Yeast cells respond to hypertonic shock by activation of a (MAP) mitogen-activated protein kinase cascade called the (HOG) high osmolarity glycerol response pathway. How yeast respond to hypotonic shock is unknown. Results of this investigation show that a second MAP kinase cascade in yeast called the protein kinase C1 (PKC1) pathway is activated by hypotonic shock. Tyrosine phosphorylation of the PKC1 pathway MAP kinase increased rapidly in cells following a shift of the external medium to lower osmolarity. The intensity of the response was proportional to the magnitude of the decrease in extracellular osmolarity. This response to hypotonic shock required upstream protein kinases of the PKC1 pathway. Increasing external osmolarity inhibited tyrosine phosphorylation of the PKC1 pathway MAP kinase, a response that was blocked by BCK1-20, a constitutively active mutant in an upstream protein kinase. These results indicate that yeast contain two osmosensing signal transduction pathways, the HOG pathway and the PKC1 pathway, that respond to hypertonic and hypotonic shock, respectively.

Osmotic stress induces specific cellular responses that include changes in the activity of solute transporters (1, 2) and enzymes involved in solute accumulation (3, 4), the expression of genes encoding enzymes required for solute synthesis (5–8), stress resistance (5, 9), and cell wall structure (10). Despite their importance for cell growth and survival, the signaling mechanisms responsible for mediating osmotic stress-specific responses are not nearly as well understood as those which mediate responses to ligands such as growth factors or hormones. Our understanding of how eukaryotic cells sense and respond to changes in osmolarity has been helped recently by studies of this problem in the budding yeast *Saccharomyces cerevisiae*.

In yeast, a protein kinase cascade called the HOG pathway (11) plays a central role in mediating cellular responses to an increase in external osmolarity. This pathway is defined by the HOG1 (11) and PBS2 (11–13) genes encoding members of the MAPK (mitogen-activated protein kinase) and MAPKK (MAP kinase kinase) family, respectively (14, 15). Addition of NaCl or sorbitol to increase the osmolarity of the medium induces yeast to accumulate glycerol (6) and thereby restore the osmotic gradient across the cell membrane. This response, which involves increased expression of the glycerol-3-phosphate dehydrogenase gene GPD1 (5–7), is blocked in a hog1Δ mutant (7). Other responses to an increase in osmolarity such as reorientation of cell growth and division (16) and induction of gene expression (17) are also defective in hog1Δ and pbs2Δ mutants. HOG pathway activation involves increased phosphorylation of a Hog1p tyrosine residue conserved among all MAP kinases which is required for growth at high osmolarity (17). Mammalian cells contain structural and functional homologs of Hog1p, suggesting that the HOG pathway is conserved among eukaryotes (18–21).

In its natural environment, yeast cells are exposed to not only increases but also decreases in osmolarity. Although the HOG pathway has a clear role in mediating cell responses to increases in osmolarity, little is known about how yeast sense and respond to decreases in osmolarity. Yeast cells contain four known MAP kinase cascades (22–24). One of these, referred to here as the PKC1 pathway, is mediated by a protein kinase C-like protein encoded by the gene PKC1 (25). Other protein kinases on the PKC1 pathway have been identified using different genetic approaches and placed into a linear pathway that proceeds downward from Pkc1p to MAPKKK (called Bck1p or Slk1p) (26–28) to two MAPKK (Mkk1p and Mkk2p) (29) to MAPK (called Mpk1p or Slt2p) (30, 31). A comparison of deletion mutants in the HOG pathway to those in the PKC1 pathway reveal opposite phenotypes. For example, a hog1Δ MAPK mutant grows in low but not high osmolarity medium while a mpk1Δ MAPK mutant grows in high but not low osmolarity medium (30), a phenotype exacerbated by growth at elevated temperature, i.e. 37 °C. Mutants in other genes of the PKC1 pathway show a phenotype similar to that of mpk1Δ (26, 29, 31–33). Although there are other possible explanations, this observation is consistent with a model in which the PKC1 pathway, like the HOG pathway, mediates an osmotic signal and induces cellular responses required for growth at the new (lower) osmolarity. At the time this work was initiated there was no known activating signal for this kinase cascade. In this report we test the hypothesis that the PKC1 kinase cascade is an osmosensing signal transduction pathway which responds to hypotonic shock as an activating signal.

**EXPERIMENTAL PROCEDURES**

Materials—The yeast strain used for most experiments was YPH102 (MATα ura3 leu2 his3 ade2 lys2) (34), into which different plasmids were introduced by LiAc-based transformation (35) with selection on uracil-deficient medium for URA3 carried on the plasmid. Other strains used in experiments are described in the legends of the figures in which they were used. Plasmids and PKC1 pathway mutant strains were obtained from Michael Snyder (Yale), Kunihiro Matsumoto (Nagoya

*This work was supported in part by National Science Foundation Grant MCB-9206462 and American Cancer Society Grant BE-224 (to M. C. G.), National Institutes of Health Predoctoral Training Grant ST2GM 07833-15 (to K. D.), and National Institutes of Health Grant GM 48533 and American Cancer Society Grant FRA-446 (to D. E. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: HOG, high osmolarity glycerol; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAP kinase kinase; PKC, protein kinase C; HA, hemagglutinin.
University, and Carl Mann (Centre d’Etudes de Saday, GIF-sur-Yvette). The MPK1-hemagglutinin (HA) plasmid (36) rescued the mpk1Δ mutant phenotype of reduced growth in low osmolarity medium indicating that addition of the HA epitope did not interfere with the normal function of the Mpk1p. A similar plasmid carrying a mutation which codes for a substitution of phenylalanine for the conserved tyrosine in the MPK1 gene (pMPK1-HA Y192F) (36) did not rescue the mpk1Δ phenotype and was deleterious to a wild-type strain transformed with the plasmid (data not shown).

Growth Conditions— Cultures of plasmid-bearing yeast were grown overnight in uracil-deficient medium and then grown to log phase in YEPD (2% Bacto-peptone, 2% glucose, 1% sodium chloride) containing 50 μm 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium. To detect the Mpk1p MAPK containing the HA epitope (39), immunoblots were probed with the D2CA5 anti-HA monoclonal antibody (Babco or Boehringer Mannheim) and then a horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham Corp.). Immune complexes were detected using an enhanced chemiluminescence procedure for detecting peroxidase activity (Amersham Corp.).

**RESULTS**

Hypotonic Shock Induces the Tyrosine Phosphorylation of Mpk1p— Specific extracellular signals activate MAP kinases by inducing their phosphorylation on a single conserved threonine and a nearby tyrosine (40, 41). For Hog1p and the PKC1 pathway MAPK Mpk1p, like other MAP kinases, phosphorylation of this conserved tyrosine residue is required for pathway function because substitution of the non-phosphorylatable residue phenylalanine for the conserved tyrosine blocks pathway-specific responses (17, 31). Therefore, to determine whether hypotonic shock activates the PKC1 pathway, we exposed the yeast strain YPH102 and YPH102 mpk1Δ:HIS3 (mpk1Δ) to decreases in external osmolarity and then assayed cell proteins for tyrosine phosphorylation by immunoblot analysis with an antibody to phosphotyrosine. One minute after the osmolarity of the medium was lowered by reducing the concentration of sorbitol from 1 M to 0.2 M, an increase in tyrosine phosphorylation of a single band with an apparent size of 68 kDa was observed in the wild type but not in the mpk1Δ strain (Fig. 1 (left)). A gene fusion coding for Mpk1p tagged at the COOH terminus with a HA epitope (38) was introduced on a high copy 2μ plasmid into an mpk1Δ strain. Decreasing the osmolarity stimulated the tyrosine phosphorylation of a band that migrated more slowly than that in the wild type strain (YPH102). In the mpk1Δ strain transformed with a plasmid identical to the MPK1-HA-
ways induce rapid (response to hypotonic shock. Anti-phosphotyrosine immunoblot analysis of Mpk1p phosphorylation at different times after hypotonic shock of YPH102 containing the 2μ MPK1-HA plasmid. Aliquots of a single culture were withdrawn before (0) and at the indicated times following a change in the sorbitol concentration of the medium from 1 M to 0.2 M (see “Experimental Procedures”) and 1 min after a final dilution of the medium in a ratio of 1 part culture to 3 parts water (30' + 1'). These aliquots were rapidly cooled, and cell extracts were prepared and assayed as described under “Experimental Procedures.”

containing plasmid with the exception of a point mutation in MPK1 which substitutes a phenylalanine for the conserved tyrosine, no band is seen in the anti-phosphotyrosine immunoblot. An immunoblot of the same cell extracts with an anti-HA epitope antibody (Fig. 1 (right)) revealed an immunoreactive protein found in the MPK1-HA and mutant MPK1-HA containing strains, which had the same mobility as the tyrosine phosphorylated band. We interpret these observations to mean that the PKC1 pathway MAPK1 p is tyrosine phosphorylated in response to hypotonic shock.

Time Course of Mpk1p Phosphorylation—MAP kinase pathways induce rapid (<1 min) changes in MAPK tyrosine phosphorylation in response to specific signals. As shown in Fig. 2, lowering external osmolarity induced an increase in Mpk1p tyrosine phosphorylation that occurred within 15 s of the stimulus and persisted for 10–15 min. After 30 min when the tyrosine phosphorylation of Mpk1p had dropped to nearly pre-stimulus level, a further reduction in the osmolarity of the medium caused by addition of water induced a second rapid increase in Mpk1p tyrosine phosphorylation. Because cells containing a single chromosomal copy of MPK1 produce a small amount of Mpk1p which makes detection of phosphotyrosine difficult, this and following experiments were carried out with cells containing multiple copies of MPK1-HA.

Osmotic Dependence of MAP Kinase Phosphorylation—To extend and confirm the observation that Mpk1p and Hog1p respond in opposite fashion to osmotic changes, the in vivo tyrosine phosphorylation of both kinases was measured after changing the external osmolarity to a range of higher and lower levels. Specifically, a culture was grown to log phase in medium containing 1 M sorbitol, and then the osmolarity decreased or increased by addition of water with varying concentrations of sorbitol. Cells were collected 1 min after the osmotic change and MAP kinase tyrosine phosphorylation measured as before using an immunoblot procedure. Compared to the control cells (Fig. 3, marked by an asterisk (*)) where external osmolarity was unchanged, decreasing osmolarity induced an increase in Mpk1p phosphorylation that was proportional to the magnitude of the osmotic shock. Increasing osmolarity induced an increase in tyrosine phosphorylation of a band that we has the same mobility relative to molecular weight standards as that which was previously identified as Hog1p and, as expected from previous results, was absent in hog1Δ cells (not shown). We noted that it was easier to detect both basal and high osmolarity-induced increases in tyrosine phosphorylation in Hog1p in cells containing a high copy Mpk1p (or Mpk1p-HA) plasmid, although anti-Hog1p immunoblot analysis (17) showed that the amount of Hog1p was unchanged under these different conditions (data not shown). This Mpk1p overexpression-induced increase in the amount of tyrosine phosphorylated Hog1p is blocked in mutants lacking protein kinases upstream of Mpk1p on the PKC pathway (see below). The physiological significance and explanation of this phenomenon is unknown.

Solute Independence of the Hypotonic Response—In experiments described above, external osmolarity was changed by altering the concentration of sorbitol. To determine if the phosphorylation of Mpk1p was due to osmotic changes or a sorbitol specific response, we tested whether changes in the concentration of other solutes, namely glucose or NaCl, would also activate Mpk1p phosphorylation. As shown in Fig. 4, cells were grown to log phase in 20% YEPD plus 0.95 M glucose for 3 h and then diluted 1:4 with H2O, 1 M glucose, or 2 M glucose. The experiment was then repeated substituting NaCl for glucose at half the stated molarities (roughly the same osmolarity).

Osmotic Regulation of Mpk1p Phosphorylation Involves Upstream Kinases in the PKC Pathway—To determine whether Mpk1p phosphorylation by hypotonic shock is mediated through the PKC1 pathway, we measured this response in different mutant strains. As shown in Fig. 5 (top), hypotonic shock-induced tyrosine phosphorylation of Mpk1p-HA was not detectable in strains containing deletions in the genes that encode protein kinases upstream of Mpk1p on the PKC1 path-

![Fig. 2.](image-url) **Fig. 2.** Transient induction of Mpk1p phosphorylation by a hypotonic shock. Anti-phosphotyrosine immunoblot analysis of Mpk1p phosphorylation at different times after hypotonic shock of YPH102 containing the 2μ MPK1-HA plasmid. Aliquots of a single culture were withdrawn before (0) and at the indicated times following a change in the sorbitol concentration of the medium from 1 M to 0.2 M (see “Experimental Procedures”) and 1 min after a final dilution of the medium in a ratio of 1 part culture to 3 parts water (30' + 1'). These aliquots were rapidly cooled, and cell extracts were prepared and assayed as described under “Experimental Procedures.”

![Fig. 3.](image-url) **Fig. 3.** Osmotic dependence of Mpk1p and Hog1p phosphorylation. Strain used was YPH102 containing the 2μ MPK1-HA plasmid. Time of incubation in different osmotic medium before chilling cells and extract preparation was 1 min. Fraction of initial osmolarity refers to the ratio of final sorbitol concentration to that present in the initial culture medium (20% YEPD plus 1 M sorbitol). Osmolarity was changed by diluting 10 ml of cell culture into 40 ml of water containing different concentrations of sorbitol.
way. These include a protein kinase C mutant \( \text{pkc1}^{\Delta} \) (25), a MAPKKK mutant \( \text{bck1}^{\Delta} \) (27), and a MAPKK mutant \( \text{mkk1}^{\Delta} \text{mkk2}^{\Delta} \) (29). The failure to detect Mpk1p-HA tyrosine phosphorylation could be explained by PKC1 pathway-dependent expression of Mpk1p-HA. However, immunoblot analysis with the anti-HA antibody (Fig. 5, bottom) showed that the amount of Mpk1p-HA was independent of the upstream kinases in the PKC1 pathway.

Tyrosine phosphorylation of Mpk1p was inhibited in cells exposed to an increase in osmolarity of the medium while Hog1p tyrosine phosphorylation increased (Fig. 6). To test whether these responses involved the PKC1 pathway, this experiment was repeated using a \( \text{BCK1-20} \) mutant which is reported to encode a constitutively active form of the Bck1p protein kinase (26). This strain no longer shows the high osmolarity-induced decrease in Mpk1p phosphorylation. Note that the high osmolarity-induced increase in Hog1p phosphorylation was relatively unaffected by this mutation.

**DISCUSSION**

**Osmosensing MAP Kinase Pathways in Yeast**—Our results show that there are two osmosensing signal transduction pathways in yeast, each containing structurally similar protein kinases (22, 24). The symmetry between the pathways is striking. The Hog1p pathway genes \( \text{HOG1} \) and \( \text{PBS2} \) are required for cell growth at high osmolarity and high osmolarity rapidly induces a transient, PBS2-dependent hyperphosphorylation of the Hog1p MAP kinase (11). The PKC1 pathway genes \( \text{PKC1}, \text{BCK1} \) (\( \text{SLK1} \)), \( \text{MKK1}/\text{MKK2} \), and \( \text{MPK1} \) (\( \text{SLT2} \)) are required for cell growth at low osmolarity (26, 29–33), and low osmolarity rapidly induces a transient, \( \text{MKK1}/\text{MKK2} \)-dependent hyperphosphorylation of the Mpk1p MAP kinase (this study). Mpk1p kinase activity is rapidly elevated in cells exposed to a hypotonic shock (36). The Hog1p pathway has a fairly well defined role in the cellular response to an increase in osmolarity. Based on the phenotypes of hog1p and \( \text{pbs2}^{\Delta} \) mutants, the Hog1p pathway is required for high osmolarity-stimulated transcription of specific genes (7, 17) leading to increased synthesis of the principal osmolyte glycerol (11) and general stress resistance (17). The PKC1 pathway is required for constructing a cell wall. Cells without PKC1 die by cell lysis (32, 33). Deletion mutations in \( \text{BCK1} \) (\( \text{SLK1} \)), \( \text{MKK1}/\text{MKK2} \), or \( \text{MPK1} \) (\( \text{SLT2} \)) have similar phenotypes: cell lysis that is accentuated by growth at higher temperatures. This temperature-sensitive cell lysis phenotype is suppressed by growth on high osmolarity medium and is correlated with a decrease in glucan content of the cell wall (10, 42). The mechanism responsible for the weakened cell walls in PKC1 pathway mutants is not known with any certainty but may involve defects in polarized vesicle secretion/cell growth (43) or changes in glucan content (10). Therefore, one possible role of the PKC1 pathway is to regulate cell wall properties in response to changes in external osmolarity. This type of physiological response has been observed in fungi. The constant growth rate of the fungus Achlya bisexualis.

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**Fig. 5.** Hypotonic shock-induced Mpk1p phosphorylation in PKC1 pathway mutants. Strains containing the indicated deletion mutations plus a high copy \( 2\mu \) MPK1-HA plasmid were grown to log phase in 20% YEPD plus 1 M sorbitol. These cultures were then diluted 1:34 with water and the cells collected 1 min later for preparation of cell extracts and immunoblot analysis of tyrosine phosphorylation (top) and Mpk1p-HAp (bottom). Strains were the following: wild-type (YPH102) (34), \( \text{mkk1,2}^{\Delta} \) (233-1B) (29), \( \text{bck1}^{\Delta} \) (Y782) (27), and \( \text{pkc1}^{\Delta} \) (DL376) (33).

**Fig. 6.** Hypertonic shock induces a BCK1-dependent decrease in Mpk1p phosphorylation. YPH102 strains containing either a high copy \( 2\mu \) MPK1 plasmid (left) or a low copy CEN BCK1-20 plasmid (26) (right) were grown separately to log phase in YEPD. The cultures were then split, and 0.4 M NaCl was added to one of the two cultures. Cells were then collected after 1 and 10 min, and protein tyrosine phosphorylation was analyzed by immunoblot.
In medium of different osmolarity is correlated with changes in the mechanical properties of their cell wall with a stronger wall at low osmolarity than at high osmolarity (44).

The complex phenotype of PKC1 pathway mutants suggests that this pathway responds to physiological signals beside changes in external osmolarity. Besides the sensitivity to low osmolarity, such mutants are altered in cell morphogenesis (27, 45). Mutants lacking the PKC1 pathway MAP kinase kinase kinase Bck1p (SLK1) are sensitive to starvation with defects indicative of a failure to exit the vegetative growth cycle (27). Compared to wild-type (BCK1+) cells, bck1Δ (slk1Δ) mutants do not accumulate glycogen, fail to undergo meiosis, are heat shock-sensitive, continue to form buds in stationary phase cultures, and lose viability in nutrient-poor medium (45). These phenotypes are independent of the osmolarity of the medium (45). These defects in growth control suggest that the PKC1 pathway has a role in nutrient sensing. The PKC1 pathway is required for growth at elevated temperature and the Mpk1p kinase activity is strongly activated by exposure of cells to higher temperature (36). How functions of the PKC1 pathway such as osmosensing, temperature-sensing, and nutrient sensing are coordinated with each other remains to be determined.

An important aspect of the two yeast osmosensing MAP kinase pathways is that similar pathways appear to exist in cells from other eukaryotes including mammals. In the case of the HOG pathway, Hog1p shows a high degree of similarity in amino acid sequences in other eukaryotes including mammals. In the case of the HOG pathway affected by overexpression of a protein kinase in the PKC1 pathway MAP kinase kinase kinase (MEKK), respectively (31, 32). Strikingly, studies in a human intestinal cell line D12. Boguslawski, G., and Polazzi, T. (1987) J. Biol. Chem. 262, 213–224.

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A Second Osmosensing Signal Transduction Pathway in Yeast: HYPOTONIC SHOCK ACTIVATES THE PKC1 PROTEIN KINASE-REGULATED CELL INTEGRITY PATHWAY

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J. Biol. Chem. 1995, 270:30157-30161.
doi: 10.1074/jbc.270.50.30157

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