Maturation of the Yeast Plasma Membrane [H+]ATPase Involves Phosphorylation during Intracellular Transport

Amy Chang and Carolyn W. Slayman

Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. In this study we show that the plasma membrane [H+]ATPase of *Saccharomyces cerevisiae* is phosphorylated on multiple Ser and Thr residues in vivo. Phosphorylation occurs during the movement of newly synthesized ATPase from the ER to the cell surface, as revealed by the analysis of temperature-sensitive *sec* mutants blocked at successive steps of the secretory pathway. Two-dimensional phosphopeptide analysis of the ATPase indicates that, although most sites are phosphorylated at or before arrival in secretory vesicles, some phosphopeptides are unique to the plasma membrane. Phosphorylation of plasma membrane–specific site(s) is associated with increased ATPase activity during growth on glucose. Upon glucose starvation, dephosphorylation occurs concomitantly with a decrease in enzymatic activity, and both are rapidly reversed (within 2 min) upon readdition of glucose. We suggest that reversible, site-specific phosphorylation serves to adjust ATPase activity in response to nutritional signals.

THE plasma membrane ATPase of *Saccharomyces cerevisiae* is a proton pump that regulates cytoplasmic pH and provides the driving force for nutrient uptake (Serrano et al., 1986, Serrano, 1989). Not surprisingly, in light of these critical physiologic functions, the [H+]ATPase is essential for cell viability. It has strong structural homology with other E_1E_2 or P-type ATPases involved in maintaining ionic homeostasis, including the [Na^+,K^+] and Ca^{2+}ATPases of mammalian cells. Like other members of its class, the yeast ATPase has a catalytic subunit of ~100 kD, hydrolyzes ATP via a transient aspartylphosphate intermediate, and is inhibited by low concentrations of vanadate. Sequence analysis of the PMA1 gene encoding the yeast [H+]-ATPase predicts that the protein consists of a large central cytoplasmic domain containing putative sites for ATP binding and hydrolysis, anchored in the membrane by four hydrophobic segments at the NH_2-terminal end and four to six hydrophobic segments at the COOH-terminal end.

One approach taken in our laboratory to understanding structure–function relationships of the [H+]ATPase has been to study the acquisition of tertiary structure and activity during enzyme biogenesis. Recent work has established that newly synthesized [H+]ATPase becomes integrated into the endoplasmic reticulum membrane without cleavage of an NH_2-terminal signal sequence, and is delivered to the cell surface via the secretory pathway (Holcomb et al., 1988). In *sec* mutants blocked at discrete steps of the pathway, membrane biogenesis is prevented (Novick and Schekman, 1983; Tschopp et al., 1984), and [H+]ATPase en route to the plasma membrane becomes trapped within the ER, Golgi compartment, or secretory vesicles (Brada and Schekman, 1988). The ER and Golgi forms of the ATPase have not yet been examined for activity, but the enzyme accumulated in secretory vesicles is capable of splitting ATP and pumping protons (Walworth and Novick, 1987; Nakamoto et al., 1991).

While the time course and mechanism of intracellular ATPase activation remain unclear, it is known that [H+]-ATPase activity is regulated at the plasma membrane in response to growth conditions, increasing severalfold during glucose metabolism (Serrano, 1983) and in an acid milieu (Erasco and Gancedo, 1987). In addition, the ATPase is thought to mediate intracellular alkalinization that prefaces DNA synthesis at the start of the cell cycle (Gillies et al., 1981). Indeed, expression of the yeast [H+]ATPase in mammalian cells induces cytoplasmic alkalinization and cell proliferation (Perona and Serrano, 1988). It has been suggested that the control of ATPase activity at the plasma membrane occurs via protein kinase–mediated phosphorylation. The ATPase is phosphorylated in vivo (McDonough and Mahler, 1982; Portillo and Mazon, 1985), and has been reported to serve as an in vitro substrate for a plasma membrane–associated casein kinase (Yanagita et al., 1987; Kolarov et al., 1988). Furthermore, incubation of purified ATPase with acid phosphatase leads to a decrease in enzymatic activity (Kolarov et al., 1988).

In this study, we show that phosphorylation at multiple Ser and Thr residues occurs during intracellular transport of newly synthesized [H+]ATPase. Significantly, arrival at the plasma membrane coincides with the phosphorylation of specific site(s), which appears to regulate ATPase activity.

Materials and Methods

Strains and Growth Media

The strains of *S. cerevisiae* used in this study were the wild-type NY 13

(MAT a; *ura 3-52*), and the temperature-sensitive mutants NY 431 (MAT a; *ura 3-52*; *sec 18-1*), NY 17 (MAT a; *ura 3-52*; *sec 6-4*), and NY 176 (MAT a; *ura 3-52*; *sec 7-1*). The strains, which are isogenic, were kindly provided by Dr. Peter Novick (Yale University, New Haven, CT). Cultures were grown in minimal medium containing 0.7% yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI), 2% glucose, and uracil (20 μg/ml).

Metabolic Labeling, Immunoprecipitation, and Enzyme Digestions

Cells were grown to midlogarithmic phase at 25°C in low-sulfate minimal medium (100 μM Na_2SO_4) containing 2% glucose, and harvested by centrifugation at 1,000 *g* for 5 min. The cells (usually 50–100 OD_{600} U) were resuspended at 1 OD_{600}/ml in minimal medium containing 25 μM Na_2SO_4 and 2% glucose, preincubated at 25 or 37°C for 30 min, and then labeled with 10-20 μCi/OD_{600} U Expre[$^{35}S^{35}S$] (New England Nuclear, Boston, MA) at 25 or 37°C. In pulse-chase experiments radiolabeling was terminated by adding an equal volume of minimal medium containing 20 mM cysteine and 20 mM methionine, adjusted to pH ~6. The cells were then centrifuged and resuspended in chase medium with 10 mM cysteine and 10 mM methionine, pH ~6. At various times of chase, 10 mM NaN_3 was added and the cells were placed on ice. The cells were centrifuged, resuspended at 200 OD_{600}/ml in buffer (10 mM Tris, pH 7.4, 0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$), and lysed with glass beads as described (Fujiyama and Tamanoi, 1986). A protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml chymostatin, 1 mM PMSF) was included in the cell lysis buffer. The cell lysate was centrifuged at 400 *g* for 5 min to remove unbroken cells, and a total membrane fraction was prepared by centrifugation of the supernatant at 100,000 *g* for 1 h (Ti 50 rotor; Beckman Instruments, Palo Alto, CA). For immunoprecipitation, aliquots of total membranes were diluted in RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40, 1% Na deoxycholate) containing protease inhibitors; insoluble material was removed by centrifugation in a microfuge (Eppendorf; Brinkmann Instruments, Westbury, NY) for 5 min. The supernatant was incubated overnight at 4°C with rabbit anti-ATPase antibody and protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The antibody, raised and affinity-purified against the plasma membrane [H^+]ATPase of *Neurospora crassa* (Hager et al., 1986), reacts specifically with the homologous 100-kD polypeptide in yeast. Immunoprecipitates were washed three times with RIPA buffer, followed by one wash with RIPA containing 0.5 M NaCl, and one wash with H_2O.

For alkaline phosphatase digestion, immunoprecipitated ATPase was liberated from protein A–Sepharose by resuspension in 15 μl buffer containing 50 mM Tris, pH 8, 1% β-mercaptoethanol, 1% SDS, and protease inhibitors. After incubation at 37°C for 5 min the samples were then diluted with 50 μl of 50 mM Tris, pH 8, containing protease inhibitors, and centrifuged for 0.5 min in the microfuge to remove the protein A–Sepharose beads. The supernatant was divided and incubated for 1 h at 37°C in the presence or absence of 1 U alkaline phosphatase (from calf intestine for molecular biology, Boehringer, Mannheim, Germany).

For digestion with endoglycosidase F, immunoprecipitated ATPase was released from protein A–Sepharose by incubation at 37°C for 5 min in 10 μl buffer containing 50 mM Tris, pH 6.8, 1% β-mercaptoethanol, 1% SDS. The supernatant was diluted with 140 μl 100 mM Na acetate, pH 5.5, 50 mM EDTA, 1% NP-40, 1% β-mercaptoethanol. Samples were centrifuged for 0.5 min and the supernatant was divided and incubated overnight at 37°C in the presence or absence of 1 μl endoglycosidase F (50 U/ml; Boehringer).

Immunoprecipitates were analyzed by electrophoresis on 8% polyacrylamide gels after resuspension in Laemmli sample buffer and heating at 37°C for 5 min (Laemmli, 1970). To increase resolution of small mobility changes, gels were run with prestained molecular weight markers (Sigma Chemical Co., St. Louis, MO) until the β-galactosidase marker had migrated through two-thirds of the gel. Fluorography was performed by impregnating the gels with Amplify (Amersham Chemical Co., Arlington Heights, IL); the gels were then dried and exposed to film (XAR-5; Eastman Kodak, Rochester, NY) at −70°C.

Cell Fractionation, Western Blotting, and ATPase Assay

Secretory vesicles were purified from *sec6-4* using the method of Walworth and Novick (1987), as modified by Nakamoto et al. (1991). Briefly, radiolabeled cells were collected by centrifugation, washed with cold 10 mM NaN_3 and converted to spheroplasts by incubation for 30–45 min at 37°C in buffer (1.4 M sorbitol, 10 mM NaN_3, 40 mM β-mercaptoethanol, 50 mM K_2HPO_4, pH 7.5) containing zymolyase (100T; ICN Radiochemicals, Irvine, CA) at 217 μg/ml. Inclusion of glucose during this incubation did not affect the phosphorylation pattern of secretory vesicle ATPase. The spheroplasts were then coated with con A to stabilize the plasma membranes so that they could be removed by sedimentation at low speed. The spheroplasts were then lysed in a Dounce homogenizer and centrifuged at 10,000 *g* for 10 min to remove unbroken cells, nuclei, and mitochondria. Finally, the supernatant fraction was centrifuged at 100,000 *g* to yield a membrane pellet, which was homogenized in 1 mM EGTA/Tris, pH 7.5, and resedimented at 100,000 *g*. This protocol results in a 36-fold purification of secretory vesicles over crude lysate, with <5% contamination by plasma membranes (Nakamoto et al., 1991).

Plasma membranes were purified essentially as described by Serrano (1988). Radiolabeled cells (50–100 OD_{600} units) were harvested by centrifugation, and lysed by vortexing with glass beads. The lysate was centrifuged at 600 *g* for 5 min to remove unbroken cells, and then further centrifuged for 20 min at 20,000 *g*. The pellet was resuspended in 1.5 ml of 20% glycerol in TED buffer (0.1 mM EDTA, 0.1 mM DTT, and 10 mM Tricine, pH 7.5) and applied to a discontinuous gradient of 1.2 ml 53% (wt/wt) sucrose in TED buffer and 2.3 ml 43% (wt/wt) sucrose/TED. After centrifugation for 6 h at 60,000 *g* (SW55 Ti rotor; Beckman Instruments), plasma membranes were collected from the sucrose interface, diluted with H_2O, and centrifuged at 80,000 *g* for 20 min.

For Western blotting, SDS gels were transferred to nitrocellulose for 2 h at 100 V in 25 mM Tris, 0.2 M glycine, 20% methanol. Nonspecific sites were blocked by incubating the filter in 10 mM Tris, pH 7.4, 150 mM NaCl, 0.05% NaN_3 containing 5% milk.

Protein concentrations were measured by the method of Bensadoun and Weinstein (1976). Vanadate-sensitive ATPase activity was measured in the presence and absence of 100 μM Na vanadate. Membrane fractions were diluted 100-fold into assay mixture containing 50 mM 2-morpholino ethane sulfonic acid, pH 6.5, 5 mM Na_2ATP, 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 5 mM KN_3. The reaction was carried out for 10–20 min at 30°C. Free phosphate was determined according to Fiske and Subbarow (1925).

Glucose Activation, In Vivo Phosphorylation, Phosphopeptide Mapping, and Phosphoamino Acid Analysis

Glucose activation experiments were done by a modification of the protocol described by Serrano (1983). Wild-type cells were grown to midlogarithmic phase in low-phosphate (50 μM KH_2PO_4) minimal medium containing 2% glucose, harvested, washed three times with H_2O, and incubated for 30 min in medium without glucose. Cycloheximide (0.02 mg/ml) was added for 5 min, followed by glucose to a concentration of 2%. At various times after glucose addition, 10 mM Na azide was added, the cells were transferred to an icebath, and a total membrane fraction was prepared as described above. Cells maintained in 2% glucose medium for the duration of the experiment were analyzed as a control.

For in vivo phosphorylation experiments, cells were grown at 25°C to midlogarithmic phase in low-phosphate minimal medium (50 μM KH_2PO_4). The cells (50–100 OD_{600} units) were harvested and resuspended at 1 OD_{600}/ml in fresh medium before labeling with 2 mCi [^{32}P]orthophosphoric acid (New England Nuclear). For labeling of *sec 6-4* cells at the restrictive temperature, cells were incubated at 37°C for 30 min before addition of isotope. Plasma membrane and secretory vesicle fractions were prepared upon termination of labeling. Phosphatase inhibitors (10 mM Na pyrophosphate, 4 mM EDTA) were included in the lysis buffers; however, ATPase phosphorylation appeared quite stable, and omission of inhibitors did not change the extent or pattern of the observed phosphorylation. To examine ATPase phosphorylation during glucose activation, wild-type cells were labeled with [^{32}P]orthophosphoric acid for 2 h, centrifuged, and either washed with H_2O or resuspended in fresh minimal medium with 2% glucose.

Two-dimensional phosphopeptide mapping was performed as described by Lai et al. (1987): ^{32}P-labeled ATPase was immunoprecipitated and isolated by SDS-PAGE; the ATPase band was excised, washed extensively with 50% methanol to remove SDS, and exhaustively digested with 50 μg/ml thermolysin at 37°C for 24 h. The resultant peptides were spotted on thin layer chromatography plates (cellulose, microcrystalline; J.T. Baker Inc., Phillipsburg, NJ) and separated by electrophoresis in the horizontal dimension followed by ascending chromatography in the vertical dimension. Samples were prepared for phosphoamino acid analysis by thermolytic digestion

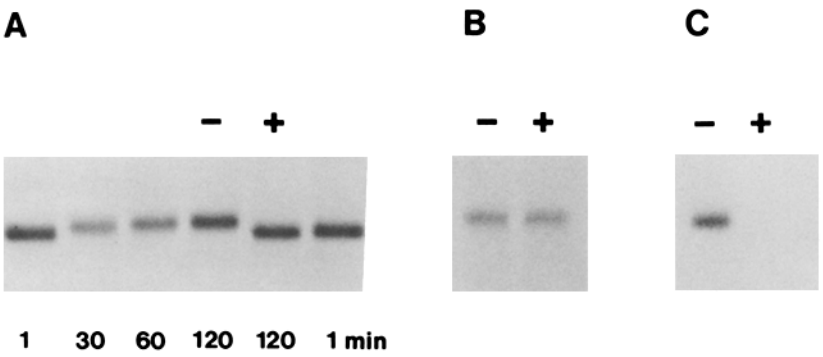

Figure 1. Kinetics of ATPase maturation. (*A*) Wild-type cells were pulse-labeled for 2 min with Expres[^{35}S^{35}S], centrifuged to remove the radiolabel, and resuspended in chase medium containing 10 mM methionine and 10 mM cysteine at 25°C. At various times of chase (1, 30, 60, and 120 min), aliquots were removed, total membrane fractions were prepared, and ATPase was immunoprecipitated and analyzed by SDS-PAGE and fluorography, as described in Materials and Methods. In lanes *4* and *5*, immunoprecipitates were incubated in the absence and presence of 1 U alkaline phosphatase for 1 h at 37°C. (*B*) Radiolabeled ATPase was immunoprecipitated from a total membrane fraction after 2 h of chase and incubated overnight at 37°C in the presence and absence of endoglycosidase F. (*C*) Wild-type cells were labeled with [^{32}P]orthophosphoric acid for 2 h at 25°C. ATPase was immunoprecipitated from plasma membranes, incubated in the presence and absence of alkaline phosphatase for 1 h at 37°C, and resolved by SDS-PAGE and autoradiography.

of the ATPase described above followed by hydrolysis of the peptides for 2 h at 110°C with 6 N HCl. The phosphoamino acids were mixed with phosphoserine, phosphothreonine, and phosphotyrosine standards (Sigma Chemical Co.) and resolved by thin layer chromatography on cellulose plates (microcrystalline; Eastman Kodak) according to Nairn and Greengard (1987). The TLC plates were stained with ninhydrin and then exposed to x-ray film with intensifying screens at −70°C.

Results

Newly Synthesized ATPase Is Phosphorylated

As a first step to studying ATPase maturation, a pulse–chase experiment was carried out in which wild-type cells were labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 2 min at 25°C, and then transferred to chase medium for varying periods of time (1 min to 2 h). A total membrane fraction was prepared for each sample, from which ATPase was immunoprecipitated and analyzed by SDS-PAGE. When the 100-kD band was allowed to run two-thirds of the way through an 8% gel, a progressive increase in the apparent relative molecular mass of newly synthesized ATPase was observed during the chase, indicating a posttranslational modification (Fig. 1 *A*). The ATPase achieved maximal size by ~2 h of chase.

It seemed possible that glycosylation could account for the observed mobility shift since the deduced amino acid sequence of the ATPase contains a consensus sequence for glycosylation (Serrano, 1986). However, digestion with endoglycosidase F had no effect on the electrophoretic mobility of ATPase immunoprecipitated after a 2-h chase (Fig. 1 *B*), although the mobility of the precipitating IgG was shifted (not shown). Furthermore, there was no detectable binding of ^{125}I-con A to immunoprecipitated ATPase, indicating an absence of associated mannose residues (not shown).

By contrast, digestion of immunoprecipitated ATPase with alkaline phosphatase converted the high molecular weight form seen after 2 h of chase to the low molecular weight form seen at 1 min of chase, suggesting that the ATPase is phosphorylated (Fig. 1 *A*, lanes *4* and *5*). To examine this possibility directly, cells were incubated with [^{32}P]orthophosphoric acid for 2 h, and ATPase was immunoprecipitated. Fig. 1 *C* shows that [^{32}P]phosphate was incorporated into the ATPase and that the radiolabel was removed upon treatment with alkaline phosphatase.

Phosphorylation Occurs on Ser and Thr

The aspartyl–phosphate catalytic intermediate formed by the [H$^+$]ATPase is known to be labile under the Laemmli gel conditions used in this study (Smith and Hammes, 1988), so it seemed likely that the observed phosphorylation was protein kinase mediated. To test this idea, ATPase was immunoprecipitated from cells labeled for 30 min with [^{32}P]orthophosphoric acid, digested exhaustively with thermolysin, and hydrolyzed with 5.7 N HCl. Phosphoamino acids were then resolved by thin-layer chromatography. As shown in Fig. 2, phosphorylation occurred predominantly on Ser residues, although some phosphothreonine was also present. No phosphorylation of tyrosine residues was seen.

ATPase Phosphorylation Occurs in Multiple Compartments along the Secretory Pathway

To determine the cellular site(s) at which the ATPase becomes phosphorylated, temperature-sensitive mutants blocked at discrete steps of the secretory pathway were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 1 h at the restrictive temperature (37°C). In this manner, it is possible to examine newly synthesized ATPase accumulated in the ER (*sec18*), Golgi cisternae (*sec7*), and secretory vesicles (*sec6*). A total membrane fraction was prepared from each strain, and the accumulated ATPase was immunoprecipitated (Fig. 3). In each case, alkaline phosphatase digestion of the immunoprecipitated sample converted the ATPase to the low molecular weight form; the ATPase mobility shift in *sec18* and *sec7* was small but reproducible, suggesting that some phosphorylation occurs in the ER and Golgi apparatus. The mobility shift was greater in the samples from *sec6* and wild-type cells, also labeled for 1 h at 37°C, indicating a greater degree of phosphorylation in the later steps of the secretory pathway. Thus, phosphorylation of newly synthesized ATPase appears to occur in multiple compartments of the secretory pathway.

Specific Phosphorylation Occurs upon Arrival at the Plasma Membrane

To investigate further the intracellular processing of newly synthesized ATPase, we compared phosphorylation of ATPase accumulated in purified secretory vesicles with that in the plasma membrane. Both secretory vesicles and plasma

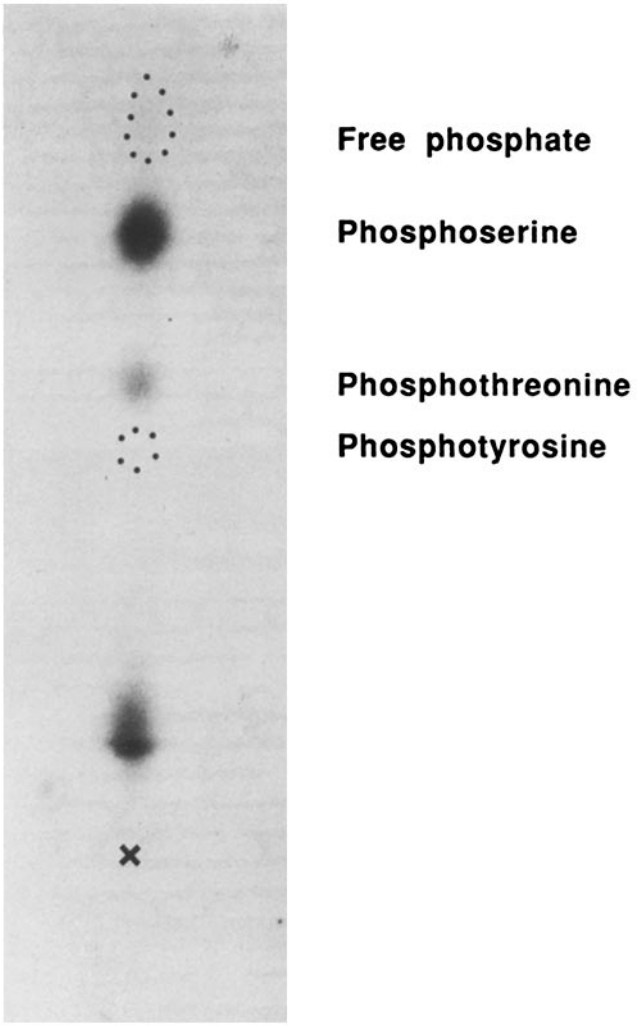

Figure 2. ATPase phosphorylation occurs at Ser and Thr. Phosphoamino acid analysis of ATPase immunoprecipitated from total membranes of cells labeled for 30 min with [^{32}P]orthophosphoric acid. The ATPase was gel-purified, exhaustively digested with thermolysin, and hydrolyzed for 2 h at 110°C with 5.7 N HCl. The hydrolysate was spotted on a cellulose thin layer chromatography plate and chromatographed as described in Materials and Methods. ^{32}P-labeled phosphoamino acids were detected by autoradiography; the positions of the standards, detected by ninhydrin staining, are indicated. The origin is depicted as *X*. The material migrating above the origin is likely partially hydrolyzed protein.

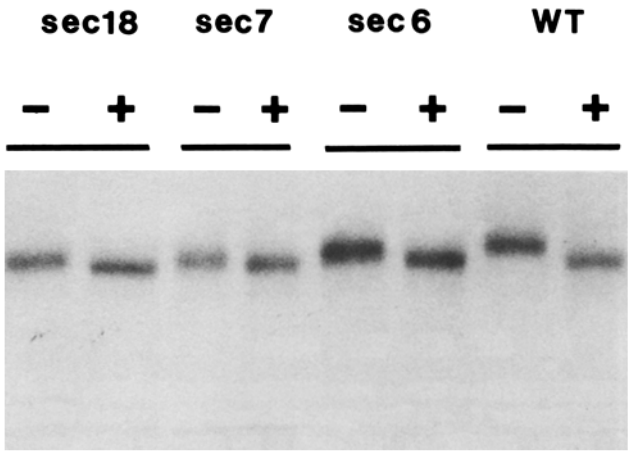

Figure 3. ATPase phosphorylation occurs during intracellular transport. Wild-type, *sec18*, *sec7*, and *sec6* cells were incubated at 37°C for 30 min before the addition of Expres[^{35}S^{35}S] for 1 h. ATPase was immunoprecipitated from a total membrane fraction, incubated in the presence and absence of 1 U alkaline phosphatase for 1 h at 37°C, and analyzed by SDS-PAGE and fluorography.

membranes were purified from *sec6* cells shifted to 37°C and labeled with [^{32}P]orthophosphate. Phosphoamino acid analysis revealed no significant difference in the ratio of phosphoserine to phosphothreonine for the two membrane fractions (not shown). Phosphopeptide mapping was then used to examine the sites of ATPase phosphorylation. ATPase immunoprecipitated from each fraction was gel-purified, exhaustively digested with thermolysin, and the peptides were then separated by two-dimensional chromatography. In Fig. 4, the distinctly resolved phosphopeptides have been arbitrarily numbered 1-7. Of them, most (1–5) appeared equally labeled in the secretory vesicle and plasma membrane forms of the ATPase. Furthermore, no change in the phosphopeptide map of the secretory vesicle form was observed when the vesicles were accumulated for 30 min or 2 h (data not shown). Thus, peptides no. 1–5 are phosphorylated before or soon after arrival at the secretory vesicles.

Two other peptides displayed significant changes in phosphorylation at the plasma membrane. Especially striking was no. 7, which was not detectable in the secretory vesicle preparation, but was labeled in the plasma membrane form of the ATPase. In addition, phosphopeptide no. 6 was visible in secretory vesicles, but its labeling rose significantly at the plasma membrane. The phosphorylation pattern was the same when plasma membranes were isolated from *sec6* or wild-type cells at 25°C (not shown).

Phosphorylation/Dephosphorylation at the Plasma Membrane Is Associated with Activation of the ATPase by Glucose

Protein kinase–mediated phosphorylation has been suggested to participate in the control of [H$^+$]ATPase activity at the yeast plasma membrane. For example, Serrano (1983) found that the specific activity, K_m, pH optimum, and vanadate sensitivity of the ATPase are rapidly modulated in response to glucose metabolism, and proposed phosphorylation/dephosphorylation of a regulatory site as a plausible mechanism. It therefore seemed worthwhile to ask whether any of the phosphorylation sites seen in the two-dimensional chromatogram of Fig. 4 might play a role in glucose regulation of the ATPase. In the experiment of Fig. 5 *A*, which was carried out to establish conditions for phosphorylation measurements, wild-type cells growing exponentially in 2% glucose were harvested, washed, and incubated in the absence of glucose for 30 min. Vanadate-sensitive ATPase activity, assayed in total membranes, fell during this period from 0.390 to 0.131 μmol/min per mg. Glucose was then added back (to 2%), producing a rapid increase in ATPase activity to 0.469 μmol/min per mg within 2 min (Fig. 5 *A*). In six such experiments, the addition of glucose to starved cells resulted in a 3.02 ± 0.52-fold increase in specific activity. Preincubation of the cells with cycloheximide did not elimi-

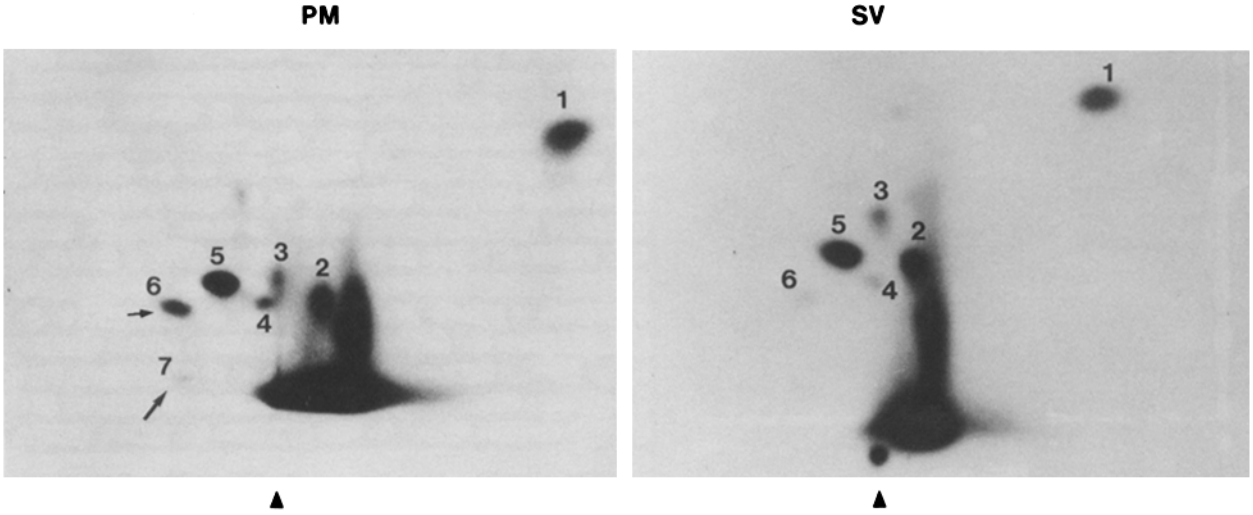

Figure 4. Specific phosphorylation occurs upon arrival of newly synthesized ATPase at the plasma membrane. Two-dimensional phosphopeptide mapping of ATPase from plasma membrane (*PM*) and accumulated secretory vesicles (*SV*) isolated from *sec6* cells labeled with [^{32}P]orthophosphate at 37°C. ATPase was immunoprecipitated and exhaustively digested with thermolysin. Phosphopeptides were spotted on cellulose thin-layer chromatography plates, separated by electrophoresis in the horizontal dimension (anode on right) followed by ascending chromatography in the vertical dimension, and visualized by autoradiography. Corresponding peptides are arbitrarily numbered 1–7. Arrowheads indicate the origins. Arrows indicate the positions of the plasma membrane-specific phosphopeptides.

nate the increase, indicating that new synthesis of ATPase was not required. These results are consistent with previous reports (Serrano, 1983; Portillo and Mazon, 1986).

To examine ATPase phosphorylation during glucose regulation, wild-type cells were labeled with [^{32}P]orthophosphoric acid for 2 h, as described in Materials and Methods. Total membranes were prepared from cells maintained in 2% glucose (control), cells incubated in glucose-free medium for 30 min, and cells that were starved and refed 2% glucose for 3 min. ATPase was then immunoprecipitated from the membranes and analyzed by phosphopeptide mapping (Fig. 5 *B*). In control cells (Fig. 5 *B*, *a*), the map was similar to that seen earlier for purified plasma membranes. By contrast, in starved cells (Fig. 5 *B*, *b*) peptide no. 7 disappeared, and phosphorylation of peptide no. 6 was diminished. And finally, 3 min after glucose readdition, peptide no. 7 became prominently phosphorylated once again and phosphorylation of peptide no. 6 increased. Together, the data support the idea that ATPase activity is regulated by phosphorylation and dephosphorylation reactions at the plasma membrane.

Discussion

We have shown in this study that the yeast [H$^+$]ATPase undergoes kinase-mediated phosphorylation during intracellular transport. In pulse–chase experiments the apparent relative molecular mass of newly synthesized ATPase increased incrementally, suggesting that all newly made ATPase molecules were modified progressively. Although ATPase biogenesis has been studied previously (Holcomb et al., 1988), phosphorylation was not noted, probably because the post-translational modification results in only a very subtle change in the electrophoretic mobility of the polypeptide. Phosphopeptide mapping of ^{32}P-labeled ATPase in purified secretory vesicles revealed that most of the phosphorylation takes place in intracellular compartments (Fig. 4). The time course for phosphorylation of newly synthesized ATPase is not rapid; by 2 h of chase, pulse-labeled enzyme achieved maximal size on SDS polyacrylamide gels, suggesting arrival at the plasma membrane (Fig. 1 *A*). Consistent with the observation that the time for different proteins to reach the cell surface varies from 3 min to >1 h (Novick and Schekman, 1983), our data suggest that ATPase transit through the secretory pathway is relatively slow.

At present, the kinase(s) involved in ATPase phosphorylation are not clear. However, one likely candidate is a casein kinase associated with the yeast plasma membrane which has been shown to phosphorylate the ATPase on Ser residues in vitro (Kolarov et al., 1988; Yanagita et al., 1987). Indeed, we have noted a number of consensus recognition sequences for casein kinase (Kemp and Pearson, 1990) distributed throughout the 100-kD polypeptide. However, other serine/threonine protein kinases have been described in yeast, including cAMP-dependent (Cannon et al., 1990), Ca^{2+}, calmodulin-dependent (Miyakawa et al., 1989), and most recently Ca^{2+}, phospholipid-dependent (Levin et al., 1990) protein kinases. Since ATPase phosphorylation occurs intracellularly as well as at the plasma membrane, it is quite possible that several different kinases play a role in modifying the ATPase during enzyme maturation.

A major new finding in this study is that glucose-mediated activation of the yeast [H$^+$]ATPase at the plasma membrane occurs by phosphorylation (Fig. 5, *A* and *B*). Previous efforts to detect an overall change in ATPase phosphorylation were unsuccessful (Serrano, 1983; Portillo and Mazon, 1985), but based on the results described above, it is now clear that such measurements are complicated by the extensive constitutive phosphorylation of the 100-kD polypeptide. Instead, we have used phosphopeptide mapping to pinpoint a major peptide (no. 7; Fig. 5 *B*, *arrow*) that is phosphorylated specifically at the plasma membrane, remains phosphorylated during growth on glucose, and is associated with enhanced ATPase activity. Dephosphorylation of this site during glucose starvation occurs concomitantly with a decline in enzy-

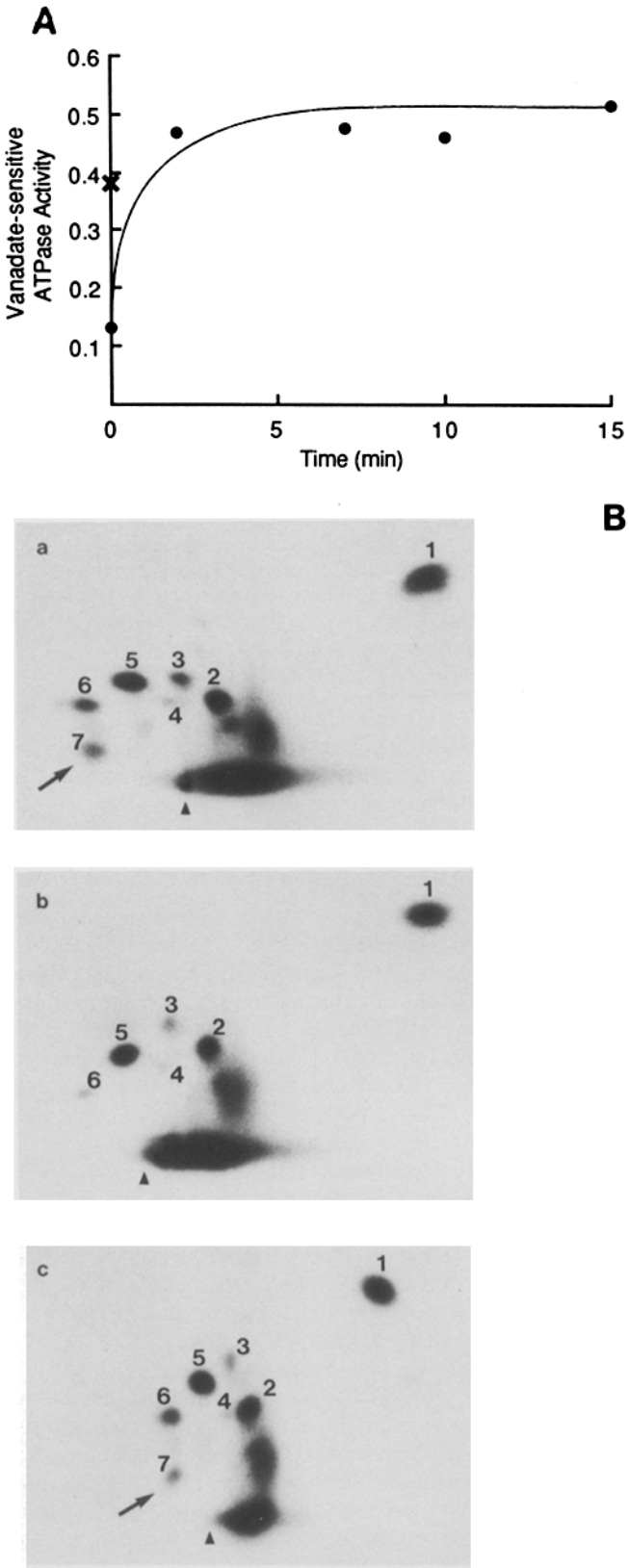

Figure 5. Phosphorylation regulates ATPase activity during glucose starvation and activation. (*A*) Time course of glucose activation. Wild-type cells were incubated in minimal medium without glucose for 30 min, glucose (2%) was added, and vanadate-sensitive ATPase activity (micromoles/milligram/minute) was measured in a total membrane fraction as a function of time (●). Cells maintained in 2% glucose medium for the duration of the experiment were analyzed as a control (*X*). (*B*) Phosphopeptide maps of ^{32}P-labeled ATPase from cells incubated in minimal medium containing 2% glucose (*a*, control), cells starved for glucose for 30 min (*b*, −glucose), and cells that were starved and then fed glucose for 3 min (*c*, +glucose). Numbers 1–7 indicate corresponding peptides. Arrowheads indicate the origins. The arrow indicates the position of the most prominent glucose-regulated phosphopeptide.

matic activity. A coordinate change in the phosphorylation of peptide no. 6 was also detected; it is unclear whether peptides nos. 6 and 7 are incomplete digestion products containing the same phosphorylation site(s), or whether there are several distinct regulatory sites. In addition, we cannot rule out other phosphorylation changes that are not detected by thermolytic mapping. Nevertheless, we believe that glucose-regulated phosphorylation at one or more specific site(s) may represent a gain control that adjusts ATPase activity in response to nutritional signals. It follows that, during glucose starvation, a specific phosphatase may be stimulated to dephosphorylate the ATPase at the regulatory site(s). Interestingly, ATPase mutants with deletion of 11 amino acids from the carboxy terminus or a point mutation in the putative ATP binding domain (Ala_{547} to Val) maintain the kinetic parameters of glucose-activated enzyme even under starvation conditions (Cid and Serrano, 1988; Portillo et al., 1989). In light of our results, it is tempting to speculate that the mutant enzymes display high ATPase activity in the absence of glucose because they cannot undergo dephosphorylation. This hypothesis does not exclude the previously proposed idea that the carboxy terminus constitutes an inhibitory domain whose interaction with the active site is regulated by glucose metabolism (Cid and Serrano, 1988; Portillo et al., 1989).

It has become clear in recent years that phosphorylation/dephosphorylation reactions regulate the activity of many membrane transport proteins. Since the yeast [H$^+$]ATPase is an E_1E_2 enzyme, our results fit well with reports that other members of the class are similarly controlled by phosphorylation (James et al., 1989; Bidwai et al., 1987; Margolis et al., 1990; Lowndes et al., 1990). The functional significance of constitutive, intracellular phosphorylation of the newly synthesized ATPase remains unknown, although we can speculate that phosphorylation at specific sites might control the onset of activity during enzyme maturation. Alternatively, since phosphorylation has been suggested to play a role in intracellular protein sorting (Herman et al., 1991), it is possible that ATPase phosphorylation may mediate its assembly or targeting to the plasma membrane.

We are grateful to Dr. Fred Gorelick for help with phosphopeptide mapping and phosphoamino acid analysis, Kenneth Allen for expert technical assistance, and Drs. Peter Arvan, Peter Novick, and Rajini Rao for helpful suggestions.

This work was supported by National Institutes of Health research grant GM 17561 to C. W. Slayman and postdoctoral fellowship GM 11967 to A. Chang, and an American Heart Association Grant-in-Aid to A. Chang.

Received for publication 26 February 1991 and in revised form 2 July 1991.

References

Bensadoun, A., and D. Weinstein. 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* 70:241–250.

Bidwai, A. P., and J. Y. Takemoto. 1987. Bacterial phytotoxin, syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma-membrane polypeptides. *Proc. Natl. Acad. Sci. USA.* 84:6755–6759.

Brada, D., and R. Schekman. 1988. Coincident localization of secretory and plasma membrane proteins in organelles of the yeast secretory pathway. *J. Bacteriol. (Lond.).* 170:2775–2783.

Cannon, J. F., R. Gitan, and K. Tatchell. 1990. Yeast cAMP-dependent protein kinase regulatory subunit mutations display a variety of phenotypes. *J. Biol. Chem.* 265:11897–11904.

Cid, A., and R. Serrano. 1988. Mutations of the yeast plasma membrane H^+-ATPase which cause thermosensitivity and altered regulation of the enzyme. *J. Biol. Chem.* 263:14134–14139.

Eraso, P., and C. Gancedo. 1987. Activation of yeast plasma membrane ATPase by acid pH during growth. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 224: 187–192.

Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66:375–400.

Fujiyama, A., and F. Tamanoi. 1986. Processing and fatty acylation of ras1 and ras2 proteins in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 83:1266–1270.

Gillies, R. J., K. Ugurbil, J. A. den Hollander, and R. G. Shulman. 1981. ^{31}P NMR studies of intracellular pH and phosphate metabolism during the cell division cycle of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 78:2125–2129.

Hager, K. M., S. M. Mandala, J. W. Davenport, D. W. Speicher, E. J. Benz, Jr., and C. W. Slayman. 1986. Amino acid sequence of the plasma membrane ATPase of *Neurospora crassa*: deduction from genomic and cDNA sequences. *Proc. Natl. Acad. Sci. USA.* 83:7693–7697.

Herman, P. K., J. H. Stack, J. A. DeModena, and S. D. Emr. 1991. A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. *Cell.* 64:425–437.

Holcomb, C. L., W. J. Hansen, T. Etchverry, and R. Schekman. 1988. Secretory vesicles externalize the major plasma membrane ATPase in yeast. *J. Cell Biol.* 106:641–648.

James, P. H., M. Pruschy, T. E. Vorherr, J. T. Penniston, and E. Carafoli. 1989. Primary structure of the cAMP-dependent phosphorylation site of the plasma membrane calcium pump. *Biochemistry.* 28:4253–4258.

Kemp, B. E., and R. B. Pearson. 1990. Protein kinase recognition sequence motifs. *Trends Biochem. Sci.* 15:342–346.

Kolarov, J., J. Kulpa, M. Baijot, and A. Goffeau. 1988. Characterization of a protein serine kinase from yeast plasma membrane. *J. Biol. Chem.* 263:10613–10619.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lai, Y., A. C. Nairn, F. Gorelick, and P. Greengard. 1987. Ca^{2+}/calmodulin-dependent protein kinase II: Identification of autophosphorylation sites responsible for generation of Ca^{2+}/calmodulin-independence. *Proc. Natl. Acad. Sci. USA.* 84:5710–5714.

Levin, D. E., F. O. Fields, R. Kunisawa, J. M. Bishop, and J. Thorner. 1990. A candidate protein kinase C gene, PKC1, is required for the *S. cerevisiae* cell cycle. *Cell.* 62:213–224.

Lowndes, J. M., M. Hokin-Neaverson, and P. J. Bertics. 1990. Kinetics of phosphorylation of Na^+/K^+-ATPase by protein kinase C. *Biochim. Biophys. Acta.* 1052:143–151.

Margolis, R. N., M. J. Schell, S. I. Taylor, and A. L. Hubbard. 1990. Hepatocyte plasma membrane ECTO-ATPase (pp120/HA4) is a substrate for tyrosine kinase activity of the insulin receptor. *Biochem. Biophys. Res. Commun.* 166:562–566.

McDonough, J. P., and H. P. Mahler. 1982. Covalent phosphorylation of the Mg^{2+}-dependent ATPase of yeast plasma membranes. *J. Biol. Chem.* 257:14579–14581.

Miyakawa, T., Y. Oka, E. Tsuchiya, and S. Fukui. 1989. *Saccharomyces cerevisiae* protein kinase dependent on Ca^{++} and calmodulin. *J. Bacteriol. (Lond.).* 171:1417–1422.

Nairn, A. C., and P. Greengard. 1987. Purification and characterization of Ca^{+2}/calmodulin-dependent protein kinase I from bovine brain. *J. Biol. Chem.* 262:7273–7281.

Nakamoto, R. K., R. Rao, and C. W. Slayman. 1991. Expression of the yeast plasma membrane $[H^+]$ATPase in secretory vesicles: a new strategy for directed mutagenesis. *J. Biol. Chem.* 266:7940–7949.

Novick, P., and R. Schekman. 1983. Export of major cell surface proteins is blocked in yeast secretory mutants. *J. Cell Biol.* 96:541–547.

Perona, R., and R. Serrano. 1988. Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. *Nature (Lond.).* 334:438–440.

Portillo, F., and M. J. Mazon. 1985. Activation of yeast plasma membrane ATPase by phorbol ester. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 192:95–98.

Portillo, F., I. F. de Larrinoa, and R. Serrano. 1989. Deletion analysis of yeast plasma membrane H^+-ATPase and identification of a regulatory domain at the carboxyl-terminus. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 247:381–385.

Serrano, R. 1983. In vivo glucose activation of the yeast plasma membrane ATPase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 156:11–14.

Serrano, R. 1988. H^+-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. *Methods Enzymol.* 157:533–544.

Serrano, R. 1989. Structure and function of plasma membrane ATPase. *Annu. Rev. Plant Physiol.* 40:61–94.

Serrano, R., M. C. Kielland-Brandt, and G. R. Fink. 1986. Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+,K^+),K^+- and Ca^{2+}-ATPases. *Nature (Lond.).* 319:689–693.

Smith, K. E., and G. G. Hammes. 1988. Studies of the phosphoenzyme intermediate of the yeast plasma membrane proton-translocating ATPase. *J. Biol. Chem.* 263:13774–13778.

Tschopp, J., P. C. Esmon, and R. Schekman. 1984. Defective plasma membrane assembly in yeast secretory mutants. *J. Bacteriol. (Lond.).* 160: 966–970.

Walworth, N. C., and P. N. Novick. 1987. Purification and characterization of constitutive secretory vesicles from yeast. *J. Cell Biol.* 105:163–174.

Yanagita, Y., M. Abdel-Ghany, D. Raden, N. Nelsen, and E. Racker. 1987. Polypeptide-dependent protein kinase from bakers' yeast. *Proc. Natl. Acad. Sci. USA.* 84:925–929.