The *Saccharomyces cerevisiae CDC25* gene product is a 180 kDa polypeptide and is associated with a membrane fraction

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In the yeast *Saccharomyces cerevisiae*, the *CDC25* gene product is supposed to interact with ras proteins and adenylate cyclase for progression through the cell division cycle. To identify the *CDC25* gene product, we raised antibodies against two hybrid proteins, encoded by in-frame fusions between the *E. coli lacZ* gene and two different parts of the *CDC25* gene. By protein immuno-blotting, we were able to identify the *CDC25* gene product as a 180 kDa polypeptide, which we named $p180^{CDC25}$. It was detected only when the *CDC25* gene was overexpressed in a proteases-deficient yeast strain. Subcellular fractionation experiments showed that $p180^{CDC25}$, as well as ras proteins, is attached to the membrane, even after treatments which release peripheral membrane proteins.

Cell cycle; Signal transduction; RAS; CAMP pathway

1. INTRODUCTION

During the cell division cycle of *Saccharomyces cerevisiae*, cAMP plays a major role for completion of the START step of the G1 phase [1]. Adenylate cyclase, the product of the *CDC35* gene [2–4], is activated by two ras proteins encoded by two distinct genes, *RAS1* and *RAS2* [5,6]. Like their mammalian homologs, yeast ras proteins bind guanine nucleotides and display a weak GTPase activity [7]. Only the GTP-bound form is able to activate adenylate cyclase [8]. Another key element in the yeast cAMP production pathway is the *CDC25* gene product [9–11]. After a shift to the non-permissive temperature, the phenotype of *cdc25* thermosensitive mutants is very similar to that of *cdc35* mutants: G1 arrest and rapid drop of the intracellular cAMP level [9]. Genetic and biochemical studies suggest that the *CDC25* gene product could interact with ras proteins, increasing the GDP dissociation rate, leading to a higher proportion of the active (ras-GTP) complex [10–14]. Ras proteins and adenylate cyclase are well characterized. By contrast, very little is known about the *CDC25* gene product. DNA sequence shows an open reading frame capable of encoding a 1589 amino acid polypeptide [9,11,15]. The *CDC25* gene is transcribed into a 5200 bases mRNA [9,16]. To identify the *CDC25* gene product, we constructed in-frame fusions between the *E. coli lacZ* gene and various portions of the *CDC25* gene and we used the encoded proteins to raise antibodies. With these antibodies, we identified the *CDC25* gene product as a 180 kDa polypeptide, which we named $p180^{CDC25}$, and showed that $p180^{CDC25}$ is tightly attached to a membrane fraction of yeast cells.

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2. MATERIALS AND METHODS

2.1. *Preparation of hybrid proteins*

The plasmids of the pUR family are designed for IPTG-inducible expression of hybrid proteins encoded by in-frame fusion between the *E. coli lacZ* gene and any coding sequence [17]. A 1100 bp *Rsa*I-*Eco*RI fragment, containing codons 86–493 of the *CDC25* open reading frame, was inserted, using octameric synthetic *Bam*HI linkers, into pUR 292. The recombinant plasmid, named pPR17, was introduced into TG1 *E. coli* cells [18] by the $CaCl_2$ transformation method [19]. A 1569 *Bgl*II-*Bgl*II fragment, containing codons 354–877, was inserted into pUR 290. The recombinant plasmid, named pE2JM7, was introduced into JM101 *E. coli* cells [20]. Transformed cells culture, IPTG induction, and cell extraction were performed as described by Simon et al. [21]. Hybrid proteins were purified by affinity chromatography on APTG-Sepharose according to Ullmann [22]. They were used for rabbit immunization and antibody purification.

2.2. *Rabbit immunization and antibody purification*

Rabbit immunization was performed essentially as described by Part et al. [23].

For antibody purification, 8 mg pE2JM7 protein and 7 mg pPR17 protein were coupled each to 5 ml CNBr-activated Sepharose 4B (Pharmacia), according to manufacturer's instructions.

Immunoglobulin fraction was purified from crude serum by ammonium sulfate precipitation (45% saturation) and chromatography on a DEAE-Sephadex A-50 column equilibrated in 50 mM sodium acetate/21 mM acetic acid, pH 5 [24]. IgG were collected in the column flow-through. The specific antibodies were purified by immunoaffinity chromatography on pE2JM7- or pPR17-Sepharose columns, according to [25].

2.3. *Overexpression of the* CDC25 *gene*

pBM272 [26,27] contains the galactose-inducible GAL1-GAL10 yeast promoter inserted into the yeast/*E. coli* shuttle vector Ycp50

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[28]. The *CDC25* gene was inserted into pBM272 cut by *Bam*HI and *Nru*I, as a 5362 bp *Xmn*I-*Pvu*II fragment. The blunt *Xmn*I end was inserted into the *Bam*HI site of pBM272 by use of synthetic octameric *Bam*HI linkers. The 5362 bp *Xmn*I-*Pvu*II fragment contains the entire *CDC25* coding sequence, 126 bp upstream the first ATG codon and 466 bp downstream the STOP codon. The recombinant plasmid, named pGAL 25.6.1, was used to transform the thermosensitive *cdc25* mutant strain OL97.1.11B (MATα, *cdc25.5, ura3, leu2,* His$^-$) [12] and the protease-deficient yeast C13ABYS86 (*pra1-1, prb1-1, prc1-1, cps1-3, ura3, leu2-3,* His$^-$) – a generous gift from D.H. Wolf – by the protoplast transformation method.

2.4. CDC25 *mRNA analysis*

Total and poly(A)$^+$ mRNA extraction, electrophoresis, transfer and molecular hybridization were performed as described previously [9], except that mRNA were transferred to Pall-Biodyne nylon membranes, according to manufacturer's instructions. As a *CDC25*-specific DNA probe, we used a 767 bp *Kpn*I-*Cla*I fragment, inserted into the M13mp18 vector. As a control, we used a 615 bp *Eco*RI-*Hin*dIII fragment of the *TRP1* gene [29] inserted into the M13mp19 vector. The single strand probes derived from M13 templates and labeled with [α-^{32}P]dCTP (800 Ci/mmol) were obtained by complementary DNA synthesis [30].

2.5. *Whole cell protein extracts and subcellular fractionation*

Yeast cells were grown on minimal medium (yeast nitrogen base 0.67%) supplemented with 0.5% casein acid hydrolysate and 20 μg/ml uracil [31], containing either 2% glucose, or 3% (w/v) glycerol plus 2% galactose. Yeast cells transformed either by pBM272 or by pGAL25.6.1, were grown on the same media, but without uracil. Cells were harvested in log phase. For total protein extraction, frozen cells, resuspended in 0.2 M Tris-HCl, pH 8, 0.1 M NaCl and 10% (v/v) glycerol, were broken by passage through a Nanton-Gaulin homogenizer. Proteases inhibitors were added just before extraction at the following final concentrations: 313 μg/ml benzamidin, 1.36 μg/ml pepstatin-A, 0.26 μg/ml leupeptin, 2 μg/ml antipain, 2 μg/ml chymostatin, 2 mM phenyl-methyl-sulfonyl-fluoride and 4 mM *o*-phenanthrolin.

For membrane preparations, yeast cells were broken by vigorous vortexing with glass beads (0.4 mm diameter) in ice-cold lysis buffer: 50 mM Mes/KOH buffer, pH 6.2, 0.1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM 2-mercapto-ethanol and proteases inhibitors as above. The mixture was centrifuged at 800 × *g* for 5 min at +4°C to remove unbroken cells and glass beads. The crude cell extract was then centrifuged at 80000 × *g* for 1 h at +4°C. The supernatant was referred to as the cytosolic fraction, the pellet as the membrane fraction. Alternatively, yeast cells were broken by enzymatic digestion of cell walls with zymolyase (100 U/ml), in 0.1 M sodium acetate, 60 mM EDTA, 1.2 M sorbitol for 30–60 min at 30°C. Protoplasts were then washed with 10 ml of 50 mM Hepes/NaOH buffer, pH 7, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM $MnCl_2$, 0.8 M sorbitol (buffer B) and resuspended in 1 ml buffer B [32]. After addition of 3 ml of 50 mM Mes/KOH buffer, pH 6.2, 0.1 mM $MgCl_2$, 0.1 mM EDTA and protease inhibitors (see above), protoplasts were homogenized by several strokes in a tightly fit Potter-Elvejheim apparatus. After elimination of unbroken cells and large debris by centrifugation at 200 × *g* for 10 min, crude membrane and cytosolic fractions were separated as above. Aliquots of this membrane fraction were incubated for 30 min at 0°C in the presence of 0.1 M Na_2CO_3, 0.5 M potassium acetate, or 2.5 M urea [33]. Integral membrane proteins (pellet) and peripheral membrane proteins (supernatant) were separated by centrifugation at 150000 × *g* for 1 h at +4°C.

2.6. *Electrophoresis and immunoblotting*

Whole cell extracts and various subcellular fractions were made 1 × Laemmli sample buffer [34] with a 5 × solution and boiled 3 min prior to SDS-polyacrylamide gel electrophoresis. After electrophoresis and transfer to nitrocellulose sheets by the 'semi-dry' method [35], the membranes were processed as described by Part et al. [23]. Immunoreactive protein bands were detected by goat anti-rabbit IgG serum labelled with alkaline phosphatase (Promega Bio-Tech).

3. RESULTS

3.1. *Very low transcription levels of the* CDC25 *gene allow suppression of* cdc25 *mutations*

Plasmid pGAL25.6.1 is a pBM272-based plasmid which contains the entire *CDC25* open-reading-frame under control of a *GAL1* promoter, and the *URA3* gene.

We checked the ability of pGAL25.6.1 to complement the thermosensitive *cdc25* mutation of OL97.1.11B yeast cells (*MAT*α, *cdc25-5, ura3*Δ, *leu2,* His$^-$). Surprisingly, all the Ura$^+$ transformants that we have tested could grow at restrictive temperature (36°C) whatever glucose or galactose is the carbon source. As expected, pBM272 transformed cells obtained at 26°C were not able to grow at 36°C whatever glucose or galactose is the carbon source. Thus even when the *GAL1* promoter is repressed by the presence of glucose, the very low residual amount of transcription leads to suppression of the *cdc25* mutation.

To address this question of *CDC25* expression on pGAL25.6.1 and overexpression in the presence of galactose, poly(A)$^+$ mRNA were isolated from glucose – and galactose – grown cells (OL97.1.11B yeasts transformed by pGAL25.6.1). After agarose gel electrophoresis and transfer to nylon membranes, these

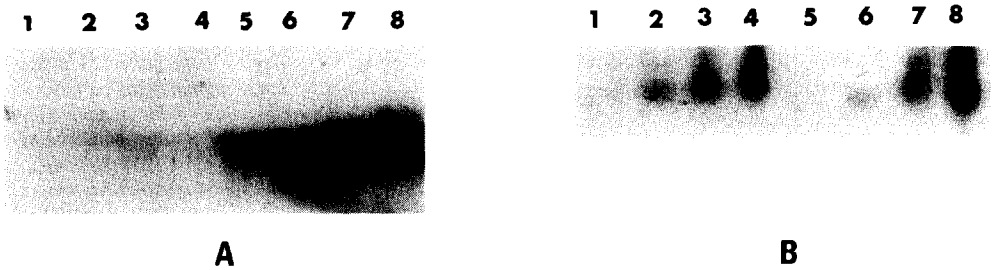

Fig. 1. Northern blot analysis of transcription of the *CDC25* gene cloned into the galactose-inducible expression vector pBM272. Poly(A)$^+$ mRNA were prepared from OL97.1.11B cells transformed with pGAL25.6.1 and grown in the presence of either glucose (lanes 1–4) or galactose (lanes 5–8). After electrophoresis, RNA were transferred to a nylon membrane and probed with a *CDC25* specific probe (part A) or a *TRP1* specific probe (part B). The following amounts were loaded on the gel: lanes 1 and 5, 10 μg; lanes 2 and 6, 20 μg; lanes 3 and 7, 40 μg; lanes 4 and 8, 80 μg.

mRNA were probed with a *CDC25*-specific, ^{32}P-labeled DNA probe. In glucose-grown cells, *CDC25* mRNA was barely detectable. In galactose-grown cells, there was an about 50-fold induction of the *CDC25* mRNA (Fig. 1). In both cases, the *CDC25* mRNA was of the expected size, around 5200 nucleotides [9]. This galactose-induced accumulation of *CDC25* mRNA was not observed in yeasts transformed by pBM272.

Did this enhancement of transcription promote an over-accumulation of the *CDC25* gene product? In a preliminary experiment, whole cell protein extracts from OL97.1.11B yeast cells transformed by pGAL25.6.1 were analysed by immunoblotting. Polyclonal rabbit antibodies were raised against two hybrid proteins: pE2JM7 hybrid protein which contains amino acids 345–877 of the *CDC25* open reading frame and pPR17 hybrid protein which contains amino acids 86–393 of the *CDC25* open reading frame. Anti-pE2JM7 antibodies were named A2.4 and anti-pPR17 antibodies were named A6.3. In galactose-grown cells, an immunoreactive polypeptide was detected, having an apparent molecular mass of 31 kDa. This 31 kDa polypeptide was not detected in glucose-grown cells (data not shown). Therefore, to rule out proteolytic artifacts, we used the strain C13ABYS86 which lacks four vacuolar proteases [36].

3.2. *In a protease-deficient yeast strain, the product of the* CDC25 *gene is a 180 kDa polypeptide*

Whole protein extracts of yeast cells (C13ABYS86) transformed by pGAL25.6.1, which contains the *CDC25* gene under control of a galactose-inducible promoter, were analyzed by immunoblotting. A2.4 antibodies detected a 180 kDa polypeptide (Fig. 2, lane d). This polypeptide, which we named p180^{CDC25}, was detected only in cells grown on galactose, but not in cells grown on glucose (Fig. 2, lane c). It was detected neither in non-transformed cells grown on glucose (Fig. 2, lane a) or galactose (Fig. 2, lane b), nor in cells transformed with pBM272, the vector without insert (data not shown). A6.3 antibodies gave the same results (data not shown).

3.3. *p180*^{CDC25} *is associated with a membrane fraction*

The *CDC25* gene product probably interacts with ras proteins, which in turn activate adenylate cyclase. Since ras proteins [37] and adenylate cyclase [38] are located in the yeast plasmic membrane, we wondered whether p180^{CDC25} could be detected either in a soluble cytosolic fraction or in a membrane fraction.

Yeast cells (C13ABYS86 strain transformed by pGAL25.6.1 grown on galactose) were homogenized by vortexing with glass beads. The whole extract was fractionated by high-speed centrifugation between a cytosolic fraction (supernatant) and a membrane fraction (pellet). After electrophoresis and transfer, proteins were probed with A6.3 antibodies raised against

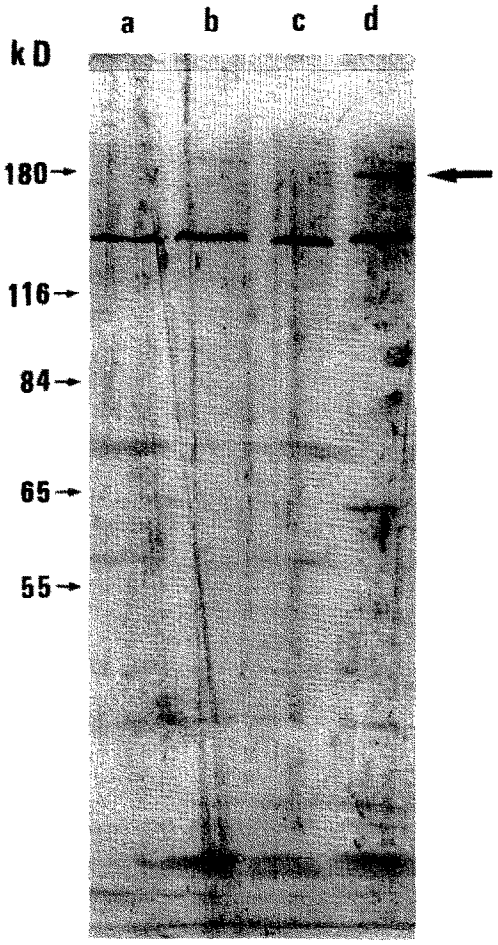

Fig. 2. Immunostaining of p180^{CDC25} in whole extracts of C13ABYS86 yeast cells. Whole cell extracts were run on 8% acrylamide-SDS gels. After transfer to nitrocellulose, proteins were probed with A2.4 antibodies raised against pE2JM7 fusion protein which contains amino acids 354–877 of the cdc25 protein. (Lane a) Non-transformed yeasts grown on glucose minimal medium; (lane b) non-transformed yeasts grown on galactose minimal medium; (lane c) yeasts transformed by pGAL25.6.1, grown on glucose minimal medium; (lane d) yeasts transformed by pGAL25.6.1, grown on galactose minimal medium.

the pPR17 hybrid protein. As shown in Fig. 3A, p180^{CDC25} was only detected in the membrane fraction (lane b). Similar results were obtained when cells were broken by zymolyase treatment followed by grinding of protoplasts in a Potter-Elvejheim apparatus (data not shown).

We further checked whether p180^{CDC25} was an integral membrane protein or a peripheral membrane protein. After vortexing with glass beads, aliquots of the 80000 × *g* membrane pellet were incubated in the presence of 0.1 M Na_2CO_3, pH 11, or 0.5 M potassium acetate, or 2.5 M urea [33]. Peripheral membrane proteins (supernatant) were separated from integral membrane protein (pellet) by centrifugation at 150000 × *g*. After electrophoresis and transfer, the different fractions were probed with A6.3 antibodies. As shown in

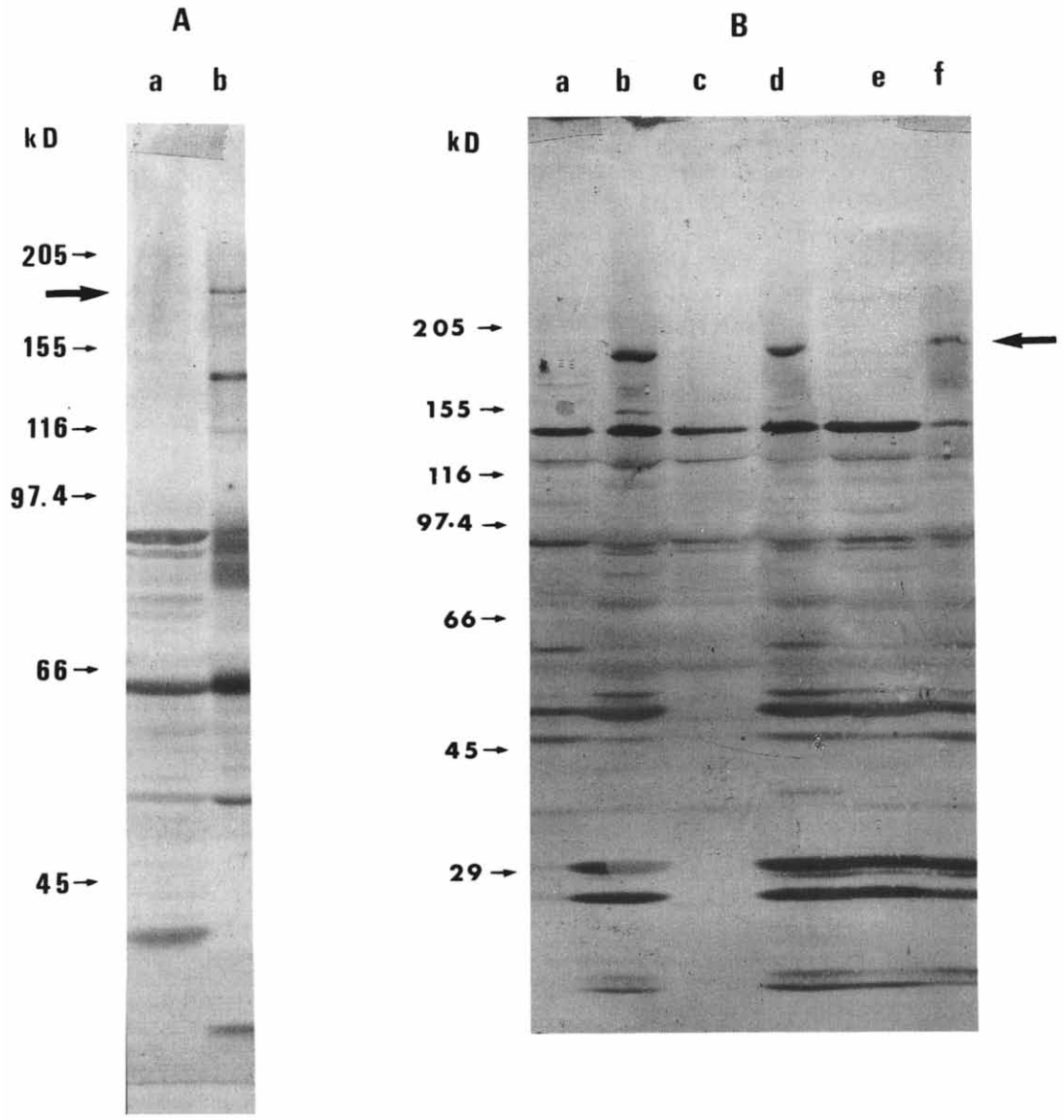

Fig. 3. Immunostaining of p180^{CDC25} in membrane fractions of C13ABYS86 yeast cells transformed by pGAL25.6.1, grown on galactose minimal medium. After electrophoresis on polyacrylamide-SDS gels and transfer to nitrocellulose membranes, proteins were probed with A6.3 antibodies raised against pPR17 hybrid protein which contains amino acids 86–493 of the cdc25 protein. (A) Crude membrane separation (8% acrylamide gel). (Lane a) 80000 × *g* supernatant (cytosolic fraction); (lane b) 80000 × *g* pellet (crude membrane fraction). (B) Aliquots of crude membrane pellet were incubated in the presence of 0.1 M Na_2CO_3, or 0.5 M potassium acetate, or 2.5 M urea. Peripheral membrane proteins (supernatant) were separated from integral membrane proteins (pellet) by a 150000 × *g* centrifugation. Equal amounts of each fraction were loaded on a 5% to 15% acrylamide gradient gel. (Lane a) Supernatant after urea treatment; (lane b) pellet after urea treatment; (lane c) supernatant after potassium acetate treatment; (lane d) pellet after potassium acetate treatment; (lane e) supernatant after Na_2CO_3 treatment; (lane f) pellet after Na_2CO_3 treatment.

Fig. 3B, p180^{CDC25} is predominantly detected in the integral membrane fraction (lanes b, d and f). As observed with whole cell extracts, p180^{CDC25} was only detected in yeasts transformed by pGAL25.6.1 grown on galactose.

In order to assess the quality of the membrane preparations, the same different fractions were probed with a ras antiserum (a generous gift from J.B. Créchet). As expected, ras proteins were only detected in the membrane fraction (Fig. 4A, lane b) and remained predominantly associated with the integral membrane fraction after Na_2CO_3, potassium acetate, or urea treatment (Fig. 4B, lanes a, c and e).

The results for ras proteins and p180^{CDC25} localization were identical, whatever method has been used for cell breakage.

4. DISCUSSION

The *CDC25* gene contains an open reading frame of 1589 codons, capable of encoding a 179 kDa protein [9,11,15]. By immunoblotting, we detected a polypep-

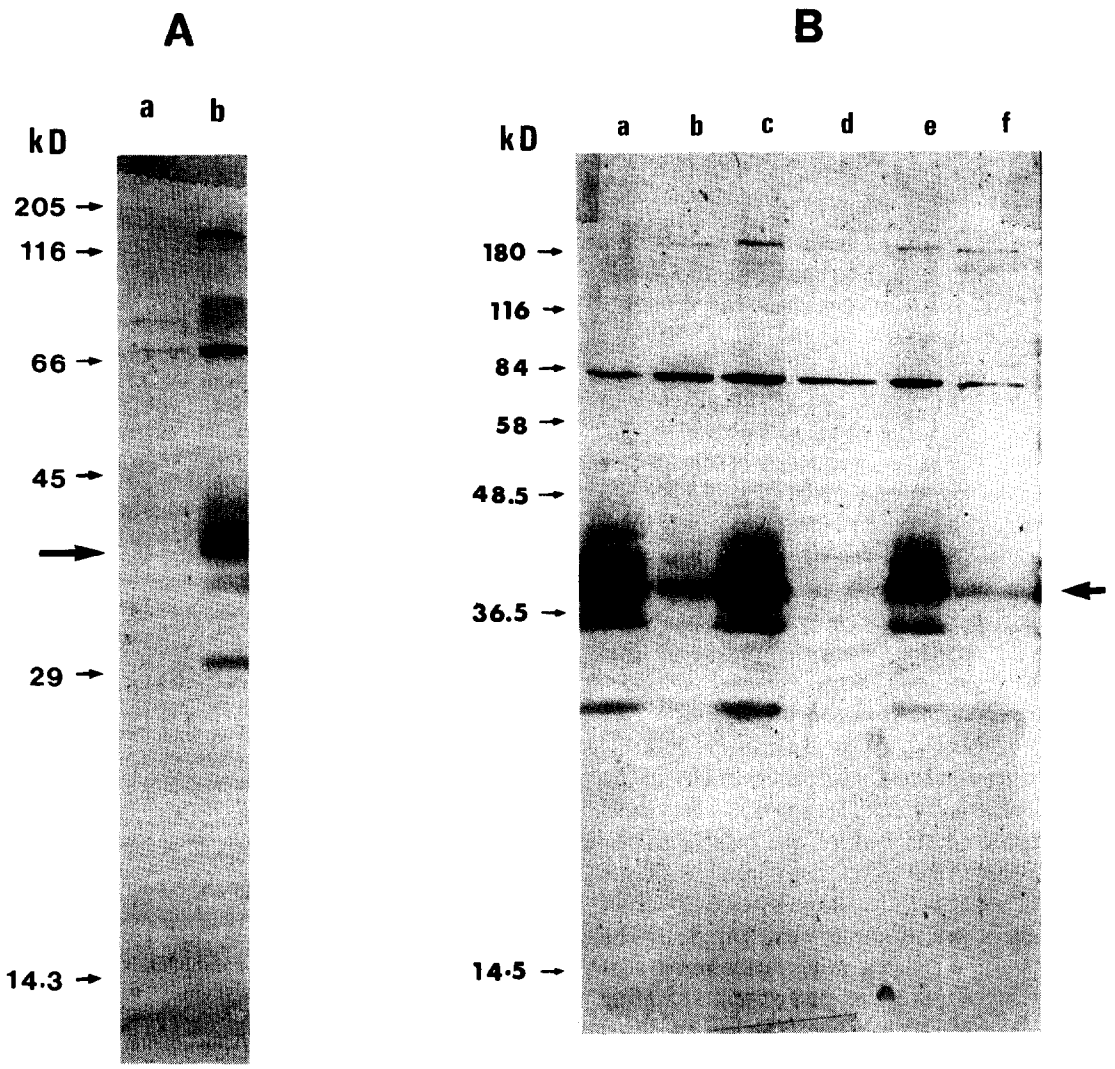

Fig. 4. Immunodetection of RAS proteins in membrane fractions of C13ABYS86 yeast cells transformed by pGAL25.6.1, grown on galactose minimal medium. The same fractions as in Fig. 3 were run on a 12% acrylamide-SDS gel, transferred to nitrocellulose sheets and probed with anti-ras antibodies. (A) Lane a, 80000 × *g* supernatant (cytosolic fraction); lane b, 80000 × *g* pellet (crude membrane fraction). (B) Lane a, pellet after Na_2CO_3 treatment; lane b, supernatant after Na_2CO_3 treatment; lane c, pellet after potassium acetate treatment; lane d, supernatant after potassium acetate treatment; lane e, pellet after urea treatment; lane f, supernatant after urea treatment.

tide of apparent molecular mass 180 ± 10 kDa which we named $p180^{CDC25}$. This 180 kDa immunoreactive polypeptide is detected by two different sets of polyclonal antibodies raised against two different hybrid proteins encoded by two different parts of the *CDC25* gene. pPR17 protein contains amino acids 86–493 and pE2JM7 contains amino acids 354–877. So these two hybrid proteins represent only the N-terminal part of the *CDC25* open reading frame. Even after purification on immunoaffinity columns, the two sets of antibodies, A2.4 and A6.3, still react with other proteins of lower apparent molecular mass than $p180^{CDC25}$. These cross-reacting species are not the same for each set of antibodies. Moreover, $p180^{CDC25}$ is the only polypeptide whose detection requires both presence of pGAL25.6.1 and galactose induction.

The pGAL25.6.1 recombinant plasmid contains the whole open reading frame of the *CDC25* gene, 126 bp upstream the first ATG codon and 466 bp downstream the STOP codon. In this construct, the first ATG codon encountered downstream the *GAL1* promoter is the initiation codon of the *CDC25* open reading frame [9]. In galactose-induced cells, the over-accumulated *CDC25* mRNA was of the right size (5200 nucleotides), in good agreement with that previously reported for the mRNA transcribed from the *CDC25* gene in its natural chromosomal location (5200 nucleotides) [9,16]. Therefore $p180^{CDC25}$ represents the primary translation product of the *CDC25* gene when cloned into the vector pGAL25.6.1. The apparent molecular weight on SDS-polyacrylamide gel is in good agreement with the molecular weight deduced from the open reading frame. That seems to exclude an important post-translational modification, such as glycosylation.

However, since the antibodies have been raised against proteins expressed in *E. coli*, they could be unable to recognize yeast specific post-translational determinants.

We were unable to detect $p180^{CDC25}$ in the absence of overexpression, which suggests $p180^{CDC25}$ is not abundant in wild type cells. This low level is relevant to the very low amount of *CDC25* mRNA detected and could explain that pGAL25 6.1 is able to complement *CDC25* deficient cells, even in the presence of glucose. Moreover, sequence analysis of the *CDC25* open reading frame shows a strong bias in the codon usage index. $p180^{CDC25}$ was only detected in the absence of proteolytic artefacts, by using the C13ABYS86 strain, which lacks four vacuolar proteases [36]. These vacuolar proteases have a broad specificity and are not likely to be involved in protein maturation. If proteolytic processing is essential for $p180^{CDC25}$ function, none of the proteases defective in the C13ABYS86 strain seems to be involved, since this strain is viable in the usual laboratory growth conditions, unlike *CDC25* deficient cells. However, our results cannot exclude a physiological proteolytic maturation of $p180^{CDC25}$

We [12,14] and others [10,11] have proposed that the *CDC25* gene product interacts with ras proteins to activate adenylate cyclase. In *Saccharomyces cerevisiae*, ras proteins and adenylate cyclase are bound to the membrane. We found $p180^{CDC25}$ associated with the membrane fraction after cell breakage by two different methods. No significant amount was found in the cytosolic fraction. To check the attachment of $p180^{CDC25}$ to the membrane, we performed stripping experiments of the crude membrane fraction by three different reagents able to release only peripheral membrane proteins. These three methods gave essentially the same results: when we loaded equal protein amounts, $p180^{CDC25}$ immunostaining was found almost exclusively in the integral membrane protein fraction. However, if we take into account the total amount of $p180^{CDC25}$ present in each fraction, a low but significant amount of $p180^{CDC25}$ has been released from the membranes by such treatments. The same repartition was observed for ras proteins.

Roadway et al. [39] pointed out the presence of a SH3 sequence between amino acids 65 and 129 of the *CDC25* gene product. This SH3 sequence is present in the N-terminal parts of several proteins bound to a submembranous actin cytoskeleton, such as c-src [40], v-crk [41], fodrin [42] and myosin IB [43]. Fodrin [44] and myosin IB [43] are released from membrane preparations by 1 M KCl or 100 mM NaOH, which release peripheral membrane proteins [45]. By contrast, $p180^{CDC25}$ is only partially released from membranes by treatments known to release peripheral membrane proteins. This suggests some other way than the presence of a SH3 sequence for anchoring $p180^{CDC25}$ into the membrane. We have shown that $p180^{CDC25}$ is bound to the membrane as tightly as ras proteins, which are anchored into the membrane via post-translational acylation [37]. How $p180^{CDC25}$ is anchored into the membrane remains to be elucidated. It could be by means of a putative transmembrane domain as already proposed by others [46,47]. Anyway, it could be functionally relevant that $p180^{CDC25}$ and ras proteins, which together activate adenylate cyclase, are both bound to the membrane.

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REFERENCES

[1] Matsumoto, K., Uno, I. and Ishikawa, T. (1983) Exp. Cell Res. 146, 151–161.
[2] Casperson, G.F., Walker, N. and Bourne, H.R. (1985) Proc. Natl. Acad. Sci. USA 82, 5060–5062.
[3] Boutelet, F., Petitjean, A. and Hilger, F. (1985) EMBO J. 4, 2631–2641.
[4] Kataoka, T., Broek, D. and Wigler, M. (1985) Cell 43, 493–505.
[5] DeFeo-Jones, D., Tatchell, K., Robinson, L.C., Sigal, I.S., Vass, W.C., Lowy, D.R. and Scolnick, E.M. (1985) Science 228, 179–184.
[6] Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J.B., Broach, J. and Wigler, M. (1984) Cell 36, 607–612.
[7] Tamanoi, F., Walsh, M., Kataoka, T. and Wigler, M. (1984) Proc. Natl. Acad. Sci. USA 81, 6924–6928.
[8] Broek, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J. and Wigler, M. (1985) Cell 41, 763–769.
[9] Camonis, J.H., Kalekine, M., Gondré, B., Garreau, H., Boy-Marcotte, E. and Jacquet, M. (1986) EMBO J. 5, 375–380.
[10] Robinson, L.C., Gibbs, J.B., Marshall, M.S., Sigal, I.S. and Tatchell, K. (1987) Science 135, 1218–1221.
[11] Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S. and Wigler, M. (1987) Cell 48, 789–799.
[12] Camonis, J.H. and Jacquet, M. (1988) Mol. Cell. Biol. 8, 2980–2983.
[13] Daniel, J., Becker, J.M., Enari, E. and Levitzki, A. (1987) Mol. Cell. Biol. 7, 3857–3861.
[14] Créchet, J.B., Poullet, P., Mistou, M., Parmeggiani, A., Camonis, J., Boy-Marcotte, E., Damak, F. and Jacquet, M. (1990) Science (in press).
[15] Jacquet, M., Camonis, J., Boy-Marcotte, E., Damak, F. and Garreau, H. (1989) in: The Guanine-Nucleotide Binding Proteins (Bosch, Kraal and Parmeggiani eds) pp. 241–249, Plenum, New York.
[16] Martegani, E., Baroni, M.D., Frascotti, G. and Alberghina, L. (1986) EMBO J. 5, 2363–2369.
[17] Rüther, U. and Müller-Hill, B. (1983) EMBO J. 2, 1791–1794.
[18] Gibson, T.J. (1984) Studies on the Epstein-Barr Virus Genome, PhD Thesis, Cambridge University, England.
[19] Morrison, D.A. (1979) Methods Enzymol. 68, 326–331.
[20] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[21] Simon, M.N., Mutzel, R., Mutzel, H. and Véron, M. (1988) Plasmid 19, 94–102.

[22] Ullman, A. (1984) Gene 29, 27–31.
[23] Part, D., De Gunzburg, J. and Veron, M. (1985) Cell Diff. 17, 221–227.
[24] Zarling, D.A., Arndt-Jovin, D.J., Robert-Nicoud, M., McIntosh, L.P.,. Thomae, R. and Jovin, T.M. (1984) J. Mol. Biol. 176, 369–415.
[25] Wittenberg, C., Richardson, S.L. and Reed, S.I. (1987) J. Cell Biol. 105, 1527–1538.
[26] Johnston, M. and Davis, R.W. (1984) Mol. Cell. Biol. 4, 1440–1448.
[27] Hovland, P., Flick, J., Johnston, M. and Sclafani, R.A. (1989) Gene 83, 57–64.
[28] Parent, S.A., Fenimore, C.M. and Bostian, K.A. (1985) Yeast 1, 83–138.
[29] Tschumper, G. and Carbon, J. (1980) Gene 10, 157–166.
[30] Hu, N. and Messing, J. (1982) Gene 17, 271–277.
[31] Sherman, F., Fink, G.R. and Lawrence, C.W (1974) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[32] Casperson, G.F., Walker, N., Brasier, A.R. and Bourne, H.R. (1983) J. Biol. Chem. 258, 7911–7914.
[33] Hicke, L. and Schekman, R. (1989) EMBO J. 8, 1677–1684.
[34] Laemmli (1970) Nature 277, 680–682.
[35] Khyse-Andersen, J. (1984) J. Biochem. Biophys. Methods 10, 203–209.
[36] Achstetter, T. and Wolf, D.H. (1985) Yeast 1, 139–157.
[37] Fujiyama, A. and Tamanoi, F. (1986) Proc. Natl. Acad. Sci. USA 83, 1266–1270.
[38] Casperson, G.F., Walker, N., Brasier, A.R. and Bourne, H.R. (1983) J. Biol. Chem. 258, 7911–7914.
[39] Wang, H.L., Malbon, C.C. and Bahout, S. (1989) J. Biol. Chem. 264, 14424–14431.
[39] Roadaway, A.R.F., Sternberg, M.J.E. and Bentley, D.L. (1989) Nature 342, 624–624???.
[40] Takeya, T. and Hanafusa, H. (1983) Cell 32, 881–890.
[41] Mayer, B.J., Hamaguchi, M. and Hanafusa, H. (1988) Nature 332, 27–2275.
[42] Wasenius, V.M., Saraste, M., Salvén, P., Eramaa, M., Holm, L. and Lehto, V.P. (1989) J. Cell Biol. 108, 79–93.
[43] Adams, R.J. and Pollard, T. (1989) Nature 340, 565–568.
[44] Bennet, V., Davis, J. and Fowler, W.E. (1982) Nature 299, 126–131.
[45] Fujiki, Y., Hubbard, A.L., Fowler, S. and Lazarov, P.B. (1982) J. Cell Biol. 93, 97–102.
[46] Daniel, J. (1986) Curr. Genet. 10, 879–885.
[47] Munder, T., Mink, M. and Küntzel, H. (1988) Mol. Gen. Genet. 214, 271–277.