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Enhancement of the GDP-GTP Exchange of RAS Proteins by the Carboxyl-Terminal Domain of SCD25

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In *Saccharomyces cerevisiae*, the product of the *CDC25* gene controls the *RAS*-mediated production of adenosine 3′,5′-monophosphate (cAMP). In vivo the carboxyl-terminal third of the *CDC25* gene product is sufficient for the activation of adenylate cyclase. The 3′-terminal part of *SCD25*, a gene of *S. cerevisiae* structurally related to *CDC25*, can suppress the requirement for *CDC25*. Partially purified preparations of the carboxyl-terminal domain of the *SCD25* gene product enhanced the exchange rate of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) of pure RAS2 protein by stimulating the release of GDP. This protein fragment had a similar effect on the human c-H-*ras*–encoded p21 protein. Thus, the SCD25 carboxyl-terminal domain can enhance the regeneration of the active form of RAS proteins.

IN *Saccharomyces cerevisiae* THE RAS PROTEINS are components of the enzymatic cascade that activates adenylate cyclase and plays a key role in cell cycle progression. They belong to a family of proteins that bind GTP and GDP tightly and display a low-turnover guanosine triphosphatase (GTPase) activity that changes the active form induced by GTP, needed for interaction with effectors, into the inactive form bound to GDP (*1–3*). The conversion of RAS·GDP to RAS·GTP therefore represents the mechanism of activation of these proteins (*1*). In *S. cerevisiae*, the *CDC25* gene product controls the RAS-mediated production of cAMP, probably by enhancing the rate of GDP-GTP exchange of RAS proteins (*4, 5*). This mechanism is implied by indirect observations that CDC25 is dispensable in cells with either a permanently activated RAS protein, such as the product of the RAS2($Gly^{19} \rightarrow Val$) allele (*5*), or a RAS protein displaying an enhanced rate of GDP-GTP exchange, such as the product of the RAS2($Thr^{152} \rightarrow Ile$) allele (*6*). In vivo the 3′-terminal third of *SCD25* is sufficient for the complementation of the *cdc25-5* mutant (*5*). The 3′-terminal part of *SDC25*, a gene of *S. cerevisiae* structurally related to *CDC25*, can suppress the requirement for *CDC25*, most likely by activation of RAS proteins (*7, 8*). Therefore, we have tested the effect of the SCD25 carboxyl-terminal domain on the RAS2–guanine nucleotide interaction in vitro.

The SCD25 COOH-terminal domain was produced in *Escherichia coli*. For this purpose a 1878-bp fragment from pLA2 starting at the Xba I site and containing the 3′-terminal part of *SCD25* was inserted into the pUC19 polylinker (Appligene). The resulting plasmid pJEL11 drives the expression of a chimeric protein composed of the first 13 amino acid residues of β-galactosidase encoded by pUC19 followed by 550 amino acids of SCD25, under control of the lacZ promoter. The plasmid was introduced in *E. coli* JM101 cells and expression of the fused protein was induced by isopropyl β-D-thiogalactoside. As a control, pJEL11ΔBam H1 was constructed by a 741-bp deletion between the two Bam H1 sites of the *SCD25* portion (*7, 8*). This deletion leads to the loss of the 247 amino acids in the SCD25 COOH-terminal domain essential for complementation; the open reading frame upstream and downstream of this fragment are fused in frame.

To test the effect of the SCD25 COOH-terminal domain on RAS2 functions, we used a pure 30-kD form of RAS2 isolated from *E. coli* (*6*). This truncated form results from the removal of the COOH-terminal end of the protein by proteolytic cleavage, a deletion that affects neither the 1:1 stoichiometric binding of GDP or GTP nor the ability to activate the adenylate cyclase in vitro. The supernatant from sonicated, pJEL11-transformed cells dramatically enhanced the release of [^{3}H]GDP from the RAS2·[^{3}H]GDP complex, whereas the supernatant from cells transformed with pUC19 or pJEL11ΔBam H1 was inactive (Table 1). The releasing activity in the supernatant could only be detected by using a filtration method on short columns (*9*). In fact, without purification, the large amount of contaminating protein in the supernatant hindered binding of the complexes of GTP or GDP and RAS2 to the nitrocellulose filters. By contrast, partial purification of the SCD25 COOH-terminal domain (*10*) allowed us to use filtration on nitrocellulose to measure the dissociation kinetics of the RAS2 complexes. The SCD25 COOH-terminal domain had neither guanine nucleotide binding activity nor GTPase activity and did not have any proteolytic effects on RAS2, as verified by immunoblots after SDS–polyacrylamide gel electrophoresis. The

Table 1. Effect on the RAS2·[^{3}H]GDP dissociation rate of the SCD25 COOH-terminal domain. GDP-free (*6*) RAS2 protein (30 pmol) in a standard buffer containing 25 mM tris-HCl, pH 7.5, 1 mM $MgCl_2$, 100 mM NH_4Cl, 1 mM dithiothreitol (DTT), and 0.1 mg/ml bovine serum albumin (BSA) was incubated in 80 μl with 35 pmol [^{3}H]GDP (10 Ci/mmol) for 30 min at 30°C to achieve equilibrium conditions. The dissociation reaction was started by adding a 1000-fold excess (35 nmol) of unlabeled GDP in 30 μl of storage buffer (50% glycerol in 50 mM tris-HCl, pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, and 7 mM 2-mercaptoethanol or 30 μl of a mixture containing 35 nmol of unlabeled GDP and 200 μg of each of the different extracts in storage buffer. After a 15-min incubation at 30°C, 90 μl of each sample were loaded onto a Sephadex G25 column (18 by 0.4 cm) equilibrated with standard buffer and eluted in 100-μl fractions, and the radioactivity was measured. To obtain the cell extracts, *E. coli* cells (9 g) were sonicated in a threefold volume of 50 mM tris-HCl, pH 7.5, 1 mM $MgCl_2$, 100 mM KCl, 0.25 M sucrose, 7 mM 2-mercaptoethanol, 2 mM phenylmethylsulphonylfluoride (PMSF), and 1 mM EDTA, and centrifuged at 100,000*g* for 90 min. The supernatant was dialyzed against the storage buffer and kept at −30°C. The mean ± SEM was calculated from four experiments for each condition.

Conditions	RAS2·[^{3}H]GDP (pmol)
No addition	22 ± 2
+ pJEL11 transformed cell extract	2.6 ± 0.3
+ pUC19 transformed cell extract	23 ± 1
+ JEL11ΔBam H1 transformed cell extract	22 ± 2.5

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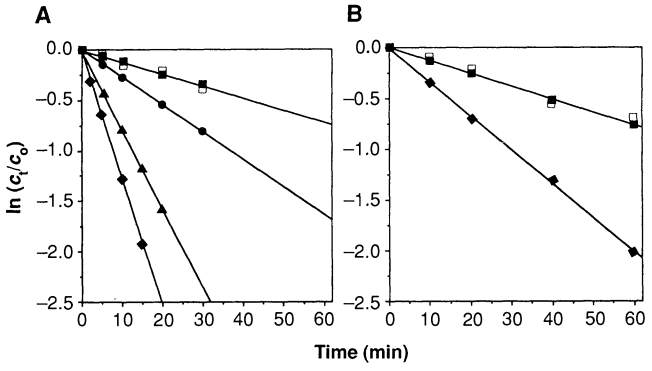

Fig. 1. Effect of SCD25 COOH-terminal domain on the kinetics of dissociation of (**A**) RAS2·[^{3}H]GDP and (**B**) RAS2·[γ^{32}P]GTP. GDP-free RAS2 (4.5 pmol) was incubated with [^{3}H]-GDP (22 pmol, 10 Ci/mmol) or [γ^{32}P]GTP (22 pmol, 5.25 Ci/mmol) for 1 hour at 0°C in 200 μl of standard buffer (Table 1). After 3 min at 30°C, the reaction was started with 20 μl of the elution buffer containing 130 mM KCl and a 1000-fold excess of unlabeled nucleotide without or with partially purified SCD25 COOH-terminal domain. At given time intervals, aliquots (40 μl) were withdrawn, filtered on nitrocellulose membranes (Sartorius SM 11306) (0.45 μm), and the retained radioactivity was determined. The apparent first-order rate of dissociation was calculated according to the equation $\ln(c_t/c_o) = k'_{-1} \cdot t$, where c_o is the initial concentration of RAS2·nucleotide complex and c_t its concentration at the time t. (A) With 4 (●), 8 (▲), and 16 μg (♦) of partially purified SCD25 COOH-terminal domain; with storage buffer substituting for the cell extract (■); and with the same amount of the corresponding fraction, purified from the extract of pUC19-transformed cells (□). (B) With 16 μg (♦) of the partially purified SCD25 COOH-terminal domain; with storage buffer substituting the cell extract (■); and with the same amount of the corresponding fraction, purified from the extract of pUC19-transformed cells (□). The results were corrected for the very low background of GTP hydrolysis occurring during the experiment.

Table 2. Effect of the SCD25 COOH-terminal domain (C-domain) on the apparent dissociation rate constants (k'_{-1}) (see Fig. 1 for calculation) of the complexes of RAS2 and H-Ras with GDP and GTP. Four experiments were conducted for each condition. Data are expressed as mean ± SEM.

Complex	$10^2 \cdot k'_{-1}$ (min^{-1})		+C-domain/ −C-domain
	−C-domain	+C-domain	
RAS2·GDP	1.10 ± 0.06	12.5 ± 0.3	11.4
H-Ras·GDP	0.50 ± 0.03	7.2 ± 0.4	14.4
RAS2·GTP	1.20 ± 0.05	3.1 ± 0.1	2.6
H-Ras·GTP	0.40 ± 0.02	1.1 ± 0.03	2.8

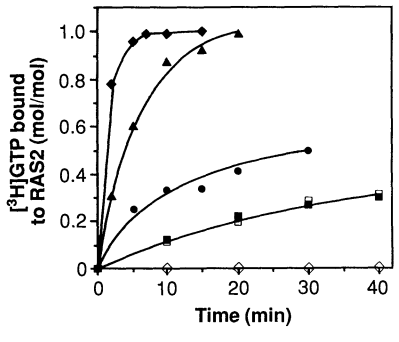

Fig. 2. Kinetics of the exchange reaction RAS2·GDP-[^{3}H]GTP in the presence of the SCD25 COOH-terminal domain. The reaction mixture (450 μl) contained, in standard buffer, 10 pmol of RAS2·GDP. The reaction at 30°C was started by the addition of 1.6 μl of [^{3}H]GTP (7 Ci/mmol) to a final concentration of 500 nM without (■) or with 7.5 (●), 15 (▲), or 30 (♦) μg of partially purified SCD25 COOH-terminal domain, and with 30 μg (□) of the corresponding fraction, purified from the extract of pUC19-transformed cells. At the times indicated, aliquots (80 μl) were withdrawn and the amount of [^{3}H]GTP bound was determined by filtration on nitrocellulose (as in Fig. 1). The same experiment was performed in the absence of RAS2 in a mixture containing 30 μg of the partially purified SCD25 COOH-terminal domain (◇).

COOH-terminal domain of SCD25 enhanced the dissociation of the RAS2·[^{3}H]-GDP complex; the effect increased with increasing SCD25 COOH-terminal domain concentration (Fig. 1A). The dissociation rate of the RAS2·[γ^{32}P]GTP complex (Fig. 1B) was also enhanced, albeit to a much lesser extent than that of the RAS2·GDP complex; the effect in Fig. 1B was obtained by adding the highest concentration of the SCD25 COOH-terminal domain used for the RAS2·GDP experiment in Fig. 1A. The same partially purified fraction from pUC19-transformed cells was devoid of any releasing activity.

As a consequence of the fast release of GDP from RAS2 induced by the SCD25 COOH-terminal domain, the GDP to [^{3}H]GTP exchange rate of RAS2·GDP was markedly enhanced (Fig. 2). The exchange rate was not influenced by the equivalent protein fraction purified from pUC19-transformed cells.

The SCD25 COOH-terminal domain also enhanced, to a similar extent, the dissociation rate of complexes of GDP or GTP and pure human c-H-*ras*–encoded p21 protein (H-Ras) (Fig. 3, A and B, and Table 2). Also in this case, the H-Ras·GDP complex was the preferred target; its dissociation was more strongly affected than that of the H-Ras·GTP complex. The GDP to GTP exchange reaction of H-Ras was stimulated to the same extent as that of RAS2. These results obtained with the intact H-Ras show that the effect of the SDC25 COOH-terminal domain is not dependent on a truncated form of the RAS2 protein.

We have characterized an effector that can enhance the GDP-GTP exchange of RAS proteins, as the result of stimulation of the GDP·RAS complex dissociation. The SCD25 COOH-terminal domain acts as a guanine nucleotide releasing factor with a specific effect on the GDP complex. The higher concentration of GTP over GDP in the cell (*11, 12*) will ultimately favor the formation of the active GTP complex. This mechanism is reminiscent of the effect of elongation factor Ts, which promotes disso-

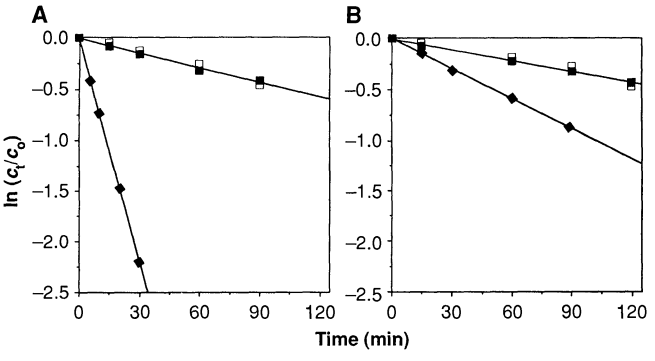

Fig. 3. Effect of SCD25 COOH-terminal domain on the dissociation rate (see Fig. 1 for calculation) of the (**A**) H-Ras·[^{3}H]GDP and (**B**) H-Ras·[γ^{32}P]GTP complexes. H-Ras·GDP (4 pmol) was incubated with [^{3}H]GDP (22 pmol, 10 Ci/mmol) or [γ^{32}P]GTP (22 pmol, 5.25 Ci/mmol) for 10 min at 30°C in 195 μl containing 25 m tris-HCl, pH 7.5, 2 mM $MgCl_2$, 100 mM NH_4Cl, 0.2 mg/ml BSA, 1 mM DTT, and 5 mM EDTA (in order to exchange the bound GDP with the labeled nucleotide). Magnesium chloride in a 2 mM excess over EDTA was then added. The dissociation reaction was monitored (as in Fig. 1) without (■) and with (♦) 16 μg of the partially purified SCD25 COOH-terminal domain, and (□) 16 μg of the corresponding fraction purified from the extract of pUC19-transformed cells. In (B) the results were corrected for the background of GTP hydrolysis occurring during the experiment. Overproduction in *E. coli* (*21*) and purification of H-Ras (*22*) were carried out as described.

ciation of the elongation factor Tu (EF-Tu)·GDP complex for the recycling of EF-Tu·GTP (*13*).

Because of the tight binding of GDP and GTP, the key steps of the RAS–guanine nucleotide cycle are: dissociation of the RAS·GDP complex, the prerequisite for the regeneration of the active complex RAS·GTP, and hydrolysis of GTP, which turns off the active form of RAS. An activating factor for H-Ras GTPase activity (GAP) has been described in mammalian cells (*14, 15*) and a related factor, the *IRA1* gene product, has been reported in yeast (*3, 16*). In the presence of these factors, the exchange of GDP and GTP is the limiting step for RAS activation and consequently for growth. Control of this step by a releasing factor is therefore a critical mechanism.

The CDC25 protein is a candidate for this role in *S. cerevisiae*. Using a similar experimental approach to the one described here for SCD25, we have been unable to detect any guanine nucleotide releasing activity of the region of CDC25 that shares 50% homology with the SCD25 COOH-terminal domain (*17*). The lack of activity may be due to technical problems, such as defective expression in *E. coli*, or CDC25 may not be active because it requires another element for activation, as might be expected in a signal transducing cascade. Activation of the releasing factor may be necessary for RAS activation, as in the mechanism by which receptors activate G proteins in response to hormone binding or rhodopsin acts on transducin upon light stimulation (*2*). For H-Ras, the mode of regulation of the GDP-GTP exchange is still unknown, although overexpression in yeast cells of the SCD25 COOH-terminal domain leads to an unregulated cAMP-activating cascade, as in the RAS2($Gly^{19} \rightarrow Val$) mutant, while overexpression of CDC25 results in a normal phenotype, as if the cascade were still regulated (*18*). The fact that the SCD25 COOH-terminal domain can enhance in vitro the GDP-GTP exchange rate of RAS2 without any activating system could explain its property to induce a phenotype of permanent activation of cAMP production.

In cells lacking a functional *CDC25* gene, the intact *SCD25* cannot reverse the lack of cAMP or the defect in growth (*19*). In contrast, the SCD25 COOH-terminal domain restores cAMP production and growth. This can be interpreted to mean that the other domains of the protein might be involved in the specification of the target. Thus, the SCD25 COOH-terminal domain acts as a releasing factor with a broad range specificity, while the true target of the *SCD25* gene product remains to be identified.

A gene (*STE6*$^+$) with similarities to the COOH-terminal domain of SCD25 has been found in *Schizosaccharomyces pombe*, which could also be involved in the activation of the *S. pombe RAS1* gene product (*20*). This observation suggests the existence of a family of proteins mediating the GDP-GTP exchange of the Ras proteins in different organisms. The presence of analogous factors in mammalian cells is suggested by the capacity of H-Ras to be activated by the COOH-terminal domain of SCD25.

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10. To purify the SCD25 product, three chromatographic steps were performed at 4°C, by fast protein liquid chromatography (Pharmacia). The active fractions were identified by their ability to stimulate the dissociation of the RAS2·[^{3}H]GDP, by gel filtration on short columns (*9*) for the first step or filtration on nitrocellulose for the last steps. The cell extract supernatant (Table 1) was applied to a MonoQ HR5/5 column equilibrated with buffer A (50 mM tris-HCl, pH 7.5, 1 mM $MgCl_2$, 50 mM KCl, 20 μM GDP, and 7 m*M* 2-mercaptoethanol). The SCD25 COOH-terminal domain was eluted in one step with buffer A containing 250 mM KCl. After concentration, the active pool was filtered on a Superose 12 Prep-grad column (Pharmacia) equilibrated with buffer A with 50 mM KCl and without GDP. The active fractions were applied once more to a MonoQ HR5/5 column equilibrated with buffer A without GDP and eluted with a linear 50 to 250 mM KCl gradient in buffer A without GDP. The GDP-releasing activity emerged as a sharp peak at about 130 mM KCl, still comprising several bands by SDS–polyacrylamide gel electrophoresis. The same chromatographic steps were carried out in parallel with the extract from pUC19-transformed *E. coli* cells.
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