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22. RNA was isolated from 20 animal caps (IF, EF) or five whole embryos and treated with deoxyribonuclease I by standard procedures (*36*). Complementary DNA was prepared, and one-twentieth of the sample was amplified by 25 cycles of PCR (*37*) with specific primers for Xbra (*21*) or *Xenopus* activin receptor (XAR) (*38, 39*). The radiolabeled PCR products were resolved by 6% nondenaturing polyacrylamide electrophoresis, fixed and dried, and autoradiographed for 2 days.
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24. Embryo injections, dissections, and cultures were as described in Fig. 1. Asparagine-17 RNA was isolated from 10 animal caps as described above and subjected to Northern blotting (*36*) or to RT-PCR analysis as described above with primers directed against muscle actin or α-globin, except that 20 cycles of PCR were used for actin and 25 cycles were used for α-globin. The primers for muscle actin were 5′-GCTGACAGAATGCA-GAAG-3′ and 5′-TTGCTTGGAGGAGTGTGT-3′, and the primers for α-globin were 5′-GCCTA-CAACCTGAGAGTGG-3′ and 5′-CAGGCTGGT-GAGCTGCCC-3′ (*23*).
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42. We thank Y. Rao and P. Wilson for critical reading of this manuscript, D. Kessler for helpful discussions, D. Gifford for assistance with computer graphics, N. Sonenberg for providing the eIF-4E alanine-53 mutant, M. Baron for mouse erythroleukemia cell complementary DNA, P. Wilson for primers of Xbra, M. Whitman for Asn-17 *ras*^{H} in pSP64T, and A. Hemmati-Brivanlou for mRNA encoding the truncated activin receptor. P.S.K. is supported by the Howard Hughes Medical Institute Post-Doctoral Fellowship for Physicians. This work is supported by a grant from the National Institutes of Health.

17 December 1993; accepted 6 July 1994

An Osmosensing Signal Transduction Pathway in Mammalian Cells

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The osmotic balance between the cytoplasmic and extracellular compartments of cells is critical for the control of cell volume. A mammalian protein kinase, Jnk, which is a distant relative of the mitogen-activated protein kinase group, was activated by phosphorylation on threonine and tyrosine in osmotically shocked cells. The activation of Jnk may be relevant to the biological response to osmotic shock because the expression of human Jnk in the yeast *Saccharomyces cerevisiae* rescued a defect in growth on hyper-osmolar media. These data indicate that related protein kinases may mediate osmosensing signal transduction in yeast and mammalian cells.

The maintenance of homeostasis is a fundamental property of the cells that form the tissues of the body. Exposure to anisotonic media initiates a response that maintains cellular volume. This response is thought to be mediated by changes in plasma-membrane ion transport (*1*) and the accumulation of nonpolar solutes (*2, 3*). The signal transduction pathway that controls this response to osmotic shock in mammalian cells is not understood. However, detailed studies of osmoregulation in enteric bacteria have established the importance of a two-component pathway involving a histidine kinase sensor (*EnvZ*) and a transcriptional regulator (*OmpR*) that is activated by phosphorylation on Asp (*4*). In the yeast *Saccharomyces cerevisiae*, both a histidine kinase sensor and a member of the MAP kinase group [high osmolarity glycerol response 1 (Hog1)] have been implicated in osmosensing (*5, 6*).

The exposure of cultured mammalian cells to anisotonic media causes an increase in the phosphorylation of several proteins (*7*). This reaction suggests that osmotic shock may cause the activation of one or more protein kinases. Consistent with this hypothesis, the exposure of Chinese hamster ovary (CHO) cells to hyper-osmolar media caused a marked increase in Jnk activity, which was detected in an immune complex protein kinase assay by the use of a polyclonal anti-Jnk1 antibody. Activation of Jnk in response to osmotic shock was also observed in CHO cells transfected with epitope-tagged Jnk1 (Fig. 1A). The increase in Jnk1 activity was observed within 5 min of exposure of the cells to hyper-osmolar media (Fig. 1B). Maximal activity was observed 15 to 30 min after osmotic shock, with a progressive decline in Jnk1 activity at later times. The activation of Jnk1 caused by ultraviolet radiation requires the phosphorylation of Thr^{183} and Tyr^{185} (*8*). Therefore, we investigated whether these phosphorylation sites were required for the activation of Jnk1 by osmotic shock. The replacement of Thr^{183} (with Ala) and Tyr^{185} (with Phe) blocked Jnk1 activation (Fig. 1C). Together, these data demonstrate that the Jnk signal transduction pathway (*8, 9*) is activated by the exposure of cultured mammalian cells to hyper-osmolar media.

Comparison of the sequence of human Jnk1 with the GenBank database revealed that this enzyme is similar to the *Saccharomyces cerevisiae* protein kinase Hog1 (Fig. 2). Yeast that are defective in Hog1 expression cannot grow after osmotic shock (*5*). This

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inability suggest that Hog1 functions as a component of a signal transduction pathway that is induced in osmotically shocked yeast (5). The sequence similarity and the activation by osmotic shock suggest that the role of Jnk1 in mammalian cells may be similar to that of Hog1 in yeast. Therefore, we tested the effect of Jnk1 expression in yeast that lack Hog1 (null mutant *hog1-Δ1*). This yeast strain grows well on YEPD (1% yeast extract, 2% bactopeptone, and 2% dextrose), but fails to grow on a medium of high osmolarity, such as YEPD supplemented with 0.9 M NaCl (5). Both Hog1 and Jnk1 complemented the growth defect of the *hog1-Δ1* mutant (Fig. 3). In contrast, the human Erk2 isoform of mitogen-activated protein (MAP) kinase did not complement *hog1-Δ1* (Fig. 3).

The complementation of the *hog1-Δ1* mutant by Jnk1 indicates that the human Jnk1 protein kinase can substitute for Hog1 in yeast. It is possible that this complementation is a nonspecific action of Jnk1. However, the finding that kinase-negative Jnk1 did not complement the defect in Hog1 expression (Fig. 3) demonstrated that Jnk1 kinase activity was required for growth of the mutant yeast on hyper-osmolar media. To test whether Jnk1 activation was required for complementation, we substituted the sites of phosphorylation activation of Jnk1 at Thr[183] and Tyr[185] with Ala and Phe, respectively (8). The phosphorylation-defective Jnk1 protein kinase (Thr/Tyr−) failed to complement the *hog1-Δ1* mutant. Further evidence indicating a role for Jnk1 phosphorylation at Thr[183] and Tyr[185] was obtained from the finding that the MAP kinase kinase Pbs2 (5, 10) was required for the complementation of the *hog1-Δ1* mutant (Fig. 4). Together, these data demonstrate that the human Jnk1 protein kinase can functionally substitute for Hog1 in an osmosensing signal transduction pathway of yeast. Thus, the activation of Jnk1 in mammalian cells may be a component of the biological response to osmotic shock (Fig. 1).

The osmotic sensor that activates the Jnk protein kinase cascade is not known. Possible mechanisms include the effect of osmotic shock to stress focal-adhesion plaques and the cytoskeleton. Alternatively, by analogy with enteric bacteria (4) and yeast (6), there may be a role for a two-component pathway. The Jnk protein kinases may regulate the function of transporter proteins for ions and nonpolar solutes. They may also regulate the expression of cell-surface transporter proteins. Indeed, an important role for increased gene expres-

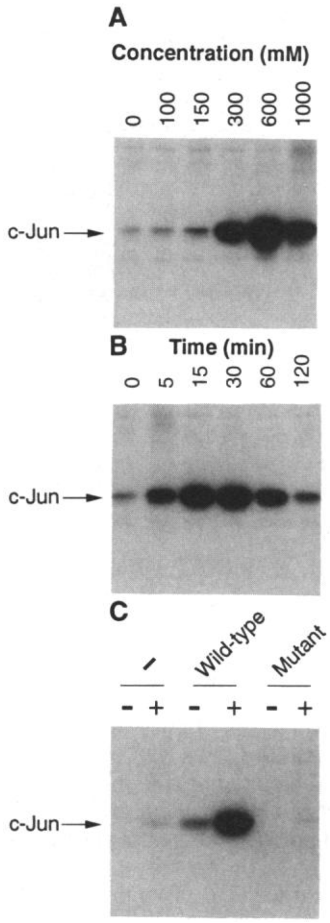

Fig. 1. Activation of the Jnk signal transduction pathway by osmotic shock. (**A**) Chinese hamster ovary cells were incubated in Ham's F12 medium supplemented with various concentrations of sorbitol for 1 hour at 37°C. The cells were harvested, and Jnk1 protein kinase activity was measured in an immune complex kinase assay with the substrate c-Jun (*14*). (**B**) The time course of Jnk1 protein kinase activation was measured in cells incubated in medium supplemented with 300 mM sorbitol (*14*). (**C**) Mutation of Jnk1 at the phosphorylation sites Thr[183] and Tyr[185] blocked the activation of Jnk1 protein kinase activity by osmotic shock. The presence (+) or absence (−) of sorbitol is indicated. CHO cells were transfected with vector (dash), wild-type Jnk1 (Thr[183]/Tyr[185]), and mutated Jnk1 (Ala[183]/Phe[185]). The cells were incubated in medium supplemented without or with 300 mM sorbitol for 15 min before measurement of Jnk1 protein kinase activity (*14*).

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                                                 I                 II            III               IV
JNK1      MSRSKRDNNFYSVEIGDSTFTVLKRYQNLKPIGSGAQGIVCAAYDAILERNVAIKKLSRPFQNQTHAKRAYRELVLMKCVNHKNIIGLLNVFTPQKSLEEF
HOG1      .T---TNEE.IRTQ.FGTV.EITN..ND.N.V.M..F.L..S.T.TLTSQP.....IMK..STAVL...T....K.L.HLR.E.L.C.QDI.-----.SPL
Consensus M        F    I    F    RY  L P G GA G VC A D      VAIKK   PF     AKR YREL L K   H N I L   F     L

                       V                 VIA                VIB              VII            * *  VIII
JNK1      QDVYIVMELMDANLCQVIQME-LDHERMSYLLYQMLCGIKHLHSAGIIHRDLKPSNIVVKSDCTLKILDFGLARTAGTSFMMTPYVVTRYYRAPEVILG-M
HOG1      E.I.F.T..QGTD.HRLL.TRP.EKQFVQ.F...I.R.L.YV....V..........LINEN.D...C......IQDPQ--..G..S........IM.TWQ
Consensus  D Y V EL    L    Q   L      Y LYQ L G K  HSAG IHRDLKPSNI     C LKI DFGLAR       MT YV TRYYRAPE  L

               IX                                X
JNK1      GYKENVDLWSVGCIMGEMVCHKILFPGRDYIDQWNKVIEQLGTPCP--EFMKKLQPTVRTYVENRPKYAGYSFEKLFPDVLFPADSEHNKLKASQARDLLS
HOG1      K.DVE..I..A...FA..IEG.P....K.HVH.FSIITDL..S.-.KDVINTICSENTLKF.TSL.HRDPIP.SER.K--TV----------EPD.V...E
Consensus  Y   VD WS GCI  EM   K LFPG D   Q       LG P P               V   P      F   F                  A DLL

                   XI
JNK1      KMLVIDASKRISVDEALQHPYINVWYDPS-EAEAPPPKIPDKQLDEREHTIEEWKELIYKEVMDLEERTKNGVIRGQPSPLAQVQQ
HOG1      ....F.PK...TAAD..A...SAPYH..TD.P--VADAKF.WHFNDADLPVDT.RVMM.S.IL.FHKIGGSDGQIDISATFDDQVA>
Consensus KMLV D  KRI    AL HPY     DP  E         D            W    Y E  D
```

Fig. 2. Similarity of Jnk1 to the yeast protein kinase Hog1. The human Jnk1 protein kinase and the yeast protein kinase Hog1 were compared with the BESTFIT program (version 7.2; P. Haeberli, Wisconsin Genetic Computer Group). Gaps introduced to optimize the alignment are illustrated with a dash, identical residues are shown with a period, and a consensus sequence is presented. The conserved protein kinase subdomains are shown with roman numerals I to XI, and the sites of Thr and Tyr phosphorylation are indicated with asterisks. The sequence of Hog1 that extends beyond the kinase domain is truncated (>). The identity (41.1%) and similarity (64.2%) were calculated with the BESTFIT program. The GenBank accession numbers for Jnk1 and Hog1 are L26318 and L06279, respectively (*15*).

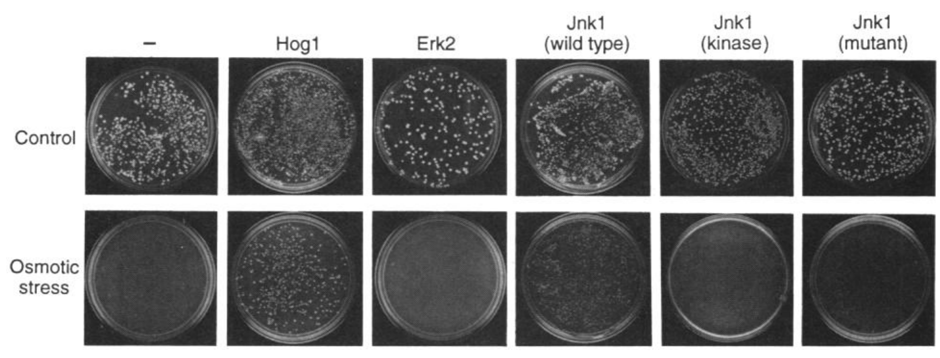

Fig. 3. Rescue of osmotically shocked yeast by Jnk1. Yeast lacking the protein kinase Hog1 (*hog1-Δ1*) were transformed with an empty expression vector (dash) and spread on agar plates supplemented without (control) or with 0.9 M NaCl (osmotic stress). Other cultures of yeast were transformed with the plasmid vector encoding Hog1, Erk2, wild-type Jnk1, a catalytically inactive Jnk1 (Lys[54] and Lys[55] substituted with Arg), or Jnk1 mutated at the sites of activating phosphorylation (Thr[183] and Tyr[185] substituted with Ala and Phe, respectively). Photographs of representative plates showing the growth of yeast colonies are presented (*16*).

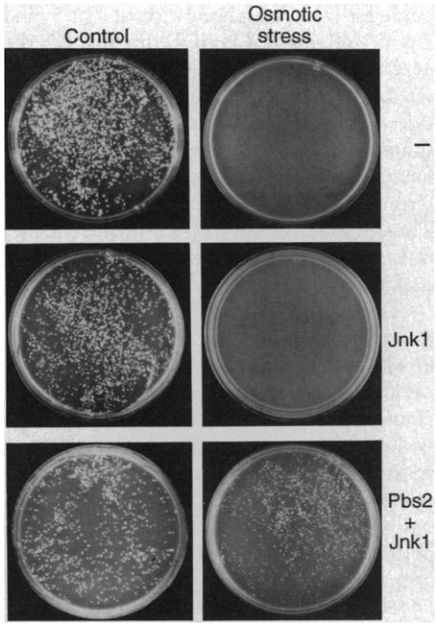

Fig. 4. Function of Jnk1 within an osmosensing signal transduction pathway requires the MAP kinase kinase Pbs2. Yeast lacking the protein kinases Hog1 (*hog1-Δ1*) and Pbs2 (*hog1-Δ1 pbs2-Δ1*) were transformed with expression vectors and spread on agar plates supplemented without (control) or with (osmotic stress) 0.9 M NaCl. Photographs of representative plates showing the growth of yeast colonies are presented (*16*).

sion in osmotically shocked cells has been established. Examples include the system A amino acid transporter (*2*) and the Na^+-inositol cotransporter (*3*). Thus, Jnk may regulate transcription factors that control the expression of these genes. The transcription factor c-Jun is known to bind to Jnk and to be phosphorylated at two sites within the NH_2-terminal activation domain (*8*, *9*), resulting in increased transcriptional activity (*11*). Furthermore, osmotic shock induces the expression of c-Jun (*12*) and, in some tissues, c-Fos (*13*). Increased activity of the transcription factor AP-1 may therefore be relevant to the osmotic shock response. Indeed, Jnk could participate in this process by activating AP-1 or other transcription factors.

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12. Osmotic shock induces the expression of c-Jun [G. Finkzeller, W. Newsome, F. Lang, D. Haüssinger, *FEBS Lett.* **340**, 163 (1994)]. The increase in c-Jun expression may be mediated by Jnk1 because activated c-Jun (phosphorylated by Jnk1) positively autoregulates c-Jun expression [P. Angel, K. Hattori, T. Smeal, M. Karin, *Cell* **55**, 875 (1988)].
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14. Chinese hamster ovary cells were co-transfected with the plasmids pCMV-Flag-Jnk1 and pRSV-Neo (*8*). A stable cell line expressing epitope-tagged Jnk1 (Flag; Immunex, Seattle, WA) was isolated by selection with Geneticin (Gibco-BRL). The cells were incubated with different concentrations of sorbitol for defined times at 37°C. The cells were collected in lysis buffer [20 mM tris (pH 7.4), 1% Triton X-100, 2 mM EDTA, 137 mM NaCl, 25 mM β-glycerophosphate, 1 mM orthovanadate, 2 mM pyrophosphate, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (10 μg/ml)], and a soluble extract was obtained by centrifugation at 100,000*g* for 30 min at 4°C. The epitope-tagged Jnk1 was isolated by immunoprecipitation with the monoclonal antibody M2 (Immunex). The immunoprecipitates were washed extensively with lysis buffer. Immunecomplex kinase assays were done in 25 μl of 25 mM Hepes (pH 7.4), 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM dithiothreitol, 100 μM orthovanadate, and 50 μM [γ-^{32}P] ATP (10 Ci/mmol) with 2.5 μg of bacterially expressed c-Jun (residues 1 to 79) fused to glutathione-S-transferase as a substrate (*8*).
15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
16. The Hog1 expression vector pJB17 and the yeast strains JBY10 (null mutant *hog1-Δ1*) and JBY1041 (null mutant *hog1-Δ1 pbs2-Δ1*) were obtained from M. Gustin (*5*). Human Erk2 [F. A. Gonzalez, D. L. Raden, M. R. Rigby, R. J. Davis, *FEBS Lett.* **304**, 170 (1992)] and Jnk1 (*8*) complementary DNAs have been described. Mutations of Jnk1 were constructed as described [S. N. Ho *et al.*, *Gene* **77**, 51 (1989)]. Expression vectors for Jnk1 and Erk2 were constructed by sub-cloning of the complementary DNAs at the Bam HI and Hind III sites of pVP16 [A. B. Vojtek, S. M. Hollenberg, J. A. Cooper, *Cell* **74**, 205 (1993)]. The structure of all plasmids was confirmed by sequencing with an Applied Biosystems (Foster City, CA) model 373A machine. The yeast were transformed with the plasmids, and equal portions were spread on agar plates supplemented without and with 0.9 M NaCl as described (*5*).

17. We thank M. Gustin for providing yeast strains and plasmids, C. Peterson for advice about complementation assays, T. Barrett for DNA sequencing, and M. Shepard for excellent secretarial assistance. We also thank M. Dickens, S. Gupta, and H. Sluss for discussions. Supported by a grant from the National Cancer Institute. R.J.D. is an Investigator of the Howard Hughes Medical Institute.

26 April 1994; accepted 21 June 1994

A MAP Kinase Targeted by Endotoxin and Hyperosmolarity in Mammalian Cells

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Mammalian cells respond to endotoxic lipopolysaccharide (LPS) by activation of protein kinase cascades that lead to new gene expression. A protein kinase, p38, that was tyrosine phosphorylated in response to LPS, was cloned. The p38 enzyme and the product of the *Saccharomyces cerevisiae HOG1* gene, which are both members of the mitogen-activated protein (MAP) kinase family, have sequences at and adjacent to critical phosphorylation sites that distinguish these proteins from most other MAP kinase family members. Both HOG1 and p38 are tyrosine phosphorylated after extracellular changes in osmolarity. These findings link a signaling pathway in mammalian cells with a pathway in yeast that is responsive to physiological stress.

Few extracellular stimuli elicit systemic responses in mammals that are as immediate and profound as those resulting from exposure to LPSs of Gram-negative bacteria. LPSs are endotoxins. They are prototypical activators of cells of the immune and inflammatory systems and initiate the systemic changes known as septic shock (*1*). Cells of monocytic origin [monocytes and macrophages (MOs)] respond to LPS by releasing mediators that produce septic shock (*2*). CD14, a glycosylphosphatidylinositol-anchored membrane glycoprotein of MOs, serves as an LPS receptor controlling cell activation under physiological conditions (*3*, *4*).

Binding of LPS to CD14 causes rapid protein tyrosine phosphorylation of a number of intracellular proteins that in some, but not all, LPS-responsive cells include the 44- and 42- or 40-kD isoforms of the mitogen-activated protein kinases (MAPK1 and MAPK2, respectively) (*5*, *6*). LPS also

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