Effects of Methylphosphonate, a Phosphate Analogue, on the Expression and Degradation of the High-Affinity Phosphate Transporter Pho84, in *Saccharomyces cerevisiae*[†]

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ABSTRACT: In *Saccharomyces cerevisiae*, the Pho84 high-affinity transport system is the major phosphate transporter activated when the cells experience a limitation in external phosphate. In this study, we have compared the phosphate-responsive mechanism of cells expressing *PHO84* with a Δ*pho84* strain by use of a phosphate analogue, methylphosphonate, which was judged to be suitable for assessment of phosphate homeostasis in the cells. Intracellular levels of the analogue, which in several respects mimicks phosphate, were monitored by ^{31}P NMR spectroscopy. Results show that methylphosphonate is a nonhydrolyzable and nonutilizable analogue that cannot be used to replenish phosphate or polyphosphate in yeast cells grown under conditions of phosphate limitation. However, the presence of methylphosphonate under such conditions represses the Pho5 acidic phosphatase activity of *PHO84* cells, a finding that implies a direct role of the analogue in the regulation of phosphate-responsive genes and/or proteins. Likewise, accumulation of the Pho84 protein at the plasma membrane of the same cells is inhibited by methylphosphonate, although the derepressive expression of the *PHO84* gene is unperturbed. Thus, a post-transcriptional regulation is suggested. Supportive of this suggestion is the fact that addition of methylphosphonate to cells with abundant and active Pho84 at the plasma membrane causes enhanced internalization of the Pho84 protein. Altogether, these observations suggest that the Pho84 transporter is regulated not only at the transcriptional level but also by a direct molecule-sensing mechanism at the protein level.

Phosphate acquisition of the yeast *Saccharomyces cerevisiae* from the surrounding environment, an essential prerequisite for ensuring the growth and survival of the cells, relies on the transduction of extracellular phosphate signals across the plasma membrane into the cell and the nutrient-controlled transcriptional regulation of the phosphate transport systems. The mechanism involved in the cellular phosphate response forms part of a complex cascade pathway, the *PHO* regulon, a genetic regulatory circuit composed of structural and functional components encoded by ∼30 genes (*1*, *2*). Cells are able to catalyze the uptake of inorganic phosphate from the environment through at least five phosphate transporters, i.e., two high-affinity permeases, Pho84p (*3*) and Pho89p (*4*), and three low-affinity permeases, Pho87p (*5*), Pho90p, and Pho91p (*6*). A quintuple deletion of *PHO84*, *PHO87*, *PHO89*, *PHO90*, and *PHO91* completely abolishes phosphate transport and is therefore lethal (*6*). When phosphate becomes limiting, the cells respond with an increase in the level of synthesis of the high-affinity transport system and of secreted acid phosphatases (Pho3p, Pho5p, Pho11p, and Pho12p) (*1*, *7*) to scavenge phosphate from the environment. Once the cells have taken up phosphate, its intracellular compartmentalization in the yeast vacuole in the storage form of phosphate and polyphosphate (polyP),[1] a linear polymer of phosphate in an anhydrous linkage (*8*), allows for the pivotal involvement in phosphate homeostasis and interplay with gene products of the *PHO* pathway (see ref *2*). Much information about the *PHO* signaling pathway has been obtained through the use of *PHO5* gene expression as its extracellular phosphatase activity can easily be monitored. Transcription of the *PHO5* gene, as well as several other *PHO* genes, is regulated through a system consisting of the cyclin-dependent kinase (CDK), Pho85p, one of its cyclins, Pho80p, and the CDK inhibitor (CKI), Pho81p, together with transcription factors Pho4p and Pho2p (*1*, *7*, *9*). High-capacity uptake of free phosphate from outside the cell is under phosphate limitation mediated by the H^+-coupled Pho84 cotransporter (*3*, *10*, *11*). The expression of *PHO84* is derepressed when the external

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[1] Abbreviations: CDK, cyclin-dependent kinase; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; CKI, CDK inhibitor; HP_i, high-phosphate medium; LP_i, low-phosphate medium; MP, methylphosphonate; NMR, nuclear magnetic resonance; Phi, phosphite; P_i, inorganic phosphate; polyP, polyphosphate; rAPase, repressible acid phosphatase.

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phosphate concentration of the growth medium decreases to ~100 μM (*12*, *13*). Synthesis and transport activity of the Pho84p is maintained at its highest levels at a cell density corresponding to the midexponential growth phase during logarithmic batch growth, a condition under which the extracellular phosphate concentration is in the range of 30−40 μM. At lower phosphate concentrations, the protein is removed from the plasma membrane and subjected to vacuolar sorting and degradation (*12*). Like other yeast transporters which can be removed from the plasma membrane in response to high levels of their corresponding substrate (*14*), a regulation which presumably prevents an overload of excess external substrate, the Pho84p transporter expressed in cells grown at a low level of phosphate is endocytosed and sorted to the vacuole in response to added high levels of phosphate (*13*). The adaptation of the cells to phosphate limiting and surplus conditions requires that the prevailing concentration be "sensed" by the cells. The possibility that the Pho84p transporter is active in phosphate sensing has previously been investigated in a *pho84* defective mutant in which a complete derepression of *PHO5* was found to be caused by a decrease in the rate of phosphate uptake rather than the lack of the transporter molecule (*6*). Recently, evidence that expression of *PHO5* is regulated by intracellular orthophosphate levels in a manner strongly correlated with intracellular levels of phosphate and polyP was presented, in addition to evidence that orthophosphate is not the only signal of phosphate availability (*15*). In contrast to that of *PHO5*, *PHO84*, and *PHO89*, the transcription of *PHO87*, *PHO90*, and *PHO91* genes has been shown to be independent of both phosphate concentration and Pho4p activity (see refs *2* and *16*), and these genes are not expressed at a higher level in the absence of *PHO84* (*17*). However, Pho84p and Pho87p of cells starved for phosphate were recently shown to be able to sustain rapid phosphate signaling in the activation of the protein kinase A pathway in the presence of glucose, suggesting that they can act as phosphate sensors by using overlapping nutrient signal transduction pathways (*18*). In addition, evidence that Pho84p also has a role in metal ion transport has been presented (*11*, *19*). Clearly, the multiple roles of Pho84p in transport and signaling make it a central component in the regulation of cellular homeostasis.

This study addresses the substrate specificity of the phosphate sensory machinery and mobilization of the internal polyP pool of *S. cerevisiae* cells by use of the phosphate analogue, methylphosphonate (MP). The expression of Pho84p and Pho5p together with the status of the intracellular phosphate pools under different growth conditions was used to monitor the effects of MP on phosphate homeostasis.

EXPERIMENTAL PROCEDURES

Materials. [^{32}P]Orthophosphate (carrier-free), horseradish peroxidase-conjugated anti-mouse IgG antibody (from sheep), and the enhanced chemiluminescence detection kit were obtained from Amersham Biosciences. Anti-myc antibodies were obtained from Invitrogen. TaqPlus Long and TaqPlus Precision polymerases were from Stratagene. All other materials were reagent grade and were obtained from commercial sources.

Strain and Growth Conditions. S. cerevisiae strain CEN.PK113-5D (*MAT*a *MAL2-8^c SUC2 ura3-52*) harboring the chromosomal *PHO84* gene tagged with a tandem *MYC* epitope at its C-terminus (CEN.PK113-5D-Pho84-*myc*) and the strain harboring a C-terminal addition of GFP (CEN.PK113-5D-Pho84-GFP) used in this work have already been described (*13*). The CEN.PK113-5D-Δ*pho84* strain is a Pho84p null strain in which the *PHO84* gene was deleted using PCR technology essentially as described previously (*20*). The deletion cassette was PCR amplified from pU6H2MYC (*20*) with oligonucleotide primers 5′-ATCACCAGGGCACACAACAAACAAAACTCCACCACGAATACAATCCAATCCCACCACCATCATCATCAC and 5′-TGCGGCAGCAGCAAGTTCCAACTTAGCGTTAACATCCAATTGATAACTATAGGGAGACCGGCAGATC and subsequently transformed into CEN.PK113-5D cells for homologous recombination using the lithium acetate procedure (*21*). After selection on YPD-Geneticin (200 μg/mL) plates, colonies were restreaked on fresh YPD-Geneticin plates, and the deletion was verified by PCR.

Cells expressing *PHO84-MYC* or without the *PHO84* gene were precultivated aerobically for 12 h in YPD (1% yeast extract, 2% glucose, and 2% peptone) medium at 30 °C under agitation, harvested by centrifugation, washed twice with sterile water, and then inoculated in liquid high-phosphate (HP_i, 15 mM P_i) or low-phosphate (LP_i, 250 μM P_i) medium (*22*). Cells were grown aerobically at 30 °C under agitation. Samples for phosphate uptake assays, Western blot analyses, NMR analysis, and acid phosphatase activity measurements were withdrawn at the indicated time points as stated in the figure legends.

Acid Phosphatase Activity Measurements. Yeast cells were grown in either LP_i or HP_i at 30 °C to an A_{600} ranging from 0.5 to 10, and the acid phosphatase activity was assayed according to the method described previously (*4*, *23*).

Phosphate Transport Measurements. Phosphate uptake was assayed in intact *S. cerevisiae* cells expressing *PHO84-MYC* grown in LP_i. The uptake was initiated by the addition of 1 μL of [^{32}P]orthophosphate (carrier-free, 0.18 Ci/μmol, 1 mCi = 37 Mbq) to 30 μL aliquots of cells, resuspended to 3 mg of cells (wet weight)/30 μL of buffer [25 mM Tris-succinate (pH 4.5) and 3% glucose], to a final phosphate concentration of 0.22 mM essentially as described previously (*24*). The velocity of uptake was measured during the first minute in the absence or presence of 11 mM MP added to the 25 mM Tris-succinate assay buffer.

Western Blot Analysis of Pho84p Expression. Cells grown to specified A_{600} values in LP_i as described above were collected by centrifugation at 5500*g* and 4 °C for 10 min. Proteins were extracted and precipitated essentially as described previously (*25*). Equivalent concentrations of the resolubilized protein (25 μg) were mixed with sample buffer prior to separation on a 10% SDS−polyacrylamide gel (*26*). Immunoblotting to poly(vinylidene difluoride) membranes (Immobilon-P Millipore) was carried out according to the manufacturer's protocol (Amersham Biosciences). Use of anti-myc antibody and horseradish peroxidase-conjugated anti-mouse IgG antibody allowed for immunological detection of the Pho84-myc protein. After a short incubation with the chemiluminescent substrate, the blot was exposed to X-ray film for 1−2 min. The molecular masses of separated proteins were determined by the relative mobility of prestained marker proteins (Bio-Rad).

RNA Isolation and RT-PCR. Total RNA from yeast cells expressing *PHO84-MYC* in LP_i in the absence or presence of MP was isolated using RNeasy (Qiagen). The RNA samples were treated with DNase I (Amersham Biosciences) prior to RT-PCR. The SuperScript One-step RT-PCR system (Invitrogen) was used for the RT-PCR. Amplified DNA products were then separated on a 1% agarose gel. Primers (5′-ACTCTGGTGCTGAATGTGATGCTAGA and 5′-TGAGCAGTAGATCTGTAACGAGTT) designed for amplification of the *PHO84* gene were used in RT-PCR. Primers (5′-TGAGGTTGCTGCTTTGGTTA and 5′-TTCTGGGGCTCTGAATCTTT) were designed for amplification of the actin gene (*ACT1*).

Expression and Degradation of the Pho84−GFP Chimera and Microscopy Analyses. Yeast cells expressing the Pho84−GFP chimera were precultivated aerobically for 12 h in YPD medium at 30 °C, washed twice with water, and inoculated in LP_i in the absence or presence of 10 mM MP. Cells were grown aerobically at 30 °C, and samples for microscopy analyses were withdrawn at the indicated time points. Samples were mixed with an equal volume of melted agarose (1%), immobilized on a slide, and cooled prior to analysis. The Y-FL EX 465−495 epi-fluorescence filter (Nikon) was used for the excitation of the GFP. The cells were monitored using a 60/1.40 oil objective on a Nikon Eclipse E600 microscope (100 W Hg source) equipped with the C1 modular confocal microscope system (Nikon, Instech. Co., Ltd.) and a cooled CCD camera (C4742-95-12SC, Hamamatsu). For image capture, the Nikon EZ-C1 confocal microscope software was used. For analysis of Pho84−GFP degradation, yeast cells expressing the Pho84−GFP chimera were precultivated aerobically for 12 h in YPD medium at 30 °C, washed twice with water, inoculated in LP_i, and grown to an A_{600} of 2.4, corresponding to the maximal expression of the Pho84−GFP chimera. Cells were then treated with either the combination of MP (10 mM) and cycloheximide (10 μg/mL) or only cycloheximide (10 μg/mL). Protein internalization was analyzed by fluorescent micrographs as described above over the course of 80 min.

In Vitro ^{31}P NMR Measurements. Yeast cells expressing *PHO84-MYC* or without the *PHO84* gene (Δ*pho84*) grown in HP_i or LP_i, in the absence or presence of 10 mM MP, were harvested at specified A_{600} values by centrifugation at 5000*g* for 5 min, and washed twice with sterile water, and 4 or 2.5 g portions of collected cells were frozen in liquid nitrogen. Perchloric acid (PCA) extraction of cells was performed as described previously (*27*). Divalent cations, Mg^{2+} and Mn^{2+}, were chelated by addition of 50 mM CDTA, and the pH was buffered with 25 mM Hepes and adjusted to pH 7.5. The samples (3 mL) contained 10% (v/v) D_2O. The deuterium resonance of D_2O was used as a signal lock, and samples were analyzed for 1 h at room temperature. NMR spectra were recorded on a Varian NMR spectrometer (500 MHz) equipped with a 10 mm multinuclear probe tuned at 202.519 MHz. The acquisition conditions were as follows: 70° radio frequency pulses (22 μs) at 1.8 s intervals, spectral width of 11 000 Hz, and 1024 scans. Free induction decays were collected as 8K data points, zero-filled to 16K, and processed with a 5.0 Hz exponential line broadening. The specific assignments of individual resonances were based on published chemical shifts (*28*) and, additionally, on those of known compounds added to the samples during PCA extraction. The chemical shifts of all ^{31}P spectra were referenced to an 85% phosphoric acid external reference at 0 ppm (*29*).

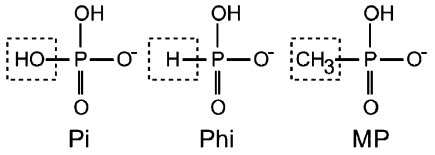

FIGURE 1: Structure of inorganic phosphate (P_i) and phosphate analogues phosphite (Phi) and methylphosphonate (MP).

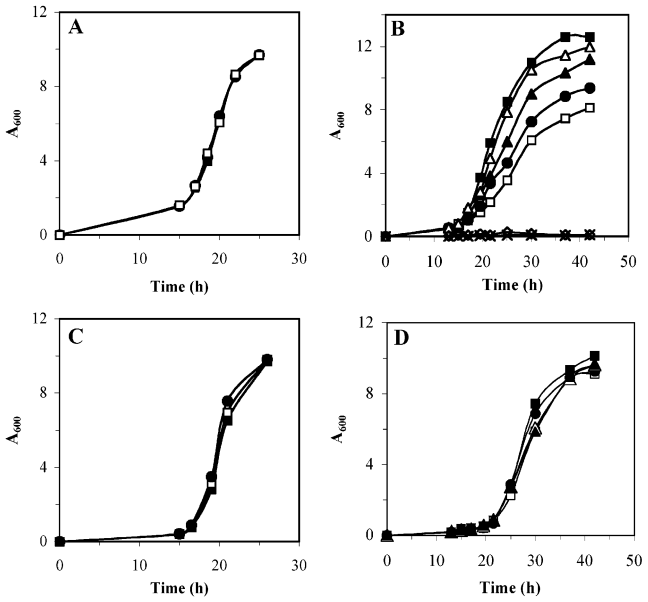

FIGURE 2: Growth analysis of cells expressing *PHO84-MYC* or without the *PHO84* gene under HP_i or LP_i growth conditions, in the absence or presence of different concentrations of MP and Phi. (A) *PHO84-MYC* cells were grown in HP_i (■), in the presence of 10 mM MP (□) or 0.1 mM Phi (●). (B) *PHO84-MYC* cells were grown in LP_i (■), in the presence of 0.1 mM MP (△), 1 mM MP (▲), 10 mM MP (□), 0.1 mM Phi (●), 1 mM Phi (◇), or 10 mM Phi (×). (C) Δ*pho84* cells were grown in HP_i (■), in the presence of 10 mM MP (□) or 0.1 mM Phi (●). (D) Δ*pho84* cells were grown in LP_i (■), in the presence of 0.1 mM MP (△), 1 mM MP (▲), 10 mM MP (□), or 0.1 mM Phi (●).

RESULTS

Cells Expressing PHO84 Show Increased Sensitivity toward Nonmetabolized Phosphate Analogues. The toxicity of phosphate analogues, methylphosphonate (MP) and phosphite (Phi) (Figure 1), on yeast cells was investigated in an effort to establish suitable cell culture conditions and analogue concentrations for treatment. Cells, *PHO84-MYC* or deleted in *PHO84* (Δ*pho84*), were grown in HP_i or LP_i in the absence or presence of the analogues at different concentrations (Figure 2). Both yeast strains grew at the same rates on HP_i (Figure 2A,C) independent of the analogue concentration that was applied. In contrast, cells expressing *PHO84-MYC* in LP_i show an inhibited growth in the presence of high concentrations of MP or Phi. Indeed, additions of 1 or 10 mM Phi completely arrest cell growth, while Phi at a concentration of 0.1 mM exerted a moderate inhibition of growth (Figure 2B). Also, treatment with MP led to inhibition of cell growth but at higher concentrations than in the case of Phi. While 10 mM MP led to a partial inhibition of growth over the cultivation time that was used (25−42 h), 0.1 and

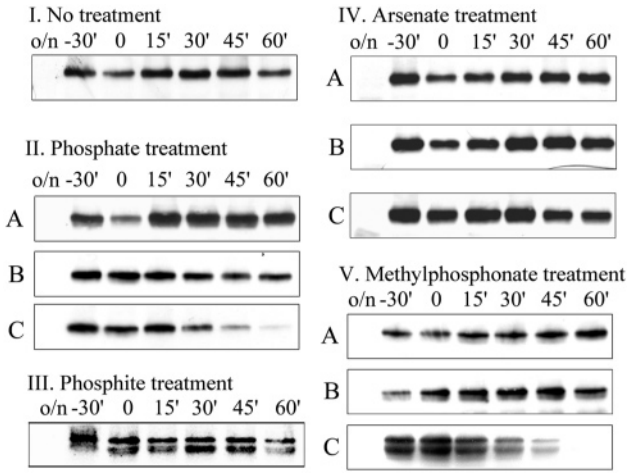

FIGURE 3: Levels of synthesis and degradation of Pho84p in cells expressing *PHO84-MYC* in the absence or presence of various concentrations of phosphate and phosphate analogues. Cells were precultured in YPD medium for 12 h (denoted o/n) and were after inoculation in LP_i allowed to grow to an A_{600} of 2.5 (denoted −30), after which the individual cultures were treated with the specified phosphate analogues (denoted 0). Samples were withdrawn at the indicated time points (o/n, 0, 15, 30, 45, and 60 min), and proteins were solubilized; 25 μg of membrane protein was subjected to SDS−polyacrylamide gel electrophoresis, immunoblotting, and detection of the Pho84-myc protein using an anti-myc antibody as described in Western Blot Analysis of Pho84p Expression: (I) no treatment, (II) phosphate treatment [addition of 0.1 (A), 1 (B), and 10 mM (C) phosphate], (III) Phi treatment (addition of 0.1 mM Phi), (IV) arsenate treatment [addition of 0.1 (A), 1 (B), and 10 mM (C) arsenate], and (V) MP treatment [addition of 0.1 (A), 1 (B), and 10 mM (C) MP].

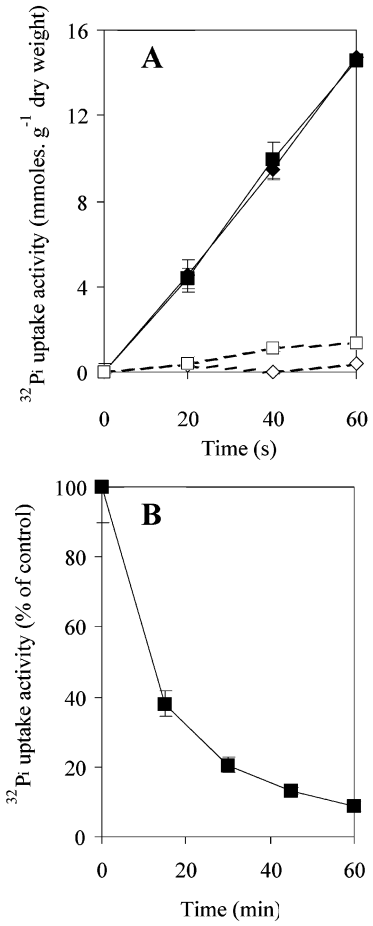

FIGURE 4: Uptake of $^{32}P_i$ into *PHO84-MYC* cells grown under LP_i conditions. (A) Cells grown in LP_i to an A_{600} of 2.5 were divided into two cultures, one subjected to continued growth for 60 min in the same medium and another in which the growth medium was supplemented with 11 mM MP during the continued growth. LP_i-grown cells were assayed for $^{32}P_i$ uptake in the absence (■) and presence (◆) of 11 mM MP, and LP_i/MP medium-grown cells were assayed for $^{32}P_i$ uptake in the absence (◇) and presence (□) of 11 mM MP. (B) LP_i-grown cells at an A_{600} of 2.5 were treated with 11 mM MP for 0, 15, 30, 45, and 60 min, harvested, and subjected to $^{32}P_i$ uptake analysis. Activities were normalized. $n = 3$.

1 mM MP affected growth of the cells only to a limited extent (Figure 2B). Interestingly, LP_i-grown Δ*pho84* cells were essentially unaffected by the presence of 10 mM MP or 0.1 mM Phi (Figure 2D). These results suggest that uptake of the analogues is mediated by the Pho84 high-affinity transporter localized in the plasma membrane under LP_i conditions. Because of the high toxicity of Phi toward LP_i-grown cells expressing *PHO84-MYC*, no concentration higher than 0.1 mM was used in this study.

Recognition of Phosphate and Its Nonmetabolized Analogue Methylphosphonate Triggers Degradation of the Pho84p Transporter. PHO84-MYC cells were grown in LP_i to an A_{600} of 2.5 with a continued agitated growth in LP_i for 60 min in the absence (Figure 3, I) or in the presence of a concentration of 0.1 (A), 1 (B), or 10 mM (C) phosphate (Figure 3, II), arsenate (Figure 3, IV), or MP (Figure, 3 V), or 0.1 mM Phi (Figure 3, III). Cells harvested at indicated time points were subjected to protein solubilization, electrophoretic separation, and Western blot analysis. As can be seen in part C of panel II of Figure 3, addition of 10 mM phosphate induced degradation of the full-length Pho84-myc protein with a close to complete removal after incubation for 60 min. Treatment with lower concentrations of phosphate, 0.1 and 1 mM over the same time, did not affect the stability of the transporter, and protein levels were fully comparable with that of the nontreated cells. This observation suggests that the removal of the Pho84 transporter occurs in a concentration-dependent manner with respect to phosphate. Importantly, since Pho84-myc is expressed at its highest level at an A_{600} of 2−3 (see Figure 5A), degradation of the protein due to cell toxicity can be ruled out since no or a minor inhibitory effect on cell growth is seen at these A_{600} values, even in the presence of MP and Phi at concentrations of 10 and 0.1 mM, respectively (Figure 2B). Also, addition of MP at a concentration of 10 mM (Figure 3, V, C) triggered degradation of the Pho84-myc protein at a rate comparable to that observed in the presence of 10 mM phosphate and, thus, lowered the high-affinity phosphate uptake mediated by Pho84p to less than 10% of the initial activity during the incubation (Figure 4B). In contrast, treatment with arsenate at a concentration of 0.1, 1, or 10 mM (Figure 3, IV) did not lead to any significant degradation of the Pho84p and Phi at a concentration of 0.1 mM exerted a moderate effect on the stability of the protein. Although not affecting the Pho84p directly, arsenate at a concentration of 10 mM arrests cell growth (not shown) due to, for example, drastic effects on cellular respiration. It is most likely that the cell machinery required for the degradation process is rendered nonfunctional by the addition of arsenate. Like arsenate, Phi is toxic to the cells and inhibits growth of *PHO84-MYC*-expressing cells at a concentration of 0.1 mM and completely arrests growth at 1 and 10 mM (Figure 2). Our data suggest that MP is not as toxic as both arsenate and Phi and did not cause cessation

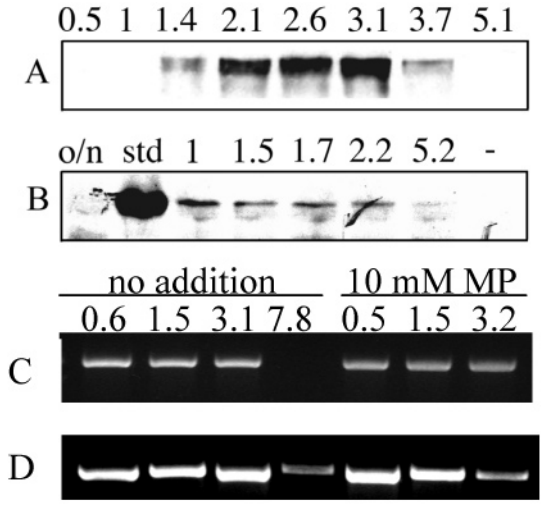

FIGURE 5: Analysis of synthesized Pho84p and the *PHO84* transcript in LP_i-grown *PHO84-MYC*. Cells were collected from the 12 h YPD preculture (denoted o/n) and from the inoculated LP_i cell culture in the absence or presence of 10 mM MP. (A) Pho84 synthesized during growth in LP_i. Samples were collected at A_{600} values of 0.5, 1, 1.4, 2.1, 2.6, 3.1, 3.7, and 5.1; 25 μg of extracted membrane proteins was analyzed by SDS−polyacrylamide gel electrophoresis followed by immunodetection as described in Western Blot Analysis of Pho84p Expression. (B) Pho84 synthesized during growth in LP_i supplemented with 10 mM MP was withdrawn at A_{600} values of 1, 1.5, 1.7, 2.2, and 5.2, and analyzed as described above. In lane 2, std corresponds to a reference sample (25 μg) of *PHO84-MYC* cells withdrawn at an A_{600} of 2.2 from an LP_i culture grown in the absence of MP. (C) *PHO84-MYC* cells were grown in LP_i in the absence or presence of 10 mM MP. RT-PCR was performed on the cell samples collected at the defined A_{600} values, using synthesized homologous primers directed toward the *PHO84* gene; 100 ng of total RNA was used for each reaction. (D) *PHO84-MYC* cells were grown and analyzed as described for panel C using synthesized homologous primers directed toward the actin gene (*ACT1*).

of cell growth, even at a concentration of 10 mM. Interestingly, samples used for MP and Phi treatment exhibited two separate immunodetected Pho84-myc protein bands, apparently equally sensitive to degradation, both prior to and following addition of 10 mM MP (Figure 3, V, C) or 0.1 mM Phi (Figure 3, III), while samples collected for control (Figure 3, I), phosphate (Figure 3, II), or arsenate (Figure 4) treatment revealed a single Pho84-myc protein band. The appearance of the two resolved Pho84-myc proteins suggests that the protein can exist in two forms equally accessible to MP and Phi treatment. The molecular properties of the two forms of the protein, probably reflecting structurally modified populations of the protein, still remain to be clarified.

The Presence of Methylphosphonate Does Not Affect Phosphate Uptake by Pho84p. The specificity of the Pho84 transporter for phosphate and MP was studied by a competitive uptake analysis. *PHO84-MYC* cells expressing the transporter were grown to an A_{600} of 2.5 in LP_i, harvested, and assayed for $^{32}P_i$ uptake in the absence and presence of 11 mM MP (Figure 4A). Although MP was present in 50-fold excess over phosphate (0.22 mM), the $^{32}P_i$ uptake velocity was unaffected by the presence of the analogue, implying that phosphate is taken up in preference over MP. Preincubation of the LP_i-grown cells with 11 mM MP for 60 min prior to the $^{32}P_i$ uptake assay resulted in an essentially abolished transport activity, in agreement with the data depicted in Figure 3. Since MP appears not to be recognized as a transport competent substrate of Pho84p, it is possible that the degradation and inactivation of Pho84p [Figure 3 (V, C) and Figure 2B, respectively] are mediated by a periplasmically located regulatory substrate recognition site on the transporter.

Methylphosphonate Does Not Repress Transcription of PHO84-MYC. Since MP appears to induce the degradation of the Pho84-myc protein in the plasma membrane, it was interesting to see whether the expression and *de novo* synthesis of the protein were affected by the presence of MP during growth in LP_i. For this reason, *PHO84-MYC* cells were grown in the absence (Figure 5A) or presence (Figure 5B) of 10 mM MP, and samples were withdrawn from the cultures at different A_{600} values. The immunodetection of solubilized Pho84-myc subjected to electrophoresis shown in Figure 5 illustrates the synthesis of Pho84-myc at A_{600} values in the range of ~1−4, with the highest protein levels detected between an A_{600} of 2−3, when grown in the absence of MP (Figure 5A). In contrast, inclusion of 10 mM MP in LP_i effectively repressed the accumulation of the protein at all A_{600} values monitored (Figure 5B). Because of the apparent downregulation mediated by a high concentration of this substrate-like molecule, we asked whether the lack of translated product was due to repressed transcription of the *PHO84-MYC* gene or events at the post-transcriptional level. We, therefore, performed a RT-PCR analysis on harvested *PHO84-MYC* cells grown in LP_i in the absence and presence of 10 mM MP (Figure 5C). As can be seen, the *PHO84-MYC* transcripts were also present in cells exposed to MP. Thus, the transcription of the *PHO84* gene appears not to be repressed by 10 mM MP present during growth. This observation together with the corresponding very low levels of synthesized protein (Figure 5B) is in support of the interpretation that MP, unlike phosphate, is not able to repress the *PHO*-regulated expression of the *PHO84-MYC* gene, and that MP apparently exerts its effect on the Pho84 transporter at the post-transcriptional level.

Methylphosphonate Alters Pho84 Accumulation and Triggers Pho84 Internalization under Phosphate-Limiting Conditions. MP present during LP_i growth conditions drastically reduces the cellular Pho84p content without causing a repression at the transcript level (Figure 5). To distinguish among three possible scenarios, (i) a decreased Pho84 translation efficiency, (ii) an alternative sorting of the protein to the vacuole excluding delivery to the plasma membrane, or (iii) unperturbed sorting of the protein to the plasma membrane where it is subjected to an MP-triggered internalization resulting in lowered membrane and cellular levels of Pho84, we analyzed the LP_i expression of a Pho84−GFP chimera in the absence and presence of 10 mM MP (Figure 6). As can be seen, the Pho84−GFP chimera is targeted to the plasma membrane at an early stage of growth in the presence of MP added to the LP_i (Figure 6A, a), although at a lower level than in the absence of MP (Figure 6B). After cells were grown for 6 h in LP_i supplemented with MP, the Pho84−GFP chimera clearly accumulates in fluorescent vesicular structures (Figure 6A, b) preceding an enhanced internalization of the protein as compared to cells grown in LP_i only (Figure 6B). The Pho84−GFP chimera is targeted to the vacuole for proteolytic breakdown (Figure 6A, c) as previously reported (*12*). In agreement with the data shown in Figure 5B, an efficient membrane accumulation of the

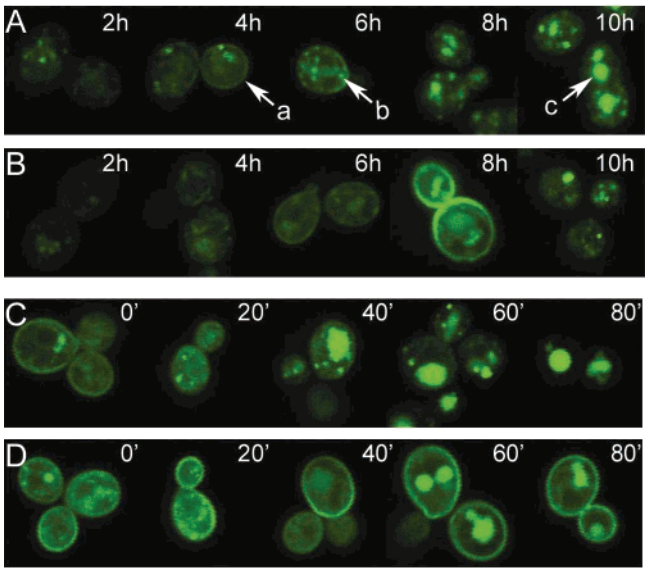

FIGURE 6: Time course study of localization and degradation of the expressed Pho84−GFP protein in the presence of MP. Fluorescent micrographs of cells expressing *PHO84-GFP* grown in LP_i in the presence (A) or absence (B) of 10 mM MP. Images were analyzed and qualitatively compared at specified time points of growth up to 10 h. Cells grown in LP_i to an A_{600} of 2.4, corresponding to the maximal expression of the Pho84−GFP chimera, were treated with either 10 mM MP and cycloheximide (10 μg/mL) (C) or only cycloheximide (10 μg/mL) (D). Expression and internalization of the proteins were analyzed by fluorescent micrographs over a time course of 80 min.

Pho84 protein is prevented by the presence of MP. Since the Pho84 mRNA transcription is unaffected (Figure 5C) and the translation functional (Figure 6A), we wished to investigate whether the degradation triggered by MP (see Figure 3, V, C) was similar to the one previously observed with addition of repressive amounts of P_i (*13*). Cells grown in LP_i to an A_{600} of 2.4, a value at which the Pho84−GFP chimera is strongly expressed and membrane localized, were treated with 10 mM MP supplemented with cycloheximide to halt protein synthesis. Pho84−GFP trafficking was analyzed over a time course of 80 min (Figure 6C). As can be seen, the presence of cycloheximide did not prevent the MP-triggered internalization of the Pho84−GFP protein, the characteristic intracellular distribution, and routing to the previously identified vacuolar compartment (*12*) (Figure 6C), while cells treated with cycloheximide only retained an unaffected peripheral fluorescence throughout the time course that was studied (Figure 6D). Treatment of cells with MP only did not reveal any detectable difference in fluorescence intensity or distribution when compared to the data shown in Figure 6C (not shown). The results indicate that the Pho84 protein is synthesized and sorted to the plasma membrane also in the presence of MP but that accumulation of the protein in the membrane is efficiently prevented by the enhanced internalization and degradation triggered by MP at the level of the plasma membrane.

Methylphosphonate Uptake Is Dependent on the PHO84 Gene Expressed under Phosphate-Limiting Conditions. To analyze the intracellular levels of phospho compounds, especially MP, phosphate, and polyP, in *PHO84-MYC* and Δ*pho84* cells grown in MP-supplemented LP_i or HP_i, ^{31}P NMR analyses of cells collected at A_{600} values of 1.5, 3, 5, and 8 were carried out. *PHO84-MYC* cells grown in MP-supplemented HP_i (Figure 7A) revealed similar and relatively constant pools of inorganic phosphate and polyP, at chemical shifts of 2.3 and −22.5 ppm, respectively. Additional peaks include (a) sugar phosphates at approximately 4 ppm, (b) glycerol-3-phosphorylcholine at approximately −0.2 ppm, and (c) glyceryl-3-phosphorylethanolamine at approximately −0.9 ppm, and the transient energy forms of NDPs, NTPs, and NADH found at positions d−f corresponding to the γ-, α-, and β-phosphates, respectively, of NDPs and NTPs (−5, −10, and −20 ppm, respectively) were also apparent. The presence of MP does not affect the status of phosphorylated compounds in these HP_i-grown cells. MP is barely detectable in any of these samples (Figure 7A, spectra I−III). Only after prolonged growth (Figure 7A, spectrum IV) is MP detected as the small peak at a chemical shift of 23 ppm. MP therefore appears to be a poor substrate for the phosphate transport system under these HP_i conditions. As expected, the ^{31}P NMR spectra recorded for Δ*pho84* cells grown under the same conditions were similar to those of the *PHO84-MYC* cells. The *PHO84* disruptant did not confer any changes in phosphate or MP acquisition during growth in MP-supplemented HP_i, but the level of polyP was lower than in *PHO84-MYC* cells.

PHO84-MYC cells grown in MP-supplemented LP_i revealed when analyzed at an A_{600} of 1.5 a drastically different spectrum (Figure 7B, spectrum I). Typically, also for cells grown in LP_i in the absence of MP (*2*), the intracellular storage of polyP (−23 ppm) and shorter chains of tri- and tetra-polyP (peaks g and h, respectively) were totally depleted. The magnitudes of the peaks corresponding to the α-, β-, and γ-phosphates of NTPs [−5, −10, and −20 ppm, respectively (peaks d−f)] were also diminished. After prolonged growth (A_{600} of 2.5, spectrum II), the *PHO84-MYC* cells had acquired an intracellular phosphate level comparable with that of cells grown in HP_i/MP medium. At later stages of growth (Figure 7B, spectra III and IV), the intracellular level of phosphate gradually decreased again as the low levels of phosphate in the medium were exhausted and the cells entered stationary phase. At this growth stage, the regulation of the cellular phosphate levels is no longer maintained by the Pho84 transporter (see Figure 5).

MP at a high concentration is found in the *PHO84-MYC* cells grown in LP_i/MP medium as noted by the presence of the large peak at 23 ppm (Figure 7B, spectra II−IV). Thus, in contrast to the situation in HP_i, MP can readily gain entry into these cells when grown in LP_i and accumulate at a high concentration. However, the spectra derived from Δ*pho84* cells grown in MP-supplemented LP_i revealed a close to abolished, slow uptake of MP in the A_{600} range of 1.5−8, and severely impaired uptake of phosphate over the entire growth phase. All cellular metabolites derived from phosphate were below the level of detection (100 μM). Two alternatives may be distinguished explaining the presence of MP in the *PHO84-MYC* cells grown in LP_i/MP medium. In the first alternative, MP is efficiently taken up in LP_i-grown cells expressing *PHO84-MYC* (Figure 7B) but not in cells lacking the *PHO84* gene (Figure 7D), suggesting that MP enters the cells via the Pho84 transporter despite the MP-mediated inhibition of the growth phase-specific accumulation of plasma membrane-localized transporter molecules (Figure 5A,B) and the preference for phosphate in Pho84p-mediated transport (Figure 4A). Another hypothetical

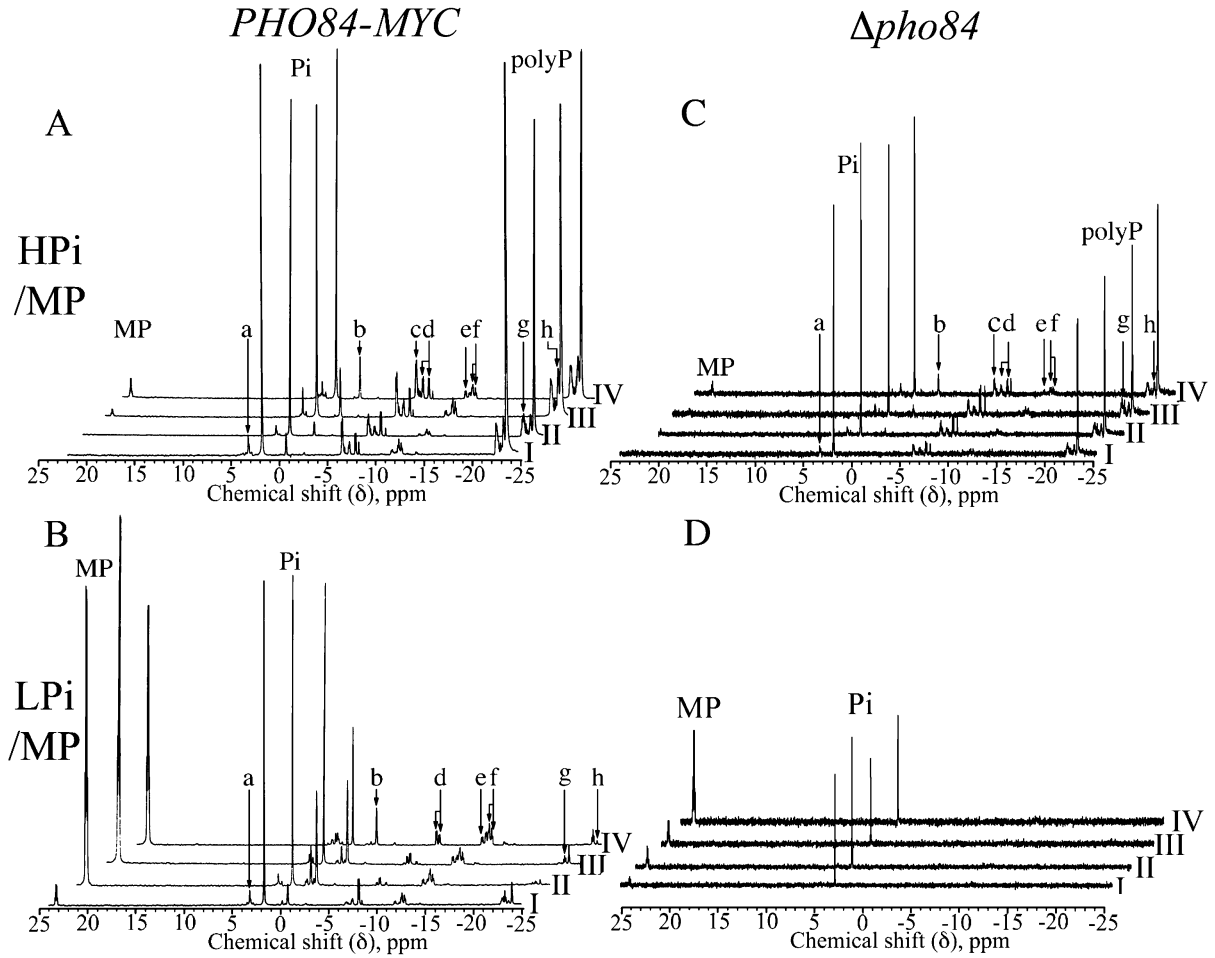

FIGURE 7: ^{31}P NMR spectra showing the intracellular levels of phosphate, polyP, and MP in *PHO84-MYC* and Δ*pho84* cells grown in LP_i or HP_i supplemented with 10 mM MP. Cells, 2.5 g (in the case of the Δ*pho84* strain) or 4 g (in the case of the *PHO84-MYC* strain), harvested at specified A_{600} values were treated with 10% (v/v) perchloric acid (PCA) and neutralized, as described in Experimental Procedures, and subjected to ^{31}P NMR analysis. Spectral peaks were assigned from the literature and by addition of known phospho compounds to the sample during PCA extraction, and are as follows: (a) phosphomonoesters, including glucose 6-phosphate and fructose 6-phosphate (at approximately 4 ppm); (b) glycerylphosphorylcholine (−0.2 ppm) and glycerylphosphorylethanolamine (at approximately −0.9 ppm); (c and d) γ-phosphate of NTPs (−5 ppm); (e) α-phosphate of NTPs (−10 ppm); (f) β-phosphate of NTPs (−20 ppm) and the terminal phosphate signals from polyP and triphosphate; (g) tri-polyP; and (h) tetra-polyP. (A) *PHO84-MYC* cells grown in HP_i supplemented with 10 mM MP. (B) *PHO84-MYC* cells grown in LP_i supplemented with 10 mM MP. (C) Δ*pho84* cells grown in HP_i supplemented with 10 mM MP. (D) Δ*pho84* cells grown in LP_i supplemented with 10 mM MP. ^{31}P NMR spectra of cells harvested at A_{600} values of 1.5 (I), 3.0 (II), 5.0 (III), and 8.0 (IV) are shown.

alternative is that MP bound to the Pho84 transporter and possibly inside the endocytic vesicles enters the cell via the turnover of the protein through degradation and internalization processes.

Pho84p-Transported Methylphosphonate Mediates Suppression of the Acidic Phosphatase. The finding that MP does not appear to affect *PHO84* at the transcriptional level (Figure 5C) urged us to assess the *PHO* derepression by monitoring the acid phosphatase activity of both strains when grown in HP_i or LP_i in the absence or presence of 10 mM MP (Figures 8 and 9). The expression and activity of the secreted phosphatase, Pho5p, have previously been used as a marker to monitor the effect of varying phosphate conditions on the expression of the genes controlled by the *PHO* regulon (*1*). *PHO5* expression is regulated in response to external phosphate availability, showing a strong increase in the level of *PHO5* transcription when phosphate becomes limiting (*7*). Similar criteria were used for the acid phosphatase assay of cells harboring the *PHO84-MYC* construct and of cells without *PHO84* (Δ*pho84*). *PHO84-MYC* cells grown in LP_i (Figure 8A) displayed a 4-fold increased Pho5p activity during early growth ($A_{600} = 1.5$), which remained constant during further growth ($A_{600} = 3-10$). However, inclusion of 10 mM MP in this growth medium (Figure 8A) reduced the phosphatase activity by 50−70% compared to that for growth in the absence of MP. The phosphate-mediated decrease in the acidic phosphatase activity has previously been suggested to be the cause of a lowered level of expression of this enzyme (*30*). The Δ*pho84* strain did not exhibit a repressed acidic phosphatase activity when grown in LP_i (Figure 8B), but displayed a linear increment of the activity of cells harvested in the A_{600} range of 0.5−7, corresponding to a 16-fold enhanced activity. In contrast to the *PHO84-MYC* strain, these cells displayed, in the presence of MP (Figure 8B), an activity close to that seen in the absence of the analogue indicating that MP is able to cause only a moderate suppression of the *PHO5*, which may be explained by the inability of the deletion mutant to sense the presence of MP. This is in agreement with the finding that Pho84p is required for the MP acquisition (Figure 7)

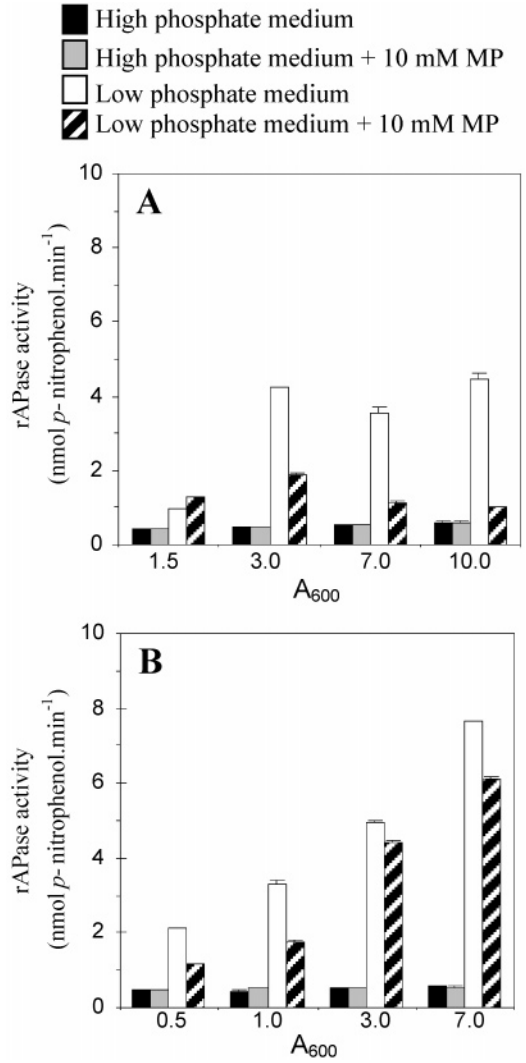

FIGURE 8: Acidic phosphatase activity of *PHO84-MYC* and Δ*pho84* cells grown in the absence or presence of 10 mM MP. (A) *PHO84-MYC* cells grown in LP_i or HP_i in the absence or presence of 10 mM MP were collected at specified A_{600} values and subjected to acidic phosphatase measurement. (B) Δ*pho84* cells grown and analyzed as described for panel A.

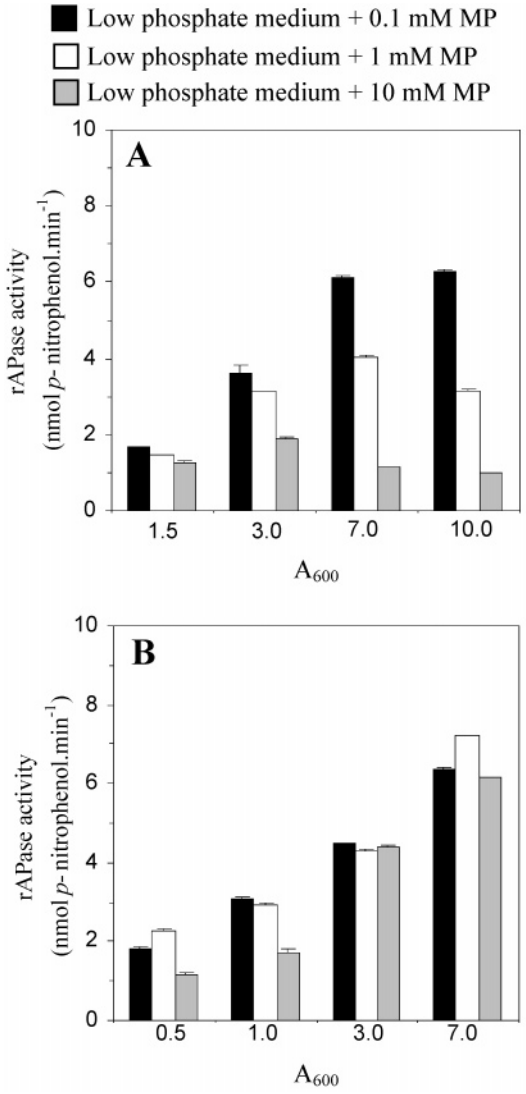

FIGURE 9: Acidic phosphatase activity of *PHO84-MYC* and Δ*pho84* cells grown in LP_i in the presence of various concentrations of MP. (A) *PHO84-MYC* cells grown in LP_i supplemented with 0.1, 1, or 10 mM MP were collected at specified A_{600} values and subjected to acidic phosphatase measurement. (B) Δ*pho84* cells, grown and analyzed as described for panel A.

and thereby for the analogue to elicit downregulation of Pho5p (Figure 8) and Pho84p (Figure 4B) activities. Both strains displayed a constant, repressed Pho5p activity when grown in HP_i, independent of whether MP was present (Figure 8A,B). In a similar fashion as observed for the induction of Pho84p degradation (Figure 3, II), different concentrations of MP also resulted in varying degrees of inhibition of the Pho5p activity in *PHO84-MYC* cells (Figure 9A). Growth in LP_i in the presence of MP at a concentration of 0.1 mM had a negligible effect on the acid phosphatase activity. However, an enhanced inhibition was observed in the presence of 1 mM, and a concentration of 10 mM rendered a fully repressed Pho5p activity (Figure 9A). The minor inhibition caused by 10 mM MP during growth of the Δ*pho84* mutant (Figure 9B) supports the requirement of Pho84p for the control of Pho5p.

DISCUSSION

The important role of the Pho84 transporter in the phosphate metabolic pathway is well-documented in studies demonstrating a greatly enhanced upregulation of the *PHO84* transcript when phosphate is limiting (*3*, *31*, *32*). In this study, the nonmetabolized analogue MP was selected for assessment of cellular phosphate homeostasis and the importance of the Pho84 transporter in this regulation. *PHO84-MYC* cells grown in LP_i supplemented with 10 mM MP were, in contrast to Δ*pho84* cells, capable of uptake of MP at A_{600} values coinciding with the highest expression levels of Pho84p observed when cells were grown in the absence of MP in LP_i (Figure 4). This suggests that Pho84p despite the barely detectable levels of synthesized protein (Figure 5), when grown in the presence of MP, maintains sufficient levels of the transporter for an accumulation of MP and phosphate. Alternatively, the entry of MP into the cells could occur via the internalization and degradation of Pho84p (*12*). In this alternative hypothetical explanation, MP molecules might be bound to Pho84p or trapped in the vesicular lumen. However, also in the absence of Pho84p, a delayed and low-level accumulation of MP takes place, suggesting that MP can gain entry also by use of the less efficient low-affinity uptake system (Figure 7). Interestingly,

in *PHO84-MYC* cells grown in MP-supplemented LP_i, a prolonged presence of phosphate was detected during late growth, suggesting that the presence of MP delayed the use of phosphate or, alternatively, that phosphate release from the vacuole was slower. This finding is in agreement with a reported reduced rate of release of phosphate in Phi-treated cells of *S. cerevisiae* (*33*). On the other hand, the inability of MP to prevent the breakdown of polyP in the cells expressing the *PHO84-MYC* construct under LP_i conditions implies that the intracellular signaling that controls the mobilization of polyP is unaffected by the presence of the analogue. The disappearance of polyP is probably due to its mobilization as a short-term response to phosphate starvation, as previously suggested by other studies (*2*, *31*). An alteration of the polyP synthesis could result in an alteration of the activity of the high-affinity plasma membrane transport system and therefore influence acquisition of phosphate from the external environment (*32*). This aspect has interesting implications for signaling between the vacuole, the main polyP storage compartment in which synthesis and breakdown of this long chain polymer occur, and the expression of genes involved in scavenging and acquisition of phosphate (*34*). The MP molecule is clearly not a favored substrate of the Pho84 transporter. However, although neither short-time exposure to MP or lower concentrations (0.1 and 1 mM) of MP affect the stability of the Pho84p, incubation of the *PHO84-MYC* cells with 10 mM MP reduces the amount and activity of the protein in the plasma membrane by more than 90% (Figures 3 and 4), a finding consistent with that seen for its natural substrate, phosphate, at the same concentration (*13*). In contrast, both arsenate at a concentration of 0.1, 1, or 10 mM and Phi at a concentration of 0.1 mM failed to mimic such a substrate-specific response of the Pho84 transporter in the membrane, possibly due to their toxic effect on cellular processes, such as respiration (Figures 2 and 3). However, in a recent study (*18*), it was shown that short-term arsenate treatment at a concentration of 0.2 mM has an effect similar to that of phosphate treatment on the induction of trehalase activity of the cells, and that the arsenate-induced activation of trehalase was Pho84p-specific. It was concluded that arsenate can be used to mimic phosphate in triggering the phosphate signaling pathway and that Pho84p was an essential component in the signaling pathway involved in trehalase activation and phosphate sensing. It can, however, not be ruled out that binding of these analogues, prior to the proteolytic event, to a degradation-sensitive structure in Pho84 is prevented by a lack of structural recognition. On the other hand, MP appears to convey a true phosphate-like response at the level of Pho84p even though its minor molecular deviation from the natural substrate is large enough to significantly decrease its affinity for binding and/or transport. It is presently not known whether the phosphate recognition preceding degradation involves a separate binding site that harbors a general phosphate sensing function. The usefulness of MP in dissecting the phosphate signaling and expression of the *PHO* genes is further emphasized by the finding that the derepressive expression of the *PHO84* gene and its translation are unperturbed by the presence of 10 mM MP during growth in LP_i, while the triggered degradation of the membrane-localized Pho84−GFP chimera does not lead to any major accumulation in the membranes (Figure 6). Such a post-transcriptional regulation of the Pho84p activity has been proposed previously (*6*, *17*). Indeed, during growth in LP_i in the presence of MP, low levels of Pho84 are observed and small amounts of the protein accumulate in the plasma membrane (Figures 5 and 6). Although our results suggest that MP triggers internalization and degradation of the membrane-localized Pho84 protein also in the presence of cycloheximide, conditions at which *de novo* synthesis of the protein is halted, the existence of an alternative sorting whereby the newly synthesized protein is directly routed to the vacuole for degradation cannot be excluded. Such a scenario has previously been reported for Tat2, a high-affinity tryptophan permease in yeast, which is regulated at the level of protein sorting and stability. In starved cells, the internal newly synthesized Tat2 is routed to the vacuole in a manner that is independent of the plasma membrane for degradation (*35*). The finding that transcription of *PHO84* is not affected by the presence of 10 mM MP (Figure 5) raised the possibility that the expression of *PHO5* was also unaffected (Figures 8 and 9). MP contained in the LP_i resulted in a concentration-dependent decrease in the acidic phosphatase activity of *PHO84-MYC* cells to a level close to that observed with HP_i-grown cells, in the absence or presence of high MP concentrations. In contrast to the situation with the *PHO84-MYC* cells, the acidic phosphatase activity of Δ*pho84* cells was close to 2-fold higher in a situation where the intracellular phosphate level was at a constant and low level. In agreement with the influence of Phi at a concentration of 0.5 mM on Δ*pho84* cells (*33*), MP-treated cells preserved ∼80% of its acidic phosphatase activity. The Δ*pho84* cells lack the tuned acid phosphatase response to changes in external MP concentrations. The impaired response of MP correlates well with the deficient uptake of the analogue in these cells.

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