

Yeast protein kinase Ptk2 localizes at the plasma membrane and phosphorylates in vitro the C-terminal peptide of the H⁺-ATPase

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Abstract

Glucose triggers posttranslational modifications that increase the activity of the *Saccharomyces cerevisiae* plasma membrane H⁺-ATPase (Pma1). Glucose activation of yeast H⁺-ATPase results from the change in two kinetic parameters: an increase in the affinity of the enzyme for ATP, depending on Ser⁸⁹⁹, and an increase in the V_{\max} involving Thr⁹¹². Our previous studies suggested that Ptk2 mediates the Ser⁸⁹⁹-dependent part of the activation. In this study we find that Ptk2 localized to the plasma membrane in a Triton X-100 insoluble fraction. In vitro phosphorylation assays using a recombinant GST-fusion protein comprising 30 C-terminal amino acids of Pma1 suggest that Ser⁸⁹⁹ is phosphorylated by Ptk2. Furthermore, we show that the Ptk2 carboxyl terminus is essential for glucose-dependent Pma1 activation and for the phosphorylation of Ser⁸⁹⁹.

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1. Introduction

The plasma membrane H⁺-ATPase of yeast, encoded by the *PM1* gene [1], is a predominant membrane protein that belongs to the family of P-type cation pumps [2]. Pma1 is a polytopic cell surface protein which generates the electrochemical proton gradient essential for nutrient uptake by secondary active transport [3,4].

Because of the central role of this enzyme in fungal physiology, its activity is finely regulated by a large number of environmental factors to match ATPase activity to the physiological requirements of the cell. Glucose, the external signal more extensively studied in yeast, controls the H⁺-ATPase at two levels [5]. First, glucose metabolism induces a moderate (2- to 4-fold) and slow (4 h) increase in *PM1* gene expression, mediated by the transcription factors Rap1 and Gcr1 [6–8]. Second, glucose triggers a rapid (5 min) and strong (5- to 10-fold) increase in ATPase catalytic activity [9].

The glucose activation of the yeast H⁺-ATPase results from a combined effect on the kinetic parameters of the enzyme, including a K_m decrease and a V_{\max} increase [9]. The mechanism proposed to explain this posttranslational regulation is similar in yeast and plants: activators overcome the negative regulation exerted by a regulatory domain located at the carboxyl-terminus [10]. In *S. cerevisiae*, mutations in this domain affecting Ser⁸⁹⁹ or Thr⁹¹², abolish the glucose-dependent K_m or V_{\max} change respectively [11].

Although the machinery mediating the activation of yeast H⁺-ATPase is not well understood, there are preliminary evidences pointing to phosphorylation of the inhibitory domain as part of the activating mechanism. Introduction of a non-phosphorylatable residue at position 899 abolishes the glucose-dependent K_m decrease and, by contrast, introduction of a negatively charged residue at this position leads to an enzyme with a K_m similar to that of the wild type activated enzyme, suggesting that Ser⁸⁹⁹ may be phosphorylated during activation [10].

Ptk2, a protein kinase of the NPR/HAL5 family [12], was initially isolated as a kinase regulating polyamine transport [13,14]. More recently, in a systematic analysis of protein kinase mutants phenotype, it was identified as a protein kinase

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which mediates glucose activation of the plasma membrane H^+ -ATPase [15,16]. Mutant strains deleted of *PTK2* are defective in the glucose-induced Ser^{899} -dependent K_m decrease of H^+ -ATPase and this phenotype is suppressed by the expression of a *PMA1* allele carrying a S899D mutation suggesting a glucose-dependent phosphorylation of Ser^{899} , which could be mediated, directly or indirectly, by Ptk2 [15].

In this report, we show that Ptk2 is located at the plasma membrane and provide biochemical evidence, based on in vitro phosphorylation assays, which suggests that Ptk2 phosphorylates the yeast H^+ -ATPase at Ser^{899} during glucose activation. We also show that the carboxyl-terminus of Ptk2 is required for this process.

2. Materials and methods

2.1. Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. Standard methods for yeast transformation, culture and manipulation were used [17]. To test the tolerance to Hygromycin B, the different strains were grown during 48 h in liquid synthetic minimal medium (SD) containing 0.67% Yeast Nitrogen Base w/o amino acids (USBiologicals), 2% glucose and the appropriate requirements. Cultures were serially diluted in water (10-, 100-, and 1000-fold) and about 3 μ l were dropped on rich medium agar plates (YPD) supplemented with Hygromycin B. Large scale production of recombinant GST-fusion proteins in yeast were performed as described [18].

2.2. Ptk2 antibodies

Antibodies directed against Ptk2 were purchased from EUROGENTEC S. A. (Herstal, BE). A 13-mer peptide (GNGRSLRKRPTSP) corresponding to amino acids 46–58 of Ptk2 was coupled to keyhole limpet hemacyanin and used to immunize two rabbits. Antibodies were purified by affinity chromatography.

2.3. Subcellular fractionation, membrane isolation and extraction

Yeast FY1679-08A was grown in YPD medium to mid-exponential phase ($OD_{660}=0.6-0.7$), pelleted by centrifugation and lysed by vortexing with glass beads in TEI buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA plus 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail) [19]. The homogenate was cleared from intact cells by centrifugation for 10 min at $300\times g$ (Beckman JA-20 rotor). The supernatant (S3) was centrifuged for 60 min at $100,000\times g$ (Beckman 60 Ti rotor). Aliquots of the supernatants (S100), pellets (P100) and total extracts (S3) were saved for immunoblot analysis.

Table 1
Genotype of yeast strains used in this study

Strain ^a	Relevant genotype	Reference ^b
FY1679-08A	<i>Mata ura3-52 leu2Δ1 trp1 Δ63 his3 Δ200</i>	EUROSCARF
FENS018-06c	<i>ptk2::KanMX4</i>	EUROSCARF
FPY1926	<i>ptk2::YIp352::PTK2</i>	
FPY2014	<i>ptk2::YIp352::ptk2Δ811</i>	
FPY2015	<i>ptk2::YIp352::ptk2Δ801</i>	
FPY2016	<i>ptk2::YIp352::ptk2Δ791</i>	
FPY2252	<i>ptk2::YIp352::ptk2Δ461-483</i>	
FPY2187	FY1679-08A [pEG(KT)::C1]	
FPY2251	FY1679-08A[pEG(KT)::C1S899D]	

^a All the strains are isogenic to FY1679-08A.

^b Unless otherwise indicated, the strains are from this study.

Subcellular localization of Ptk2 was performed as described [20]. Briefly, total membranes were suspended in 10% (w/w) sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol plus protease inhibitor cocktail and layered on top of a continuous gradient composed of 20–53% (w/w) sucrose in the same buffer. After centrifugation for 16 h at 30,000 rpm (Beckman SW 40Ti rotor) fractions of 0.75 ml were manually collected from top to bottom of the gradient. The fractionated membranes were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Immobilon P, Millipore) for immunodetection using rabbit polyclonal anti-Ptk2, mouse monoclonal anti-Pma1 [21], anti-Vph1 or anti-Dpm1 (Molecular Probes). Immunodetection was performed using the ECL Western blot analysis system (Amersham Biosciences) according to the manufacturer's protocol.

To analyze the nature of Ptk2 membrane association, plasma membranes were purified by differential and sucrose gradient centrifugation [19] and incubated on ice for 30 min in PBS, 1 M NaCl, 0.1 M $NaCO_3$ (pH 11.0), 5 M Urea or 1% Triton X-100 and centrifuged for 30 min at $14,000\times g$ (Beckman TLA100 rotor). Fractions of the pellet (P) and supernatant (S) were analyzed by Western blot analysis using rabbit polyclonal anti-Ptk2 and mouse monoclonal anti-Pma1 [21].

2.4. Fluorescence microscopy

Yeast cells were fixed as described by Pringle et al. [22] and permeabilized in PBS containing 0.1% Triton X-100 [23]. For immunofluorescence staining, polyclonal antibodies anti-Ptk2 and anti-Pma1 [1] were used as primary antibodies followed by FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Images were obtained with a Zeiss Axiofot microscope and further processed with Adobe Photoshop 7.0.

2.5. Plasmids

The yeast expression vector pEG(KT) [18] was used to express the carboxyl-terminus fragments of Pma1 fused to GST. Fragments of the different *PMA1* alleles were amplified by PCR and introduced into the plasmid by "gap repair". The wild type (fragment C1) or *pma1*-S899D (fragment C1-S899D) alleles of *PMA1* were used as DNA template [10,11]. The wild type C1 and mutant C1-S899D GST-tagged fragments contained amino acids 880–910 of Pma1. PCR fragments used for gap repair were generated using chimeric primers consisting of 30 bases homologous to the recipient plasmid and 17 bases, at the 3'-end, homologous to the desired sequence in the templates. Approximately 0.5 μ g of the PCR products and 10 ng of pEG(KT), linearized by digestion with *Sma*I and *Sal*I, were used to transform yeast cells to Ura^+ . Each recombinant plasmid was verified by sequencing. GST fusion proteins were produced and purified from yeast as described [18].

Ptk2 carboxyl terminus deletions were made by introducing termination codons by site directed mutagenesis. To this end, a 1.8-kb *Eco*RI–*Bam*HI fragment of the *PTK2* gene was subcloned into the yeast integrative plasmid YIp352 [24] and point mutations within the *PTK2* sequence were introduced by one-step PCR using the Quick-change II site directed mutagenesis kit (Stratagene). Fragments subjected to site-directed mutagenesis were sequenced completely to discard undesirable mutations. After mutagenesis, each mutagenized plasmid was linearized with *Kpn*I and used to transform the wild type FY1679-08A strain. Integration of the mutant constructs at the *PTK2* locus was confirmed by PCR and sequencing.

2.6. Enzyme assays and protein determination

To purify plasma membranes for the in vitro kinase assays, yeast were grown to mid-log phase ($OD_{660}=0.5$), harvested by centrifugation and suspended in water at 20 mg wet weight/ml. Cells were incubated with 100 mM glucose (PM-GF) or 0.9 M sorbitol (PM-GS) during 10 min and frozen in liquid nitrogen. Total yeast proteins were extracted by glass beads lysis in TEI buffer containing a cocktail of protein phosphatase inhibitors (20 mM NaF, 40 mM β -glycerolphosphate and 2 mM vanadate). Plasma membranes were purified by differential and sucrose gradient centrifugation and homogenized in kinase buffer (20 mM HEPES, pH 7.5, 2 mM DTT, 20 mM β -glycerolphosphate, 0.1 mM vanadate and 20 mM $MgCl_2$). Kinase reaction contained in a final

volume of 50 μ l: 50 μ g of recombinant GST fusion protein attached to glutathione sepharose beads (Amersham Biosciences), 5 μ g of purified plasma membranes, 20 μ M unlabeled ATP, 10 μ Ci of γ - 32 P-labeled ATP, in kinase buffer. After 30 min at 30 $^{\circ}$ C Sepharose beads were sedimented and washed three times with PBS and labeled GST fusion proteins were resolved by SDS-PAGE. GST-tagged C-terminal fragments of Pma1 were probed by immunoblot with an anti-Pma1 C-terminal polyclonal antibody [25].

ATPase activity from glucose-starved and glucose-fermenting cells was assayed in purified plasma membranes at pH 6.5 with 2 mM ATP [9,19] except for K_m determination where ATP concentrations ranging from 0.8 to 6 mM and an excess Mg^{2+} over ATP of 5 mM were used. Protein concentration was determined by the Bradford method [26] with the Bio-Rad Protein Assay reagent (Bio-Rad) and bovine IgG as standard.

3. Results

3.1. Subcellular localization of Ptk2

To assist in the molecular analysis of Ptk2 function, we generated antibodies against the N-terminus of the enzyme. A 13-residue synthetic peptide, corresponding to amino acids 46–58 of Ptk2 was synthesized, coupled to keyhole limpet hemacyanin and used to immunize rabbits. Antibodies that recognized the peptide were affinity-purified and used in Western blot experiments. Cell lysates from both wild type and *ptk2* Δ mutant strains were subjected to SDS gel electrophoresis and probed with anti-Ptk2 antibodies. Ptk2 is predicted to be a 91.4 kDa protein. As shown in Fig. 1, a prominent band of about 91 kDa was detected in extracts from wild type cells that was absent in *ptk2* Δ mutant cells indicating that anti-Ptk2 antibodies specifically recognize the Ptk2 protein. As a first step to localize Ptk2, cell fractionation experiments were done. A cell lysate from wild type cells was subjected to differential centrifugation and fractions were analyzed by immunoblotting using antibodies against Ptk2 or Pgk1 and Pma1 as markers for soluble and membrane proteins respectively. Ptk2 was found in low-

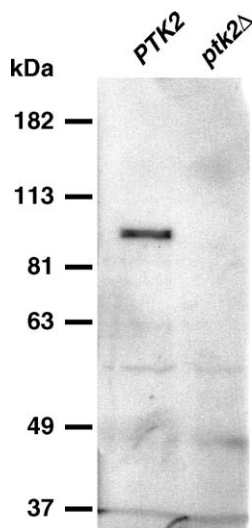


Fig. 1. Specificity of the Ptk2 antibodies. Protein extracts were prepared from wild type (*PTK2*) or mutant cells carrying a deletion of the *PTK2* gene (*ptk2* Δ). Aliquots of 20 μ g of protein from each extract were resolved on a SDS gel and probed with anti-Ptk2 polyclonal antibodies. The position and molecular weight of prestained standards are indicated on the left.

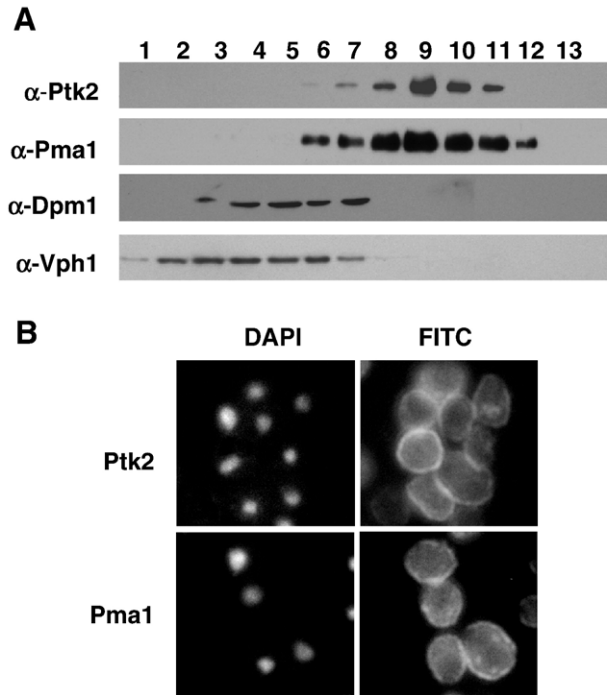


Fig. 2. Subcellular localization of Ptk2 determined by sucrose gradient centrifugation and immunofluorescence staining of yeast cells. (A) Total membranes were isolated from wild type yeast and separated in a linear sucrose gradient as described under Materials and methods. The gradient was fractionated from top (fraction 1) to bottom (fraction 13) and analyzed by immunoblot using anti-Ptk2 antibodies (α -Ptk2) or the antibodies listed on the left: α -Pma1 (plasma membrane), α -Dpm1 (endoplasmic reticulum), α -Vph1 (vacuolar membrane). (B) Wild type cells were processed for indirect immunofluorescence microscopy as described under Materials and methods. Panels show staining of the nucleus (DAPI) and corresponding fluorescence images (FITC).

speed (300 \times g) supernatant and high-speed (100,000 \times g) pellet fractions and was not detected in the 100,000 \times g supernatant, indicating its association with the membrane fraction (data not shown). To further identify the intracellular localization of Ptk2, total yeast membranes were fractionated by sucrose gradient centrifugation under isopycnic conditions. Fractions were subsequently analyzed by immunoblotting using antibodies against Ptk2 and marker proteins specific for the distinct membrane systems of the cell. The results shown in Fig. 2A suggest that Ptk2 is located at the plasma membrane. This result was confirmed by immunofluorescence detection of Ptk2 in formaldehyde-fixed yeast spheroplasts (Fig. 2B). The anti-Ptk2 antibodies decorated the cell periphery thus confirming that Ptk2 is a plasma membrane protein.

To determine the nature of the association of Ptk2 with the membrane fraction, we treated purified plasma membranes with different reagents prior to centrifugation at 14,000 \times g. The distribution of Ptk2 and the integral plasma membrane Pma1 between the pellet and soluble fractions was examined by Western blot analysis (Fig. 3). Similarly to Pma1, Ptk2 was not extracted into the supernatant fraction neither with the nonionic detergent Triton X-100 nor reagents that disrupt protein–protein interactions. This result suggests that Ptk2 is strongly associated to the plasma membrane. The presence of a predicted signal

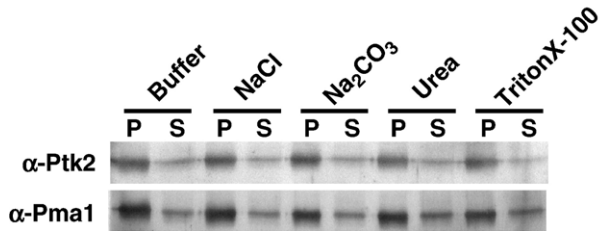


Fig. 3. Ptk2 fractionates as an intrinsic plasma membrane protein. Aliquots of 20 μ g of purified plasma membranes were treated with the indicated reagent for 30 min on ice and then centrifuged for 30 min at $14,000 \times g$. The resultant pellet (P) and supernatant (S) fractions were analyzed by Western blotting using anti-Ptk2 (α -Ptk2) or anti-Pma1 polyclonal antibodies (α -Pma1).

sequence (first 49 amino acids) and a putative transmembrane domain (from amino acid 461 to 483) in Ptk2 (“Saccharomyces Genome Database” <http://www.yeastgenome.org>) prompted us to test the possibility that Ptk2 is inserted into the plasma membrane using the predicted membrane spanning region. To test whether the predicted transmembrane domain acts as a membrane anchor, we proceeded to delete the protein segment encompassing the putative transmembrane domain (*ptk2* Δ 461–483) and examined the location of the resultant mutant protein. Cell lysates from wild type and *ptk2* Δ 461–483 mutant strains were subjected to differential centrifugation and fractions were analyzed by immunoblotting. Elimination of the predicted transmembrane domain did not abolish the membrane association of Ptk2 (data not shown). Taken together these results suggest that Ptk2 is an intrinsic plasma membrane protein although the exact nature of its membrane association is not clear.

3.2. In vitro phosphorylation of Pma1 carboxyl-terminus

Analysis of the phosphorylation of the full-length Pma1 by an individual protein kinase is complicated by the fact that the H^+ -ATPase is phosphorylated at numerous residues during its transport along the secretory pathway [27]. In addition, Pma1 is phosphorylated at Ser⁵⁰⁷ by the glucose-regulated casein kinase I, and this phosphorylation prevents glucose activation of the enzyme [28]. The putative phosphorylation of the inhibitory domain could therefore be masked by the presence of casein kinase. To overcome these difficulties we analyzed the ability of Ptk2 to phosphorylate in vitro a GST-tagged C-terminal fragment of Pma1 containing amino acids 880–910 (GST-C1). Ptk2 was expressed in yeast, as a fusion to GST, under the regulatable promoter GAL1, but every attempt to get a solubilized and active Ptk2 fraction was unsuccessful. The same result was obtained when Ptk2 was expressed in *E. coli* cells where the enzyme accumulated in inclusion bodies. Therefore, the recombinant C-terminal fragment of Pma1 was purified from yeast and incubated in the presence of [γ -³²P]ATP and plasma membranes purified from wild type or *ptk2* Δ strains grown on glucose. Phosphorylation of the GST-C1 fusion protein was only observed in the presence of plasma membranes purified from the wild type strain (Fig. 4A, lanes 1 and 2). The phosphorylation was dependent on the presence of Ser⁸⁹⁹ in the

Pma1 fragment since it was greatly diminished when the assay was performed using a C-terminal fragment in which Ser⁸⁹⁹ was replaced by an aspartate residue (GST-C1S899D) (Fig. 4A, lanes 3 and 4). The results presented so far point to Ptk2 as the protein kinase responsible for the phosphorylation of the regulatory site defined by Ser⁸⁹⁹. We then asked if this phosphorylation is influenced by the growth conditions of the

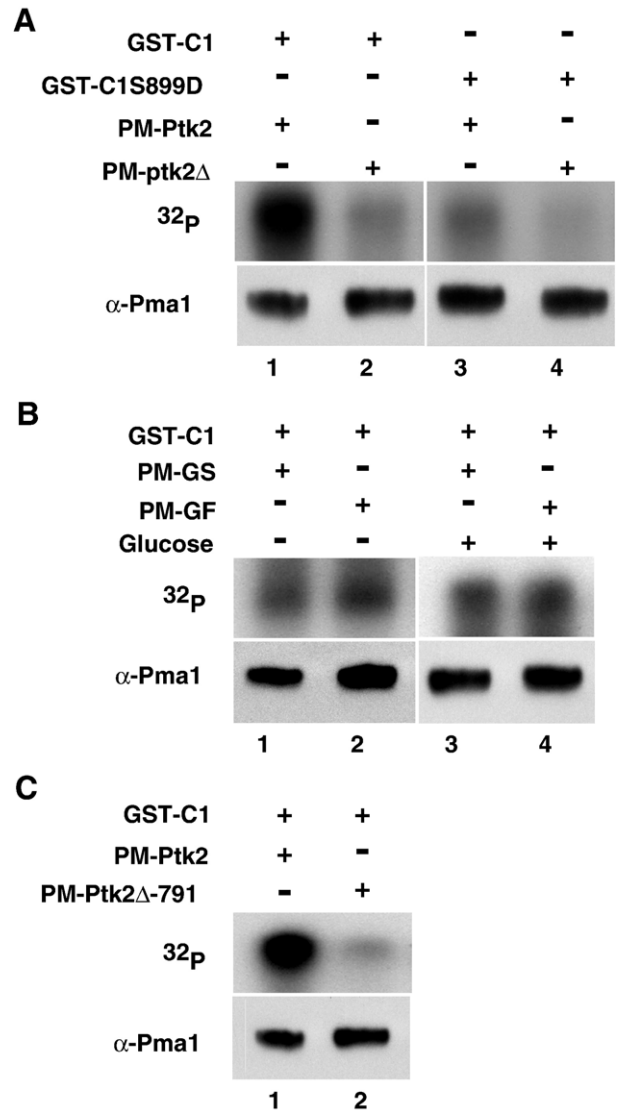


Fig. 4. In vitro phosphorylation of GST-tagged C-terminal fragments of Pma1. Recombinant GST-tagged proteins including Pma1 amino acids 890–910 (GST-C1) or a mutated version in which Ser⁸⁹⁹ was replaced by aspartic acid (GST-C1S899D) were produced and purified from yeast as described under Materials and methods. (A) Agarose-bound GST fusion proteins were incubated with plasma membrane purified from either wild type (PM-Ptk2) or *ptk2* Δ (PM-ptk2 Δ) mutant strains. (B) Purified plasma membranes from glucose starved (PM-GS) or glucose fermenting (PM-GF) wild type cells were incubated with recombinant GST-fusion protein (GST-C1) in the absence or presence of 25 mM glucose. (C) Recombinant GST-C1 fusion protein was incubated with purified plasma membranes from either wild type (PM-Ptk2) or *ptk2* Δ -791 (PM-ptk2 Δ -791) mutant strains growing on glucose. In all cases proteins were resolved by SDS-gel electrophoresis and the phosphorylated proteins visualized by autoradiography (³²P). GST-tagged C-terminal fragments of Pma1 were detected by immunoblot using anti-Pma1 C-terminal polyclonal antibodies (α -Pma1).

cells used to purify the plasma membranes. To answer this question we compared the activities of membranes purified from glucose-fermenting and glucose-starved wild type cells. In addition, we also tested the effect of the presence of glucose in the *in vitro* assays. As shown in Fig. 4B, the phosphorylating capacity was very similar in both membrane preparations (Fig. 4B, lanes 1 and 2) indicating that the protein kinase responsible for the phosphorylation of the C-terminal fragment of Pma1 is equally active under both growing conditions. The results also show that the activity is not altered when glucose is included in the assay (Fig. 4B, lanes 3 and 4).

3.3. The carboxyl terminus of Ptk2 is required for kinase activity

The catalytic domain of Ptk2 is located at the central part of the enzyme extending from amino acid 261 to 562. To identify additional regions of the enzyme, required for glucose activation of H⁺-ATPase, serial 10-residues deletions of the C-terminal moiety of the enzyme (from residues 818 to 771) were performed. To analyze the effect of the deletions on Ptk2 function, we exploited the fact that a nonfunctional Ptk2 renders the yeast cells resistant to Hygromycin B [15]. As shown in Fig. 5, deletion of the last 18 amino acids of the protein (*ptk2Δ-801*) had no consequences on the growth phenotype, but deletion of the last 28 amino acids (*ptk2Δ-791*) rendered a yeast strain resistant to Hygromycin B suggesting that the region encompassing residues 791 to 801 is required for Ptk2 to be active. To discard the possibility that deletions of the carboxyl-terminus alter the localization of the *ptk2* mutant proteins we examined the amount of protein in purified plasma membranes by immunoblot analysis and found similar levels of Ptk2 in all

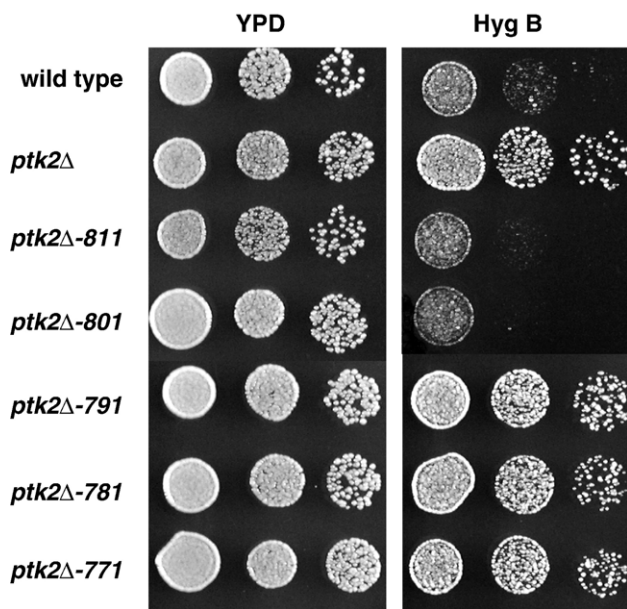


Fig. 5. Ptk2 carboxyl-terminus is essential for function. Wild type strain and *PTK2* mutants were grown in SD medium and serial dilutions were dropped on YPD plates with Hygromycin B (HygB, 50 μg/ml). Growth was scored after two days. Similar results were observed in two separate experiments.

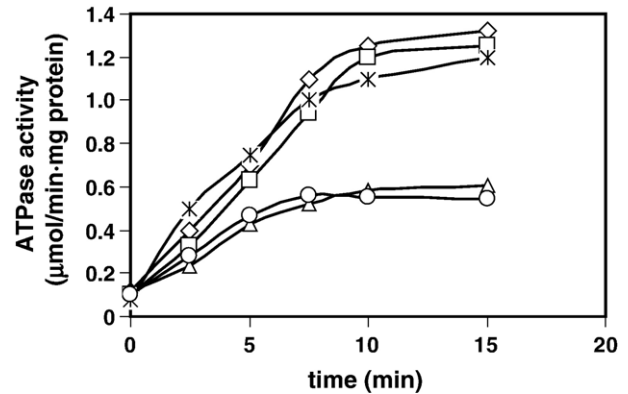


Fig. 6. ATPase activity in purified plasma membranes from wild type and *ptk2* mutant strains. Cells were grown in SD medium until mid-exponential phase ($OD_{660}=0.6-0.7$), pelleted by centrifugation and suspended in water. At time zero, glucose was added to wild type (◇), *ptk2Δ* (○), *ptk2Δ-811* (□), *ptk2Δ-801* (*) and *ptk2Δ-791* (Δ) mutant strains. Samples were taken at the indicated times and plasma membranes purified. The ATPase activity of the purified plasma membrane fraction was determined at pH 6.5 with 2 mM ATP. Values are the average of two independent experiments.

the strains (data not shown). To analyze whether *ptk2* deletions altered Pma1 regulation, we examined the activation of the ATPase by glucose in some of the mutant strains (Fig. 6). After 15-min incubation with glucose, a 12-fold increase in ATPase activity was observed in the wild type, *ptk2Δ-811* and *ptk2Δ-801* mutant strains. By contrast, under the same conditions glucose caused a 4-fold activation of the *ptk2Δ-791* mutant strain, activation similar to that observed in the yeast strain deleted of *PTK2*. Furthermore, we determined the kinetic properties of H⁺-ATPase in purified plasma membranes from the wild type or *ptk2Δ-791* mutant strains. As expected, in the case of *ptk2Δ* and *ptk2Δ-791* mutant strains only the Ptk2-independent V_{max} increase was produced while the typical Ptk2-dependent K_m decrease caused by glucose was not observed (Table 2). To analyze whether mutant Ptk2 was still able to phosphorylate Pma1 C-terminus, kinase assays were performed. Purified plasma membranes from *ptk2Δ-791* were unable to phosphorylate the GST-tagged fusion protein (Fig. 4C, lane 2) as opposed to the phosphorylation observed with plasma

Table 2

Kinetic parameters of ATPase from glucose-starved (GS) and glucose fermenting (GF) wild type and mutant cells

Strain	Genotype	GS		GF	
		K_m^a (mM)	V_{max}^a (μmol/min/mg)	K_m^a (mM)	V_{max}^a (μmol/min/mg)
FY1679-08A	wild type	4.5	0.5	1.2	1.6
FPY2014	<i>ptk2Δ</i>	5.0	0.5	4.0	1.5
FPY2015	<i>ptk2Δ811</i>	4.0	0.4	1.4	1.8
FPY2016	<i>ptk2Δ801</i>	4.0	0.6	1.2	1.5
FPY1933	<i>ptk2Δ791</i>	4.0	0.5	4.5	1.6

^a ATPase activity of purified plasma membranes was assayed at pH 6.5 with a range of Mg²⁺ and ATP concentrations from 0.8 to 6 mM and an excess Mg²⁺ over ATP of 5 mM. The apparent K_m and V_{max} were extrapolated from double-reciprocal plots fitted using a standard least-squares method (linear regression coefficients, $r = 0.96$ to 0.98). Values are the average of two independent experiments.

membranes from wild type cells (Fig. 4C, lane 1). These results suggest that *ptk2* Δ -791 mutant protein maintains its capacity to interact with the membrane but is a nonfunctional enzyme. We can therefore conclude that the segment of the protein between amino acids 791 and 800 is required for Ptk2 to be active.

4. Discussion

As a first step in the molecular analysis of Ptk2 function, we investigated the subcellular location of the enzyme. Using differential and sucrose gradient centrifugation together with immunofluorescence microscopy, we found that Ptk2 localization is confined to the plasma membrane. Previous studies reported a cytosolic and nuclear localization for Ptk2 [28,29]. These studies were performed using a C-terminal GFP-tagged version of Ptk2 and the possibility exists that the introduction of the tag in the carboxyl-terminus of the enzyme disturbs its localization. In fact, we found that the introduction of an HA-epitope in the C-terminus of Ptk2 rendered an enzyme unable to complement the phenotype of a *ptk2* Δ mutant strain (data not shown). We show that the association of Ptk2 to the plasma membrane must be strong, since it cannot be disrupted by high-salt, alkaline sodium carbonate, urea or Triton X-100 treatment. Deletion of the putative transmembrane segment does not alter Ptk2 localization, indicating some other type of association with the membrane. Consensus sequences for GPI linkage, myristoylation or farnesylation are not detected within Ptk2 protein sequence, but a lipid anchor such as palmitoylation of an internal cysteine as is the case for yeast Rgs16 and Ste18 could be possible [30,31].

Our previous studies established a genetic link between Ptk2 and the Ser⁸⁹⁹-dependent part of the Pma1 glucose activation [15]. Now we report biochemical evidence supporting that Ser⁸⁹⁹ is a target for Ptk2. Evidence is based on in vitro kinase assays using a recombinant GST-fusion protein containing a C-terminal fragment of Pma1 as substrate and purified plasma membranes as the source of phosphorylating activity. The results obtained with this in vitro assay clearly show that plasma membranes lacking Ptk2 are unable to phosphorylate the GST-fusion protein and that the replacement of Ser⁸⁹⁹ by aspartic acid in the Pma1 moiety of the fusion protein also abolished phosphorylation. These data together with the previous genetic evidence [15] lead us to propose Ptk2 as the kinase responsible for the in vivo phosphorylation of Ser⁸⁹⁹ during glucose activation of Pma1.

The exact molecular mechanisms and events leading to Pma1 activation by glucose are not yet completely understood. It has been suggested that glucose activation of ATPase is modulated by a combination of a down-regulating CK1-dependent phosphorylation of the MgATP-binding domain and another up-regulating phosphorylation(s) of the C-terminal domain [32]. CK1 action on Pma1 is glucose-dependent being the activity of the enzyme lower in glucose-fermenting cells than in glucose-starved cells. Two potential phosphorylation sites, essential for glucose activation of Pma1, are present in the C-terminal domain [10]: the phosphorylation site for Ptk2 (Ser⁸⁹⁹), required for the K_m change, and a proposed one for

calmodulin-dependent protein kinase II (Thr⁹¹²), involved in V_{max} increase. Experiments using calmodulin antagonists [33] or a *clk1* Δ *rck1* Δ *cmk1* Δ *cmk2* Δ quadruple mutant (F. Portillo, unpublished) indicate that this kinase is not involved in glucose activation of ATPase. Moreover, a systematic analysis of protein kinase mutants failed to identify a protein kinase mediating the Thr⁹¹²-dependent part of the Pma1 activation [15]. These results suggest that the kinase acting on Thr⁹¹², if any, should be either an essential or a redundant protein kinase. Thus, Ptk2 is the only kinase described so far acting on the C-terminus of the ATPase that mediates glucose activation of the enzyme. Nevertheless the mechanism by which glucose triggers Ptk2 action on ATPase remains to be elucidated. The fact that Ptk2 exhibits the same phosphorylating activity in vitro when purified from glucose-starved or from glucose-fermenting cells and that the presence of glucose in the kinase assay did not increase the phosphorylation level of the GST-fusion protein, discards glucose itself as the direct activator of Ptk2. One plausible hypothesis is that one of the glucose-derived metabolites acts as activator of Ptk2. This possibility is supported by the fact that a triple yeast mutant in the glucose phosphorylating enzymes Hxk1, Hxk2 and Glk1, devoid of the first step in glucose metabolism, is defective in Pma1 activation (Belinchón, M and Gancedo J.M. submitted; F. Portillo, unpublished results). A similar metabolite-dependent protein kinase activation has been recently proposed for TOR kinases [34]. The in vitro kinase assay developed in this work will help in the identification of such metabolite. It is also possible that, concomitantly, Ptk2 phosphorylation of Pma1 could depend on the phosphorylation state of the protein at other regulatory site(s) such as the one described for CK1. In this scenario the glucose-dependent decrease of CK1 activity could allow for Ptk2 phosphorylation of Pma1.

Deletion analysis of Ptk2 led to the identification of a C-terminal region required for Ptk2 activity. This region comprises a stretch of positively charged amino acids (791-KKKKVIHHHL). The analysis of the role of this domain, the identification of the glucose-derived metabolite and the relationship between CK1 and Ptk2 deserve further investigation that will help in the elucidation of Ptk2 function and regulation.

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