Tandem Phosphorylation of Ser-911 and Thr-912 at the C Terminus of Yeast Plasma Membrane H\(^{+}\)-ATPase Leads to Glucose-dependent Activation*\^[5]

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In recent years there has been growing interest in the post-translational regulation of P-type ATPases by protein kinase-mediated phosphorylation. Pma1 H\(^{+}\)-ATPase, which is responsible for H\(^{+}\)-dependent nutrient uptake in yeast (Saccharomyces cerevisiae), is one such example, displaying a rapid 5–10-fold increase in activity when carbon-starved cells are exposed to glucose. Activation has been linked to Ser/Thr phosphorylation in the C-terminal tail of the ATPase, but the specific phosphorylation sites have not previously been mapped. The present study has used nanoflow high pressure liquid chromatography coupled with electrospray electron transfer dissociation tandem mass spectrometry to identify Ser-911 and Thr-912 as two major phosphorylation sites that are clearly related to glucose activation. In carbon-starved cells with low Pma1 activity, peptide 896–918, which was derived from the C terminus upon Lys-C proteolysis, was found to be singly phosphorylated at Thr-912, whereas in glucose-metabolizing cells with high ATPase activity, the same peptide was doubly phosphorylated at Ser-911 and Thr-912. Reciprocal \(^{14}N/\(^{15}N\) metabolic labeling of cells was used to measure the relative phosphorylation levels at the two sites. The addition of glucose to carbon-starved cells led to a 3-fold reduction in the singly phosphorylated form and an 11-fold increase in the doubly phosphorylated form. These results point to a mechanism in which the stepwise phosphorylation of two tandemly positioned residues near the C terminus mediates glucose-dependent activation of the H\(^{+}\)-ATPase.

Pma1 H\(^{+}\)-ATPase of Saccharomyces cerevisiae belongs to the widely distributed family of P\(_{2}\)-type cation pumps, which include the sarcoplasmic reticulum and plasma membrane Ca\(^{2+}\)-ATPases and the Na\(^{+}\),K\(^{+}\)-ATPase of animal cells and the plasma membrane H\(^{+}\)-ATPase of higher plants (1). Like other members of the P\(_{2}\) family, the 100-kDa yeast ATPase is folded into 3 cytoplasmic domains (N, P, and A) that form the catalytic portion of the molecule, anchored in the lipid bilayer by 10 transmembrane helices that comprise the ion transport pathway (2). Pma1 ATPase acts physiologically to pump protons out of the cell, creating the electrochemical gradient that drives solute uptake by an array of H\(^{+}\)-coupled cotransporters. Its activity is strongly regulated, reflecting both its essential role in cell growth and the fact that, as the most abundant protein in the plasma membrane, it consumes at least 20% of cellular ATP (3).

The first evidence for Pma1 regulation was reported in 1983 by Serrano (4), who found that the addition of glucose to carbon-starved yeast cells led within minutes to a 5–10-fold increase in ATPase activity accompanied by a severalfold lowering of the \(K_{m}\) for MgATP and an alkaline shift in pH optimum. Given the rapidity of the activation, it seemed likely to occur at the post-translational level. Direct evidence for this idea came in 1991, when Chang and Slayman (5) immunoprecipitated Pma1 ATPase from \(^{32}P\)-labeled yeast cells and analyzed it by two-dimensional phosphopeptide mapping. Against a background of “constitutive” phosphorylation, which occurred in a stepwise fashion as newly synthesized ATPase moved from the endoplasmic reticulum through the Golgi and secretory vesicles to the cell surface, additional phosphorylation took place when glucose was added to carbon-starved cells. Complete digestion of the \(^{32}P\)-labeled ATPase revealed both phosphoserine and phosphothreonine but no detectable phosphotyrosine (5). These results strongly supported the idea that glucose activation of the ATPase was related to kinase-mediated phosphorylation at one or more Ser/Thr residues, although the location of the phosphorylation sites had yet to be determined.

In parallel, studies in several laboratories sought to identify the domain(s) of the 100-kDa Pma1 ATPase that participates in glucose-dependent regulation (for review, see Ref. 6). Attention soon focused on the 45-amino acid C-terminal tail, which is known to protrude from the plasma membrane into the cytoplasm (7). Removal of the last 18 amino acids by mutagenesis led to constitutive activation of the ATPase even in the absence of glucose (8, 9). Consistent with this observation, immuno-...
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blotting with anti-C-terminal antibody revealed that the C terminus was relatively inaccessible during carbon starvation but could be rapidly digested by trypsin upon the addition of glucose (10). Of added significance was the fact that amino acid substitutions within the tail, especially at Thr-912, reduced glucose activation in a manner that could be suppressed by second-site mutations elsewhere in the 100-kDa protein (11, 12). Replacing Thr-912 with Ala (to prevent phosphorylation) arrested the ATPase in a trypsin-resistant state characteristic of carbon-starved wild-type cells, whereas replacing it with Asp (to mimic phosphorylation) locked the ATPase in a trypsin-sensitive state typical of glucose-activated wild-type cells (10). Taken together, these results pointed to a key regulatory role of the C-terminal tail, which appears to act in an autoinhibitory fashion during glucose starvation, binding tightly to regions of the N and/or P domains to inhibit ATPase activity. Upon the addition of glucose, the C-terminal tail is released from autoinhibition, most likely through phosphorylation, and allows the ATPase to assume an open, activated conformation.

In the work described below, we have used an ensemble of state-of-the-art mass spectrometric methods to map phosphorylation sites throughout the 100-kDa Pma1 ATPase, with the aim of directly identifying the site(s) responsible for glucose activation. The results point conclusively to Ser-911 and Thr-912 in the C-terminal tail, acting in concert as phosphorylation sites that mediate the glucose effect.

EXPERIMENTAL PROCEDURES

Yeast Strains—Strain NY13 of S. cerevisiae (MATa ura3-52) was used in this study. For metabolic labeling (see below) the wild-type LRA3 allele was integrated downstream from the PMA1 locus to confer growth in the absence of added uracil.

Preparation of Plasma Membranes from Carbon-starved and Glucose-metabolizing Cells—Cells were grown to mid-exponential phase at 30 °C in minimal medium containing 4% glucose, harvested, washed twice with water, and incubated with a nitrogen atmosphere at 30 °C in minimal medium containing 4% glucose (10). Of added significance was the fact that amino acid substitutions within the tail, especially at Thr-912, reduced glucose activation in a manner that could be suppressed by second-site mutations elsewhere in the 100-kDa protein (11, 12). Replacing Thr-912 with Ala (to prevent phosphorylation) arrested the ATPase in a trypsin-resistant state characteristic of carbon-starved wild-type cells, whereas replacing it with Asp (to mimic phosphorylation) locked the ATPase in a trypsin-sensitive state typical of glucose-activated wild-type cells (10). Taken together, these results pointed to a key regulatory role of the C-terminal tail, which appears to act in an autoinhibitory fashion during glucose starvation, binding tightly to regions of the N and/or P domains to inhibit ATPase activity. Upon the addition of glucose, the C-terminal tail is released from autoinhibition, most likely through phosphorylation, and allows the ATPase to assume an open, activated conformation.

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Preparation of Plasma Membranes from Carbon-starved and Glucose-metabolizing Cells—Cells were grown to mid-exponential phase at 30 °C in minimal medium containing 4% glucose, harvested, washed twice with water, and incubated with 1 h in water without glucose. Glucose (4%) was then added back to an aliquot of the culture for 30 min (glucose-metabolizing cells (GM)), whereas another aliquot was kept in the nitrogen atmosphere at 30 °C in minimal medium containing 4% glucose (10). Of added significance was the fact that amino acid substitutions within the tail, especially at Thr-912, reduced glucose activation in a manner that could be suppressed by second-site mutations elsewhere in the 100-kDa protein (11, 12). Replacing Thr-912 with Ala (to prevent phosphorylation) arrested the ATPase in a trypsin-resistant state characteristic of carbon-starved wild-type cells, whereas replacing it with Asp (to mimic phosphorylation) locked the ATPase in a trypsin-sensitive state typical of glucose-activated wild-type cells (10). Taken together, these results pointed to a key regulatory role of the C-terminal tail, which appears to act in an autoinhibitory fashion during glucose starvation, binding tightly to regions of the N and/or P domains to inhibit ATPase activity. Upon the addition of glucose, the C-terminal tail is released from autoinhibition, most likely through phosphorylation, and allows the ATPase to assume an open, activated conformation.

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Sample Preparation for MS Analysis—Plasma membranes (100 μg) from CS and GM cells were individually subjected to SDS-PAGE and stained with Coomassie Blue, and the 100-kDa Pma1 band was excised, cut into 1-mm² pieces, and placed in a siliconized tube. The gel pieces were destained with brief washes in 100 mM NH₄HCO₃, 50% methanol, then dehydrated for 5 min in 25 mM NH₄HCO₃, 50% acetonitrile and for 30 s in 100% acetonitrile and dried in a vacuum centrifuge for 5 min. Samples were rehydrated in a solution containing 25 mM dithiothreitol in 25 mM NH₄HCO₃ and reduced for 20 min at 56 °C. Protein alkylation was performed by adding freshly prepared iodoacetamide in 25 mM NH₄HCO₃ and incubating for 20 min at room temperature in the dark. Gel pieces were dehydrated and dried again as described above.

To perform in-gel digestion, the gel pieces were rehydrated in 25 mM NH₄HCO₃, 3% acetonitrile containing 20 ng/μl of trypsin (Promega sequencing grade modified), Lys-C (Roche Applied Science sequencing grade), chymotrypsin (Worthington), Glu-C, or Asp-N (Sigma) and incubated overnight at 37 °C. Digested peptides were extracted by vortexing for 15 min in 0.1% trifluoroacetic acid. A second extraction was performed using 70% acetonitrile, 25% H₂O, 5% trifluoroacetic acid. Extracted peptides from survey samples were dried in a vacuum centrifuge and reconstituted in 0.1% trifluoroacetic acid (15). The samples were then solid phase-extracted using C18-Zip-Tips (Millipore).

Metabolic Labeling with a Stable Isotope—Non-labeled cells (for the 15N samples) were grown in minimal medium prepared with regular yeast nitrogen base lacking amino acids. For the 15N-labeled samples, cells were grown in minimal medium prepared with yeast nitrogen base lacking amino acids and ammonium sulfate to which [15N]ammonium sulfate (99 atom%; Isotec) had been added. The rest of the experiment was carried out as described under “Results.”

MALDI TOF/TOF Mass Spectrometry—A Sciex 4800 MALDI TOF/TOF (Applied Biosystems) was used to analyze the in-gel digests. An MS scan was conducted from 700 to 4000 m/z, and the 10 most abundant peaks with signal to noise ratios >10 were selected for tandem mass spectrometry (MS/MS) experiments. Data were analyzed using Explorer 4000 software (Version 3.6) and searched with Mascot 2.1 (Matrix Science) against a list of yeast protein sequences downloaded from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) (16). Searches allowed up to two missed cleavages for a given enzyme and MS and MS/MS tolerances of ±0.4 Da. Variable modifications included carbamidomethylation of cysteine, deamidation of asparagine and glutamine, oxidation of methionine, phosphorylation of serine, threonine, and tyrosine, and N-terminal acetylation of the protein.

Three-dimensional Ion-trap Mass Spectrometry—In-gel-digested samples were analyzed with an Agilent 1100 series LC/MSD ion trap mass spectrometer. Samples were loaded with an autosampler (Agilent) onto a C18 reverse-phase trap cartridge (Agilent). After loading, the trap cartridge was switched in-line with an analytical 75-μm × 150-mm column packed with 3.5-μm Zorbax C₁₈ reversed-phase particles (Agi-
lent) and a gradient conducted with an Agilent 1100 series capillary HPLC system. Peptides were eluted with a gradient of buffer A (0.1% v/v formic acid) and buffer B (95% v/v acetonitrile and 0.1% v/v formic acid) by 5–60% buffer B in 90 min. The instrument was operated in a data-dependent fashion with an MS survey scan (300–2200 m/z) followed by MS/MS or MS/MS/MS analysis (300–2200 m/z) of as many as the five most intense peaks with dynamic exclusion for 120 s of fragmented m/z values. All ion trap MS2 and MS3 data were converted to Mascot generic files with DataAnalysis 2.2 (Agilent) using default settings. Mascot generic format (MGF) files were then searched using Mascot with the same parameters as described above except that MS and MS/MS tolerances were set to ±0.8 Da.

**QTOF Mass Spectrometry**—Trypsin, Lys-C, chymotrypsin, and Glu-C digests were analyzed using a QTOF2 mass spectrometer (Micromass) coupled to an HP 1100 HPLC (Agilent). Separations were conducted using home-pulled fused silica columns (100 μm × 10 cm) packed with Eclipse C18 particles (Agilent) and eluted at ~500 nl/min with a gradient of buffer A (0.1% formic acid v/v) and buffer B (95% v/v acetonitrile, 0.1% v/v formic acid). After loading samples in 5% buffer B, the gradient was 5–12% buffer B in 10 min, 12–50% buffer B in 105 min, 50–60% buffer B in 5 min, and 60–100% buffer B in 5 min. The instrument was operated in a data-dependent fashion with an MS scan (400–2200 m/z) with as many as 2 m/z values selected for MS/MS experiments (50–2200 m/z). After being selected for tandem MS, individual m/z peaks within 2 Da were dynamically excluded for 120 s. QTOF MS/MS data were converted to peak list files using ProteinLynx Global Server 2.15 (Waters). The MS and MS/MS scans were smoothed twice with a Savitzky-Golay smooth. MS scans were background-subtracted using the standard method with a first-order polynomial, whereas MS/MS data were background-subtracted with the adaptive method and a fifth-order polynomial, and the data were saved as peak lists. Peak list files were then searched with Mascot using the same parameters as described above except that MS and MS/MS tolerances were set to ±0.3 Da.

**Isotopic Measurements**—Metabolizing cells were grown in standard (14N) medium and starving cells in 15N-enriched medium (two experiments), and a third experiment was performed but with the labels reversed. Combined samples were analyzed by QTOF mass spectrometry (see above), and data were processed as previously described (17). Briefly, samples were searched using Mascot 2.0 with amino acid masses corresponding to natural abundance (14N) and then searched again with amino acid masses corresponding to 15N-labeling. Both sets of searches were conducted with the same modifications as those used for isotopically unlabeled samples except that deamidation was not allowed in the 15N searches because this modification would be indistinguishable due to the isotopic label. The results from heavy and light searches were combined, and extracted ion chromatograms were generated 0.25 m/z wide for the monoisoitopic m/z value and the 100% 15N incorporation of identified peptides. In addition, extracted ion chromatograms were generated for m/z values corresponding to the double-, triple-, quadruple, and quintuple-charged forms of the 390aSTRSVEDFMAAMQRVSTQHET3918 peptide with zero, one, and two phosphate moieties. Linear regression was used to calculate a 15N/14N ratio using extracted ion chromatogram values for the monoisotopic peak and the peak corresponding to the peptide with 100% 15N incorporation using Mathematica 5.1 (Wolfram Research). Ratios with an R-value greater than 0.85 were used to determine GM:CS measurements. For a given phosphorylation state of the peptide, the measurements from all charge states were averaged. Because the monoisotopic peak and the 100% 15N incorporation peak represent different fractions of their respective isotopic envelopes, a correction was calculated and applied to the measured ratio.

**Localization of Phosphorylation Sites by Electron Transfer Dissociation (ETD)-MS/MS**—An ETD-enabled ThermoFisher linear ion trap-orbitrap hybrid mass spectrometer was employed to determine the exact sites of phosphorylation on peptides P1 and P2 from CS and GM samples (18). Peptide separations were performed on a reversed-phase, self-prepared capillary column. The separation column consisted of a precolumn that was butt-connected to an analytical column using a 0.012-inch inner diameter Teflon sleeve (Zeus Industrial Products, Orangeburg, SC). The analytical column was 360 μm × 50 μm (outer diameter × inner diameter) fused silica (Polymerico Technologies, Phoenix, AR) and was prepared by pulling a bottle neck and integrated ESI tip using a laser puller (Sutter Instrument Co., Novarto, CA, model P-2000) as described elsewhere (19). Approximately 1–2 mm of 5–20-μm C18 particles (YMC, Milford, MA) followed by 7 cm of 5-μm C18 particles (Alltech Associates Inc., Deerfield, IL) were packed into the analytical column. The precolumn was made of 360-μm × 75-μm (outer diameter × inner diameter) fused silica and incorporated a LiChrosorb frit (EMD Chemicals Inc., Gibbstown, NJ) of ~2 mm in length. The precolumn was then packed with 5 cm of 5-μm C18 particles.

Approximately 2 pmol of Lys-C-digested Pma1 were loaded onto the column, and on-line peptide separations were performed with an Agilent 1100 Series binary HPLC system coupled to the ETD-enabled orbitrap. The sample was eluted from the column at a flow rate of 60 nl/min into the mass spectrometer using a linear gradient of buffer A (0.1 M acetic acid) and buffer B (70% v/v acetonitrile in 0.1 M acetic acid) at 0–100% B in 60 min. The mass spectrometer was operated in a targeted ion fashion. First, a full scan (300–2000 m/z) mass spectrum was acquired using the orbitrap at a nominal resolving power of 30,000. The following precursor cations were isolated and subjected to a 100-ms ETD reaction: 668.1, 676.6, 688.1, 696.6, 708.1, 716.6 m/z. These ions corresponded to the unphosphorylated, singly phosphorylated, and doubly phosphorylated forms of the 896–918 peptide that were 14N- and 15N-labeled in the [M + 4H]4+ charged state. Relevant scans were manually sequenced.

**RESULTS**

**Recovery of Peptide Fragments for Analysis**—As described in the Introduction, the central goal of this study was to identify sites of protein phosphorylation that are mechanistically related to the activation of Pma1 ATPase by glucose. The first step was to prepare plasma membranes from CS and GM cells, purify the ATPase, subject it to in-gel proteolytic digestion, and perform mass spectrometric analyses.

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To maximize coverage of the protein, five different proteases were used: trypsin, Lys-C, chymotrypsin, Glu-C, and Asp-N. Fig. 1 is a topological map of the ATPase showing that peptides corresponding to 78% of the cytoplasmic part of the protein were identified (see the supplemental Appendix for a complete table of results). Most of the undetected peptides were located in the 10 transmembrane segments of the ATPase, where the predicted proteolytic fragments are generally too large and/or too hydrophobic to be observed by MS analysis. Two regions of the N-terminal extension (Asp-31—Gly-70 and Asp-83—Lys-98) and the short loop between transmembrane segments 6 and 7 were also missing from the list of recovered peptides.

Differential Phosphorylation in the C-terminal Tail Correlates with Glucose Activation of Pma1 —As illustrated in Fig. 2, peptide 896–918 (P0; \(m/z\) 890.5, 2671.5 Da), obtained after digestion with Lys-C, was identified by LC-MS/MS using the three-dimensional ion trap mass spectrometer in both CS and GM samples. This peptide corresponds to the C-terminal tail of the ATPase: \(\text{pHis}^\text{6-STRSVEFMAAMQRVSTQHKET}^{918}\). Both CS and GM samples also contained peptide 896–916 (2437.15 Da), in which Lys-C cleavage had occurred at Lys-916. The signal for 896–916 was much less intense than that for 896–918, presumably reflecting the fact that cleavage at Lys-916 was strongly inhibited by the flanking Glu residues (20). Furthermore, peptide 896–916 is found in Pma2, a poorly expressed version of peptide 896–918, presumably reflecting the fact that cleavage at Lys-916 was strongly inhibited by the flanking Glu residues (20). Therefore, peptide 896–918 is unique to Pma1. For both reasons, most of the analysis focused on 896–918.

In addition to P0, both the carbon-starved and glucose-metabolizing samples contained a peptide whose mass was −80 Da heavier than the unmodified 896–918, as expected for a singly phosphorylated form (P1; \(m/z\) 917.2). The glucose-metabolizing sample also contained a peptide whose mass was consistent with a doubly phosphorylated form of 896–918 (P2; \(m/z\) 943.8). Neither sample gave any detectable evidence for higher multiples of phosphorylation of peptide 896–918.

When peptides P1 and P2 were analyzed by MS/MS on the three-dimensional ion trap, the spectrum for P1 displayed a single dominant peak corresponding to a neutral loss of 98 Da from the parent peptide, whereas the spectrum for P2 showed two abundant peaks, corresponding to the loss of one and two phosphate moieties (Fig. 3). This is the expected signature for phosphopeptides. Unlike most peptides that undergo random protonation along the backbone in the gas phase to generate a series of sequence-informative fragments by collisional activation, phosphopeptides are preferentially cleaved at the phosphate group, leaving the peptide backbone intact (22).

Taken together the results are consistent with the idea that the C-terminal tail of Pma1 ATPase is singly phosphorylated in carbon-starved cells and undergoes a second phosphorylation during glucose activation. However, the lack of peptide backbone fragmentation upon collisional activation made it impossible to locate the sites of phosphorylation in the P1 and P2 peptides by this approach.

Locating the Phosphorylation Sites —We, therefore, turned to a new peptide fragmentation method known as ETD (18, 22–25) to identify the two phosphorylation sites. ETD is performed in an ion-trap mass spectrometer by reacting isolated peptide cation precursors with small-molecule radical anions. The reaction results in the transfer of an electron to the peptide cation, which then dissociates randomly to generate a series of peptide backbone cleavages while preserving labile posttranslational modifications such as phosphorylation. The present study employed a new instrument equipped with a secondary orbitrap mass analyzer, allowing the intact peptide mass to be recorded with high \(m/z\) resolution and accuracy.

![Topological diagram of yeast Pma1 H\textsuperscript{+}-ATPase illustrating the proteolytic peptides recovered in this study. The 100-kDa ATPase was gel-purified from yeast plasma membranes, in-gel digested with trypsin, Lys-C, chymotrypsin, Glu-C, or Asp-N, and analyzed by three-dimensional ion-trap, MALDI TOF/TOF, and QTOF mass spectrometry. Identified peptides (highlighted in gray) represent 78% of the cytoplasmic portion of the ATPase.](image)
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Ser-911 and Thr-912, the C-terminal tail of Pma1 ATPase contains two more serine residues (Ser-896, Ser-899) and two more threonines (Thr-897 and Thr-918). ETD MS/MS sequencing of P1 and P2 gave no evidence for phosphorylation at any of these sites. Furthermore, although phosphorylation at Ser-896 may have inhibited cleavage by Lys-C protease at Lys-895, MS data analysis showed no sign of the corresponding peptide. Thus, Thr-912 appears to be the only site of C-terminal phosphorylation under carbon-starved conditions, and Ser-911, the only site that becomes phosphorylated during glucose activation.

**Stable Isotope Labeling to Determine the Extent of Phosphorylation at Ser-911 and Thr-912**—Neither Ser-911 nor Thr-912 appears to be fully phosphorylated in vivo, since the unmodified peptide (P0) was present in both carbon-starved and glucose-metabolizing samples despite the presence of phosphatase inhibitors at each step of membrane isolation. We examined the extent of phosphorylation in greater detail using stable isotope labeling, in which \(^{15}\)N was incorporated metabolically into CS or GM cells. Differentially labeled CS and GM samples were combined and analyzed together with the known mass difference making it possible to distinguish CS- and GM-derived peptides and compare each pair of peptides quantitatively.

Fig. 6 illustrates the experimental design in which two yeast cultures were grown; that is, one in standard medium (\(^{14}\)N) and the other in medium containing isotopically labeled (NH\(_4\))\(_2\)SO\(_4\) as the only nitrogen source (\(^{15}\)N). CS and GM cells were then prepared from both cultures. Before isolating plasma membranes, CS cells from the \(^{14}\)N culture were mixed with an equal quantity of GM cells from the \(^{15}\)N culture to give sample A. Conversely, GM cells from the \(^{14}\)N culture were mixed with an equal quantity of CS cells from the \(^{15}\)N culture to give sample B. Plasma membranes were prepared from both samples and used for gel purification of Pma1 ATPase, which was then digested with Lys-C for MS analysis.

Fig. 7 displays the non-phosphorylated (P0), singly phosphorylated (P1), and doubly phosphorylated (P2) forms of peptide 896–918 that were seen in sample A and sample B. Each phosphorylation state was observed as a pair of isotopic envelopes, the lower m/z component corresponding to the unlabeled peptide and the higher m/z component corresponding to the \(^{15}\)N-labeled peptide. As can be seen in the figure, the incorporation of \(^{15}\)N was very efficient (99% when calculated as described in Nelson et al. 17), resulting in the complete separation of the two clusters. The CS and GM envelopes can, thus, be clearly distinguished from one another based on their content of either the lighter \(^{14}\)N or heavier \(^{15}\)N isotope. Because pairs of \(^{14}\)N- and \(^{15}\)N-containing peptides are otherwise chemically identical, the intensities of the two envelopes could be compared to obtain information about the relative abundance of CS and GM forms in the sample. The mixing ratio of CS and GM samples was verified to be 1:1 by comparing multiple pairs of unphosphorylated Pma1 peptides.

MS analysis of the 896–918 peptide confirmed that a fraction of Pma1 ATPase was not phosphorylated at either Ser-911 or Thr-912 (P0 form); this fraction was equal in CS and GM cells (Fig. 7, left-hand panels). By contrast, the singly phosphorylated peptide (P1) was ~3-fold more abundant in the CS sample than in the GM sample (Fig. 7, middle panels), whereas the doubly

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**FIGURE 3. Tandem mass spectra (MS/MS) from three-dimensional ion-trap MS of P1 and P2 confirm that they are phosphorylated versions of peptide 896–918.** Lys-C-digested samples from GM cells were used for this experiment. The MS/MS spectrum for the triply charged version of peptide P1 (917.1 m/z) shows a dominant ion (884.4 m/z), which corresponds to the neutral loss of 98 Da (phosphoric acid) from the parent peptide; the neutral loss is seen here as a shift of 32.7 m/z on a triply charged peptide: 32.7 × 3 = 98.1. The MS/MS spectrum for the triply charged version of P2 shows two abundant ions corresponding to the loss of one and two phosphoric acids. Int., intensity.

Peptide P1 was sequenced from an \(^{14}\)N-labeled CS sample and an \(^{15}\)N-labeled GM sample that had been mixed together before the isolation of plasma membranes (see next section). Inspection of the ETD-MS/MS spectra revealed that \(^{14}\)N-labeled peptide P1 from CS cells was phosphorylated at Thr-912 (Fig. 4A). Identical results were seen for the \(^{15}\)N-labeled P1 from GM cells (Fig. 4B). By contrast, peptide P2, which was found only in samples from GM cells, was phosphorylated at both Ser-911 and Thr-912 (Fig. 5A–C). In parallel, the shorter peptide 896–916 from GM samples was also found to be phosphorylated at both Ser-911 and Thr-912 (Fig. 5D). Thus, Thr-912 appears to be a constitutive site of phosphorylation in Pma1 ATPase, whereas Ser-911 becomes phosphorylated during glucose activation.

To make certain that Thr-912 was the only phosphorylation site in peptide P1, the c- and z-type ions were carefully examined along the peptide backbone. The c-type ion series did not exhibit the +80 addition until c\(_{17}\); likewise, the z-type ion series exhibited a +80 addition at z\(_{17}\). To rule out the possibility of a second isoform, singly phosphorylated at Ser-911, we searched for the presence of unmodified c\(_{2}\) and z\(_{17}\), but no m/z peaks corresponding to those values were detected. In addition to
phosphorylated peptide (P2) was \(11\)-fold more abundant in GM than in CS (Fig. 7, right-hand panels). Because the amount of P0 was the same in the two samples, the total amount of phosphorylated peptide (P1 + P2) must also have been the same. One can, therefore, calculate that \(93\%\) of the modified fraction of the ATPase was singly phosphorylated under carbon-starving conditions, whereas \(70\%\) was doubly phosphorylated during glucose metabolism. Thus, most of the singly phosphorylated form was converted to a doubly phosphorylated form upon glucose activation of the protein.

**Phosphorylation Sites Elsewhere in Pma1 ATPase**—Two other possible phosphorylation sites were discovered in Pma1 ATPase during the course of this study. In one case, evidence came from the initial three-dimensional ion trap MS/MS analysis, which pointed to post-translational phosphorylation near the N-terminal end of the protein. The situation was clearest in samples from CS cells, where the N-terminal peptide was detected by three-dimensional ion-trap MS/MS after digestion with trypsin (peptide 2–28; \(m/z\) 893.7; 2678.2 Da) (supplemental Appendix and Table S1A). As shown in the same table, a singly phosphorylated version of this peptide, characterized by a neutral loss of 98 Da, was also readily detected in CS samples. However, the presence of a long stretch of Ser and several Thr residues in the sequence (2TDTSSSSSSASSVSHQPTQEKPAK) has so far prevented the identification of the phosphorylated residue.

Peptide 2–28 was never seen in samples from GM cells, suggesting the possibility of a second glucose-dependent phosphorylation that inhibited proteolysis in the N-terminal region.
Indeed, when GM samples were treated with phosphatase before proteolytic digestion, peptide 2–28 was now observed. One attractive candidate for phosphorylation is Thr-29, where the introduction of a negatively charged phosphoryl group might be expected to hinder cleavage at Lys-28; however, trypsinolysis of a GM sample from a T29A mutant strain still failed to show any evidence of cleavage at Lys-28. Thus, the putative glucose-dependent phosphorylation site appears to lie elsewhere in the N-terminal region, and further work will be required to locate it.

**DISCUSSION**

**Ser/Thr Phosphorylation Sites Identified in Pma1 ATPase**—As described in the Introduction, it has been known for more than a decade that yeast Pma1 H^{+}-ATPase is phosphorylated at multiple serine and threonine residues (5). The present study set out to map the phosphorylation sites, with the specific goal of identifying a site(s) responsible for the activation of the enzyme by glucose. To maximize coverage of the ATPase, the gel-purified 100-kDa band was digested with four different proteases, and the resulting peptides were analyzed by MALDI TOF/TOF, ion-trap, and QTOF mass spectrometry. Overall, peptides representing 78% of the cytoplasmic part of the protein were recovered and identified.

The most prominent family of phosphopeptides came from the C-terminal tail of the ATPase and gave direct evidence that phosphorylation in this region mediates glucose activation. Using ETD, it was possible to pinpoint Thr-912 as a constitutive site of phosphorylation and the immediately adjacent residue, Ser-911, as a regulated, glucose-dependent site. The roles of Ser-911 and Thr-912 in activation of the ATPase will be discussed below.

In addition, a constitutive phosphorylation site was detected near the amino terminus of Pma1 ATPase along with indirect evidence for a second site in the same region that may be phosphorylated (or otherwise modified) in the presence of glucose. The N terminus is unlikely to play a major role in glucose activation, however, since the deletion of residues 1–27 has no significant effect on the responsiveness of the ATPase to glucose (8). No clear sign of phosphorylation was seen elsewhere in Pma1 ATPase, but it should be noted that coverage of the Pma1 protein was not complete and that detection of phosphopeptides can be difficult due to low stoichiometric abundance and low ionization efficiencies in the positive mode.

**Mechanism of Glucose Activation by Phosphorylation at Ser-911 and Thr-912**—The major outcome of this study has been to demonstrate a key regulatory role for Ser-911 and Thr-912, located at positions 8 and 7 from the C terminus of yeast Pma1 ATPase. Both residues are conserved in all known fungal Pma-type ATPases, and mutagenesis studies have suggested Thr-912 as a possible regulatory site (11). Mass spectrometric data now provide direct evidence that they are both phosphorylated; Thr-912, constitutively, and Ser-911 within minutes after the addition of glucose to the growth medium. As described previously, glucose addition leads to a 5–10-fold stimulation in the rate of ATPase hydrolysis accompanied by a severalfold increase in the apparent affinity for MgATP (4). Thus, the sequential phosphorylation of Thr-912 and Ser-911 appears to shift the ATPase from a low activity, low affinity form under energy-restricted conditions to a high activity, high affinity form when ATP is no longer rate-limiting.

To look quantitatively at the behavior of Ser-911 and Thr-912 during glucose regulation, 14N/15N metabolic labeling was employed to measure the relative amounts of unphosphorylated, singly phosphorylated, and doubly phosphorylated forms of peptide 896–918 in ATPase prepared from carbon-starved and glucose-metabolizing cells. The results revealed a pool of the non-phosphorylated form, present in equal amounts under both conditions. By contrast, striking differences could be seen for the singly phosphorylated peptide (3-fold more abundant in carbon-starved cells) and especially for the doubly phosphorylated peptide (11-fold more abundant in glucose-metabolizing cells). This is consistent with a stepwise model in which the non-phosphorylated form represents a silent “storage” pool of ATPase, which becomes partially mobilized in carbon-starved cells by an initial phosphorylation at Thr-912 and further activated in a glucose-dependent way by phosphorylation at Ser-911.

When the new phosphorylation results are viewed in the context of previously published trypsinolysis data (10), one can begin to picture the mechanism by which dual phosphorylation of Thr-912 and Ser-911 activates the ATPase. In the earlier work plasma membranes were treated with trypsin and immunoblotted with anti-C-terminal antiserum. Significantly, the C-terminal tail of Pma1 ATPase was relatively inaccessible to trypsin in samples from carbon-starved cells but rapidly degraded in samples from glucose-metabolizing cells. Substitution of Thr-912 by Ala led to arrest of the ATPase in the former state, whereas substitution by Asp led to arrest in the latter state (10). Fig. 8 depicts a mechanism that is consistent with both the trypsinolysis data and the new phosphorylation data. Starting with a storage form of the ATPase in which a tightly folded C-terminal tail blocks ATP hydrolysis, phosphorylation of Thr-912 shifts the tail to create a low activity, low affinity form of the ATPase in carbon-starved cells. Additional phosphorylation at Ser-911 then loosens the tail completely to yield a high activity, high affinity form during glucose metabolism. A key feature of this model is that the initial phosphorylation of Thr-912 is necessary to trigger the phosphorylation of Ser-911, since no evidence was found for a singly modified Ser-P peptide.

For a more detailed understanding of the mechanism at the molecular level, it will be essential to have a three-dimensional structure of yeast Pma1 ATPase at atomic resolution. Such a structure is not yet available, and the C-terminal tails of Pma1 and the Ca^{2+}-ATPase of sarcoplasmic reticulum are not sufficiently similar to build a homology model based on recently published crystal structures for the latter enzyme (26). Useful clues can come, however, from a published 8Å structure of the Neurospora crassa Pma1 ATPase (2) combined with the use of standard algorithms to evaluate secondary structural characteristics of the polypeptide sequence. Both suggest that the proximal portion of the tail folds as a 13-amino acid α-helix (H35477STRSVEDFAAMQ), which is relatively flexible and hydrophilic at the N-terminal end but more rigid and hydrophobic (and, therefore, less likely to be exposed) at the C-ter-
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minal end. Next comes a kink or hinge region (RST) containing Ser-911 and Thr-912 followed by another short helix (QHEKET). Thus, one can readily imagine that the introduction or removal of negatively charged phosphate groups in the hinge region may control the interaction of the first and/or the second helix with the catalytic center of the protein, promoting autoinhibition (when neither Ser-911 nor Thr-912 is phosphorylated), partially relieving the inhibition (when Thr-912 is

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**A)**

isotopic clusters in isolation window:

- peptide 1 (+4)
- peptide 2 (+2)

**B)**

ETD MS/MS

|  |  |
|---|---|
| **C** |  |

peptide 1 within 5 ppm mass error of proposed sequence

**C)**

716.271 theoretical

**D)**

650 m/z

Intensity: 2.9 x 10^5
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ATPase, serves to reverse the C-terminal autoinhibition (29). Importantly, this is not a direct effect; rather, it is mediated by a family of 25-kDa binding proteins (14-3-3 proteins), whose interaction with the ATPase is promoted by phosphorylation at Thr-947 (29).

Related mechanisms have been described in plant and animal plasma membrane Ca\(^{2+}\)-ATPases, where either the N-terminal region (in the former case) or the C-terminal region (in the latter case) acts in an autoinhibitory fashion. In both cases, calmodulin functions as a small regulatory protein, and the inhibition is reversed by kinase-mediated phosphorylation within the calmodulin binding domain of the ATPase (30).

Other P-type ATPases are down-regulated, not by an inhibitory sequence within the ATPase itself, but by a small interacting protein. Here again, kinase-mediated phosphorylation plays a prominent role. The best-studied example is sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), which is inhibited by a 52-amino acid protein known as phospholamban (PLB). In its unphosphorylated form, PLB lowers calcium pumping by reducing the apparent affinity of SERCA ATPase for Ca\(^{2+}\). During \(\beta\)-adrenergic stimulation of cardiac cells, however, PLB becomes phosphorylated by protein kinase A and Ca\(^{2+}\)/calmodulin kinase, relieving the inhibition and stimulating Ca\(^{2+}\) uptake (31). The two phosphorylation sites lie immediately adjacent to one another at Ser-16 (protein kinase A) and Thr-17 (Ca\(^{2+}\)/calmodulin kinase), both located in cytosolic domain Ia of PLB (for review, see Ref. 32). Of them, Ser-16 appears to be the primary regulatory site, since substitution by Ala at this position reduced the \(\beta\)-adrenergic stimulation of SERCA ATPase in transgenic mice, whereas substitution of Thr-17 by Ala had little or no effect (33, 34). At the molecular level, scanning mutagenesis and cross-linking studies followed by model-building based on crystal structures of SERCA (28) have suggested that the phosphorylated segment of PLB must unwind to interact with the complementary face of SERCA (35). This idea is supported by recent EPR and NMR studies, which have resolved both a “tense” conformation and a “relaxed” conformation of PLB (36, 37). In the proposed mechanism, phosphorylation at Ser-16 partially unwinds the helical content of the domain, shifting the equilibrium toward the more disordered state and diminishing the interaction with SERCA (38). Further work will be needed to determine whether similar conformational changes follow the phosphorylation of Ser-911 and Thr-912 of yeast Pma1 ATPase.

Future Steps—An obvious direction for future research on the yeast ATPase will be to carry out MS studies of strains bearing mutations at Ser-911 and/or Thr-912; such studies will

FIGURE 5. ETD-MS/MS spectra of peptide P2 from the C-terminal tail of Pma1 ATPase, demonstrating phosphorylation on both Ser-911 and Thr-912. GM cells were grown for this experiment in \(^{15}\)N-enriched medium and doubly phosphorylated forms of peptide 896–918 (panels A–C) and peptide 896–916 (panel D) were studied. A, within the isolation window of the target peptide precursor cation, a co-eluting peptide cation could be seen with the same nominal \(m/z\) value but a different charge state (peptide 2, \(m/z\) value 716.349). The isotopic cluster \(m/z\) peaks corresponding to the doubly phosphorylated form of Pma1 peptide 896–918 are marked with open circles, whereas those corresponding to the co-eluting peptide are marked with closed circles. B, upon ETD fragmentation, c- and z-type product ions were generated from both peptide precursor cations. From these fragments, the target peptide and sites of phosphorylation (Ser-911 and Thr-912) could readily be determined. Fragment ions arising from the co-eluting peptide cation were also observed and have been denoted with an asterisk. C, calculation of the theoretical mass and isotopic distribution for the identified phosphopeptide revealed an identical match to the observed full MS spectrum and differed by only 5 ppm. Note that the y axis was magnified 50-fold in regions marked by brackets. D, ETD-MS/MS spectra of the doubly phosphorylated form of peptide 896–916. Analysis of consecutive backbone fragment ions from either the C terminus (z-type) or N terminus (c-type) made it possible to identify the phosphorylation site on this peptide as Ser-911 and Thr-912.

FIGURE 6. Method for determining the relative levels of P0, P1, and P2 in CS and GM samples. Yeast cultures were grown in either standard medium (\(^{15}\)N) or medium containing \(^{14}\)N-labeled \((\text{NH}_4\)\()_2\text{SO}_4\) as the sole nitrogen source (\(^{15}\)N) and CS and GM cells were prepared from both cultures. Before isolating plasma membranes, CS cells from the \(^{15}\)N culture were mixed with an equal quantity of GM cells from the \(^{15}\)N culture to give sample A, and conversely, GM cells from the \(^{15}\)N culture were mixed with an equal quantity of CS cells from the \(^{15}\)N culture to give sample B. Plasma membranes were prepared from both samples and used for gel-purification of Pma1 ATPase, which was then digested with Lys-C for MS analysis. In MS plots, each peptide is represented by a pair of signals, heavy and light, corresponding to the CS and GM samples. Starting from the same amount of CS and GM cells, pairs of unmodified peptides should have equally intense signals (or a constant ratio of intensity). On the other hand, when phosphorylation occurs (\(+80 \text{Da} = \text{HPO}_3\)), a difference in phosphorylation level between CS and GM samples should appear as a change in the ratio of intensity of the corresponding peaks.

phosphorylated), and allowing maximal activity (when Ser-911 becomes phosphorylated as well).

Role of Phosphorylation in Regulating Other P-type ATPases—Several other P-type ATPases exhibit similar regulatory mechanisms, with autoinhibition brought about by either the N or C terminus in a manner that can be counteracted by kinase-mediated phosphorylation at a nearby site. In the AHA family of plant plasma membrane H\(^+\)-ATPases, for example, Palmgren and co-workers (27) have mapped autoinhibition to a region midway along the 90-amino acid C-terminal tail and found that a synthetic 28-amino acid peptide corresponding to that region can inhibit proton pumping after removal of the C terminus by trypsinolysis (28). The same group has found that phosphorylation at Thr-947, the penultimate residue of the
yield quantitative information about the interaction between these two sites and the consequences for the regulation of ATPase activity by glucose. In parallel, it will be important to search for small binding proteins that may modulate the auto-inhibitory action of the C-terminal tail. Worth mentioning in this regard is that yeast contains two genes encoding 14-3-3 proteins, BMH1 and BMH2 (39), although there is as yet no evidence that they are involved in Pma1 regulation.

Another promising direction, also beyond the scope of the present study, will be to identify the kinase(s) and phosphatase(s) that are responsible for the glucose-dependent regulation of Pma1 ATPase. Thr-912 is part of a classical recognition motif for casein kinase II ((S/T)XXE) and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (RXX(S/T)), whereas Ser-911 may be recognized by a kinase belonging to the AGC group. To date, studies with mutants have implicated two specific protein kinases (Ptk2 and Hrk1 (40)) and one protein phosphatase (PP1, encoded by the Glc7 gene (41)) in Pma1 regulation, but further work will be needed to understand the regulatory process in detail. This will be a major undertaking, since yeast is known to possess at least 119 protein kinases and 43 protein phosphatases, including at least 6 calmodulin-activated kinases and two members of the casein kinase II family (42).

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