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YEAST cAMP-DEPENDENT PROTEIN KINASE CAN BE ASSOCIATED TO THE PLASMA MEMBRANE

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SUMMARY : Using an anti-yeast regulatory subunit antibody and the synthetic peptide Kemptide as specific substrate we show in this work that purified preparations of yeast plasma membrane have an associated form of the regulatory subunit and cAMP-dependent protein kinase activity. Treatment of the plasma membrane "in vitro" with 1 μM cAMP releases cAMP-independent protein kinase activity while regulatory subunit remains on the membrane as revealed by immunoblotting. Incubation of the plasma membrane with [γ-^{32}P]ATP results in the phosphorylation of the regulatory subunit.

Modulation of biological systems by phosphorylation-dephosphorylation of specific target proteins is an important regulatory mechanism. An increasing number of protein kinases appears to play a crucial role in signal transduction for a variety of biologically active substances [1]. Many of these protein kinases are integral membrane proteins or are somehow associated with the plasma membrane a position in which they may sense and transduce the presence of external stimuli [2].

In the yeast *Saccharomyces cerevisiae* control of growth and cell division is largely exerted by nutritional signals [3,4] such as glucose. Addition of glucose to yeast is known to produce a very rapid increase in cAMP [5,6,7], an effect similar to that produced by several hormones in higher organisms, but it is not known whether glucose plays another role in yeast besides its use as a carbon source. Our purpose in this work has been to identify membrane-associated protein kinases whose activity could be involved in the early response of yeast cells to changes in the nutritional situation. This approach has led us to the finding that purified yeast plasma membrane shows a cAMP-dependent protein kinase (cAMP-PK) activity that varies along growth on glucose. Results concerning the enzymatic activity, solubilization and autophosphorylation of the membrane associated protein kinase are presented.

MATERIALS AND METHODS

Organism and growth conditions. *Saccharomyces cerevisiae* strain X2180 was used throughout this work. Yeasts were grown at 30 °C in rich medium containing 1% Yeast Extract

Abbreviations: cAMP-PK, cAMP-dependent protein kinase; R, regulatory subunit of cAMP-PK; C, catalytic subunit of cAMP-PK.
Enzymes: cAMP-dependent protein kinase (EC 2.7.1.37); plasma membrane ATPase (EC 3.6.1.35).

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1% Peptone and 2% Glucose. Growth was followed by measuring the turbidity at 660 nm and corresponding dry weights were calculated from a calibration curve of dry weight versus absorbance.

Homogenization and Plasma-membrane purification. Yeasts were collected at the desired optical density by filtering through Millipore and immediately frozen in liquid nitrogen. Cell free extracts were prepared as in [8] and centrifuged at 27000 x g for 30 min .Supernatant was purified through DE-52 for soluble fraction preparation, as described below. Pellet was used as the starting material for plasma membrane purification through a discontinous sucrose gradient as described in [8] except that Antipain was added during the homogenization of the 27000 x g pellet at a concentration of 50 µg/ml and mantained at this concentration in subsequent purification steps.

cAMP-dependent protein kinase assay. Protein kinase activity was measured as in [9] using Kemptide as the substrate and not more than 3 µg of purified plasma-membrane protein as the source of enzyme. After 4 min incubation at 37°C reactions were stopped and phosphate incorporation was analysed as described [10]. Endogenous activity without substrate was substracted from the total radiactivity incorporated.

Autophosphorylation of plasma-membrane proteins. Autophosphorylation was carried out as described in [11] except that manganese was used as the working cation,because of the cleaner phosphoprotein pattern obtained with Mn^{+2} as compared with Mg^{+2} in autophosphorylating conditions.

Membrane-bound R subunit solubilization. Purified plasma-membranes were diluted in 50 mM Hepes/Tris pH 8.5, 10 mM EDTA, 1 mM EGTA, 1 % Triton X-100 to give a final protein concentration of 0.2 mg/ml and incubated at room temperature for 10 min, shaking occasionally. After centrifugation at 100000 x g for 30 min, the supernatant was used directly (for immunoprecipitation procedures) or was purified through DE-52 as described below (for affinity chromatography). Pellets were resuspended in buffer A: 50 mM Tris, 5 mM EDTA, 5mM dithioerytritol, 20 % glycerol and 50 µg/ml Antipain.

DE-52 ion exchange chromatography. Plasma membrane solubilized proteins were applied to a DE-52 column (0.5 ml) equilibrated in 25 mM Mes/NaOH pH 6.5, 1 mM EGTA, 1 mM EDTA, 10 mM Iodoacetate, 100 µg/ml Soybean Trypsin inhibitor, 1 mM Phenylmethylsulphonyl fluoride and 50 µg/ml Antipain. After washing with ten volumes of buffer B lacking Antipain protein was eluted with 2.5 ml of buffer B plus 0.15 M NaCl. These conditions were also used for soluble fraction purification.

cAMP-agarose affinity chromatography. Affinity chromatography was performed on DE-52 purified solubilized plasma-membrane fractions in a batch-wise fashion as described in [11], using cAMP- agarose instead of cAMP-Sepharose.

In-vitro cAMP treatment of plasma-membranes. Stationary phase cells plasma-membranes were diluted to 1 mg/ml of protein in 25 mM Hepes/NaOH pH 7.5, 25 mM 2-mercaptoethanol, 50 µg/ml Antipain and 1 µM cAMP, incubated for 20 min at 4°C, and centrifuged at 100000 x g for 30 min to separate plasma membrane . Membrane pellets were homogenized in buffer A. Supernatant and membrane fractions were assayed for protein kinase as described above.

Western blots and immunoprecipitation of R subunit. Anti-Yeast Regulatory subunit antibody was a kind gift from Dr Edwin G. Krebs. Western blot analysis was carried out according to [12] after separation of soluble or plasma-membrane proteins by 10% SDS-PAGE as described [13].

Immunoprecipitations were performed in buffer C with the addition of 0.1 % SDS and 0.1 M NaCl. Usually,150 µg of autophosphorylated and solubilized plasma-membrane proteins were incubated with specific or control anti-sera. After an overnight incubation at 4°C, 30 µl of a 50 % Protein-A Sepharose preparation were added and the mixture was further incubated for 90 min. Immunoprecipitates were sedimented at 12000 rpm, washed six times with immunoprecipitation buffer (1 ml each), resuspended in 50 µl of Laemmli's sample buffer and heated for 5 min at 100 °C. After this, the Sepharose was decanted and the sample subjected to SDS-PAGE.

Protein determination. Protein was measured by a modified Bradford procedure [14] using bovine serum albumin as standard.

Materials. Kemptide and Antipain were from Sigma, [γ-^{32}P]ATP was from Amersham.

RESULTS

cAMP-PK in the plasma membrane

We observed that purified yeast plasma membrane phosphorylated Kemptide, a substrate highly specific for cAMP-PKs, in a cAMP-dependent manner. This observation prompted us to investigate this kinase activity since yeast cAMP-PK was reported to be a soluble enzyme [15].

A first step was to measure the kinase activity of plasma membrane prepared from yeasts grown to different stages on rich medium . Table 1 shows that the ability of the plasma membrane to phosphorylate Kemptide , in a cAMP-dependent maner, largely increases during growth on glucose, reaching a specific activity in stationary cells that is about seven fold higher than in logarithmically growing cells, and 50% of that found in the soluble fraction.

To further asses the nature of the membrane-bound kinase, membrane proteins were separated on polyacrylamide gels under denaturing conditions and immunoblotted with an anti-R antibody prepared against the regulatory subunit of yeast soluble cAMP-PK [16]. As shown in Fig.1 (lanes 1&2) a protein band reacting with the antibody was detected on the membrane that was indistinguishable from that detected on the soluble fraction with respect to mobility in SDS-polyacrylamide gels, giving an apparent Mr of 53 Kd, a size reported for the yeast soluble R subunit [16]. Both this immunoreactive material and the catalytic activity were shown to comigrate in continuous sucrose gradients with the ATPase localized in the yeast plasma membrane [17] (data not shown). Material crossreacting with the anti-R antibody was not solubilized by washing the membrane preparation with NaCl up to 0.5 M, indicating that the detected regulatory subunit is not a loosely bound membrane protein (see below).

Table 1 ***Variations of plasma membrane-bound cAMP-PK activity during growth.*** To determine enzymatic activity along growth, cultures between 0.8 and 1.4 mg dry weight/ml were considered to be in the early logarithmic phase of growth, cultures between 2.8 and 3.3 mg dry weight/ml were considered transitional and cultures between 4.0 and 4.5 mg dry weight/ml were taken as stationary. cAMP-PK activity was assayed in the presence or absence of 10 µM cAMP. Data are representative of three separate experiments. 1 Unit (U) is defined as the number of picomoles of phosphate incorporated into Kemptide in 1 minute.

Growth phase	cAMP-PK activity (U/ mg)	
	+ cAMP	- cAMP
Exponential	194	20
Transitional	647	20
Stationary*	1520	150

* Step-wise DE-52 purified soluble fraction showed an specific activity of 3000 U/mg.

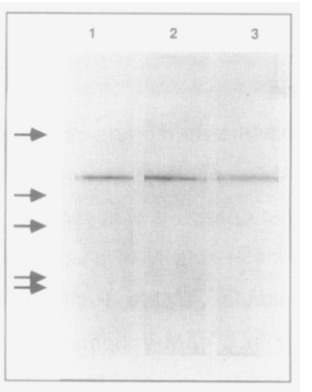

Figure 1. ***Western blot analysis of R subunit in soluble , plasma membrane and solubilized plasma membrane fractions.*** Proteins of soluble and membrane fractions were separated on 10% SDS-polyacrylamide slab gels, transferred to nitrocellulose paper and immunoblotted using anti-R antibody. Lane 1: 50 µg of DE-52 purified soluble fraction , Lane 2: 15 µg of purified plasma membrane from stationary phase grown cells, and Lane 3: EGTA/EDTA, Triton X-100 solubilized protein from plasma membrane of stationary phase cells.(Arrows indicate molecular weight markers: 67, 45, 36, 29,24 Kd.).

Solubilization of the membrane-bound form of the cAMP-dependent protein kinase

To our knowledge this is the first report of cAMP-PK in yeast plasma membrane although the presence of cAMP-binding proteins was initially reported in [18] based on the reaction with the photoaffinity ligand 8-N_3-[^{32}P]-cAMP.

We found that the incubation of the purified plasma membrane with 1% Triton X-100, 10 mM EDTA ,1 mM EGTA and 20 mM Tris pH 8,5, solubilized a 53 KD protein that was recognized by anti-R antibody (Fig.1, lane 3) and that was retained by cAMP-agarose thus indicating that the solubilized protein is a cAMP-binding protein (fig.3,lane 2).

On the other hand , cAMP treatment of the plasma-membrane was sufficient to release the catalytic subunit of the cAMP-PK. As shown in Fig.2 when plasma membrane was treated with 1 µM cAMP for 15 minutes at 4 °C, Kemptide phosphorylating activity was released while anti-R antibody crossreacting material sedimented with the plasma membrane after centrifugation These results indicate that the kinase is anchored to the membrane through the R subunit .

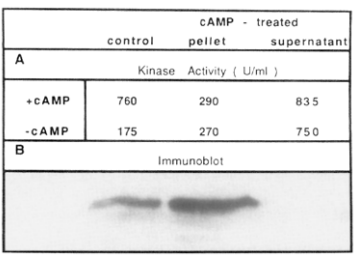

Figure 2. ***in-vitro cAMP treatment of plasma membrane.*** Purified plasma membrane was treated with 1 µM cAMP as described under Materials & Methods. A : Phosphorylating activity was measured in the presence or absence of cAMP. The 100000 x g supernatant was precipitated with 40% ammonium sulphate before assay . B: R subunit was detected by immunoblot on the same fractions as in A.

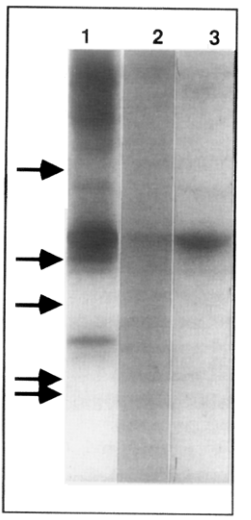

Figure 3. ***Purification of the phosphorylated form of plasma membrane bound R subunit.*** Purified plasma membrane proteins from transitional phase cells were autophosphorylated (Lane 1), solubilized and purified trough cAMP-agarose (Lane 2) or solubilized and immunoprecipitated with anti-R antibody (Lane 3) as described under Materials & Methods. (Arrows indicate molecular weight markers as described in legend to fig. 1)

The cAMP-independent Kemptide phosphorylating activity released by cAMP treatment had to be immediately precipitated with ammonium sulphate to preserve it and to eliminate soluble cAMP . Table 2 shows that the activity thus recovered was completely cAMP-independent and that nearly no cAMP-dependent activity remained in the membrane . In other experiments cAMP-independent activity was further purified through DE-52 before ammonium precipitation, but activity was rapidly lost.

Phosphorylation of membrane bound regulatory subunit

When yeast plasma membrane was incubated under autophosphorylation conditions, that is, low ATP concentration and low temperature, R subunit incorporated [^{32}P]-phosphate as shown by solubilization and immunoprecipitation with the antibody (Fig. 3, lane 3). However this was not the only membrane protein of 53 Kd that was phosphorylated. The supernatants of the immunoprecipitates contained other protein(s) of equal mobility on SDS-gels that were also heavily labeled under these conditions. Phosphoaminoacid analysis of the "in vitro" [^{32}P]-phosphate labeled 53 Kd protein bands gave phosphothreonine and phosphoserine.

DISCUSSION

The results presented in this paper show that yeast plasma membrane presents cAMP-PK activity. In most cells this enzyme is predominantly in the soluble fraction, but membrane-bound cAMP-PK has also been found in other systems such as cardiac tissue and brain [19,20 and for review see 21]. In many of these cases the holoenzyme is anchored to the membranes through the R subunit and the catalytic activity can be released by dissociating the enzyme with cAMP, as seems to occur also with the yeast enzyme. The fact that C subunit is

removed from the membrane upon "in vitro" treatment with cAMP provides an explanation for the observed increase in specific activity of the plasma membrane-bound enzyme along growth on glucose. As yeast cells progress to the late logarithmic and stationary phases of growth, glucose is being exhausted and the intracellular cAMP is reaching the lowest levels [22] thus allowing reassociation of the holoenzyme. Since R remains membrane bound after catalytic subunit is released it seems clear that the maximum cAMP-PK enzymatic activity associated to the membrane will always be limited to the amount of R in that fraction.

The plasma membrane bound R subunit appears to be identical with its soluble counterpart because it is recognized by antibodies prepared against cytosolic R subunit purified from *Saccharomyces cerevisiae* and comigrates with cytosolic R on SDS-gels. cAMP-binding proteins have been reported previously in plasma membrane of yeast [18]. The authors detected other bands of smaller size probably due to proteolytic fragments conserving the cAMP-binding domain. We find very convenient the addition of antipain along membrane preparation in order to obtain the full-sized 53Kd band.

The cloning of yeast R subunit has been recently reported by three different laboratories [23-25]. They find that there is only one gene for R thus excluding the possibility that the membrane bound form is codified by a different gene. The possibility of two mRNAs, one coding for the cytosolic and another one coding for the membrane form, is also discarded by the fact that only one messenger was found [23], indicating that both forms of R subunit are codified by the same gene. The protein sequence deduced from the cloned gene does not give any clue regarding the way of attachment of the protein to the membrane . An N-terminal glycine residue typical of miristylated proteins is absent as is the acyltransferase recognition sequence Cys-A-A-X at the C-terminus in palmitylated proteins. Recently the presence of cAMP-PK activity has been shown in yeast mitochondria [26] raising the interesting question of how the product of one gene is sorted to three different subcellular compartments.

It has been reported that yeast R subunit can be phosphorylated by catalytic subunit from mammalian origin [16] and also it has been shown that yeast C phosphorylates cytosolic R subunit [27]. We show in this paper that plasma membrane incubated under autophosphorylation conditions incorporate [^{32}P] into R subunit . It is known for mammalian cAMP-PKs that an intramolecular, cAMP-independent reaction catalyzes the phosphorylation of R subunit by its associated C subunit. The result obtained with yeast plasma membrane is taken as evidence that yeast C subunit is able to phosphorylate the membrane bound R subunit.

Although we cannot, at the moment, asign a specific function to the presence of this protein kinase in the yeast plasma membrane it is tempting to speculate that it may have a role in signal transduction since it is in a position where even small local variations of cAMP levels in response to nutrients such as glucose [6] will activate its catalytic activity. It also exists the possibility that R subunit is playing a role by itself.

In summary, we have detected a membrane associated cAMP-PK in yeast. The role that this kinase may fulfill is unknown at present but future work will be aimed towards the

understanding of how the enzyme is attached to the membrane and whether the "in vivo" phosphorylation state of R affects its subcellular localization. Also detection of the endogenous substrates for this enzyme may shed light on its function.

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