New Components of a System for Phosphate Accumulation and Polyphosphate Metabolism in *Saccharomyces cerevisiae* Revealed by Genomic Expression Analysis

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The PHO regulatory pathway is involved in the acquisition of phosphate (P\textsubscript{i}) in the yeast *Saccharomyces cerevisiae*. When extracellular P\textsubscript{i} concentrations are low, several genes are transcriptionally induced by this pathway, which includes the Pho4 transcriptional activator, the Pho80-Pho85 cyclin-CDK pair, and the Pho81 CDK inhibitor. In an attempt to identify all the components regulated by this system, a whole-genome DNA microarray analysis was employed, and 22 PHO-regulated genes were identified. The promoter regions of 21 of these genes contained at least one copy of a sequence that matched the Pho4 recognition site. Eight of these genes, PHM1–PHM8, had no previously defined function in phosphate metabolism. The amino acid sequences of PHM1 (YFL004w), PHM2 (YPL019c), PHM3 (YJL012c), and PHM4 (YER072w) are 32–56% identical. The *phm3* and *phm4* single mutants and the *phm1 phm2* double mutant were each severely deficient in accumulation of inorganic polyphosphate (polyP) and P\textsubscript{i}. The phenotype of the *phm5* mutant suggests that PHM5 (YDR452w) is essential for normal catabolism of polyP in the yeast vacuole. Taken together, the results reveal important new features of a genetic system that plays a critical role in P\textsubscript{i} acquisition and polyP metabolism in yeast.

INTRODUCTION

Phosphate (P\textsubscript{i}) is an essential nutrient for all organisms, used in the biosynthesis of diverse cellular components, including nucleic acids, proteins, lipids, and sugars. It is therefore essential for organisms to have evolved regulatory mechanisms for acquisition, storage, and release of this molecule (Torriani-Gorini et al., 1994).

In *Saccharomyces cerevisiae*, the PHO regulatory pathway regulates expression of the “PHO” genes, involved in the scavenging and specific uptake of P\textsubscript{i} from extracellular sources (Johnston and Carlson, 1992; Oshima, 1997). The PHO regulatory system consists of at least five PHO-specific regulatory proteins, the Pho2 and Pho4 transcriptional activators, the Pho80-Pho85 cyclin-cyclin dependent protein kinase (CDK) complex, and the Pho81 CDK inhibitor (Figure 1). Pho84 is a high-affinity P\textsubscript{i} transporter localized on plasma membrane, which has been shown to contribute to P\textsubscript{i} uptake from culture medium (Bun-ya *et al.*, 1991). *PHO84* gene expression is activated by a P\textsubscript{i}-starvation signal mediated by the PHO regulatory system. Additionally, the *PHO5* gene encodes a repressible acid phosphatase which is localized to the periplasmic space.

When the P\textsubscript{i} concentration in the medium is low (~ 0.2 mM P\textsubscript{i}), the Pho81 protein inhibits the Pho80-Pho85 kinase activity, which in its active state catalyzes a hyperphosphorylation of Pho4 (Schneider *et al.*, 1994; Ogawa *et al.*, 1995). The hypophosphorylated form of Pho4 is preferentially localized to the nucleus, where together with Pho2, it activates target gene transcription (Kaffman *et al.*, 1998; Komelli and O’Shea, 1999). Alternatively, when the P\textsubscript{i} concentrations are high (~ 10 mM P\textsubscript{i}), the Pho80-Pho85 kinase phosphorylates Pho4. In addition to having a lower affinity for Pho2 and the nuclear import protein Pse1/Kap121, phosphorylated Pho4 is a preferred substrate of the nuclear export protein Msn5, resulting in extranuclear localization. Phosphorylated Pho4 is thus unable to activate target gene expression.

Besides *PHO5* and *PHO84*, seven additional genes are known to be regulated by the PHO regulatory system; these include *PHO11* and *PHO12* (homologs of *PHO5*), *PHO8* (vacuolar alkaline phosphatase, Kaneko *et al.*, 1987), *PHO89* (Na/P\textsubscript{i} cotransporter, Martinez and Persson, 1998), *PHO86*...
**PhO regulation system. A simplified schematic of the PHO regulation mechanism showing the five main regulator proteins is illustrated (Johnston and Carlson, 1992; Oshima, 1997). Ovals and boxes represent proteins and genes, respectively. Thick lines mean that the signals are transduced to the downstream component, while dotted lines indicate the absence of an interaction with the downstream component. Open ovals and boxes indicate active states, gray oval and boxes indicate inactive state. The PHO5 constitutive mutants used in this study are listed to the right of the corresponding mutated protein with the nature of the mutation indicated by "gain" or "loss" of function.**

**Figure 1.**

The preparation of the yeast ORF DNA microarray, RNA preparation from yeast cells, probe preparation with fluorescence dye, hybridization, scanning of the hybridized array, and data processing were performed as described previously (Chu et al., 1998; Spellman et al., 1998). To compare gene expression patterns between the mutants and wild-type, yeast strains NBDB2-1 (PHO4-1), NBDB0-1 (pho88Δ), NBDB85A-1 (pho853Δ), NOFB (PHO5-1), and NBW7 (wild-type) were precultivated in 25 ml of YPAD overnight, the grown cells were collected washed with YPAD and inoculated into 500 ml of YPAD at an OD₆₀₀ of 0.1. The remainder of the procedure was the same as above.

**Materials and Methods**

**Strains and Media**

*S. cerevisiae* strains used in this study are listed in Table 1. YPAD medium (Adams et al., 1997) was used as rich media. A Pₐ-depleted YPAD medium (YPAD-Pi) was prepared as described (Kaneko et al., 1982), and a high-Pᵢ YPAD (YPAD + Pi) medium was prepared by addition of sodium phosphate (10 mM, pH 5.8) to the YPAD-Pi medium. High-Pᵢ low- Pᵢ and Pᵢ-free synthetic media were prepared as described (Yoshida et al., 1989). YPAD medium buffered at pH 7.5 supplemented with 50 mM CaCl₂ was prepared as described (Zhang et al., 1998).

**Growth Conditions for RNA Preparation**

For comparison of the gene expression pattern between the low- and high-Pᵢ conditions, yeast strain NBW7 or DBY7286 was grown in 25 ml of the YPAD + Pi medium overnight, the cells were collected, washed with the YPAD-Pi medium two times, and inoculated into 500 ml of the YPAD + Pi and -Pi media to an OD₆₀₀ of 0.05. The two cultures were shaken at 30°C to an OD₆₀₀ of 0.5, the grown cells were harvested, and then frozen by immersion in liquid nitrogen. The cells were stored in −80°C until RNA was prepared. For comparison of the gene expression pattern between the mutants and wild-type, yeast strains NBDB2-1 (PHO4-1), NBDB0-1 (pho88Δ), NBDB85A-1 (pho853Δ), NOFB (PHO5-1), and NBW7 (wild-type) were precultivated in 25 ml of YPAD overnight, the grown cells were collected washed with YPAD, and inoculated into 500 ml of YPAD to an OD₆₀₀ of 0.1. The remainder of the procedure was the same as above.

**DNA Microarray Analysis**

The preparation of the yeast ORF DNA microarray, RNA preparation from yeast cells, probe preparation with fluorescence dye, hybridization, scanning of the hybridized array, and data processing were performed as described previously (Chu et al., 1998; Spellman et al., 1998). To compare gene expression patterns between the low- and high-Pᵢ conditions, Cy3- or Cy5-labeled cDNA probes were prepared as described. The preparation of the yeast ORF DNA microarray, RNA preparation from yeast cells, probe preparation with fluorescence dye, hybridization, scanning of the hybridized array, and data processing were performed as described previously.

**Construction of the S. cerevisiae Disruption Mutants**

To make disruption mutants of PHM1, PHM2, PHM3, PHM4, PHM5, PHM6, and CTF19, PCR-mediated gene disruptions were performed as described (Sakamoto et al., 1997). PCR fragments on pUC19, respectively, were obtained from Dr. Y. Mukai (Osaka University), and used as templates to generate the PCR fragments for the gene disruptions. Deletion regions in the target gene were amplified by PCR and cloned into pUC19, respectively.
Table 1. S. cerevisiae strains used in this study

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<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
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<td>Ogawa and Oshima, (1990)</td>
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<tr>
<td>CRXb</td>
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<td>NB91-6b</td>
<td>MATa his3 leu2 trp1 pep4 (=pho9-1)</td>
<td>Our stock</td>
</tr>
</tbody>
</table>

* These strains were derived from strain NBW7.

** These strains were derived from strain CRY.

**Polyphosphate Overplus**

Polyphosphate overplus (Harold, 1966) culture was performed as follows: The yeast strains indicated were grown in the YPAD-Pi media overnight, the grown cells were collected, washed with water, and resuspended in 10 ml of the synthetic P,-free media to an OD600 of 0.5. The cultures were shaken for 2 h at 30°C, then potassium phosphate (pH 5.8) was added (10 mM final concentration). After 2 more hours of cultivation, the cells were harvested, and then frozen by immersion in liquid nitrogen. For the polyP overplus at low pH, yeast strains were grown in the YPAD-Pi media overnight, the grown cells were collected, washed with water, and resuspended in 10 ml of the YPAD media supplemented with 10 mM (final) KH2PO4 and/or 10 mM (final) sodium acetate buffer (pH 4.0) to an OD600 of 0.5. The cultures were shaken for 2 h at 30°C, and then the cells were harvested, and frozen by immersion in liquid nitrogen.

**Cell Extract Preparation From Yeast**

Cells in 150 μl of extraction buffer (50 mM Tris-HCl [pH 7.4], 100 mM KCl, 1 mM EDTA) were mixed in a vortex mixer with 150 mg of acid-washed glass beads (0.5-mm diameter) for 2 min at 4°C, and microcentrifuged at 14,000 rpm for 10 min at 4°C. The aqueous phase was extracted by phenol:chloroform followed by chloroform and ether extractions. After removing ether in the samples by evaporation, their A260 values were measured to calculate their total RNA concentrations.

**PolyP Detection by PAGE**

PolyP analysis by PAGE was performed as described in Wurst et al. (1995) with the following modifications. Samples containing the indicated amounts of RNA were loaded with 7 μl of loading dye solution (1X TBE buffer [Sambrook et al., 1989], 10% sucrose, 0.05% bromophenol blue) on a 20% polyacrylamide gel (270-mm height x 165-mm wide x 0.5-mm thick) with 1X TBE buffer. Ten μg of sodium phosphate glass type 5, 15, or 35 (Sigma, St. Louis, MO) were loaded as a PolyP size markers. Radioactive [γ-32P]-ATP was also used as an additional size marker. The electrophoresis was run at 20 V/cm for ~1.5 or 3 h, and the ATP-loaded gel piece was analyzed by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The non-radioactive piece of gel was soaked into 10% acetate, 10% methanol for 15 min, stained with the staining solution (0.5% Toluidine Blue O [Sigma], 25% methanol, 5% glycerol, 5% acetate) for 15 min, and destained with destaining solution (same as the staining without Toluidine Blue O) for 10 min several times. ATP migrates at a position corresponding to polyP of between seven and eight P residues long. PolyP bands of sodium phosphate glass type P35 with 55 and 65 chain length on PAGE were extracted and used as markers to estimate longer chain lengths of polyP.

**Enzymatic polyP Assays**

PolyP was assayed by the nonradioactive method as described (Ault-Riché et al., 1998), without the Glassmilk purification steps. Since the major population of yeast polyP is < 60 P residues in length (Figure 4B), they are not able to be effectively trapped by Glassmilk. Concentrations of polyP in the samples were shown as mol of P, residues per mg of total RNA.

**PHM2-GFP Fusion Experiment**

pPHM2-GFP was constructed by insertion of the PCR fragment corresponding to nucleotide positions from ~393 to +2505 of PHM2 (relative to A of initiation codon of ATG) into an EcoRI-
HindIII gap of pTS395, a GFP-expressing yeast vector provided by Dr. D. Botstein. Yeast transformant NBM-4L19H1[pPHM2-GFP] was grown in synthetic low-P medium lacking uracil for > 3 h at 30°C. Fluorescent dye, FM4–64 (Molecular Probes, Eugene, OR), which has been reported to stain vacuolar membrane (Vida and Emr, 1995), was added to 30 μM, and incubated for an additional 15 min. Ten-microliter samples were examined by fluorescent microscope Axiosplan2 (Carl Zeiss, Thomwood, NY) following the procedure of Vida and Emr (1995) and Adams et al. (1997).

P. Uptake Assay

A P. uptake assay was performed following the procedure of Bunya et al. (1991). Yeast strains were grown in the YPAD-P, media overnight, the grown cells were collected, washed with water, and resuspended in 50 ml of synthetic P.,free media to an OD₆₆₀ of 0.1. The cultures were shaken for 2 h at 30°C, and their OD₆₆₀ values were remeasured. Potassium phosphate (0.1 mM, pH 5.8) and radioactive phosphate (32PO₄; 1 Ci/ml) were added to cultures, and shaken. Five-milliliter samples were taken at indicated intervals and immediately filtered through a HA filter (3.5-mm diameter, Millipore). The cells trapped on the filter were washed with 10 ml of synthetic P.,free media. The radioactivity trapped on the membrane filters was quantitated by a liquid scintillation counting.

RESULTS

Whole Genome Survey for the PHO-Regulated Genes

In order to identify all of the genes regulated by the PHO regulatory pathway, we used DNA microarrays fabricated as described previously (DeRisi et al., 1997, Chu et al., 1998; DeRisi et al., 1997, Chu et al., 1998; Spellman et al., 1998). A cDNA probe prepared from poly(A)⁺ RNA isolated from a wild-type yeast strain, NBW7, cultured in low-P media (YPAD-Pi), was labeled with Cy5 fluorescent dye, while a cDNA probe prepared from the same strain cultivated in high-P media (YPAD+Pi) was labeled with Cy3 fluorescent dye. The Cy5- and Cy3-labeled cDNA probes were mixed and hybridized to the microarray. As expected, the transcript levels of both PHO5 and PHO84 were elevated in low-P, media in two independent experiments (Figure 2A). In a repeat experiment using strain DBY7286, derived from S288C, (the standard S. cerevisiae strain for the genome database), most of the highly derepressed genes we found in NBW7 were consistent with those identified in DBY7286 (Figure 2) suggesting similar PHO regulation in the two strains.

To evaluate if these gene expression changes were dependent on the known PHO regulatory factors, further DNA microarray analyses were performed comparing the parental strain NBW7 to four strains carrying mutations in components of the PHO regulatory system (Figure 1): 1) a gain-of-function mutation of the PHO4 gene encoding transcriptional activator named PHO4⁺-1 (Ogawa and Os-hima, 1990); 2) a pho80 deletion mutant; 3) a pho85 deletion mutant; and 4) PHO81⁻¹ a gain-of-function mutation in the PHO81 gene, encoding the CDK inhibitor for Pho80-Pho85 (Ogawa et al., 1995). In all cases PHO5 was expressed (Figure 2A) irrespective of P. concentration (YPAD media). A total of eight DNA microarray experiments were performed, including two independent repeated experiments. Cluster analysis of the combined data is shown in Figure 2B. Transcript levels of > 80 genes changed significantly in response to one or more of the conditions we total.

All nine genes that have previously been reported to be PHO-regulated (PHO5, PHO11, PHO12, PHO8, PHO84, PHO89, PHO86, PHO81 and SPL2) were successfully identified (Figure 2B). PHO5, PHO11, PHO12, PHO8, PHO84, and PHO89 had high differential expression ratios (more than five-fold) whereas PHO8 and PHO86 had lower differential ratios (between two and fivefold), consistent with previous results from Northern analysis (Kaneko et al., 1987; Bunya et al., 1991; Yompaakdee et al., 1996). The nine known PHO-regulated genes can be categorized into three groups according to their function (Table 2). A third member of the PHO81 family, YPL110c, also seems to be regulated by the PHO regulatory pathway (Figure 2B) in a manner similar to PHO81. Twelve additional genes showed clear evidence of PHO regulation in our experiments (Figure 2B and Table 2). Three of these genes (YPL019c, YJL012c and YER072w) had levels of differential expression similar to PHO5 and PHO84, in nearly all of the eight experiments. Interestingly, the putative proteins encoded by these three genes share homology with YFL004w as described later (Figure 3A). The expression of YFL004w was also regulated by the PHO pathway, with differential expression levels similar to PHO8 and PHO86 (Figure 2B). Because of their homology and high differential expression ratios, we speculated that these four genes, which we have named PHM1 through PHM4 (phosphate metabolism genes), might have important functions in P. metabolism.

Among the other newly identified PHO-regulated genes listed in Table 2, the functions of three had been previously described: HOR2/GPP2 (YER062c), CTF19 (YPL018a), and HIS1 (YER055c). The HOR2/GPP2 gene encodes glyceral phosphatase (Norbeck et al., 1996) which hydrolyzes the phosphate bond of glycerolphosphate, releasing free P. The His1 enzyme (ATP phosphoribosyltransferase)-catalyzed re-action can produce pyrophosphate from ATP and phospho-ribosylpyrophosphate. While both HIS1 and HOR2 encode enzymes that catalyze reactions that release P., the special significance of these reactions for the cell’s P. economy is not obvious. The Ctf19 protein has previously been identified as a subunit of the centromere-binding complex (Ortiz et al., 1999), and has no recognized role in P. metabolism or regulation. A possible explanation for the observed differential expression could be that CTF19 shares promoter sequences with the “head-to-head” oriented adjacent gene YPL019c/PHM2, which was highly differentially expressed under PHO regulation. More plausibly, it may have an additional, unrecognized role in P. metabolism.

Two “core” Pho4 binding sequences, CACGTG and CACGTT have previously been described. At least one of these sequences is found within 500 bp upstream of the coding sequence of 21 of the 22 putative PHO-regulated genes listed in Table 2, a fraction significantly higher than the 20% of all yeast ORFs that have one of these sequences within 500 bp upstream (1287 ORFs out of 6282). Of the 14 genes with more than two putative Pho4 binding sites, 9 had the high derepression ratios described above. Higher levels of differential expression showed some correlation to the presence of both of the two types Pho4 binding sites. Of the 22 putative PHO-regulated genes, only YER038c had no consensus Pho4-binding sites in its promoter region. The 3’
end of the predicted coding region of the adjacent gene, YER037w, is located only 4 bp from the YER038c ORF, which had a very similar observed expression pattern (Figure 2). It is therefore likely that the apparent PHO regulation of YER038c reflects hybridization to the 3' untranslated region of YER037w.

PHM1, PHM2, PHM3, and PHM4 Are Involved in polyP Accumulation

The predicted coding regions Phm1 and Phm2 are similar in length (828 and 835 amino acid residues for Phm1 and Phm2, respectively) and 58% identical in amino acid sequence. The predicted coding region of Phm3 is smaller (648 amino acids) and has 33% identity to the N-terminal region of Phm1 (Figure 3A). Phm4 is predicted to encode a protein of 129 amino acids, with 32% identity to the C-terminal region of Phm1, which contains three putative transmembrane spanning regions. Phm3 and Phm4 share no homology. Interestingly, the N-terminal regions (amino acids 1–135, Figure 3A) of Phm1, Phm2, and Phm3 have significant similarity to the N-terminal regions of Pho81, and its homolog Ypl110c (Figure 3B). Furthermore, this similarity is shared among a total of nine S. cerevisiae ORFs, including the putative phosphate transporter Pho87 (Bun-ya et al., 1996), two proteins homologous to Pho87, Yjl198w and Ynr013, and Syg1 (Spain et al., 1995), a multi-copy suppressor for a GPA1 deletion. The similarities were confined to the N-terminal region (< 300 amino acids) in all of the nine proteins.
Homologous sequences can also be found in the databases of *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, mouse, and human (Battini et al., 1999).

To address the function of the *PHM1*, *PHM2*, *PHM3*, and *PHM4* genes, strains carrying deletions of each of these genes were constructed (see MATERIAL AND METHODS). All five mutant strains, including a *phm1 Δ phm2 Δ* double mutant, were viable in rich media (YPAD), and no significant growth defects were observed in low-Pi synthetic media. All five were able to produce acid phosphatase in response to low Pi. When we analyzed polyP accumulation, however, the mutants showed striking phenotypes.

It is known that *S. cerevisiae* accumulates a large amounts of polyP in vacuoles under conditions of high Pi, preceded by a period of Pi starvation. This is referred to as the “polyphosphate overplus” phenomenon (Harold, 1966). PolyP chains in extracts from yeast were analyzed by PAGE followed by staining with a Toluidine Blue dye, which stains polyP as well as nucleic acids. The extract from wild-type cells, NBW7, after the polyP overplus treatment (see MATERIAL AND METHODS) resulted in two distinct populations of stained molecules as is shown in Figure 4A. The upper population represents RNA, whereas the lower “ladder-like” population is polyP, confirmed by pretreatment with either RNase A or ex-
opolyphosphatase (our unpublished results). The chain lengths of the polyP bands were determined by comparing them with polyP marker ladders run in a nearby lane. The distribution of the polyP chain length in the NBW7 strain was broad (100 Pi residues) with a median length of 60 Pi residues (Figure 4B). The cellular concentration was measured at 19.4 μmol (1.55 mg) of polyP/mg total RNA by an enzymatic assay (Ault-Riche et al., 1998) (Table 3).

The effects of PHM1-PHM4 on polyP accumulation following the polyP overplus treatment were analyzed by gel electrophoresis (Figure 4) and enzymatic assay (Table 3). Total polyP was slightly reduced in the phm1Δ mutant. The phm2Δ mutant had significantly reduced total polyP (14% of wild-type), and the median size of the polyP molecules was significantly smaller than that in the wild-type. PolyP was undetectable in the phm1Δ phm2Δ double mutant. This clearly demonstrates that the PHM1 and PHM2 genes have redundant functions in polyP accumulation. Both the phm3Δ and phm4Δ mutants lacked detectable polyP. These results suggest that Phm1, 2, 3, and 4 proteins are involved in the accumulation of polyP and that the products of Phm3, Phm4 and either Phm1 or Phm2 are required for polyP accumulation.

Phm2 is Localized to the Vacuole
More than 90% of total polyP in yeast is localized to the vacuole (Urech, 1978). Since the Phm1–Phm4 proteins are involved in polyP synthesis, we speculated that the Phm1–Phm4 proteins were also localized to the vacuole. To address this question, a PHM2-GFP fusion gene, which encodes the full PHM2 ORF fused to the GFP ORF, was constructed and
expressed in wild-type and phm1Δ phm2Δ double mutant strains. The fusion protein was active; PHM2-GFP was able to complement the polyP deficient phenotype of the phm1Δ phm2Δ mutant (our unpublished results). Phm2-GFP indeed appeared to be localized to the vacuoles (Figure 5) by colocalization with the vacuolar membrane marker FM4-64 (Vida and Emr, 1995).

While this study was in progress, Cohen et al. (1999) reported on a gene family involved in a vacuolar transporter chaperon, VTC1, VTC2, VTC3, and VTC4 in S. cerevisiae. These four genes are identical to PHM4, PHM1, PHM2, and PHM3, respectively. In their report, the Phm4/Vtc1 protein was originally found in the same fraction as a Vma10 protein, a subunit of vacuolar H+-ATPase (v-ATPase) localized on the vacuolar membrane. Furthermore, Phm3/Vtc4, which does not have a putative transmembrane domain, was found in the membrane fraction only in the presence of Phm4/Vtc1 and either Phm1/Vtc2 or Phm2/Vtc3. These finding support our hypothesis that the Phm1 through 4 proteins form a complex on the vacuolar membrane. In addition, an S. pombe protein, Nrf1, with an amino acid sequence 78% identical to that of Phm4/Vtc1, was recently reported as a vacuolar membrane protein (Murray and Johnston, 2000).

Table 3. PolyP amounts in the various mutants

<table>
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<th>Strain</th>
<th>Mutation</th>
<th>PolyP (µmol/mg of RNA)</th>
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<td>wild type</td>
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<tr>
<td>NBD8184-1B</td>
<td>phm84Δ</td>
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Figure 5. Localization of Phm2-GFP. The identical frames of the yeast transformant NBM4L19H1[pPHM2-GFP] were shown for Nomarsky (left column), FM 4–64 fluorescence (center column), and GFP fluorescence (right column) images.

Figure 6. (A) PolyP PAGE analysis of vma4 and pep4 mutants. PolyP samples were prepared from the strains, CRY-V4 (vma4Δ), CRY (WT), CRX (ppx1Δ), CB024 (Pro), and NB91–6A (pep4Δ) cultivated by the polyP overplus method. The other conditions were as described in Figure 4A. (B) PolyP PAGE analysis of vma4 and phm mutants cultivated at low pH. PolyP samples were prepared from the strains, CRY (WT), CRY-V4 (vma4Δ), NBM4L19H2 (phm1Δ phm2Δ), NBM12W2 (phm3Δ), and NBM72W7 (phm4Δ) cultivated in YPAD-Pi media, followed by cultivation in YPAD supplemented with either 10 mM Pi (Pi), or 10 mM Pi and 10 mM sodium acetate buffer (Ac), for 2 h. The other conditions were as described in Figure 4A.

PolyP Synthesis Is Influenced by v-ATPase Activity but It Is Not Essential

Previous work has shown that polyP accumulation is dependent upon v-ATPase activity (Wurst et al., 1996). A vma4Δ mutant strain, in which v-ATPase activity is completely deficient (Zhang et al., 1998), was reproducibly found to have no accumulation of polyP under the polyP overplus conditions (Figure 6A). Cohen et al., (1999) reported that the
v-ATPase activity in a phm4Δ/vtc1Δ mutant was 10–30% of that in wild-type cells. Cells with mutations in v-ATPase are characteristically deficient in respiration and sensitive to media containing 60 mM CaCl2 at pH 7.5 (Zhang et al., 1998). To address the possibility that the Phm1 through 4 proteins could be necessary for v-ATPase activity, and thus indirectly for the accumulation of polyP, the phm disruptants were tested for growth on nonfermentable carbon sources and in the presence of CaCl2. Growth of the phm1Δ phm2Δ, phm3Δ, and phm4Δ mutants in 60 mM CaCl2 was similar to that of the wild-type strain (Figure 7), whereas the vma4Δ mutant did not grow. Moreover, phm1Δ phm2Δ, phm3Δ, and phm4Δ mutants were able to grow on rich media with ethanol or glycerol as a sole carbon source, while the vma4Δ mutant was not (our unpublished results). These data suggest that the phm mutants retain v-ATPase activity.

It is known that incubating yeast in low pH media can, in part, reverse the phenotype seen with v-ATPase mutants by lowering the intravacuolar pH via fluid-phase endocytosis (Nelson and Nelson, 1990). A low but significant level of polyP accumulation was detected when the vma4Δ mutant was incubated at low pH (Figure 6B). In contrast, the phm1Δ phm2Δ, phm3Δ and phm4Δ mutants failed to accumulate detectable polyP with the low pH incubation (Figure 6B). These results strongly suggest that the PHM1-PHM4 gene products are directly involved in polyP accumulation and that v-ATPase activity is not strictly essential for polyP synthesis.

PolyP Formation Prevents Short-term Saturation of Cellular Pi Accumulation

Phm1 through 4 proteins clearly play a role in polyP synthesis. The apparently paradoxical increase in the cell’s ability to convert Pi into polyP in response to Pi starvation might represent a strategy for accumulating and holding precious Pi, We therefore measured Pi uptake in the phm1Δ-phm4Δ mutants (Figure 8). After cultivation in Pi-free synthetic media for 2h, NBW7 wild-type cells displayed a linear (nonsaturable) Pi uptake for up to 25 min when incubated in 0.1 mM Pi, (twofold less concentration of synthetic low-Pi, medium). In contrast, the pho84Δ mutant, which is deficient in H+ /Pi, cotransporter activity, showed very low uptake, as reported previously (Bun-y et al., 1991). The phm1Δ, 2, 3, and 4 mutants each had unique saturable Pi-uptake profiles. The phm1Δ single mutant had an uptake profile similar to wild-type. The phm2Δ single mutant showed a rate of uptake similar to wild-type from 0 to 8 min, but after 8 min uptake was minimal. The Pi-uptake profiles of the phm1Δ phm2Δ double mutant, the phm3Δ, and the phm4Δ mutants were similar to wild-type for the first 5 min, after which uptake appeared to cease. This cessation of uptake in the phm mutants correlated well with the overall ability of each mutant to accumulate polyP. These data suggest that polyP accumulation is required, presumably as a sink, to sustain a high rate of Pi uptake.

PHM5 May Encode a Polyphosphatase in the Vacuole

The previously uncharacterized ORFs YDR452w, YDR281c, YOL084w, and YER037w were also found to be PHO-regulated. They are therefore named PHM5 through PHM8, respectively. YDR281c/PHM6 and YER037w/PHM8 were previously noted by Gray et al. (1998) to be induced by treatment with drugs that inhibit Cdc28 and Pho85 kinases. Among those four genes, Phm5 has significant homology to human and C. elegans acid sphingomyelinases (Figure 3C) which function in hydrolyzing phosphodiester bonds in sphingomyelin. When phm5Δ, ctf19Δ, and phm6Δ single disruptants were constructed and tested for polyP accumulation levels, we observed that the phm5Δ mutant had a unique distribution of longer chain length polyP when compared with wild-type (Figure 4A). PolyP levels and size in the ctf19Δ and phm6Δ mutants were indistinguishable from wild-type. The average polyP chain length in the phm5Δ mutant was over 150 P residues (Figure 4B). By enzymatic
measurement, the total amount of polyP in the phm5Δ mutant was similar to that of wild-type (Table 3), indicating that polyP synthesis activity in phm5Δ was unaffected. These data suggest that the Phm5 protein is associated with, or is a polyphosphatase.

The deduced amino acid sequence of Phm5 (Figure 3C) includes a putative N-terminal membrane spanning domain, similar in size (27 amino acids), position and flanking amino acid sequences (rich in serine and positive charged residues) to that of the Pho8 protein, a PHO-regulated vacuolar alkaline phosphatase, which is anchored to the vacuolar membrane by a transmembrane domain at its N-terminus (Klionsky and Emr, 1989).

Wurst et al. (1995) reported that a mutant deficient in three vacuolar proteinases: proteinase A, proteinase B, and carboxypeptidase Y, has a longer polyP chain length distribution than the wild-type. This phenotype was reproducibly observed in our PAGE assay (Figure 6A). Furthermore, the phenotype was observed in a pep4Δ single mutant, which has a defect only in Proteinase A, which is required for maturation of several vacuolar enzymes, including Pho8. Together with Phm5's similarity to sphingomyelinases, these data suggest that PHM5 encodes a polyphosphatase, which is matured by the vacuolar proteinase. Kumble and Kornberg (1996) purified a processed endopolyphosphatase of 35 kDa from yeast. Recently, it has been found that the 35-kDa protein contains amino acid sequences identical to the deduced Phm5 sequence (Sethuraman and Kornberg, personal communication).

Mutations in the PHO Regulator Genes Affect polyP Accumulation

Since the genes for polyP processing are regulated by the PHO regulatory system, polyP accumulation should be affected by mutations which affect this system. To investigate this, polyP levels under polyP overplus conditions were measured in pho4Δ, pho80Δ, and pho84Δ mutants (Figure 4A). The pho4 mutant, which is incapable of derepression of its target genes (Figure 1) accumulates a lower level of polyP than wild-type. The pho84Δ mutant, lacking a high-affinity Pi transporter, accumulates a very low but detectable level of polyP. The pho80Δ mutant, surprisingly, did not accumulate detectable levels of polyP, despite the fact that PHO84, and PHM1 through 4 are highly expressed in this mutant. This paradoxical phenotype may be a consequence of an abnormal vacuole in this mutant (Nicolson et al., 1995).

DISCUSSION

The Proteins Involved in the Pi Acquisition and Storage System in Yeast

Using DNA microarray technology, we have identified at least 22 genes, including 13 novel genes, whose expression is regulated by the PHO pathway. Based on the premise that PHO-regulated genes are integral components of Pi metabolism, further molecular genetic approaches were undertaken to search for the function of the previously-uncharacterized genes. Our work has identified five of these genes as being involved in polyP metabolism. Thus, a total of 17 genes have now been found to function in Pi acquisition in yeast (Figure 9).

It is interesting to consider how the products of those 17 genes work together as a physiological system for Pi acquisition and storage. When yeast encounter conditions of Pi starvation, the low Pi signal initiates Pho81 activity, which suppresses Pho80-Pho85 kinase activity. The ankyrin domain of the Pho81 protein is sufficient to inhibit Pho80-Pho85 activity (Schneider et al., 1994; Ogawa et al., 1995). The PHO-regulated Pho81 homologs YPL110c and SPL2 share this domain, but their involvement in Pho80-Pho85 inhibition remains obscure (Flick and Thorner, 1998). Inhibition of the Pho80-Pho85 kinase results in an active Pho4 protein (hypophosphorylated form), which is localized to the nucleus where it acts as a specific transcriptional activator of PHO-regulated genes. Transcribed genes include PHO81, providing a positive feedback loop, which acts to keep Pho4 in its active form (Ogawa et al., 1995), resulting in high, continued expression of all PHO-regulated genes.

The Pi starvation signal triggers increased production of at least four types of phosphatases; 1) the acid phosphatases Pho5, Pho11, and Pho12, which are localized in periplasmic space; 2) the alkaline phosphatase Pho8, which is localized to the vacuole, 3) the glycerol phosphatase Hor2; 4) the putative polyphosphatase Phm5, localized in the vacuole. The acid and alkaline phosphatases are nonspecific, and
Hydrolysis of a variety of phosphorylated substrates, including nucleic acids, phosphoglycerides, phospholipids, and phospho-proteins. The glycerol phosphatase hydrolyzes the phosphate ester bond of glycerolphosphate, which is found in many sugar and lipid metabolites. All of these enzymes can contribute to increased levels of free P_i.

Under conditions of P_i starvation, expression of genes encoding the phosphate transporters, Pho44 and Pho69, are induced. Their optimal conditions are quite different: Pho44 transports P_i optimally at pH 4 and cotransports Na^+. Pho69, on the other hand, has optimal activity at pH 9.0 and co-transporters Na^+. This pair of P_i transporters work well in a wide range of environmental conditions in which yeast live.

Expression of PHO86, a P_i transporter-related gene, is also increased in P_i starvation. The Pho86 protein was originally thought to form a complex with Pho44 and Pho87 (Bun-ya et al., 1996), however Lau et al. (2000) recently found that Pho86 is localized to the endoplasmic reticulum, where it functions in the proper localization of the Pho84 protein to the plasma membrane. Thus, Pho86 is now thought to act indirectly in P_i uptake.

The PHM1 through 4 genes, which we have shown to be involved in polyP synthesis, contribute to the P_i accumulation by a unique mechanism. Our results suggest that polyP synthesis is required for proper P_i accumulation. When polyP synthesis is critically slow, it can control the rate at which P_i is taken up by Pho84 membrane transporter. When polyP synthesis is slow, intracellular free P_i levels become high, which in turn acts as a direct negative feedback on the Pho84 membrane transporter. This critical intracellular P_i level is achieved after approximately 5 min of incubation in media containing 0.1 mM P_i. The phm mutants that lacked detectable polyP synthesis activity (phm1Δ phm2Δ double, phm3Δ and phm4Δ single disruptants) showed rapid initial uptake of P_i (like wild-type) but were incapable of further P_i uptake after ~5 min. Interestingly, the phm2 mutant, which had some residual polyP synthesis activity (~10% of wild-type) could continue to accumulate P_i after the initial 5-min period, but did so at a very reduced rate. In this mutant, it appears that the rate-determining step in P_i uptake in the first 5 min was controlled by the Pho84 membrane transporter, and after this time the rate was controlled by P_i to polyP conversion.

In this study, we have shown that polyP plays an important role in P_i accumulation and metabolism in yeast. The evidence for this involvement is not only metabolic but also genetic. Similar genetic interactions between polyP and P_i metabolism have been observed in E. coli. The promoter of the ppk-ppx operon, containing the genes for polyP kinase and exopolyphosphatase, includes a pho box, the response element for the P_i starvation signal mediated by the phoB- phoR two-component regulator (Kato et al., 1993). This suggests that the corresponding bacterial genes are regulated in a manner analogous to PHM1 through 4 and PHM5, respectively. Vibrio cholerae, a Gram-negative bacterium, has a similar ppp-ppx operon, with a pho box in its promoter, and its ppp mutant was unable to sustain a high rate of P_i accumulation (Ogawa et al., 2000). Thus, regulation by P_i appears to be a physiologically conserved feature of the genes for polyP metabolism, in both bacteria and yeast. Moreover, these results suggest that a major physiological role of polyP may be to promote long-term uptake and accumulation of P_i.

**PHM5 Encodes a Vacuolar Polyphosphatase**

Prior to this study, the PPX1 gene, encoding an exopolyphosphatase, was the only yeast gene implicated in polyP processing (Wurst et al., 1995). The expression of the PPX1 gene in this study showed no detectable response to P_i, levels or perturbation of PHO regulation. PPx1 protein is believed to be localized to the cytoplasm, which contains negligible amounts of polyP. Since ~90% of total cellular polyP is accumulated in vacuoles (Urech et al., 1978), the principal physiological polyphosphatase is likely to be vacuolar. The predicted vacuolar localization and PHO-regulation of Phm5 suggest that it is likely to contribute significantly to polyP degradation in vivo. Indeed, the phm5Δ mutation resulted in a marked increase in polyP chain lengths, whereas the ppx1Δ mutation resulted in a much smaller change (Figure 6A).

**Phm1 through 4 Proteins Represent a New Type of polyP Synthesis System**

Every Gram-negative bacterium for which the genome has been sequenced, has genes homologous to E. coli PPK (Tzeng and Kornberg, 1998). No genes homologous to PPK have been found in Gram-positive bacteria, archa, or eukaryotes. This PPK is the only reported enzyme capable of synthesizing polyP, despite the fact that polyP has been found in every organism in which it has been sought, including Gram-positive bacteria, eukaryotes, and archa (Kornberg, 1999). Kornberg (1999) has speculated that mammalian cells synthesize polyP directly from the incorporated P_i, without an ATP intermediate. Our results suggest that eukaryotic cells have an enzyme system for polyP synthesis completely different from the PPK of Gram-negative bacteria. PolyP synthesis in yeast requires v-ATPase activity, which produces proton motive force across the vacuolar membrane. We therefore speculate that the high-energy phosphoanhydride bonds in polyP are directly synthesized from P_i, using the proton motive force as a source of energy, by a vacuolar membrane-bound enzyme(s), analogous to the F-type ATPase in mitochondria. Based on this hypothesis, the Phm1 through 4 protein complex is the best candidate for this polyP synthesis enzyme in yeast.

Does a P_i acquisition system similar to the yeast system exist in higher eukaryotic cells? Kido et al. (1999) reported that expression of type II Na^+/P_i cotransporter gene, NPT2, in rat kidney is derepressed by a dietary P_i starvation, and that the P_i signal-responsive element in its promoter contains the sequence, CACGTG, which is identical to the Pho4-binding site in yeast. Moreover, the N-terminal regions of Phm1, Phm2, and Phm3 contain conserved domains shared with six additional S. cerevisiae proteins, and similar protein structures can be found in genomes of many eukaryotes. Eight of the nine yeast proteins with this domain are related to P_i metabolism. We therefore refer to this domain as the “phosphate (P_i) domain” (Figure 3B). Since most of the homologous genes in other species have little or no functional characterization (except for human XPR1, gene encoding xenotropic and polyporphic retrovirus receptor [Battini et al., 1999]), the function of the P_i domain may provide a useful lead for further functional investigations. Thus, further studies of the yeast system for P_i metabolism are likely.
to provide fundamental insights into $P_i$ metabolism in all eukaryotes.

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