The Rsp5 E3 Ligase Mediates Turnover of Low Affinity Phosphate Transporters in Saccharomyces cerevisiae

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In an effort to identify novel components of the PHO regulon in Saccharomyces cerevisiae, we have isolated and characterized suppressors of the Pho− phenotype associated with deletion of the Pho4 transcriptional activator. Here we report that either a defective form of the Rsp5 E3 ubiquitin ligase or deletion of the End3 component of the endocytic pathway restores growth of the pho4Δ mutant in the presence of limiting inorganic phosphate (P_i). The spa1-1 suppressor allele of RSP5 encodes a phenylalanine-to-valine replacement at position 748 (F748V) within the catalytic HECT domain of Rsp5. Consistent with suppression due to impaired ubiquitin ligase activity, the heat-sensitized mutant is suppressed either by overexpression of ubiquitin or by osmotic stabilization. Western blot analyses revealed that the cellular levels of the Pho87 and Pho91 low affinity P_i are markedly increased in the spa1-1 mutant, yet Pho84 high affinity P_i transporter levels are unaffected. Furthermore, Pho87 and Pho91 are ubiquitinated in vivo in an Rsp5-dependent manner, and the Pho+ phenotype of the spa1-1 suppressor is dependent upon Pho87 and Pho91. We conclude that turnover of the low affinity P_i transporters is initiated by Rsp5-mediated ubiquitination followed by internalization and degradation by the endocytic pathway.

The yeast Saccharomyces cerevisiae has evolved an elaborate system to sense, acquire, and store inorganic phosphate (P_i) in response to its availability in the extracellular environment (for review, see Refs. 1 and 2). The PHO system consists of (i) the Pho3, Pho5, Pho11, and Pho12 acid phosphatases that are localized to the periplasmic space, (ii) the Pho8 and Pho13 alkaline phosphatases that are localized to the vacuole and periplasm, respectively, (iii) the high affinity plasma membrane P_i transporters Pho84 and Pho89, which are regulated in response to P_i availability, and the low affinity, constitutively expressed P_i transporters Pho87, Pho90, and Pho91, (iv) Git1, a transporter that scavenges glycerophosphoinositol derived from secreted phosphatidylinositol, thereby replenishing inositol, P_i, and glycerol (3), and (v) the PHM proteins that are involved in the synthesis and breakdown of polyphosphate, a storage form of P_i in the vacuole (for review, see Ref. 4).

The PHO regulon is responsible for scavenging P_i and has been most extensively studied with regard to regulation of PHOS expression. The Pho84 transporter and the Pho80–Pho85 cyclin/cyclin-dependent kinase are negative regulators of PHOS transcription, whereas the Pho81 inhibitor of Pho80–Pho85 and the Pho4 transcriptional activator are positive regulators. In the presence of high external concentrations of P_i, Pho85 phosphorylates Pho4, resulting in Pho4 nuclear export via the Msn5 exportin and cytoplasmic retention such that PHOS is repressed (5). Conversely, when P_i concentrations are low, Pho81 inhibits Pho85 activity. Pho4 remains unphosphorylated and has high affinity for the Pse1 importin, resulting in its nuclear retention where the Pho2–Pho4 complex induces PHOS transcription (5). The high affinity transporter genes, PHO84 and PHO89, are also components of the PHO regulon and are controlled in a manner similar to PHOS. In contrast, expression of the low affinity transporter genes, PHO87, PHO90, and PHO91, are independent of Pho4.

Although the downstream events in the PHO signal transduction pathway have been well characterized, the upstream components that sense P_i concentrations and transduce that information are not well understood. Indeed, the direct sensor of P_i has not been identified. Recently, it was proposed that the PHO regulon is controlled by two different regulatory signals, one sensing internal P_i concentrations by an unidentified protein, the other sensing external P_i concentrations by the low affinity P_i transporters (6). In contrast to pho2Δ, pho4Δ, or pho81Δ mutants that fail to activate PHO genes in response to P_i depletion, pho87Δ, pho90Δ, or pho91Δ deletion mutations result in constitutive derepression of PHO genes (6, 7). Furthermore, pho87Δ, pho90Δ, and pho91Δ are hypostatic to pho2Δ, pho4Δ, and pho81Δ (6). These results place the low affinity P_i transporters in the PHO signal transduction pathway, upstream of Pho2, Pho4, and Pho81. As such, Pho87, Pho90, and Pho91 appear to function not only as P_i transporters but as signal transducers that link the extracellular environment with the regulation of PHO gene expression (6).
Recently, it was reported that the protein kinase A signal transduction pathway is required for degradation of Pho4 (8), although there are no reports describing turnover of the low affinity Pᵢ transporters. A number of cell surface proteins, including transporters, permeases, and signaling receptors, are known to be down-regulated by ubiquitin-mediated endocytosis (9–11). As examples of the ubiquitin-endosome pathway, the yeast Rsp5 E3 ubiquitin ligase is involved in turnover of the Gap1 general amino acid permease (12, 13), the Fur4 uracil permease (14–16), the Mal61 maltose transporter (20, 21). Accordingly, ubiquitin-mediated endocytosis appears to be a general mechanism to respond to changes in nutrient availability.

In an effort to identify proteins that regulate phosphate metabolism, we sought suppressors that would bypass the Pho4 requirement for cell growth in limiting Pₙ medium. We anticipated finding (i) proteins that act downstream of Pho4 either to repress Pho4-regulated genes or to mediate Pho4 activation and (ii) factors that affect Pᵢ uptake independent of Pho4. Suppressors of the former class, which includes components of the RNA polymerase II mediator complex were found and will be described elsewhere. Here we describe suppressors of the latter class that includes the Rsp5 E3 ubiquitin ligase and the End3 component of the endocytic pathway. Our results identify the low affinity phosphate transporters Pho87 and Pho91 as novel targets of the Rsp5 endocytic pathway and define an important role for these proteins in sensing Pᵢ availability.

### EXPERIMENTAL PROCEDURES

**Yeast Strains**—All yeast strains used in this study are listed in Table 1. YMH623 is a segregant derived from sporulation of a diploid strain created by a cross between EY131 and FY121. Strains YMH613 (pho4::URA3) and YMH624 (pho4::TRPI) were derived from strains W303-1A and YMH623, respectively, by one-step gene disruption (22) of the PHO₄ chromosomal locus. YMH842, YMH843, and YMH844 were derived from W303-1B, FY121, and YMH655, respectively, by one-step gene disruption of END3 using the pFA6a-His3MX6 cassette. YMH827, YMH828, and YMH829 were derived from W303-1B, FY121, and YMH655, respectively, by one-step gene disruption of PHO91 using the pFA6a-His3MX6 cassette. YMH854, YMH855, and YMH856 were derived from W303-1B, FY121, and YMH655, respectively, by integration of 3xHA::his5 at the PHO84 chromosomal locus. YMH854, YMH855, and YMH856 were derived from W303-1B, FY121, and YMH655, respectively, by integration of 3xHA::his5 at the PHO87 chromosomal locus. YMH860, YMH861, and YMH862 were derived from W303-1B, FY121, and YMH655, respectively, by one-step gene disruption of PHO87 using the pFA6a-KanMX6 cassette. YMH860, YMH861, and YMH862 were derived from W303-1B, FY121, and YMH655, respectively, by one-step gene disruption of PHO91 using the pFA6a-HIS3MX6 cassette. One-step gene disruptions and marker integrations at each locus were performed as described initially (23). YMH690 was constructed as described above ("Allelism Test").

**Plasmids**—Plasmid pN1688 [RSP5 CEN URA3] was derived from the vector pRS416 (24). Plasmids pM1847 [rsp5-1 CEN URA3] and pM1848 [rsp5-CA CEN URA3] encode the Rsp5 L733S and C777A replacements, respectively, and were derived from pN1688 by site-directed mutagenesis. Plasmid pM1739 [CUP1p-LIB4 2µ TRPI] was derived from pM1738 [CUP1p-
myc-UBI4 2 μ TRP1]) by deleting the Myc tag, pM1761 [CLIP1p- UBI4 2 μ LEU2] was derived from pM1739 by a TRP1 to LEU2 marker swap (25) using plasmid pDP207, and plasmid pRS425 was used as the vector control.

Growth Media—Rich (YPD),5 synthetic complete (SC), and omission media (– Ura and – Leu) were prepared according to standard procedures (26). Media of defined Pi concentrations was crossed with YMH655 (MATα ura3 trp1 pho4Δ::TRP1 RSP5::URA3), and a diploid strain was sporulated and dissected. All phenotypes segregated 2:2 (n = 5 four-spore tetrads) and all Ura+ (RSP5::URA3) segregants were Pho−, Tsm−, and Slg+, whereas all Ura– segregants were Pho+, Tsm+, and Slg. Thus, the spa1-1 suppressor segregates opposite RSP5. Combined with complementation of spa1-1 by RSP5, these results confirm that spa1-1 is allelic to RSP5.

Isolation of spa1-1 by Gap-repair—The spa1-1 allele was cloned from strain YMH655 by gap-repair (22). The RSP5::URA3-CEN plasmid pN1688 was digested with BstEII, creating a gap in the RSP5 open reading frame, and introduced into YMH655 (spa1-1). Ura+ transformants were selected and screened for retention of the Pho+ and Tsm– suppressor phenotypes. Plasmid DNA was retrieved, screened by restriction analysis for recovery of gapped DNA, and reintroduced into YMH655. Whereas undigested pN1688 complemented YMH655, restoring the Pho− and Tsm+ phenotypes, transformants derived from the gap-repaired plasmid (pN1704), retained the Pho+ and Tsm– suppressor phenotypes.

Extract Preparation and Western Blot Analyses—Yeast whole cell extracts were prepared as described previously (28). Briefly, 2 A600 units of yeast cells were collected by centrifugation and lysed by resuspension in 100 μl of 1.85 M NaOH plus 7% 2-mercaptoethanol. Proteins were precipitated by the addition of 100 μl of 50% trichloroacetic acid and collected by centrifugation at 13,000 rpm (Sorvall Biofuge) for 5 min. The pellet was rinsed in 0.5 ml of 1 M Tris base without resuspension, dissolved in 100 μl of 2X sample buffer (4% SDS, 0.1 M Tris HCl, pH 6.8, 4 mM EDTA, 20% glycerol, 2% 2-mercaptoethanol), and dissociated by heating for 10 min at 37 °C. Protein samples corresponding to 0.1–0.2 A600 units were separated in 8% SDS-polyacrylamide gels, and Western blot analyses were performed as described previously (29). Pho87-3xHA and Pho91-3xHA were assayed by incubation using a 1:3000 dilution of anti-HA antibody followed by incubation with horseradish peroxidase-conjugated anti-mouse antibody. Control anti-Ess1 antibody (a gift from S. Hanes) was used at 1:10,000 dilution. Immunoprecipitated Pho87-3HA and Pho91-3HA proteins were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and assayed by Western blot using 1:3000 dilution of anti-ubiquitin antibody (Sigma). Control anti-Rpa1 antibody (a gift from S. Brill) was used at a 1:10,000 dilution. Antigen-antibody complexes were detected using the western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences).

Immunoprecipitation of Membrane Proteins—Proteins were extracted and immunoprecipitated as described previously (30) with minor modifications. Briefly, 1 A600 units of cells were lysed by incubation for 10 min on ice with 40 μl of 1.85 M NaOH plus 2% 2-mercaptoethanol. Proteins were precipitated by the addition of 40 μl of 50% trichloroacetic acid, collected by centrifugation for 5 min at 13,000 rpm. Samples were resuspended in 30 μl of 2X sample buffer (without 2-mercaptoethanol or bromphenol blue) plus 20 μl of 1 M Tris base followed by heating for 10 min at 37 °C. TNET buffer (0.6 ml, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) was added, and the insoluble material was removed by centrifugation at 13,000 rpm for 30 min. The supernatant was incubated overnight at 4 °C with 10 μl of anti-HA antibody (Sigma) plus 40 μl of protein A-Sepharose beads (Sigma). Immunoprecipitates were washed five times with TNET buffer, and proteins were eluted in 30 μl of 2X sample buffer (4% SDS, 0.1 M Tris-HCl, pH 6.8, 4 mM EDTA, 20% glycerol, 2% 2-mercaptoethanol, 0.02% bromphenol blue) for 10 min at room temperature. Proteins were immunoprecipitated using anti-ubiquitin (1:50) and anti-HA (1:50) antibodies.

RESULTS

Isolation of pho4Δ Suppressors—To identify genes that bypass the Pho4 activator requirement, we isolated suppressors of the Pho− phenotype associated with a pho4Δ deletion in strain EY131. One Pho− revertant (YMH655) also exhibited a pronounced Tsm+ phenotype at 37 °C (Fig. 1). YMH655 was backcrossed to a pho4Δ mutant of opposite mating type

5 The abbreviations used are: YPD, yeast extract/peptone/dextrose; SC, synthetic complete; WT, wild type; Ub, ubiquitin.
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(YMH613), and the resulting diploid strain was Pho− and Tsm−, indicating that the revertant phenotypes are due to a recessive mutation(s). After sporulation and dissection, the Pho−/Pho− and Tsm−/Tsm− phenotypes segregated 2:2 among all four-spore progeny (n = 12), and the Pho−/Tsm− and Pho−/Tsm− phenotypes co-segregated. Thus, YMH655 harbors a recessive, single-gene suppressor of pho4Δ that confers a pleiotropic Tsm− phenotype. We designated this suppressor mutation spa1-1 for suppressor of phosphate auxotrophy.

The spa1-1 Suppressor Is Allelic to RSP5—The Tsm− phenotype associated with the spa1-1 suppressor was used to clone the wild type gene. A YCP50 genomic library (31) was introduced into strain YMH655, and transformants were selected on −Ura medium at 37°C. A single Tsm− Pho− transformant was recovered from 23,500 transformants. Plasmid DNA was isolated, amplified, and reintroduced into YMH655, confirming plasmid dependence of the Tsm− and Pho− phenotypes. DNA sequence analysis from either end of the vector insert identified a 15-kilobase fragment of chromosome V encompassing five open reading frames, including RSP5, and none of the flanking open reading frames fully complemented spa1-1 (data not shown). Genetic linkage analysis confirmed that spa1-1 is allelic to RSP5 (see “Experimental Procedures”).

Characterization of the spa1-1 Suppressor Mutation—Rsp5 is a member of the Nedd4 class of E3 ubiquitin ligases (for review, see Refs. 11, 32, and 33). The structure of Rsp5 includes a C2 domain, implicated in membrane association, two WW domains that mediate enzyme-substrate recognition, and a catalytic HECT domain that includes the active site cysteine. To address how the spa1-1 allele of RSP5 suppresses pho4Δ, we isolated and sequenced the spa1-1 allele. The spa1-1 allele was cloned from strain YMH655 by gap-repair. DNA sequence analysis of the entire open reading frame from plasmid pN1704 identified a single base pair substitution encoding a phenylalanine-to-valine replacement at position 748 (F748V), located within the HECT domain of the Rsp5 C terminus (Fig. 2A).

The HECT domain alone is sufficient for E3 ligase catalytic activity (24). A crystal structure of the human E6AP HECT domain is available, revealing a larger N-terminal lobe and a smaller C-terminal lobe (34). Phe-748 is located within the smaller C-terminal lobe, in the same hydrophobic environment as the catalytically impaired L733S replacement encoded by the rsp5-1 allele (24). The smaller C-terminal lobe also includes the active site cysteine residue (Cys-777) that is essential for E3 ligase activity and cell viability (35, 36). To test whether loss of Rsp5 E3 ligase activity is responsible for suppression of pho4Δ, plasmid-borne rps5 alleles encoding the L733S and C777A replacements were introduced into the spa1-1 mutant and scored for complementation of the Tsm− phenotype. In contrast to the wild type RSP5 plasmid, neither of the catalytically defective rps5 plasmids complemented the Tsm− phenotype of spa1-1 (Fig. 2B). Thus, diminished Rsp5 E3 ligase activity compensates for the Pho− phenotype associated with loss of the Pho4 transcriptional activator.

Suppression of spa1-1 by Overexpression of Ubiquitin or by Osmotic Stabilization—The growth phenotypes of several rps5 ubiquitin ligase-defective alleles are suppressed by elevated expression of UBI4, the gene encoding ubiquitin (37). If the spa1-1 suppressor affects a ubiquitin-mediated process, then the Tsm− phenotype of spa1-1 might be suppressed by UBI4 overexpression. To test this possibility, we introduced plasmid DNA (pM1761) carrying the UBI4 gene under control of the copper-inducible CUP1 promoter (CUP1p-UBI4) into wild type, pho4Δ, and pho4Δ spa1-1 strains. The LEU2 vector pRS425 was included as a control. Transformants were streaked on −Leu medium and −Leu plus 0.05 mM CuSO4 and incubated at 37°C. The Tsm− phenotype of the spa1-1 mutant was weakly suppressed by CUP1p-UBI4 in the absence of copper and markedly suppressed in the presence of copper, whereas no effect of CUP1p-UBI4 was observed in the wild type or pho4Δ strains (Fig. 3A). Weak suppression in the absence of copper is presumably a consequence of leaky expression of UBI4 from the CUP1p promoter (38). Thus, overexpression of ubiquitin effectively compensates for diminished Rsp5 E3 ligase activity.

The Tsm− phenotype of ligase-defective rps5 mutants is also suppressed by osmotic stabilization in the presence of 1 m sorbitol (39). The Tsm− phenotype of the spa1-1 suppressor is likewise suppressed by 1 m sorbitol (Fig. 3B). Taken together, these results indicate that loss of Rsp5 E3 ligase activity bypasses the normal Pho4 requirement for growth in low phosphate medium. We conclude that Rsp5-mediated protein ubiquitination plays a negative role in regulating the response to P_{i} availability.
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FIGURE 3. Suppression of spa1-1 by overexpression of ubiquitin or by osmotic stabilization. A, the Tsm phenotype of the pho4Δ spa1-1 mutant is suppressed by overexpression of UBI4-encoded ubiquitin. The isogenic WT (W303-1B), pho4Δ (YE131), and pho4Δspa1-1 (YM6655) strains were transformed with the high copy number CUP1-UBI4 LEU2 plasmid (pM1761) or vector alone (pRS5425). 10-Fold serial dilutions of each strain were spotted onto either −Leu −CuSO4 medium or onto −Leu + CuSO4 medium to induce UBI4 expression. Plates were photographed after 2 (30 °C) or 3 (37 °C) days of incubation. B, the Tsm phenotype of the pho4Δspa1-1 mutant is suppressed by osmotic stabilization in the presence of 1 M sorbitol. 10-Fold serial dilutions of wild type (W303-1B), pho4Δ (YE131), and pho4Δspa1-1 (YM6655) strains were spotted onto SC medium or SC medium containing 1 M sorbitol. Plates were photographed after 2 (30 °C) or 3 (37 °C) days of incubation. The synthetic media depicted in panels A and B contain normal (high) Pi levels; accordingly, none of the growth phenotypes can be attributed to Pi auxotrophy.

The Endocytic Pathway Regulates Phosphate Metabolism—The End3-Pan1-Sla1 complex is essential for the internalization step of endocytosis (40), and End3 has been implicated in Rsp5-mediated endocytosis and vacuolar degradation of plasma membrane proteins (20, 41). To test whether the endosomal pathway is involved in phosphate metabolism, we asked whether an end3Δ deletion would suppress pho4Δ in a manner similar to the spa1-1 suppressor. We deleted the END3 gene in the WT, pho4Δ, and pho4Δspa1-1 backgrounds and scored the resulting strains for growth in the presence of high and low concentrations of Pi. Consistent with earlier results (40), the end3Δ deletion was lethal at 30 °C but viable at 24 °C, albeit exhibiting a slow-growth (Slg−) phenotype in the presence of 1 mM Pi. Accordingly, all growth phenotypes were scored at 24 °C. Results are shown in Fig. 4. First, the Pho− phenotype of the pho4Δ mutant is suppressed by end3Δ (cf. sectors 3 and 4 on 25 μM Pi). Presumably, end3Δ suppression of pho4Δ is even more pronounced than it appears due to the Sgl− phenotype associated with end3Δ even in the presence of high Pi at 24 °C (cf. sectors 2, 4, and 6 with sectors 1, 3, and 5 on 1 mM Pi). Second, suppression of pho4Δ by spa1-1 and end3Δ are not additive, as the pho4Δspa1-1 end3Δ triple mutant exhibited the same growth phenotype on low Pi medium as either the pho4Δspa1-1 or pho4Δ end3Δ double mutants (cf. sectors 4, 5, and 6 with sector 3 on 25 μM Pi), consistent with the idea that Rsp5 and End3 function in the same pathway. Interestingly, the Sgl− phenotype of the end3Δ mutant on high Pi medium was suppressed by Pi limitation (cf. sectors 2 on 1 mM and 25 μM Pi). This observation suggests that the Sgl− phenotype of all end3Δ mutants in the presence of 1 mM Pi might be an adverse consequence of the inability of end3Δ mutants to down-regulate the low affinity Pi transporters in the presence of excess Pi (see below). Taken together, these results define end3Δ as a suppressor of the pho4Δ deletion and implicate the End3 endosomal pathway in regulation of Pi metabolism.

Loss of Rsp5 Stabilizes the Low Affinity Pi Transporters—What is the proteolytic substrate(s) of the Rsp5 E3 ligase responsible for pho4Δ suppression? Given that Rsp5 ubiquititates and stimulates the turnover of several plasma membrane proteins, including permeases, transporters, and receptors (for review, see Ref. 11), we asked whether diminished Rsp5 activity might overcome the Pho− phenotype of the pho4Δ mutant by stabilizing plasma membrane Pi transporters.

To determine whether the Pho− phenotype of the pho4Δspa1-1 revertant is due to stabilization of Pi transporters, we assayed the levels of the low affinity Pho87 and Pho91 transporters and the high affinity Pho84 transporter from wild type, pho4Δ, and pho4Δspa1-1 strains. Transporter levels were assayed by Western blot using cell extracts from isogenic strains that had been 3xHA epitope-tagged at the normal chromosomal loci of PHO84, PHO87, and PHO91 (repeated efforts to generate 3xHA-tagged derivatives of the Pho89 high affinity and Pho90 low affinity Pi transporters were unsuccessful). Results are shown in Fig. 5. The Pho87 and Pho91 transporters were barely detectable by Western blot in either the wild type or pho4Δ strains, whereas the steady-state levels of both proteins were clearly elevated in the spa1-1 strains (panel A). By contrast, Pho84 levels were detectable in the wild type strain and induced upon phosphate depletion (panel B). Consistent with the Pho4 dependence of PHO84 expression (42), the Pho4 protein was undetectable in the pho4Δ mutant under either inducing or repressing conditions, and this defect was not suppressed by spa1-1. Thus, the Pho87 and Pho91 low affinity Pi transporters accumulate in the absence of normal Rsp5 E3 ligase activity. In contrast, the high affinity Pho84 transporter is uninducible in the absence of Pho4 and is unaffected by loss of Rsp5. These results implicate Rsp5-mediated ubiquitin conju-
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**FIGURE 5.** The spa1-1 allele of RSP5 enhances the steady-state levels of the Pho87 and Pho91 low affinity P_\text{i} transporters but not the Pho84 high affinity P_\text{i} transporter. A, Western blot analysis was performed using extracts from WT (YMH854), *pho4\Delta* (YMH855), and *pho4\Delta spa1-1* (YMH856) strains carrying the PHO87-3xHA tagged allele or from WT (YMH860), *pho4\Delta* (YMH861), or *pho4\Delta spa1-1* strains carrying the PHO91-3xHA tagged allele. The blot was probed with antibody to the HA epitope (*α*-HA) or to Rpa1 (*α*-Rpa1) as a loading control. B, Western blot analysis was performed using extracts from WT (YMH 829), *pho4\Delta* (YMH830), or *pho4\Delta spa1-1* (YMH856) strains carrying the PHO84-3xHA tagged allele or WT (YMH860), *pho4\Delta* (YMH861), or *pho4\Delta spa1-1* strains carrying the PHO91-3xHA-tagged allele. Cells were grown in liquid medium containing either 1 mM P_\text{i} (H) or 25 \mu M P_\text{i} (L) to repress or induce PHO84-3xHA expression. The blot was probed with antibody to the HA epitope (*α*-HA) or to Rpa1 (*α*-Rpa1) as a loading control.

The Pho87 and Pho91 Transporters Are Ubiquitinated in an Rsp5-dependent Manner—To determine whether the Pho87 and Pho91 transporters are directly ubiquitinated, we immunoprecipitated (IP) the Pho87-3xHA and Pho91-3xHA proteins from cell extracts of the WT, *pho4\Delta*, and *pho4\Delta spa1-1* strains using *α*-HA antibody followed by immunoblot (IB) analysis using *α*-ubiquitin (*α*-Ub) antibody. As shown in Fig. 6, immunoprecipitated Pho87-3xHA and Pho91-3xHA from the wild type strains were readily detected using *α*-Ub antibody (lanes 4 and 7). The ubiquitinated levels of both proteins were lower in the *pho4\Delta* mutants (lanes 5 and 8), implicating Pho4 in ubiquitination of the low affinity P_\text{i} transporters, although the mechanism of this effect is unknown. Importantly, the levels of ubiquitinated Pho87-3xHA and Pho91-3xHA were also diminished in the *pho4\Delta spa1-1* strains (lanes 6 and 9) even though the levels of these two proteins are higher in this background (Fig. 5A, lanes 3 and 6). Immunoblot analysis (*α*-HA) of HA-immunoprecipitated protein confirmed that the recombinant proteins are intact (Fig. 6, second panel). Analysis of the same strains that had not been 3x-HA tagged confirmed that the observed bands do indeed correspond to Pho87 and Pho91 (Fig. 6, lanes 1–3). We also performed the reciprocal experiment, first immunoprecipitating ubiquitinated proteins (*α*-Ub) from cells extracts, followed by immunodetection (*α*-HA) of Pho87-3xHA and Pho91-3xHA. The results confirmed that Pho87 and Pho91 are indeed ubiquitinated (Fig. 6, third panel).

Taken together, the results in Figs. 5 and 6 demonstrate that the Pho87 and Pho91 low affinity P_\text{i} transporters are ubiquitinated in an Rsp5-dependent manner that affects the steady-state levels of both proteins.

The spa1-1 Suppressor Phenotype Is Pho87- and Pho91-dependent—To confirm that stabilization of the Pho87 and/or Pho91 low affinity P_\text{i} transporters associated with diminished Rsp5 E3 ligase activity is responsible for reversion of the *pho4\Delta* mutant phenotype, we deleted the PHO87 and PHO91 genes. If the Pho^- suppressor phenotype of the *pho4\Delta spa1-1* strain is dependent upon the low affinity P_\text{i} transporters, then deletion of the encoding genes should at least partially restore the Pho^- mutant phenotype. Indeed, deletion of either *PHO87* or *PHO91* resulted in loss of the Pho^- phenotype of the *pho4\Delta spa1-1* suppressor strain (Fig. 7, cf. sectors 3, 5, and 6 for both the upper
(pho87Δ) and lower (pho91Δ) panels on 5 μM Pi (medium). This effect is not due to impaired growth associated with either pho87Δ or pho91Δ alone as neither deletion impaired growth on low phosphate medium in the wild type background to the same extent as in the suppressor strain (cf. sectors 1, 2, and 6). These results confirm that suppression of the Pho phenotype by defective Rsp5 requires the Pho87 and Pho91 low affinity Pi transporters. Repeated attempts to construct double pho87Δ pho91Δ deletions in the pho4Δ spa1-1 strain failed, even though both genes could readily be deleted in the wild type and pho4Δ strains. It is, therefore, likely that loss of both low affinity Pi transporters is lethal in the absence of functional Rsp5. These results underscore the functional relationship between the Rsp5 E3 ligase and the low affinity Pi transporters and are consistent with our conclusion that turnover of the low affinity Pi transporters is mediated by the Rsp5-endosome pathway.

DISCUSSION

In this study we have uncovered a role for Rsp5-mediated substrate ubiquitination in regulation of the Pho87 and Pho91 low affinity membrane Pi transporters of *S. cerevisiae*. First, the Pho phenotype associated with the absence of the Pho4 activator of the PHO regulon is suppressed by mutation in the Rsp5 E3 ubiquitin ligase. Suppression is presumably due to diminished Rsp5 ligase activity because the spa1-1 suppressor encodes a single amino acid replacement within the Rsp5 catalytic HECT domain. Also, two different *rsps* alleles encoding catalytically defective Rsp5 proteins (L733S and C777A) fail to complement the spa1-1 growth defect. Second, the *spa1-1* Tsm- phenotype is suppressed by overexpression of ubiquitin, shown previously to suppress catalytically defective *rsps* mutants (39). In addition, *spa1-1* is phenotypically suppressed by 1 M sorbitol, which is also known to suppress *rsps* catalytic mutants (37). Third, deletion of the End3 component of the endosome, which functions in Rsp5-mediated turnover of plasma membrane proteins in yeast (43), suppresses the *pho4Δ* deletion. Fourth, the levels of the Pho87 and Pho91 low affinity Pi transporters are clearly elevated in the *spa1-1* mutants. Fifth, Pho87 and Pho91 are ubiquitinated, and ubiquitination of these proteins is diminished in the *spa1-1* background. Finally, the Pho revertant phenotypes associated with loss of Rsp5 E3 ligase activity is dependent upon the Pho87 and Pho91 transporters. To our knowledge, this is the first study to implicate the ubiquitin-endosome pathway in turnover of plasma membrane Pi transporters.

In contrast to its effects on the low affinity Pi transporters, the *spa1-1* allele of *RSPS* is without effect on the high affinity Pho84 Pi transporter. *PHO84* is a component of the PHO regulon, whose components are regulated by the Pho4 transcription factor in response to Pi availability. Accordingly, Pho84 is inducible in response to low Pi, in the *pho4Δ* deletion mutant, and this defect is not overcome by the *spa1-1* suppressor (Fig. 5B). Moreover, Pho84 appears to be degraded by a pathway different from that of Pho87 and Pho91. Add-back of Pho7 to Pho84-starved cells normally results in rapid degradation of the Pho84 transporter. However, inhibition of protein kinase A was reported to stabilize Pho84, thereby defining a role for the protein kinase A signal transduction pathway in Pho84 turnover (8). Nonethe-
magnitude greater than the $K_m$ of the high affinity system ($K_m = 8 \mu M$) (49), it seems unlikely that the transporter activities of Pho87 and Pho91 account for growth on limiting Pi medium. It seems more likely that the low affinity Pi transporters function as signal transducers that detect external Pi concentrations and transmit this information to the intracellular PHO pathway. This possibility is supported by the recent report that the low affinity Pi transporters regulate PHO gene expression independently of internal Pi concentrations (6). Our results are consistent with the suggestion that Pho87, Pho90, and Pho91 function as both Pi transporters and sensors. Furthermore, this external Pi signal transduction pathway can function independently of the Pho4 transcriptional activator, as our results demonstrate that stabilization of Pho87 and Pho91 bypass Pho4 for growth on limiting Pi. It remains to be determined how the low affinity Pi transporters function as signal transducers and how Rsp5 responds to changes in Pi concentrations.

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