In *Saccharomyces cerevisiae*, the phosphate signal transduction pathway (PHO pathway) is known to regulate the expression of several phosphate-responsive genes, such as *PHO5* and *PHO84*. However, the fundamental issue of whether cells sense intracellular or extracellular phosphate remains unresolved. To address this issue, we have directly measured intracellular phosphate concentrations by $^{31}$P NMR spectroscopy. We find that phosphate signal transduction is known to regulate the expression of several phosphate-responsive genes, such as *PHO5* and *PHO84*. However, the fundamental issue of whether cells sense intracellular or extracellular phosphate remains unresolved. To address this issue, we have directly measured intracellular phosphate concentrations by $^{31}$P NMR spectroscopy. We find that 

PHO5 expression is strongly correlated with the levels of both intracellular orthophosphate and intracellular polyphosphate and that the signaling defect in the Δpho84 strain is likely to result from insufficient intracellular phosphate caused by a defect in phosphate uptake. Furthermore, the Δphm1Δphm2, Δphm3, and Δphm4 strains, which lack intracellular polyphosphate, have higher intracellular orthophosphate levels and lower expression of PHO5 than the wild-type strain. By contrast, the Δphm5 strain, which has lower intracellular orthophosphate and higher polyphosphate levels than the wild-type strain, shows repressed expression of PHO55, similar to the wild-type strain. These observations suggest that PHO5 expression is under the regulation of intracellular orthophosphate, although orthophosphate is not the sole signaling molecule. Moreover, the disruption of PHM3, PHM4, or of both PHM1 and PHM2 in the Δpho84 strain suppresses, although not completely, the PHO5 constitutive phenotype by increasing intracellular orthophosphate, suggesting that Pho4p affects phosphate signaling largely by functioning as a transporter.

Inorganic phosphate is an essential nutrient needed in large amounts for nucleic acid and phospholipid biosynthesis, as well as for energy metabolism. It is therefore essential that organisms have appropriate regulatory mechanisms to respond and adapt rapidly to changes in phosphate availability (1). In *Saccharomyces cerevisiae*, the phosphate signal transduction pathway (PHO pathway) regulates the expression of several phosphate-responsive genes that are involved in the scavenging and specific uptake of phosphate from extracellular sources, including *PHO5*, which encodes repressible acid phosphatase (rAPase), and *PHO84*, which encodes a high affinity phosphatase transporter that acts in response to phosphate levels (2–4). Under high phosphate conditions, the transcription factor Pho4p is phosphorylated by the cyclin-dependent kinase (CDK) complex Pho80p-Pho85p (5) and exported from the nucleus to the cytoplasm (6), thereby turning off expression of the *PHO5* and *PHO84* genes. When yeast cells are starved of phosphate, the CDK inhibitor Pho81p inactivates Pho80p-Pho85p (7, 8). Under these conditions, Pho4p is unphosphorylated and active (9), leading to the induction of *PHO5* and *PHO84* expression to scavenge phosphate from the environment (2–4, 10). Although many components and the molecular mechanism of the PHO pathway have been extensively studied, fundamental questions, such as whether cells sense intracellular or extracellular phosphate and what phosphate metabolites function as signals for regulating this pathway, remain unanswered.

A previous study demonstrated that disruption of *PHO84* results in constitutive expression of *PHO5* (10), suggesting that a defect in phosphate uptake in the Δpho84 strain may lead to a lower level of intracellular phosphate (or another metabolite) that could serve as a regulation signal for the PHO pathway, which in turn would mimic the effects of phosphate starvation. A recent study has shown that the overexpression of other phosphate transporter genes, such as *PHO87*, *PHO90*, and *PHO91*, increases phosphate uptake ability and suppresses constitutive expression of *PHO5* in the Δpho84 strain (11). Because Pho87p, Pho90p, and Pho91p share no significant amino acid similarity with Pho84p, it seems unlikely that these transporters contain common signal-responsive domains similar to those of *PHO84*. It is therefore likely that the signaling defect of Δpho84 cells is suppressed by the increase in the intracellular phosphate level, suggesting that there is a sensing system for intracellular phosphate rather than for extracellular phosphate.

There is, however, no experimental evidence showing that phosphate uptake ability accurately reflects the intracellular phosphate level; in addition, the possibility remains that each phosphate transporter possesses different signaling domains and thus has a role not only in phosphate transport but also in phosphate sensing and possibly in extracellular phosphate sensing. An example of a transporter that also functions as a sensor is Mep2p, an ammonium transporter that generates a signal to regulate filamentous growth in response to ammonium starvation, in addition to its role in ammonium uptake (12). On the other hand, it is known that several transporter-like transmembrane proteins that do not participate in nutrient uptake have nutrient-sensing activities (13). Examples of such transporter-like homologues include Snf3p and Rgt2p, which share significant identity with hexose transporters but instead function as glucose sensors (14–16). Dominant gain-of-function mutations in *SNF3* and *RGT2* cause constitutive glucose-inde-
Intracellular Phosphate Serves as a Signal for PHO Pathway

TABLE I
S. cerevisiae strains used in this study

| Strain          | Relevant genotype | Source |
|-----------------|-------------------|--------|
| NBW8            | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 | Ref. 20 |
| SH8333          | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 pho84::HIS3 | Our stock |
| NMY4L19H2       | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 pho1::CgLEU2 phm2::CgHIS3 | Ref. 31 |
| PHY22           | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 phm3::CgTRP1 | This study |
| NBM72W7         | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 phm4::CgTRP1 | This study |
| PHY25           | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 phm5::CgTRP1 | This study |
| PHY46           | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 pho84::HIS3 phm1::CgLEU2 phm2::CgHIS3 | This study |
| PHY24           | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 pho84::HIS3 phm3::CgTRP1 | This study |
| PHY44           | MATa ade2 leu2–3,112 ura3–1,2 trp1–289 his3–532 can1 pho3–1 pho84::HIS3 phm4::CgTRP1 | This study |

RESULTS
PHO5 Expression Is Strongly Correlated with the Levels of Intracellular Orthophosphate and Polyphosphate—To investigate whether the PHO pathway responds to the intracellular or extracellular phosphate level, we directly measured the intracellular phosphate concentration by 31P NMR spectroscopy, a noninvasive method for the analysis of in vivo intracellular phosphorus-containing metabolites, which has been extensively used in studies of phosphate metabolism in yeast (26–29). We first focused on the Δpho84 strain, which is defective in phosphate signaling and therefore exhibits a phenotype of constitutive PHO5 expression even under high phosphate conditions (Fig. 1A). We found that in the Δpho84 strain grown in a phosphate-rich medium, both orthophosphate and polyphosphate, the major forms of intracellular phosphate in yeast (30), were reduced to approximately one-half (14 and 98 mM, respectively) of the levels in the wild-type strain (23 and 230 mM, respectively) (Fig. 1B). It should be noted that the phosphate concentrations in this study are given in terms of phosphate residues, not polymer molecules, which are the relevant indicator of polyphosphate availability.

To explore further the relationship between intracellular phosphate and PHO5 expression, we measured intracellular phosphate and rAPase activity in the wild-type strain grown in medium containing an initial phosphate concentration of 11 (high phosphate condition), 2.2, 0.88, 0.44, 0.22, 0.11, or 0.044 mM. At initial extracellular phosphate concentrations above 0.44 mM, intracellular orthophosphate and polyphosphate levels in the wild-type strain were constant at ~25 and 210 mM, respectively (Fig. 2A), and no rAPase activity was detected (Fig. 2B). By contrast, when the initial extracellular phosphate concentrations were lower than 0.22 mM, the intracellular orthophosphate and polyphosphate levels markedly declined (Fig. 2A) and rAPase activity inversely increased in proportion to the intracellular phosphate concentration (Fig. 2B). These observations indicate that PHO5 expression is tightly coupled to both intracellular orthophosphate and intracellular polyphosphate over a range of extracellular phosphate levels and that PHO5 expression is derepressed when the intracellular phosphate levels are lower than a certain threshold (orthophosphate and polyphosphate concentrations of ~25 and 210 mM, respectively).

PHO5 Expression Is Under the Regulation of Intracellular Orthophosphate—To distinguish between the role of intracellular orthophosphate and that of intracellular polyphosphate in phosphate signaling, we measured intracellular orthophosphate and rAPase activity in the Δphm1Δphm2, Δphm3, and Δphm4 strains, which are defective in vacuolar polyphosphate accumulation (31). Phm1p/Vtc2p, Phm2p/Vtc3p, Phm3p/Vtc4p, and Phm4p/Vtc1p are vacuolar membrane proteins that are involved in vacuolar polyphosphate accumulation (31) and function as regulators of vacular H⁺-ATPase activity and vacuolar transporter chaperones (32). In agreement with results from polyphosphate PAGE analysis (31), intracellular polyphosphate in the Δphm1Δphm2, Δphm3, and Δphm4 strains was undetectable by NMR analysis (data not shown). Interestingly, under both high phosphate (11 mM) and low phosphate (0.22 mM) conditions, intracellular orthophosphate levels were higher in these disruptants than in the wild-type strain (Fig. 3A). Next, we measured intracellular orthophosphate levels in the Δphm3 strain grown in an initial extracellular phosphate concentration of 11, 2.2, 0.88, 0.44, 0.22, 0.11, or 0.044 mM, as in Fig. 2. Under all of the phosphate conditions examined, the intracellular orthophosphate levels were higher in the Δphm3 strain than in the wild-type strain (Fig. 3B). These results suggest that the defect in polyphosphate synthe-
sis in the Δphm1Δphm2, Δphm3, and Δphm4 strains leads to an excess accumulation of intracellular orthophosphate.

Despite the lack of polyphosphate, the disruptants exhibited repressible PHO5 expression, similar to the wild-type strain (Fig. 3C). Consistent with the increase in intracellular orthophosphate, the Δphm1Δphm2, Δphm3, and Δphm4 strains showed lower PHO5 expression than the wild-type strain grown under low phosphate conditions (Fig. 3C). When PHO5 expression was examined in more detail in the Δphm3 strain grown in phosphate concentrations lower than 0.22 mM, we found that PHO5 expression was lower in the Δphm3 strain than in the wild-type strain (Fig. 3D). These findings suggest that polyphosphate is not essential for PHO5 repression, at least under conditions of modestly elevated orthophosphate, and that orthophosphate is a more efficacious signaling molecule, further strengthening the idea that PHO5 expression is under the regulation of intracellular orthophosphate.

Intracellular Orthophosphate Is Not the Only Signal of Phosphate Availability—To distinguish further between the role of intracellular orthophosphate and that of intracellular polyphosphate in phosphate signaling, we next examined intracellular orthophosphate and polyphosphate levels and rAPase activity in the Δphm5 strain, a disruptant of a gene encoding vacuolar endopolyphosphatase (33), which has been shown to cause a significant increase in polyphosphate chain length (31). We anticipated that the Δphm5 strain may show an increase not only in polyphosphate chain length but also in polyphosphate quantities. If this is the case, it is possible that the intracellular phosphate serves as a signal for the PHO pathway.
The above results indicate that the intracellular orthophosphate level may decrease because most of the phosphate that has been taken up into the cells is stored as excess polyphosphate in the vacuole. In fact, when we measured intracellular orthophosphate and polyphosphate levels in this disruptant, we found that the Δphm5 strain grown in high phosphate medium had an intracellular polyphosphate level of ~280 mM, which was 1.3-fold higher than that of the wild-type strain (Fig. 4). By contrast, the intracellular orthophosphate level of the Δphm5 strain was 12 mM; that is, one-half of the level of the wild-type strain (23 mM) (Fig. 4) and similar to that of the Δpho84 strain (14 mM) (Fig. 1B).

If intracellular orthophosphate is the only signal for regulating the PHO pathway, PHO5 expression in the Δphm5 strain should be derepressed under high phosphate conditions. Under these conditions, however, PHO5 expression in the Δphm5 strain was repressed, similar to that in the wild-type strain (Fig. 4). These observations suggest that intracellular orthophosphate is not the only signal of phosphate availability and that another phosphate signal could be polyphosphate itself, although orthophosphate seems to be more efficient at repressing PHO5 expression. Alternatively, the authentic phosphate signal may be an as yet unidentified phosphate compound that in the Δphm5 strain is elevated to a level that is sufficient to compensate for the decrease in intracellular orthophosphate.

Pho84p Affects Phosphate Signaling Mainly as a Transporter—The above results indicate that the Δpho84 strain, which exhibited constitutive PHO5 expression even under high phosphate conditions, has reduced levels of both intracellular orthophosphate and intracellular polyphosphate. To investigate whether Pho84p acts as a phosphate sensor in addition to its transporter function, we examined intracellular phosphate and PHO5 expression in the Δpho84Δphm1Δphm2, Δpho84Δphm3, and Δpho84Δphm4 strains. We assumed that deletion of PHM3, PHM4, or both PHM1 and PHM2 may suppress the constitutive expression of PHO5 in the Δpho84 strain by increasing intracellular orthophosphate. As expected, we found that the intracellular orthophosphate levels in the Δpho84Δphm1Δphm2, Δpho84Δphm3, and Δpho84Δphm4 strains (33, 25, and 30 mM, respectively) are 2-fold higher than those in the Δpho84 strain (Fig. 5A) and that these strains do not accumulate polyphosphate (data not shown), similar to the Δphm1Δphm2, Δphm3, and Δphm4 strains. Interestingly, rA-Pase activity data revealed that the constitutive expression of PHO5 observed in the Δpho84 strain is suppressed in the Δpho84Δphm1Δphm2, Δpho84Δphm3, and Δpho84Δphm4 strains (Fig. 5B). The suppression of PHO5 expression in these disruptants is not complete, however, as these disruptants exhibit weak PHO5 expression under high phosphate conditions, although they contain higher intracellular phosphate levels than the wild-type strain. These results suggest that the signaling defect in the Δpho84 strain is a consequence of insufficient intracellular phosphate levels and that Pho84p functions mainly as a transporter in the PHO pathway, although a role as a sensor in phosphate signaling cannot be completely ruled out.

DISCUSSION

The downstream events in the PHO pathway and the transcription control mechanisms of phosphate-responsive genes, such as PHO5, have been extensively studied; however, the initial events that take place in sensing phosphate availability are poorly understood. In this study, by using 31P NMR spectroscopy, we have shown that the expression of PHO5 is tightly correlated with the intracellular concentration of inorganic phosphate. We have also found that PHO5 expression is under
the regulation of intracellular orthophosphate and that polyphosphate is dispensable for phosphate signaling, at least under conditions of modest orthophosphate elevation (Fig. 3). Intracellular orthophosphate is not the only signal of phosphate availability, however, because the Δphm5 strain, which has slightly elevated polyphosphate, but a reduced level of intracellular orthophosphate similar to that in the Δpho84 strain, shows normal repression of PHO5 (Fig. 4). Polyphosphate does not seem to be a direct signaling molecule of the PHO pathway because it is relatively ineffective as a repressing signal in comparison with orthophosphate.

So what other candidates are there for a phosphate signaling
molecule? If Phm5p, the vacuolar endopolyphosphatase, could also dephosphorylate other phosphate compounds, including one that is an authentic signaling molecule other than polyphosphate, then it is possible that a lack of PHM5 would also lead to an increase in the phosphorylation of this authentic signaling molecule. If this is the case, such an authentic signal could compensate for the reduced intracellular orthophosphate level observed in the Δphm5 strain. At present, however, polyphosphate is the only known substrate of Phm5p (33).

In yeast, several examples of transporter-like nutrient sensors are known. Mep2p has been suggested to act as an ammonium sensor in addition to its role in ammonium uptake (12). By contrast, Snf3p and Rgt2p, which are glucose carrier homologues that do not directly support glucose transport, act as receptors for sensing glucose (14, 15). Our results show that deletion of PHM3, PHM4, or both PHM1 and PHM2 can suppress constitutive expression of PHO5 in the Δpho84 strain by increasing intracellular orthophosphate, suggesting that PHO84 affects phosphate signaling largely by its transporter function (Fig. 5). However, the suppression of PHO5 is incomplete in these disruptants, although they contain higher levels of intracellular orthophosphate than the wild-type strain (Fig. 5). Although this observation suggests that Pho84p may have activity for sensing extracellular phosphate availability, the intracellular phosphate sensing system would seem to be more effective than the extracellular system because increasing the intracellular orthophosphate level in the Δpho84 strain by deleting PHM3, PHM4, or both PHM1 and PHM2 suppressed the derepression of PHO5 almost completely.

What, then, is the intracellular phosphate sensor? A possible candidate is the CDK inhibitor Pho81p. If this is the case, Pho81p would have to localize to the nuclear membrane to sense phosphate in the cytoplasm because it has been shown to localize to the nucleus under both high phosphate and low phosphate conditions (34). If Pho81p localizes to the nuclear lumen, but not to the nuclear membrane, the quantitative alteration of phosphate in nucleus should directly reflect that in the cytoplasm. On the other hand, a minimum domain of Pho81p containing 80 amino acids has been shown to be both necessary and sufficient to inhibit Pho80p-Pho85p CDK activity (34). It seems unlikely, however, that such a small region of Pho81p is responsible not only for inhibiting Pho80p-Pho85p activity but also for sensing phosphate directly, by binding to phosphate itself. Taking these observations together, we do not favor the idea that Pho81p is a phosphate sensor. Further studies are necessary to identify the intracellular phosphate sensor(s).

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Intracellular Phosphate Serves as a Signal for the Regulation of the PHO Pathway in Saccharomyces cerevisiae

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