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Pho85 Kinase, a Cyclin-Dependent Kinase, Regulates Nuclear Accumulation of the Rim101 Transcription Factor in the Stress Response of *Saccharomyces cerevisiae*▽

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The budding yeast *Saccharomyces cerevisiae* alters its gene expression profile in response to changing environmental conditions. The Pho85 kinase, one of the yeast cyclin-dependent kinases (CDK), is known to play an important role in the cellular response to alterations in parameters such as nutrient levels and salinity. Several genes whose expression is regulated, either directly or indirectly, by the Rim101 transcription factor become constitutively activated when Pho85 function is absent,. Because Rim101 is responsible for adaptation to alkaline conditions, this observation suggests an interaction between Pho85 and Rim101 in the response to alkaline stress. We have found that Pho85 affects neither *RIM101* transcription, the proteolytic processing that is required for Rim101 activation, nor Rim101 stability. Rather, Pho85 regulates the nuclear accumulation of active Rim101, possibly via phosphorylation. Additionally, we report that Pho85 and the transcription factor Pho4 are necessary for adaptation to alkaline conditions and that *PTK2* activation by Pho4 is involved in this process. These findings illustrate novel roles for the regulators of the *PHO* system when yeast cells cope with various environmental stresses potentially threatening their survival.

Environmental conditions, including the concentrations of nutrients, the temperature, salinity levels, and the presence of toxic agents, are signals to which microorganisms, such as the budding yeast, *Saccharomyces cerevisiae*, respond by adjusting gene expression. Changes in these external cues are sensed by yeast via molecular alterations or interactions that are transmitted internally, ultimately to evoke appropriate responses that adapt the cells to the new conditions. The ability to sense and respond to change is essential for cell survival.

Various protein kinases function in these signaling processes and constitute a complex network that coordinates responses (36). In yeast, Tor and protein kinase A (PKA) are nutrient-sensing kinases; when nutrient levels are sufficient for growth, Tor inactivates the Yak1 kinase via PKA, leading to activation of ribosomal protein genes (17). Tor also phosphorylates the Sch9 kinase, a member of the AGC protein kinase family, to regulate ribosome biogenesis and prevent entry into G_0 phase (33). The Pho85 kinase is a member of the yeast cyclin-dependent kinase (CDK) family and functions in the responses to phosphate (P_i) starvation (25), alterations in nutrient status (23), and environmental stresses, including elevated extracellular Ca^{2+} and Na^+ levels (10). When nutrients are sufficient, for example, one role of Pho85 is the phosphorylation of Rim15 to prevent the cells from entering G_0 phase, an outcome reinforced by the action of Tor and PKA (35). When the level of P_i in the medium is sufficient, Pho85 also phosphorylates the Pho4 transcription factor to exclude it from the nucleus, resulting in repression (i.e., the absence of transcription) of the P_i-responsive (*PHO*) genes, whose products are needed only under conditions of P_i starvation (11, 12). Similarly, when the levels of amino acids are sufficient, this CDK stimulates the degradation of the transcription factor Gcn4, which activates the genes involved in amino acid synthesis (19). Pho85 is one of the kinases that phosphorylate the transcription factor Crz1 (31), a mediator of the stress response to high levels of Ca^{2+} and Na^+ and to elevated temperatures (3). When Crz1 is dephosphorylated by calcineurin, it accumulates in the nucleus to activate its target genes (3). Thus, whenever environmental conditions are satisfactory for cell growth, Pho85 functions so that the yeast cells express only genes appropriate to the conditions (2).

An alkaline pH stresses yeast by disruption of the plasma membrane H^+ gradient, which powers the transport of cations and of diverse nutrients (34). To enhance survival under this critical condition, yeast cells express two genes that encode ion pumps, *ENA1* and *VMA4*, which require transcriptional activation by functional Rim101 (13). The Ca^{2+}-mediated signaling pathway is also known to influence the transcriptional response to alkaline pH conditions (29). Interestingly, *PHO84* and *PHO12*, which are normally activated upon P_i starvation and which encode a P_i transporter and the repressible acid phosphatase, respectively (25), are also activated under alkaline conditions (29). Since Pho4 is the activator of these two genes and Pho85 negates the activity of Pho4, this observation suggests an impairment of Pho85 function under conditions of alkaline stress. Our previous global expression analysis of yeast

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TABLE 1. Yeast strains and plasmids used in this work

Strain	Genotype	Source or reference
W303-1A	*MAT***a** *ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100*	YGRC[a]
MFY115	*MATα leu2 his1 ade1 trp1 ura3*	21
MFY116	MFY115 Δ*pho85*::*LEU2*	21
MFY129	MFY115 Δ*pho80*::*LEU2*	21
MFY276	W303-1A Δ*pho85*::*LEU2*	This work
MFY359	W303-1A Δ*rim101*::*URA3*	This work
MFY361	MFY276 Δ*rim101*::*URA3*	This work
MFY362	MFY115 Δ*pcl1*::*LEU2* Δ*pcl2*::*TRP1*	This work
MFY363	MFY115 Δ*pcl5*::*URA3*	This work
MFY364	MFY115 Δ*clg1*::*URA3*	This work
MFY371 (BY4741)	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	1
MFY373	MFY371 Δ*pho85*::*URA3*	24
MFY395	W303-1A Δ*rim13*::*TRP1*	This work
MFY413	MFY276 Δ*rim13*::*TRP1*	This work
MFY414	W303-1A Δ*msn5*::*CgHIS3*	This work
MFY415	MFY413 Δ*msn5*::*CgHIS3*	This work
MFY416	W303-1A Δ*ptk2*::*TRP1*	This work
MFY417	MFY276 Δ*ptk2*::*TRP1*	This work
MFY418	W303-1A Δ*pho4*::*LEU2*	This work
MFY419	MFY276 Δ*pho4*::*URA3*	This work

[a] YGRC, Yeast Genetic Resource Center (http://yeast.lab.nig.ac.jp/nig/).

genes showed that the absence of Pho85 leads to reduced transcription of genes, such as *NRG1* and *SMP1*, that are negatively regulated by Rim101 (14) and consequently to elevated expression of genes, such as *ENA2* and *CWP1*, that are normally repressed by *NRG1* and *SMP1* (23). In other words, it appears that the Rim101 repressor becomes active in the absence of Pho85; this prompted us to investigate the possible existence of a genetic interaction between *RIM101* and *PHO85*. Here we report that Pho85 influences Rim101 repressor activity by regulating its accumulation in the nucleus. The resulting transcriptional responses, which include decreased *CLN2* transcription, allow the cell to cope with the stress brought about by alkaline conditions. We also demonstrate that the Pho4 transcription factor plays an important role in the cellular response to alkaline stress.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study, except for the *vps* deletion strains, are listed in Table 1. The Δ*vps20*, Δ*vps27*, and Δ*vps37* strains were from the collection of *Saccharomyces cerevisiae* deletion strains (Open Biosystems) (strain numbers 6211, 4890, and 2730, respectively). *Escherichia coli* strain DH5α was used as a host for plasmids. Media for growth of *E. coli* and the complete (YPAD) and synthetic dextrose (SD) media for yeast were prepared as described previously (27). Alkaline SD media containing appropriate nutrients and 0.01% bromothymol blue were prepared by amending the medium with 20 mM Tris HCl (pH 9.0).

DNA manipulation. Standard *E. coli* and yeast genetic methods, as well as DNA manipulations, were carried out as described previously (27, 28). To construct a reporter plasmid bearing the *CLN2* promoter and *lacZ*, the promoter fragment (positions −970 to +30) was first cloned by PCR and then incorporated into plasmid pMF1152 (22). The QuikChange II site-directed mutagenesis kit (Stratagene) and the primers listed in Table 2 were used to mutagenize the putative Rim101 binding sequence in the *CLN2* promoter and the Pho4 binding site in the *PTK2* promoter, and to substitute Ala for the Ser and Thr residues at the possible phosphorylation sites of the Rim101 protein (residues 42, 76, 98, 107, 120, 287, and 309). Successful mutagenesis was confirmed by DNA sequencing. MFY362 (MFY115 Δ*pcl1* Δ*pcl2*) was constructed by transformation of MFY171 (Δ*pcl2*) with the Δ*pcl1*::*LEU2* fragment. MFY363 (MFY115 Δ*pcl5*) and MFY364 (MFY115 Δ*clg1*) were constructed by replacing the +97-to-+365 region of *PCL5* and the +390-to-+930 region of C*LG1*, respectively, with a *URA3* fragment. The other strains with individual *pcl* deletions have been described previously (21). To disrupt the *RIM101* or *RIM13* locus of W303-1A, either a *URA3* fragment was used to replace the PpuMI (+735)-StyI (+1056) fragment of *RIM101* or a *TRP1* fragment was used to replace the BglII (−293)-SmaI (+1597) fragment of *RIM13*. To disrupt the *MSN5* gene, a *HIS3* fragment of *Candida glabrata* (*CgHIS3*) was used to replace the *MSN5* open reading frame (ORF); similarly, for the *PTK2* gene, a *TRP1* fragment was employed to replace the BglII (+235)-KpnI (+1240) fragment. To disrupt the *PHO4* locus, its SmaI (+484)-to-PmaCI (+933) region was replaced by either a *URA3* or a *LEU2* fragment. Successful disruption of these loci was confirmed by PCR. Plasmid pMF1406, producing Flag-tagged Rim101, was constructed by introduction of the PCR-cloned NcoI-BamHI fragment of Rim101 (amino acids [aa] 1 to 536) into the pFLAG-MAC plasmid (Sigma-Aldrich). Plasmid pMF1577, producing the Flag-Rim101 7A mutant protein, in which the codons for seven Ser or Thr residues were replaced by Ala codons, was constructed by replacing the BglII-XhoI fragment of pMF1406 with the corresponding mutant sequence. Plasmids producing the full-length (aa 1 to 626) or truncated (aa 1 to 536) form of Rim101 fused to green fluorescent protein (GFP) were constructed by inserting an XhoI-BamHI fragment encoding four consecutive GFP sequences into the site corresponding to the first codon of the full-length or truncated form, or the 7A mutant, of Rim101, followed by transfer of the respective fusion fragment into pRS316.

Analytical methods. Genomewide expression analysis by means of GeneChip has been described previously (23). Northern blot analysis was performed essentially as described previously (23). Digoxigenin (DIG)-labeled probes were pre-

TABLE 2. Primers used in this work

Primer	Description[a]	Sequence
MN393	*CLN2* ΔRim101BS	CTGAGGTTCAAAAGTGTCGACTTATCAATTCATGCGC
MN394	*CLN2* ΔRim101BS	CGCGCATGAATTGATAAGTCGACACTTTTGAACCTCA
MN1217	*PTK2* ΔPho4BS	GCAAATAGTTGCCCCGGGCGAGTGCGGTG
MN1218	*PTK2* ΔPho4BS	CACCGCACTCGCCCCGGGGCAACTATTTGC
MN1227	Rim101 S42A sense	GGACGGGCTGCCCGCGCCTAACCTATCTAA
MN1228	Rim101 S42A AS	TTAGATAGGTTAGGCGCGGGCAGCCCGTCC
MN1229	Rim101 S76A sense	GATGAACGGATGGCCCCGGGCAGCACTTCT
MN1230	Rim101 S76A AS	AGAAGTGCTGCCCGGGGCCATCCGTTCATC
MN1231	Rim101 T95A sense	CTTCACACTTGAACGCGCCTCCATACGAT
MN1232	Rim101 T95A AS	ATCGTATGGAGGCGCGTTCAAGTGTGAAG
MN1233	Rim101 S107A S120A sense	GGCGCTTCGGCAGTCGCGCCCACCACATCATCTTCCT CTGACTCGTCCTCCTCCGCGCCATTGGCAC
MN1234	Rim101 S107A S120A AS	GTGCCAATGGCGCGGAGGAGGACGAGTCAGAGGAA GATGATGTGGTGGGCGCGACTGCCGAAGCGCC
MN1235	Rim101 S287A sense	GTCACATTCTACCGCGCCACAGATATTACC
MN1236	Rim101 S287A AS	GGTAATATCTGTGGCGCGGTAGAATGTGAC
MN1237	Rim101 S309A sense	GTATAAGCCGGTATACGCGCCACAATTGAG
MN1238	Rim101 S309A AS	CTCAATTGTGGCGCGTATACCGGCTTATAC

[a] BS, binding site; AS, antisense.

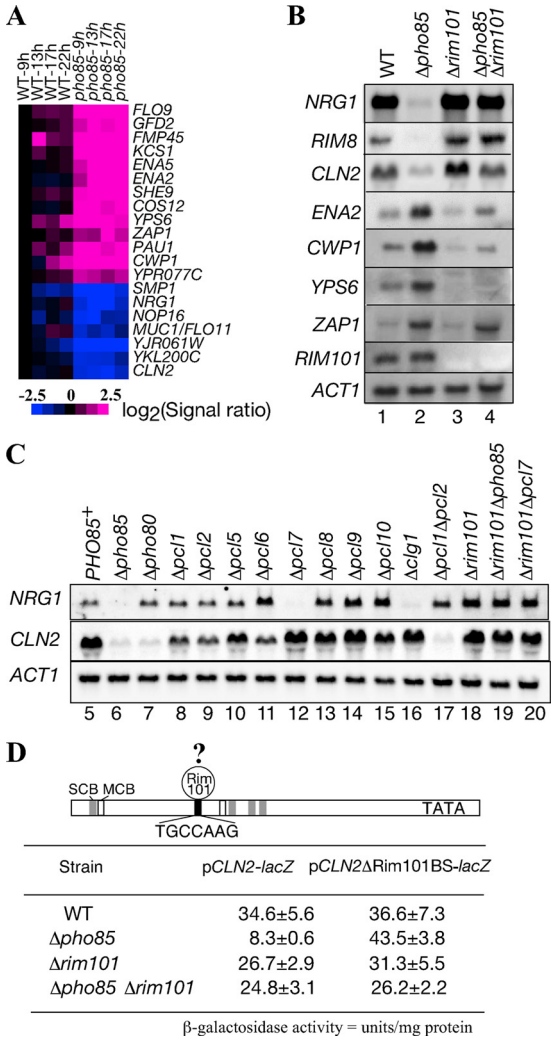
pared by PCR using chromosomal DNA as a template and gene-specific primers, except that GenePair forward and reverse primers (Invitrogen) were used for *CLN2* (*YPL256C*), *CWP1* (*YKL096W*), *YPS6* (*YIR039C*), and *ZAP1* (*YJL056C*). RNA was then visualized by probing with an anti-DIG antibody conjugated with alkaline phosphatase and CSPD (Roche) and was captured using a LAS3000 image analyzer (Fujifilm, Tokyo, Japan). β-Galactosidase activity was assayed as described previously (22). Immunoblot analysis was carried out essentially as described previously (21). For induction of Rim101 processing, yeast cells grown to the mid-log growth phase in an appropriate selective medium at pH 4 and 30°C were collected by centrifugation, divided into two equal portions, and then suspended in the medium at pH 4 or pH 7, followed by incubation at 30°C for 30 min. The cells were then collected by centrifugation, washed, and used to prepare cell extracts (8). For immunoprecipitation of HA-Rim101 from cell extracts, the cells were first precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 6%, then resuspended in 6 M urea, and finally vortexed in the presence of glass beads to produce the cell extracts. The extracts were adjusted to IP buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 0.1% NP-40, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), followed by the addition of anti-HA antibody beads (Sigma-Aldrich). After incubation of the mixtures on ice for 1 h, the beads were collected, washed with the IP buffer, and subjected to immunoblot analysis. For the treatment of Rim101 with lambda protein phosphatase (New England Biolabs), the immunoprecipitated beads were incubated with the enzyme at 30°C for 30 min, according to the protocol suggested by the manufacturer, and subjected to Western blot analysis. *In vitro* Pho85 phosphorylation of bacterial Flag-Rim101 was carried out essentially as described previously (21). Briefly, glutathione *S*-transferase (GST)-Pho85 or the GST-Pho85 E53A mutant (kinase knockout), immunoprecipitated from yeast extracts with an anti-GST monoclonal antibody and collected by absorption to protein A-Sepharose 4B beads, was added to recombinant Flag-Rim101 (0.5 μg/ml) in 40 μl of the kinase assay buffer containing 0.1 mM ATP and 5 μCi of [γ-^{32}P]ATP. The reaction mixture was incubated at 30°C for 30 min, and the reaction was stopped by addition of 4× sodium dodecyl sulfate (SDS) loading buffer; products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography with a BAS5000 Bioimage Analyzer (Fujifilm).

Cytoplasmic pH. Cytoplasmic pH was measured by ^{31}P NMR spectroscopy. ^{31}P NMR spectra were acquired at 202.3 MHz with a Varian VXR-500S spectrometer operating in the Fourier transform mode 10 times in 24 min with 4,096 transients. The yeast cell suspension was transferred to a 10-mm (in diameter) nuclear magnetic resonance (NMR) tube at a density of 4×10^8 cells/ml in Tris-buffered saline (TBS) containing 10% (vol/vol) D_2O. Pure oxygen was bubbled through the suspension at a rate of 4.7 ml/min to maintain an aerobic condition in the NMR tube. The temperature was kept at 30°C, and methylene diphosphonate was used as an external standard. The cytoplasmic pH was estimated from a calibration curve of chemical shift established as a function of pH.

Fluorescence microscopy. Cells harboring GFP-fused proteins were photographed by using a BX52 fluorescence microscope (Olympus, Tokyo, Japan) with an UPlanApo 100_/1.45 objective (Olympus) equipped with a CSU20 confocal scanner unit (Yokogawa Electric, Tokyo, Japan) and an electron-multiplying charge-coupled device (EMCCD) camera (Hamamatsu Photonics, Bridgewater, NJ). DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), which was incubated with cell suspensions at a concentration of 0.5 μg/ml at room temperature for 10 min prior to microscopic observation. Fluorescence images were captured with LSM 510 software and processed with Photoshop, version 7.0 (Adobe Systems).

RESULTS

Expression of Rim101-dependent genes is altered in the absence of Pho85. Our previous global expression analysis of yeast genes showed that the absence of Pho85 caused a set of genes to become activated and a second set to be repressed (23) (Fig. 1A). Among them, *NRG1* and *SMP1* were downregulated, whereas *ENA2*, *ENA5*, *YPS6*, and *CWP1* were constitutively expressed (Fig. 1A). *ENA2* and *ENA5* are structural and functional homologues of *ENA1*, which is repressed by Nrg1 (13). *YPS6*, *ENA2*, and *ENA5* each have a putative Nrg1 binding sequence in their upstream regions, which makes it highly likely that Nrg1 represses them as well. Consequently, the observed transcriptional activation of these genes can stem from a reduction in the amount of the Nrg1 repressor. *NRG1*



Strain	p*CLN2-lacZ*	p*CLN2*ΔRim101BS-*lacZ*
WT	34.6±5.6	36.6±7.3
Δ*pho85*	8.3±0.6	43.5±3.8
Δ*rim101*	26.7±2.9	31.3±5.5
Δ*pho85* Δ*rim101*	24.8±3.1	26.2±2.2

β-galactosidase activity = units/mg protein

FIG. 1. Genetic interaction between *PHO85* and *RIM101*. (A) Temporal gene expression profiles of the wt and a Δ*pho85* mutant analyzed with a GeneChip, showing constitutively induced (a change of 2 or higher in the transcript level) (magenta) or repressed (a change of −2 or lower or higher in the transcript level) (blue) genes in the absence of *PHO85* during the incubation period up to 22 h. A change in the transcript level is shown as $\log_2$(signal ratio) relative to the level in the wt at 9 h. Typical *PHO* genes were excluded. (B) Effects of Δ*pho85* and Δ*rim101* mutations on gene expression analyzed by Northern blotting as described in Materials and Methods. *ACT1* provides the loading control. (C) Effects of a deletion of each Pho85-cyclin gene and combinations of deletions on the expression of *NRG1* and *CLN2*, analyzed by Northern blotting. (D) Reporter assay for *CLN2* promoter activity. (Top) Schematic representation of the *CLN2* promoter region showing a putative Rim101 binding site (TGCCAAG) (filled box), the Swi4-cell cycle box (SCB) (shaded box), and the MluI-cell cycle box (MCB) (open box). The TATA sequence is also shown. (Bottom) Activities of the wt (p*CLN2*) promoter and of the mutant *CLN2* promoter lacking the Rim101 binding site (p*CLN2*ΔRim101BS), expressed as β-galactosidase activities in the designated strains.

and *SMP1* are, in turn, repressed by the Rim101 transcription factor, which functions in alkaline pH response, salt tolerance, and cell differentiation (13). *CWP1* has a putative Smp1 binding site in its upstream region, but its regulation by Smp1 is somewhat controversial. Although overproduction of Smp1 is reported to activate *CWP1* (4), the fact that *CWP1* is activated under alkaline conditions (29) and at elevated Na^+ levels (38)

but is repressed in the absence of Rim101 (14) favors the hypothesis that Rim101 regulates the transcriptional regulation of *CWP1* through repression of *SMP1* expression. Thus, the observed alterations in gene expression in the absence of Pho85 could be explained by the activity of Rim101. We examined possible genetic interactions between *RIM101* and *PHO85* and biochemical interactions between their products.

Genetic interaction between *PHO85* and *RIM101*. We used Northern blot analysis to investigate the question of genetic interaction between the genes. Most of the biological functions of *RIM101* are exerted through transcriptional repression (13). *NRG1* and *RIM8*, both subject to Rim101 repression (14), were repressed in the absence of Pho85 (Fig. 1B, lane 2), and this repression was relieved by introducing a Δ*rim101* mutation (Fig. 1B, lane 4). On the other hand, *ENA2* and *CWP1*, genes that are repressed by Nrg1 and Smp1, respectively, were highly expressed in a Δ*pho85* mutant (Fig. 1B, lane 2), and their expression was reduced to the wild-type (wt) level in a Δ*pho85* Δ*rim101* double mutant (Fig. 1B, lane 4). In the absence of Rim101, their expression levels appeared lower than those in the wt (lane 3). These results suggest that Rim101 can act to repress *SMP1* and *NRG1* in the absence of Pho85. Having a putative Nrg1 binding site in its upstream sequence, *YPS6* was regulated by *PHO85* and *RIM101* in the same way that was observed for *CWP1* and *ENA2* (Fig. 1A and B), suggesting that this gene is also regulated by Rim101 through Nrg1. *ZAP1* expression, which was activated in a Δ*pho85* mutant (Fig. 1A), was not reduced to the wt level when both Pho85 and Rim101 were absent (Fig. 1B), indicating that *ZAP1* is not under the control of *RIM101*. These results point to Rim101 becoming a highly active repressor when functional Pho85 is absent. The fact that *RIM101* expression itself was not affected by a Δ*pho85* mutation (Fig. 1B, lanes 2 and 4) suggests that Pho85 exerts its effect on Rim101 at a posttranscriptional stage.

Since Pho85 is known to interact with 10 different cyclin partners to exert its kinase function on different substrates (18), we determined which Pho85-interacting cyclins were responsible for the interaction with *RIM101* by analyzing *NRG1* expression in cyclin deletion strains. No *NRG1* transcript was detected in a Δ*pcl7* mutant, and an *NRG1* transcript was barely detectable in a Δ*clg1* mutant (Fig. 1C, lanes 12 and 16), and repression of *NRG1* in the absence of Pcl7 was suppressed by introducing a Δ*rim101* mutation (lane 20). These results suggested that these two cyclins can partner with Pho85 to regulate Rim101 repression of *NRG1*. Conversely, the absence of *PCL6*, *PCL8*, *PCL9*, or *PCL10* stimulated *NRG1* expression (Fig. 1C, lanes 11 and 13 to 15), suggesting that their gene products play a negative role in *NRG1* regulation.

Rim101 downregulates *CLN2* expression in the absence of Pho85. The expression of the *CLN2* gene, encoding one of the G_1 cyclins, appears repressed in the absence of Pho85 (Fig. 1A). Northern blot analysis revealed that *CLN2* expression was actually reduced in a Δ*pho85* mutant (Fig. 1B, lane 2) but was restored to the wt level by introduction of a Δ*rim101* mutation (lane 4). A Δ*rim101* mutation alone appeared to stimulate *CLN2* expression to some extent (Fig. 1B, lane 3). These results suggest that *CLN2* is also a target of the Rim101 repressor and that Pho85 is required to counteract the repression. There is a putative Rim101 binding site (TGCCAAG) in the *CLN2* promoter (Fig. 1D), and we used a *lacZ* reporter assay to analyze whether this site mediated the repression. In the absence of Pho85, the promoter activity was only about 25% of the level in the presence of Pho85 (Fig. 1D). A Δ*rim101* mutation alone did not have a significant effect on the wt promoter activity, and a Δ*pho85* Δ*rim101* double mutant recovered about 72% of the wt level (Fig. 1D). When the sequence of the putative Rim101 binding site was altered, the mutant promoter activity was not reduced at all in the absence of *PHO85* or *RIM101* (Fig. 1D). Taken together, these results indicate that Rim101 functions as a repressor of *CLN2* expression in the absence of functional Pho85 and that repression is mediated through its putative binding site in the promoter region.

When we looked for the cyclin partner for Pho85 to sustain *CLN2* expression, *PHO80* and the presence of both *PCL1* and *PCL2* were required; individually, Δ*pcl1*, Δ*pcl2*, and Δ*pcl6* exerted less effect (Fig. 1C). Interestingly, the absence of *PCL7* stimulated *CLN2* expression, in contrast to its effect on *NRG1* expression. These different cyclin requirements raise the possibility of competition among the cyclins to direct Pho85 antagonism of the Rim101 repressor on different target genes.

Pho85 can phosphorylate Rim101 *in vitro*. Rim101 becomes active through the processing of its C-terminal region, which requires *RIM8*, *RIM9*, *RIM13*, *RIM20*, and *RIM21* (16, 26). *Aspergillus nidulans* PacC, an orthologue of Rim101, undergoes a similar activation process in a two-step fashion, which regulates the nuclear accumulation of PacC (20). We asked whether a Δ*pho85* mutation could stimulate the processing to generate the active Rim101 repressor. Since the processing is facilitated in the cells grown at neutral to alkaline pHs, we analyzed whether Pho85 affected Rim101 processing efficiency by cultivating yeast cells at pH 4 and then shifting to pH 7, followed by immunoblot analysis of cell extracts (Fig. 2A). Processing of Rim101 proteins was determined using strains that are normal (Δ*vps37*), defective (Δ*vps20*), or hyperactive (Δ*vps24*) in the processing activity (Fig. 2A, lanes 9 to 14; positions of processed and unprocessed species are indicated by the lower and upper arrows, respectively). No significant difference in the relative amount of processed Rim101 was detected between the wt and the Δ*pho85* mutant at pH 7 (Fig. 2A, lanes 1 to 4, lower arrows); this was also true in another strain set with a different genetic background (lanes 5 to 8). These data also exclude an effect of Pho85 on the stability of Rim101. Another mechanism of control must exist.

PKA-mediated phosphorylation of Rim101 has been suggested for transcriptional activation of *IME1* at the basal level (32). In the N-terminal half of the Rim101 protein, there are seven Ser/Thr-Pro amino acid sequences that can be phosphorylated by Ser/Thr kinases, including CDK. We speculated that Pho85 could phosphorylate Rim101 to modulate its transcriptional repressor function. To test this hypothesis, we carried out *in vitro* phosphorylation reactions using bacterially produced Rim101 and yeast extracts containing GST-Pho85 as the substrate and kinase, respectively (Fig. 2B). The phosphoprotein signal of the band corresponding to the approximate molecular weight of bacterial Flag-Rim101 was observed when the reaction mixture contained the substrate (Fig. 2B, lane 15), but it was barely observable when the Rim101 7A mutant with the seven possible phosphorylation sites converted to alanine or threonine residues was used as the substrate (lane 17). The same was true when either a Pho85 mutant without kinase

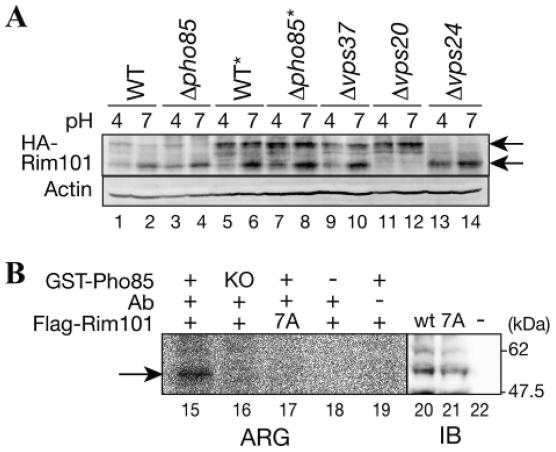


FIG. 2. Pho85 and the status of Rim101. (A) Pho85 did not appear to affect the processing of Rim101. The statuses of HA-Rim101 protein in two wt strains (MFY115 and W303 [*]) with different genetic backgrounds and their Δ*pho85* derivatives (MFY116 and MFY276 [*]) were analyzed by immunoblotting with an anti-HA monoclonal antibody (lanes 1 to 8). The cells were grown at pH 4 and were divided into equal portions, which were then incubated at either pH 4 or pH 7 for 30 min to induce processing of Rim101 prior to preparation of cell extracts for Western blot analysis. The positions of the full-length (upper arrow) and processed (lower arrow) forms of Rim101 were determined by those of Rim101 in *vps* mutants that provided normal (Δ*vps37*), defective (Δ*vps20*), or hyperactive (Δ*vps24*) processing (lanes 9 to 14). The amounts of actin in each cell extract are also shown as a loading reference. (B) *In vitro* phosphorylation of Flag-Rim101 by Pho85 kinase. GST-Pho85 was immunoprecipitated with an anti-GST polyclonal antibody (Ab) and subjected to a kinase assay using bacterially produced Flag-tagged Rim101 (wt) or Rim101 7A mutant proteins as a substrate. Phosphoprotein was visualized by autoradiography (ARG) (lanes 15 to 19). KO stands for the Pho85 E53A mutant, which is devoid of kinase activity. The arrow indicates the position of phosphorylated Flag-Rim101. Bacterial Flag-Rim101 preparations were also analyzed by immunoblotting (IB) with an anti-Flag antibody (lanes 20 to 22).

activity was used (lane 16), the kinase source was not included in the reaction mixture (lane 18), or the kinase had not been immunoprecipitated (lane 19). The position of the phosphoprotein band coincided with that of bacterial Flag-Rim101 detected by Western blot analysis with an anti-Flag antibody (lanes 20 and 21). These results indicate that Pho85 can phosphorylate Rim101 *in vitro*.

Rim101 accumulates in the nucleus in the absence of Pho85 or Msn5. Given that Pho85 regulates the activity of the Pho4 transcription factor by controlling its nuclear localization (11, 12), we next used fluorescence microscopy to analyze whether Pho85 similarly affected the cellular location of Rim101. GFP-Rim101 could rescue the alkali sensitivity of a Δ*rim101* mutant (data not shown), indicating that the fusion protein that was the basis for the localization study was functional. Usually we observed 200 to 300 cells. When GFP-Rim101 was produced in wt cells grown in complete medium (pH 5 to 6, conditions under which processing of Rim101 was not efficient), fluorescence was evenly distributed throughout each cell, i.e., no significant accumulation of Rim101 was observed in any compartment (Fig. 3A, row 1). On the other hand, the GFP-dRim101 mutant, a constitutively active form of Rim101 without the C-terminal 95 amino acids, generated a signal in a cell (arrows in Fig. 3A, row 2) coincident with the nucleus visualized with DAPI (row 2). Thus, the active (processed) form of Rim101 accumulates predominantly in the nucleus. When Pho85 was absent, GFP-Rim101 colocalized with the nucleus in about 30% of the cells we observed (arrows in Fig. 3A, row 3), and this was not the case for the wt (row 1), indicating that Rim101 accumulates in the nucleus when functional Pho85 is absent. The processed form of Rim101 (dRim101) similarly accumulated in the nucleus in a Δ*pho85* mutant (row 4). Since Lamb and Mitchell reported that the full-length form could localize in the nucleus as well and bind DNA (14), our observation raised the question of whether the nuclearly accumulated Rim101 in a Δ*pho85* mutant contained full-length Rim101. Therefore, we analyzed Rim101 localization in a Δ*rim13* mutant lacking the protease activity responsible for the processing of Rim101 (15); the cells should have only full-length Rim101. The fluorescence signal of GFP-Rim101 in the Δ*rim13* mutant was distributed evenly throughout the cytoplasm, with some exclusion from the area at the bud-mother junction that contained the nucleus (Fig. 3A, row 5, arrowhead). This observation suggests that full-length Rim101 is largely absent or is promptly exported from the nucleus. On the other hand, GFP-dRim101 accumulated in the nucleus of a Δ*rim13* mutant (row 6), confirming that nuclear transport of Rim101 functioned normally in the absence of Rim13. In a Δ*rim13* Δ*pho85* double mutant, the fluorescence signal of full-length Rim101 was distributed evenly in the cytoplasm without detectable exclusion from the nucleus (compare rows 5 and 7). This observation suggests that Pho85 function is also required for nuclear exclusion of full-length Rim101.

Once the Pho4 transcription factor is phosphorylated by Pho85, it has an increased affinity for the Msn5 exportin, which mediates its export from nucleus (12). We hypothesized that Pho85 regulated the nuclear localization of Rim101 by a similar mechanism, and we tested whether Msn5 could affect Rim101 localization. When Msn5 was absent, an accumulation of GFP-Rim101 in the nucleus was observed (Fig. 3A, row 8), indicating that Msn5 was involved in nuclear export of the repressor protein. In the absence of both Pho85 and Msn5 in Δ*rim13* mutant cells, a significant accumulation of Rim101 was detected (row 9) compared to that in Δ*rim13* Δ*pho85* mutant cells (row 7). Since only the full-length form of Rim101 was present in the nuclei of Δ*rim13* Δ*pho85* mutant cells, this observation demonstrated that Msn5 also functions in the export of unprocessed Rim101. Thus, Msn5 is required for the nuclear exclusion of both processed and unprocessed Rim101.

Phosphorylation is important for the regulation of Rim101 nuclear localization. We next analyzed the nuclear localization of the Rim101 7A mutant in order to examine the effect of phosphorylation on the behavior of Rim101. When the 7A mutant was produced in wt cells, it accumulated predominantly in the nucleus (Fig. 3A, row 10). In Δ*rim13* cells, nuclear accumulation of the mutant protein was also observed (row 11), indicating that phosphorylation is important for nuclear exclusion of both the processed and full-length Rim101 proteins. Immunoblot analysis showed that the 7A mutant was processed normally, although the efficiency with which it was processed appeared to be somewhat lower than that for the wt protein (Fig. 3B, lanes 12 to 15). The blots also provided evidence that the stability of the mutant protein was not significantly different from that of the wt (Fig. 3B). The 7A mutant protein showed higher mobility (arrows on the right-

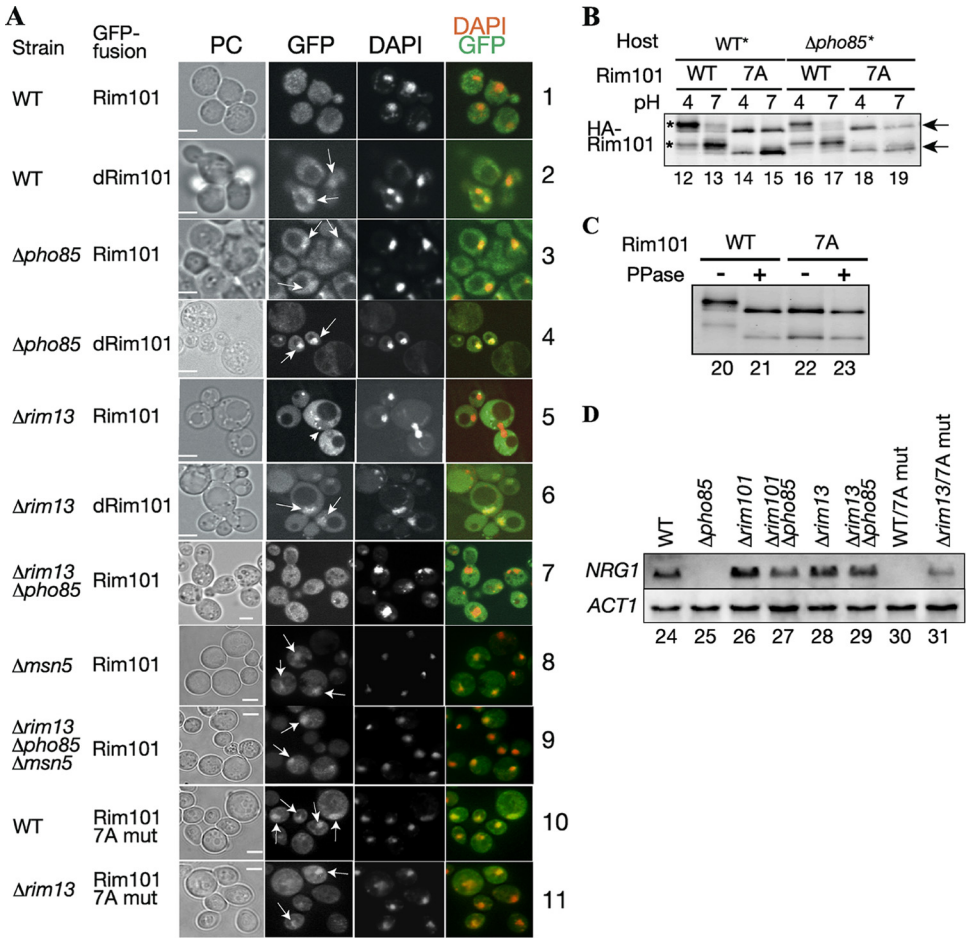


FIG. 3. Pho85 regulates the nuclear accumulation of Rim101. (A) Localization of Rim101 in the wt, Δ*pho85*, Δ*rim13*, Δ*pho85* Δ*rim13*, Δ*msn5*, or Δ*pho85* Δ*rim13* Δ*msn5* strain. Either the full-length (wt) or the truncated (aa 1 to 536) (dRim101) form of Rim101, or the Rim101 7A mutant (7A), fused with GFP at the N terminus, was produced in the strains indicated. PC, phase-contrast view. DNA was stained with DAPI. Photographs were taken through a fluorescence microscope. Colocalization of GFP-Rim101 signals (indicated by arrows) with DNA indicates nuclear accumulation of Rim101 (rows 2 to 4, 6, and 8 to 11). The arrowhead in row 5 indicates a reduction in the intensity of the GFP-Rim101 signal in a Δ*rim13* mutant. (B) Status of wt Rim101 or the 7A mutant in wt or Δ*pho85* cells. To avoid nonspecifically stained bands, the cell extracts were immunoprecipitated with an anti-HA antibody prior to Western blot analysis. The positions of the full-length and processed forms of wt Rim101 (WT) (lanes 12, 13, 16, and 17) and the 7A mutant (7A) (lanes 14, 15, 18, and 19) are shown by asterisks (left) and arrows (right), respectively. (C) Effect of lambda protein phosphatase (PPase) treatment on Rim101. Extracts prepared from wt cells producing HA-Rim101 (WT) or the HA-Rim101 7A mutant (7A) were partially purified by immunoprecipitation with an anti-HA antibody and were then incubated at 30°C for 20 min in the presence (+) or absence (−) of the phosphatase, followed by Western blot analysis. (D) Northern blot analysis of *NRG1* in the wt, Δ*pho85*, Δ*rim101*, Δ*pho85* Δ*rim101*, Δ*rim13*, and Δ*rim13* Δ*pho85* strains (lanes 24 to 29) and in wt or Δ*rim13* cells producing the Rim101 7A mutant (lanes 30 and 31). *ACT1* is shown as a loading control.

hand side of Fig. 3B) than the wt protein (asterisks), a shift likely to reflect the difference in phosphorylation status. Contrary to our expectations, in Δ*pho85* cells, wt Rim101 showed an electrophoretic mobility similar to that in wt cells, whereas Rim101 7A showed a higher mobility (Fig. 3B, lanes 16 to 19). To test the idea that the observed mobility shift reflects the different phosphorylation state of Rim101, we treated the two proteins with lambda protein phosphatase and found that the mobilities of the 7A mutant did not change detectably after the phosphatase treatment (Fig. 3C, lanes 22 and 23), while the wt bands shifted to the positions of the mutant after the treatment (Fig. 3C, lanes 20 and 21). These results indicate that Rim101 is phosphorylated *in vivo* and that after the removal of the 7 putative phosphorylation sites (7A mutant protein), no additional unsuspected sites are available for phosphorylation.

We next analyzed by Northern blot analysis whether the full-length Rim101 present in the nucleus at low levels was active as a repressor (Fig. 3D). The observation that *NRG1*, which is repressed by Rim101, was normally expressed in a Δ*rim13* mutant (lane 28) and in a Δ*rim101* mutant (lane 26) provides genetic corroboration for the nuclear exclusion of Rim101 observed by fluorescent microscopy (Fig. 3A, row 5). In a Δ*rim13* Δ*pho85* double mutant, *NRG1* expression was almost at a level similar to that in a Δ*rim13* mutant (lanes 28 and 29), indicating that full-length Rim101 has little, if any, repressor function against *NRG1*. When the 7A mutant protein entered the nucleus (Fig. 3A, row 8), it could repress *NRG1* expression (lane 30); even the full-length form of the 7A mutant protein appeared to repress *NRG1* (lane 31) when it was present in the nucleus in excess. Taken together, phosphory-

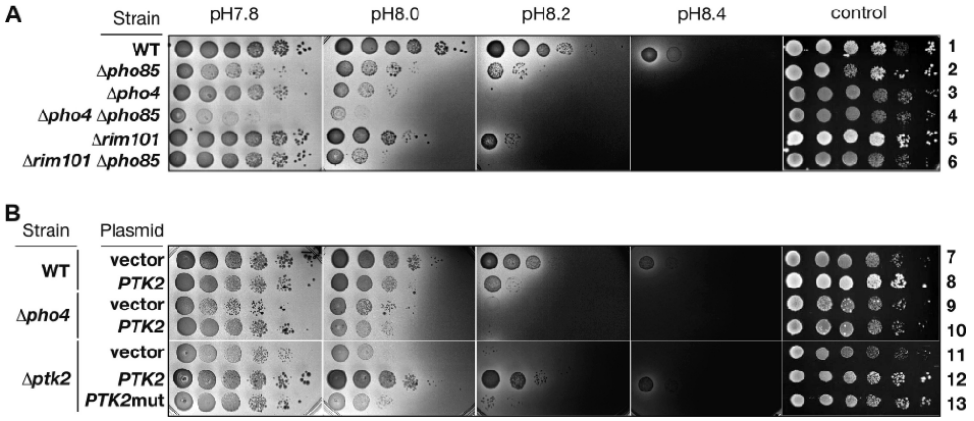


FIG. 4. Either Pho85, Pho4, or Ptk2 is required for yeast cells to adapt to alkaline conditions. (A) Alkali sensitivities of the wt, Δ*pho85*, Δ*pho4*, Δ*rim101*, Δ*pho85* Δ*pho4*, and Δ*pho85* Δ*rim101* strains. Overnight cultures of yeast strains were adjusted to ca. 5×10^7 cells/ml, and serial dilutions of the suspension were plated onto SD medium that had been adjusted to the designated pH with 20 mM Tris-HCl buffer (pH 9.0) and contained 2% glucose, appropriate nutrients, and 0.01% bromothymol blue. The plates were incubated at 30°C for 4 days before being photographed. The growth of each strain on a YPD plate (pH 6) is also shown as a control. (B) Pho4 may act through Ptk2 in the alkali response process. The effect of Ptk2 overproduction on the growth of wt or Δ*pho4* cells under alkaline conditions was examined by spotting the strains harboring the vector alone or the *PTK2* expression plasmid as indicated (rows 7 to 10). The requirement of the Pho4-binding site in the *PTK2* promoter for *PTK2* function under alkaline conditions was tested by spotting a Δ*ptk2* strain harboring either the vector alone (row 11), the plasmid containing the wt *PTK2* gene (row 12), or the plasmid containing the mutant gene lacking the Pho4-binding site (row 13). The growth of the tested strains on SD medium (pH 6) supplemented with appropriate nutrients is also shown as a control.

lation plays an important role in the regulation of Rim101 nuclear export and/or import, for both the full-length and the processed form. In this way the Pho85 kinase can control the repressor activity of Rim101.

Pho85 is required for the cellular response to alkaline conditions. Rim101, Pho4, and Crz1 are involved in the cellular response to alkaline conditions (29). Deletions of these transcription factors and of Pho85 in various combinations were tested for their effects on yeast growth under alkaline conditions (Fig. 4A). The absence of Pho4 or Rim101 conferred alkali sensitivity on yeast cells, as previously reported (Fig. 4A, rows 3 and 5) (6, 13). The absence of Pho85 should allow Rim101 and Pho4 to exert their activities and conceivably stimulate alkali tolerance, but a Δ*pho85* mutant showed a growth defect even at pH 7.8, where Δ*rim101* cells could grow almost normally (Fig. 4A, row 2). We assume that the deregulation of Pho4, Rim101, and Crz1 in the absence of Pho85 could disrupt the coordinated expression of alkali-responsive genes needed under alkaline conditions. The introduction of a Δ*rim101* mutation could suppress the growth defect of Δ*pho85* cells at pH 7.8 (Fig. 4, rows 2 and 6), suggesting that the mechanisms by which Pho85 participates in the alkali stress response involves the regulation of Rim101. However, this suppression was no longer observed at or above pH 8.0, probably because the presence of Rim101 became crucial for alkali tolerance under high pH conditions. In addition to the deregulation of the transcription factors, the observation that cytoplasmic pH became high in a Δ*pho85* mutant, irrespective of its genetic background (Fig. 5), indicates that normal ion homeostasis is disturbed in the absence of Pho85. This notion further supports a more general role of Pho85 in the cellular response to alkaline stress.

A Δ*pho4* mutant appeared more sensitive to alkaline stress than Δ*rim101* or Δ*pho85* mutant cells (Fig. 4A, row 3), and a Δ*pho4* Δ*pho85* double mutant became supersensitive (row 4). This suggests that Pho4 may play a rather crucial role in the cellular response to alkaline conditions. We observed that yeast growth on an alkaline medium was accompanied by acidification of the medium surrounding the colony, as indicated by the change in the color of the pH indicator (Fig. 4, white areas), suggesting that proton export is important for adaptation to alkaline conditions. Pma1 is a plasma membrane H^+-ATPase that pumps protons out of the cell and is a major regulator of both the cytoplasmic pH and the plasma membrane potential that is crucial to resistance to alkaline stress (1a). Pma1 is activated through phosphorylation by Ptk2 protein kinase (7), and we have reported that Pho4 activates *PTK2* expression (24). In addition, a Δ*ptk2* mutant grows poorly at pH 8 (6). These facts raise the possibility that Pho4, in addition to activating P_i transporters (29), also participates in the cellular alkaline response by activating *PTK2* expression and consequently Pma1. If this is the case, the alkali sensitivity of a Δ*pho4* strain could be suppressed by overproducing Ptk2. When Ptk2 was overproduced under the direction of the *TDH3* promoter, the growth of wt cells under alkaline conditions

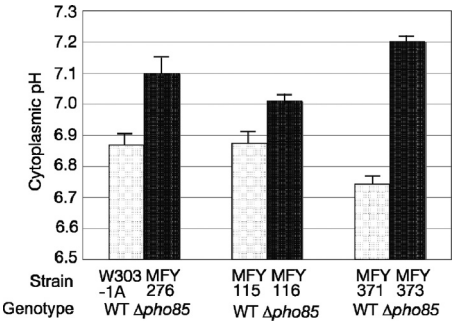


FIG. 5. Pho85 is required for the maintenance of cytoplasmic pH. Cytoplasmic pH was measured by ^{31}P NMR spectroscopy in three different sets of wt (open bars) and Δ*pho85* (filled bars) strains as designated. Error bars, standard errors.

became defective, probably because of the toxic effect of overproduced Ptk2 (Fig. 4B, rows 7 and 8). Overproduced Ptk2, however, allowed the growth of a Δ*pho4* strain at pH 7.8 but not at higher pH values (rows 9 and 10), suggesting that Ptk2 could rescue the growth defect of Δ*pho4* cells, but only when alkaline stress was not too severe. Next, we tested whether Pho4 is required for *PTK2* activation by using a *ptk2* mutant (*PTK2*mut) in which a possible Pho4 binding site (at −242) in the promoter had been mutated. In the absence of Ptk2, yeast cells became alkali sensitive (Fig. 4B, row 11), and this could be remedied by introducing wt *PTK2* (row 12). The mutant appeared to recover the growth defect of Δ*ptk2* cells to some extent at pH 7.8 but could no longer do so at pH 8.0 and above (row 13). These results indicate that the Pho4 binding site in the *PTK2* promoter is required for *PTK2* activation so that yeast cells can cope with alkaline stress. Thus, *PTK2* activation appears to be another pathway in which Pho4 functions in the cellular response to alkaline conditions, adding another clue as to how Pho85 functions in the alkali response of yeast.

DISCUSSION

In this paper, we have shown that Pho85 regulates the activity of the Rim101 repressor, at least in part, by controlling its cellular location and that this regulation could be one of the mechanisms by which Pho85 functions in the alkali stress response. This conclusion was drawn from the observations that Rim101-dependent genes are strongly repressed in the absence of Pho85 (Fig. 1A and B), that Pho85 affected neither the transcription (Fig. 1B), the processing, nor the stability of Rim101 (Fig. 2A), and that the absence of Pho85 kinase activity resulted in accumulation of the repressor in the nucleus (Fig. 3A). We have demonstrated that phosphorylation of Rim101 plays an important role in determining its cellular location (Fig. 3A). Since Msn5 exportin can remove Rim101 from the nucleus (Fig. 3A), phosphorylation of Rim101 would enhance its interaction with the exportin and conversely reduce its interaction with importin, the latter also contributing to the exclusion of Rim101 from the nucleus. *A. nidulans* PacC is also phosphorylated (9). In this case, however, it is speculated that phosphorylation leads to recruitment of the proteasome machinery that carries out the processing of the transcription factor (9). We found that Pho85 could phosphorylate Rim101 *in vitro* (Fig. 2B), and we expected that it would do so *in vivo*. However, we observed that Rim101 has the same electrophoretic mobility whether it is derived from wt cells or from a Δ*pho85* mutant (Fig. 3B, lanes 12, 13, 16, and 17), whereas a difference in mobility was observed when wt Rim101 and Rim101 7A were compared (lanes 16 to 19). Treatment with protein phosphatase eliminates this difference (Fig. 3C). This unanticipated result may mean that comparison of wt Rim101 and Rim101 7A conveys a misleading notion of how the actual phosphorylation state of Rim101 *in vivo* could be observed, or that Pho85 may not be the major kinase responsible for Rim101 phosphorylation, even though it clearly affects Rim101's cellular location. Pho85 may regulate Rim101 in an indirect fashion. For instance, Pho85 might phosphorylate a yet unknown protein, which in turn might modulate the affinity of Rim101 for nuclear transport proteins. Alternately, Pho85 could also exert its effect by disturbing organelle function. In the absence of Pho85, activated Rim101 might induce plasma membrane Na^+-ATPase, which pumps out Na^+ (14), and activated Pho4 might induce *PHO84*, which encodes a symporter of P_i and H^+ (25). If the events cause simultaneous ion fluxes, the resulting disturbance in ion homeostasis could result in defective structure and function of both the vacuole and the endosome. In fact, in the absence of Pho85, cytoplasmic pH is elevated (Fig. 5), and disorders of vacuoles and endosomes have been reported (10). Since Rim101 is processed to the active form on the endosome, these structural as well as functional defects may abnormally stimulate Rim101 activity.

Alkaline pH is a stress that affects the plasma membrane proton gradient, leading to abnormal transport of cations and diverse nutrients (34). Yeast cells alter gene expression profiles via Rim101, Pho4, and Ca^{2+} signaling pathways to respond to this critical stress condition (29). Our results suggested that Pho85 functions to coordinate pathways responding to alkaline stress by regulating the activities of these transcription factors. In addition, Serrano et al. reported that copper and iron are necessary for tolerance to alkaline conditions (30). We have demonstrated previously that Pho85 is required for proper expression of *MAC1* and *RCS1*, the products of which activate *CTR1* and *FTR1*, which are important for the uptake of copper and iron, respectively (23). Thus, the sensitivity of a Δ*pho85* mutant to alkaline conditions may stem from the pleiotropic function of Pho85 kinase. Our observation that a Δ*rim101* mutation could rescue the growth of Δ*pho85* mutant cells at pH 7.8, whereas a Δ*pho4* Δ*pho85* mutant exhibited more sensitivity (Fig. 4A), suggests that Pho4 plays a rather crucial role in the alkali response process. Our results demonstrate that Ptk2 can be a target of Pho4 in this process, but Pho4 should have more targets that remain to be identified. In this context, it is noteworthy that our previous genomewide ChIP-on-chip analysis has demonstrated that Pho4 binds to the promoter regions of *ARO2*, *GLY1*, *BOP2*, *VTC1*, and *YPS1* (24a). Deletions of these genes cause growth defects at pH 7.5 (30) or at pH 8 (6).

When yeast cells encounter environmental stress, progression through the cell cycle is delayed, providing time for adaptation to the stress conditions. Hog1-mediated G_1 arrest under high osmolarity exemplifies this phenomenon; the arrest involves the downregulation of Cln1 and Cln2 and the stabilization of Sic1 (5). A recent report demonstrates that Pho85 participates in relieving G_1 checkpoint arrest after DNA damage (37). We found that Rim101 was involved in the downregulation of *CLN2* expression (Fig. 1) and that *CLN2* was downregulated under alkaline conditions in a Rim101-dependent manner (M. Nishizawa et al., unpublished observation). These findings provide another clue to cell cycle regulation requiring Pho85 function in response to environmental stress.

Pho85 phosphorylation of transcriptional activators can regulate their activities by causing a change in cellular location (e.g., Pho4) or the degradation of the regulatory factor (e.g., Gcn4). The results of this work identify another role of Pho85: it can regulate the activity of a transcriptional repressor protein. While the Pho85-Pho80 complex is responsible for phosphorylation of Pho4 in response to a P_i signal (11, 12), different effects of deleting genes for the cyclins that complex with Pho85 were seen for *NRG1* and *CLN2* expression (Fig. 1C), suggesting that different Pho85-cyclin complexes are formed, depending on environmental signals, to regulate the activity of the Rim101 repressor on dif-

ferent target genes. In this context, it is noteworthy that a deletion of *PCL6* renders the cells sensitive to alkali; the mutant cells are defective for growth at pH 8 (6). The activity of the Pho85-Pcl6 complex is required for yeast growth under alkaline conditions where Rim101 activity is also needed (Fig. 4A) (14). In fact, a reduction in Rim101 repressor activity occurs in the absence of Pcl6, judging from the increase in *NRG1* expression that we found (Fig. 1C). The diversity of substrate specificity of Pho85 that is realized by complex formation with 10 different cyclins enables the kinase to function in many aspects of the regulation of cellular function responding to changes in various environmental conditions. Pho85 presents an ideal system for the study of the molecular mechanisms underlying cellular responses to various external signals.

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