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The *Saccharomyces cerevisiae CDC25* Gene Product Binds Specifically to Catalytically Inactive Ras Proteins In Vivo

THOMAS MUNDER AND PETER FÜRST*

Department of Biotechnology, CIBA-GEIGY Ltd., K-681.3.04, CH-4002 Basel, Switzerland

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Genetic data suggest that the yeast cell cycle control gene *CDC25* is an upstream regulator of *RAS2*. We have been able to show for the first time that the guanine nucleotide exchange proteins Cdc25 and Sdc25 from *Saccharomyces cerevisiae* bind directly to their targets Ras1 and Ras2 in vivo. Using the characteristics of the yeast Ace1 transcriptional activator to probe for protein-protein interaction, we found that the *CDC25* gene product binds specifically to wild-type Ras2 but not to the mutated Ras2^{Val-19} and Ras2Δ^{Val-19} proteins. The binding properties of Cdc25 to Ras2 were strongly diminished in yeast cells expressing an inactive Ira1 protein, which normally acts as a negative regulator of Ras activity. On the basis of these data, we propose that the ability of Cdc25 to interact with Ras2 proteins is strongly dependent on the activation state of Ras2. Cdc25 binds predominantly to the catalytically inactive GDP-bound form of Ras2, whereas a conformational change of Ras2 to its activated GTP-bound state results in its loss of binding affinity to Cdc25.

The Ras proteins of *Saccharomyces cerevisiae* belong to a large family of GTP-binding proteins. In yeast cells, Ras exhibits a crucial function in the nutrient signal transduction pathway, which indicates the availability of nutrients to cellular processes. Ras accomplishes its biological function through a cycle of GDP-GTP exchange and subsequent GTP hydrolysis (3, 26) (Fig. 1). It stimulates adenylate cyclase (AC) in a GTP-dependent manner via the cyclase-associated protein Cap1 (also called Srv2) (17, 20, 25), which finally results in the formation of the second messenger cyclic AMP (cAMP). The activation state of Ras is controlled by two classes of regulatory proteins encoded by the genes *IRA1/IRA2* and *CDC25/SDC25*, respectively. The Ira1 or Ira2 protein can convert Ras to the catalytically inactive, GDP-bound state (54, 56) by stimulation of the intrinsic GTPase activity of Ras (53). It has been shown that the reverse reaction, a GDP-to-GTP exchange, is catalyzed by the *CDC25* and *SDC25* gene products (13, 32) (Fig. 1). The *CDC25* gene was originally identified as a temperature-sensitive mutation leading to an arrest of mitotic cell growth in G_1, which is accomplished by an altered intracellular level of cAMP at the restrictive temperature (46). Genetic data indicate that the *CDC25* gene product acts as a regulatory element upstream of Ras (4) because the dominant activating *RAS2*$^{Val-19}$ and *RAS2*Δ mutants are able to bypass the growth arrest of the conditionally lethal *cdc25-1* mutation as well as lethal *cdc25* disruptions (37, 47). Recently, Powers et al. (45) described the isolation of two dominant lethal *RAS* mutations (*RAS2*$^{Ala-22}$ and *RAS2*$^{Pro-25}$). It has been proposed that the encoded mutant Ras proteins form a strong complex with the product of the *CDC25* gene.

To further investigate the relationship between Cdc25 and Ras proteins, we established a system in *S. cerevisiae* that makes use of fusion proteins to detect protein-protein interactions. This system is based on the activity of the yeast transcription factor Ace1 (59), also known as Cup2 (61). This protein induces the expression of the metallothionein gene *CUP1* by binding to *cis*-acting control sequences upstream of the transcription initiation site of *CUP1* in the presence of Cu or Ag ions (24, 59). Metallothionein protects the yeast cell against copper toxicity by tightly chelating copper ions (for a review, see reference 29). The *ACE1* gene product is composed of two domains acting independently of each other: the N-terminal half specifically binds to DNA in the presence of metal ions (24), whereas the C-terminal half codes for an acid blob-type transactivator function (11, 24). These observations are the basis for the protein-protein interaction scheme shown in Fig. 2. As reporters for Ace1 activity, we used either the β-galactosidase gene from *Escherichia coli* fused to the *CUP1* promoter (59) or metallothionein expression, which leads to copper-resistant growth (23). Copper has no effect on reporter gene transcription in yeast cells that separately express the two domains of Ace1. The creation of two hybrid proteins between the Ace1 domains and two proteins X and Y results in reporter gene expression in the presence of copper if and only if X and Y interact with one another, as this brings the transactivation part of Ace1 close to the DNA-binding domain and hence its normal site of action (Fig. 2C).

In this report, we demonstrate that under the conditions described above, the carboxy-terminal half of the *CDC25* gene product binds to both Ras1 and Ras2 proteins in vivo. As expected from genetic data, Sdc25 sequences can substitute for Cdc25. Furthermore, using an *ira1*-disrupted yeast strain and defined mutations in the *RAS2* gene, we were able to show that Cdc25 specifically binds to the catalytically inactive GDP-bound form of Ras2.

MATERIALS AND METHODS

Yeast strains and transformation. Yeast strains used in this study are described in Table 1. The Bio-Rad gene pulser was used for transformation of yeast cells by electroporation (1). For verification of correct integration and gene replacement events, polymerase chain reactions (PCRs) were performed with primers flanking the insertion sites. Subsequently, the amplified fragments were analyzed by agarose gel electrophoresis.

Recombinant DNA techniques. Manipulation of DNA was carried out by using standard procedures throughout this study (36). *E. coli* MC1061 was used for the construction and

* Corresponding author.

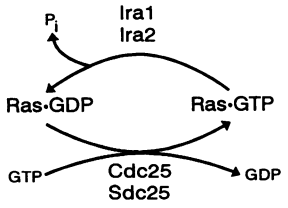

FIG. 1. Regulation of Ras activity by Ira1/Ira2 and Cdc25/Sdc25.

propagation of plasmids. Double-stranded DNA was sequenced by the dideoxynucleotide termination method (50), using the Pharmacia T7 sequencing system.

Construction of plasmids that express the Ras-Ace1(C) fusion. A 393-bp truncated version of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter ($GAPCL_P$ [38]) was cloned as a 670-bp *Sal*I-*Eco*RI fragment into pUC19. Subsequently, the synthetic polylinker composed of *Eco*RI, *Nsi*I, *Sph*I, and *Bgl*II sites, 5′-AATTCATGCATG CATGCAGATCT-3′ 3′-GTACGTACGTACGTCTAGATT AA-5′, was inserted into the *Eco*RI site of the former plasmid to give pTM2 and, for the reverse linker orientation, pTM2R. The coding sequence for the transactivation domain of Ace1, starting at residue 123, was taken as a 968-bp *Bgl*II fragment from plasmid pRI3 (59) and cloned in the *Bgl*II site of pTM2, yielding pTM3. pTM4 was constructed by cloning the 1,166-bp *Sal*I-*Sna*BI fragment of pTM3 between the unique *Bam*HI-*Sal*I sites of the 2μm circle-derived expression plasmid pDP34 (38) after filling in the *Bam*HI site with Klenow enzyme (Fig. 3). The *Eco*RI-*Bgl*II sites downstream of $GAPCL_P$ were used as cloning sites for the insertion of *RAS* genes.

The wild-type *RAS1* and *RAS2* genes were obtained by PCR, using total yeast genomic DNA as the template. The sequences were verified by dideoxy sequencing. The 5′ oligonucleotides were designed to generate an *Eco*RI site directly upstream of the ATG codon. The 3′ oligonucleotides generated a *Bgl*II site at the end of the *RAS* coding sequence, removed the stop codon, and conveniently allowed in-frame fusions with the transactivation (C-terminal) domain of Ace1 (Ace1C).

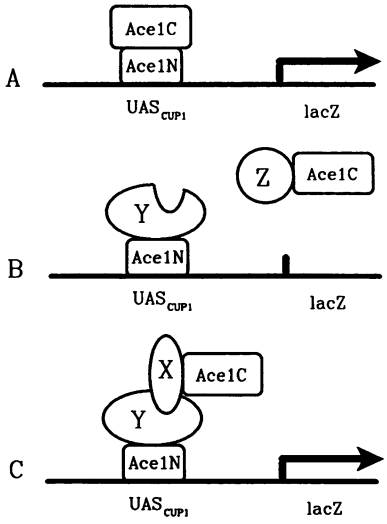

FIG. 2. The Ace1-based protein-protein interaction system. (A) Native Ace1 binds to the upstream activation sequence of *CUP1* (UAS_{CUP1}) and induces transcription of the *lacZ* reporter gene. (B) Fusions between the Ace1 domains and the unrelated proteins Y and Z result in an inhibition of *lacZ* gene expression. (C) Fusions between both Ace1 domains and the interacting proteins X and Y stimulate expression of β-galactosidase.

Derivatives of plasmid YCp50 containing various *RAS2* mutations were kindly provided by J. Gibbs. From these plasmids, the *RAS2* mutations $RAS2^{Val\text{-}19}$, *RAS2*Δ, and $RAS2\Delta^{Val\text{-}19}$ were amplified by PCR with primers containing the *Eco*RI and *Bgl*II restriction sites as described above and transferred into the pTM4 expression vector.

The $RAS2^{Ala\text{-}22}$ mutation was obtained by performing two independent PCRs with yeast genomic DNA as the template. Amplification with the primers 5′-GCGAATTCATGCCTT TGAACAAGTCG and 5′-TGGTTAACGCAGATTTAGCA ACACC (restriction sites underlined) resulted in the 5′-terminal 80-bp fragment of *RAS2*, starting with an *Eco*RI site at the start codon and ending with a newly created *Hpa*I site downstream of the introduced Ala-22 mutation. A second PCR with the primers 5′-TCTGCGTTAACCATACAAT TGACCCA and 5′-CCAGATCTAAACTTATAATACAA CAGCC resulted in a second fragment of 894 bp flanked by a *Hpa*I site and a *Bgl*II site. Finally a three-way ligation between the two digested PCR fragments and the *Eco*RI-*Bgl*II-digested pTM4 DNA resulted in plasmid pTM40. The $RAS2^{Ile\text{-}152}$ mutation was obtained analogously, using the internal primers 5′-GCTTGCTTAGCAGATATCTCCAAG AAAGG and 5′-CCTTTCTTGGAGATATCTGCTAAGCA AGC, which generated an *Eco*RV site. The sequences of all DNA fragments obtained by PCR have been verified by sequencing using custom-made primers.

Construction of plasmids that express the Ace1(N)-Cdc25 and -Sdc25 fusions. After destruction of the *Xba*I site in pUC19 (resulting in pTM5), a 423-bp *Eco*RI-*Bam*HI fragment of pTM2R was inserted into the corresponding sites of pTM5. pRI3 (59) was digested with *Eco*RV and *Bgl*II to obtain a 544-bp fragment containing the coding sequence for the DNA-binding (N-terminal) domain of Ace1, including residues 1 to 122 (Ace1N) and its entire promoter region. Cloning of this DNA into the *Bgl*II and blunt-ended *Eco*RI sites of pTM6 yielded pTM7. The 405-bp *Bgl*II-*Bam*HI fragment of pTM7 was replaced by the corresponding fragment of pTM2 to obtain an *Eco*RI site just downstream of the N-terminal portion of Ace1 (pTM8). Finally, a 1,479-bp *Eco*RI-*Bgl*II fragment of plasmid pYSK7 (6) containing the yeast *CYC1* gene (coding for iso-1-cytochrome c_1), including its termination sequences, followed by the *TRP1* gene was inserted between the *Eco*RI-*Bam*HI sites of pTM8, yielding the yeast integrating plasmid pTM9 (Fig. 3).

The carboxy-terminal part of the yeast *CDC25* gene (*CDC25*C), including residues 877 to 1588, was amplified as a PCR *Bgl*II-*Nsi*I fragment (primers 5′-GGAGATACTGGA GAATTTAGATCTGTCAATT and 5′-GCAAATTGTAT GCATTTTTATCGAA) from yeast genomic DNA and inserted between the unique *Bgl*II-*Nsi*I sites of pTM9. pTM9-CDC25C was integrated into the *trp1* allele of strain TFY2 after *Xba*I linearization, producing TFY3.

The carboxy-terminal part of the yeast *SDC25* gene (*SDC25*C) from residues 701 to 1251 was cloned analogously by PCR, using primers creating a *Pvu*II site flanking the *SDC25* coding sequence. Digestion of the PCR fragment with *Pvu*II and ligation with blunt-ended *Bgl*II-*Eco*RI-digested pTM9 DNA resulted in plasmid pTM9-SDC25C, which was then integrated into the *ace1-1* allele of TFY2 after linearization with *Nco*I (TFY58).

Cloning of *BCY1*. The yeast *BCY1* gene was cloned by

TABLE 1. Yeast strains used

Strain	Genotype	Fusion
KL1b[a]	*MAT***a** *cdc25-1 ura3*	
BR10[b]	*MAT***a** *his4 trp1-285 gal1 ade CUP1*^{r}	
DTY23[b,c]	*MAT*α *his ura3-52 ace1 LEU2*::YipCL *CUP1*^{r}	
TFY2[d]	*MAT*α *his ura3-52 trp1-285 ace1 LEU2*::YipCL *CUP1*^{r}	
TFY13	*MAT*α *his ura3-52 ace1 LEU2*::YipCL *TRP1*::pTM9-BCY1 *CUP1*^{r}	*ACE1N-BCY1*
TFY14	TFY13/pTM4	*ACE1C*
TFY15	TFY13/pTM4-BCY1	*BCY1-ACE1C*
TFY20	TFY13/pTM4-RAS2	*RAS2-ACE1C*
TFY3	*MAT*α *his ura3-52 ace1 LEU2*::YipCL *TRP1*::pTM9-CDC25C *CUP1*^{r}	*ACE1N-CDC25C*
TFY7	TFY3/pTM4	*ACE1C*
TFY52	TFY3/pTM37	*RAS1-ACE1C*
TFY5	TFY3/pTM4-RAS2	*RAS2-ACE1C*
TFY32	*MAT*α *his ura3-52 ace1 ira1*::*ILV2*^{r} *LEU2*::YipCL *TRP1*::pTM9-CDC25C *CUP1*^{r}	*ACE1N-CDC25C*
TFY33	TFY32/pTM4	*ACE1C*
TFY56	TFY32/pTM37	*RAS1-ACE1C*
TFY34	TFY32/pTM4-RAS2	*RAS2-ACE1C*
TFY59	TFY3/pTM40	*RAS2*$^{Ala-22}$*-ACE1C*
TFY67	TFY3/pTM47	*RAS2*$^{Ile-152}$*-ACE1C*
TFY68	TFY3/pTM49	*RAS2*$^{Val-19}$*-ACE1C*
TFY69	TFY3/pTM51	*RAS2Δ-ACE1C*
TFY70	TFY3/pTM53	*RAS2Δ*$^{Val-19}$*-ACE1C*
TFY58	*MAT*α *his ura3-52 ace1 LEU2*::YipCL *TRP1*::pTM9-SDC25C *CUP1*^{r}	*ACE1N-SDC25*
TFY60	TFY58/pTM4	*ACE1C*
TFY61	TFY58/pTM37	*RAS1-ACE1C*
TFY62	TFY58/pTM4-RAS2	*RAS2-ACE1C*

[a] Described in reference 42.
[b] BR10 and DTY23 are described in references 49 and 59, respectively.
[c] YipCL contains a fusion between the *CUP1* promoter and the *E. coli lacZ* gene (59).
[d] Derived from a cross between BR10 and DTY23.

using PCR as described above. The primers contained *Eco*RI and *Bgl*II restriction sites, which allowed the insertion of the amplified fragments into pTM4 and pTM9. The multicopy plasmid pTM4-BCY1 expresses a fusion protein, which is composed of the first 161 residues of Bcy1 followed by the transactivation domain of Ace1, whereas pTM9-BCY1 expresses the fusion between the Ace1 DNA-binding domain and the first 161 Bcy1 residues. The latter plasmid was linearized with *Xba*I and integrated into the yeast *trp1* allele of TFY2.

***IRA1* gene disruption.** A central portion of the yeast *IRA1* gene starting at codon 1202 and extending to codon 2078 was amplified by PCR from yeast genomic DNA, using the primers 5′-GCACTAGTAAACTCGAGTTAACG and 5′-CGGTCGACACGGTAACCTTGTACAAC. After digestion of the 2,628-bp fragment with *Spe*I and *Sal*I, the DNA was cloned into the appropriate sites of pBluescript KS(+). The *IRA1* gene was disrupted by replacement of an 1,101-bp fragment starting at the *Bgl*II site (codon 1428) and extending to the *Hin*dIII site (codon 1795) with a 2,765-bp fragment encoding a mutated version of the yeast *ILV2* gene (*ILV2*^{r}; kindly provided by F. Buxton) which confers resistance to the herbicide sulfometuron methyl at a concentration of 30 μg/ml (10). The 4,292-bp *Xho*I-*Bst*EII fragment containing the disrupted *ira1* gene was introduced into the yeast genome of strain TFY3 by single-step replacement (48), which gave rise to strain TFY32.

β-Galactosidase assay. Yeast cells were grown in SD medium (2% glucose, 0.67% yeast nitrogen base without amino acids) supplemented with 40 mg of histidine per liter at 30°C to a density of 2×10^7 cells per ml. Induction of reporter gene transcription with $CuSO_4$ and permeabilization of cells were performed as described previously (24). β-Galactosidase activity, measured as *o*-nitrophenyl-β-D-galacto-

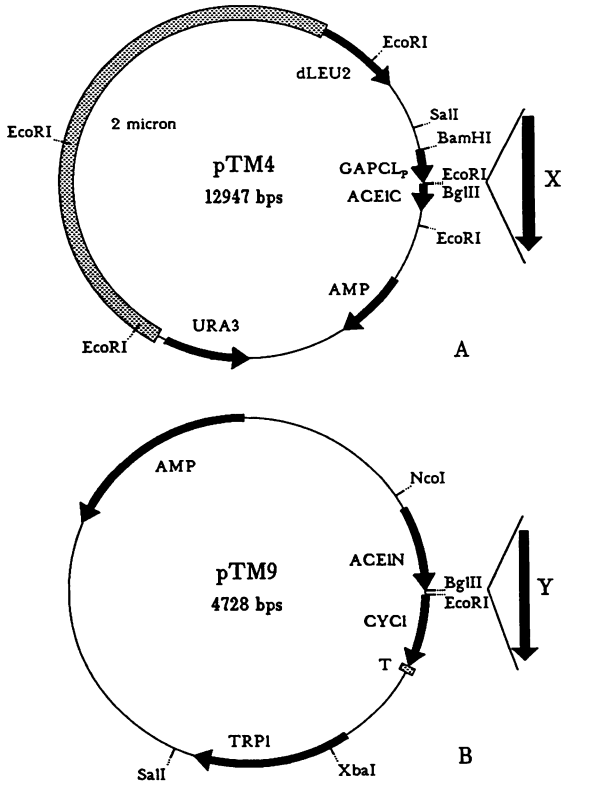

FIG. 3. Expression plasmids pTM4 and pTM9. Positions of target gene insertion are indicated. X, *BCY1*, *RAS1*, *RAS2*, *RAS2*$^{Val-19}$, *RAS2Δ*, *RAS2Δ*$^{Val-19}$, *RAS2*$^{Ile-152}$, and *RAS2*$^{Ala-22}$; Y, *BCY1*, *CDC25C*, and *SDC25C*.

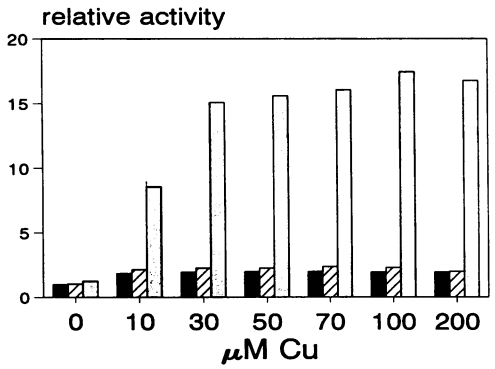

FIG. 4. Validation of the Ace1 protein interaction system. Shown is Cu-dependent β-galactosidase expression in yeast strains containing the hybrid proteins Ace1N-R subunit and Ace1C (TFY14; black bars), Ace1N-R subunit and R subunit-Ace1C (TFY15; shaded bars), and Ace1N-R subunit and Ras2-Ace1C (TFY20; hatched bars).

pyranoside hydrolysis at 420 nm, was normalized to cell culture density and expressed as arbitrary units. The activity in control strain TFY7, TFY14, or TFY60 was set to the value of 1.

RESULTS

Validation of the protein-protein interaction system. To validate the protein-protein interaction assay, we analyzed the ability of the regulatory (R) subunit of the yeast cAMP-dependent protein kinase, encoded by the gene *BCY1* (9, 35, 57, 60), to form dimers. Since it is known that the formation of a stable R-subunit dimer requires only a segment within the amino terminus (60), we fused the N-terminal half of the R subunit to the Ace1 DNA-binding domain (plasmid pTM9-BCY1), integrated the DNA into the yeast strain TFY2, and subsequently transformed this strain (TFY13) with the multicopy plasmid pTM4-BCY1, expressing the transactivation domain of Ace1 fused to the N terminus of the R subunit (strain TFY15; see Materials and Methods).

The induction of *lacZ* gene transcription by addition of $CuSO_4$ is shown in Fig. 4. Copper has no effect on β-galactosidase expression in strain TFY14 carrying the Ace1N-R subunit fusion alone. In contrast, strain TFY15 showed a highly copper dependent expression of the reporter gene, resulting in a maximal stimulation of about 14-fold. Coexpression of both fusion proteins also rendered TFY14 highly copper resistant (not shown). As a negative control we used strain TFY20, which expresses the Ace1N-R subunit and the Ras2-Ace1C fusions. No copper-dependent induction of β-galactosidase was measured in TFY20. The two calpactin I subunits (31) also showed strong interaction when fused individually to both domains of Ace1 (not shown). These data indicate that we were able to detect direct and specific protein-protein interactions in yeast cells in vivo.

Interaction of Cdc25 with wild-type Ras2 protein. Genetic data suggest that Ras2 might be the substrate of Cdc25. If that is the case, both proteins have to associate directly. To probe for such an interaction, we expressed the C-terminal 711 residues of Cdc25, which are sufficient to suppress the lethal phenotype of *cdc25* null mutants (4), as an Ace1N-Cdc25C fusion protein. Figure 5A shows the Cu-inducible expression of the *lacZ* gene in strain TFY5, expressing both the Ace1N-Cdc25C and Ras2-Ace1C hybrids, and TFY7,

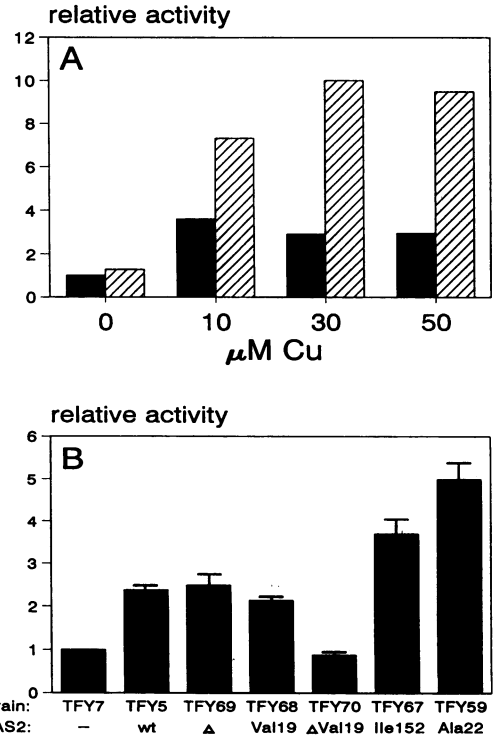

FIG. 5. (A) Cu-dependent β-galactosidase expression in strains TFY7 (*CDC25C*; black bars) and TFY5 (*CDC25C-RAS2*; hatched bars); (B) *lacZ* gene induction by addition of 30 μM Cu in strains TFY7, TFY5, TFY69 (*CDC25C-RAS2Δ*), TFY68 (*CDC25C-RAS2*$^{Val-19}$), TFY70 (*CDC25C-RAS2Δ*$^{Val-19}$), TFY67 (*CDC25C-RAS2*$^{Ile-152}$), and TFY59 (*CDC25C-RAS2*$^{Ala-22}$). Data are expressed as means ± standard deviations from at least three independent experiments.

which expresses the Ace1N-Cdc25C fusion and the transactivation domain of Ace1 alone. Copper induces *lacZ* gene expression to a stimulation of about two- to threefold in strain TFY5 compared with TFY7, which suggests that the C terminus of Cdc25 binds directly to the Ras2 protein in vivo. Addition of more than 50 μM $CuSO_4$ results in reduced *lacZ* expression due to copper toxicity (see Discussion). To address the specificity of this interaction, we examined the ability of Cdc25 to bind to mutated Ras2 proteins.

Mutations in Ras2 critically affect binding to Cdc25. The induction of β-galactosidase expression after addition of 30 μM Cu in strains expressing various Ace1C-mutant Ras2 hybrids is shown in Fig. 5B. The deletion of Ras2 residues 175 to 300 (*RAS2Δ*) in strain TFY69 has no effect on *lacZ* expression in comparison with wild-type Ras2 (strain TFY5). Similar results were obtained with a Ras2 mutant that lacks the whole C-terminal portion starting at residue 175, including the membrane localization signal Cys-Ile-Ile-Ser (data not shown). Expression of the dominant activated form of Ras2 (Ras2^{Val-19}) in strain TFY68 resulted in a weak but reproducible reduction of β-galactosidase activity (*CDC25C-RAS2* = 2.38 ± 0.11; *CDC25C-RAS2*$^{Val-19}$ = 2.14 ± 0.08). In strain TFY70 containing the double mutant Ras2Δ^{Val-19}, no enhanced *lacZ* gene expression was observed in comparison with strain TFY7. The opposite pattern of β-galactosidase induction was obtained in strain TFY59 expressing the Ras2^{Ala-22}-Ace1C hybrid. Here the expression level of the *lacZ* gene is much higher than in

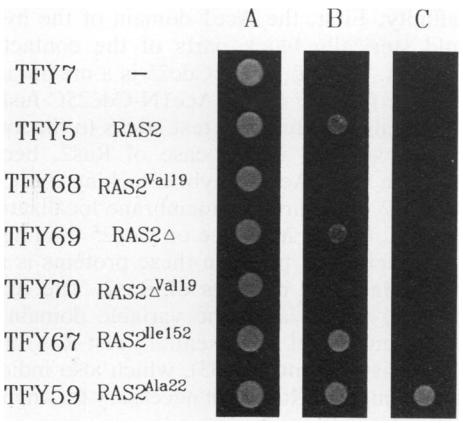

FIG. 6. Induction of metallothionein expression. Equal numbers of cells were spotted onto SD plates containing no (A), 110 μM (B), and 200 μM (C) $CuSO_4$ and incubated for 3 days at 30°C.

strain TFY5 containing the wild-type Ras2-Ace1C fusion. Finally, in a strain expressing the $Ras2^{Ile-152}$-Ace1C hybrid protein, we measured β-galactosidase activity intermediate to the levels detected in TFY5 (wild-type Ras2) and TFY59 ($Ras2^{Ala-22}$). To exclude the possibility that the Ras-Ace1C fusions interact themselves with the DNA-binding domain of Ace1 and induce transcription of the reporter genes, we performed β-galactosidase assays in strains expressing the various Ras2-Ace1C proteins and Ace1N. We observed no significantly enhanced β-galactosidase activity compared with the strain expressing Ace1N and Ace1C alone (not shown).

To confirm our data, we tested the ability of various strains to grow in the presence of copper. Figure 6 shows the viability of yeast strains after 3 days of incubation on $CuSO_4$-containing minimal plates. At 110 μM $CuSO_4$, the copper resistance is restricted to cells expressing Ace1C hybrids with wild-type Ras2, Ras2Δ, $Ras2^{Ile-152}$, and $Ras2^{Ala-22}$, whereas the $Ras2^{Val-19}$ and $Ras2\Delta^{Val-19}$ hybrids did not grow any more. At 200 μM $CuSO_4$, strain TFY59 (*CDC25C-RAS2*$^{Ala-22}$) is still copper resistant, in contrast to all other *RAS2* variants analyzed. In summary, copper resistance and β-galactosidase levels are correlated. To prove that the Cdc25- and Ras2-Ace1 hybrid proteins retains their biological activity within the nutrient signal transduction pathway, we examined their abilities to complement the temperature-sensitive *cdc25-1* mutation. Expression of the $Ras2^{Val-19}$-Ace1C hybrid fully suppresses the growth lesion of *cdc25-1* at the nonpermissive temperature of 37°C, whereas Ras2-Ace1C does not (data not shown). This finding is in accordance with previous results showing that the activity of dominant activated $Ras2^{Val-19}$ is independent of an active Cdc25 protein, in contrast to wild-type Ras2 (4, 37, 47). Additionally, we found that expression of the Ace1N-Cdc25C hybrid under the control of the Ace1 promoter restores growth of the *cdc25-1* mutant at 37°C (not shown).

Taken together, our data clearly demonstrated that in this hybrid-protein interaction system, the C-terminal half of the *CDC25* gene product binds much more strongly to the $Ras2^{Ala-22}$ and $Ras2^{Ile-152}$ proteins than to wild-type Ras2. We were not able to detect an interaction between Cdc25 and the constitutively activated Ras2 variant $Ras2\Delta^{Val-19}$. Binding of Cdc25 to $Ras2^{Val-19}$ was weak, particularly with copper resistance used as a reporter. In the $Ras2^{Val-19}$ and especially the $Ras2\Delta^{Val-19}$ mutants, a higher amount of GTP

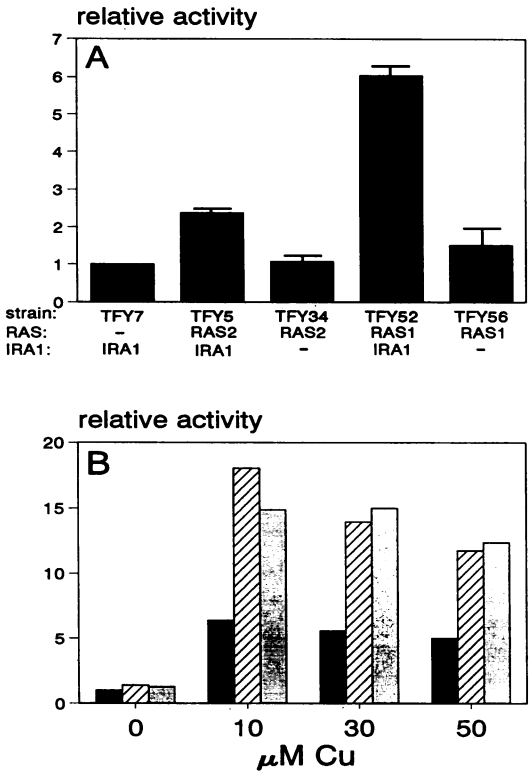

FIG. 7. (A) Ira1-dependent expression of β-galactosidase after addition of 30 μM $CuSO_4$ in strains TFY7 (*CDC25C*), TFY5 (*CDC25C-RAS2*), TFY34 (*CDC25C-RAS2 ira1*), TFY52 (*CDC25C-RAS1*), and TFY56 (*CDC25C-RAS1 ira1*). Data are expressed as means ± standard deviations from at least three independent experiments. (B) Sdc25-Ras interaction. Shown is Cu-dependent expression of β-galactosidase activity in strains TFY60 (*SDC25C*; black bars), TFY61 (*SDC25C-RAS1*; hatched bars), and TFY62 (*SDC25C-RAS2*; shaded bars).

is complexed to the protein than in wild-type Ras2 (27, 55). These findings lead us to speculate that the level of phosphorylation of Ras2-bound guanine nucleotides controls binding to Cdc25. In *ira*1-disrupted strains, Ras2 is constitutively active because Ras2 is complexed with GTP, thus remaining in the activated state (54, 55). We therefore checked for Ras2-Cdc25 interaction in an *ira*1-disrupted strain.

Cdc25 and Ras do not associate in *ira*1-disrupted yeast strains. We disrupted the *IRA1* gene by deleting an essential fraction of its coding region showing homology to the C terminus of the mammalian GTPase-activating protein (GAP) (see Materials and Methods). Figure 7A shows that in the *ira1*-disrupted strain TFY34, no copper-induced expression of β-galactosidase occurred, in contrast to the wild-type *IRA1* strain TFY5. This result showed that in Ira1-deficient yeast cells, no interaction between Cdc25 and wild-type Ras2 takes place, probably because Ras2 exists in the activated state, although an additional role of Ira1 in Cdc25-Ras2 interaction cannot be excluded.

Furthermore, we examined whether Cdc25 is also able to bind to the Ras1 protein. As shown in Fig. 7A, we were able to measure a strong copper-dependent induction of *lacZ* gene expression in strain TFY52, containing the *ACE1N-CDC25C* and *RAS1-ACE1C* hybrid genes. Interestingly, we did not observe any β-galactosidase expression in the iso-

genic but *ira1*-disrupted strain TFY56, indicating that Ira1 uses Ras1 as a substrate as well as Ras2. The level of copper resistance of these strains correlated with the respective β-galactosidase activities. Ira1$^-$ strains, expressing the Ace1-Cdc25C and -Ras1 or -Ras2 hybrids, were not able to grow on minimal plates containing 110 μM $CuSO_4$ (data not shown).

Interaction of Sdc25 with Ras proteins. Since we were successful in detecting direct protein-protein interaction between Cdc25 and Ras proteins, it was interesting to examine the ability of the extragenic suppressor of *cdc25* mutants called *SDC25* for binding to Ras proteins. Recent data indicate that the carboxy-terminal portion of Sdc25 is able to enhance GDP-GTP exchange of the Ras2 protein (13). We have fused the C-terminal half of Sdc25, composed of 550 residues, to the Ace1 DNA-binding domain and integrated this hybrid gene into the yeast genome (strain TFY58). Coexpression of Ras1-Ace1C (TFY61) or Ras2-Ace1C (TFY62) led to copper-inducible β-galactosidase expression (Fig. 7B). We conclude that the *SDC25* gene product, like Cdc25, can form complexes with both Ras proteins.

As in the case of *CDC25*, we observed slightly elevated β-galactosidase expression in strains expressing the Ace1N-Sdc25C hybrid in comparison with the Ace1C domain alone. This finding reflects an artificial transactivation function of either one of the proteins due to the targeting of Cdc25 and Sdc25 to the nuclear environment.

DISCUSSION

In this report, we demonstrate for the first time biochemically that the carboxy-terminal half of the *CDC25* gene product binds to Ras proteins in vivo.

The system that we used to determine this specific protein-protein interaction, outlined in Fig. 2, is based on the characteristics of the yeast transcription factor Ace1. It allows to qualitatively determine binding affinities between two given proteins simply by varying the copper concentration in the medium. Copper induces the binding of Ace1 to DNA. Fusion proteins with a high affinity to each other give rise to higher levels of reporter gene expression than do low-affinity complexes. The instability of the latter complexes results in lower β-galactosidase activities and in an enhanced sensitivity to copper. A similar approach based on Gal4 has been used by Fields and Song to show interaction between Snf1 and Snf4 (22). Dimerization of the R subunit of the cyclic AMP-dependent protein kinase, an example of a stable and high-affinity protein-protein interaction, was used to validate the approach. The binding affinity of the Cdc25-Ras interaction is considerably lower than that of the R-subunit dimerization, indicated by a reduced β-galactosidase activity and an enhanced sensitivity to copper. We conclude from this observation that Cdc25 is weakly bound to Ras. Survival as a reporter is a very stringent and sensitive marker that overestimates the strength of weak interactions. Quantification of growth is difficult. Cells that would grow no more in the presence of a certain amount of copper might find escape routes, e.g., via inducing *CUP*1 expression (7, 63). We therefore consider *lacZ* to be a more accurate reporter. The rather low affinity between Cdc25 and Ras may be due to a high dissociation rate of the protein complex, which is necessary for transient activation of Ras by Cdc25. Our lack of successful coimmunoprecipitation and coelution experiments supports this interpretation. However, we cannot exclude other reasons, which might contribute to the low binding affinity. First, the Ace1 domain of the hybrid proteins could sterically block parts of the contact sites of Cdc25 and Ras. Second, since Cdc25 is a membrane-bound protein, only a fraction of the Ace1N-Cdc25C fusion might enter the nucleus, whereas the rest sticks to the membrane. This can be excluded in the case of Ras2, because the expression of a Ras2-Ace1N hybrid lacking the C-terminal portion of Ras2, including the membrane localization signal Cys-Ile-Ile-Ser, has no influence on Cdc25 binding, indicating that the interaction between these proteins is restricted to the N-terminal 174 residues of Ras2. The mammalian H-Ras protein, which lacks the variable domain of yeast Ras, complements all of the essential yeast Ras functions in a *ras*1 *ras*2 yeast mutant (15, 33), which also indicates that the variable domain of Ras is not necessary for an interaction with Cdc25.

Cdc25 functions as a catalyst for GDP-GTP exchange by Ras proteins. In cells carrying the dominantly activated mutations *RAS2*$^{Val\text{-}19}$ and *RAS2*Δ, the mutated Ras proteins have a reduced rate of GTP hydrolysis, which renders the cells independent of regulation by Cdc25 (4, 37, 47). In the double mutant Ras2Δ$^{Val\text{-}19}$ a much higher amount of GTP is complexed to the protein than in the single mutant alone (27). This reflects the enhanced biological potency of Ras2Δ$^{Val\text{-}19}$ to bypass the *cdc25* lethality (37). In strains expressing the Ras2$^{Val\text{-}19}$-Ace1C protein, we observed a reduced affinity of Cdc25 for Ras2$^{Val\text{-}19}$, and the double mutant Ras2Δ$^{Val\text{-}19}$ did not interact with Cdc25 at all. In vegetatively growing cells, the equilibrium of bound guanine nucleotides to wild-type Ras is strongly shifted to the GDP-complexed form (27). We propose that Cdc25 interacts preferentially with the GDP-bound Ras protein, implying a rapid dissociation of the protein complex after the conformational change of Ras to the GTP-bound form. The structural changes of Ras after release of GDP and binding of a new GTP molecule would therefore be responsible for the different affinity of Cdc25 to Ras proteins. X-ray studies revealed that two regions of the H-Ras protein are the main elements whose conformation change when GTP replaces GDP: the switch I domain, including residues 30 to 38 of loop L2, and the switch II domain, consisting of residues 60 to 76 of loop L4 and the α2 helix downstream of it (5, 40, 44). After binding of GTP, both loops interact with the γ phosphate of the guanine nucleotide, which induces the conformational change of H-Ras. Both regions are located at the surface of the molecule, as shown by X-ray analysis of the crystal structure. Therefore, they are good candidates for interaction with other proteins (40). It has been suggested from genetic experiments that the switch I region and parts of the switch II region are possible contact sites with mammalian GAP (reviewed in references 2 and 40). Furthermore, it was hypothesized that the switch II region is one possible recognition site for a putative upstream regulator of H-*ras*. The coupling of both switch domains by the γ phosphate leads to a model predicting that the putative guanine nucleotide release protein binds to the switch II domain of H-Ras, which leads to the release of GDP by a subsequent binding of GTP, finally inducing changes in the switch I domain, which is now able to interact with GAP and/or other effector molecules (40). In view of the high structural and functional similarities between the yeast and mammalian Ras proteins, it is very likely that their topological structures are similar and that the conformational transition between GDP- and GTP-bound forms involve the same structural domains (44). We therefore expect that at least the residues around 67 to 83

of yeast Ras2 (identical with the switch II domain of H-Ras) interact with Cdc25.

Our suggestion is supported by the fact that the switch II domain is the main element whose conformation is changed in the oncogenic GTP-bound H-Ras^{Val-12} mutant (34). The equivalent Ras2^{Val-19} protein does not bind to Cdc25, possibly because of poorly accessible contact sites in switch II for Cdc25. It cannot be excluded that other elements of Ras participate in the interaction with Cdc25. The guanine nucleotide release proteins of EF-Tu and G-protein α chains bind to specific regions involved in guanine ring complexing, which are homologous to H-Ras residues 112 to 119, 144 to 146, and 152 to 166. It has been postulated that these regions are potential binding sites for putative H-*ras* guanine nucleotide release proteins (reviewed in reference 2).

Feig et al. reported a decreased affinity of the H-Ras$^{Ile-144}$ mutant for GTP (19). We found that the analogous substitution of Thr-152 to Ile in the yeast Ras2 protein leads to increased binding to Cdc25. Ras2$^{Ile-152}$ versus Ras2 exhibits a moderately reduced affinity for GTP and a strongly decreased affinity for GDP (12). Overall, this leads to enhanced GDP-GTP exchange. Cdc25 probably interacts with the GDP-GTP pocket of Ras2 in order to perform the nucleotide exchange reaction. The Ras2$^{Ile-152}$ mutation is located within this pocket. The altered binding properties of Ras2$^{Ile-152}$ toward Cdc25 are therefore not surprising. However, the phenotype of Ras2$^{Ile-152}$ does not have to be accounted for by the altered binding properties, since Ras2$^{Ile-152}$ suppresses temperature-sensitive *cdc25* mutations (8).

The *RAS2*$^{Ala-22}$ mutation (a replacement of Gly) was identified as a dominant-interfering inhibitor of wild-type Ras2 function (45). Those mutations are known to be functional with respect to binding to an appropriate target but nonfunctional in producing any cellular responses (30). The temperature-sensitive growth inhibition of yeast strains carrying the *RAS2*$^{Ala-22}$ allele was found to be suppressed by overexpression of Cdc25, but only in the presence of wild-type Ras2. These results were interpreted as a formation of an irreversible complex between Cdc25 and the mutant Ras2 protein, resulting in the depletion of endogenous Cdc25 and thereby blocking its activity (45). Our data demonstrate that such a complex indeed exists. Enhanced reporter gene expression shows that this complex is stabilized compared with the interaction of Cdc25 with wild-type Ras2. In mammalian systems, homologous dominant-interfering mutations in the H-Ras protein are known (H-*ras*$^{Ala-15}$, H-*ras*$^{Asn-16}$, and H-*ras*$^{Asn-17}$), which inhibit wild-type H-Ras and/or yeast Ras function (18, 45, 51, 52). These mutations are located in the highly conserved phosphate-binding loop L1, where they affect their ability to bind guanine nucleotides: in H-Ras^{Asn-16} the affinity for both GDP and GTP is reduced (51), whereas H-Ras^{Asn-17} has a preferential affinity for GDP (18, 52). The Ras2^{Ala-22} mutant is not able to convert from the biologically inactive to the active form either because it is locked in the GDP-bound off conformation or because it is impaired in the ability to bind guanine nucleotides at all, resulting in a permanent association with Cdc25 (45). Since it is extremely unlikely that Ras proteins exist in a guanine nucleotide-free state for an extended time period (28), we favor the first explanation.

The yeast homologs to mammalian GAP are proteins encoded by the *IRA1* and *IRA2* genes. Disruption of the *IRA1* or *IRA2* gene results in an enhanced production of cAMP in response to addition of glucose due to reduced activity of the endogenous Ras GTPase, which in turn results in a significantly increased amount of Ras-GTP (54, 55). We found that in an *ira1*-disrupted strain, no interaction between Cdc25 and Ras2 as well as Ras1 proteins occurs. One interpretation is that Cdc25 does not bind to the GTP-bound form of Ras, in agreement with the Ras2 point mutation analysis. Our data support the suggestion of Tanaka et al. that Ira1 negatively regulates the activity of both Ras proteins (55). This view is in contrast to a previous report, which revealed that a *ras2* but not a *ras1* mutation can suppress the phenotype of an *ira1* disruption (54). However, it is possible that the presence of Ira1 is somehow necessary for the Cdc25-Ras interaction (see below).

Créchet et al. demonstrated recently that the C-terminal portion of the Sdc25 protein enhances the GDP-GTP exchange of Ras2 by acting especially on GDP-bound Ras2 (13). This finding leads to the suggestion that at least the carboxy-terminal half of Sdc25 (Sdc25C) interacts like Cdc25 with Ras2. Indeed, our results clearly exemplify this proposal. We are able to directly detect binding of Sdc25C to Ras proteins in vivo. However, there is evidence that the full-length Sdc25 protein is not able to undertake the biological function of Cdc25 (14). Since the *SDC25* gene is dispensable for cell growth, it is not known whether Ras2 is really a physiological target for Sdc25 despite its binding and guanine nucleotide exchange capacity.

In summary, our studies have shown that the carboxy terminus of Cdc25, starting at residue 877, interacts directly with the N-terminal 174 residues of the Ras2 protein in vivo. This interaction is restricted to the catalytically inactive GDP-bound form of Ras2. We cannot exclude the possibility that other proteins or protein domains such as Ira1 are directly involved in the formation or stabilization of the Cdc25-Ras complex. There is accumulating evidence that protein-protein interactions play an important role in the yeast nutrient signal transduction pathway. The ability of dominant-interfering mutations of AC to suppress the heat shock sensitivity of a Ras2^{Val-19} mutant has led to the idea that the mutated AC acts by sequestering Ras2 proteins, either by a direct binding to Ras via the leucine-rich repeats or with participation of other proteins, which combine with AC to form a complex capable of interacting with Ras, such as the cyclase-associated protein (21).

Engelberg et al. suggested a biochemical link between Cdc25 and AC, since overexpression of Cdc25 relocalizes AC to the plasma membrane in a *ras1 ras2 bcy1* mutant, in which the cyclase activity is found in the soluble fraction (16). Data obtained with Ira1-specific antibodies, which inhibit binding of AC to the plasma membrane and comigration of Ira1 and AC in gel filtration chromatography on Sepharose 4B, indicated that both proteins are associated in an oligomeric complex (41). This complex might also include Ira2 and at least transiently Ras proteins. We suggest that there exists a large multienzyme complex located at the inner surface of the plasma membrane, composed at least of the proteins Cdc25, Ras1, and Ras2, their negative regulators Ira1 and Ira2, Cap1, AC, and maybe Sdc25. This functional unit is activated by phosphorylated glucose via a still putative glucose-repressible protein (reviewed in reference 58). Finally, we want to stress the possibility that at least parts of this large protein complex, namely, Cdc25 (43), Ras (39, 62), and Cap (17, 20, 25), could act as branch points between other signal transduction pathways in yeast cells. This protein-protein interaction system reveals a powerful way to probe for such cross-talks.

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