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OVEREXPRESSION OF THE *CDC25* GENE, AN UPSTREAM ELEMENT OF THE RAS/ADENYLYL CYCLASE PATHWAY IN *Saccharomyces cerevisiae*, ALLOWS IMMUNOLOGICAL IDENTIFICATION AND CHARACTERIZATION OF ITS GENE PRODUCT

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ABSTRACT The product of the START gene *CDC25*, an upstream element of the RAS/adenylyl cyclase pathway in *Saccharomyces cerevisiae*, was identified using specific antibodies raised against a chimeric β-galactosidase/CDC25 protein. The CDC25 protein is poorly expressed and can be detected only when the *CDC25* gene is overexpressed under the control of the galactose-inducible *GAL1-10* strong promoter elements. It has a molecular weight of 180,000, is not glycosylated and is strongly associated with the particulate fraction. After deletion of residues 1255-1550 the protein is found in the soluble fraction.

The major regulatory area in *Saccharomyces cerevisiae* cell cycle is located in the G_1, unbudded phase and is called START (1,2). At START a yeast cell monitors different metabolic and enviromental signals, such as the nutrient level (3), the presence of mating factors, the attainement of a critical protein level (4), the presence of specific gene products (5), the cAMP level (3), etc. in order to decide whether enter a new mitotic cycle or enter alternative differentiation pathways, such as sporulation or stationary phase. Of paramount importance in the START area are the genes involved in the cAMP metabolism, such as *CDC35* (*CYR1*) encoding adenylyl cyclase (6-8) the *ras* oncogene homologues *RAS1* and *RAS2* (9-11) and *CDC25* (12-14). The CDC25 protein is required for mitotic growth, its inactivation bringing to a G_1/G_0 arrest (15-17), and to derepressed sporulation (18). Available genetic and biochemical evidence indicates that the *CDC25* function acts upstream of *RAS* (14, 19-21). CDC25 may directly interact with the RAS proteins, possibly by modulating the GDP/GTP exchange rate of the RAS proteins (21, 22). As a tool towards understanding the biochemical and molecular basis of the *CDC25* function, we present the

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generation of anti-CDC25 antibodies and their use in the identification and subcellular localization of the CDC25 protein.

MATERIALS AND METHODS

Strains, media and growth conditions The yeast strain X4004-3A (*MAT a lys5 met2 ura3 trp1*) was obtained from the Yeast Genetic Stock Center. [ ] denotes plasmid-carrier state. Synthetic media contained 0.67 % Yeast Nitrogen Base (Difco) supplemented with 50 mg/l of each required nutrient. Media were supplemented with either 2 % glucose, 2 % raffinose or 2 % galactose. Cultures of transformed strains were grown in synthetic media lacking uracil in order to mantain selection for plasmid-carrying cells. Solid media contained 2 % agar. *Escherichia coli* JM101 (23) was used as an host for recombinant DNA manipulations.

Recombinant DNA procedures Standard DNA manipulations were performed according to Maniatis et al. (23). Plasmid DNA was isolated from JM101 cultures by the alkaline lysis method (24). Restriction endonucleases, DNA polymerase and T4 DNA ligase were purchased from Boeheringer and used according to the manufacturers procedures. Plasmid pIND25-2 (G. Frascotti Ph.D. Thesis, Università di Milano 1990) in which *CDC25* expression is controlled by the strong *GAL1-10* promoter elements, was constructed by subcloning the whole *CDC25* coding region in plasmid pBCL26, a derivative of pLGSD5 (25) whose *lacZ* gene has been deleted (26). Plasmid pIND25-2Δ was derived by pIND25-2 by deleting a 885 bp *Bam*HI-*Sph*I fragment (spanning codons 1255-1550) followed by self-ligation after filling protruding ends with the Klenow fragment of DNA polymerase I. Plasmids pMV31 and pMV32 were obtained by ligating an internal *Bgl*II-*Bgl*II and a *Bgl*II-*Sal*I fragment (extending into the Tet^R gene), respectively, with the plasmid pUR290 (27), as outlined in Fig. 1A. The resulting plasmids contain codons 324-877 (pMV31) and 878-1547 (pMV32) of the *CDC25*-encoded ORF fused in frame with the whole *lacZ* gene. The plasmid pDGEm-1 (12) was used as a source of *CDC25* DNA.

Isolation of β-galactosidase/CDC25 fusion proteins and immunization Cultures of *Escherichia coli* JM101 bearing either the pMV31 or the pMV32 plasmid were grown to late log phase in L medium containing 50 mg/l of ampicillin . Synthesis of fusion proteins was induced with 1mM IPTG for 60 minutes. Inclusion bodies were prepared essentially as described (28) and the hybrid proteins further purified by preparative 5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gel slices containing ca. 150 μg of each hybrid protein were homogeneized with sterile PBS and directly injected in rabbits at multiple intradermal sites. After two subsequent injections at monthly intervals, each with about 75 μg of protein, both antisera, referred to as serum 1 (pMV31) and serum 2 (pMV32) gave a positive reaction at a 1:200 dilution when tested by an immunoblot assay versus their cognate antigen. Antibodies specific for CDC25 epitopes are present in both sera, since they preferentially recognised the antigen used for immunization (Fig. 1, panels A and B).

Electrophoresis and immunoblotting SDS-PAGE was performed essentially as described by Laemmli (29). Molecular weight standards were: rabbit muscle myosin, 205 KDa; *E. coli* β -galactosidase, 116 KDa; rabbit muscle phosphorylase b, 97 KDa; Bovin albumin, 66 KDa; egg albumin, 45 KDa; bovine carbonic anhydrase, 29 KDa. Proteins were electrophoretically transferred to nitrocellulose in a Bio-Rad trans-blot cell. Bound antibodies were detected with either[^{125}I]protein A or with an horseradish peroxidase second antibody stain. Bovine serum albumin (5 %) in 20 mM TRIS, 150 mM NaCl pH 7.4 was used as a blocking agent.

Preparation of protein extracts and cell fractionation All the preparation were carried out in the presence of 10 mM pepstatin and 2 mM phenylmethylsulfonylfluoride. Total protein extracts were prepared by vortexing yeast cells with glass beads. Glass beads and cell

debris were removed by low-speed centrifugation. Crude membrane fractions were prepared by centrifugation (100,000 x g, 30 min). The resulting pellet (crude membrane fraction) and supernatant (cytoplasmic fraction) were directly mixed with SDS-sample buffer, denatured and loaded on SDS-PAGE. In order to test solubility of the CDC25 protein, the crude membrane fraction was incubated for 30 min at 0^{o} C in the presence of various agents, centrifuged at 100,000 x g, mixed with SDS-sample buffer, denatured and loaded on SDS-PAGE. Differential centrifugation of mechanically broken cells was performed by sedimenting at 5,000 x g, 7 min, 20,000 x g, 30 min and 100,000 x g, 30 min. Insoluble cytoplasmic matrices were obtained following a previously reported method (30-31). Yeast spheroplasts from 2×10^{9} cells were lysed in 0.5 % Triton X-100 and the detergent soluble and insoluble fraction were obtained after a brief centrifugation at low speed. Samples were treated with RNase (100 μg/ml) and DNase (50 μg/ml) before centrifugation. The soluble fraction was lyophilized before SDS-PAGE analysis while the insoluble fraction was dissolved directly in SDS-sample buffer. The proteins extracted from $7*10^{7}$ cells were loaded on all reported immunoblots.

RESULTS AND DISCUSSION

Identification of the CDC25 protein Lacking a specific functional assay for a protein, the development of immunological reagents is essential. In order to raise antibodies specific for the CDC25 protein, *in frame* fusions between the *E. coli lac*Z gene, encoding β-galactosidase, and *CDC25* fragments have been constructed as detailed in Materials and Methods and summarized in Fig.1A. Using immunoblotting techniques, neither serum 1 (Fig.2A, lane 2) nor serum 2 (data not shown) recognized any specific polypeptide of the expected molecular weight in yeast cell-free extracts prepared from strain X4004-3A, most likely because of the very low levels of the *CDC25*-encoded mRNA (12, 32). Thus cell-free extracts were prepared from X4004-3A cells transformed with plasmid pIND25-2. Using serum 1 it was possible to identify in galactose-grown X4004-3A[pIND25-2] transformants a polypetide of 180 KDa (p180), i.e. the size expected for the primary translation product of the *CDC25* gene (Fig. 2A, lane 1). As expected, the levels of the 180 KDa polypeptide were lower in glucose than in galactose (Fig.2B, lanes 1 and 2 respectively), as previously reported for *CDC25* mRNA levels (G. Frascotti Ph.D Thesis, Università di Milano 1990). The fairly high levels of expression under repressing conditions, i.e. glucose media, are in keeping with the derepressing effects of *CDC25* overexpression on invertase (J.M. Thevelein, personal communication), a glucose-repressible enzyme. Formal identification of the p180 as the product of *CDC25* was achieved by overexpressing an inactive *CDC25* gene. A plasmid called pIND25-2Δ was obtained by deleting codons 1225-1550 of the CDC25 ORF in plasmid pIND25-2. Immunoblot analysis with serum 1 of cell-free extracts prepared from galactose-grown X4004-3A[pIND25-2] transformants identified a 150 KDa protein (i.e. the size expected for the *CDC25* deletion derivative), while the p180 was no longer present (Fig.2C, lane 1). Since serum 2 did not recognize the p180 protein even in X4004[pIND25-2] strain, strain X4004-3A[pIND25-2] and serum 1 were used in all the following experiments, unless otherwise indicated.

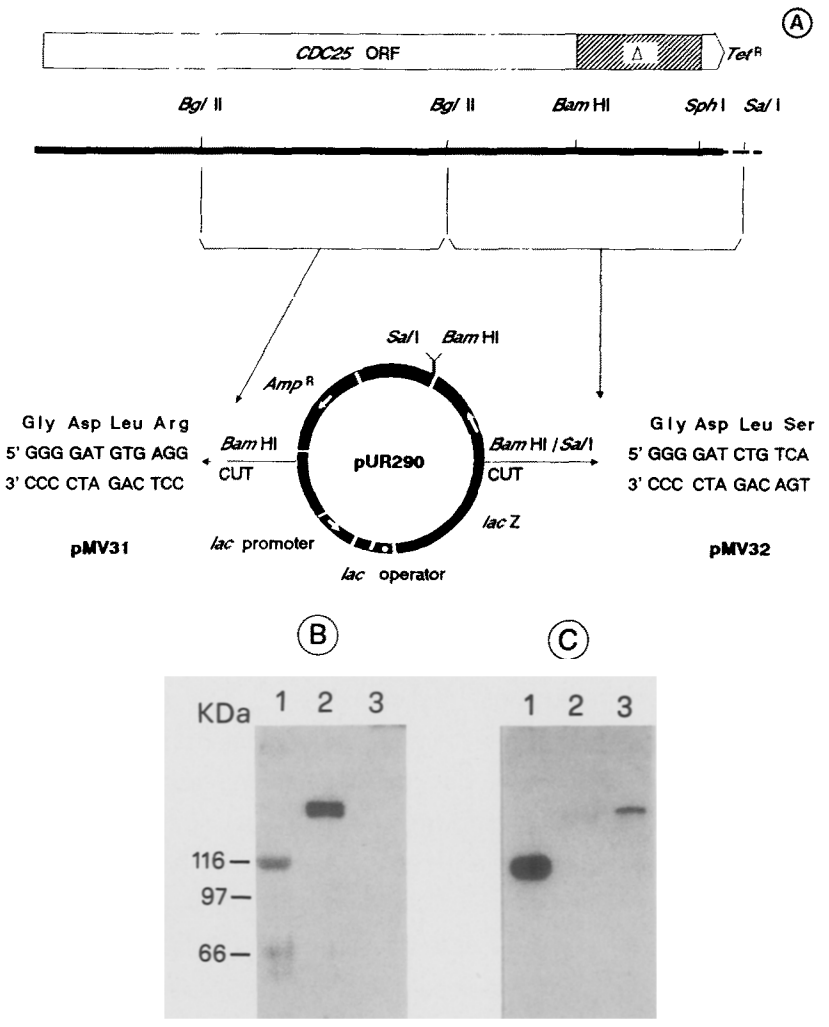

Figure 1. (A) Construction of *lacZ/CDC25* gene fusions. The white arrow indicates the *CDC25* ORF. The hatched area represents the *Bam*HI-*Sph*I fragment deleted in plasmid pIND25-2Δ . (B and C) Specificity of anti-fusion protein antibodies. Purified β-galactosidase/CDC25 fusion proteins prepared from JM101[pMV31] (lane 2) and JM101[pMV32] (lane 3) strains were separated by 6% SDS-PAGE, electroblotted to nitrocellulose and immunodecorated with anti-pMV31 (panel B) or anti-pMV32 (panel C) serum (1:200 dilution). Molecular weight markers were loaded on lane 1.

A 90 or 180 min treatment of X4004-3A[pIND25-2] cells exponentially growing in synthetic galactose medium with 10 μg/ml of tunicamycin, a specific inhibitor of the first step of protein *N*-glycosylation, had no effect on p180 electrophoretic mobility (data not shown). This finding indicates that despite the presence of 28 potential *N*-glycosylation sites (13,19), CDC25 is not glycosylated and suggests that the protein does not follow the secretory pathway, in keeping with the lack of a typical amino-terminal signal sequence.

Localization of CDC25 In order to determine the localization of the CDC25 protein, cell-free extracts were fractionated for 30 min at 100,000xg, the resulting pellet (crude particulate

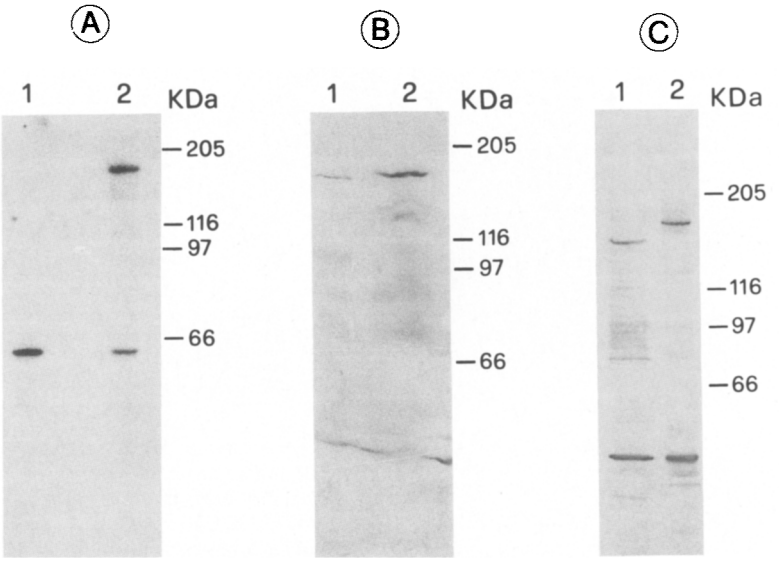

Figure 2. Identification of the CDC25 protein. The proteins extracted from 7 x 10^7 cells were separated by 7% SDS-PAGE, electroblotted to nitrocellulose and immunodecorated with serum 1 (1:200 dilution). The following strains were used, X4004-3A (panel A, lane 1); X4004-3A[pIND25-2] grown in synthetic medium supplemented with either galactose (lane 2 of panels A, B and C) or glucose (panel B, lane 1); X4004-3A[pIND25-2Δ] grown in synthetic galactose medium (panel C, lane 1).

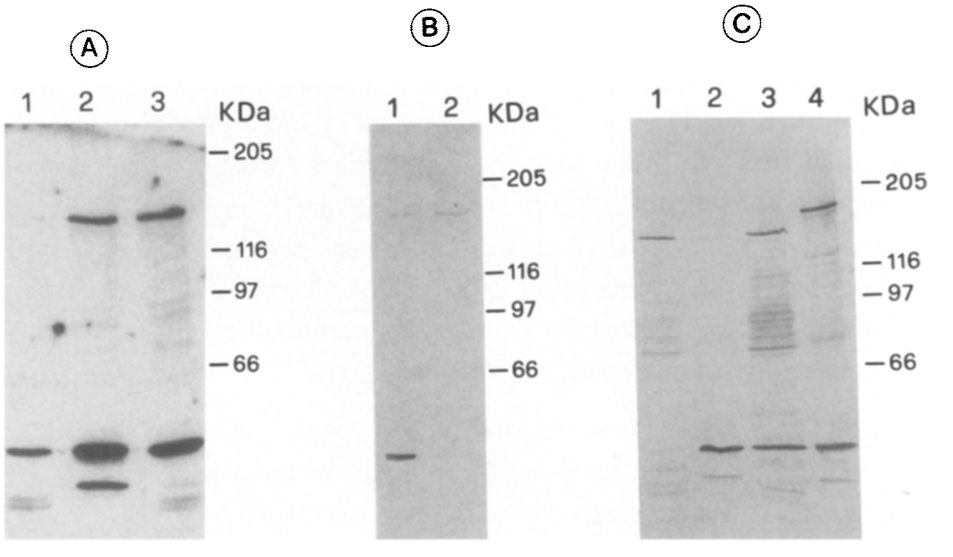

Figure 3. Solubilization of CDC25. Cell-free extracts of strain X4004-3A[pIND25-2] (panels A, B and panel C, lane 4) or X4004-3A[pIND25-2Δ] (panel C, lanes 1-3) were prepared by high-speed vortexing and fractionated by centrifugation (100,000xg, 30 min). Total (lanes A3 and C3), soluble (lanes A1 and C1) and pellet (lanes A2, C2 and C4) proteins were analyzed by 7% SDS-PAGE and immunoblotting. In panel B CDC25 was solubilized by treating the 100,000xg pellet with 5M urea at 0^o C for 30 min. The resulting soluble (lane 1) and insoluble (lane 2) proteins were fractionated by centrifugation at 100,000xg for 30 min. The proteins extracted from 7 x 10^7 cells were loaded on each lane.

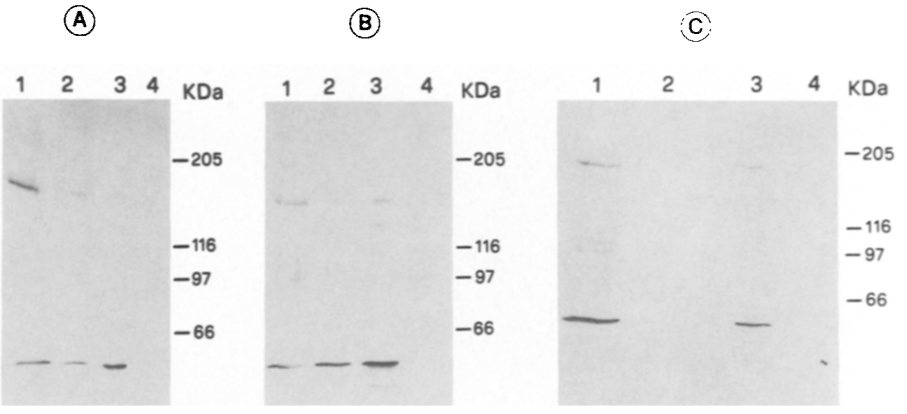

Figure 4. Analysis of CDC25 protein in particulate and soluble fraction in mechanically and enzymatically lysed X4004-3A[pIND25-2] cells. Yeasts were disrupted by vortexing with glass beads in the absence (panel A) or in the presence (panel B) of 0.5% triton X-100. The insoluble fractions obtained after spins of 5000 x g (lane 1), 20,000 x g (lane 2), 100,000xg, (lane 3) and the 100,000 x g supernatant (lane 4) were analyzed by immunoblotting as described above. In panel C detergent-insoluble (lane 1) and soluble (lane 2) proteins were extracted from spheroplasts. Pellet proteins were further extracted with 300 mM KI and fractionated by centrifugation. Residual, not extracted proteins were loaded on lane 3, extracted proteins on lane 4.

fraction) and supernatant (crude soluble fraction) were analyzed by immunoblotting. Most CDC25 protein was found to be associated with the particulate fraction (Fig.3A, lane 2), while little, if any, was found in the soluble fraction (Fig.3A, lane 1). Several treatments, including 1% triton X-100, 1% sodium deoxycholate, 1 M sodium chloride, 0.5 M sodium carbonate pH 11.5, failed to reproducibly release CDC25 from the 100,000xg pellet (data not shown). In fact only 5 M urea was able to partially release the p180 from the 100,000xg pellet (Fig. 3B). Interestingly, fractionation of a cell-free extract of strain X4004-3A[pIND25-2Δ] showed that most, if not all, of the CDC25 deletion derivative was found in the soluble fraction (Fig.3C), thus suggesting that a topogenic sequence, or a domain involved in protein/protein interaction with other, as yet unidentified protein(s), is contained within the deleted sequence. It is interesting to note that a hydrophobic region (residues 1452-1473) predicted to form a transmembrane helix according to the method of Rao and Argos (33) is contained within the deleted region.

CDC25 localization was further investigated by differential centrifugation. Even after low-speed centrifugation (5,000xg) most of the CDC25 protein was associated with the particulate fraction, both in the presence and in the absence of 0.5 % triton X-100 (Fig. 4, lane 1 of panels A and B respectively). The remaining CDC25 protein was associated with the 20,000xg and 100,000xg pellets (Fig.4A and B, lanes 2 and 3 respectively); in keeping with the results reported in Fig.3A, no CDC25 protein was detectable in the 100,000xg supernatant (Fig.4A and B, lane 4). This behaviour is reminescent of proteins associated with yeast cytoplasmic matrices, a structure resembling the cytoskeleton of mammalian cells (30, 31). To further assess this point X4004-3A[pIND25-2] detergent-soluble proteins

were extracted from yeast spheroplasts with 0.5 % triton X-100 in the presence of DNase and RNase as described (30, 31). The CDC25 protein was reproducibly found in the insoluble fraction, even after potassium iodide treatment (30) that is known to release loosely bound cytoskeletal proteins (Fig. 4B, lanes 1 and 3, respectively), thus suggesting a strong interaction between the CDC25 protein and the cytoskeletal matrix.

It is worth noting that CDC25 association with cytoskeletal elements may be consistent with the proposed receptor-like role of this molecule (34). In recent years it has become increasingly clear that in a variety of cells, plasma membrane proteins are linked to a surface lamina, or structural matrix, which persist upon removal of membrane lipids with non-ionic detergents (35, 36). Among the proteins that constitutively or transiently interact with cytoskeletal elements there are several plasma membrane receptors: these include the epidermal growth factor receptor from A431 cells (37, 38), the chemotactic cAMP receptor of *Dictyostelium discoideum* (39), the *N*-formylated peptide receptor of neutrophils (40) and the nerve growth factor receptor from PC12 cells (41). The possibility that *CDC25* overexpression affects the intracellular distribution of the CDC25 protein cannot be excluded since the protein is not detectable under normal expression conditions. However the normal phenotype of X4004[pIND25-2] cells (G. Frascotti Ph.D Thesis, Università di Milano 1990) argues against mislocalization of CDC25, since an altered localization of a protein with such a key regulatory role might well hamper normal cellular metabolism. Moreover the cytoplasmic localization of the CDC25 deletion derivative imply that the reported membrane localization is not dependent on overexpression per se, but depends on specific protein sequences, strongly suggesting that our results are not artefactual, but reflect the physiological localization of the CDC25 protein.

In conclusion, CDC25-specific antibodies have been raised against a β-galactosidase/CDC25 hybrid protein. Formal identification of an unglycosylated 180 KDa protein associated with the particulate fraction as the product of the *CDC25* gene was achieved by overexpressing the *CDC25* gene, or a deletion derivative, under the control of the galactose-inducible promoter elements. These results are the first identification and preliminary characterization of the CDC25 protein. It is expected that the antibodies will be a powerful tool to analyze the molecular and biochemical role played by CDC25 in the signal transduction pathway in *S. cerevisiae*. Experiments along this line are currently under way.

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