Saccharomyces cerevisiae Histidine Phosphotransferase Ypd1p Shuttles between the Nucleus and Cytoplasm for SLN1-Dependent Phosphorylation of Ssk1p and Skn7p

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An important aspect of regulation of many, if not all, eukaryotic signal transduction pathways is the subcellular localization or compartmentalization of signaling molecules. In Saccharomyces cerevisiae, the osmotic stress-regulated mitogen-activated protein (MAP) kinase Hog1p is predominantly cytoplasmic in unstressed cells but rapidly concentrates within the nucleus in response to hyperosmotic conditions (7). Nuclear accumulation depends on Hog1p phosphorylation by the cytoplasmic MAP kinase Pbs2p (7, 31), interaction with the Ptp2 protein (which has been proposed to be a nuclear anchor [22]), and the presence in the nucleus of the Msn2p and Msn4p transcription factors, which are involved in the stress response (31). The nuclear localization of Msn2p and Msn4p is regulated by some of the more global consequences of exposure of cells to stress, such as changes in cyclic AMP levels (10). Like Hog1p, Msn2p and Msn4p lack an apparent nuclear localization sequence (NLS), and a localization mechanism based on cytoplasmic anchoring has been proposed (10). Another example of a transcription factor that relocates to the nucleus in response to stress is Yap1p. Nuclear accumulation of Yap1p is accomplished via modification of the Yap1p cysteine-rich domains in the presence of an oxidant, which perturbs the Yap1p interaction with the exportin Crm1p as well as other interactions that favor export (16, 38). The Hog1 pathway is regulated by a two-component phosphorelay pathway that consists of a dimeric sensor-kinase, Slp1p; the histidine phosphotransfer (Hpt) molecule, Ypd1p; and two response regulators, Ssk1p and Skn7p (14, 18, 21, 28, 29). Slp1p autokinase activity is modulated in response to changes in osmotic pressure. The phosphoryl group is transferred from a histidine residue near the kinase domain of Slp1p to a conserved aspartate within the Slp1p receiver domain, from there to a histidine on Ypd1p, and finally to an aspartate residue of one of two response regulators, Ssk1p and Skn7p (Fig. 1A) (18, 29). Since activation of the Hog1 MAP kinase pathway requires unphosphorylated Ssk1p, the pathway is inactive under normal osmotic conditions by virtue of the presence of a basal level of phosphorylated Ssk1p. Hypertonic conditions reduce Slp1p pathway activity, allowing Ssk1p to accumulate in the unphosphorylated form and thus triggering the activity of the Hog1 pathway (21, 29). In contrast, hypertonic conditions and certain mutations (e.g., fps1 or snl1*) stimulate Slp1p pathway activity and cause hyperphosphorylation of both the Ssk1p and Skn7p response regulators (5, 18, 36). Skn7p is a DNA binding protein, and phospho-Skn7p activates expression of the mannosyltransferase gene OCH1 and other genes, including but not limited to those involved in cell wall integrity and cell cycle progression (19; J. S. Fassler et al., unpublished data). Skn7p is also involved in the activation of oxidative stress response genes; however, Skn7p aspartyl phosphorylation is not required (18, 24).

The Ssk1p response regulator is expected to localize to the cytoplasm, since molecules in the Hog1 pathway, including Hog1, are cytoplasmic in unstressed cells. The Skn7p response regulator, in contrast, is nuclear (4, 30). Hence, a detailed molecular understanding of yeast two-component regulation must account for the subcellular localization of the molecules in the pathway. Phosphotransfer to the nuclear Skn7p response regulator requires at least the transient presence of other two-component molecules in the nucleus or movement of the Skn7 protein out of the nucleus. In this report we show that there are no apparent changes in Ssk1p or Skn7p localization in response to different types of stress. In contrast, we find that the histidine phosphotransferase, Ypd1p, is normally present in both cytosol and nucleus, suggesting that Ypd1p may be responsible for delivering the phosphoryl group...
FIG. 1. SLN1 pathway components are compartmentalized in yeast. (A) Fluorescence microscopy of GFP fusions with Sln1p (pCLM814 in JF1455), Ypd1p (pJF1414 in JF2153), Ssk1p (pJL1465 in JF1919), and Skn7p (pJL1380 in JF1904). (B) Confocal microscopy of JF2153 (ypd1Δ) expressing GFP-Ypd1p fusion protein. DNA was stained with propidium iodide. The merged image is shown on the right.
to Ssk1p in the cytosol and to Skn7p in the nucleus. The presence of Ypd1p in both the cytosol and the nucleus may reflect the need for simultaneous phosphorylation of the Ssk1p and Skn7p response regulators under normal growth conditions.

**MATERIALS AND METHODS**

**Strains.** All strains used are isogenic and derivatives of S288C. Strains were constructed for these experiments or are from the Fassler laboratory collection (Table 1). Disruption of YPD1 was achieved by transforming yeast with a PCR fragment containing a kanamycin resistance gene amplified with disruption oligonucleotides YPD-KAN-F (5'-TTGAATACCGGGATGATATTTGTATTGACGCAATGCAAAATCTTAGCCAGCTGAAGTTGT) and YPD-KAN-R (5'-AGCTGGATCCCAGACATTATTGTGTGTAT-3') and YPD-KAN-R (5'-GAATTCGTGTGAAGAATTATTC-3'). The YPD1 gene was one-step replacement with the pRS426 by using genomic PCR and subsequent restriction analysis of the amplified fragment. ypd1Δ::kan transformants of JF2148 carrying pRS426-PPT2 were screened by sensitivity to fluoro-orotic acid (FOA) before PCR and restriction analyses. shn1Δ::TRP1 disruption was carried out as described previously (38). Deletion of the SKN7 gene was achieved by one-step replacement with the PstI-XbaI fragment from plasmid pDSS14 from H. Saito (28). To disrupt HOG1, JF1565 was achieved by one-step replacement with the disruption fragment linearized from pGy150 (39) by BamHI-ClaI digestion. Transformants were screened by sensitivity to 0.9 M NaCl before Southern hybridization analysis. JF2123 (fps1Δ::LEU2) is a plasmid-free derivative of JF1532 (36).

**Media.** Solid and liquid media were prepared as described by Sherman et al. (32) and included synthetic complete (SC) medium lacking one or more specific amino acids and rich medium (yeast extract-peptone-dextrose [YPD]). Yeast transformation was performed by a modified lithium acetate method (9, 13). Yeast strains were grown to log phase and streaked or spotted onto various media after serial dilution. The viability of ypd1Δ strains carrying pRS426-PPT2 was assayed on SC medium containing 0.1% 5-FOA to test functional complementation by various YPD1 plasmids. All plate assays were carried out at 30°C.

**Stress treatment.** All localization studies were carried out with log-phase cultures grown at 28°C. Green fluorescent protein (GFP) fusion plasmids were introduced into strains with deletions in the corresponding gene.

(i) Osmotic stress. Hypo-osmolality was achieved by adding sorbitol to 1 M or NaCl to 0.5 or 0.9 M in log-phase cultures. Samples were taken and fixed at 5, 15, 30, 60, and 90 min after addition of the osmoticum. A HOGL-GFP construct (7) was used as a control. Hypo-osmolality was generated by using the fps1 mutation (strain JF2123), which prevents glycerol efflux and causes accumulation of intracellular glycerol (20). We previously showed that the fps1 mutation activates the SKN7-Skn7 pathway (36), and we conclude, based on these studies, that Skn1p kinase activity is increased under hypotonic conditions.

(ii) Heat stress. Log-phase cells expressing GFP-SKN7 or GFP-YPD1 were shifted to elevated temperature (37 and 42°C), and samples were taken at 5, 15, 30, 60, 120, and 180 min.

(iii) Oxidative stress. Log-phase cells expressing GFP-SKN7 were treated with 0.6 or 1 mM tert-butylhydroperoxide (Sigma) or 0.05% hydrogen peroxide (Sigma), and samples were taken at 5, 15, 30, 60, and 90 min. Activation of the oxidative response gene TRA2 was monitored in parallel to confirm the treatment conditions.

**Plasmids.** The plasmids used in this study are summarized in Table 2. Construction schemes are described below. The nucleotide positions are referred as +1 if downstream of the ATG start codon and as -1 upstream. All PCR fragments were amplified with the high-fidelity Pfu or Turbo Pfu DNA polymerase (Stratagene).

(i) Reporters. The OCH1 reporter plasmid was created by subcloning a 3-kb fragment containing UASOCH1 (-336 to +26)–lacZ from pZL1320 (19) into pRS314 by using EcoRI and SalI sites to create pJL1416.

(ii) GFP fusions. The SLN1-GFP plasmid pCLM814 was created by insertion of a NcoI fragment containing the GFP open reading frame (ORF) at the stop codon of YEplac112-SLN1 (B12). The GFP fragment was amplified by PCR with oligonucleotides B568 (5'-TCAAGTCCGCGCCGATGTTCAAAGGTGAAAGATTATTC-3') and B569 (5'-ATACGACTGAGCCGCGCATTGCATTTATTTATGCATAATTCCATGACC-3') and GFP mut3 (S65G S72A, GenBank accession number U73901) as a template. The pCLM814 plasmid was shown to be functional by complementation of the 2α invisibility phenotype in the strain shn1Δ::LEU2 heterozygote, JF1545, into which it was transformed.

The 2 μm GFP-SKN7 plasmid pJL380 was created by insertion of the SKN7 ORF downstream of UASOCH1-GFP (B564L S567T) cassette previously cloned into pRS425 (pJL1363). The pJFI380 plasmid was shown to be functional by complementation of the oxidative stress and hygroycin B sensitivities of a shn1Δ strain.

To construct the GFP-YPD1 fusion, a BamHI-HindIII polynucleotide was engineered by PCR (QuickChange site-directed mutagenesis kit [Stratagene]) beyond the translation start site of YPD1 in pCLM669 (a pRS316-YPD1 plasmid) by using oligonucleotides YPD1 + 3 BamHI-HindIII (5'-CGGATCCCAAGTACATCGTCTTACTATCCCCACGAAA-3') and YPD1 + 6 BamHI-HindIII (5'-CGGTGCTACGTGGATCGAGATGAAAACCTGATGCTTACTATCCCCACGAAA-3'). A GFP PCR fragment

### Table 1. Yeast strains used in this study

| Strain* | Relevant genotype | Derivation (reference) |
|---------|------------------|------------------------|
| JF1455  | MATaα his4-917 lys2-1286 trplΔ1 ura3-52 leu2 sln1Δ::LEU2/SLN1 | Diploid strain |
| JF1565  | MATaα his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴ | Can⁴ Cys⁴ derivative of FY834 (37) |
| JF1592  | MATa kar1-1 ade2-101 his2 leu2 trplΔ1 or -62 ura3-52 | sln1-22 derivative of JF1565; two-step replacement (36); plasmid free |
| JF1904  | MATa sln1Δ::TRP1 his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴ | sln1-22 derivative of JF1565; two-step replacement (36); plasmid free |
| JF1910⁸ | MATa sln1-22 his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴ | sln1-22 derivative of JF1565; two-step replacement (36); plasmid free |
| JF1919  | MATa skn1Δ::LEU2 his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴ | sln1-22 derivative of JF1565; two-step replacement (36); plasmid free |
| JF1920  | MATa sln1-22 skn1Δ::LEU2 his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴ | sln1-22 derivative of JF1565; two-step replacement (36); plasmid free |
| JF1974  | MATa hog1Δ::TRP1 his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴ | sln1-22 derivative of JF1565; two-step replacement (36); plasmid free |
| JF2123  | MATa fps1Δ::LEU2 his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴ | Plasmid-free version of JF1732 (36) |
| JF2148  | MATa his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴; pRS426-PPT2 | JF1565 carrying pRS426-PPT2 |
| JF2150  | MATa ypd1Δ::kan skn1Δ::LEU2 his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴; pRS426-PPT2 | ypd1Δ::kan derivative of JF1919; one-step replacement |
| JF2153  | MATa ypd1Δ::kan his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴; pRS426-PPT2 | ypd1Δ::kan derivative of JF1918; one-step replacement |
| JF2219⁸ | MATa sln1-22 ypd1Δ::kan skn1Δ::LEU2 his3Δ200 leu2Δ1 ura3-52 trplΔ1 lys2-201 can⁴ cyr⁴ | ypd1Δ::kan derivative of JF1920; one-step replacement |

* All strains used in this study except JF1455 were created by transformation of FY834 (37) or its can⁴ cyr⁴ derivative JF1565, both of which are isogenic to S288C. The JF1455 strain is congenic with S288C.

*sln1-22* is one of several activating alleles of SLN1 collectively referred to as sln1*⁺* alleles. The sln1-22 mutation causes a change of proline 1148 to serine (5).
**TABLE 2. Plasmids used in this study**

| Plasmid | Description |
|---------|-------------|
| pCLM814 | YEplac112-UAS_SNL1-SLN1-GFP |
| pCLM816 | YCplac112-GFP/YPD1-GFP |
| pCLM136 | pRS425-UAS_SNL1-GFP |
| pCLM138 | pRS425-UAS_SNL1-GFP-SNL7 |
| pCLM144 | pRS316-UAS_YPD1-GFP-C-YPD1 |
| pCLM145 | pRS316-UAS_YPD1-GFP-C-YPD1/H64Q |
| pCLM146 | pRS314-ODH1 (~336 to +20)-lacZ |
| pCLM147 | pRS315-UAS_YPD1-GFP |
| pCLM149 | pRS416-UAS_YPD1-GFP-C-YPD1/H64Q |
| pCLM143 | pRS315-UAS_YPD1-GFP-C-YPD1/H46Q |
| pCLM147 | pRS416-UAS_YPD1-GFP-C-YPD1/SnaX(SSHIS) |
| pCLM148 | pRS415-UAS_YPD1-GFP/C-YPD1-SnaX(SSHIS) |
| pCLM149 | pRS316-UAS_YPD1-GFP-NLS-C-YPD1 |
| pCLM154 | pRS316-UAS_YPD1-GFP-NLS-C-YPD1 |
| pCLM154 | pRS315-UAS_YPD1-GFP-NLS-C-YPD1 |
| pCLM156 | pRS316-UAS_YPD1-GFP-NLS-C-YPD1 |
| pCLM156 | pRS314-UAS_YPD1-GFP-NLS-CYDP1 |
| pCLM157 | pRS313-UAS_YPD1-NLS-CYPD1 |
| pCLM153 | pRS313-UAS_SLP1-GFP-HTBI |

*All plasmids except HOG1-GFP (7) were constructed for this study. See Materials and Methods for construction details. Plasmids used solely as intermediates in various construction schemes are not listed here.*

amplified by using primers GFBamHI-BglII (5'-GGGATCCCTGAGATCT GTGCTAAATGGAATT-3') and GFP Nhel-R (5'-CCGAGTCGAC ATCTTGGATCCATTGACACTACACC-3') was then digested with BamHI and Nhel and cloned to generate pJL1356, in which GFP is fused to the N terminus of the YPD1 ORF. To create GFP*-YPD1, a 1.8-kb fragment containing the YPD1 coding sequence (positions +3 to +1072) was fused via synthetic BamHI and SalI sites downstream of GFP, replacing the YPD1 ORF with GFP-YPD1 in pJL1356 and generating a pRS316-GFP*-YPD1 plasmid called pJL1414. Plasmid pJL1414 was shown to be functional by complementation of the snl1Δ activation defect in J2219 (snl1Δ sod1Δ pda1Δ) and the inviability phenotype of JF2153 (pda1Δ pdr4Δ PTP2) on 5-FOA plates selecting against the presence of the PTP2 plasmid.

The GFP*-SK1 plasmid pJL1456 was constructed by replacing the YPD1 ORF and downstream sequence in pJL1414 with a PCR fragment containing the YPD1 coding region to create pJL1440, which was released from pJF1454 by use of BamHI and Nhel and cloned into pRS315 to create pJL1454 and pJL1461, respectively.

**Localization of the Ypd1p Histidine Phosphotransferase**

**Yeas protein extracts and immunoblot analysis.** Yeast cultures were grown to log phase in selective media. Cells were pelleted and resuspended in 10 mM Tris-50 mM EDTA, washed once in lysin buffer (50 mM Tris, 140 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 1 mM dithiobitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail [Sigma]), pelleted, and frozen at -80°C. The pellets were resuspended in lysin buffer and broken by vigorous vortexing in the presence of 425- to 600-μm diameter glass beads (Sigma), and lysates were prepared by centrifugation and stored at -20°C. The protein concentration was determined by using the Bio-Rad Microassay. GFP protein levels were examined by using rabbit anti-GFP antibody and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antisera (Sigma). Immune complexes were visualized by using the ECL enhanced chemiluminescence kit (Amersham Corp.).

**Fluorescence microscopy.** Log-phase yeast cultures expressing GFP fusions were fixed in 70% ethanol, washed and resuspended in phosphate-buffered saline (PBS), and stained with 0.5 μg of 4',6-diamidino-2-phenylindole (DAPI) (Sigma) per ml to visualize nuclei. Images were processed by using IP-LAB Spectrum software and edited in Adobe Photoshop. For confocal microscopy, log-phase cells were fixed in cold 75% methanol, washed twice in PBS and three times in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]), and treated with DNA-free RNase A (100 μg/ml) (Sigma) for 20 min at 37°C. Samples were then rinsed several times in 2× SSC and incubated with 500 nM propidium iodide (Molecular Probes) prepared in 2× SSC for 5 min at room temperature. Samples were washed five times and resuspended in 2× SSC prior to microscopy with an MRC-600 laser scanning confocal microscope (Bio-Rad).

**Heterokaryon assay.** The heterokaryon assay was modified from published protocols (20, 22). JF2213 (pda1Δ) carrying a pRS426-PTP2 plasmid as well as pJL1514 (pRS313-UAS_SLP1-GFP-NLS-CYPD1) was grown to late log phase in synthetic medium (2% glucose) before dilution into medium containing

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RAS2 + 969 XbaI-R (5'-GCTCTAGACCTAATCTTATAATACGACCCCGCCCGCTCT-3') and
2% raffinose as the sole carbon source. The culture was grown overnight to 10^6 cells/ml, and galactose was added to a final concentration of 2%. The culture was then grown for 3 h, washed three times in YPD (2% glucose), and resuspended in YPD for an additional 2 h of growth. Cells (2 × 10^7) were mixed with an equal number of JF1592 (kar1-1) cells from a YPD-grown log-phase culture. The cell mixture was concentrated on a 25-mm-diameter, 0.45-μm-pore-size nitrocellulose filter and incubated at 28°C for 2 h on solid YPD. Cells were washed by gentle vortexing in a microcentrifuge tube containing 0.5 ml of YPD, collected by brief centrifugation, fixed in cold 80% methanol for 30 min, washed twice in PBS, and resuspended in DAPI (0.5 μg/ml in PBS). The nonshuttling control, JF1565 (wild-type strain) bearing pJL1532 (pRS313-UAS GAL10-GFP-HHTB1), was mated to kar1-1 cells in the same manner.

**RESULTS**

Ypd1p resides in both nuclear and cytosolic compartments. The subcellular localization of Sln1p, Ypd1, Ssk1p, and Skn7p was examined by using GFP fusions on high-copy (Sln1p and Skn7p) or low-copy (Ssk1p and Ypd1p) yeast expression plasmids under the control of the native promoters (with the exception of Ssk1p, which was expressed by using the stronger YPD1 promoter). In all cases, expression of the GFP fusions from these plasmids was able to complement the phenotypes of the corresponding mutants (data not shown). Sln1p-GFP localized to the rim of the cell, consistent with the expected plasma membrane localization of the sensor kinase (Fig. 1A). In contrast, GFP-Skn7p was present exclusively in the nucleus. GFP-Skk1p appears to be localized to the cytoplasm (Fig. 1A); however, the relatively weak intensity of the GFP-Ssk1p signal precludes our ruling out its presence in the nucleus. These observations suggest that phosphotransfer from Ypd1p to the two response regulators occurs in distinct intracellular compartments and that the localization of Ypd1p could be a regulated step in the pathway, as has been previously reported for plants (12).

Analysis of GFP<sub>Δ2</sub>-Ypd1p revealed that the Ypd1p protein is distributed throughout the cytoplasm and in the nucleus under normal growth conditions. There is no evidence of plasma membrane localization, suggesting that its interaction with the plasma membrane-localized Sln1p is transient. Nuclear Ypd1p is seen as a more intense spot of fluorescence that coincides with the DAPI-staining material (Fig. 1A and 2) within the cytoplasm. The presence of Ypd1p in the nucleus was confirmed by confocal microscopy (Fig. 1B). Since two copies of GFP were fused in frame with Ypd1p, the resulting fusion protein was 74 kDa in size, which is too large for passive import into the nucleus. We therefore conclude that the presence of Ypd1p in the nucleus could be due to active transport.

Localization of Ypd1p and Skn7p is unchanged in response to osmotic stress. To determine whether localization of Ypd1p or Skn7p changes in response to fluctuations in osmotic pressure, cells carrying GFP<sub>Δ2</sub>-SKN7 and GFP<sub>Δ2</sub>-YPD1 were shifted to hyperosmotic medium. A HOG1-GFP construct was used as a control, since the rapid translocation of Hog1p to the nucleus upon exposure to a hyperosmotic stimulus has been well studied (7). As previously reported, Hog1p-GFP rapidly moved to the nucleus following salt addition; however, neither the GFP-Skn7p nor GFP-Ypd1p localization profile was altered following exposure of the cells to hyperosmotic conditions (Fig. 2A).

Skn7p undergoes Sln1p-dependent phosphorylation when cells are exposed to hypo-osmotic stress (19, 36). To examine the effect of a hypo-osmotic environment on localization, we
introduced a mutation in the \textit{FPS1} gene, which encodes the major glycerol channel in yeast. Reduced glycerol efflux in the \textit{fps1} mutant results in the accumulation of intracellular glycerol under normal growth conditions, which is interpreted by cells as a hypo-osmotic signal and increases activity of the SLN1-SKN7 pathway (20, 36). However, neither Hog1p, Skn7p, nor Ypd1p localization was affected in the \textit{fps1} mutant (Fig. 2B). The effects of heat shock and oxidative stress, two conditions that activate Skn7p in an \textit{SLN1}-independent fashion (15, 24, 30), were also examined. Again, no apparent changes in localization of Skn7p or Ypd1p were observed under these conditions (data not shown).

The Ypd1p localization profile is independent of phosphorylation. The Snl1p osmotic sensor is an integral membrane protein, and phosphotransfer from Snl1p to Ypd1p occurs on the cytoplasmic surface of the plasma membrane. Ypd1p is an obligatory intermediate in both the \textit{SLN1}-YPD1-SSK1 and \textit{SLN1}-YPD1-SKN7 pathways. The phosphorylation of Ypd1p might therefore play a role in its localization. To test this hypothesis, the histidine (H64) phosphoacceptor of Ypd1p was changed to glutamine. In comparisons of the wild type and the H64Q mutant, no differences were observed in the distribution of Ypd1p (Fig. 3), suggesting that Ypd1p localization to both cytosol and nucleus is not regulated by changes in osmotic pressure or by the phosphorylation state of the histidine.

Increased localization of Ypd1p to the nucleus or to the cytosol has no detectable effect on signaling. To test the hypothesis that movement of Ypd1p between the nucleus and the cytoplasm is important for phosphorylation of the response regulators residing in each compartment, an NLS or NES was added to the \textit{YPD1} construct. The effect of manipulating the localization of Ypd1p on Skn1p phosphorylation was evaluated by looking at the viability of a \textit{YPD1} strain carrying the NLS- or NES-tagged \textit{YPD1} construct and expressing \textit{PTP2} from a high-copy \textit{URA3}-marked plasmid. Phosphorylation of the Skn1p response regulator is essential for viability; however, overexpression of the Hog1p phosphatase gene, \textit{PTP2}, suppresses the phenotype (21, 25, 26). The ability of Ypd1p to phosphorylate Skn1p is conveniently evaluated by testing for growth on medium containing 5-FOA, which selects against the presence of the \textit{URA3}-marked \textit{PTP2} plasmid.

NES tagged \textit{GFP}\textsubscript{NLS}-Ypd1p was predominantly cytoplasmic (Fig. 4A), and a \textit{YPD1} strain carrying this construct was viable in the absence of the \textit{PTP2} plasmid (Fig. 4B). As expected, the NLS-tagged \textit{GFP}\textsubscript{NLS}-Ypd1p was predominantly nuclear (Fig. 4A). Surprisingly, nuclear localization of Ypd1p had no adverse effect on viability (Fig. 4B). This implies that Skn1p was phosphorylated despite the apparent nuclear localization of Ypd1p.

The ability of the NES- and NLS-tagged \textit{GFP}\textsubscript{NES}-Ypd1p to phosphorylate Skn7p was evaluated by measuring the activity of the \textit{SLN1}-SKN7-dependent target gene reporter, \textit{OCH1-lacZ} (19). Activating mutations in the \textit{SLN1} gene (called \textit{sln1}\textsuperscript{*} mutations) increase \textit{OCH1-lacZ} expression 2.5- to 3-fold ((19) Fig. 4C), and \textit{sln1}\textsuperscript{*} activation is dependent on Skn7p phosphorylation, since mutation of the phosphorylated aspartate residue in the \textit{SKN7} receiver domain abolishes SKN7-dependent activation of the reporter (19). \textit{sln1}\textsuperscript{*} activation also depends on phosphotransfer from Ypd1p to Skn7p (18). In the presence of NLS-tagged Ypd1p, both Ypd1p and Skn7p are present in the nucleus, and therefore \textit{sln1}\textsuperscript{*} activation of the \textit{OCH1-lacZ} reporter was normal, as expected (Fig. 4C). In the presence of the NES-tagged Ypd1p, which we found to be largely cytoplasmic, \textit{sln1}\textsuperscript{*} activation was expected to be defective; however, no measurable decline in activation of a \textit{SKN7}-dependent target gene was observed (Fig. 4C).

\textbf{Tethering Ypd1p to the plasma membrane prevents signaling to Skn7p.} The absence of observable effects of the NES and NLS Ypd1p tags on signaling might suggest that phosphotransfer between Ypd1p and the Skn1p and Skn7p response regulators is not limited to any particular subcellular compartment. Alternatively, the results might reflect the presence of efficient native trafficking signals that allow Ypd1p to circulate in and out of the nucleus. To better discriminate between these possibilities, we tethered Ypd1p to the plasma membrane by using the \textit{CaaX} box prenylation motif from Ras2p. Sequences containing a \textit{CaaX} box prenylation motif were fused to the 3’ end of GFP-\textit{YPD1}. A control construct (\textit{SaaX}) in which the \textit{CaaX} box cysteines were changed to serine, thus preventing plasma membrane localization, was also generated. Western analysis showed that each Ypd1p derivative was expressed at levels roughly comparable to that of the wild type (Fig. 5B, rightmost panel [data not shown for \textit{SaaX} construct]). As expected, Ypd1p-\textit{CaaX}, but not Ypd1p-\textit{SaaX}, was partially localized to the plasma membrane. Some cytoplasmic staining is still apparent in the Ypd1p-\textit{CaaX} strain, possibly reflecting trafficking intermediates (Fig. 5A). The ability of each construct to phosphorylate \textit{Ssk1p} was assessed by examining viability. Strains carrying Ypd1p-\textit{CaaX} or Ypd1p-\textit{SaaX} were both viable, suggesting that tethering Ypd1p to the plasma membrane does not inhibit phosphotransfer to \textit{Ssk1p} (Fig. 5B). A more quantitative analysis of Ypd1p phosphotransfer to \textit{Ssk1p} was carried out by examining the effect of the \textit{CaaX} and \textit{SaaX} mutants on expression of the \textit{HOG1} pathway target, \textit{GPD1}, after a shift to high-salt medium. The \textit{CaaX} mutant exhibited 75% of wild-type induction, while the \textit{SaaX} control was nearly normal at 94% (data not shown). Thus, the effect of Ypd1p tethering on \textit{SLN1}-SSK1 pathway activity was minimal.

The ability of tethered Ypd1p to signal to nuclear Skn7p was
examined by assessing the effect of Ypd1p-CaaX on sln1\(^*\) activation. As seen previously, wild-type YPD1 exhibited threefold activation in this assay (Fig. 5C), whereas the ydp1 H64Q mutant exhibited no activation. The ydp1-CaaX allele resembled the H64Q mutant in this assay, exhibiting no sln1\(^*\) activation. In contrast, the YPD1-SaaX construct was nearly fully active. These results indicate that nuclear localization of Ypd1p is necessary for signaling to Skn7p.

**NLS-Ypd1p is capable of nucleo-cytoplasmic shuttling.** The finding that nuclear targeting of Ypd1p is essential for Skn7p activation together with the lack of apparent effect of NLS and NES Ypd1p tags on signaling activity led us to propose that Ypd1p may shuttle between compartments to regulate Ssk1p and Skn7p. This was tested by using a heterokaryon assay (8).

In this assay, a kar1 mutant defective in nuclear fusion is mated with cells carrying a transcriptionally regulated NLS-tagged GFP-Ypd1 fusion protein. If the GFP fusion is capable of nuclear export, it will move out of the KARI nuclei and into the kar1 nucleus, causing GFP staining in both nuclei of the heterokaryon. The nuclear-localized NLS-tagged GFP-YPD1 allele was placed under the control of the galactose-inducible GAL10 promoter and transformed into a ypd1\(\Delta\) strain kept alive by a PTP2 overexpression plasmid. Expression of the NLS-tagged GFP-YPD1 construct was highly inducible by galactose and was repressed within an hour of switching to glucose (Fig. 6B). Following mating with the kar1-1 strain, the
majority of heterokaryons showed GFP fluorescence in both nuclei, indicating nuclear export of Ypd1p (Fig. 6A). In contrast, fluorescence from a GFP fusion with the yeast histone H2B gene (GFP-H2B) was restricted to a single nucleus in control heterokaryons (Fig. 6A). These data strongly support the conclusion that Ypd1p moves between the nucleus and the cytoplasm.

DISCUSSION

Eukaryotic two-component pathways share many features with the more prevalent bacterial two-component pathway. However, some accommodations are assumed to be necessary due to the spatial constraints imposed by the compartmentalized intracellular environment. In this study we have examined the subcellular localization of the two-component signaling molecules in the yeast SLN1 pathway, and we conclude that the distribution of the histidine phosphotransfer protein, Ypd1p, in both the cytosol and the nucleus is one strategy by which eukaryotic two-component pathways have adapted to a compartmentalized environment.

The SLN1 pathway bifurcates downstream of the histidine phosphotransferase, Ypd1p, and culminates in the phosphorylation of two very different types of response regulators. Phosphorylation of the cytosolic response regulator, Ssk1p, is essential for viability, whereas phosphorylation of the nuclear response regulator, Skn7p, is not. Thus, the existence of separate nuclear and cytosolic pools of Ypd1p may be a reflection of the need to retain a pool of cytoplasmic Ypd1p even under conditions that call for Skn7p phosphorylation.

Under normal conditions, the small size of Ypd1p (19.2 kDa) may allow diffusion in and out of the nucleus. However, fusion of Ypd1p to two tandem copies of GFP did not alter the...
pancellular distribution, suggesting the possible involvement of more active translocation mechanisms. When strong heterologous NLS and NES tags were added to the protein, the localization profile became nuclear in the case of the NLS fusion and cytoplasmic in the case of the NES fusion. These results suggest that any localization signals embedded within the Ypd1p sequence or present within proteins that normally interact with Ypd1p are relatively weak or that the strength of the intrinsic import and export signals are perfectly balanced, giving an even distribution of the protein both inside and outside the nucleus.

Even in the presence of strong NLS and NES signals, Ypd1p levels in the nucleus and cytosol were sufficient to sustain near-normal levels of signaling in both compartments. This suggests that very little Ypd1p is needed in each compartment or that the protein is very efficiently recruited to the appropriate signalosome. We used a heterokaryon assay to validate the conclusion that Ypd1p is capable of moving out of the nucleus in spite of the NLS tag. Heterokaryon assays were used first to demonstrate nuclear-to-cytoplasmic protein movement for Npl3p (8) and later to show nucleocytoplasmic shuttling of Gal80p (27) and Los1p (6). We conclude that native Ypd1p is fully capable of trafficking into and out of the nucleus to accomplish its dual signaling missions.

Although signaling to Ssk1p, as measured by viability and, quantitatively, by induction of a HOG1-dependent target gene, was not strongly affected when Ypd1p was plasma membrane associated, signaling to Skn7p was eliminated, presumably by curtailing the transit of Ypd1p to the nucleus. This is consistent with the view that Skn7p has a strict nuclear localization and that Ypd1p must enter the nucleus to carry out phosphorylation with the Skn7p response regulator.

In our current working model for the spatial organization of the yeast two-component pathway molecules (Fig. 7), SLN1 pathway signaling is regulated by the intracellular shuttling of the phosphorelay intermediate Ypd1p and by the differential compartmentalization of the cytoplasmic Ssk1p and the nuclear Skn7p response regulators. In step one (phosphotransfer) of the pathway, plasma membrane-associated Sln1p transfers a phosphoryl group to the cytoplasmic Ypd1p. In step two (shuttling), phospho-Ypd1p translocates into the nucleus. In step three (phosphorylation), Ypd1p-P phosphorylates Skn7p in the nucleus as well as Ssk1p in the cytoplasm. This model is
supported by several lines of evidence. First, localization studies show the presence of Ypd1p in both the cytoplasm and the nucleus. Second, the fact that alteration of Ypd1p levels in either compartment by addition of NES or NLS tags had a minimal impact on signaling to Ssk1p or Skn7p suggests that even under these conditions Ypd1p is not confined to one compartment. Third, heterokaryon assays show that NLS- tagged Ypd1, and presumably native Ypd1p, is indeed capable of nuclear export. Finally, since tethering of Ypd1p to the plasma membrane via a CaaX box caused a severe impairment in Skn7p signaling, we conclude that Skn7p is restricted to the nucleus and that its activation requires nuclear targeting of Ypd1p. The temporal relationship of these events is not clear. Since the intracellular distribution of the nonphosphorylatable Ypd1p H64Q is indistinguishable from that of normal Ypd1p, it is possible that both phospho and dephospho forms of Ypd1p move in and out the nucleus. However, there is no known role for dephospho-Ypd1p in the nucleus.

An alternative model in which Skp1p is phosphorylated inside the nucleus before moving out to the cytoplasm to regulate the HOG1 pathway seems unlikely, since Skp1p appears to be cytoplasmically localized under all osmotic conditions tested. This localization pattern is consistent with the known cytoplasmic localization of the MAP kinase kinase, Pbs2p (7), which is activated by interaction with Skp1p, and with the observation that the MAP kinase, Hog1p, translocates to the nucleus upon osmotic treatment. In addition, both the NES- Ypd1p and plasma membrane-tethered Ypd1p-CaaX exhibited near-normal signaling to Skp1p, indicating that nuclear Ypd1p is not required for Skp1p signaling.

Recruitment of cytoplasmic signaling proteins into the nucleus is an essential step in the activation of gene expression in response to extracellular signals. In MAP kinase pathways, it is frequently the MAP kinase that transits to the nucleus. In eukaryotic two-component signaling, it appears that independent histidine phosphotransferase (Hpt) proteins such as Ypd1p may have evolved for this purpose. In Arabidopsis thaliana, AHP1 to -5 encode homologs of Ypd1p that function in the cytokinin signal transduction pathway (12, 34, 35). A subset of AHP molecules are translocated into the nucleus in a cytokinin-dependent manner, where they can signal to a nuclear family of response regulators (11). The regulated transport of the AHPs in Arabidopsis and the constitutive cycling of yeast Ypd1p into and out of the nucleus appear to represent distinct mechanisms for two-component signaling in compartmentalized cells. The basis for the difference between the two systems may lie with the tolerance and/or requirement of the cell for expression of pathway targets. For example, expression of cytokinin genes in the absence of cytokinins may be harmful, whereas modest expression of SLN1-SKN7 target genes such as OCH1, encoding a mannosyltransferase, is likely to be important even in the absence of stress. It will be of interest to examine the localization of molecules in other eukaryotic two-component pathways to determine whether Hpt-based localization is a general principle in eukaryotic two-component signaling pathways.

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