Phosphorylation of Yeast Plasma Membrane H\textsuperscript{+}-ATPase by Casein Kinase I*

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Enrique Estrada‡, Patrizia Agostinis§, Jackie R. Vandenheede§, Jozef Goris§, Wilfried Merlevedes§, Jean François§, André Goffeau‡, and Michel Ghislain‡

From the ‡Unité de Biochimie Physiologique, Université Catholique de Louvain, Place Croix du Sud 2-20, B-1348 Louvain-la-Neuve, Belgium, the §Laboratory of Biochemistry, Faculty of Medicine, Catholic University, B-3001 Leuven, Belgium, and the ¶Département de Génie Biochimique et Alimentaire, Institut National des Sciences Appliquées, F-31077 Toulouse Cedex, France

The plasma membrane H\textsuperscript{+}-ATPase of Saccharomyces cerevisiae is subject to phosphorylation by a casein kinase I activity in vitro. We show this casein kinase I activity to result from the combined function of YCK1 and YCK2, two highly similar and plasma membrane-associated casein kinase I homologues. First, H\textsuperscript{+}-ATPase phosphorylation is severely impaired in the plasma membrane of YCK-deficient yeast strains. Furthermore, the wild-type level of the phosphoprotein is restored by the addition of purified mammalian casein kinase I to the mutant membranes. We used the H\textsuperscript{+}-ATPase as well as a synthetic peptide substrate that contains a phosphorylation site for casein kinase I to compare kinase activity in membranes prepared from yeast cells grown in the presence or absence of glucose. The addition of glucose results in increased H\textsuperscript{+}-ATPase activity which is associated with a decline in the phosphorylation level of the enzyme. Mutations in both YCK1 and YCK2 affect this regulation, suggesting that H\textsuperscript{+}-ATPase activity is modulated by glucose via a combination of a “down-regulating” casein kinase I activity and another, yet uncharacterized, “up-regulating” kinase activity. Biochemical mapping of phosphorylated H\textsuperscript{+}-ATPase identifies a major phosphopeptide that contains a consensus phosphorylation site (Ser-507) for casein kinase I. Site-directed mutagenesis of this consensus sequence indicates that Glu-504 is important for glucose-induced decrease in the apparent $K_m$ for ATP.

When glucose is added to starved yeast cells, there is a transient rise in cAMP which induces a protein phosphorylation cascade (1). Among the many metabolic changes observed within minutes after glucose addition is an increased plasma membrane H\textsuperscript{+}-ATPase activity (2), which is encoded by the essential PMA1 gene (3). This observation initially led to the hypothesis that the cAMP-dependent protein kinase might be involved in regulation of the PMA1 protein during growth in glucose, an hypothesis consistent with the finding that the C terminus constitutes an inhibitory domain whose interaction with the active site is regulated by glucose via protein kinase-mediated phosphorylation. That the H\textsuperscript{+}-ATPase activity is indeed regulated by phosphorylation has recently been confirmed by the finding that in vivo phosphorylation of PMA1 is associated with increased H\textsuperscript{+}-ATPase activity during growth in glucose (9). However the identity of the kinase(s) involved in this regulation and the target sites in PMA1 remain to be identified.

On the other hand, PMA1 is known to be phosphorylated by a plasma membrane-bound kinase both in vitro and in vivo (10, 11). Accordingly, treatment of PMA1 with acid phosphatase leads to a decrease in H\textsuperscript{+}-ATPase activity (12). Although PMA1 is a major in vitro substrate for a protein kinase of the plasma membrane-associated casein kinase type I (12), there is no demonstration of a functional relationship between casein kinase I (CKI)\textsuperscript{1} activity and glucose regulation of H\textsuperscript{+}-ATPase activity.

In this study we show that the plasma membrane-bound CKI activity described by Kolarov et al. (12) results from the combined function of YCK1 and YCK2, two yeast homologues of mammalian CKI which together are essential to mitotic growth (13). We also provide evidence for the regulation of CKI activity by glucose and show that loss of YCK function results in impaired regulation of H\textsuperscript{+}-ATPase activity by glucose. Finally we show that PMA1 is phosphorylated in vitro at a consensus site for CKI1, which is located in the putative MgATP-binding domain of the enzyme.

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| To whom correspondence should be addressed. Tel.: 32-10-473614; Fax: 32-10-473872; E-mail: goffeau@fysa.ucl.ac.be.

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1 The abbreviations used are: CK1, casein kinase I; CK2, casein kinase II; DT, dithiothreitol; HPLC, high pressure liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; p90k, ribosomal S6 protein kinase p90; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SPP, small phosphorylated peptide.

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Yeast Strains—The S. cerevisiae strains Σ1278b and W303-1B (MATa leu2 his3 ade2 trpl ura3) were used as wild-type strains. The congenic mutant strains, LRB341 (HIS3 inserted into pALTER-1) and LRB346 (MATα leu2 ura3-52 2cky1-1::HIS3) and LRB346 (MATα leu2 ura3-52 2cky1-1::HIS3) have been described elsewhere (14). The pma1E504A and pma1S507A mutant strains were derived from the YAK2 strain (MATa ade2-101 leu2α1 his3α200 ura3-52 trplα1363 lys2-801 pma1α1::HIS3 pma2::STRTRP) which contains PMA1 under the control of the GAL1 promoter on plasmid pGAL1 PMl (49).

Construction of pma1E504A and pma1S507A Mutant Strains—Site-directed mutagenesis of the 2.5-kilobase HindIII fragment of the PMA1 gene was made in pALTER-1 using the Promega (Madison, Wi) mutagenesis kit and either the oligonucleotide 5'-GAAGCCCAATQCACGACAATTTG-3' for the E504A mutation or the oligonucleotide 5'-GAACCTCTATGCCAATCATTG-3' for the S507A mutation. The mutant genes were introduced into the corresponding yeast strain by transformation using the lithium acetate method. Yeast strains expressing the mutated H".ATPase were selected on glucose medium containing 5-fluoroorotic acid as described (49). The resulting pma1E504A and pma1S507A alleles were isolated from the corresponding mutant strains and sequenced to check the presence of the expected mutation and to exclude any secondary mutation that might have occurred during yeast transformation.

Growth Sensitivity to Hygromycin B—Cells of the LRB341, LRB343, and LRB346 strains were grown at 28°C for 3 days on rich YPD plates. Aliquots of 100 μl were streaked on solid medium containing 100 μg/ml hygromycin B (Sigma) and incubated at 30°C for 3 days. Growth was scored as described in Ref. 18.

Purification of Plasma Membranes—Cells of the LRB341, LRB343, and LRB346 strains were grown at 28°C for 3 days on rich YPD medium containing 2% (w/v) yeast extract (Difco), 2% (w/v) agar (Sigma), 2% (w/v) glucose, and 25, 75, and 100, 100 μg/ml hygromycin B (Sigma). To assess the additional effect of KCl, the cells were plated on solid medium containing 100 μg/ml hygromycin B and KCl at a concentration ranging from 25 to 100 mm.

Purification of Plasma Membranes—Cells grown aerobically at 28°C in 2% (w/v) yeast extract (KAT, Ohli, Hamburg), 5.8% (w/v) glucose were harvested in the late exponential phase of growth (250 × 10^6 cells/ml) and washed three times in cold water. Half of the cells were then resuspended in either 250 mM sorbitol (glucose-starved cells) or sorbitol (starved cells). The next steps of plasma membrane purification are described in Goffeau and Dufour (15) except that the two Triton X-100 washes were omitted. For simplicity's sake, we shall use the term "sorbitol membranes" to designate plasma membranes from glucose-starved cells and the term "glucose membranes" when referring to plasma membranes from glucose-activated cells. Protein concentrations were measured by the method of Peterson (16), using bovine serum albumin as the standard.

ATPase Activity Measurements—ATPase activity was assayed by incubating a volume of plasma membrane suspension corresponding to 5 μg of protein for 5 min at 30°C in a reaction mixture (100 μl final volume) containing 6 mM ATP (sodium salt, Sigma), 9 mM MgCl₂, 10 mM NaN₃, 50 mM MES-NaOH at pH 6.0. The reaction was stopped by addition of 0.1% SDS. Released Pi was measured by colorimetry of the molybdate-P₂O₅ complex as described (15). Each assay was performed in the presence or absence of 100 μM vanadate, and the difference between the two measurements was used to estimate ATPase activity. Measurements of apparent K_m for ATP and V_max were determined using an ATP-regenerating system (49). Typical Michaelis-Menten kinetics were not obtained in those assays since ATP hydrolysis was found to deviate from a first-order kinetics. Similar complexity has been illustrated by the recent kinetic analysis of some pma1 mutant enzymes (50).

Purification of Peptides from Phosphorylated Plasma Membrane Proteins—Purification of peptides was performed according to Ref. 19. Peptides from purified plasma membrane proteins for biochemical analysis were purified with a 0.2% (w/v) solution of polyvinylpyrrolidone (PVP-K40, Merck), and excess polyvinylpyrrolidone was washed away with 2% (w/v) methanol. The pieces were then resuspended in 100 μl of a solution containing 25 mM Tris-Cl, 1 mM EDTA at pH 8.2, and blotted with 0.1% SDS-polyacrylamide gel electrophoresis at 200, 100, 50, and 25 μg. The blots were then exposed to X-ray film (Kodak). The bands were excised, and the gel pieces were digested with 3 μg of endo-Lys-C (sequencing grade, Boehringer Mannheim) for 24 h at 37°C. The supernatant and three additional washes of the proteins with methanol were pooled, and the radioactivity was measured by scintillation counting.

Purification of the CK1 Peptide by Plasma Membranes from Wild-type and CK1-defective Strains—Sorbitol or glucose membranes from strain Σ1278b were resuspended at 0.5 mg/ml in a cold phosphorylation medium containing 100 mM Tris-Cl (pH 7.2) or MOPS-NaOH (pH 7.2) at 4°C. Membranes (50 μg/ml) were added to 20 μl of a reaction mixture containing 2 mM of the specified peptide and 200 μM [γ-32P]ATP. The incubation was continued for an additional 15 min at 30°C. A 25-μl aliquot was then spotted on P81 phosphocellulose paper and analyzed as described above.

Purification of Plasma Membrane H".ATPase by Mammalian CK1—Sorbitol membranes from strain LRB346 were resuspended at 0.5 mg/ml in a medium containing 50 mM MOPS-NaOH, 12 mM MgCl₂, 1 mM DTT, and 8 millinits of CK1. CK1 was purified from porcine spleen (specific activity, 0.4 units/mg; one unit of CK1 incorporates 1 μmol of [32P]P-phosphate per min into casein (2 mg/ml) at 37°C (21)). The reaction was started by adding [γ-32P]ATP to a final concentration of 100 μM and was carried out at 30°C. At the times indicated, aliquots containing 6.25 μg of protein were boiled for 3 min in Laemmli sample buffer, electrophoresed, and analyzed by autoradiography as described above.

Kinetics of CK1 Peptide Phosphorylation—Sorbitol and glucose membranes from strain Σ1278b were cold-phosphorylated as described above and then depleted of ATP by two successive washes and centrifugations at 100,000 × g for 1 h in a Beckman table-top ultracentrifuge. Membranes were resuspended in washing buffer (5 mM MOPS-NaOH, pH 7.2), and 6.25 μg of protein was added to the reaction mixture containing 50 mM MOPS-NaOH, pH 7.2, 6 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 8 μM PK-I peptide inhibitor, 10 μM calmidazolium, and 100 μM PK-I peptide inhibitor, 10 μM calmidazolium, and 100 μM. The reaction was started by addition of [γ-32P]ATP (5–400 μM). The reaction was carried out for 3 min at 30°C and stopped by spotting 20-μl aliquots onto P81 phosphocellulose filters as described above. Blanks were incubated under the same conditions without the peptide. All reactions were done in duplicate.

Purification of Peptides from Phosphorylated Plasma Membrane Proteins—Purification of peptides was performed according to Ref. 19. Peptide samples (2 μg of plasma membrane proteins from glucose-starved LRB341 cells) were resuspended to a final concentration of 0.6 mg/ml in a reaction buffer (6 mM MgCl₂, 10 mM Na₃PO₄, 50 mM MES-NaOH, pH 6.0) and labeled with 100 μM [γ-32P]ATP (2000 cpm/μM) at 30°C for 15 min. The reaction was stopped by adding 5 × Laemmli sample buffer and by boiling for 3 min. The solubilized membranes were deposited in a well 7 cm long and 3 mm wide and electroblotted on polyvinylidene difluoride membranes (Millipore) in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol. After autoradiography, the heavily phosphorylated bands corresponding to the H".ATPase and to an unidentified 50-kDa protein were excised and cut into 1-mm² pieces. Free active sites on the polyvinylidene difluoride membranes were blocked with 0.2% (w/v) solution of polyvinylpyrrolidone (PV-P40, Merck), and excess polyvinylpyrrolidone was washed away with 2% (v/v) methanol. The pieces were then resuspended in 100 μl of a solution containing 25 mM Tris-HCl, 1 mM EDTA at pH 8.2, and blotted with 0.1% SDS-polyacrylamide gel electrophoresis at 200, 100, 50, and 25 μg. The blots were then exposed to X-ray film (Kodak). The bands were excised, and the gel pieces were digested with 3 μg of endo-Lys-C (sequencing grade, Boehringer Mannheim) for 24 h at 37°C. The supernatant and three additional washes of the proteins with digestion buffer were pooled, and the radioactivity was measured by scintillation counting.

Casein Kinase I and Plasma Membrane H".ATPase
concentrated in a C-18 SepPac cartridge (Amicon), and eluted with 70% (v/v) isopropyl alcohol, 0.1% trifluoroacetic acid. The organic solvent was evaporated, and the purified peptides were injected into a C-18 reverse-phase HPLC column (Vydac, 218TP52) mounted on an ABI dual pump. A linear gradient was formed by mixing solvent B (50% acetonitrile, J. T. Baker Inc.) with solvent A (0.05% trifluoroacetic acid (ABI), 1 mM NaH2PO4) and gradually increasing the proportion of solvent B (from 4 to 98% v/v) as the mixture was run through the column, at a flow rate of 425 μl/min, over a 150-min period. The absorbance of the eluent was recorded at 214 nm, and the fractions corresponding to absorbance peaks were collected and dried in a Speed Vac (Savant). Radioactive peptides were solubilized in 70% (v/v) isopropyl alcohol, 0.1% trifluoroacetic acid, blotted onto Polybrene membranes, and subjected to automated Edman degradation in an ABI 477 sequencer.

RESULTS

Time and pH Dependence of Yeast Plasma Membrane Phosphorylation—Plasma membranes were prepared from glucose-starved cells of the wild-type strain Σ1278b. Fig. 1 shows that the addition of [γ-32P]ATP to the purified membranes resulted in the intense labeling of a 100-kDa protein, which was identified as PMA1, the catalytic subunit of the plasma membrane H⁺-ATPase (3). Two additional proteins, a 50-kDa protein (p50) and a small polypeptide (SPP), were also phosphorylated in vitro. The phosphorylation level of PMA1 increased linearly with time (over a 15-min incubation) and was more pronounced at pH 6.0 than at pH 7.2, whereas p50 and SPP were better phosphorylated at pH 7.2. The phosphorylation level of all three polypeptides increased approximately linearly with protein concentrations in the range of 0.125 to 1.5 mg/ml (data not shown).

Effect of Growth Conditions on the Level of Plasma Membrane Phosphorylation—We examined the phosphorylation pattern of plasma membranes isolated from LRB341 cells that had been incubated in glucose instead of sorbitol before membrane homogenization. The wild-type strain LRB341 which has a genetic background different from that of Σ1278b is the parental strain of the yck mutants (21, 22) used in this study (see below).

Fig. 2A shows that the major proteins phosphorylated in sorbitol membranes of LRB341 cells were the same three as observed in Σ1278b cells. Phosphorylation of PMA1 in both strains was also higher at pH 6.0 than at a more alkaline pH as shown in Fig. 1. The three phosphoproteins showed different pH optima for phosphorylation. The phosphorylated residue(s) of PMA1 appeared to be more accessible at pH 6.0 (the optimum pH for ATPase activity) than at more alkaline pH values. This result suggests that the sequence including the phosphorylation site(s) is prone to undergo pH-dependent conformational changes, such as those reported by Blanpain et al. (23) in

![Fig. 1. Time and pH dependence of the in vitro phosphorylation of plasma membrane proteins.](image)

**CONCLUSION**

The results presented in this study demonstrate that the phosphorylation of the plasma membrane H⁺-ATPase is influenced by both time and pH. The phosphorylation level of PMA1 increased linearly with time and was more pronounced at pH 6.0 than at pH 7.2. The phosphorylation level of all three polypeptides increased approximately linearly with protein concentrations in the range of 0.125 to 1.5 mg/ml (data not shown). The phosphorylation pattern of plasma membranes isolated from LRB341 cells that had been incubated in glucose instead of sorbitol before membrane homogenization was similar to that of Σ1278b cells. However, the pH optima for phosphorylation of the three phosphoproteins were different. This result suggests that the sequence including the phosphorylation site(s) is prone to undergo pH-dependent conformational changes, such as those reported by Blanpain et al. (23) in

**Fig. 2. Phosphorylation of plasma membranes from cells defective in casein kinase I activities.** Glucose membranes or sorbitol membranes were purified from wild-type strain LRB341 and mutant strains LRB343 and LRB346. The membranes were incubated for 15 min at 30 °C as in the experiment depicted in Fig. 1, although a broader pH range (pH 6.0 and 6.6 MES-NaOH or pH 7.2 and 8.5 MOPS-NaOH) was examined. A, YCK1 YCK2 strain LRB341. B, YCK1 yck2 strain LRB343. C, yck1 yck2 strain LRB346. The YCK1 and YCK2 genes encode two yeast isoforms of mammalian CK1 (13). The yck2" mutation affects the catalytic kinase domain of YCK2, conferring growth thermosensitivity to the mutant cells.
addition of the various peptide substrates and sorbitol membranes). In both membranes, CK1 peptide phosphorylation was observed because of the high background of endogenous phosphoproteins (data not shown). To lower this endogenous background, the membranes were preincubated with 100 μg of ATP at 30°C for 10 min, before the addition of the various peptide substrates and [γ-32P]ATP. Under these conditions only the peptide DDEESITRR, a specific synthetic substrate of CK1 (20), was phosphorylated (Fig. 3B).

Using the CK1 peptide as a specific phosphorylation substrate, we showed that CK1 activity decreased in glucose membranes (61% remaining relative to the amount seen in the sorbitol membranes). In both membranes, CK1 peptide phosphorylation was higher at pH 7.2 than at pH 6.0 (Fig. 3B, see also Fig. 4).

**Physiology of the CK1 Peptide by Plasma Membranes Defective in CK1 Activity**—In the yeast *S. cerevisiae*, three isoforms of CK1 are currently recognized. Two of these, YCK1 and YCK2, are tightly associated with the plasma membrane and together are required for cell viability (13, 25). In contrast, the third casein kinase I isoform is found predominantly in the nucleus (25). We therefore examined the phosphorylation of the CK1 peptide by plasma membranes isolated from yck mutant strains. The YCK1Δyck2 strain LRB343 contains a null mutation of the YCK2 gene (Δyck2), whereas the Δyck1 yck2–1ts strain LRB346 bears a gene deletion of YCK1 (Δyck1) and another mutation in YCK2 (yck2–1ts) that confers growth thermosensitivity to the mutant cells (14).

In comparison to the wild-type W303 and S1278b strains (YCK1 YCK2), a 2-fold decrease in the phosphorylation level of the CK1 peptide was found in sorbitol membranes of the YCK1Δyck2 strain LRB343 lacking YCK2 (Fig. 4). This result suggests that YCK1 and YCK2 contribute equally well to the CK1 activity seen in wild-type membranes. Strikingly, the amount of 32P incorporated in the CK1 peptide by glucose membranes of the LRB343 strain was slightly higher than the amount measured in sorbitol membranes of the same mutant strain. In contrast, no phosphorylation of the CK1 peptide could be detected in either membranes of the Δyck1 Δyck2–1ts strain LRB346.

Taken together, these results indicate that the plasma membrane kinase activity which specifically phosphorylates the CK1 peptide is mediated by YCK1 and YCK2. Consistently, these two similar and functionally interchangeable isoforms of CK1 are tightly associated to the plasma membrane through posttranslational prenylation of their C terminus (25).
In sorbitol membranes of the YCK1 Δyck2 strain LRB343 (Fig. 2B), loss of YCK2 function had little effect on the level of PMA1 phosphorylation in comparison to membranes of the wild-type YCK1 YCK2 strain LRB341 (Fig. 2A). Strikingly, the high phosphorylation level of PMA1 was maintained in glucose membranes of the mutant strain although it decreased in the parental strain. Loss of YCK2 function has no effect on pH dependence of PMA1 phosphorylation in either membrane. Concerning the remaining other two proteins that are phosphorylated by wild-type membranes (see Fig. 1), we found that the phosphorylation of p50 was quite low in either membrane of the mutant strain, whereas the phosphorylation level of the small polypeptide (SPP) was higher in glucose membranes than in sorbitol membranes (Fig. 2B).

Fig. 2C shows the phosphorylation of plasma membranes from the Δyck1 yck2−Δ strain LRB346, which is defective in CK1 activity (see Fig. 4). Only SPP exhibited some appreciable phosphorylation and only in sorbitol membranes. A very weak phosphorylation of p50 could be detected after a longer exposure of the gels (data not shown). Similar results were obtained if the mutant cells were incubated at the more permissive temperature of 22 °C (data not shown).

These results indicate that the in vitro phosphorylation of PMA1 is due solely to CK1 activity. Both YCK1 and YCK2 contribute to this kinase activity which increases upon glucose starvation, as already shown by using the CK1 peptide as a phosphorylation substrate.

In vitro Phosphorylation of PMA1 by Purified Mammalian Casein Kinase I—The addition of purified mammalian CK1 (20) to sorbitol membranes of the CK1-deficient strain LRB346 (Δyck1 yck2−Δ) restored wild-type level of PMA1 phosphorylation in the mutant membranes. As shown in Fig. 5, PMA1 was readily phosphorylated by the exogenous kinase. The level of PMA1 phosphorylation reached a plateau 10 min after the addition of [γ-32P]ATP and then slightly decreased after 30 min of incubation. This is another evidence confirming that PMA1 is subject to phosphorylation by yeast CK1 homologues in vitro. This result also indicates that PMA1 contains a species-conserved consensus motif for phosphorylation by CK1.

Regulation of Yeast CK1 Activity by Glucose—We have shown that the phosphorylation level of the CK1 peptide increases upon glucose starvation (Fig. 3B). As a first step for characterizing this regulation of CK1 activity, ATP dependence of the CK1 peptide phosphorylation were compared under initial rate conditions in sorbitol membranes and glucose membranes of the wild-type strain S1278b. The membranes were first cold-phosphorylated and then depleted of ATP before fixed concentrations of the CK1 peptide were added and incubated in the presence of increasing concentrations (5–400 μM) of [γ-32P]ATP (blanks were without the CK1 peptide).

The double-reciprocal plot of the data shown in Fig. 6 revealed that the apparent $K_m$ for ATP was similar for sorbitol and glucose membranes. This apparent $K_m$ value obtained is close to that described by Vancura et al. (26) for soluble YCK2. The predicted $V_{\text{max}}$, however, was about two times higher for sorbitol membranes than glucose membranes (Fig. 6). One explanation for decreased phosphorylation of the synthetic CK1 peptide at the onset of glucose metabolism is that glucose somehow induces down-regulation of CK1 activity.

H^+-ATPase Activity in Plasma Membranes of yck Mutant Strains—We have shown that the level of PMA1 phosphorylation in glucose membranes is low relative to sorbitol membranes (Fig. 2A). On the other hand, glucose is known to stimulate H^+-ATPase activity (2). To determine whether this regulation is a function of the extent of phosphorylation, we analyzed the effects of yck mutations on H^+-ATPase activity.

The addition of glucose to starved cells of the wild-type strain LRB341 results in a 3-fold increase of H^+-ATPase activity (Fig. 7), as previously shown (2). In contrast, there is no effect of glucose on H^+-ATPase activity in the YCK1 Δyck2 strain LRB343 (Fig. 7), which shows abnormally high levels of PMA1 phosphorylation both in the presence or absence of glucose (see Fig. 2). This result suggests that CK1-mediated phosphorylation may prevent PMA1 from being activated by glucose. When CK1 activity is strongly decreased by the double Δyck1 yck2^ts mutation, glucose-starved cells (strain LRB346) show increased H^+-ATPase activity (Fig. 7), comparable with the value obtained with glucose-incubated cells of the wild-type strain LRB341. However, the addition of glucose to the Δyck1 yck2^ts mutant cells results in further activation of the H^+-ATPase activity, suggesting another regulation distinct from CK1-mediated phosphorylation.

Effect of yck Mutations on Sensitivity of Yeast Cells to Hygromycin B—Hygromycin B is an antibiotic that affects protein synthesis by inhibiting the peptidyl transferase activity of 70S ribosomes. The sensitivity of the yck1 strains to hygromycin B was analyzed. As shown in Fig. 8, the Δyck1 yck2 strain is more sensitive to hygromycin B than the parental strain. This is consistent with the reduced levels of PMA1 phosphorylation in the Δyck1 yck2 strain, which is expected to result in decreased H^+-ATPase activity.
Translation. The transport of this aminoglycoside into yeast cells is thought to require the plasma membrane electric potential generated through the PMA1 function. This belief primarily stems from the correlation between increased growth resistance to hygromycin B and a depolarization of the potential generated through the PMA1 function. This belief is thought to require the plasma membrane electric potential. In an independent control experiment, we purified and sequenced a strain LRB341, YCK1 YCK2 cells (strain LRB341), YCK1 Δyck2 cells (mutant strain LRB343), and Δyck1 yck2Δ cells (mutant strain LRB346). The main peptide revealed two peptides contiguous in PMA1. The main peptide of peak 1 failed to yield an identifiable sequence, for lack of a sufficient amount of peptide (data not shown). Peak 3 was a complex mixture of at least three peptides present in various amounts. The more abundant peptide (120 pmol) contained no phosphorylation nor regulation of H+-ATPase activity by glucose was monitored by replacing the wild-type PMA1 gene with pma1-E504A or pma1-S507A, two mutant alleles with, respectively, Glu-504 or Ser-507 replaced by Ala. Neither PMA1 phospho-

Fig. 7. H+-ATPase activity in plasma membranes of yck mutant strains. Bar plot shows specific H+-ATPase activity in sorbitol membranes and glucose membranes from YCK1 YCK2 cells (wild-type strain LRB341), YCK1 Δyck2 cells (mutant strain LRB343), and Δyck1 yck2Δ cells (mutant strain LRB346). H+-ATPase activity was assayed as described under "Materials and Methods." The amount of PMA1 in membranes of the wild-type and mutant strains was shown to be identical as determined from SDS-polyacrylamide gel electrophoresis and scanning densitometry.

Identification of a CK1 Phosphorylation Site in PMA1—In vitro phosphorylation of PMA1 occurs exclusively on serine residues. We attempted to identify those residues in vitro. Phosphorylation of PMA1 occurs exclusively on serine residues (11, 12). Interestingly, Glu-504 and Ser-507 are located in the ATP-binding domain of the enzyme. The effect of phosphorylating Ser-507 on H+-ATPase activity was determined by replacing the wild-type PMA1 gene with pma1-E504A or pma1-S507A, two mutant alleles with, respectively, Glu-504 or Ser-507 replaced by Ala. Neither PMA1 phosphorylation nor regulation of H+-ATPase activity by glucose was found to be dependent on Ser-507 (data not shown). Surprisingly, the pma1-E504A mutant enzyme showed a smaller reduction in the apparent Km for ATP, which was induced by glucose (a 2-fold decrease in comparison to a 12-fold decrease for the wild-type enzyme). However, the Vmax values of the pma1-E504A H+-ATPase were not significantly different from...
phorylation could no longer be detected in a yeast strain deficient in both YCK kinases. In particular, loss of YCK2 function results in a 2-fold reduction of CK1 peptide phosphorylation by sorbitol membranes, consistent with YCK1 and YCK2 contributing equally well to plasma membrane CK1 activity. The addition of glucose to starved yeast cells results in decreased CK1 activity as assayed with the specific CK1 peptide substrate. Although the molecular mechanisms of this down-regulation are unknown, we can imagine that, by analogy to the situation in mammals (41), YCK1 and YCK2 are regulated by phosphorylation of their C terminus, which may serve to create an autoinhibitory domain. Alternatively, the amount of these kinases may be regulated at the plasma membrane, through selective prenylation. The possibility that one YCK isoform may be involved in the inactivation of the second isoform is suggested by the finding that glucose has no inhibitory effect on CK1 activity in a mutant strain that only expresses YCK1. Actually, this CK1 activity is comparable with the activity measured in glucose membranes of wild-type cells.

As shown for the CK1 peptide, the phosphorylation level of PMA1 decreases during growth in glucose. It is known that ATPase activity is regulated at the plasma membrane in response to glucose (2). Accordingly, we have found that a decline in CK1-mediated phosphorylation of PMA1 is associated with increased H\(^{-}\) -ATPase activity during glucose metabolism.

When compared with the wild-type strain, H\(^{-}\) -ATPase activity increases 3-fold in starved cells of a CK1-deficient strain. Furthermore, glucose has no effect on H\(^{-}\) -ATPase activity in a mutant strain that maintains abnormally high levels of PMA1 phosphorylation, due to the loss of YCK2 function. All together these results suggest several mechanisms for the regulation of the H\(^{-}\) -ATPase. One possibility is that H\(^{-}\) -ATPase activity is inhibited through phosphorylation by the YCK kinases whose functions are stimulated upon glucose starvation. It is also possible that YCK-mediated phosphorylation of PMA1 may prevent the enzyme from being activated by glucose, through another kinase-dependent phosphorylation. This second explanation is supported by our results that the H\(^{-}\) -ATPase activity of a YCK-deficient strain is still stimulated by glucose and by the finding that increased H\(^{-}\) -ATPase activity correlates with the phosphorylation of specific sites in vivo (9).

It appears likely that PMA1 contains at least two antagonistic regulatory sites (Fig. 10). YCK-mediated phosphorylation of one site inhibits H\(^{-}\) -ATPase activity; phosphorylation of the second site by a different protein kinase, however, influences the H\(^{-}\) -ATPase activity. When one of the two YCK isoforms is missing, glucose has no effect on H\(^{-}\) -ATPase activity, showing the dominance of the inhibitory phosphorylation over the activating one. This model is probably too simple since more than one target site may be phosphorylated by each kinase. Moreover, it does not consider other ways by which PMA1 might be regulated, such as dephosphorylation of CK1-phosphorylated sites by a protein phosphatase (12, 14).

We have identified the Ser-507 residue of PMA1 as a major site for Yckp-mediated phosphorylation. The sequence surrounding Ser-507 conforms with a bona fide consensus motif for CK1 (29, 30, 31) and is fully conserved in fungal H\(^{-}\) -ATPases but not in plant enzymes (45). However, mutagenesis analysis revealed that the overall level of PMA1 phosphorylation is not affected by the pma1S507A mutation, which replaces Ser-507 with Ala. It is therefore likely that PMA1 contains more than one CK1 phosphorylation site, consistent with the identification of three phosphopeptides by biochemical mapping.

Interestingly enough, we have found that the Glu-504 residue of PMA1 is important for regulation of H\(^{-}\) -ATPase activity by glucose. The pma1E504A mutation has a strong inhibitory
The ATP-binding domain since this residue is labeled by fluorescein isothiocyanate. Glu-504 is close to Lys-474 that is part of the ATP-binding domain since this residue is labeled by fluorescein isothiocyanate. Glu-504 mediates the regulation of H^+\text{ATPase} activity by glucose (47) which is suppressed by the replacement of Pro-536 (47). Ouabain, an inhibitor of the Na^+-\text{K}^+-\text{ATPase}, inhibits glycolysis in tumor cells. Moreover, a protein kinase of Ehrlich ascites tumor cells phosphorylates the Na^+-\text{K}^+-\text{ATPase}, which decreases Na^+ pump efficiency (52). The pH-sensing mechanism related to growth control of eukaryotic cells remains to be determined (47). However, the nature of these pH sensors could be investigated by a genetic approach using yeast as a model system.

In conclusion, we have shown that PMA1 is phosphorylated by two plasma membrane-associated isoforms of CK1 in vitro. This is the first evidence of a biochemical function for CK1 in yeast. CK1-phosphorylation of PMA1 also provides a coherent starting basis for further in vivo studies of the regulation of H^+\text{ATPase} activity and may prove to be a useful system for understanding the role of CK1-mediated proton transport for cell growth.

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| Yeast casein kinase I phenotype | Yeast casein kinase I activity | Unknown kinase activity | Subpopulation of active ATPase molecules |
|-------------------------------|-----------------------------|------------------------|-----------------------------------------|
| Yck1p                         | Starvation                  | Starvation             | Starvation                              |
| Yck2p                         | Glucose                     | Glucose                | Glucose                                 |
| Yck1p                         | Starvation                  | Starvation             | Starvation                              |
| yck2ts                        | Glucose                     | Glucose                | Glucose                                 |
| yck2ts                        | Starvation                  | Starvation             | Starvation                              |

FIG. 10. Regulation of H^+\text{ATPase} activity by phosphorylation. PMA1 is regulated through phosphorylation by a plasma membrane-bound CK1 and another unknown protein kinase. Steady-state kinase activity is represented by a double arrow, with the plus or minus sign indicating activation of inhibition of phosphorylation, respectively. Ki- expresses the YCK1 isoform is shown in the first row; the strain is represented in the second row; and the Δyck2-1 strain which only expresses a mutant version of YCK2 is shown in the third row.

The effect on glucose-induced decrease of the apparent K_m for ATP. Glu-504 is close to Lys-474, which is part of the ATP-binding domain since this residue is labeled by fluorescein isothiocyanate and protected from this labeling by ATP (46). Substitution of Arg for Lys-474 results in the inhibition of H^+\text{ATPase} activity (47) which is suppressed by the replacement of Pro-536 by Leu (48). Interestingly, the F536L mutation was previously isolated as a suppressor of the double mutation S911A/T912A which affects glucose-induced activation of the H^+\text{ATPase} (8).

These and other results strongly indicate that the ATP-binding region of the H^+\text{ATPase} is prone to structural rearrangements and interacts with the C terminus whose phosphorylation may control H^+\text{ATPase} activity. It is therefore possible that Glu-504 mediates the regulation of H^+\text{ATPase} activity by glucose either directly, through the control of ATP binding, or indirectly, via the interaction between the catalytic domain and the C-terminal regulatory domain of the enzyme.

The plasma membrane H^+\text{ATPase} is rate-limiting for yeast growth, which is optimal at neutral intracellular pH and decreases as the cell interior becomes more acidic (47). One of the major regulatory functions of the H^+\text{ATPase} is the control of intracellular pH by ejection of protons from the cytosol. Active nutrient transport is another mechanism of growth control since the proton gradient generated by the enzyme is the driving force for nutrient uptake. As glucose metabolism produces intracellular acidification, the increase in ATPase activity can be rationalized as the basis of the need for intracellular pH homeostasis (47). Expression of yeast PMA1 in mouse fibroblasts increases intracellular pH and induces cell proliferation, suggesting that similar mechanisms may be involved in growth control by proton transport in all eukaryotic cells (47). A common early response to stimuli that activate cell proliferation is an intracellular alkalization which, in animal cells, is caused by the activation of a Na^+-K^+-ATPase system. An increase in intracellular pH is known to activate glycolysis. Consistently, one of the most prominent properties characteristic of rapidly growing cancer cells is their capacity to sustain high rates of aerobic glycolysis. Type II hexokinase, which is expressed at high levels in such cells and bound to the outer mitochondrial membrane, plays a key role in the aberrant glucose metabolism (51).

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Casein Kinase I and Plasma Membrane H+-ATPase