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Phosphorelay-Regulated Degradation of the Yeast Ssk1p Response Regulator by the Ubiquitin-Proteasome System

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In *Saccharomyces cerevisiae*, a phosphorelay signal transduction pathway composed of Sln1p, Ypd1p, and Ssk1p, which are homologous to bacterial two-component signal transducers, is involved in the osmosensing mechanism. In response to high osmolarity, the phosphorelay system is inactivated and Ssk1p remains unphosphorylated. Unphosphorylated Ssk1p binds to and activates the Ssk2p mitogen-activated protein (MAP) kinase kinase kinase, which in turn activates the downstream components of the high-osmolarity glycerol response (HOG) MAP kinase cascade. Here, we report a novel inactivation mechanism for Ssk1p involving degradation by the ubiquitin-proteasome system. Degradation is regulated by the phosphotransfer from Ypd1p to Ssk1p, insofar as unphosphorylated Ssk1p is degraded more rapidly than phosphorylated Ssk1p. Ubc7p/Qri8p, an endoplasmic reticulum-associated ubiquitin-conjugating enzyme, is involved in the phosphorelay-regulated degradation of Ssk1p. In *ubc7*Δ cells in which the degradation is hampered, the dephosphorylation and/or inactivation process of the Hog1p MAP kinase is delayed compared with wild-type cells after the hyperosmotic treatment. Our results indicate that unphosphorylated Ssk1p is selectively degraded by the Ubc7p-dependent ubiquitin-proteasome system and that this mechanism downregulates the HOG pathway after the completion of the osmotic adaptation.

In eukaryotic cells, the mitogen-activated protein kinase (MAPK) cascades are activated by a variety of external stimuli such as growth factors, cytokines, UV irradiation, and osmotic stress. In the yeast *Saccharomyces cerevisiae*, the high-osmolarity glycerol (HOG) response MAPK pathway is activated by increased external osmolarity (Fig. 1) (6, 18, 20, 49, 54). An upstream His-Asp phosphorelay signaling system, which is homologous to bacterial two-component systems, regulates the downstream HOG MAPK cascade (38, 45, 50). Under normal osmolarity conditions, Sln1p, a membrane-associated histidine kinase, phosphorylates a histidine residue in itself. The phosphate group is in turn transferred to an aspartic acid residue in its receiver domain. Subsequently, the phosphate group is transferred to the Ypd1p phosphotransmitter and then to the receiver domain of the Ssk1p response regulator. High osmolarity inactivates Sln1p and thus lowers the phosphorylation level of Ssk1p (26, 27, 38, 44, 50). Unphosphorylated Ssk1p binds to the Ssk2p, and perhaps the Ssk22p, MAPK kinase kinases (MAPKKKs) through its receiver domain and activates them (48). Activated Ssk2p phosphorylates and activates the Pbs2p MAPK kinase, which in turn phosphorylates and activates the Hog1p MAPK (36, 48, 49). Phosphorylated Hog1p activates the transcription of genes required in the response to high osmolarity (1, 12, 53). Deletion of *SLN1* or *YPD1* induces constitutive activation of the HOG pathway as the consequence of failure in phosphorylating Ssk1p (38, 50). In addition to Ssk1p, *S. cerevisiae* has another response regulator, Skn7p, that is also phosphorylated via the Sln1p-Ypd1p phosphorelay (8, 29, 32). Skn7p has a DNA-binding domain homologous to heat shock transcription factors, as well as a receiver domain, and in fact acts as a transcription factor involved in multiple physiological processes, such as maintenance of cell wall integrity, cell cycle regulation, and oxidative stress response (4, 7, 8, 31, 39, 40). Under hypo-osmotic conditions, the receiver domain of Skn7p remains as phosphorylated as that of Ssk1p, and induction of the target genes, including *OCH1*, is dependent on the phosphorylation (7, 32, 33, 63). In contrast, induction of the target genes for oxidative stress response is independent of the phosphorylation (31). Skn7p is not implicated in the regulation of the HOG pathway except for some marginal genetic interaction (29).

In the absence of osmotic stress, or once the adaptation process to high osmolarity is completed, the HOG pathway is negatively regulated by protein phosphatases. Upon activation of Hog1p, a tyrosine residue and a threonine residue in the activation loop are phosphorylated by Pbs2p (6, 25, 37, 38, 64, 66). Hog1p is inactivated by dephosphorylation of the tyrosine residue by the tyrosine phosphatases Ptp2p and Ptp3p (25, 37, 66) or of the threonine residue by the type 2C serine-threonine protein phosphatase Ptc1p (37, 64). The dephosphorylation of constituent kinases by protein phosphatases is a common mechanism for the negative regulation of MAPK cascades. On the other hand, recent reports have shown that the ubiquitin-proteasome system also negatively regulates MAPK cascades. The human extracellular signal-regulated protein kinase (ERK) pathway is downregulated by the degradation of the ERK MAPKs (35), and the yeast pheromone-induced MAPK cascade is downregulated by the degradation of the Ste11p MAPKKK (14).

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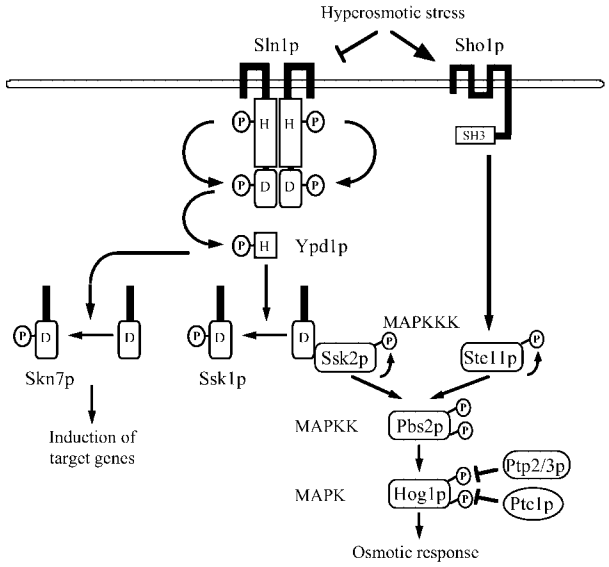


FIG. 1. The osmosensing signal transduction pathway in yeast. Hyperosmotic stress inhibits the phosphorelay system initiated from the Sln1p membrane-associated histidine kinase, which functions as a homodimer, to cause reduced levels of phosphorylation of Ypd1p and the response regulator Ssk1p. Unphosphorylated Ssk1p activates the Ssk2p MAPKKK, which in turn activates the downstream HOG MAPK cascade. Skn7p is another response regulator phosphorylated by the phosphorelay system, which is a transcriptional factor. Another sensor system that is dependent on the membrane anchor Sho1p also functions, in which Pbs2p is activated by Ste11p (36, 47, 51, 52). Msb2p is also implicated in osmosensing (not shown) (43). MAPKK, MAPK kinase.

Here, we report that the yeast osmosensing pathway is also downregulated by the ubiquitin-proteasome system. In this case, the Ssk1p response regulator is the target for degradation. Unphosphorylated Ssk1p is selectively degraded, and Ubc7p/Qri8p, a ubiquitin-conjugating enzyme (E2) implicated in endoplasmic reticulum (ER)-associated degradation (ERAD) (3, 10, 15, 21), is involved in the degradation. This mechanism downregulates the HOG pathway after the completion of the osmotic adaptation. We propose that phosphorelay-regulated degradation of response regulators can be a general regulatory mechanism of eukaryotic His-Asp phosphorelay signaling systems.

MATERIALS AND METHODS

Bacterial and yeast methods. Standard *Escherichia coli* and yeast manipulations were performed as described previously (2). *E. coli* strain XL10-Gold (Stratagene, San Diego, Calif.) was used for plasmid propagation. SGal and SRaf media are modified synthetic complete media with dextrose (SD media) in which glucose is replaced by galactose and raffinose, respectively.

Yeast strains. The yeast strains used in this study are listed in Table 1. *UBC7* was disrupted by transformation with pRH1186 (17), *SKN7* was disrupted by transformation with pNS396 (see below), and *RPN9* was disrupted by transformation with pJUN180 (61).

Plasmids. The plasmids used in this study are listed in Table 2.

To induce the C-terminally epitope-tagged *SSK1* from the galactose-inducible GAL1 promoter, pNS252, pNS114, or pNS462 was used. A three-hemagglutinin (HA)–six-His composite epitope tag was fused to the C terminus of Ssk1p as follows. A *Sal*I site was introduced just before the termination codon of *SSK1* by PCR. The 2.2-kb *Cla*I-*Sal*I fragment covering the *SSK1* open reading frame (ORF) and the 0.2-kb *Sal*I-*Asc*I (blunted) fragment containing a three-HA–six-His composite epitope tag derived from pFA6a-3HA-6His-His3MX6 (T. Maeda, unpublished data), which has the three-HA–six-His tag in the common pFA6a-His3MX6 backbone (34), were cloned into the *Cla*I and *Hin*cII sites of p424GAL1 (41) to produce pNS252. The 0.7-kb *Sal*I-*Sma*I fragment containing the GAL1 promoter from pGSS1 (38), the 2.2-kb *Cla*I (blunted)-*Sal*I fragment described above, and the 0.4-kb *Sal*I-*Sac*I fragment containing the three-HA–six-His tag and the alcohol dehydrogenase terminator derived from pFA6a-3HA-6His-His3MX6 were cloned into the *Sal*I and *Sac*I sites of pRS414 (58) to produce pNS114. pNS462 is essentially the same as pNS114 except that the parental vector is pRS416 (58). pNS476 is essentially the same as pFP57 (50) except that the insert is wild-type *YPD1*.

To induce N-terminally three-FLAG-tagged *SSK1* from the GAL1 promoter, pTB426 was constructed as follows. An *Eco*RI site was introduced just in front of the initiation codon of *SSK1* by PCR. The 2.6-kb *Eco*RI-*Bst*XI segment covering the entire ORF and the 3′ flanking region of *SSK1*, together with a vector-derived linker sequence adjacent to the *Bst*XI site, was isolated as an *Eco*RI fragment. The *Eco*RI fragment was cloned into the *Eco*RI site of pcDNA3.1/N-3×FLAG (T. Maeda, unpublished), which has a three-FLAG tag in the common pcDNA3.1(+) backbone (Invitrogen Corp., Carlsbad, Calif.), to produce pTB422. The 2.7-kb *Hin*dIII-*Xho*I fragment, containing the N-terminally three-FLAG-tagged ORF and the 3′ flanking region of *SSK1*, was cloned into the *Hin*dIII and *Xho*I sites of p416GAL1 (41) to produce pTB426.

To express C-terminally FLAG-tagged *SSK1*, pNS454 was constructed as follows. The 2.2-kb *Cla*I-*Sma*I fragment containing the *SSK1* ORF from pNS252 and the 0.1-kb *Bam*HI (blunted)-*Apa*I (blunted) fragment containing a single FLAG epitope from pcDNA3-FLAG were cloned into the *Cla*I and *Hin*cII sites of p426TEF (42) to produce pNS454. The ubiquitin-like (UBL) domain of Ssk1p

TABLE 1. Yeast strains used in this study

Strain	Relevant genotype	Source or reference
TM141	*MAT***a** *leu2-Δ1 his3-Δ200 trp1-Δ63 ura3-52*	38
NS318	Same as TM141 except *ssk1*::*HIS3 hog1*::*kanMX6*	This study
NS320	Same as TM141 except *ypd1*::*LEU2 ssk1*::*HIS3 hog1*::*kanMX6*	This study
NS325	Same as TM141 except *skn7*::*TRP1 ssk1*::*HIS3 hog1*::*kanMX6*	This study
NS327	Same as TM141 except *ubc7*::*LEU2*	This study
NS329	Same as TM141 except *ubc7*::*LEU2 ssk1*::*HIS3 hog1*::*kanMX6*	This study
NS333	Same as TM141 except *ubc7*::*LEU2 ypd1*::*URA3 ssk1*::*HIS3 hog1*::*kanMX6*	This study
NS336	Same as TM141 except *rpn9*::*LEU2 ypd1*::*URA3 ssk1*::*HIS3 hog1*::*kanMX6*	This study
NS337	Same as TM141 except *rpn9*::*LEU2 ssk1*::*HIS3 hog1*::*kanMX6*	This study
NS354	Same as TM141 except *ssk1*::*HIS3 ssk2*::*TRP1 hog1*::*kanMX6*	This study
NS355	Same as TM141 except *ypd1*::*LEU2 ssk1*::*HIS3 ssk2*::*TRP1 hog1*::*kanMX6*	This study
TM107	*MAT***a** *leu2-Δ1 trp1-Δ63 ura3-52 ptp2*::*LEU2*	37
NS358	Same as TM107 except *ubc7*::*TRP1*	This study
NS362	Same as TM107 except *ubc7*::*TRP1 hog1*::*kanMX6*	This study
W303-1A	*MAT***a** *leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-101 can1-100*	61
J43	Same as W303-1A except *rpn9*::*LEU2*	61
YK109	Same as W303-1A except *rpn12-1*	30

TABLE 2. Plasmids used in this study

Plasmid	Description[a]	Source or reference
p416GAL1	P_{GAL1} *URA3 CEN*	41
p424GAL1	P_{GAL1} *TRP1* 2μm	41
p426TEF	P_{TEF} *URA3* 2μm	42
p426GPD	P_{GPD} *URA3* 2μm	42
pRS414	*TRP1 CEN*	58
pRS416	*URA3 CEN*	58
pRS424	*TRP1* 2μm	11
pFA6a-3HA-6His-His3MX6	3HA-6His-T_{ADH} *His3MX6*	T. Maeda, unpublished
pFA6a-TRP1	*TRP1*	34
pRH1186	pBluescript KSII *ubc7*::*LEU2*	17
pJUN180	pBluescript KS(−) *rpn9*::*LEU2*	61
pRS315-SKN7	*SKN7 LEU2 CEN*	8
pNS396	pBluescript KS(+) *skn7*::*TRP1*	This study
pNS252	p424GAL1 *SSK1-3HA-6His*	This study
pNS114	pRS414 P_{GAL1}-*SSK1-3HA-6His*	This study
pNS476	pRS416 *YPD1*	This study
pFP57	pRS416 *ypd1* (*H64Q*)	50
pcDNA3.1/N-3×FLAG	*3×FLAG*	T. Maeda, unpublished
pTB422	pcDNA3.1 *3×FLAG-SSK1*	This study
pTB426	p416GAL1 *3×FLAG-SSK1*	This study
pNS462	pRS416 P_{GAL1}-*SSK1-3HA-6His*	This study
pcDNA3-FLAG	*FLAG*	Gift from M. Kawabata
pNS454	p426TEF *SSK1-FLAG*	This study
pNS456	p426GPD *SSK1ΔUBL-FLAG*	This study
pNS473	pRS416 P_{GAL1}-*SSK1*	This study
pHG11	pRS424 *HOG1*	38

[a] P_{GAL1}, P_{TEF}, and P_{GPD} are, respectively, GAL1, TEF, and GPD promoters. T_{ADH} is an alcohol dehydrogenase terminator.

was deleted as follows. An *Eco*RV restriction site was introduced at nucleotides 709 to 714 of the *SSK1* ORF (mutated from GAA TCA to GAT ATC) by PCR. The 0.7-kb *Cla*I-*Eco*RV fragment containing the 5′ portion of *SSK1*, the 1.3-kb *Eco*RV-*Sma*I fragment containing the 3′ portion of *SSK1* from pNS358, and the 0.1-kb *Bam*HI (blunted)-*Apa*I (blunted) fragment described above were cloned into the *Cla*I and *Hin*cII sites of p426GPD (42) to produce pNS456 containing *SSK1ΔUBL* (amino acids 243 to 290 were deleted).

pNS396, the plasmid used for the deletion of *SKN7*, was constructed as follows. The 3.5-kb *Sma*I-*Xba*I fragment containing the *SKN7* locus from pRS315-SKN7 (8) was cloned into the *Hin*cII and *Xba*I sites of pBluescript KS(+). The 1.5-kb *Pst*I-*Hin*cII fragment within the *SKN7* ORF was then replaced with the 1.0-kb *Pst*I-*Pme*I fragment containing the *TRP1* cassette from pFA6a-TRP1 (34).

Detection of epitope-tagged Ssk1p. Cells expressing tagged *SSK1* were cultured in SD medium and then transferred to SRaf medium. Cells were grown to an optical density at 600 nm of ~0.8, and galactose was added to the medium to a final concentration of 2%. After 3 h, cells were transferred to SD medium containing cycloheximide (0.5 mg/ml). Aliquots were collected at the indicated times and centrifuged. Cell pellets were suspended in the Laemmli sample buffer and boiled for 5 min. Proteins were resolved by sodium dodecyl sulfate–7 to 10% polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane, and the blot was incubated with the mouse anti-HA monoclonal antibody 12CA5, peroxidase-conjugated sheep anti-mouse immunoglobulin (Amersham Biosciences, Piscataway, N.J.), and then enhanced chemiluminescence detection reagent (Amersham Biosciences). Actin was detected with the mouse anti-actin monoclonal antibody C4 (ICN Biomedicals, Costa Mesa, Calif.). In some experiments (see Fig. 3B), the primary antibody was replaced with the mouse anti-FLAG monoclonal antibody M2 (Sigma, St. Louis, Mo.).

Coimmunoprecipitation of Ssk1p with the proteasome. NS318 and NS320 strains expressing *SSK1* (wild type or Δ*UBL*)-*FLAG* were grown to an optical density at 600 nm of ~1.0. Cells were collected by centrifugation, suspended in lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 3 mM phenylmethylsulfonyl fluoride, 40 μg of aprotinin/ml, 10 μg of pepstatin A/ml, 20 μg of leupeptin/ml, 0.1 mM MG-132), and mixed with an equal volume of glass beads (Sigma). After being vortexed 10 times with 30-s pulses, samples were centrifuged at 17,000 × *g* for 15 min at 4°C. The supernatant was then incubated with anti-FLAG M2-agarose beads (Sigma) for 4 h at 4°C. Beads were collected and washed twice with lysis buffer and then suspended in Laemmli sample buffer and boiled for 5 min. Western blotting was performed essentially as described above, with 12CA5, M2, and rabbit anti-20S proteasome core subunit polyclonal antibody (62).

Detection of phosphorylated Hog1p. TM141 and NS327 strains expressing multicopy *HOG1* from pHG11 (38) were grown overnight in SD medium without sorbitol. Cells were transferred to yeast extract-peptone-dextrose medium without sorbitol and grown for 2.5 h to an optical density at 600 nm of ~0.8. Cells were then resuspended in yeast extract-peptone-dextrose medium containing 1.5 M sorbitol and collected at the indicated times. Cell pellets were suspended in Laemmli sample buffer containing 1 mM sodium vanadate and boiled for 5 min. Cell extract was prepared with glass beads as described above. Western blotting was performed essentially as described above, with rabbit polyclonal anti-phospho-p38 antibody (New England Biolabs, Beverly, Mass.) and the goat polyclonal anti-Hog1p antibody yC-20 (Santa Cruz Biotechnology, Santa Cruz, Calif.).

RESULTS

Ssk1p is stabilized in proteasome mutants. A recent paper on the high-throughput mass spectrometric protein complex identification applied to *S. cerevisiae* (22) reported that Ssk1p binds to the Ssk2p MAPKKK, Ubi4p (ubiquitin), and Ubc7p. Ubc7p is a ubiquitin-conjugating enzyme (E2) responsible for ERAD, which involves the ubiquitination of misfolded proteins retrotranslocated from the ER to the cytoplasm (15, 21, 46). Ubc7p also functions in the regulatory degradation of the following proteins: Hmg2p, a yeast isozyme of the cholesterol-biosynthesis enzyme HMG-coenzyme A reductase (3, 17, 19); Matα2p, a transcriptional repressor (10, 60); and Ole1p, an ER-bound Δ-9 fatty-acid desaturase (5).

This discovery prompted us to investigate the relationship between Ssk1p and the ubiquitin-proteasome system. We examined the stability of Ssk1p in *rpn9*Δ (61) and *rpn12-1* (30) mutants, which are defective in a 19S regulatory subunit of the proteasome. C-terminally epitope-tagged Ssk1p, which had

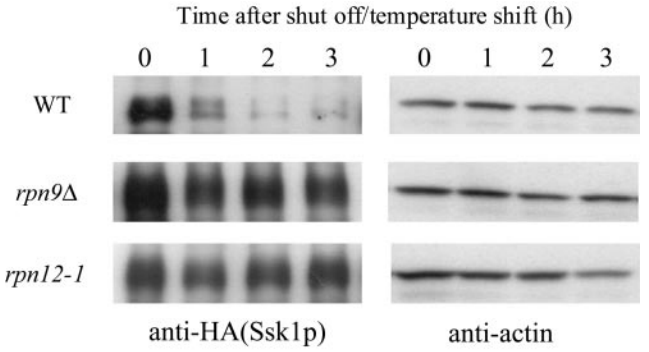


FIG. 2. Ssk1p is stabilized in proteasome mutants. HA-tagged Ssk1p was expressed from the high-copy-number plasmid pNS252 in the wild-type (WT) (W303-1A), *rpn9*Δ (J43), and *rpn12-1* (YK109) strains cultured in SGal medium at 30°C. Transcription and translation were shut off in SD medium containing cycloheximide at 37°C. Cells were collected at the indicated times, the proteins were extracted, and the Ssk1p content was monitored by Western blotting with anti-HA antibody. Actin was detected as the loading control.

been shown to complement the deletion of *SSK1* (data not shown), was expressed under the GAL1 promoter in wild-type (W303-1A), *rpn9*Δ, and *rpn12-1* strains cultured in SGal medium at 30°C, the permissive temperature. Then the GAL1 promoter was shut off in SD medium, and novel protein synthesis was inhibited by the addition of cycloheximide to the medium. Simultaneously, cells were shifted to 37°C, the nonpermissive temperature, and sampled every hour. The Ssk1p content was monitored by Western blotting. In wild-type cells, Ssk1p content constantly decreased over time, whereas Ssk1p was more stable in *rpn9*Δ and in *rpn12-1* cells than in wild-type cells (Fig. 2). These results indicate that Ssk1p is degraded by the activity of the proteasome system.

The stability of Ssk1p is dependent on both the phosphotransfer from Ypd1p and the proteasome system. In the absence of hyperosmotic stress, Ypd1p phosphorylates and inactivates Ssk1p. Deletion of *YPD1* keeps Ssk1p constitutively unphosphorylated (50). To determine whether the phosphorylation state of Ssk1p affects its stability, we examined the stability of Ssk1p in strains in which *YPD1* was intact or deleted. To circumvent the lethality of *ypd1*Δ, the experiments were performed on a *hog1*Δ background. C-terminally tagged Ssk1p was expressed in both *YPD1*$^+$ and *ypd1*Δ cells, and Ssk1p content was monitored by Western blotting as described above, except that *SSK1* was expressed from a low-copy-number plasmid and the cells were kept at 30°C throughout the experiment. In *ypd1*Δ cells, in which Ssk1p was unphosphorylated, the Ssk1p content decreased more rapidly than in *YPD1*$^+$ cells, in which Ssk1p was kept phosphorylated (Fig. 3A). These results indicate that unphosphorylated Ssk1p is less stable than phosphorylated Ssk1p. The instability of Ssk1p in *ypd1*Δ cells was almost negated by the deletion of *RPN9* (Fig. 3A). This observation suggests that the instability of unphosphorylated Ssk1p is caused by its preferred degradation by the proteasome system. Alternatively tagged Ssk1p, with a different epitope tag at the other terminus, was also degraded in a phosphorelay-regulated manner as was the C-terminally tagged Ssk1p used in the experiments thus far, which indicates that the degradation was unlikely to be affected by the epitope tagging (Fig. 3B). Thus, we used the C-terminally tagged version in the following experiments.

The possibility was tested that the instability of Ssk1p in *ypd1*Δ cells was caused by phosphorelay-independent effects of Ypd1p deficiency, such as the loss of a physical and stabilizing complex between Ypd1p and Ssk1p. In *ypd1*Δ cells, expression of mutant Ypd1^{H64Q}p, which is incapable of phosphotransfer but expected to be able to interact with Ssk1p (26, 50), did not significantly stabilize Ssk1p, while wild-type Ypd1p did (Fig. 3C). These results suggest that the phosphorylation state of Ssk1p itself is the determinant of the stability.

Instability of unphosphorylated Ssk1p is independent of the *SKN7* pathway. In *ypd1*Δ cells, not only is Ssk1p unphosphorylated and/or activated but Skn7p is also unphosphorylated and/or inactivated (8, 29, 32) (Fig. 1). To test the possible involvement of the *SKN7* pathway in Ssk1p stability, we examined the stability of Ssk1p in strains with intact or deleted *SKN7*. The deletion of *SKN7* did not decrease the stability of Ssk1p in *YPD1*$^+$ cells (Fig. 3D), indicating that Ssk1p instability in *ypd1*Δ cells is not caused by the inactivation of the *SKN7* pathway. Conversely, Ssk1p was marginally, but reproducibly, more stable in *skn7*Δ cells than in *SKN7*$^+$ cells. This may be due to increased phosphorylation of Ssk1p caused by the absence of Skn7p, which potentially competes with Ssk1p for phosphotransfer from Ypd1p.

Ubc7p is involved in the degradation of unphosphorylated Ssk1p. In light of the results of high-throughput mass spectrometric protein complex identification applied to *S. cerevisiae* as mentioned above (22), Ubc7p was expected to be involved in the degradation of Ssk1p. To test the possibility, we examined the stability of Ssk1p in *ubc7*Δ cells. In *YPD1*$^+$ cells, deletion of *UBC7* did not significantly affect the stability of Ssk1p. In *ypd1*Δ cells, on the other hand, disruption of *UBC7* stabilized Ssk1p cells in comparison to *UBC7*$^+$ cells (Fig. 4). These results indicate that unphosphorylated Ssk1p is selectively degraded by the Ubc7p-dependent degradation pathway.

Ssk2p-independent degradation of Ssk1p. To examine the possible involvement of the Ssk1p-Ssk2p interaction in the regulation of the Ssk1p stability, the level of Ssk1p was monitored in *ssk2*Δ cells. Both in *YPD1*$^+$ and *ypd1*Δ cells, deletion of *SSK2* did not significantly affect the stability of Ssk1p (Fig. 5). These results indicate that the Ssk1p-Ssk2p interaction is not required for the degradation of Ssk1p.

The UBL domain of Ssk1p is not required for the interaction with the proteasome. We found a UBL domain in the N-terminal region (amino acids 243 to 290) of Ssk1p (Fig. 6A). Recent reports showed that the UBL domains of Rad23p and Dsk2p bind to Rpn1p, a component of the base complex of the 26S proteasome (13, 16, 56, 65). Thus, we examined the interaction between the UBL domain of Ssk1p and the proteasome. Wild-type or UBL domain-deleted Ssk1p (Ssk1$^{\Delta UBL}$p) fused to a FLAG tag was expressed in both *YPD1*$^+$ and *ypd1*Δ cells. When expressed from *SSK1*'s own promoter, the expression level of Ssk1$^{\Delta UBL}$p was much lower than that of wild-type Ssk1p, probably because of the gross structural perturbation of this mutant protein (data not shown). To minimize the difference between the expression levels in this experiment, Ssk1$^{\Delta UBL}$p was overproduced from the strong GPD promoter while wild-type Ssk1p was overproduced from the weaker TEF

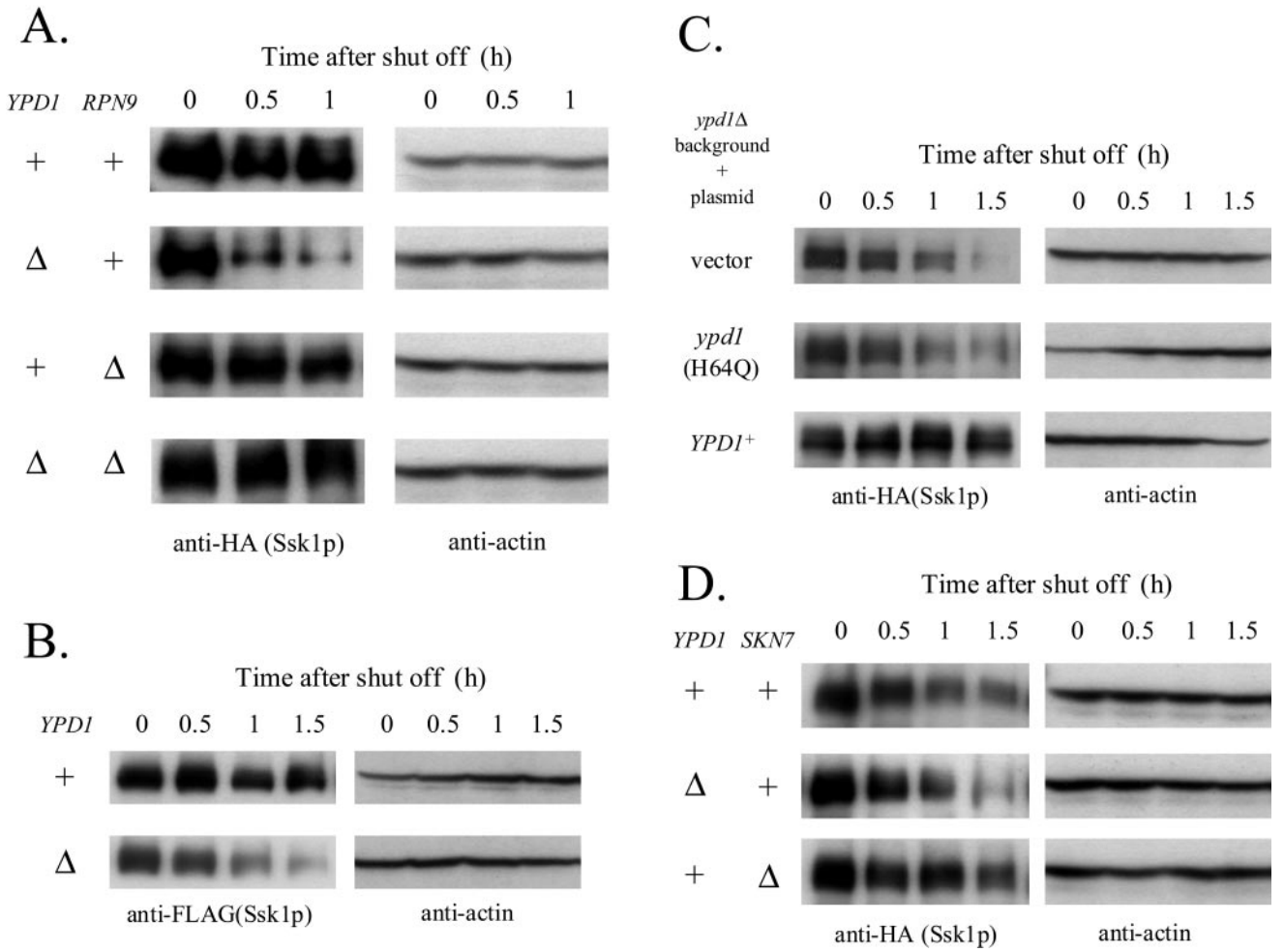


FIG. 3. The stability of Ssk1p is dependent on both the phosphotransfer and the proteasome system but not on the *SKN7* pathway. (A) The stability of Ssk1p is dependent on the phosphotransfer from Ypd1p and the proteasome system. C-terminally tagged Ssk1p was expressed from the low-copy-number plasmid pNS114 in the *YPD1*$^+$ *ssk1*Δ *hog1*Δ *RPN9*$^+$ (NS318), *ypd1*Δ *ssk1*Δ *hog1*Δ *RPN9*$^+$ (NS320), *YPD1*$^+$ *ssk1*Δ *hog1*Δ *rpn9*Δ (NS337), and *ypd1*Δ *ssk1*Δ *hog1*Δ *rpn9*Δ (NS336) strains, and the Ssk1p content was monitored by Western blotting. (B) N-terminally three-FLAG-tagged Ssk1p is also degraded in a phosphorelay-regulated manner. N-terminally three-FLAG-tagged Ssk1p was expressed from the low-copy-number plasmid pTB426 in NS318 and NS320 strains, and the Ssk1p content was monitored. (C) Ypd1p-Ssk1p interaction does not affect the stability of Ssk1p. NS320 strains with pNS114 were transformed with pRS416 (vector), pFP57 (*ypd1* [*H64Q*]), or pNS476 (*YPD1*$^+$), and the Ssk1p content was monitored. (D) Deletion of *SKN7* does not reduce the stability of Ssk1p. Tagged Ssk1p was expressed by pNS462 in NS318, NS320, and *skn7*Δ *ssk1*Δ *hog1*Δ (NS325) strains, and the Ssk1p content was monitored. +, present; Δ, deleted.

promoter. Ssk1p was immunoprecipitated with anti-FLAG antibody, and proteasomes bound to Ssk1p were detected by Western blotting with anti-20S proteasome antibody. Even when the UBL domain was deleted, Ssk1p coprecipitated with the proteasome (Fig. 6B). These results indicate that the UBL domain of Ssk1p is atypical in that it is not required for interaction with the proteasome.

The Hog1p MAPK is ectopically activated in *ubc7*Δ cells. In *ubc7*Δ cells, the delay of the Ssk1p degradation was expected to cause ectopic activation of the Hog1p MAPK. To examine the possibility, we overexpressed *SSK1* under the strong GAL1 promoter in wild-type, *ubc7*Δ, *ptp2*Δ, and *ubc7*Δ *ptp2*Δ cells on SGal medium. Overexpression of *SSK1* was slightly toxic to *ubc7*Δ cells compared to wild-type cells, as observed by poorer growth of *ubc7*Δ cells than of wild-type cells. More remarkably, in *ptp2*Δ cells, in which the inactivation process of Hog1p is partially defective (66), the overexpression of *SSK1* was lethal when *UBC7* was deleted (Fig. 7A). This lethality was suppressed by deletion of *HOG1* (Fig. 7B). These results suggest that the Ssk1p degradation by the Ubc7p-dependent mechanism represses the activity of the HOG pathway.

Next, we monitored the phosphorylation level of Hog1p in wild-type and *ubc7*Δ cells. To improve the sensitivity of our assay, *HOG1* was expressed from a high-copy-number plasmid. Cells were treated with or without 1.5 M sorbitol then sampled at the indicated times, and phosphorylated Hog1p was detected by Western blotting with anti-phospho-p38 antibody. After the hyperosmotic treatment, the dephosphorylation and/or inactivation process of Hog1p was delayed in *ubc7*Δ cells compared with wild-type cells. On the other hand, in the absence of osmotic stress, no significant increase in the basal activity of Hog1p was observed in *ubc7*Δ cells compared with

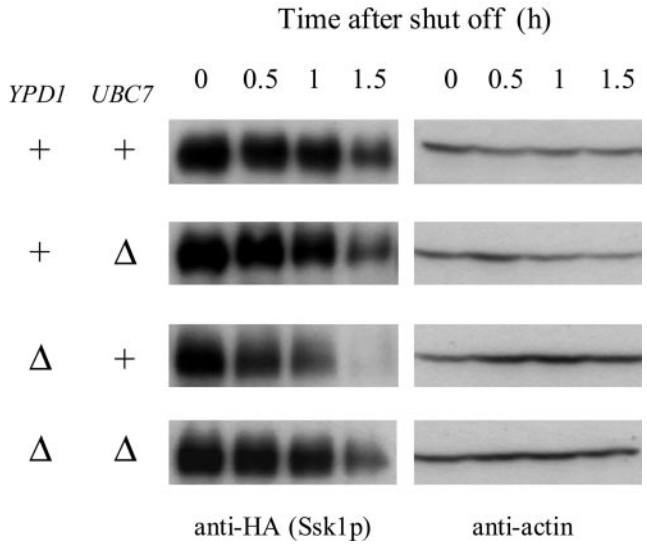


FIG. 4. Ubc7p-dependent degradation of unphosphorylated Ssk1p. Ubc7p is involved in the degradation of Ssk1p. Tagged Ssk1p was expressed from pNS114 in the *YPD1*$^+$ *ssk1*Δ *hog1*Δ *UBC7*$^+$ (NS318), *YPD1*$^+$ *ssk1*Δ *hog1*Δ *ubc7*Δ (NS329), *ypd1*Δ *ssk1*Δ *hog1*Δ *UBC7*$^+$ (NS320), and *ypd1*Δ *ssk1*Δ *hog1*Δ *ubc7*Δ (NS333) strains, and the Ssk1p content was monitored as described in the legend to Fig. 3. +, present; Δ, deleted.

wild-type cells (Fig. 7C). These results indicate that the Ubc7p-dependent degradation mechanism, by degrading an unphosphorylated pool of Ssk1p, downregulates the HOG pathway after the completion of the osmotic adaptation, in cooperation with the protein phosphatases that inactivate the pathway (25, 37, 64, 66).

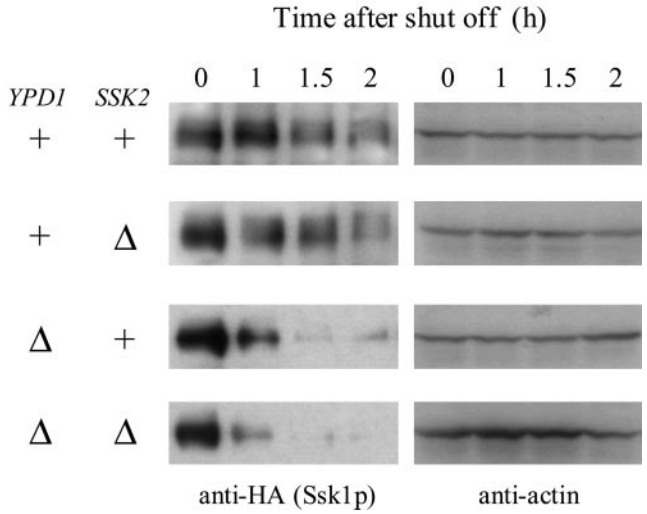


FIG. 5. Ssk2p-independent degradation of Ssk1p. Tagged Ssk1p was expressed from pNS462 in the *YPD1*$^+$ *ssk1*Δ *hog1*Δ *SSK2*$^+$ (NS318), *ypd1*Δ *ssk1*Δ *hog1*Δ *SSK2*$^+$ (NS320), *YPD1*$^+$ *ssk1*Δ *hog1*Δ *ssk2*Δ (NS354), and *ypd1*Δ *ssk1*Δ *hog1*Δ *ssk2*Δ (NS355) strains, and the Ssk1p content was monitored. +, present; Δ, deleted.

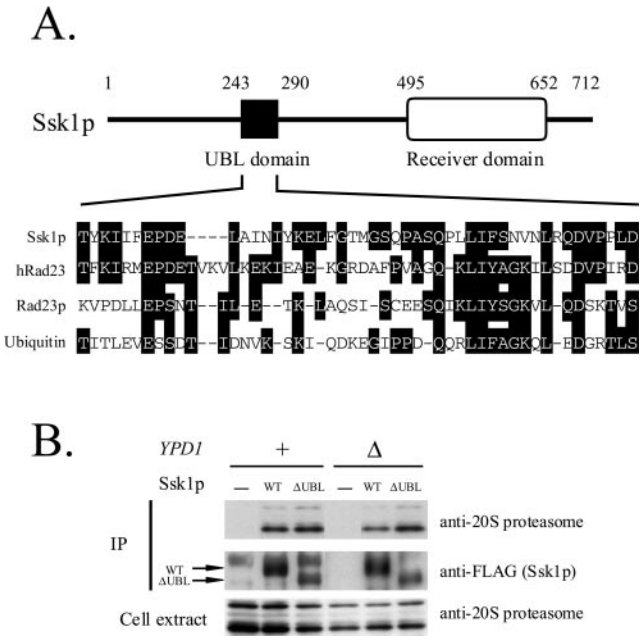
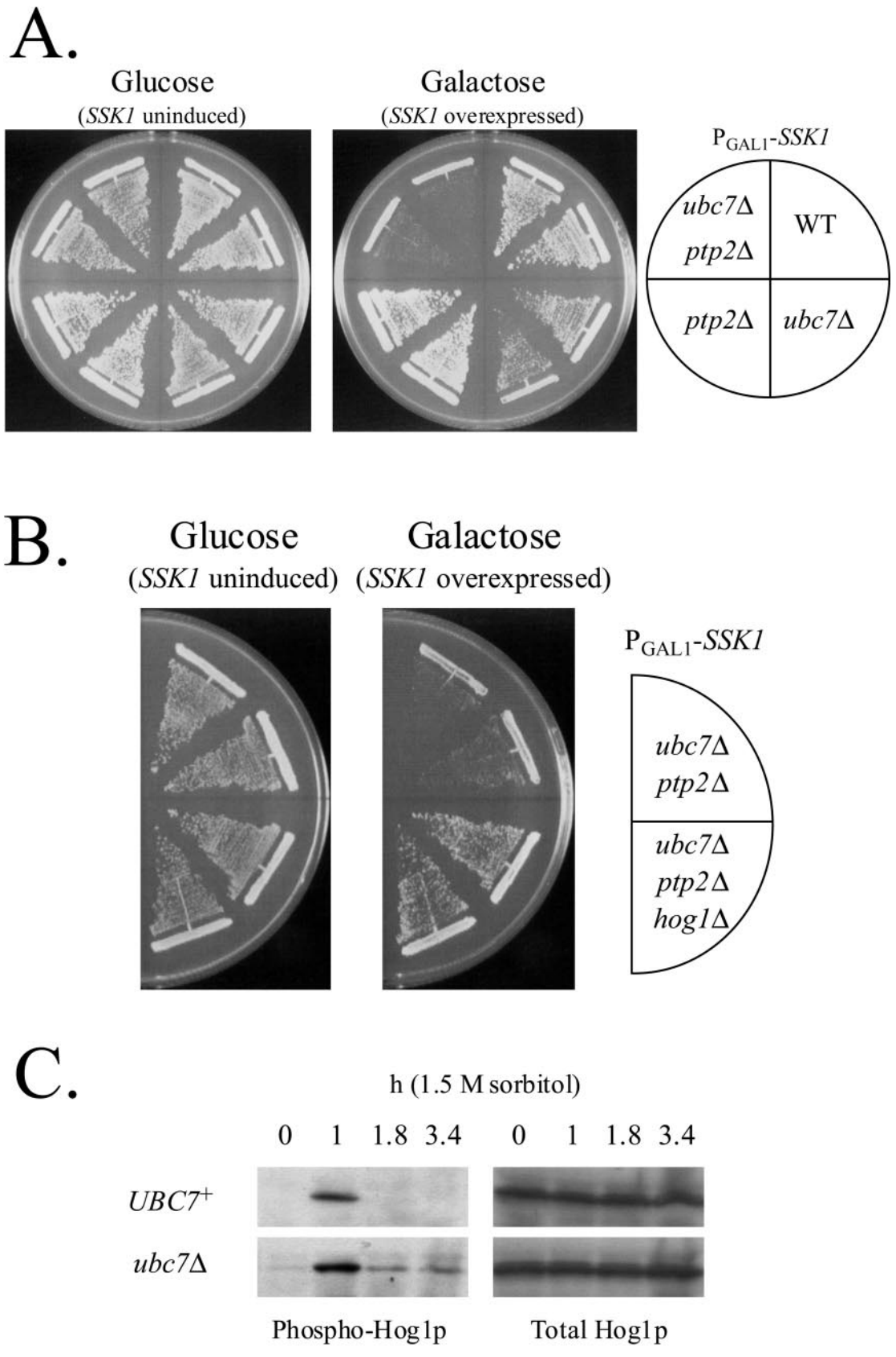


FIG. 6. The UBL domain of Ssk1p is not required for the interaction with the proteasome. (A) UBL domain of Ssk1p. In the middle of the molecule (amino acids 243 to 290), Ssk1p contains a small domain homologous to ubiquitin and the UBL domains of human and yeast Rad23p. The UBL domain of Ssk1p shares amino acid identities of 42.6% with human Rad23p, 25.5% with yeast Rad23p, and 25.5% with ubiquitin. (B) Coimmunoprecipitation of Ssk1p with the proteasome. *SSK1* (wild type [WT] or Δ*UBL*)-*FLAG* was expressed from pNS454 or pNS456, respectively, in the *YPD1*$^+$ *ssk1*Δ *hog1*Δ (NS318) and *ypd1*Δ *ssk1*Δ *hog1*Δ (NS320) strains. Ssk1p (WT or ΔUBL)-FLAG was immunoprecipitated with anti-FLAG M2 beads, and proteasomes bound to Ssk1p were detected by Western blotting. +, present; Δ, deleted; −, not present.

DISCUSSION

Activation of the yeast HOG MAPK cascade is essential in the organism's adaptation to high extracellular osmolarity (6, 36, 38, 48). On the other hand, proper inactivation of the HOG cascade is also essential for growth because hyperactivation of this cascade is toxic to cells (36, 37, 38, 66). Therefore, the HOG cascade must remain inactive unless cells are exposed to high osmolarity and must be inactivated promptly once the adaptation process to high osmolarity is completed. Dephosphorylation of a phosphotyrosine or a phosphothreonine residue in active Hog1p by protein phosphatases is a well-characterized inactivation mechanism (25, 37, 38, 64, 66). Our results demonstrate an additional downregulation mechanism in the pathway: unphosphorylated Ssk1p response regulator is degraded by the ubiquitin-proteasome system.

Two distinct physiological roles are conceivable for this downregulation mechanism. One is to maintain the low basal activity of the HOG pathway in the absence of osmotic stress. This aids cells in avoiding ectopic induction of osmotic responses under normal osmotic conditions, which is not only unnecessary but also deleterious. The other is to downregulate the HOG pathway after a sufficient signal is transmitted to complete the adaptation process. This ensures that adequate adaptation is achieved and that the immediate resumption of


A.
Glucose
(SSK1 uninduced)
Galactose
(SSK1 overexpressed)
PGAL1-SSK1
ubc7Δ
ptp2Δ
WT
ptp2Δ
ubc7Δ
B.
Glucose
(SSK1 uninduced)
Galactose
(SSK1 overexpressed)
PGAL1-SSK1
ubc7Δ
ptp2Δ
ubc7Δ
ptp2Δ
hog1Δ
C.
h (1.5 M sorbitol)
0 1 1.8 3.4 0 1 1.8 3.4
UBC7+
ubc7Δ
Phospho-Hog1p
Total Hog1p

growth occurs once the adaptation is complete. Here, we show that the latter is the main role for the degradation mechanism because, as shown in Fig. 7C, the deletion of *UBC7* caused the prolonged activation of the HOG pathway after the hyperosmotic treatment but did not significantly increase the basal activity of Hog1p in the absence of osmotic stress.

The reaction steps in the ubiquitination of substrates to be degraded require three kinds of enzymes: ubiquitin-activating enzyme (E1), E2, and ubiquitin ligase (E3). E1 activates ubiquitin by forming a thiol-ester bond between ubiquitin and E1. Activated ubiquitin is then transferred to E2. E3 interacts with E2 and the substrate and facilitates ubiquitination. The E3 involved in Ssk1p ubiquitination has yet to be identified. In the case of the human ERK pathway, MEKK1 (MAPKKK), which contains a RING finger-like PHD domain, functions as E3 for the ERK1/2 MAPKs and downregulates the pathway (35). However, our observation that deletion of *SSK2* does not affect the stability of Ssk1p excludes the possibility that the Ssk2p MAPKKK acts as E3 for Ssk1p. Consistently, Ssk2p does not appear to have any of the motifs frequently found in E3s, such as a RING, HECT, or U-box domain. The yeast Ste11p MAPKKK is activated by both mating pheromone and osmotic stress (18, 20, 47, 49). When activated by pheromone, Ste11p is degraded by the ubiquitin-dependent system (14). However, E3 for Ste11p has also yet to be identified. In the case of ERAD, Hrd1p (3, 19) and Doa10p (60) have been defined as ER-associated E3s that act together with Ubc7p, whereas degradation of Ole1p is independent of these E3s (5). Whether Hrd1p and/or Doa10p is also an E3 in the ubiquitination of Ssk1p awaits further investigation.

The domains in Ssk1p required for its interaction with E2, E3, or proteasomes, if any, have yet to be determined. We found a UBL domain in the N-terminal region (amino acids 243 to 290) of Ssk1p (Fig. 6A). Recent reports showed that the UBL domains of Rad23p and Dsk2p bind to Rpn1p, a component of the base complex of the 26S proteasome (13, 16, 56, 65). In contrast, the UBL domain of Ssk1p is dispensable for its interaction to proteasomes. In accord with the difference in function, there are some structural differences between the UBL domains of Ssk1p and Rad23p/Dsk2p. The UBL domain of Ssk1p is shorter than those of Rad23p and Dsk2p, and its location is in the center of the molecule, whereas those of Rad23p and Dsk2p are near the N termini. The UBL domain of Ssk1p may bind to degradation-related proteins other than components of the 26S proteasome, such as E2 and/or E3.

A model for the functional connection between Ssk1p and the ubiquitin-proteasome system is shown in Fig. 8. Once the osmotic adaptation is completed, unphosphorylated Ssk1p, which potentially activates Ssk2p, is ubiquitinated by the Ubc7p E2 and an unidentified E3 on the surface of the ER.

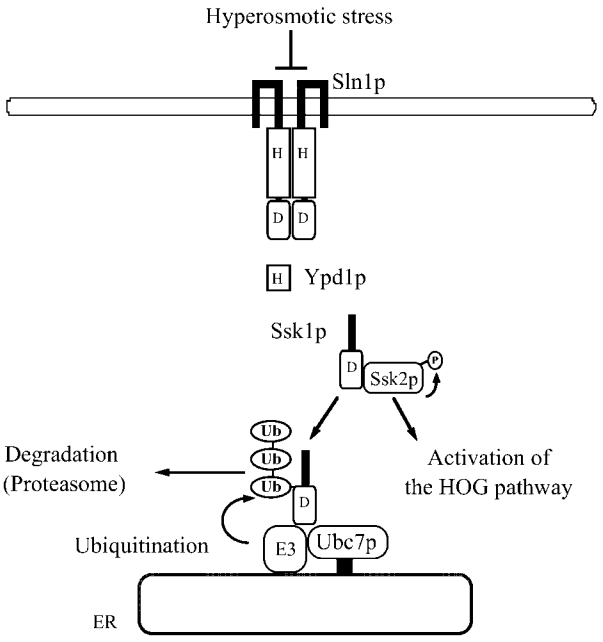


FIG. 8. Model for the functional connection between Ssk1p and the ubiquitin-proteasome system. See Discussion for explanation.

Then unphosphorylated and ubiquitinated Ssk1p is targeted to the 26S proteasome and degraded, which ensures the timely inactivation of the HOG pathway after the completion of the osmotic adaptation.

Phosphorelay-regulated degradation of response regulators by the ubiquitin-proteasome system may be a general regulatory mechanism in eukaryotic His-Asp phosphorelay signaling systems. Higher plants make use of His-Asp phosphorelay signaling systems in the signal transduction of plant hormones ethylene and cytokinin (9, 23, 24, 28, 55, 57, 67). Recently, a mutant of an Rpn12p homolog in *Arabidopsis* was reported to show altered responses to cytokinin (59). Although response regulators for cytokinin signaling are still to be identified, impaired degradation of them may cause the altered responses to the hormone in this proteasome mutant.

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FIG. 7. Ectopic activation of the HOG pathway in *ubc7*Δ cells. (A) Overexpression of *SSK1* is toxic to *ubc7*Δ cells. Wild-type (TM141), *ubc7*Δ (NS327), *ptp2*Δ (TM107), and *ubc7*Δ *ptp2*Δ (NS358) strains were transformed with pNS473. Transformants were grown on SD and SGal plates. *SSK1* was overexpressed under the GAL1 promoter on an SGal plate. Each plate was incubated at 30°C for 4 days. (B) Deletion of *HOG1* suppresses the lethality caused by *SSK1* overexpression. *SSK1* was overexpressed from pNS473 in the NS358 and *ubc7*Δ *ptp2*Δ *hog1*Δ (NS362) strains. Each plate was incubated at 30°C for 5 days. (C) Prolonged phosphorylation of the Hog1p MAPK in *ubc7*Δ cells after the hyperosmotic treatment. TM141 and NS327 strains expressing *HOG1* from pHG11 were treated with or without 1.5 M sorbitol, and phospho-Hog1p was detected by Western blotting.

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