,J.B. (1989) *Proteins*, **6**, 306–315.
Scherer,A., John,J., Linke,R., Goody,R.S., Wittinghofer,A., Pai,E.F. and Holmes,K.C. (1989) *J. Mol. Biol.*, **206**, 257–259.
Schlichting,I. *et al.* (1990) *Nature*, **345**, 309–315.
Shih,T.Y., Stokes,P.E., Smythers,G.W., Dhar,R. and Oroszlan,S. (1982) *J. Biol. Chem.*, **257**, 11767–11773.
Simon,M.A., Bowtell,D.D.L., Dodson,S.G., Laverty,T.R. and Rubin,G.M. (1991) *Cell*, **67**, 701–716.
Tong,L., Milburn,M.V., de Vos,A. and Kim,S.-H. (1989) *Science*, **245**, 244.
Tong,L., de Vos,A.M., Milburn,M.V. and Kim,S.-H. (1991) *J. Mol. Biol.*, **217**, 503–516.
Trahey,M. and McCormick,F. (1987) *Science*, **238**, 542–545.
Tucker,J., Sczakiel,G., Feuerstein,J., John,J., Goody,R.S. and Wittinghofer,A. (1986) *EMBO J.*, **5**, 1351–1358.
Valencia,A., Chardin,P., Wittinghofer,A. and Sander,C. (1991a) *Biochemistry*, **30**, 4637–4648.
Valencia,A., Kjeldgaard,M., Pai,E.F. and Sander,C. (1991b) *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 5443–5447.
Willumsen,B.M., Vass,W.C., Velu,T.J., Papageorge,A.G., Schiller,J.T. and Lowy,D.R. (1991) *Mol. Cell. Biol.*, **11**, 6026–6033.
Wittinghofer,A. and Pai,E.F. (1991) *Trends Biochem. Sci.*, **16**, 382–387.
Wolfman,A. and Macara,I.G. (1990) *Science*, **248**, 67–69.

Received on February 17, 1992; revised on March 25, 1992