FEBS Letters 577 (2004) 322–326 FEBS 28954

Ycf1-dependent cadmium detoxification by yeast requires phosphorylation of residues Ser^{908} and Thr^{911}

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Received 10 August 2004; revised 8 October 2004; accepted 11 October 2004

Available online 21 October 2004

Edited by Ulf-Ingo Flügge

Abstract Yeast cadmium factor (Ycf1), an ATP-binding cassette (ABC) protein of the multidrug resistance protein subfamily, is a vacuolar GS-conjugate transporter required for heavy metal and drug detoxification. There is evidence that phosphorylation may play a critical role in the function of ABC transporters from higher organisms. In this work, the possibility of Ycf1 phosphorylation was examined using site-directed mutagenesis. We demonstrate that Ser^{908} and Thr^{911}, within the regulatory domain (R domain), are functionally important for Ycf1 transport activity and likely sites for phosphorylation. Mutation of these residues to alanine severely impaired the Ycf1-dependent cadmium detoxification capacity and transport activity, while replacement by acidic residues (mimicking phosphorylation) significantly suppressed the cadmium resistance and transport defects. Both in vitro treatment of Ycf1 with alkaline phosphatase and changes in the electrophoretic mobility of the S908A, T911A and double mutant S908A/T911A proteins supported the conclusion that Ycf1 is a phosphoprotein. The screening of the yeast kinome identified four protein kinases affecting cadmium detoxification, but none of them was involved directly in the phosphorylation of Ycf1. Our data strongly implicate Ycf1 phosphorylation as a key determinant in cadmium resistance in yeast, a significant finding given that very little is known about phosphorylation of ABC transporters in yeast.

Keywords: Yeast; Cadmium detoxification; *YCF1*; ATP-binding cassette transporter; ATP-dependent vacuolar pump; Phosphorylation; Site-directed mutagenesis

1. Introduction

Yeast cells have developed a variety of detoxification mechanisms to eliminate toxic compounds. In these mechanisms, glutathione *S*-conjugate (GS-X) pumps, belonging to the family of ATP-binding cassette (ABC) transporters, play an essential role. In *Saccharomyces cerevisiae*, two proteins, yeast cadmium factor (Ycf1) [1] and Bpt1 [2], have been shown to be GS-X pumps that catalyze the transport of glutathione conjugates into the vacuole [3,4]. Through the action of these two transporters, cadmium ions, complexed to GSH, are removed from the cytosol and transported into the vacuole.

Yeast ABC proteins have been classified into six subfamilies on the basis of phylogenetic analysis and sequence comparisons [5–7]. Ycf1p belongs to one of these, the multidrug resistance protein (MRP) subfamily, together with Bpt1, Ybt1 [8], Vmr1p (Wawrzycka, D., unpublished), all of them showing vacuolar membrane localization, Yor1 [9], located at the plasma membrane, and Nft1, recently shown to code for a new MRP-type transporter [10].

Members of the MRP subfamily of transporters show two features that distinguish them from other ABC proteins. First, all yeast MRPs except for Yor1 show an approximately 200 amino acid residue N-terminal extension. In the case of Ycf1, this N-terminal region is proteolytically processed in a Pep4-dependent manner [11], and is required both for vacuolar trafficking and transport activity [12], providing a convenient marker for its vacuolar localization. The second feature, common to the MRP and cystic fibrosis transmembrane conductance regulator (CFTR) subfamilies, is the presence of an additional hydrophilic domain that, because of a limited but significant homology with the regulatory domain of CFTR (R domain), has been designated CFTR-like "regulatory" domain or R domain. The regulatory significance of the R domain in CFTR is well established and several serine residues within it have been shown to be required for the channel activation by cAMP-dependent protein kinase (PKA) [13]. Ycf1 and its close homolog Bpt1 show the dibasic consensus sequence R[R/K]X[S/T] in the R domain, corresponding to RRAS^{908} in Ycf1 and RRAS^{931} in Bpt1. On the basis of the cadmium sensitivity of a *S908A* mutant of *YCF1*, it was proposed that Ser^{908} could be a site of PKA phosphorylation [1]. In the present work, we have studied the transport activity of a S908A Ycf1 mutant protein and have examined the role of PKA.

Our results demonstrate that the phosphorylation of the Ser^{908} residue is necessary for Ycf1 transport activity of glutathione conjugates into vacuolar membrane vesicles and suggest that PKA is not the kinase responsible for this phosphorylation. Furthermore, the results presented here show that Thr^{911} phosphorylation is also required for cadmium detoxi-

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Abbreviations: Ycf1, yeast cadmium factor; MRP, multidrug resistance protein; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; PKA, cAMP-dependent protein kinase; R domain, regulatory domain; GS-X, glutathione *S*-conjugate; LTC_4, glutathione *S*-conjugated leukotriene C_4; MIC, minimal inhibitory concentration; HA, hemagglutinin

0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.030

fication and transport activity by Ycf1, thus pointing to the involvement of still another kinase in the regulation of the Ycf1 protein by phosphorylation. Efforts aimed at the identification of the protein kinases involved in Ycf1 phosphorylation are also discussed.

2. Materials and methods

2.1. Yeast strains and growth media

The strains used are Δ*ycf1* derivatives of the *S. cerevisiae* strain W303-1A (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ycf1:: URA3*), described in [14], except for experiments with the *pka* mutant where strains S7-7A (*MATa his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1*) and S18-1D (S7-7A *tpk1^{w1}*) were used [15]. The EUROSCARF mutant strains were constructed in the BY4741 strain background. Yeast were grown at 30 °C on glucose minimal medium (SD) containing 6.7 g/l yeast nitrogen base without amino acids and 2% glucose. SD medium was supplemented with the appropriate requirements (added at 0.1 mg/ml). Solid media for Cd^{2+} resistance assays contained 2% agar and was supplemented with drop-out complete mix (SC).

2.2. Site-directed mutagenesis

Point mutations were constructed by one-step PCR, using the Quickchange II site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. A 2.1 kb *YCF1 Stu*I–*Sal*I fragment was cloned into pSL301 (Invitrogen) and mutagenic oligonucleotides were designed to create the desired changes. All *YCF1* mutant fragments subjected to mutagenesis were sequenced and used as a source to exchange the *Stu*I–*Sal*I fragment with the wild-type fragment. The plasmids used for the exchange, pRS315 [16] and pRS425 [17] carrying a hemagglutinin (HA)-tagged version of *YCF1*, have been described [14]. All plasmids were sequenced to verify the exchange. The resultant plasmids, containing either wild-type or mutant *YCF1*, were transformed into the appropriate strain.

2.3. Cadmium resistance assays

Qualitative and quantitative determinations of Cd^{2+}resistance were performed essentially as described [14]. Cells were cultured for two days in solid medium, suspended in water to an A_{660} nm of 0.2, dropped on plates without or with $CdCl_2$, and growth scored after three days. Determination of the minimal inhibitory concentration (MIC) was performed in microtiter plates containing medium with $CdCl_2$ concentrations ranging from 0 to 1 mM, inoculated to a cell density of 6×10^5 cells/ml. The optical density of each well was determined after two days. Data were fitted to a sigmoidal dose–response equation using GraphPad Prism 4.0 Software. The MIC value is defined as the lowest Cd^{2+} concentration at which prominent inhibition of cell growth (90–95%) is observed.

2.4. Measurement of [^{3}H]LTC_4 uptake

Standard uptake experiments were performed as described [18]. Briefly, uptake was measured at 30 °C in TS buffer (250 mM sucrose, 25 mM Tris–MES, pH 8.0) containing 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 20 units/ml creatine kinase, and 50 nM [^{3}H]LTC_4 (13 nCi/pmol) in a final volume of 55 μl. Uptake was initiated by the addition of vesicles (10 μg of protein). Aliquots (10 μl) were removed, diluted in 1 ml of ice-cold TS buffer, filtered through nitrocellulose filters (pore size 0.45 μm, Millipore, Bedford, MA) and washed twice with 5 ml of ice-cold TS buffer. The retained radioactivity was counted and initial rates were calculated from the first 1.5 min of uptake.

2.5. Vacuolar membrane preparation and immunoblot analysis

Vacuolar membrane-enriched fraction was prepared from spheroplasts lysates as described in [18]. For immunoblot analysis, upper gradient fractions of 230 μl were collected, subjected to trichloroacetic acid precipitation as in [19], the protein solubilized in sample buffer [20] for 15 min at 37 °C, resolved by 8% SDS–PAGE, transferred to nylon membranes and blots probed with the anti-HA monoclonal antibody. During alkaline phosphatase treatment, a mixture of protease inhibitors (1μg/ml of each aprotinin and leupeptin, 0.1μg/ml pepstatin and 1mM PMSF) was added.

3. Results and discussion

3.1. Ycf1 residue Ser^{908} is essential for cadmium resistance in vivo and transport activity in vitro

The predicted amino acid sequence of Ycf1 revealed a putative phosphorylation site, RRAS^{908} in the R domain, compatible with the consensus phosphorylation site of PKA. Mutation of the serine in this sequence to alanine, a non-phosphorylatable amino acid, was shown to negatively affect yeast Cd^{2+} resistance [1]. To further investigate the role of this putative phosphorylation site on the function of Ycf1, we generated by site-directed mutagenesis new mutants, in addition to S908A, in which Ser^{908} was replaced by either aspartate (S908D) or glutamate (S908E). The latter residues could potentially mimic the phosphorylated serine, due to the negatively charged carboxyl group. The functionality of the mutant proteins was tested in vivo by assessing their capacity to allow growth in a Cd^{2+} containing medium. The ability of a S908T mutant protein to detoxify Cd^{2+} was also analyzed. This mutation, Ser^{908} to the phosphorylatable threonine, was found among the revertants isolated during a search for intragenic suppressors of the S908A mutation (Eraso et al., unpublished results). The Cd^{2+} resistance assays (Fig. 1A) showed that the Ser^{908} to Ala change strongly impairs the sensitivity of the mutant strain, while mutation to threonine, aspartate or glutamate produced Ycf1 variants displaying MIC values close to that of the wild-type protein. These data show that Ser^{908} is an important residue for Ycf1 biological activity and that its function can be mimicked by negatively charged amino acids, suggesting that Ycf1 may be phosphorylated at position 908. The fact that a mutant carrying a S908T change was selected as a suppressor of the defect of the S908A mutation further supports the conclusion.

Ycf1 activity can be measured in vitro by its capacity to transport the GS-conjugated leukotriene C_4 (LTC_4) [14]. To assess the effect of the changes in Ser^{908} on the activity of Ycf1, we measured [H^3]LTC_4 uptake into vacuolar membrane vesicles prepared from a Δ*ycf1* strain expressing either wild-type Ycf1 or the different mutated versions. The results (Fig. 1A) showed that [H^3]LTC_4 uptake was strongly reduced by the S908A mutation. Thus, the decreased in vivo tolerance to Cd^{2+} ions shown by the mutant is paralleled by a decreased activity of the transporter in vitro. Determination of the Ycf1 transport activity of the variants S908T, S908E and S908D indicated that both the introduction of a negative charge or the replacement by threonine were able to partially rescue the deleterious effect of the change to alanine.

The Ser^{908} mutations could affect the biogenesis of the protein, its sorting to the vacuolar membrane, or its stability, thus contributing to the observed effects in cadmium resistance and transport activity. The Ycf1 protein has been shown to suffer a proteolytic cleavage of its N-terminal extension that is Pep4-dependent and can serve as a marker for proper vacuolar localization [11,12]. Comparison of the apparent molecular masses of the Ycf1 variants with that of the wild-type, in total membrane preparations, showed in each case that correct processing had occurred (data not shown), indicating correct vacuolar localization. While no significant changes in the amount of Ycf1 in vacuolar membrane preparations were found, the Western blots unexpectedly revealed the presence of forms of the protein with differing mobility

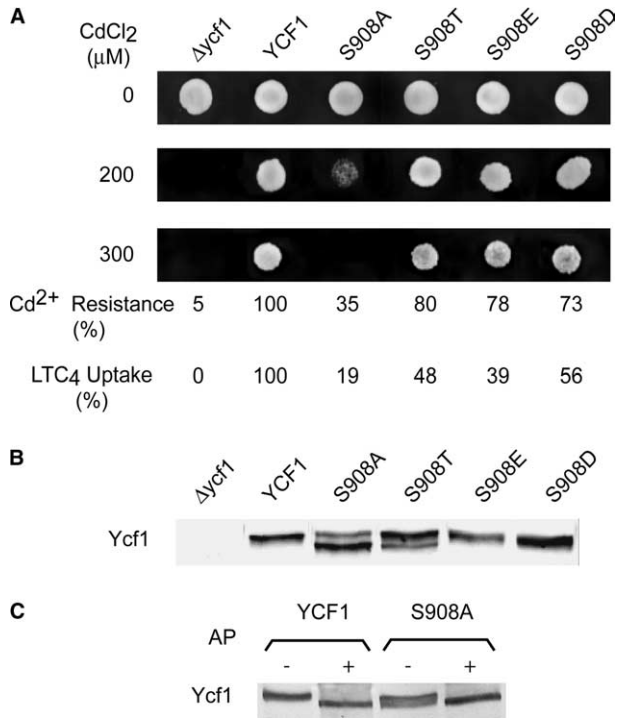

Fig. 1. Analysis of the activity and the electrophoretic mobility of wild-type and Ser^{908} mutants of Ycf1. (A) Cd^{2+} resistance and LTC_4 transport activity of Δ*ycf1* strains expressing the wild-type or the mutant forms of Ycf1. Δ*ycf1* strain was transformed with empty vector (Δycf1) or vector harbouring each of the indicated *YCF1-HA* alleles (YCF1, S908A, S908T, S908E and S908D). Each strain was dropped onto SC plates containing the indicated $CdCl_2$ concentrations, grown for three days and photographed. MIC measurement was performed as described (see Section 2) after growth on microtiter plates containing medium with increasing concentrations of $CdCl_2$. MIC value for wild-type Ycf1-HA was 763 μM. Vacuolar membrane vesicles prepared from the same strains were incubated with 50 nM [^{3}H]LTC_4 and 4 mM ATP (see Section 2) to determine relative rates of LTC_4 transport. MIC and [^{3}H]LTC_4 uptake values shown are the average of two independent experiments. (B) Immunodetection of Ycf1-HA in Δ*ycf1* strains expressing the wild-type or the mutant forms of Ycf1. 10 μg of membrane protein from the indicated mutants was subjected to SDS–PAGE and immunodetected with anti-HA monoclonal antibody. (C) 5 μg of vacuolar membrane protein from wild-type and S908A was incubated in the absence (− lanes) or presence (+ lanes) of 20 U of alkaline phosphatase for 1 h at 37 °C in the presence of Laemmli solubilizer, subjected to SDS–PAGE and immunodetected with anti-HA.

(Fig. 1B). This observation is suggestive of wild-type Ycf1 being phosphorylated at Ser^{908} producing the slow mobility form. The introduction of alanine at this position may result in a dephosphorylated form of the protein with higher mobility. The double band pattern found for the S908A mutant protein points to the existence of a second modification of the protein. This second modification is presumably a phosphorylation as indicated by the fact that treatment of the protein samples with alkaline phosphatase before gel fractionation caused the conversion of the S908A slow migrating forms to a unique and more rapidly migrating band (Fig. 1C).

Altogether, these data support the conclusion that Ser^{908} is phosphorylated in vivo and that the phosphorylation of this residue is essential for full activity of Ycf1.

3.2. Identification of a second Ycf1 phosphorylation site

The double band pattern and its elimination by alkaline phosphatase treatment suggested the existence of a second modification of the protein. In addition, a match exists between the sequence in Ycf1 $RRAS^{908}(P)DAT^{911}$ and the casein kinase 1α (CK1α) type II consensus phosphorylation site S/T(P)X_n**S/T(P)**, where prior phosphorylation at a serine or threonine residue N-terminal to the CK1α site is required [21]. *S. cerevisiae* have four casein kinase I (CKI) homologs and one of them, Yck3, has been recently shown to localize to the vacuolar membrane [22], making it a candidate for the phosphorylation of Ycf1. These observations prompted us to perform new site-directed mutagenesis of *YCF1* generating four alleles: *T911A*, *T911D*, *S908AT911A* and *S908DT911D*. Plasmids carrying these new *YCF1* alleles were transformed into the Δ*ycf1* strain and the resultant transformants tested for growth in Cd^{2+} containing medium. As shown in Fig. 2A, the Thr^{911} to Ala change decreases Cd^{2+} resistance, although to a lesser extent than the Ser^{908} mutation, and this decrease is reverted when Thr^{911} is replaced by an aspartyl residue. Among all the variants tested, the mutant carrying the *S908AT911A* allele exhibited the lowest cadmium resistance, while the introduction of aspartyl residues at both 908 and 911 Ycf1 positions reverted the effect of the double mutation to alanine. To assess the effect of the changes in Thr^{911} on the activity of Ycf1, we measured [H^3]LTC_4 uptake into vacuolar membrane vesicles prepared from a Δ*ycf1* strain expressing the wild-type Ycf1 or the different versions carrying single changes at position 911 or double changes at Ser^{908} and Thr^{911}. The results (Fig. 2A) showed that [H^3]LTC_4 uptake was strongly reduced by the T911A mutation and that this decrease was partially reverted by the introduction of an aspartyl residue. Finally, the double mutation of Ser^{908} and Thr^{911} to Ala renders a mutant protein with extremely low transport activity, while the introduction of aspartyl residues at these two positions allows for the recovery of the transport activity. These mutations did not affect protein levels or proper localization at the vacuolar membrane (Fig. 2B). Interestingly, the change at position 911 altered the electrophoretic mobility of the Ycf1 protein as the mutation at Ser^{908} did. T911A-Ycf1 migrated as a doublet, and, as it can be seen in the immunoblot, the main band is in this case the upper one. The behavior of the T911A mutant protein with respect to Cd^{2+} detoxification, [H^3]LTC_4 transport activity and in gel mobility strongly indicates that Thr^{911} is phosphorylated in vivo and that this phosphorylation affects the activity of the transporter. Moreover, the low detoxification capacity and transport activity conferred by the S908A/T911A mutant protein, and its electrophoretic migration as a single band, reminiscent of the alkaline phosphatase treated wild-type protein, points to these two Ycf1 residues, 908 and 911, as being the sites whose phosphorylation contributes to full activity of the transporter and to the band pattern observed in the immunoblots.

3.3. Screening of the complete Saccharomyces cerevisiae collection of protein kinase viable deletion mutants

To identify the protein kinase/s potentially involved in the regulation of cadmium detoxification, we took advantage of the EUROSCARF collection of yeast viable deletion mutants constructed during the systematic gene deletion project. As pointed out before, the primary sequence surrounding Ser^{908} in Ycf1 is compatible with the consensus phosphorylation site of

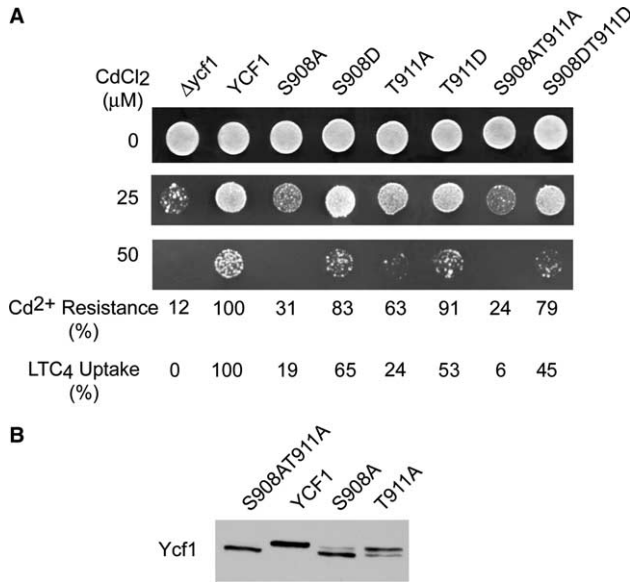

Fig. 2. Analysis of the activity and the electrophoretic mobility of wild-type and Ser^{908} and Thr^{911} single and double mutants. (A) Cd^{2+} resistance and LTC_4 transport activity of Δ*ycf1* strains expressing the wild-type or the mutant forms of Ycf1. Δ*ycf1* was transformed with empty vector (Δycf1) or with vector harboring each of the indicated *YCF1-HA* alleles (YCF1, S908A, S908D, T911A, T911D, S908AT911A and S908DT911D). Each strain was dropped onto SC plates with increasing $CdCl_2$ concentrations, grown for three days and photographed. MIC measurement was performed as described (see Section 2) after growth on microtiter plates containing medium with increasing concentrations of $CdCl_2$. Vacuolar membrane vesicles prepared from the same strains were incubated with 50 nM [^{3}H]LTC_4 and 4 mM ATP (see Section 2) to determine relative rates of LTC_4 transport. MIC and [^{3}H]LTC_4 uptake values shown are the average of two independent experiments. (B) Immunodetection of Ycf1-HA in Δ*ycf1* strains expressing the wild-type or the mutant forms of Ycf1. 10 μg of vacuolar membrane protein from the indicated mutants was subjected to SDS–PAGE and immunodetected with anti-HA monoclonal antibody.

PKA. Therefore, prior to the screening, it was important to elucidate the implication, if any, of PKA. Since *S. cerevisiae* has three A kinase catalytic subunits encoded in the *TPK* genes, with greater than 75% identity and redundant for cell viability, analysis of single mutants would not be informative. To analyze whether phosphorylation by PKA had any effect on Ycf1 activity, we sought to correlate the in vivo activity of PKA with the sensitivity to Cd^{2+} ions. To this end, we used two strains containing either a wild-type *TPK1* allele (strain S7-7A) or an attenuated *TPK1* allele, *tpk1^{w1}* (strain S18-1D), encoding the sole PKA catalytic subunit. The latter strain is viable and exhibits undetectable PKA activity [15]. Both strains were disrupted for *YCF1* and transformed with plasmids carrying either wild-type *YCF1* or the *S908A* mutant. We reasoned that if PKA was the kinase phosphorylating Ser^{908}, we would find growth of the attenuated strain in Cd^{2+} containing medium to be independent of the S908A mutation. We found, however, that although growth of the attenuated strain was more affected by increasing Cd^{2+} concentrations than that of the control strain, it responded to the presence of the different *YCF1* alleles in the same way as the wild-type strain did (Fig. 3A). In addition, no change in the electrophoretic mobility of Ycf1 was observed in the *tpk1^{w1}* strain. These results suggest that PKA is not the kinase involved in Ycf1 phosphorylation at position 908. In this regard, it is worth mentioning that upon examination of the mapped phosphorylation sites in several of the yeast PKA substrates, none of them shows an acidic residue at position +1 of the phosphorylated serine, as would be the case in the Ycf1 sequence ($RRAS^{908}D$). Proteins like FbPase, Adr1, Pyk1, Cki1, Ura7, Opi1 and Sso1, with well characterized PKA sites, exhibit hydrophobic residues at this position in most cases. An effect of PKA on *YCF1* gene expression was discarded based on the finding that comparable amounts of Ycf1 were found in Western blots performed with vacuolar membranes obtained from both strains (Fig. 3B).

The complete collection of viable protein kinase mutants was then checked for cadmium tolerance. We found that four protein kinase mutants, deleted for *YIL042c*, *YPL150w*, *YNR047w* and *YNL307c*/*MCK1*, respectively, but not the *YCK3* mutant, exhibited altered cadmium sensitivity (data not shown). If any of the deleted kinases was responsible for phosphorylating at either Ser^{908} or Thr^{911} in Ycf1, we should find that the low ability to detoxify cadmium ions would be paralelled by an increase in the electrophoretic mobility of Ycf1 and the appearance of the characteristic double-band pattern in the immunoblots. The results showed, however, that in each mutant, Ycf1 mobility was indistinguishable from that in the wild-type strain background (data not shown), indicating that none of the protein kinases selected in the screening is responsible for the phosphorylation of Ycf1 at Ser^{908} or Thr^{911}.

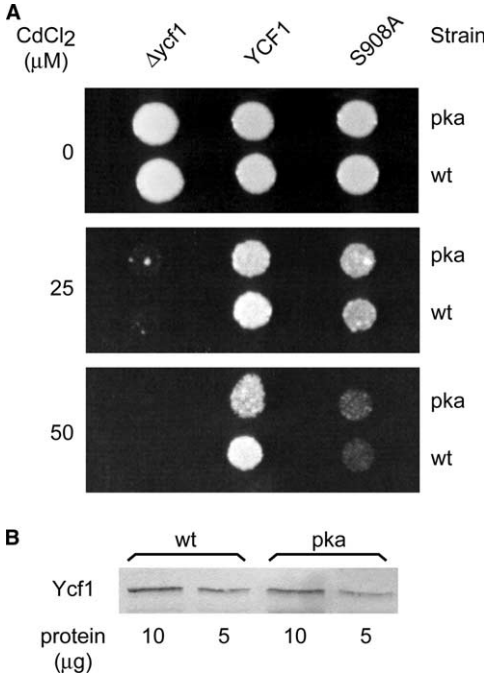

Fig. 3. Analysis of the activity and the electrophoretic mobility of wild-type and S908A mutant Ycf1 in wild-type and *pka* attenuated strains. (A) Cd^{2+} resistance of wild-type and *tpk1^{w1}* strains expressing the wild-type or the S908A mutant forms of Ycf1. Strains S7/7A and S18/1D were transformed with empty vector (Δycf1) or with vector harboring the indicated *YCF1-HA* alleles (YCF1 and S908A). Each strain was dropped onto SC plates containing the indicated $CdCl_2$ concentrations, grown for three days and photographed. (B) Immunodetection of Ycf1-HA in wild-type and *tpk1^{w1}* strains expressing the wild-type or the S908A mutant forms of Ycf1. 5 and 10 μg of vacuolar membrane protein from the indicated mutants were subjected to SDS–PAGE and immunodetected with anti-HA monoclonal antibody.

The fact that screening the yeast kinome did not allow the identification of the kinases involved in Ycf1 phosphorylation suggests that either the kinases are redundant in function or they are encoded in essential genes.

In summary, our analysis shows that two residues, Ser^{908} and Thr^{911}, located in the regulatory domain of Ycf1, are phosphorylated in vivo. The results presented here clearly indicate that Ycf1 phosphorylation at both positions is required for the biological activity of the transporter.

Acknowledgements: We thank Eulalia Morgado for her skillful help with membrane preparations and immunoblots; Dr. Paula Portela for kindly supplying the strains S7-7A and S18-1D; and Prof. Carlos Gancedo for helpful comments on the manuscript. This work was supported by funding from the Dirección General de Investigación (BMC2001-1517).

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