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Molecular Mechanisms Controlling Phosphate-Induced Downregulation of the Yeast Pho84 Phosphate Transporter†

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ABSTRACT: In *Saccharomyces cerevisiae*, phosphate uptake is mainly dependent on the proton-coupled Pho84 permease under phosphate-limited growth conditions. Phosphate addition causes Pho84-mediated activation of the protein kinase A (PKA) pathway as well as rapid internalization and vacuolar breakdown of Pho84. We show that Pho84 undergoes phosphate-induced phosphorylation and subsequent ubiquitination on amino acids located in the large middle intracellular loop prior to endocytosis. The attachment of ubiquitin is dependent on the ubiquitin conjugating enzymes Ubc2 and Ubc4. In addition, we show that the Pho84 endocytotic process is delayed in strains with reduced PKA activity. Our results suggest that Pho84-mediated activation of the PKA pathway is responsible for its own downregulation by phosphorylation, ubiquination, internalization, and vacuolar breakdown.

The acquisition of inorganic phosphate in *Saccharomyces cerevisiae* is mediated via a low-affinity system composed of the phosphate transporters Pho87, Pho90, and Pho91 (*1*, *2*) and a high-affinity system composed of the inducible phosphate transporters Pho84 and Pho89 (*3*, *4*). The activity of the high-affinity system, and those of specific secreted acid phosphatases, are transcriptionally controlled by the availability of phosphate via the well-characterized regulatory *PHO* pathway (*4*, *5*). The uptake of free phosphate in this coordinated cellular response to scavenge extracellular phosphate at phosphate limiting conditions is mainly mediated by the H^+-coupled Pho84 cotransporter and to some extent by the Na^+-coupled Pho89 transporter (*5*−*9*). A model has been proposed suggesting a positive feedback mechanism in the *PHO* pathway which under phosphate-limiting conditions downregulates the low-affinity transporter and thereby further activates the *PHO* pathway, including derepression of the Pho84 transporter (*1*).

The *PHO*-regulated *PHO84* gene is derepressed when the external phosphate concentration of the growth medium decreases to ∼100 μM (*10*, *11*). The control of the Pho84 activity is however not limited to transcriptional regulation. At external phosphate concentrations lower than 30−40 μM, the protein is removed from the plasma membrane and is subjected to vacuolar sorting and degradation (*11*). Although the existence of an endocytotic pathway for Pho84 in *S. cerevisiae* is now well established (*10*−*12*), the molecular mechanisms involved in this degradative pathway remain to be fully elucidated.

A widely accepted mechanism for selective degradation of plasma membrane proteins is via ubiquitination and/or phosphorylation, modifications that targets the proteins to the vacuole and/or lysosome (*13*−*17*). In this process, the highly conserved 76-amino acid protein, ubiquitin, is linked to the substrates by a regulated mechanism through the action of three enzymes, the ubiquitin activating enzyme, E1, the ubiquitin conjugating enzyme, E2 or Ubc, and a ubiquitin ligase, E3 (*15*). Most eukaryotes have one single E1 enzyme and several different E2 and E3 enzymes. Of the 11 existing E2/Ubc[1] enzymes in *S. cerevisiae*, at least one of the Ubc1/Ubc4/Ubc5 family members is

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[1]Abbreviations: DTT, dithioerythriol; MES, 2-(*N*-morpholino)-ethanesulfonic acid; GFP, green fluorescent protein; MP, methylphosphonate; PKA, protein kinase A; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMS, transmembrane segment; Ubc, ubiquitin conjugating enzyme.

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required for the ubiquitination of a large number yeast transporters and receptors (*18*) whereas one specific E3 ligase, Rsp5, has been suggested to be required for all plasma membrane proteins (*18*, *19*). Recently, the low-affinity transporters Pho87 and Pho91 were identified as targets of the Rsp5 endocytic pathway (*20*).

Studies have shown that phosphorylation at specific regions or sequence motifs of distinct plasma membrane proteins is needed prior to ubiquitination and internalization (*21*, *22*). The PEST sequence is a well-known phosphorylation target that is linked to ubiquitination (*23*). Another well-characterized sequence motif that is important in the internalization process is the SINNDAKSS motif initially found in the C-terminal tail of the membrane-bound Ste2 α-factor pheromone receptor (*24*). Analysis of the potential involvement of a SINNDAKSS-like sequence in the C-terminus of the Pho84 did not, however, reveal any alteration in the Pho84 degradation pattern (*11*).

More recently, a proteomics approach has identified putative sites in the amino acid sequence of Pho84 for ubiquitination (lysine residues K6 and K298) and for serine and threonine phosphorylation (S303, S321, S581, T302, and T317) (*25*). Interestingly, most of these predicted target residues are gathered in a central loop that connects two bundles of six helical transmembrane segments each that combined make up the Pho84 structure (*26*), and this proximity of the residues may facilitate cooperative modification which has been found for other plasma membrane proteins (*22*, *27*).

The PKA pathway controls several important processes in the cell by essential phosphorylation events in response to environmental cues, such as changes in nutrient availability. Addition of phosphate to phosphate-limited yeast cells is known to trigger rapid activation of the PKA pathway, and this process depends on the Pho84 transporter which apparently in this case also acts as a phosphate receptor, or "transceptor" (*28*). Downregulation of receptors by feedback inhibition involving phosphorylation and internalization is well-known, for instance, in the case of G-protein-coupled receptors (*29*). Whether Pho84-mediated activation of the PKA pathway plays a similar role in the phosphate-induced downregulation of Pho84 is unclear. Studies using the PKA inhibitor H89 have suggested that PKA influences the downregulation of Pho84 from the plasma membrane by delaying its internalization (*30*), but it is unclear whether this response is a direct and/or specific effect on Pho84 or rather a general plasma membrane protein turnover delay.

In this study, we have investigated the sequential process of phosphate sensing and signaling, and up- and downregulation of the Pho84 transporter. We show that the Pho84 protein is phosphorylated prior to being ubiquitinated and that the two events are essential for the subsequent internalization. Moreover, we show that the reduced PKA activity delays phosphate-induced internalization of Pho84, strongly suggesting that the Pho84 transceptor is responsible for its own downregulation via a negative feedback loop.

MATERIALS AND METHODS

Materials. [^{32}P]Orthophosphate (carrier-free, 0.18 Ci/μmol, 1 mCi = 37 MBq), horseradish peroxidase-conjugated anti-mouse Ig antibody (from sheep), the enhanced chemiluminescence kit, and Hi-Trap resin were obtained from GE Healthcare. Horseradish peroxidase-conjugated anti-rabbit Ig antibody (from sheep) and molecular weight marker proteins were obtained from Bio-Rad. Anti-Myc and anti-GFP antibodies and FM4-64 were from Invitrogen. Anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine antibodies were obtained from Zymed Laboratories, Inc. Anti-Pho84 antibodies were produced in rabbit against a peptide corresponding to the C-terminal end of the Pho84 protein (*31*). Zymolyase was purchased from Seikagaku Corp. Protein G-agarose was from Roche, and Protein G HP SpinTrap columns were from GE Healthcare. All other materials were reagent grade and were obtained from commercial sources.

Strains and Growth Conditions. *S. cerevisiae* strains used in this study are listed in Table 1. Cells used were precultivated aerobically under agitation (200 rpm) at 30 °C for 12 h in YPD medium (1% yeast extract, 2% glucose, and 2% peptone) or Synthetic Complete (SC) medium lacking uracil. Cells were harvested by centrifugation, washed twice with sterile water, and inoculated in low-phosphate medium (5.7 g/L yeast nitrogen base without phosphate) supplemented with 200 μM KH_2PO_4. Cells were grown aerobically at 30 °C under agitation (200 rpm). Samples for phosphate uptake assays, Western blot analyses, and microscope analyses were collected at the indicated time points as stated.

Starvation Conditions. Cells were cultured as described above into an exponential phase (A_{600} = 1.0−1.5) in YPD medium. Midexponential grown cells were harvested and transferred to phosphate starvation medium at pH 8 (5.7 g/L yeast

Table 1: Yeast Strains Used in This Study

strain	genotype	source
CEN.PK 113-5D	*MATa MAL2-8^C suc2 ura3-52*	*11*
CEN.PK 113-5D/*PHO84-MYC*	*MATa MAL2-8^C suc2 ura3-52 PHO84-MYC*	*11*
RH1800	*MATa ura3 leu2 his3 lys2 bar1-1*	*48*
RH3136	*MATa ubc1::HIS3 ura3 leu2 his3 lys2 trp1 bar1-1*	*48*
RH3130	*MATa ubc2::HIS3 ura3 leu2 his3 lys2 bar1-1*	*48*
RH3132	*MATa ubc4::TRP1 ura3 leu2 his3 lys2 ade2 bar1-1*	*48*
RH3142	*MATa ubc5::LEU2 ura3 leu2 his3 his4 lys2 trp1 bar1-1*	*48*
RH3143	*MATa ubc7::LEU2 ura3 leu2 his3 lys2 trp1 bar1-1*	*48*
SP1	*MATa leu2 his3 trp1 ade8 ura3 can1*	*49*
JT122	SP1 *tpk1^{w1}tpk2::HIS3tpk3::TRP1*	*50*
S-15-5D	SP1 *MATa his3 leu2 ura3 trp1 ade8 tpk1::ura3 tpk2^{w1} tpk3::TRP1 cat3::LEU2Tail*	*50*
S-22-5D	SP1 *MATa his3 leu2 ura3 trp1 ade8 tpk1::LEU2 tpk2::HIS3 tpk3^{w1}*	*50*
EY917	*MATapho84Δ::HIS3 pho87Δ::CgHIS3 pho89Δ::CgHIS3 pho90Δ::CgHIS3 pho91Δ ADE2*	*3*
JP11	*MATa MAL2-8^C suc2 PHO84-GFP*	*10*
EY57	*MATa ade2-1*	*3*

nitrogen base without phosphate) supplemented with 4% glucose and appropriate amino acids. Cells were starved of phosphate for 3 days at 30 °C under continuous agitation with a daily change of starvation medium.

Plasmids, Site-Directed Mutagenesis, and c-Myc and GFP Fusions. Genomic DNA from strain JP11 (Table 1) was used as a template in construction of the *PHO84-GFP* chimeric gene in yeast vector pRS406. A DNA fragment containing the promoter region of *PHO84* (940 bp upstream of the *PHO84* start codon) and the coding sequence of *PHO84* in frame with the sequence encoding the *GFP* was amplified by PCR using primers KY48 (5′-CCGCTCGAGTACCCTGTTATCCCTAGCGG) and KY49 (5′-AATCCGCGGGAGTGATAAAGAAGAG GCGG) containing restriction sites *Xho*I and *Sac*II (underlined regions), respectively. The DNA fragment was thereafter ligated into the polylinker of pRS406, thereby generating plasmid pRS406-*PHO84-GFP*, which in turn was transformed into yeast cells using the lithium acetate method (*32*). Yeast transformants were selected on SC medium lacking uracil.

Construction of Pho84 Site-Directed and Truncation Mutants. Plasmid EB1368 carrying wild-type *PHO84* under the *ADH1* promoter (*3*) was used as a template. To generate the central loop truncation, two overlapping upstream and downstream fragments conveying the deletion were generated and fused by PCR amplification. The *Bam*HI primer containing the start codon (5′-TATTGGATCCATGAGTTCCG TCAATAA AGAT) and *Pvu*I downstream primer (3′-CCTGTGTTTGGG CGATCGAACCAGCACCTGCTTCATGTTGAAGAGATGG GCTGGAGA) were used as flanking primers. The 5′-AAAATT CACGACA CCAGTAAGGCTTCGTTCAAAGAT and 3′-TT TTAAGTGCTGTGGTCATTCCGAAGCAAGTTTCTA primers were used as internal primers for construction of the 304−327Δ deletion of Pho84. To construct the K298A mutation, chromosomal DNA purified from the EY57 strain was used as a template. The *Bam*HI primer (5′-CGCGTATTTTGGATCCT CACTTCGTT TTTTTACCGTTTA GTAGACA) overlapping the promoter region of the *PHO84* gene and the *Pvu*I downstream primer (described above) were used as flanking primers. The 5′-CCGCACAAGAACAAGATGG CGAAAAGGCAAT TCACGA CACCAGTGATGA AGACATGGC and 3′-GGCG TGTTCTTGTTCTACCGCTTTTCCGTTA AGTGCTGTG GTCACTACTTCTGT ACCG primers were used as internal primers to generate the K298A mutation. The K298A and truncation mutants were subcloned prior to fluorescence analyses into the pRS406 vector harboring the *PHO84-GFP* construct described above by use of restriction enzymes *Hin*dIII and *Xba*I. The authenticity of all DNA constructs was verified by DNA sequencing.

Phosphate Transport Measurements. Phosphate uptake was assayed in cells expressing Pho84−Myc protein grown in low-phosphate medium. 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 and resuspended to 3 mg of wet weight/30 μL of 25 mM Tris-succinate and 3% glucose (pH 4.5), to a final phosphate concentration of 0.22 mM as described previously (*33*). 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.

Phosphate-starved cells were subjected to phosphate uptake measurements in collected cells washed twice with 25 mM MES buffer (pH 6); 120 mg of freshly washed cells was resuspended in 1.5 mL of phosphate starvation medium (pH 8). Aliquots of 80 μL were incubated for 15 min at 30 °C under continuous agitation, after which 20 μL of the [^{32}P]orthophosphate stock solution (1 mM) was added to a final concentration of 0.2 mM. Transport was terminated after 5 min by addition of 5 mL of ice-cold water.

Purification of Pho84. Cells were grown in low-phosphate medium to an A_{600} of 2.5; then 10 mM KH_2PO_4 was added to the culture, and samples were collected by centrifugation at 5500*g* and 4 °C for 10 min and stored at −80 °C until protein purification. Cells were resuspended in 50 mM Tris-HCl (pH 7.8), 1 M sorbitol, 10 mM $MgCl_2$, 2 mM DTT, and 2 mM PMSF and incubated for 10 min under gentle agitation at room temperature, followed by centrifugation at 4600*g* and 4 °C for 5 min. Cells were lysed in 5 mL of resuspension buffer supplemented with 1 mg of Zymolyase 20T during incubation for 1.5 h at room temperature under gentle agitation. The cell lysate was centrifuged at 4600*g* for 5 min at 4 °C, and the pellet was resuspended in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 2.5% Triton X-100 and incubated for 30 min at 4 °C under gentle agitation. Finally, the solution was centrifuged at 4600*g* for 5 min at 4 °C, and the supernatant was passed through a 0.2 μm filter prior to being loaded on a 1 mL Hi-Trap chelating HP column pretreated with 50 mM $NiSO_4$. Pho84 protein was eluted with 300 mM imidazole, 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 0.1% Triton X-100. The protein was further purified by immunoprecipitation using anti-Myc antibody. Briefly, 1 mL of the protein eluted from the Hi-Trap column was incubated with 3 μL of anti-Myc antibody (1 mg/mL) at 4 °C for 12 h under constant agitation; 50 μL of protein G-agarose was then added to the protein suspension at 4 °C and further incubated for 12 h. Precipitates were washed according to the manufacturer's protocol, suspended in SDS−PAGE sample buffer, and subjected to electrophoresis. The Pho84−GFP fusion protein was incubated with GFP antibodies (1 mg/mL) at 25 °C for 2 h under constant agitation. The immunoprecipitation was preformed with Protein G HP SpinTrap columns according to the manufacturer's protocol (GE Healthcare).

Western Blot Analysis of Pho84 Expression. Equivalent concentrations of solubilized and purified protein (10 or 25 μg, as stated in the figure legends) were separated on a 10% SDS−polyacrylamide gel (*34*). Immunoblotting to poly(vinylidene difluoride) membranes was carried out according to the manufacturer's protocol (GE Healthcare), and the immunoblots were probed with antibodies. After a short incubation with chemiluminescent substrate, the blot was exposed to X-ray film for 1−2 min.

Sampling, Extraction, and Determination of Trehalase Activity. The phosphate-starved glucose-repressed cells were rapidly cooled on ice and harvested by centrifugation. The pellet was washed twice with ice-cold 25 mM MES buffer (pH 6) and resuspended in phosphate starvation medium (pH 8) supplemented with 4% glucose and incubated at 30 °C while being agitated. After incubation for 30 min, 10 mM KH_2PO_4 was added to the culture. Samples of 75 mg of cells/mL collected at the indicated time points were rapidly cooled by addition of ice-cold water, centrifuged, and resuspended in 500 μL of ice-cold 25 mM MES buffer (pH 7) for extraction. Crude cell extracts were prepared as described previously (*35*) and dialyzed (BRL microdialysis system) at 4 °C against 25 mM MES buffer (pH 7) supplemented with 50 μM $CaCl_2$. Trehalase activity in dialyzed cell extracts was determined using a coupled enzymatic reaction of glucose oxidase and peroxidase with glucose as described previously (*35*).

Microscopy and Image Analysis. Samples of cells expressing Pho84−GFP fusion protein were collected at indicated time points as stated and mixed with an equal volume of melted agarose (1%), immobilized on a glass slide, and cooled prior to analysis. To visualize the vacuolar membrane, cells were incubated at the indicated time points in medium supplemented with 20 μM FM4-64 (*36*) for 15 min at 30 °C. Cells were washed once with fresh medium prior to microscope analysis. The Y-FL epi-fluorescence filter EX 465−495 (Nikon) was used for the excitation of the GFP. Fluorescence of the cells was 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. The images presented are representative of the overall picture of the cell population, and all fluorescence microscopy experiments were repeated at least twice. The final images were produced using Adobe Photoshop with no editing of signal intensity.

RESULTS

Downregulation of Pho84 Is Associated with Phosphorylation and Ubiquitination. The addition of large amounts of phosphate (10 mM) to low-phosphate-grown cells stimulates Pho84 downregulation and degradation (*11*, *37*). We have investigated the molecular signals involved in Pho84 downregulation using a version of the endogenous Pho84 transporter tagged with six histidine residues and a tandem c-Myc epitope. Membrane proteins were isolated from cells grown in low-phosphate medium before and after the addition of 10 mM phosphate. Pho84-Myc-His and Pho84-GFP were purified by Ni^{2+} affinity chromatography followed by immunopurification with anti-Myc antibody and by immunopurification with anti-GFP antibody, respectively, prior to Western blot analysis with anti-phosphothreonine, anti-phosphoserine, anti-phosphotyrosine, and anti-ubiquitin antibodies. Signals corresponding to the size of the tagged versions of Pho84 were immunodetected only with anti-phosphothreonine and anti-ubiquitin antibodies (Figure 1). Figure 1A shows that the wild-type ubiquitin signal was detected 5 min after phosphate addition, suggesting that Pho84 downregulation and removal from the plasma membrane are mediated via ubiquitination of the protein. The magnitude of the immunodetected signal increased gradually until the time point 30 min after phosphate addition. With the phosphospecific antibodies, we could detect a signal using the phosphothreonine antibodies (Figure 1A), whereas no modified serine or tyrosine residues were detected (not shown). Figure 1A shows that a weak signal of phosphothreonine is present before phosphate addition (time points −30 and 0) and that it strongly increases 5 min after phosphate addition which indicates that a phosphorylation event acts as a signal in the downregulation process of Pho84. Our results show that phosphorylation and subsequent ubiquitination of the Pho84 protein are associated with the Pho84 downregulation process. In Figure 1B, it is shown that, unlike the situation with the wild-type Pho84−GFP fusion protein, phosphate provisions fail to promote ubiquitination and to some extent also phosphorylation in the Pho84 304−327Δ−GFP truncation mutant.

Deletion of Ubc1 and Ubc7 Prevents Targeting of Pho84 to the Plasma Membrane. We investigated the significance of

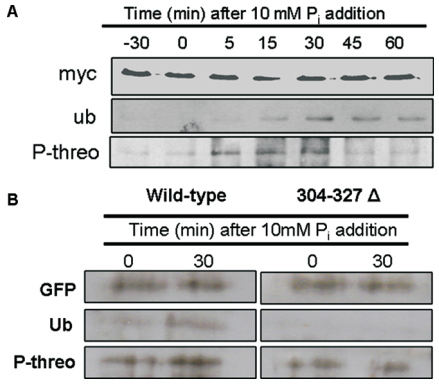

FIGURE 1: Pho84−Myc protein is phosphorylated and ubiquitinated in a phosphate-dependent manner. (A) The wild-type strain expressing Pho84−Myc protein was precultured in YPD medium for 12 h and was allowed to grow after inoculation in low-phosphate medium to an A_{600} of 2.5 (denoted −30), after which the individual cultures were treated with 10 mM phosphate (denoted 0). Samples were collected at the indicated time points, and proteins were solubilized. Immunopurification of Pho84−Myc protein was performed as described in Materials and Methods before immunodetection analysis; 25 μg of membrane protein was subjected to SDS−polyacrylamide gel electrophoresis, immunoblotting, and detection of the Pho84−Myc protein using anti-Myc antibody (myc), anti-ubiquitinated protein antibody (ub), and anti-phosphothreonine antibody (P-threo). (B) The wild-type strain expressing Pho84−GFP and Pho84 304−327Δ−GFP proteins was grown in synthetic complete medium lacking uracil under the same conditions described for panel A. Pho84−GFP protein was immunopurified using anti-GFP antibody as described in Materials and Methods.

the ubiquitination process in the downregulation of Pho84 by the use of *ubc* deletion strains (see Table 1). First, we followed the trafficking of Pho84 in the *ubc*Δ strains during growth in low-phosphate medium using a vector-based chimeric Pho84−GFP construct. The level of expression of the Pho84−GFP protein from the *PHO84* promoter on plasmid pRS406 in wild-type strain RH1800 (Figure 2A) was similar to the level of expression of genomic Pho84−GFP constructs previously described (*11*, *30*, *37*). Cellular trafficking of the Pho84−GFP protein under low-phosphate growth conditions was identical in the wild-type strain and the *ubc2*Δ, *ubc4*Δ, and *ubc5*Δ strains (data not shown). In contrast, expression of the Pho84−GFP protein was severely altered in *ubc1*Δ and *ubc7*Δ strains compared to the wild-type strain (Figure 2A). In these strains, the Pho84−GFP protein was not targeted to the plasma membrane but instead mainly found in vesicular bodies. A key role for ubiquitin in sorting proteins to and into multivesicular bodies has been demonstrated (*36*), and in particular, a ubiquitin-dependent sorting signal is required at the *trans*-Golgi network for newly synthesized proteins (*19*). The vesicular location of the Pho84−GFP protein was observed throughout growth until the cells reached an A_{600} of 2.0 in the low-phosphate medium representing the maximum density of *ubc1*Δ and *ubc7*Δ strains under these conditions, a finding that may indicate that the Ubc1 and Ubc7 enzymes are important in the sorting of Pho84 to the plasma membrane. However, since Pho84 is not correctly sorted in these mutant strains, we excluded the *ubc1*Δ and *ubc7*Δ strains in the phosphate-induced downregulation study of Pho84.

Ubiquitination as a Signal for Endocytosis and Degradation of Pho84. We have used the *ubc2*Δ, *ubc4*Δ, and *ubc5*Δ strains to study the role of these ubiquitin-conjugating enzymes in the ubiquitination and downregulation process of the Pho84

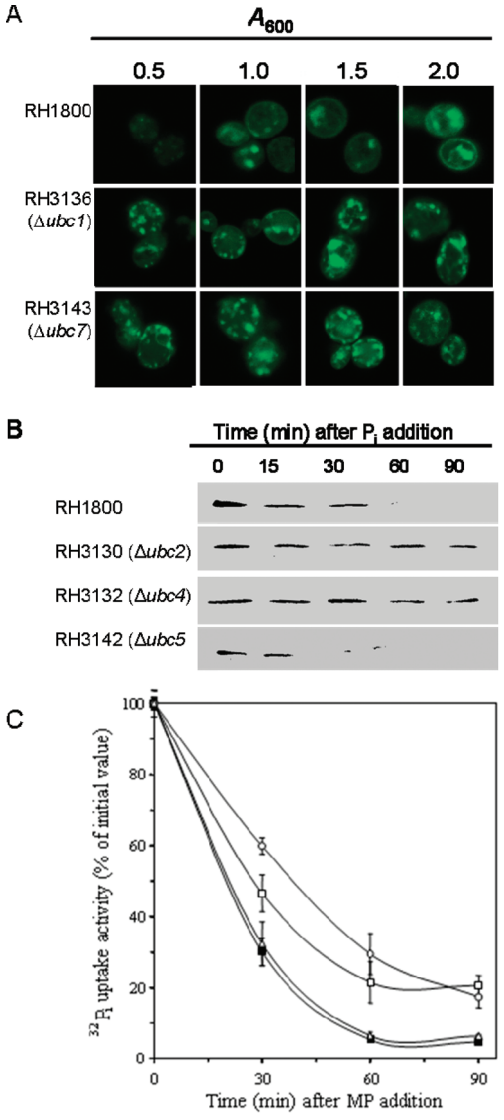

FIGURE 2: Localization and internalization of Pho84−GFP protein are dependent on ubiquitin-conjugating proteins. (A) Expression and localization of Pho84−GFP protein in wild-type, *ubc*1Δ, and *ubc*7Δ strains were monitored by fluorescence microscopy during growth to an A_{600} of 2.0 in low-phosphate medium. (B) Time course of removal of the Pho84−GFP protein from the plasma membrane in response to addition of 10 mM phosphate to wild-type, *ubc2*Δ, *ubc4*Δ, and *ubc5*Δ cells grown in low-phosphate medium to an A_{600} of 2.5 (denoted 0). Samples were collected at the indicated time points and membrane proteins solubilized; 10 μg of membrane protein was subjected to SDS−polyacrylamide gel electrophoresis, immunoblotting, and detection of the Pho84−GFP protein using an anti-GFP antibody as described in Materials and Methods. (C) The ability of *ubc*Δ mutants to downregulate Pho84 phosphate uptake activity was assayed when 10 mM methylphosphonate (MP) was added to cells grown in low-phosphate medium to an A_{600} of 2.5. Samples of wild-type (■), *ubc2*Δ (○), *ubc4*Δ (□), and *ubc5*Δ (△) cells were collected after MP addition at the indicated time points and subjected to $^{32}P_i$ uptake analysis. Normalized average and standard error values of triplicate assays are shown.

transporter. For that purpose, we added 10 mM phosphate or methylphosphonate to low-phosphate-grown cells and followed both the removal of the Pho84−GFP protein from the plasma membrane (using the vector-based *PHO84-GFP* expression system described above) and the reduction in phosphate uptake activity. Total membrane proteins were isolated from samples taken at indicated time points after phosphate addition, and immunodetection of the Pho84−GFP chimera was performed using the anti-GFP antibody (Figure 2B). Internalization of the Pho84−GFP chimera from the plasma membrane in the wild-type strain occurred within 30 min of phosphate addition as previously observed (*11*, *37*). The *ubc5*Δ strain showed an even higher rate of Pho84−GFP protein internalization with almost no signal observed 30 min after phosphate addition (Figure 2B). In contrast, internalization of the Pho84−GFP protein was strongly delayed in the *ubc2*Δ and *ubc4*Δ strains. In both cases, the signal was still detected 90 min after phosphate addition (Figure 2B). Only a slight reduction in the magnitude of the signal could be observed over the time course experiment in the two mutant strains, strongly suggesting that these enzymes are directly involved in the ubiquitination and degradation of Pho84.

Next, we followed the downregulation of the Pho84 phosphate transport activity in the same *ubc*Δ strains (Figure 2C). Methylphosphonate (MP) was used instead of phosphate to trigger the downregulation since an equimolar concentration of this phosphate analogue triggers the degradation of Pho84 at a rate comparable with that observed for phosphate without interfering with the phosphate uptake measurement (*37*). The *ubc5*Δ strain showed a downregulation of the Pho84 transport activity similar to that of the wild-type strain (Figure 2C). This is in agreement with the comparable internalization rate of the Pho84−GFP protein in the two strains (Figure 2B). The *ubc2*Δ and *ubc4*Δ strains exhibited a slower downregulation of the Pho84 transport activity (Figure 2C). Although this fits with the delayed internalization of Pho84−GFP protein (Figure 2B), the inactivation of transport activity seems to occur faster than the internalization of Pho84 from the membrane. Phosphate uptake activity in the *ubc2*Δ and *ubc4*Δ strains 90 min after methylphosphonate addition represents only ~20% of the initial activity. The lower rate of downregulation of Pho84 transport activity and the delay observed in the *ubc2*Δ and *ubc4*Δ strains suggest that the enzymes encoded by *UBC2* and *UBC4* are required for the ubiquitin-mediated endocytosis of Pho84.

The Central Intracellular Loop of Pho84 Is Required for Phosphate-Induced Internalization and Degradation. The evidence that Pho84 is phosphorylated and ubiquitinated after addition of phosphate prompted us to investigate whether Pho84 internalization is dependent on modification of the predicted target residues in the large central intracellular loop (*25*). For this purpose, we constructed two Pho84 mutant alleles fused with GFP. In the first allele, K298A, the predicted ubiquitinated lysine is mutated to alanine, and in the second allele, we deleted a major part of the intracellular loop, 304−327Δ, to remove the predicted phosphorylation sites T317 and S321 together with their flanking sequences. We investigated the cellular trafficking of the mutant chimeras in comparison with that of the wild-type Pho84−GFP chimera. Both K298A and the partial loop deletion mutant 304−327Δ display clear membrane localization after growth to mid-exponential phase in low-phosphate medium (250 μM) comparable to the localization observed with the wild-type Pho84−GFP chimera. After addition of a large amount of phosphate (10 mM), we followed localization of the protein as a function of time (Figure 3A). The vacuolar membrane was visualized by the fluorescent marker FM4-64 (*38*) at the end of the time course (120 min after addition of phosphate). The K298A mutant allele of Pho84 appeared to be internalized at the same rate as wild-type Pho84, although the vacuolar sorting was strongly affected (Figure 3A, panel a). After 120 min, the major part of wild-type Pho84 was sorted to the vacuolar lumen in agreement with

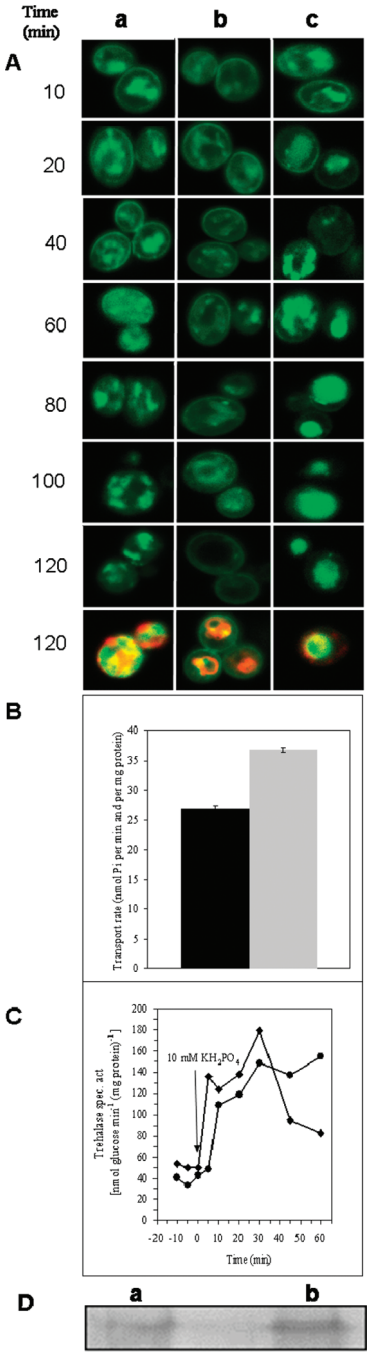

FIGURE 3: Mutations in the central loop of a functional Pho84−GFP chimera affect internalization and vacuolar targeting. (A) Strains expressing Pho84(K298A)−GFP (a), Pho84 304−327Δ−GFP (b), and wild-type Pho84−GFP (c) proteins were grown to an A_{600} of 2.5 in low-phosphate medium. Cellular localization of wild-type and mutants alleles of the Pho84−GFP chimera was monitored after addition of 10 mM phosphate by GFP fluorescence for 120 min. The vacuolar membrane was probed after 120 min with the red fluorescent dye FM4-64. The functionality of the Pho84 truncation allele was assessed by (B) Pho84-mediated phosphate uptake measurements in EY917 cells expressing wild-type Pho84 (black bar) and in cells expressing the Pho84 304−327Δ truncation allele (gray bar) (average and standard error values of triplicate assays are shown) and (C) activation of trehalase in response to phosphate-induced stimulation of PKA. Activation was measured after addition of 10 mM phosphate to phosphate-starved cells expressing wild-type Pho84 (◆) and Pho84 304−327Δ (●). (D) Immunodetection of wild-type Pho84 (a) and Pho84 304−327Δ (b) using the anti-Pho84 antibody.

previous studies (Figure 3A, panel c) (*10*). The cytosolic punctuated structures seen after internalization of the K298A allele of Pho84 most likely represent endosomal compartments unable to fuse with the vacuolar membrane. The Pho84 304−327Δ internal deletion mutant displayed a severely delayed internalization process compared to the wild type (Figure 3A, panel b), suggesting that the removed residues are needed to aid in rapid internalization of the protein.

Because the deletion of a significant part of the intracellular loop connecting TMS 6 and TMS 7 in Pho84 potentially would affect the overall structure and activity of the protein, we used a quintuple deletion strain, EY917, devoid of all five phosphate transporters (*3*) to assess the transport and signaling capacity of the 304−327Δ Pho84 allele (Figure 3B,C). Apparently, the transport activity of the Pho84 304−327Δ allele was even higher than that of wild-type Pho84, indicating that the internal deletion in the central loop does not severely compromise activity (Figure 3B). An immunodetection of membrane preparations of wild-type Pho84 and the 304−327Δ Pho84 allele (Figure 3D) clearly demonstrates that the truncation allele has a higher membrane expression level, which is in agreement with the phosphate uptake experiment presented in Figure 3 B. We also tested whether the central loop internal deletion mutant had maintained its signaling capacity for activation of the PKA pathway as described previously (*29*). We measured the activity of a well-known PKA target, trehalase, after addition of 10 mM phosphate to phosphate-starved cells. The results indicated that the Pho84 304−327Δ allele had maintained signaling capacity comparable to that of wild-type Pho84 (Figure 3C). In conclusion, the Pho84 304−327Δ allele seems to have maintained a proper overall structure required for transport and signaling, which supports the conclusion that this internal sequence is specifically required for the regulated internalization of the protein.

PKA Activity, Especially of the Tpk3 Catalytic Subunit, Is Required for Rapid Internalization of Pho84. To investigate a possible role of PKA in the internalization process of Pho84 as suggested previously (*30*), we have made use of strains expressing only one weakened *TPK* allele, tpk^{w1} (see Table 1). These strains all exhibit a low PKA phenotype (*39*). Since the *TPK* genes act redundantly for maintenance of viability, these strains are also useful in identifying specific roles of the different Tpk's in cellular processes. The Pho84−GFP construct was transformed into the tpk^{w1} strains. The cells were grown to midexponential phase in low-phosphate medium (250 μM), and the Pho84−GFP protein localization was analyzed before and after addition of 10 mM phosphate using fluorescence microscopy (Figure 4). All *tpk* attenuated strains displayed a normal Pho84 membrane localization after growth under low-phosphate conditions. After addition of phosphate, only the strain expressing the attenuated $tpk3^{w1}$ allele displayed an internalization pattern equal to that of the wild type. After 120 min, the Pho84−GFP allele was completely localized in the vacuolar lumen in the wild-type and $tpk3^{w1}$ strains (Figure 4, panels a and c). In contrast, the $tpk1^{w1}$ and $tpk2^{w1}$ attenuated strains showed a strongly delayed internalization of Pho84 (Figure 4, panels b and d). Noteworthy is the fact that the $tpk2^{w1}$ mutant strain also exhibited a severely reduced growth rate during the initial growth period on low-phosphate medium. Our results indicate that PKA activity is required for rapid internalization of Pho84 and that Tpk3 appears to be the most essential of the three catalytic subunits.

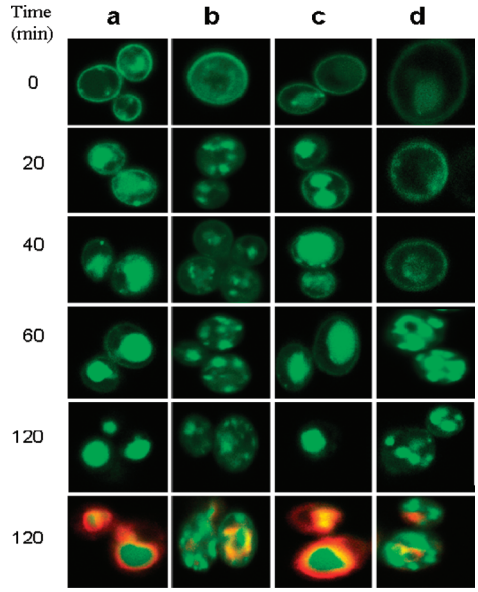

FIGURE 4: In vivo localization and vacuolar internalization are dependent on the functionality of the catalytic subunits of TPK. Localization of the Pho84−GFP protein in wild-type cells (a) and cells expressing a single partially inactivated ("weakened") TPK allele [*tpk1*Δ *tpk2*^{w1}*tpk3*Δ (b), *tpk1*Δ *tpk2*Δ *tpk3*^{w1} (c), and *tpk1*^{w1}*tpk2*Δ *tpk3*Δ (d)] grown to an A_{600} of 2.0 in low-phosphate medium was monitored by GFP fluorescence microscopy for 120 min after addition of 10 mM phosphate to the cultures. The vacuolar membrane was probed after 120 min with the red fluorescent dye FM4-64.

DISCUSSION

Phosphate-Induced Internalization of Pho84 Is Associated with Phosphorylation and Ubiquitination. The internalization and cellular trafficking of several plasma membrane proteins in yeast have often been shown to be regulated by post-translational events such as phosphorylation and ubiquitination. It has previously been demonstrated that Pho84, the main proton-coupled phosphate transporter in *S. cerevisiae*, is internalized after addition of phosphate to cells grown under phosphate-limited conditions and further sorted to the vacuole for destruction (*10*, *37*). In this study, we have for the first time investigated the molecular processes involved in downregulation of a phosphate transporter in *S. cerevisiae*. To start, we purified the Pho84 protein and demonstrated by immunodetection that the Pho84 transporter is both phosphorylated and ubiquitinated after phosphate is added back to low-phosphate-grown cells. The phosphothreonine band was detected on Pho84 before addition of phosphate, but the magnitude of the band had already been strongly increased in intensity 5 min after phosphate addition. This suggests that Pho84 is phosphorylated in its functional form in the plasma membrane and that additional phosphorylation events occur when the external phosphate concentration is increased. Extensive phosphorylation has previously been suggested to be a common post-translational event prior to protein degradation of other yeast transporters (*24*). On the other hand, the immunodetected band using anti-ubiquitin antibodies was visualized only after addition of phosphate. This correlates well with the post-translational events observed prior to internalization of several other transporters and receptors investigated in yeast (*13*, *18*). Since no ubiquitination was detected in the central loop truncation mutant Pho84 304−327Δ and only a background phosphorylation, present also prior to addition of phosphate, it appears that the target residues for these modifications indeed are located in the truncated sequence.

Phosphorylation and Ubiquitination Are Required for Internalization of Pho84. The major part of previously investigated plasma membrane proteins in yeast has been shown to have a ubiquitin-dependent downregulation process dependent on the ubiquitin ligase Rsp5. The ubiquitin conjugation enzymes, Ubc's, in yeast have been less extensively characterized in that respect. In this study, we have made use of different strains, each lacking one specific ubiquitin-conjugating enzyme, in which we have expressed a GFP- or c-Myc-tagged version of Pho84 and followed its localization and activity in these different backgrounds. The results demonstrate that the Ubc's affect both the protein localization of newly synthesized Pho84 and the internalization process after phosphate addition. In the strains with *UBC1* or *UBC7* deleted, Pho84 expression and targeting to the plasma membrane were strongly affected. It has previously been shown that Ubc1 is implicated in the degradation of misfolded proteins in the cytosol (*40*). Indeed, Ubc1 and Ubc7 contribute to the principal conjugating step in the polyubiquitination of endoplasmic reticulum-associated degradation (ERAD) substrates (*41*). Although Pho84 was classified among the 211 candidate membrane-associated ubiquitinated proteins (*42*), Pho84 was not found to belong to the identified candidate ERAD substrate proteins. The large numbers of ubiquitin-conjugating enzymes, E2's, that exist in all eukaryotic cells display several different biological functions despite their structural similarity. The interaction between these and the E3 ligase is believed to regulate the final destination of the ubiquitin molecule with respect to the substrate (*43*, *44*). In addition, the ubiquitin ligase Doa10 has been shown to ubiquitinate both membrane and soluble proteins passing the ER, and this process requires the presence of Ubc6 and Ubc7 (*45*). The three related enzymes Ubc1, Ubc4, and Ubc5 have been suggested to function together with the E3 Rsp5 (*46*, *47*), and it has been suggested that Ste6 is dependent on Ubc4 and Ubc5 in the endocytotic process (*48*).

In this study aimed at identification of the E2 enzyme involved in the ubiquitination process of Pho84 prior to internalization, we focused on the *ubc2*Δ, *ubc4*Δ, and *ubc5*Δ strains. A clearly delayed proteolytic breakdown was observed in the *ubc2*Δ and *ubc4*Δ strains as compared to the wild-type and *ubc5*Δ strains (Figure 2), suggesting that Pho84 is ubiquitinated with the help of the ubiquitin conjugation enzymes Ubc2 and Ubc4. The delayed internalization of the Pho84 protein in the *ubc2*Δ and *ubc4*Δ strains was paralleled by a slower disappearance of the phosphate uptake activity as compared to the situation in the wild-type and *ubc5*Δ strains. The Pho84 activity was nevertheless downregulated in the *ubc2*Δ and *ubc4*Δ strains but at a much slower rate. Target sites for ubiquitination and phosphorylation have been predicted to be mainly located in the large intracellular loop connecting TMS 6 and TMS 7 of Pho84 (*25*). The lysine at position 298 is an attractive target for modification by ubiquitination due to its close connection to several of the predicted phosphorylation sites. We therefore constructed a Pho84 K298A−GFP mutant and followed its expression, membrane localization, and degradation in a wild-type background. As one can see in Figure 3, the mutant is expressed and sorted to the plasma membrane like the wild-type Pho84−GFP protein. Although high-phosphate-triggered internalization of the K298A−GFP allele was only slightly slower than that of the wild-type protein, the vacuolar targeting was strongly affected and the protein was localized after 120 min to nonvacuolar punctated structures, most likely late endosome vesicles unable to

be correctly targeted to the vacuolar lumen by the help of the endocytotic protein machinery. It is known that ubiquitination influences not only the internalization process but also the multivesicular body (MVB) sorting and trafficking to the vacuole (*14*). In the case of the Pho84 304−327Δ−GFP truncation allele, the internalization of the protein from the membrane appeared to be relatively slow after addition of phosphate, suggesting that the truncated part of the central large loop confers properties for endocytotic internalization. The simplest explanation is that the phosphorylation events at the intracellular loop are most likely needed for ubiquitin-dependent endocytocis. The higher transport rate of the Pho84 304−327Δ allele compared to the wild-type rate appears to be paralleled by accumulation of more copies of Pho84 in the plasma membrane due to halted or delayed protein internalization and turnover. However, the possibility that phosphorylation may downregulate transport activity cannot be excluded, and removal of the phosphorylation target residues would then contribute to a higher transport activity. The truncation mutant also retained its ability to sense and convey phosphate signaling to the PKA target with an activity similar to that of the wild-type protein. It can therefore be concluded that neither of the residues located in the truncated sequence is required for the dual activities of the Pho84 protein, i.e., transport and phosphate sensing/signaling, and that possible structural rearrangements of the truncated protein do not negatively affect these functions.

Phosphate-Induced Activation of the PKA Pathway Plays a Role in the Internalization of Pho84. The internalization of Pho84 has been shown in a previous study to be influenced by protein kinase A (*30*). In the study presented here, we have further analyzed the Pho84−GFP protein internalization process in different mutant strains affected in the catalytic subunits of PKA (Figure 4). By analyzing the internalization processes in strains expressing only *tpk1*^{w1}, *tpk2*^{w1}, or *tpk3*^{w1}, we have been able to define the individual contribution of each catalytic subunit of PKA. In previous studies, Tpk2 has been shown to be the most active subunit in phosphate-induced PKA activation (*28*). For the Pho84 PKA-dependent internalization process, we have shown here that this is dependent especially on the activity of Tpk3. In contrast to a situation in which the activity of the entire PKA complex was inhibited (*30*), the absence of Tpk3 did not halt the internalization process but strongly affected the intracellular sorting to the vacuole via the MVB pathway. It is therefore likely that the catalytic subunit Tpk3 has an effect on essential phosphorylation events in putative unidentified upstream targets of the MVB pathway or, alternatively, directly affects phosphorylation and ubiquitination events on the Pho84 transporter. Taken together, these results further support an existing link between Pho84 and the PKA pathway. Since Pho84 has previously been shown to act as the sensor for phosphate-induced activation of well-known PKA targets (*28*), it is likely that the downregulation and degradation processes are initiated and controlled by sensing and signaling through Pho84 itself.

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