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The *CDC25* Protein of *Saccharomyces cerevisiae* Promotes Exchange of Guanine Nucleotides Bound to Ras

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The product of the *CDC25* gene of *Saccharomyces cerevisiae*, in its capacity as an activator of the *RAS*/cyclic AMP pathway, is required for initiation of the cell cycle. In this report, we provide an identification of Cdc25p, the product of the *CDC25* gene, and evidence that it promotes exchange of guanine nucleotides bound to Ras in vitro. Extracts of strains containing high levels of Cdc25p catalyze both removal of GDP from and the concurrent binding of GTP to Ras. This same activity is also obtained with an immunopurified Cdc25p–β-galactosidase fusion protein, suggesting that Cdc25p participates directly in the exchange reaction. This biochemical activity is consistent with previous genetic analysis of *CDC25* function.

Yeast *RAS* proteins are members of a large family of structurally related GTP-binding proteins which mediate information flow, ensure fidelity of specific macromolecular interactions, and provide coupling of temporally or spatially distinct processes. These proteins accomplish their biological functions through a cycle of GTP-GDP exchange and GTP hydrolysis (1, 15). The GTP-bound forms of the proteins are active. Hydrolysis of GTP to GDP inactivates the proteins, and reactivation requires replacement of the bound GDP with GTP (14, 17, 19). Given the low dissociation rate of GDP from the proteins, reactivation requires a second agent that stimulates nucleotide exchange. For some G proteins, such as transducin and G_s, the agent is a receptor protein, which promotes GTP-GDP exchange only when stimulated by interaction with a specific agonist (15, 22). GTP-GDP exchange can also be catalyzed by a cytoplasmic protein, as is the case for eIF2B activation of mammalian initiation factor eIF2 (18). The factor that catalyzes exchange of guanine nucleotides bound to mammalian $p21^{h\text{-}ras}$ has not been identified, although exchange activity has been detected in membrane preparations of bovine brain and in cytosolic extracts from rat brain (29, 30).

The product of the *CDC25* gene is the most likely candidate for the guanine nucleotide exchange factor for yeast *RAS* proteins (4, 10, 20). In *Saccharomyces cerevisiae*, Ras proteins stimulate adenylate cyclase, a process required for initiation of the cell cycle. Inactivation of *CDC25* results in cell cycle arrest in G1, a phenotype identical to that obtained by inactivation of the *RAS* genes. Dependency of cell growth on *CDC25* function is alleviated by conditions that increase GTP-Ras levels in vivo (4, 13, 20). Such conditions obtain in strains carrying activated *RAS* alleles or in strains lacking *IRA1* or *IRA2*, genes whose products stimulate the intrinsic GTPase of Ras (24–26). In addition, hyperexpression of a truncated version of a gene, *SCD25*, suppresses null mutations of *CDC25* (2). Recently, Créchet et al. showed that the carboxyl-terminal portion of the *SCD25* gene product can catalyze in vitro exchange of guanine nucleotides bound to yeast Ras and mammalian $p21^{h\text{-}ras}$ (7). However, Scd25p is not likely to be the authentic in vivo exchange factor for Ras protein. The full-length *SCD25* gene cannot suppress *cdc25* mutations, and inactivating mutations of *SCD25* yield no apparent phenotype (2). While all of these data are consistent with Cdc25p acting as a nucleotide exchange factor, they could also be explained by ascribing some other role to Cdc25p, such as inhibiting the GTPase activity of Ras. To clarify the function of Cdc25p in *RAS* activation, we have examined its biochemical properties. In this paper we report that Cdc25p promotes exchange of guanine nucleotides bound to Ras.

MATERIALS AND METHODS

Yeast strains and plasmids. Yeast strains used in these experiments are listed in Table 1. Yeast transformations were performed by the lithium acetate method described by Ito et al. (16). Plasmid YEp13-*CDC25* was constructed by inserting an *Aat*II-*Pvu*II fragment spanning the *CDC25* gene into the *Bam*HI site of the high-copy-number vector YEp13 (3). Deletion of the internal *Bgl*II fragment in this plasmid yielded an in-frame deletion allele, YEp13-*cdc25Δ1*. Plasmid YEp62-*CDC25-lacZ* was constructed by inserting the *Bgl*II-*Sph*I fragment from *CDC25*, sufficient for $Cdc25^+$ activity, into YEp62 (3) which had been digested with *Bam*HI and *Sal*I. In this plasmid, *CDC25* and *lacZ* are fused in frame and are under the control of the inducible *GAL10* promoter. Plasmid YEp13-*TPK1* has been previously described (27). YEp352-*TPK1* was constructed by inserting a 2.4-kb *Hin*dIII-*Sph*I fragment spanning *TPK1* into YEp352 digested with the same enzymes.

Culture conditions. Yeast strains were grown in synthetic medium (0.67% yeast nitrogen base without amino acids [Difco], supplemented with the appropriate auxotrophic requirements as required [21]). Carbon sources were added to 2% (wt/vol).

Production of antibodies. Polyclonal antibodies were raised in rabbits against a 15-amino-acid carboxyl-terminal peptide (TDDKNGNFLKLGKKK) conjugated to KLH and against a hybrid protein synthesized from a gene composed of the 735-bp *Bam*HI-*Hin*dIII fragment from the 3′ portion of the *CDC25* coding region fused in frame to the 3′ end of the *Escherichia coli trpE* gene in the plasmid vector pATH2 (9).

Preparation of yeast extracts. Yeast strains were grown to a density of 1×10^7 to 3×10^7 cells per ml. In the case of strains MLV201 and MLV202, cells were grown initially in the presence of raffinose to a density of 0.5×10^7 cells per ml, at which point galactose was added to a concentration of

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TABLE 1. Yeast strains

Strain	Genotype
Y294	*MATα his3Δ leu2-3,112 ura3-52 trp1-289*
SJ18[a]	*MATα his3Δ leu2-3,112 ura3-52 trp1-289* [YEp13-*CDC25*]
SJ22[a]	*MATα his3Δ leu2-3,112 ura3-52 trp1-289 cdc25::HIS3* [YEp13-*TPK1*]
SJ103[a]	*MATα his3Δ leu2-3,112 ura3-52 trp1-289 cdc25::HIS3* [YEp13-*cdc25Δ1*]
SJ24-1B	*MATα his3Δ leu2-3,112 ura3-52 trp1-289 cdc25::HIS3* [YEp352-*TPK1*]
MLV201[b]	*MATα his3Δ leu2-3,112 ura3-52 trp1-289 cdc25::HIS3* [YEp352-*TPK1* YEp62-*CDC25-lacZ*]
MLV202[b]	*MATα his3Δ leu2-3,112 ura3-52 trp1-289 cdc25::HIS3* [YEp352-*TPK1* YEp62]

[a] Derived from strain Y294 by transformation.
[b] Derived from strain SJ24-1B by transformation.

2% (wt/vol). Cells were harvested by centrifugation and washed once in MOPS-sorbitol buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$, 50 mM morpholinopropanesulfonate [MOPS], pH 7.5); resuspended to 10^9 cells per ml in the same buffer containing 1 mM phenylmethylsulfonyl fluoride, 100 U of aprotinin per ml, and 1 μM pepstatin A; and broken by vortexing at 4°C for 5 min with an equal volume of glass beads. For large volumes, a Braun homogenizer was used. Cellular debris was removed by centrifugation at 500 × *g* for 5 min. Protein concentrations were determined with the Bio-Rad protein assay kit with bovine plasma gamma globulin as a standard.

For labeling experiments, cells were grown as described above, harvested, washed once with the same (but lacking methionine) synthetic medium, and then resuspended to a concentration of approximately 2×10^8 cells per ml in methionine-free medium. [^{35}S]methionine (250 to 500 μCi; Translabel; ICN) was added, and cells were incubated at 30°C for 1 h.

Immunoprecipitation. For fluorography, [^{35}S]methionine-labeled extracts were adjusted to 0.5% sodium dodecyl sulfate (SDS), boiled for 5 min, cleared by centrifugation, and diluted to 0.1% SDS. Immediately prior to immunoprecipitation, extracts were cleared again by centrifugation and incubated with fixed *Staphylococcus aureus* cells (Immunoprecipitin; BRL) for one to several hours at 4°C. Following this incubation, extracts were centrifuged again and the cleared extracts were used for immunoprecipitation as follows. Extract from approximately 10^8 cells was incubated for 8 h with 2 to 4 μl of antibody and 10 to 20 μl of 50% protein A-Sepharose (Zymed) plus 100 to 400 μl of RIPA (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate). Immunoprecipitates were washed extensively with RIPA plus 0.5% SDS prior to gel fractionation.

For exchange assays, 3 mg of supernatant (100,000 × *g*) was incubated for 2 h in the presence of 25 μl of the appropriate antiserum and 60 μl of 50% protein A-Sepharose at 4°C. Prior to initiation of the exchange reaction, immunoprecipitates were washed 10 times with MOPS-sorbitol buffer.

Cell fractionation. Extracts were prepared as described above, except prior to the addition of detergent, they were centrifuged at 100,000 × *g* for 1 h to yield cytoplasmic (supernatant [100,000 × *g*]) and total-membrane (pellet [100,000 × *g*]) fractions. Pellets were suspended in MOPS-sorbitol buffer, recentrifuged at 100,000 × *g*, and finally resuspended in MOPS-sorbitol buffer.

GDP-GTP exchange assays. Extracts were prepared as described above. Yeast Ras2 protein was purified from *E. coli* as described previously (23).

GDP loss assays. Ras2p was preloaded with [^{3}H]GDP by incubating 25 pmol of Ras2p in 20 mM Tris-HCl (pH 7.5)–20 mM NaCl–5 mM 2-mercaptoethanol–7.5 mM EDTA with 0.6 nmol of [8-^{3}H]GDP (10 Ci/mmol; Amersham) at 30°C for 30 min. Exchange reactions were performed at 30°C and were initiated by the addition of 2.5 pmol [^{3}H]GDP-Ras to the appropriate extract plus 15 μM GTP, 25 μM GDP, and 5 mM 2-mercaptoethanol in a final volume of 230 μl. To assay activity of immunopurified Cdc25p–β-galactosidase, the reaction was started by the addition of 100 μl of the complete reaction mixture to the washed immune complexes.

To monitor the loss of [^{3}H]GDP from Ras by nitrocellulose filtration, 10-μl aliquots were applied to type HA filters (0.45-μm pore size; Millipore), which were then washed with 10 ml of ice-cold 20 mM Tris-HCl (pH 7.5)–20 mM NaCl–5 mM 2-mercaptoethanol–3 mM $MgCl_2$. Alternatively, Ras was immunoprecipitated by incubating 20-μl samples with 1 μg of antibody Y13-259 plus 25 μl of protein A-Sepharose prepared as described elsewhere (11) and resuspended as a 50% slurry in RIPA plus 5 mM $MgCl_2$. Samples were incubated for 1 to 2.5 h at 4°C and washed with RIPA plus 5 mM $MgCl_2$. Radioactivity was quantified by liquid scintillation spectrometry with Ecoscint scintillation cocktail (National Diagnostics).

GTP uptake assays. Ras2 protein (2.5 pmol), preloaded with nonradioactive GDP as described above, was incubated in an exchange reaction mixture containing [α-^{32}P]GTP (Amersham) (2.2 μM, 36 Ci/mmol or as noted) and the appropriate extract or immunoprecipitate in a final volume of 250 μl. Prior to incubation, extracts were chromatographed over G25 Sepharose to deplete endogenous pools of guanine nucleotides. After 15 min of incubation at 30°C, Ras2p was immunoprecipitated from 10- or 20-μl aliquots as described above, except cold GTP (0.5 nmol) was added to prevent nonspecific adsorption of [^{32}P]GTP to the protein A-Sepharose beads and 1.0 mg of extract from strain SJ22 was added to block nonspecific absorption of non-Ras GTP-binding protein.

RESULTS

Antibodies raised against Cdc25p recognize a 180-kDa protein present in soluble and total-membrane fractions. Antisera raised against a synthetic, 15-amino-acid peptide corresponding to the carboxyl terminus of Cdc25p (4, 6) and antisera raised against a chimeric protein made by fusing the carboxyl-terminal one-third of the *CDC25* gene to the *trpE* gene of *E. coli* precipitated a 180-kDa protein present in extracts from a yeast strain harboring *CDC25* on a high-copy-number plasmid (Fig. 1, lanes 3 and 5). This protein was absent from extracts of a strain lacking *CDC25* and was of the size predicted by the nucleotide sequence of the gene. Precipitation of the 180-kDa protein by the antipeptide antibody (AbSJ1) was inhibited by addition of the peptide against which the antibody was raised (Fig. 1, lanes 8 and 9). In addition, both antibodies precipitated the predicted 117-kDa protein produced by a high-copy-number plasmid carrying an allele of *CDC25* with an internal, in-frame deletion (Fig. 1, lane 1, and data not shown). Finally, the anti-Cdc25p-TrpE fusion antibody (AbSJ2) also precipitated a 185-kDa Cdc25p–β-galactosidase fusion protein (Fig. 1, lane

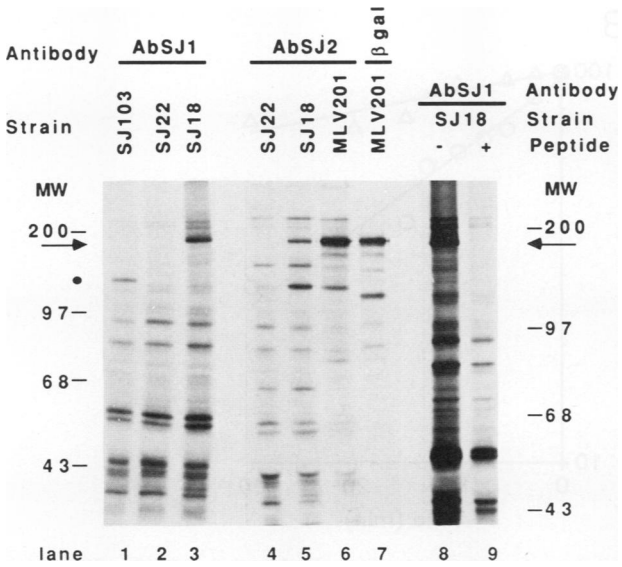

FIG. 1. Identification of Cdc25p. Immunoprecipitates of [^{35}S] methionine-labeled extracts of yeast strains (see Table 1) obtained with anti-Cdc25p antibodies (AbSJ1 or AbSJ2) or anti-β-galactosidase antibodies were fractionated on 7.5% polyacrylamide gels and processed for fluorography with dimethyl sulfoxide and 2,5-diphenyloxazole. MW, molecular weight standards (10^3). Arrows indicate the position of migration of the protein we identify as Cdc25p as well as that of the Cdc25p–β-galactosidase fusion protein, which has a predicted molecular mass of 185 kDa. The dot marks the position of migration of the 117-kda protein encoded by *cdc25Δ1*.

6). These results argue that the 180-kDa protein precipitated by our antisera is the authentic product of the *CDC25* gene.

To determine the intracellular location of Cdc25p, [^{35}S]methionine-labeled extracts were fractionated into soluble and total-membrane fractions. Partitioning of Cdc25p was assessed by immunoprecipitation. As evident in Fig. 2,

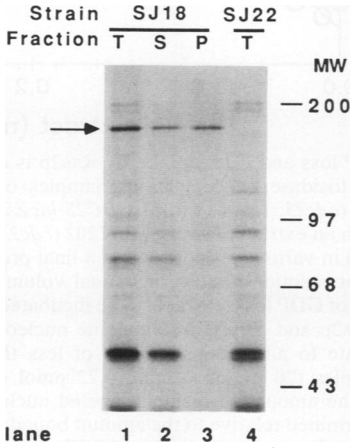

FIG. 2. Cellular localization of Cdc25p. [^{35}S]methionine-labeled extract of strain SJ18 ([YEp13-*CDC25*]) was prepared as described in the text and fractionated by centrifugation at 100,000 × *g* for 1 h to yield cytoplasmic (supernatant [100,000 × *g*]) and total-membrane (pellet [100,000 × *g*]) fractions. Cdc25p was immunoprecipitated with AbSJ1. Lane 1, total, unfractionated extract; lane 2, supernatant (100,000 × *g*); lane 3, pellet (100,000 × *g*); lane 4, strain SJ22, total extract. MW, molecular weight markers (10^3).

Cdc25p is present in essentially equal amounts in both the soluble and membrane fractions of these extracts. In contrast, Ras is found primarily in the membrane fraction of such preparations (8). Thus, in strains overexpressing Cdc25p, the protein appears to reside in both the cytoplasm and membrane fractions of the cell.

Extracts containing Cdc25p or Cdc25p–β-galactosidase catalyze exchange of guanine nucleotides bound to yeast Ras2p. We assayed guanine nucleotide exchange activity in extracts from isogenic strains that differed only in the amount of Cdc25p they produced. We compared the exchange activity of extracts with high levels of Cdc25p (obtained from strain SJ18 [Table 1]) with that of extracts lacking Cdc25p (obtained from the *cdc25* deletion strain, SJ22). We also assayed exchange activity in extracts of strain MLV201, which expresses at high level a *CDC25-lacZ* fusion carrying a fragment of *CDC25* that complements *cdc25* mutations in *S. cerevisiae* (Fig. 1, lane 6 [6]), and strain MLV202, which overexpresses *lacZ* alone. Both strains MLV201 and MLV202 carry a *cdc25* deletion allele and a multicopy *TPK1* plasmid, which suppresses the growth defect caused by the *cdc25* deletion (27).

To examine guanine nucleotide exchange, we measured the rate of dissociation of GDP bound to Ras as well as the concurrent rate of association of free GTP with Ras. In all cases, we used yeast Ras2 protein, which had been purified from *E. coli* and charged with either unlabeled or ^{3}H-labeled GDP. The rate of loss of GDP bound to Ras was determined either by measuring the [^{3}H]GDP retained after filtering reaction mixtures through nitrocellulose filters or by assessing the amount of label specifically precipitated by the anti-p21$^{h\text{-}ras}$ monoclonal antibody Y13-259 (11). Both methods yielded essentially identical results (cf. Fig. 3A and B). Extracts containing either the full-length Cdc25p or the Cdc25p–β-galactosidase fusion protein stimulated the rate of exchange of guanine nucleotides bound to Ras2p, compared with that obtained with strains devoid of Cdc25p (Fig. 3). The rate of loss of [^{3}H]GDP was approximately sixfold greater with extracts from the strain overexpressing full-length *CDC25* than that with extracts from the *cdc25* deletion strain and four- to fivefold greater in extracts from the strain producing the Cdc25p–β-galactosidase fusion than in extracts from the control *cdc25* deletion strain producing β-galactosidase. The time course for loss of GDP from Ras2p is consistent with a first-order reaction. In addition, initial rates of GDP loss were essentially linear with increasing amounts of Cdc25p–β-galactosidase-containing extract present in the reaction mixture (Fig. 4).

To confirm that the reaction stimulated by Cdc25p and Cdc25p–β-galactosidase was, in fact, exchange of GTP for GDP bound to Ras2p, we performed the exchange reaction in the presence of exogenous [α-^{32}P]GTP. Binding of GTP to Ras2p was determined by measuring the amount of label present in immunoprecipitates obtained with anti-p21$^{h\text{-}ras}$ monoclonal antibody Y13-259. The amount of [α-^{32}P]GTP bound to Ras2p was at least seven- to eightfold higher following incubation in an extract from strain MLV201 relative to that following incubation in an extract from strain MLV202 (Table 2). Immunoprecipitation of [α-^{32}P]GTP was blocked by a peptide corresponding to the epitope recognized by antibody Y13-259 but was not blocked by an unrelated peptide. This indicates that immunoprecipitable [^{32}P]GTP was bound specifically to Ras2p and not to some other guanine nucleotide-binding protein. In addition, initial rates of uptake of GTP by Ras2p were essentially linear with increasing amounts of Cdc25p–β-galactosidase-containing

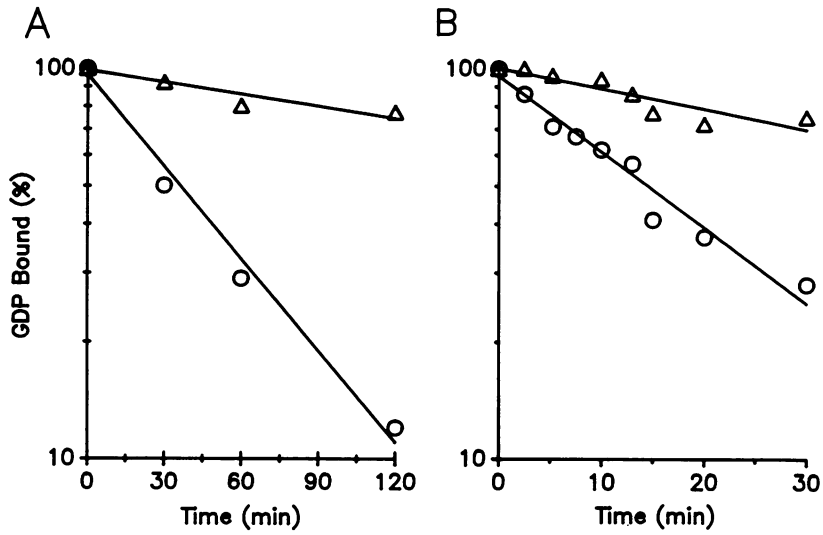

FIG. 3. Extracts containing Cdc25p or CdC25p–β-galactosidase promote guanine nucleotide exchange from yeast Ras2p. Shown is the time course for loss of [^{3}H]GDP from GDP-Ras2p during incubation in crude extracts from yeast strains. Values represent percent label retained at the indicated time, relative to that obtained at zero time, as measured by retention on nitrocellulose (A) or by immunoprecipitation with anti-p21$^{h\text{-}ras}$ monoclonal antibody Y13-259 (B). (A) Extracts (supernatant [100,000 × *g*]) of strain SJ18 [YEp13-*CDC25*] (circles) and SJ22 (*cdc25*::*HIS3* [YEp13-*TPK1*]) (triangles); (B) total extracts of strain MLV201 (*cdc25*::*HIS3* [YEp62-*CDC25-lacZ* YEp352-*TPK1*]) (circles) and strain MLV202 (*cdc25*::*HIS3* [YEp62 YEp352-*TPK1*]) (triangles). All measurements were performed in triplicate, with values agreeing to within 5% of the average. Similar experiments performed on four separate occasions using three different extract preparations yielded equivalent results.

extract and were comparable to the rates of GDP loss from Ras2p during incubation under the same conditions.

The enhanced exchange activity observed in strains overexpressing *CDC25* or *CDC25-lacZ* is not an indirect effect resulting from phenotypic differences among the various strains examined. First, both the deletion and overexpresser strains used in these experiments harbor a multicopy plasmid carrying *TPK1*, a gene encoding the catalytic subunit of the cyclic AMP (cAMP)-dependent protein kinase. This renders these strains phenotypically equivalent. Second, assays were also performed with extracts from *bcy1 tpk1*^{w1} *tpk2 tpk3* strains in which cAMP-dependent protein kinase is maintained at a low, but constitutive, level (5). In these strains as well, in which the phenotypic consequences of the *CDC25* genotype are completely masked, *CDC25* overexpression yielded extracts with enhanced exchange activity (data not shown). Third, the loss of [^{3}H]GDP from Ras2p was not due to degradation of Ras2p during incubation in extracts from strains overexpressing *CDC25*. Quantification of immunoprecipitated Ras2p from the various reaction mixtures demonstrated that the amount of Ras2p did not decline in any of the extracts during the course of the exchange assay (data not shown). Finally, as shown below, exchange activity is immunoprecipitable with anti-Cdc25p and anti-β-galactosidase antibodies.

Immunopurified Cdc25p–β-galactosidase catalyzes GTP-GDP exchange. Cdc25p–β-galactosidase fusion protein was immunoprecipitated from extracts of strain MLV201 by using anti-Cdc25p serum AbSJ2. Control immunoprecipitations were performed with the same serum from an extract of the *cdc25* deletion strain MLV202 and with either preimmune serum or no antiserum from an extract of MLV201. The extensively washed immune precipitates were incubated with GDP-Ras2p and [α-^{32}P]GTP. As shown in Fig. 5, the Cdc25p–β-galactosidase fusion protein immunoprecipitated from strain MLV201 catalyzed uptake of GTP by Ras2p.

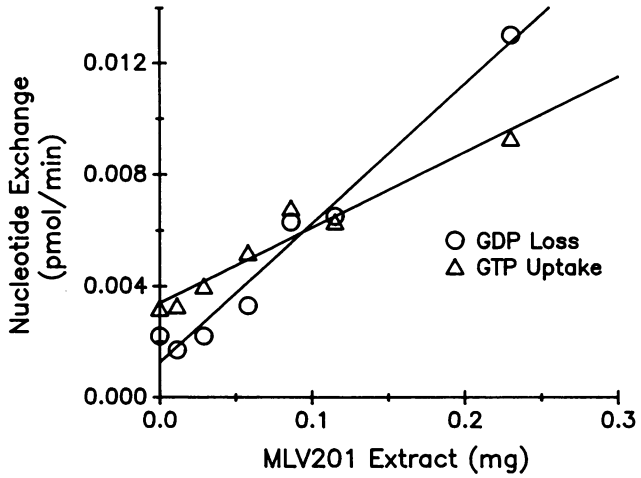

FIG. 4. GDP loss and GTP uptake by Ras2p is dependent upon Cdc25p–β-galactosidase concentration. Samples of an extract of strain MLV201 (*cdc25*::*HIS3* [YEp62-*CDC25-lacZ* YEp352-*TPK1*]) were mixed with an extract of strain MLV202 (*cdc25*::*HIS3* [YEp62 YEp352-*TPK1*]) in various proportions to a final protein concentration of 1 mg per reaction mixture in a final volume of 115 μl. To determine rates of GDP loss, extracts were incubated with 1.25 pmol of [^{3}H]GDP-Ras2p and 40 μM free guanine nucleotide at 30°C for times appropriate to allow consumption of less than 40% of the substrate. Samples (20 μl, containing 0.22 pmol of Ras2p) were removed, and the amount of residual labeled nucleotide bound to Ras2p was determined relative to the amount bound at the beginning of the incubation. For measuring rates of GTP uptake, extracts were incubated with 1.25 pmol of GDP-Ras2p and 4.5 μM [α-^{32}P]GTP (19 Ci/mmol) and incubated for 10 min. The amount of guanine nucleotide bound to Ras2p was measured by immunoprecipitation with anti-p21$^{h\text{-}ras}$ monoclonal antibody Y13-259, as described in Materials and Methods. Values of replicates agreed to within 10%. Extracts used in these experiments were prepared by Braun homogenization (see Materials and Methods) and were significantly more active than those used for experiments presented in Fig. 3 and Table 2.

TABLE 2. Cdc25p-catalyzed uptake of exogenous GTP by Ras[a]

Competing peptide	GTP uptake (pmol) catalyzed by extracts of:	
	MLV201[b]	MLV202[c]
None	0.075	0.012
Unrelated	0.088	0.009
Ras	0.005	0.002

[a] For those reactions indicated, immunoprecipitations were performed in the presence of 10 μg of synthetic peptide, corresponding either to the epitope recognized by monoclonal antibody Y13-259 (Ras peptide) or to an unrelated sequence. All measurements were performed at least in duplicate, with values agreeing to within 5%. This experiment was performed twice, using two separate extract preparations, with equivalent results. For details, see Materials and Methods.

[b] *cdc25::HIS3* [YEp352-*TPK1* YEp62-*CDC25-lacZ*].

[c] *cdc25::HIS3* [YEp352-*TPK1* YEp62].

Rates of association were essentially linear with increasing amounts of immunoprecipitate. In contrast, exchange activity was not present in any of the control immunoprecipitates.

We also examined the ability of immunoprecipitates to catalyze dissociation of [^{3}H]GDP from [^{3}H]GDP-Ras2p. Exchange activity was observed with immunoprecipitates of extracts from strain MLV201 but no extracts from strain MLV202 (data not shown). Identical results were obtained when either AbSJ2 or anti-β-galactosidase serum was used in the immunoprecipitation. A small amount of activity was detected in immunoprecipitates from MLV201 extract when preimmune sera were used. We attribute this to nonspecific binding of Cdc25p–β-galactosidase protein to the protein A-Sepharose beads.

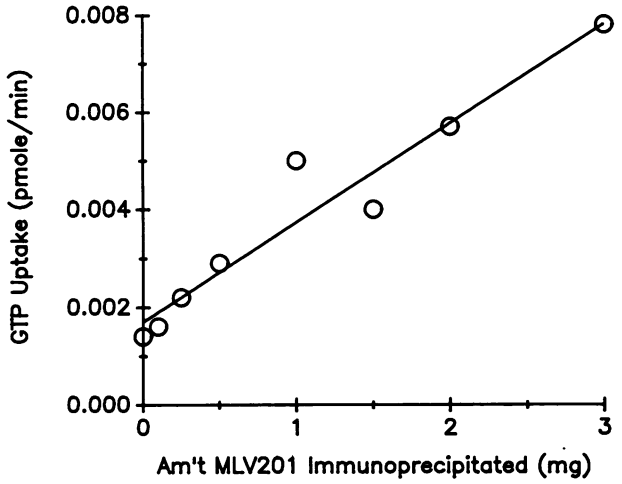

FIG. 5. Immunopurified Cdc25p–β-galactosidase catalyzes uptake of GTP by Ras2p in a dose-dependent manner. Cdc25p–β-galactosidase was immunoprecipitated from extracts of strain MLV201 (*cdc25::HIS3* [YEp62-*CDC25-lacZ* YEp352-*TPK1*]) with anti-Cdc25p antiserum AbSJ2. After thorough washing, immune complexes were mixed in various proportions with AbSJ2-coated protein A-Sepharose beads to keep the volume of beads constant while varying the amount of immunoprecipitated material. A reaction mixture containing [α-^{32}P]GTP (2.2 μM, 45 Ci/mmol), 1.1 pmol of GDP-Ras2p, and MOPS-sorbitol buffer was prepared, and 100-μl aliquots were added to each immunoprecipitate to start the reaction. Reaction mixtures were incubated at 30°C with constant mixing for 30 min. The extent of the reaction was determined by immunoprecipitation of Ras2p as described in the text. Controls—3 mg of MLV201 immunoprecipitated with preimmune serum, 3 mg of MLV201 immunoprecipitated with no antibody, and 3 mg of MLV202 (*cdc25::HIS3* [YEp62 YEp352-*TPK1*]) immunoprecipitated with AbSJ2—yielded a rate of GTP uptake of 0.0014 pmol/min. All measurements were performed in duplicate, and the values presented are representative of three independent assays.

DISCUSSION

The *RAS*/cAMP pathway of yeast is an essential component in the control of the cell growth. Although the components and interactions that compose the *RAS*/cAMP pathway have been well described, the nature of the signals to which this pathway responds has not been clearly identified. One likely source of input into the system is the guanine nucleotide exchange factor. Genetic evidence has shown that *CDC25* plays a role in the activation of Ras, and one hypothesis is that it carries out its function by acting as an exchange factor. We have assayed Cdc25p activity in vitro, and the results constitute the first direct biochemical evidence that Cdc25p, the product of the *CDC25* gene, activates Ras by stimulating guanine nucleotide exchange.

We have shown that crude extracts containing overexpressed Cdc25p or Cdc25p–β-galactosidase catalyze exchange of guanine nucleotides bound to Ras2p, while extracts of strains that do not produce Cdc25p do not show activity. Initial rates of both uptake and loss of guanine nucleotides were essentially linear with the amount of Cdc25p–β-galactosidase containing extract and were within a factor of two of each other. The absolute GTP uptake rate in crude extracts is difficult to determine, because of possible interference by guanine nucleotides and guanine nucleotide-binding proteins present in the extract. Given this potential error, the measured values are consistent with the assumption that loss of GDP and uptake of GTP are concerted processes.

We have been able to immunoprecipitate an exchange-promoting activity from extracts containing Cdc25p–β-galactosidase using anti-Cdc25p and anti-β-galactosidase sera. Immunoprecipitates of Cdc25p–β-galactosidase contain a number of contaminating proteins, and we therefore cannot state unequivocally whether Cdc25p itself catalyzes the exchange or whether Cdc25p stimulates or acts in conjunction with some other factor that coprecipitates with it. Two points would argue that Cdc25p acts directly as the exchange factor, however. First, the sequence of *CDC25* is similar to that of *SCD25*, the product of which can act directly to catalyze the exchange of guanine nucleotides bound to Ras in vitro (7). Second, as noted above, equivalent exchange activity was seen with Cdc25p–β-galactosidase immunoprecipitates obtained with two different sera. The fact that these immunoprecipitates show essentially nonoverlapping spectra of coprecipitating proteins reduces the probability that exchange is catalyzed by a protein other than Cdc25p. Créchet et al. (7) have reported that extracts of an *E. coli* strain designed to express the carboxyl-terminal portion of *CDC25*, which complements *cdc25* mutations in yeast strains, were incapable of promoting guanine nucleotide exchange in vitro. While this contrasts with the observations presented in this report, the results by Créchet et al. may be attributable to inefficient expression of the Cdc25 polypeptide in *E. coli* or to the absence of some essential posttranslational modification.

Our anti-Cdc25p sera recognized a 180-kDa protein that fractionated in both the supernatant and the pellet of a centrifugation at 100,000 × *g*, suggesting that Cdc25p, when expressed at high levels, localizes to both the membrane and the cytoplasm. This contrasts with observations made by

Garreau et al. (12) and Vanoni et al. (28), who report that their anti-Cdc25p sera recognize a 180-kDa protein that fractionates exclusively in the membrane fraction of a strain overexpressing *CDC25*. This difference may reflect different properties of the various strains used or of the extract preparation.

In its catalytic activity Cdc25p resembles a variety of nucleotide exchange factors, such as elongation factor EF-Ts, mammalian hormone and sensory receptors, and the exchange factor for eIF-2. In none of these cases has the mechanism of nucleotide exchange fully been clarified. Cdc25p could stimulate nucleotide exchange simply by chelating the Mg^{2+} atom normally resident at the GTP-binding site within Ras. Alternatively, Cdc25p could induce an open configuration of the Ras molecule to allow rapid equilibrium with exogenous guanine nucleotides. Another possibility is that Cdc25p stabilizes the GTP-bound configuration of Ras. The ability to combine biochemical and genetic analysis of Cdc25p function should allow us to distinguish among these possible mechanisms, an advancement that should shed light on these other critical biological processes as well.

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