FEBS Letters 354 (1994) 117–122

FEBS 14717

Expression of the sarcoplasmic reticulum Ca^{2+}-ATPase in yeast

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Received 23 September 1994

Abstract We describe here an easy system for the production of mg amounts of the rabbit Ca^{2+}-ATPase SERCA 1a in the yeast *S. cerevisiae*. The protein is present in several membranes, including the plasma membrane of the yeast, in a native conformation. It can be purified by immunoprecipitation and can be phosphorylated from ATP in a Ca^{2+}-dependent manner. Using a temperature-sensitive secretion mutant strain, the fully active protein can also be obtained in secretory vesicles.

Key words:

1. Introduction

The 110,000-Da SR Ca^{2+}-ATPase from skeletal muscle is a membrane protein that actively transports Ca^{2+} within the sarcoplasmic reticulum as a result of ATP hydrolysis. This P-type ATPase plays an essential role in muscle relaxation. Ca^{2+}-ATPase from rabbit and chicken fast twich muscle has been expressed mainly in mammalian cells [1,2], resulting in the description of a number of mutations or chimeras with interesting properties (e.g. see [1–7]). However, a problem with this system is the relatively low yield of Ca^{2+}-ATPase expression, which barely suffices for detailed biochemical analysis. More recently, the SR Ca^{2+}-ATPase has been expressed in the baculovirus/Sf9 system [8]. Another good and cheap alternative for large scale production of foreign membrane proteins (e.g. [9–11]), including ATPases (e.g. [12]) is the yeast *S. cerevisiae*.

We describe here a procedure with the potential for production of mg amounts of the native and fully active rabbit Ca^{2+}-ATPase SERCA.

2. Material and methods

2.1. Plasmidic constructions and yeast cultures

2.1.1. Yeast expression vector. The yeast expression vector pYeDP1/8-10 was a gift of Dr. D. Pompon [9]. Inserted cDNA was placed under the control of a GAL10 promoter and a PGK (phosphoglycerate kinase) terminator.

2.1.2. Ca^{2+}-ATPase cDNA isolation. Part of the skeletal muscle Ca^{2+}-ATPase cDNA was isolated as previously described [13] by screening a rabbit neonatal skeletal muscle cDNA library constructed in a λZap II vector (Stratagene). The bluescript plasmid DNA was excised from the selected clone by co-infection with the helper phage R408. RT-PCR was performed on total RNA from adult rabbit skeletal muscle to obtain missing 5′ and 3′ coding regions. DNA fragments obtained by both screening and RT-PCR were ligated at the appropriate sites to obtain the full-length coding cDNA which was inserted at the *Spe*I and *Apa*I sites of the pBS II KS vector (Stratagene). The DNA was sequenced on both strands using the Sequenase Kit (United States Biochemicals). The amino acid sequence obtained was identical to the sequence of the adult isoform published by Brandl et al. [14,15]; only a few silent mutations were observed at the nucleotide level.

2.1.3. Insertion of Ca^{2+}-ATPase cDNA in pYeDP1/8-10. Ca^{2+}-ATPase cDNA was inserted in *Eco*RI/*Sac*I cloning sites of pBS. The cDNA was first modified by addition of an *Eco*RI site in the 5′ end by use of *Eco*RI linker. Using the *Sac*I linker, a unique *Sac*I restriction site was added to the 3′-terminus of the cDNA. Ca^{2+}-ATPase cDNA was then excised from pBS by double digestion with *Eco*RI and *Sac*I and ligated to pYeDP1/8-10 at the same sites.

2.1.4. Yeast strains, transformation and culture. *Saccharomyces cerevisiae* W303.1B (α, leu2, his3, trp1, ura3, ade2–1, can^R, cyr$^+$) was transformed according to the method described in [16]. Transformant selection (based on ura3 complementation) and culture were performed as previously described [17]. To induce heterologous Ca^{2+}-ATPase expression, a preculture was made in glucose medium until the OD reached 2 at 660 nm and the cells were then diluted to 0.1 OD in galactose medium. The cells, shaken at 30°C, were harvested after 16–20 h.

Temperature-sensitive *Saccharomyces cerevisiae* NY17 (a, ura 3, sec6–4) [18] was transformed and selected as indicated above. Precultures and cultures were made at 25°C and the yeast was then submitted to a final heat-shock at 37°C for 2 h before harvesting, allowing the accumulation of secretory vesicles.

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Abbreviations: SR, sarcoplasmic reticulum; SERCA, sarco(endo)-plasmic reticulum Ca^{2+}-ATPase; PM, plasma membrane; $C_{12}E_8$, octaethyleneglycol monododecylether; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; pBS, plasmid bluescript (Stratagene); MOPS, 3-[*N*-morpholino]propanosulfonic acid; EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) *N,N′*-tetra-acetic acid; pAb, polyclonal antibody; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; TCA, trichloroacetic acid; FITC, fluorescein isothiocyanate; ATP, adenosine-5′-triphosphate; NADH, nicotinamide adenine dinucleotide, reduced form; RT-PCR, reverse transcriptase-polymerase chain reaction, DAPI, 4′,6-diamidino-2-phenylindole.

SSDI 0014-5793(94)01104-4

2.2. Total extract, microsome, plasma membrane and secretory vesicle preparation

2.2.1. Cell homogenization. Yeasts were pelleted and resuspended in 10 ml of ice-cold 'break' buffer (50 mM TES-KOH, pH 7.5, 0.6 M sorbitol, 5 mM EDTA, 1 mM EGTA, 100 μg/ml bacitracin, 1 mM benzamidine, 1 mM PMSF and 10 μg/ml trypsin inhibitor). The cells were homogenized with glass beads, shaken either manually or with a BeadBeader cell disrupter (Biospec). For preparation of yeast total protein extract, aliquots corresponding to 5 OD were taken out after the first resuspension, pelleted, resuspended in 2% TCA and homogenized with glass beads.

2.2.2. Preparation of microsomes, plasma membranes and secretory vesicles. Microsomal fractions were prepared immediately after cell homogenization, as described in [17]. Plasma membranes were purified by selective pH precipitation [19], followed in some cases by a discontinuous density gradient centrifugation step. A total membrane fraction was prepared by the same procedure [19] without selective pH precipitation. Secretory vesicles were prepared according to [20] except that the final S-1000 Sephacryl step was omitted. For control experiments, rabbit skeletal muscle sarcoplasmic reticulum vesicles were prepared as described in [21].

2.2.3. Protein concentration determination. Protein concentrations were determined either by the Lowry method [22] or with bicinchoninic acid [23] using bovine serum albumin as a standard.

2.3. Immuno-detection and purification of expressed protein

2.3.1. Immunoblotting. Proteins separated by SDS-PAGE [24] were either revealed by Coomassie blue staining or electrotransferred onto PVDF membranes [25]. For immunodetection, different polyclonal antibodies raised against the fast twich muscle SR Ca^{2+}-ATPase were used. pAb 78(7), 79(A) and 79(B), which have been described in [26], are directed against the entire molecule, whereas pAb (809–827) described in [27] is directed against the peptide 809–827. After further incubation with ^{35}S-labelled protein A, immunoreactive proteins were revealed by exposing PVDF membranes to X-Omat film or using a PhosphorImager detector (Molecular Dynamics).

2.3.2. Immunoprecipitation of Ca^{2+}-ATPase. Immunopurification of heterologously expressed Ca^{2+}-ATPase was performed after membrane solubilization with $C_{12}E_8$, essentially as described in [8] except that the polyclonal antibody 79(B) was used.

2.3.3. Immunolocalization of expressed Ca^{2+}-ATPase in yeast cells.. Yeasts were grown as indicated above and after induction of expression we proceeded as indicated in [28]; basically, the yeasts cells were fixed with 3.5% formaldehyde, washed, and spheroplasts, obtained by digestion with zymoliase, were labelled with the pAb 79(B) and visualized with FITC-conjugated secondary antibody.

2.4. Phosphoenzyme formation

Phosphoenzyme formation during ATP hydrolysis was carried out using immunopurified Ca^{2+}-ATPase linked to protein A-Sepharose–pAb complex. Phosphorylation was performed for 10 s in an ice-cold solution containing: 20 mM MOPS-KOH (pH 7.0), 80 mM KCl, 10 mM NaN_3, 1 mM $MgCl_2$, 2 μM [γ-^{32}P]ATP and either 100 μM $CaCl_2$ or 2 mM EGTA [29]. After addition of Laemmli buffer to stop the reaction and solubilize the membranes, samples were fractionated on SDS-PAGE and the E-^{32}P intermediate was visualized with a PhosphorImager detector.

2.5. ATPase activity

ATPase activity was measured using a coupled enzyme assay, as described in [30]. The reaction buffer (1.6 ml) contained: 10 mM TES-Tris (pH 7.5), 1 mM $MgCl_2$, 5 mM Mg-ATP, 0.1 mM $CaCl_2$, 1 mM phosphoenolpyruvate, 0.15 mM NADH, 0.1 mg/ml pyruvate kinase, 0.1 mg/ml lactate dehydrogenase and 1 mg/ml $C_{12}E_8$. We also added 5 mM NaN_3, 0.2 mM molybdate, 0.05 mM ouabain and 50 mM KNO_3 to the reaction mixture, to inhibit F_1F_0-ATPases, phosphatases, Na^+,K^+-ATPases and pyrophosphatases, respectively.

3. Results

3.1. Rabbit Ca^{2+}-ATPase is expressed in yeast membranes

Yeasts were transformed with plasmid pYeDP1/8-10 [9], containing the rabbit Ca^{2+}-ATPase cDNA insert, and the expression of protein was followed by SDS-PAGE and Western blot analysis (Fig. 1). A single band at 110 kDa was revealed by two different polyclonal antibodies specific for the rabbit fast twich skeletal muscle Ca^{2+}-ATPase, 78(7) (Fig. 1A, lane 5) and 809–827 (Fig. 1A, lane 7), at the same position as the SR Ca^{2+}-ATPase control (Fig. 1A, lane 1). It is interesting to note that Ab 78(7), raised against the complete SR Ca^{2+}-ATPase, mainly recognizes the N-terminal half of the protein, while Ab 809–827 was raised against the synthetic peptide Phe809-Pro827, located in the C-terminal half of the Ca^{2+}-ATPase. Immunological data thus indicate that the full-length polypeptide has been synthesized. No immunoreactive band was observed with these pAbs in total extracts from yeasts containing wild-type pYeDP, showing that the expression is specifically related to the presence of SERCA 1 cDNA (Fig. 1A, lanes 4 and 6). Moreover, induction by galactose was necessary for Ca^{2+}-ATPase expression since no band at 110 kDa was detected when the culture was carried out in glucose-containing medium (data not shown).

Fractionation of the yeast membranes into microsomes and plasma membranes, following published procedures [17,19], indicated that heterologously synthesized Ca^{2+}-ATPase was recovered in the plasma membrane fraction (Fig. 1A, lanes 9 and 11), and, to a lesser extent in the microsomes (Fig. 1A, lane 8): despite similar amounts of membrane proteins loaded in the microsomes and plasma membranes lanes the signal from the antibodies was weaker in the case of microsomes. The mitochondrial fraction was also tested by immunoblotting, and no Ca^{2+}-ATPase was present there (data not shown).

The targetting of SERCA Ca^{2+}-ATPase to the yeast plasma membrane was further assessed by the use of differential labeling with antibodies specific for the yeast H^+-ATPase, which is located in the plasma membrane: after linear sucrose gradient centrifugation of the total membranes and Western blotting of each fraction, the localization of Ca^{2+}-ATPase and H^+-ATPase appeared similar, sedimenting mainly in the high density region of the gradient (Fig. 2A), as expected for the plasma membrane [31]. A minor peak at 30% sucrose probably represents internal membranes. Immunolocalization experiments on yeast cells (Fig. 2B) showed that, although a significant part of Ca^{2+}-ATPase was located in membranes surrounding the nucleus, the enzyme was also present in secretory vesicles and in the plasma membrane.

Comparison of the Western blot signals from the Ca^{2+}-ATP-ase expressed in yeast (lanes 4–11 in Fig. 1A) with different amounts of Ca^{2+}-ATPase from rabbit sarcoplasmic reticulum (lanes 1–3 in Fig. 1A) shows that the expressed Ca^{2+}-ATPase is only a minor fraction of the total amount of protein. In the case of yeast total extract, Ca^{2+}-ATPase represent about 0.1% of the proteins, i.e. a total of 900 μg of Ca^{2+}-ATPase per liter of culture. In plasma membranes, this percentage rises to ~0.3%.

3.2. Rabbit Ca^{2+}-ATPase expressed in yeast is active

Despite its clear expression, the Ca^{2+}-ATPase band (110 kDa) cannot easily be identified by the Coomassie blue staining of a sample of plasma membranes: this is due to the low percentage of the expressed Ca^{2+}-ATPase compared to the large amount in these membranes of two major endogenous proteins of similar mass, the H^+-ATPase (105 kDa) and the associated glycoprotein (115 kDa) [32] (Fig. 1B, lanes 9–11). Probably for

the same reason we could not convincingly measure stimulation by Ca^{2+} of total ATPase activity or phosphorylation from [γ-^{32}P]ATP in yeast plasma membrane containing rabbit Ca^{2+}-ATPase. Nevertheless, the overall conformation of expressed Ca^{2+}-ATPase seems to be similar to that of active SR Ca^{2+}- ATPase: proteolytic digestion of the yeast plasma membrane by trypsin or protein radiolysis by γ-rays [33,34] yielded fragments identical to those of the native SR, as revealed by Western blotting (data not shown). These results are in favour of a correct folding of the heterologously expressed protein in the plasma membrane.

To demonstrate Ca^{2+}-ATPase activity we followed two kinds of strategies. In the first one, we purified Ca^{2+}-ATPase by membrane solubilization with $C_{12}E_8$ and immunoprecipitation. Fig. 3A shows that, after gel-electrophoresis, the immunoprecipitate prepared from yeast expressing Ca^{2+}-ATPase presents a distinct Coomassie blue-staining band at 110 kDa (Fig. 3A, lanes 5 and 6), which is absent when immunoprecipitation is performed from total membranes of yeast not containing the Ca^{2+}-ATPase cDNA (Fig. 3A, lanes 3 and 4). Immunopurification also allowed us to observe a calcium-dependent phosphorylation of yeast-expressed Ca^{2+}-ATPase: Ca^{2+}-ATPase was phosphorylated from [γ-^{32}P]ATP in the presence of 100 μM Ca^{2+} (Fig. 3B, lane 5), whereas phosphorylation was inhibited in the presence of 2 mM EGTA (Fig. 3B, lane 6). With control yeast no phosphorylation was observed, either in Ca^{2+} or in EGTA (Fig. 3B, lane 3 and 4). A similar immunopurification procedure performed with SR Ca^{2+}-ATPase, also yielded a Coomassie blue-stained band at 110 kDa (Fig. 3A, lanes 1 and 2), which could be phosphorylated in the presence of Ca^{2+} (Fig. 3B, lane 1), but not in EGTA (Fig. 3B, lane 2).

In the second approach, Ca^{2+}-ATPase was expressed in a yeast mutant strain altered in the secretory pathway (*sec* mutant), allowing the accumulation of secretory vesicles enriched in Ca^{2+}-ATPase. The percentage of Ca^{2+}-ATPase expressed in the vesicle fraction was measured to be about 0.6% of the protein content, i.e. larger than in the plasma membrane fraction (0.3%, see above). As shown in Fig. 4, the kinetics of ATP hydrolysis of vesicles containing the expressed Ca^{2+}-ATPase (trace b) clearly reveal an ATPase activity which is Ca^{2+}-dependent and inhibited by the specific inhibitor of SR Ca^{2+}-ATPase, thapsigargin. Vesicles prepared from control yeast do not display such dependence (trace a). In addition, the difference between control and Ca^{2+}-ATPase containing vesicles (Fig. 4, trace c) is quite similar to ATP hydrolysis by SR Ca^{2+}-ATPase (Fig. 4, trace d) when the latter is present at the same concentration as that of expressed Ca^{2+}-ATPase (as estimated by immunodetection). Under the assay conditions used here (30°C, 1 mg/ml $C_{12}E_8$) the specific activity of expressed Ca^{2+}-ATPase was 21 ± 5 μmol/mg/min (based on a calculated 20% error in the quantitation of the expressed protein by Western blotting), while the specific activity of SR was 16 μmol/mg/min. We thus conclude that rabbit Ca^{2+}-ATPase expressed in yeast is fully active.

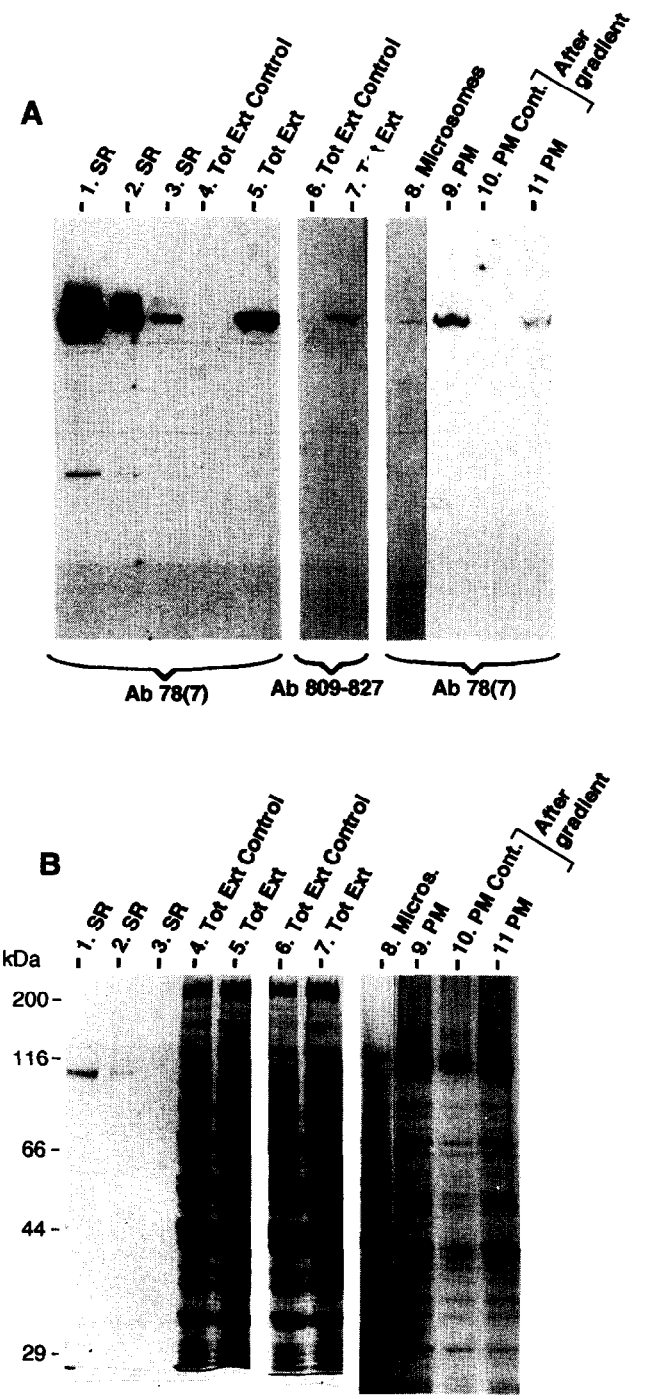
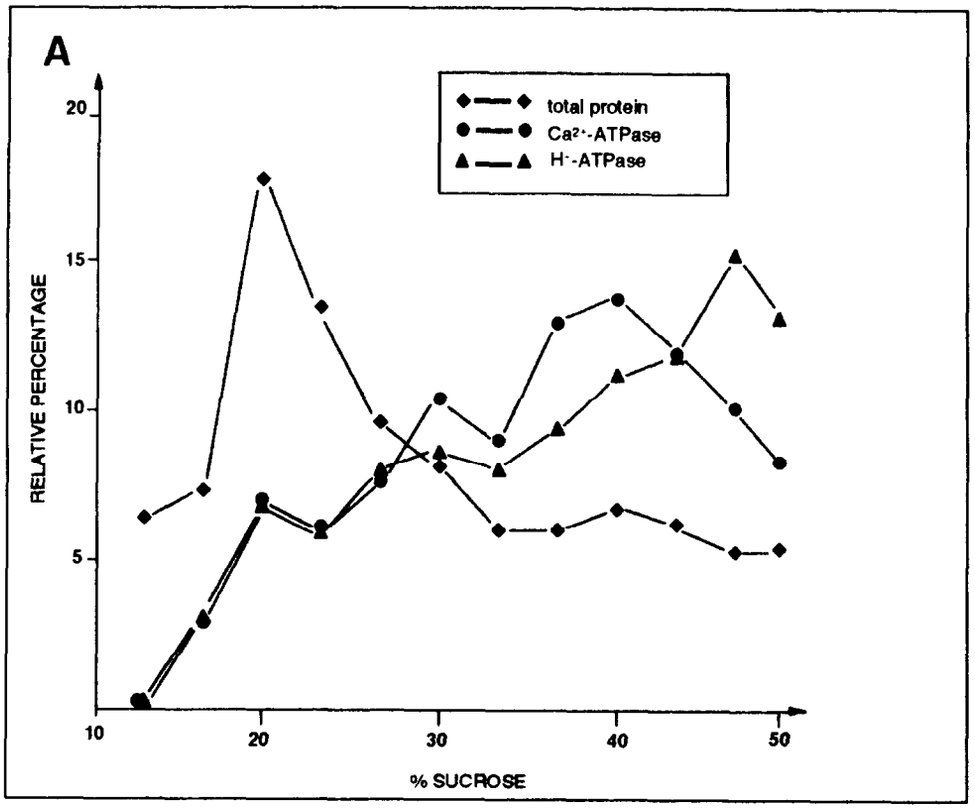


Fig. 1. Expression of rabbit SR Ca^{2+}-ATPase in yeast. Yeast containing either the Ca^{2+}-ATPase cDNA inserted in the pYeDP1/8-10 vector or the pYeDP1/8-10 vector without insert (control) were grown in a galactose culture medium. Total protein extract from yeast was obtained by cell disruption in the presence of TCA (lanes 4–7); microsomes (lane 8) and plasma membranes before or after discontinuous sucrose gradient purification (lanes 9–11, PM or PM control without Ca^{2+}-ATPase cDNA insert) were prepared as described in section 2. Proteins of these various preparations and of rabbit skeletal sarcoplasmic reticulum (lanes 1–3, SR) were separated by 7.5% SDS-PAGE. Gels were either (A) transferred to PVDF membranes and probed with two kinds of antibodies, 78(7) (lanes 1–5 and 8–11) or 809–827 (lanes 6 and 7), or (B) stained with Coomassie blue. The following amounts of protein were loaded in the wells: lane 1, 0.5 μg; lane 2, 0.1 μg; lane 3, 0.01 μg; lane 4–7, ~75 μg; lane 8, 18 μg; lane 9, 16 μg; lane 10, 5 μg; lane 11, 8 μg. Note that some proteolysis occured in lane 8.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Ca²⁺-ATPase
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RELATIVE PERCENTAGE
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15
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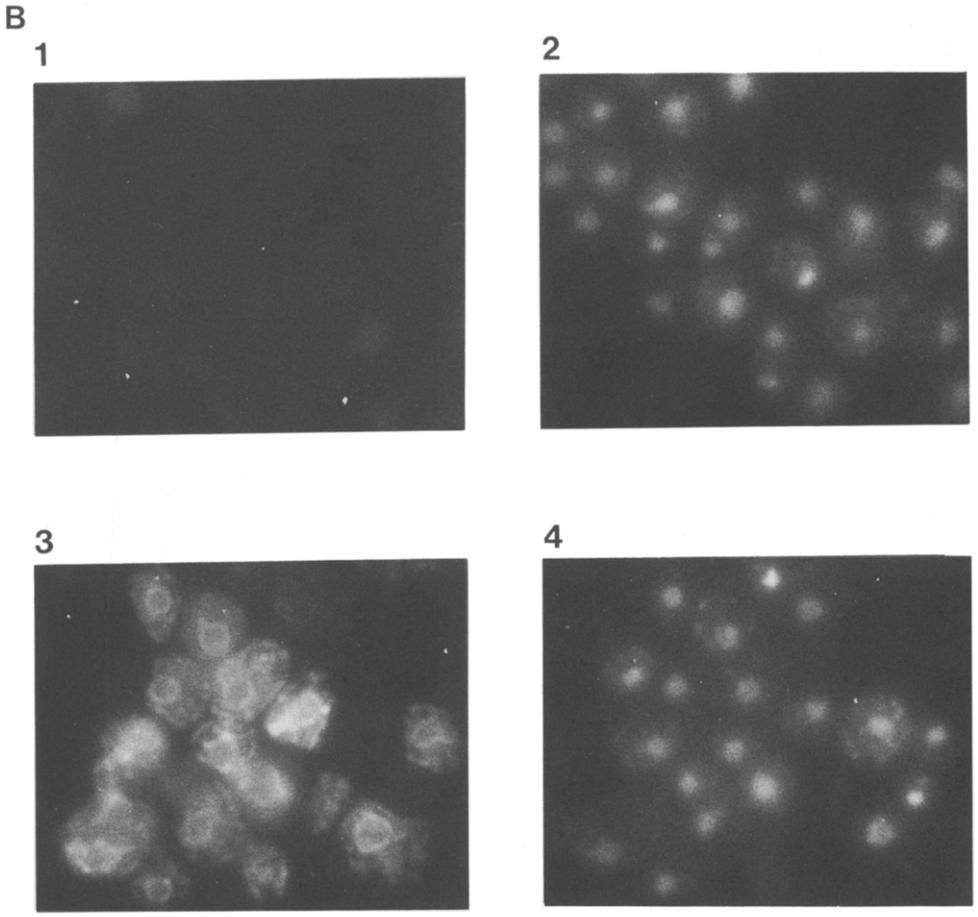
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Fig. 2. Localization of heterologous SR Ca^{2+}-ATPase in yeast. (A) Distribution of expressed Ca^{2+}-ATPase and endogenous H^+-ATPase in sucrose gradient fractions of yeast membranes. Total membranes were isolated from yeast expressing rabbit SR Ca^{2+}-ATPase and loaded on a linear 20–53% sucrose gradient. After an overnight centrifugation, 1 ml fractions were collected from the top of the gradient. The protein concentration of each fraction was measured and expressed as relative percentage (◆). Western blots of 20 μl aliquots of gradient fractions were stained with antibodies against rabbit Ca^{2+}-ATPase or yeast H^+-ATPase. After further incubation with ^{35}S-labelled protein A, immunoreactive proteins were revealed and the radioactivity of each ATPase band measured with a PhosphorImager detector. The radioactivity increased linearly with increasing amounts of membranes preparation loaded on the gel. As for total protein content of the gradient, the amount of Ca^{2+}-ATPase (●) and H^+-ATPase (▲) are expressed as relative percentage. (B) Immunocytolocalization of heterologous SR Ca^{2+}-ATPase in yeast cells. Fluorescence images of a control yeast cell (1 and 2) and of a yeast cell expressing SR Ca^{2+}-ATPase (3 and 4). Cells were DAPI-stained for nuclei (2 and 4) or immunolabelled with pAb 79 (B) against Ca^{2+}-ATPase (1 and 3).
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4. Discussion

We have shown here that the Ca^{2+}-ATPase cDNA inserted in the yeast vector pYeDP1/8-10 containing a GAL promoter allowed heterologous expression of rabbit Ca^{2+}-ATPase in *Saccharomyces cerevisiae*. The whole protein was expressed correctly folded, as suggested by immunological results, and it was active, as demonstrated by phosphorylation from [γ-^{32}P]ATP and by the observation that Ca^{2+}-ATPase specific activity was quite similar to the one measured in sarcoplasmic reticulum. We have also obtained indirect evidence for in vivo activity of the heterologous enzyme; in separate experiments (not reported here), performed with a different yeast strain (RS-72) and expression vector (pYE351) [35], where Ca^{2+}-ATPase cDNA was cloned under the control of the constitutive and powerful PMA1 promoter, we failed to obtain stable Ca^{2+}-ATPase expression but did observe transient expression: yeast grew very slowly and died after a few generations, suggesting that Ca^{2+}-ATPase over-expression may induce a toxic effect. This toxicity might arise because of an over-excretion of Ca^{2+} from the yeast cytosol or, more likely, from an indirect effect such as an increased pH inside the cell [36]. We are currently investigating this hypothesis.

When the plant plasma membrane H^+-ATPase is expressed in *Saccharomyces cerevisiae* it was found to be localised to the endoplasmic reticulum [35]. We initially expected to find the Ca^{2+}-ATPase in the same location, which is also its natural location in mammalian cells. As reported here, we found that the heterologously expressed Ca^{2+}-ATPase was localized in several membranes, including the plasma membrane of the yeast. In the case of H^+-ATPase from plant expressed in yeast, the C-terminal sequence was found to play an important role, resulting in the targetting of the heterologous protein to the endoplasmic reticulum [12]. By contrast, the N-terminal domain of the endogenous yeast H^+-ATPase is required for the functional insertion of the enyme into the plasma membrane [37]. It is a current challenge to understand the mechanism which directs a membrane protein to a given type of membrane (see e.g. [38]).

Using the pYeDP plasmid with GAL10 promoter, Ca^{2+}-ATPase is expressed in relatively low amounts (about 0.1% of the total proteins) and this may well be the reason why the yeast can tolerate the protein. Despite this low percentage, the total amount that can be produced is quite substantial since about 1 mg can be obtained per liter of culture. Moreover, the combination of GAL10 promoter and *sec* mutant enables us to purify secretory vesicles useful for functional studies. Immunopurification can be successfully applied but we are currently developing easier purification schemes such as those based on the addition of a C- or N-terminal poly-histidine cluster to the expressed protein [39].

Acknowledgements: We are grateful to Dr. D. Pompon for the gift of the yeast strain and vector, and to him and Drs. Ph. Urban and C. Cullin for their help in starting up the project. We also thank Dr. R. Serrano for the gift of anti-yeast H^+-ATPase antibody, and Dr. F. Kepes for the gift of the strain NY17 and stimulating discussions. We are grateful to Dr. M. Potier for irradiating our yeasts cells. We thank Drs. P. Champeil, A. Viel, F. Guillain and E. Mintz for helpful discussions. Finally, we thank A. Gomez de Gracia for her

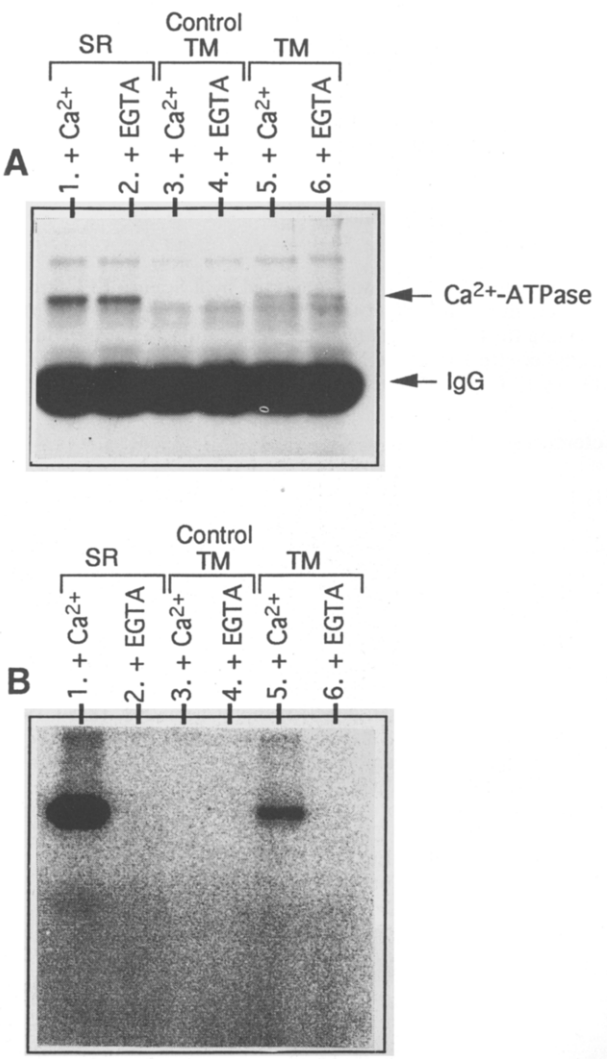

Fig. 3. Immunopurification of Ca^{2+}-ATPase (A) and phosphoenzyme formation (B). About 1 mg of total membrane from yeast, expressing or not SR Ca^{2+}-ATPase, or 10 mg of SR were solubilized by incubation in $C_{12}E_8$/azolectin in a ratio of 5:1. Solubilization was performed for 1 h at 4°C at a ratio of total protein to $C_{12}E_8$ of 1:5. After immunoprecipitation [8], the complexes were resuspended in a buffer containing 2 μM [γ-^{32}P]ATP and either 100 μM $CaCl_2$ or 2 mM EGTA and loaded on SDS-PAGE (see section 2). The gel was Coomassie blue-stained (A) and the radioactivity revealed using a PhosphorImager (B).

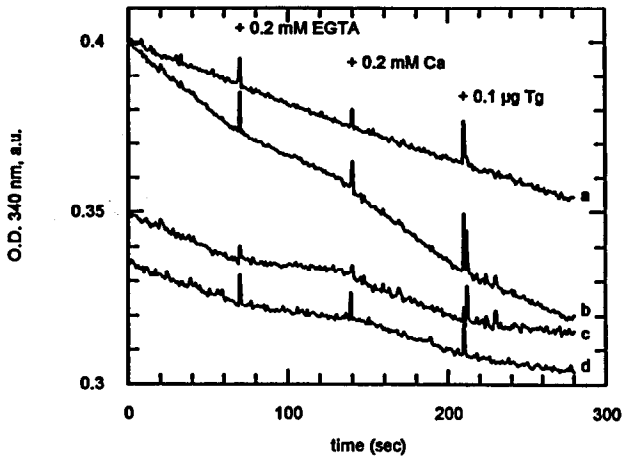

Fig. 4. ATPase activity of yeast secretory vesicles and sarcoplasmic reticulum. Experimental conditions were described in section 2. The different traces correspond to: (a) 50 µg of secretory vesicles fraction purified from control yeast; (b) 50 µg of secretory vesicles fraction purified from yeast expressing the Ca^{2+}-ATPase. This corresponds to 320 ± 60 ng of Ca^{2+}-ATPase expressed, as estimated by quantitative Western blotting; (c) is the difference between (b) and (a) +0.35; the arbitrary addition of 0.35 allows a direct comparison in the ordinate; (d) 320 ng of Ca^{2+}-ATPase from sarcoplasmic reticulum. At times indicated by a pulse, 0.2 mM EGTA, 0.2 mM $CaCl_2$ and 0.1 µg thapsigargin were successively added.

technical help. This work was supported by a Science EEC program grant (contract ERB SC1*-CT92-0783), a MRT and a MEC/MRT fellowship (to F.C.), grants from the Association Française contre les Myopathies (to Drs. M. le M. and S.D.) and in part (to M.J.M. and Y.D.) by EEC Grant BIO2-CT93-0422.

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