vious observations that relate extreme leanness with delayed puberty (*4*) and obesity with acceleration of puberty (*5*). Thus, leptin may be a factor involved in signaling to neuroendocrine pathways the attainment of a critical fat mass, a determinant for triggering puberty (*2–4*). Kennedy first postulated that the hypothalamus receives a puberty-triggering signal related to metabolic rate or food intake (*2*, *15*), and later studies showed that the attainment of a critical percentage of body fat is necessary for initiation of puberty (*16*, *17*). Although the critical fat hypothesis has been challenged (*18*) and the metabolic signal postulated by Kennedy has remained elusive, our study suggests that leptin may be that signal.

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8. Human recombinant leptin was prepared (*7*) and quantitated with antibodies specific for human leptin (Linco Research, St. Louis, MO). C57BL/6J adult females (n = 24) weighing 24.1 ± 0.7 g (mean ± SEM) were divided into control (n = 6) and experimental groups (n = 18) and injected intraperitoneally (ip) with PBS and recombinant human leptin (4 μg per gram of body weight), respectively. PBS-treated mice were killed immediately after injection and leptin-treated mice were killed at 1, 2, 3, 4, 5, and 7 hours (three mice per time point). Blood was collected by cardiac puncture and the plasma was separated by centrifugation and frozen at −20°C. Leptin in 5 μl of plasma was quantitated by radioimmunoassay as described above.
9. All procedures were approved by the University of California, San Francisco (UCSF) Committee on Animal Research. Two-week-old lactating prepubertal C57BL/6J female pups were obtained from Jackson Laboratories (Bar Harbor, ME) and allowed to recover for 1 week before initiation of experiments. Male pups were removed from the litters at 2 weeks of age. Mice were housed at the UCSF Animal Care Facility and maintained at 20°C with a 12-hour light/12-hour dark cycle (lights on at 6:00 a.m. and off at 6:00 p.m.). Recombinant human leptin (2 μg/g) was prepared, quantitated (*7*, *8*), and injected ip once daily; control animals received equal amounts of PBS. Prepubertal C57BL/6J mice (n = 25) born on the same day were weaned at 21 days of age and housed in eight cages (three or four mice per cage; littermates were placed in different cages). Leptin-treated (n = 13) and PBS-treated (n = 12) mice weighed 10.2 ± 0.4 and 10.3 ± 0.3 g (mean ± SEM), respectively. Mice were monitored continuously for body weight, food intake, and vaginal opening. Unless indicated, P values in this study were calculated by unpaired Student's t test.
10. Young female mice were housed away from males and had no contact with males or their urine until day 27, when C57BL/6J breeder males were housed with the females (1 male per cage) to initiate mating. Plugged females were then housed individually in separate cages to assess whether the copulatory plug was associated with a successful pregnancy. PBS or leptin treatment was continued for 20 days after detection of the plug.
11. C57BL/6J females (n = 24) weighing 9.9 ± 0.2 g (mean ± SEM) were weaned at 21 days and divided equally into PBS and leptin groups. The mice were treated (*9*) for 8 days and killed in the afternoon of day 29; then blood, ovaries, oviducts, and uteri were collected. Each organ was dissected under a binocular microscope to ensure removal of contaminating tissues. Organs were weighed on a Mettler AE160 high-precision analytical balance.
12. In another experiment (*10*) not shown in Fig. 2A, 7 of 13 leptin-treated mice and 3 of 12 PBS-treated mice showed vaginal opening at 26 days, whereas the remaining mice in both groups showed vaginal opening at 27 days. The variability between experiments may be due to differences in initial body weights of the mice. Vaginal smears were examined daily after vaginal opening, and the relative abundance of leukocytes, nucleated epithelial cells, and cornified cells was determined independently by two investigators.
13. Plasma samples were assayed for LH (50 μl) and 17β-estradiol (200 μl) with, respectively, a mouse-rat immunoassay (Peninsula Laboratories, Belmont, CA) and a radioimmunoassay (Diagnostic System Laboratories, Webster, TX).
14. C57BL/6J females (n = 24) were weaned at 24 days of age and divided equally into PBS- and leptin-treated groups. Blood was collected by cardiac puncture at 30, 35, and 39 days of age about 20 hours after the last leptin injection (four mice per time point; one mouse from each cage). Plasma was separated from the cells and frozen at −20°C until use. Endogenous leptin was measured by a mouse-specific radioimmunoassay (Linco Research).
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Induction of Apoptosis by ASK1, a Mammalian MAPKKK That Activates SAPK/JNK and p38 Signaling Pathways

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Mitogen-activated protein (MAP) kinase cascades are activated in response to various extracellular stimuli, including growth factors and environmental stresses. A MAP kinase kinase kinase (MAPKKK), termed ASK1, was identified that activated two different subgroups of MAP kinase kinases (MAPKK), SEK1 (or MKK4) and MKK3/MAPKK6 (or MKK6), which in turn activated stress-activated protein kinase (SAPK, also known as JNK; c-Jun amino-terminal kinase) and p38 subgroups of MAP kinases, respectively. Overexpression of ASK1 induced apoptotic cell death, and ASK1 was activated in cells treated with tumor necrosis factor–α (TNF-α). Moreover, TNF-α–induced apoptosis was inhibited by a catalytically inactive form of ASK1. ASK1 may be a key element in the mechanism of stress- and cytokine-induced apoptosis.

The MAP kinase signaling cascade, a signal transduction pathway well conserved in cells from yeasts to vertebrates, consists of three distinct members of the protein kinase family, including MAP kinase (MAPK), MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK) (*1*). MAPKKK phosphorylates and thereby activates MAPKK, and the activated form of MAPKK in turn phosphorylates and activates MAPK. Activated MAPK may translocate to the cell nucleus and regulate the activities of transcription factors and thereby control gene expression (*1*). At least two defined MAPK signaling modules function in mammalian cells: the Raf-MAPKK-MAPK and the MEKK-SEK1 (or MKK4)–SAPK (or JNK) pathways (*2–7*). MKK3/MAPKK6 (or MKK6, a close relative of MKK3) that corresponds to MAPKK and p38 MAPK form another MAPK signaling unit (*2*, *8*); however, the biological consequence and mechanism of activation of the p38 signaling cascade are poorly understood. We identified a mammalian MAPKKK that activates the MKK3/MAPKK6-p38 as well as the SEK1-SAPK signaling pathways.

We used a degenerate polymerase chain reaction (PCR)–based strategy to identify serine-threonine kinases (*9*). One PCR fragment, obtained with a set of PCR primers oriented from the conserved subdomains VI and VIII of the serine-threonine kinase

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family (*10*), was used to isolate a corresponding nearly full-length cDNA clone (*11*). Because of its characteristics, we refer to the putative serine-threonine kinase encoded by this cDNA as apoptosis signal-regulating kinase (ASK)1. The deduced amino acid sequence revealed that ASK1 consists of 1375 amino acids with a calculated relative molecular mass of 154,715 (Fig. 1A). Another clone that is truncated in the NH_2-terminal 374 amino acids was also obtained (clone 27) (*12*). The serine-threonine kinase domain of ASK1 is in the middle part of the molecule and has long NH_2- and COOH-terminal flanking sequences. RNA blot analysis revealed a single 5-kb transcript that was expressed in all human tissues tested (Fig. 1B).

A database search of the ASK1 sequence outside its kinase domain showed that a short amino acid sequence in the NH_2-terminal part contains a motif for an FK506-binding protein (FKBP)–type peptidyl-prolyl *cis-trans* isomerase, of which the functional importance is unknown (Fig. 1A). The kinase domain of ASK1 has sequence similarity with members of the MAPKKK family including MEKK1 (30.0%) in mammal and SSK2 (32.3%) and STE11 (30.4%) in *Saccharomyces cerevisiae*. Phylogenetic comparison suggested that ASK1 is distantly related to RAF-1, KSR1, TAK1, and TPL-2 mammalian MAPKKKs but most closely related to the SSK2 or SSK22 family of yeast MAPKKKs, which are upstream regulators of yeast HOG1 MAPK (*13*).

Despite differences in the overall structures of ASK1 and SSK2 or SSK22 (*13*), it was of interest to examine whether ASK1 might act as a functional kinase in yeast and thereby complement the loss of SSK2 or

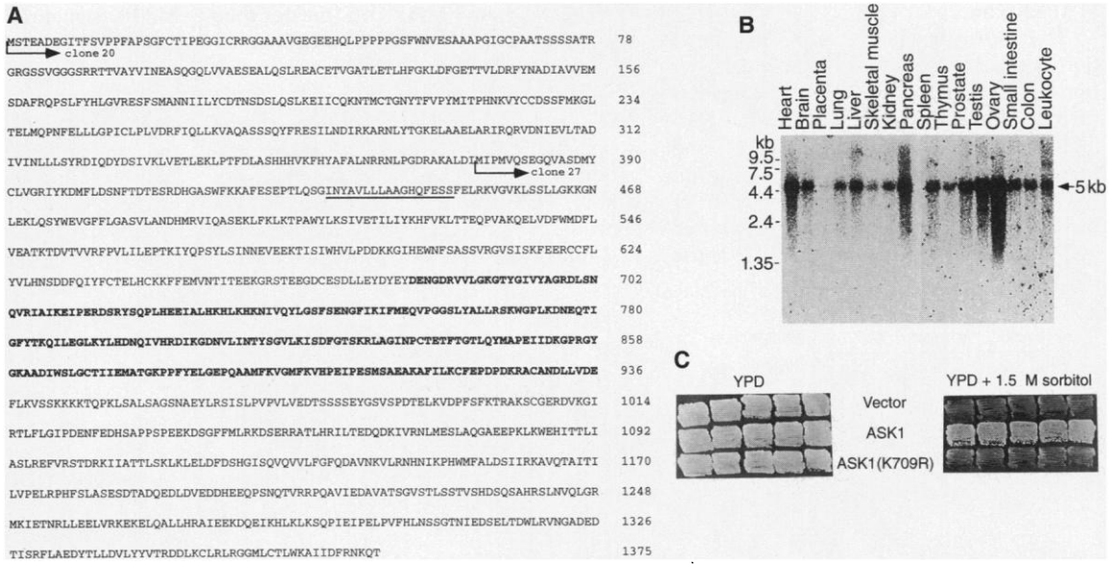

Fig. 1. Primary structure of ASK1 and complementation of SSK2/SSK22/SHO1 deficiency in *S. cerevisiae*. (**A**) Predicted amino acid sequence of human ASK1. The putative translation start sites for two independent cDNA clones, clone 20 and clone 27, are indicated by arrows. The protein kinase domain is shown in boldface. An FKBP-type peptidyl-prolyl *cis-trans* isomerase motif present in the NH_2-terminal noncatalytic portion is underlined. The cDNA sequence of ASK1 has been deposited in EMBL-GenBank data library (accession number D84476). 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. (**B**) Tissue distribution of ASK1. Immunoblots with mRNAs from various human tissues (Clontech) were probed with ASK1 cDNA labeled by random priming. (**C**) Complementation of SSK2/SSK22/SHO1 triple mutant strain in *S. cerevisiae*. Complementary DNA fragments encoding ASK1 were subcloned into the yeast expression vector pNV11 and transformed into TM257-H1 yeast strain (*13*), which is defective in SSK2, SSK22, and SHO1. Five independent transformants were selected and grown on YPD plates in the presence or absence of 1.5 M sorbitol. Photographs were taken after 6 days at 30°C. Transformants with pNV11 vector alone or ASK1(K709R) were also tested.

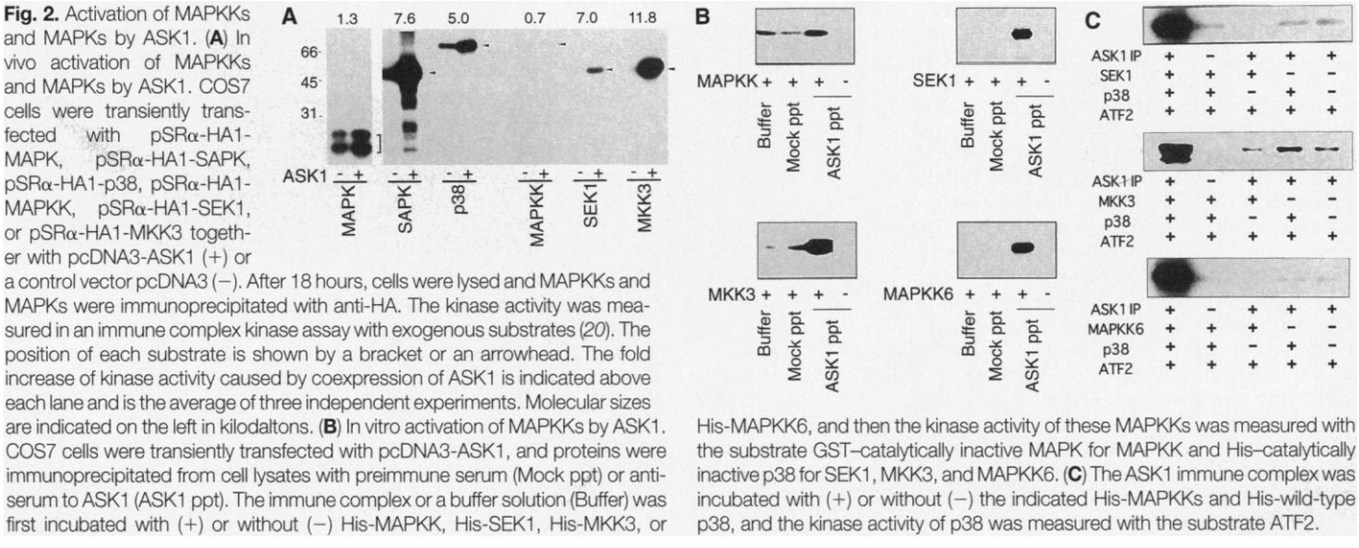

Fig. 2. Activation of MAPKKs and MAPKs by ASK1. (**A**) In vivo activation of MAPKKs and MAPKs by ASK1. COS7 cells were transiently transfected with pSRα-HA1-MAPK, pSRα-HA1-SAPK, pSRα-HA1-p38, pSRα-HA1-MAPKK, pSRα-HA1-SEK1, or pSRα-HA1-MKK3 together with pcDNA3-ASK1 (+) or a control vector pcDNA3 (−). After 18 hours, cells were lysed and MAPKKs and MAPKs were immunoprecipitated with anti-HA. The kinase activity was measured in an immune complex kinase assay with exogenous substrates (*20*). The position of each substrate is shown by a bracket or an arrowhead. The fold increase of kinase activity caused by coexpression of ASK1 is indicated above each lane and is the average of three independent experiments. Molecular sizes are indicated on the left in kilodaltons. (**B**) In vitro activation of MAPKKs by ASK1. COS7 cells were transiently transfected with pcDNA3-ASK1, and proteins were immunoprecipitated from cell lysates with preimmune serum (Mock ppt) or antiserum to ASK1 (ASK1 ppt). The immune complex or a buffer solution (Buffer) was first incubated with (+) or without (−) His-MAPKK, His-SEK1, His-MKK3, or His-MAPKK6, and then the kinase activity of these MAPKKs was measured with the substrate GST–catalytically inactive MAPK for MAPKK and His–catalytically inactive p38 for SEK1, MKK3, and MAPKK6. (**C**) The ASK1 immune complex was incubated with (+) or without (−) the indicated His-MAPKKs and His-wild-type p38, and the kinase activity of p38 was measured with the substrate ATF2.

SSK22. We used yeast strain TM257-H1 (*ssk2Δ ssk22Δ sho1Δ*) (*13, 14*), which grows in a normal YPD medium but not in hyperosmotic medium. Transformants were tested for growth in the presence of 1.5 M sorbitol (Fig. 1C). Expression of ASK1, but neither vector alone nor ASK1(K709R, a catalytically inactive mutant in which Lys^{709} was changed to Arg), complemented TM257-H1 growth in this hyperosmotic environment (*15*). These observations, together with the fact that the mammalian counterpart of yeast HOG1 is p38 MAP kinase (*16–18*), suggested that ASK1 may be a mammalian MAPKKK that functions in a signaling cascade to activate MKK3/MAPKK6 and p38.

To investigate whether ASK1 may function as a MAPKKK in mammalian cells, we transfected ASK1 into COS7 cells together with MAPK and MAPKK expression plasmids (Fig. 2A). All the MAPK and MAPKK constructs were hemagglutinin (HA) epitope–tagged, expressed with or without ASK1, and immunoprecipitated with antibody to HA (anti-HA) (*19*); the immune complexes were subjected to a kinase assay with exogenous substrates (*20*). ASK1 expression induced 7.6- and 5.0-fold activation of SAPK and p38 MAPKs, respectively, but only weakly activated MAPK. ASK1 activated SEK1 and MKK3 up to 7.0- and 11.8-fold, respectively, whereas no detectable activation of MAPKK was observed. To investigate whether the SEK1 and MKK3 were directly phosphorylated and activated by ASK1, we used an in vitro–coupled kinase assay (*21*) with recombinant SEK1, MKK3, MAPKK6, and recombinant catalytically inactive p38 (MPK2) proteins (Fig. 2B). ASK1 immunoprecipitates from COS7 cells activated SEK1, MKK3, and MAPKK6 (greater than 40-fold for each) (Fig. 2B), and phosphorylation of p38 was observed only when ASK1 was present in the kinase assay. ASK1-dependent phosphorylation of p38 was further confirmed to result in the activation of p38 in a coupled kinase assay with wild-type p38 and ATF2 (*21*) (Fig. 2C). In contrast, ASK1 weakly activated MAPKK (2.2-fold) (Fig. 2B). When Raf was used as MAPKKK for a positive control, a 27.8-fold activation of MAPKK was observed (*22*). These results indicate that ASK1 selectively activates the SEK1-SAPK and MKK3/MAPKK6-p38 pathways.

We determined the biological activity of ASK1 in mink lung epithelial (Mv1Lu) cell lines that were stably transfected with metallothionein promoter–based expression plasmids (*23*). Immunoprecipitation of ASK1 with antiserum to ASK1 (*24*) after metabolic labeling of the cells revealed that ASK1 was highly expressed only when induced by $ZnCl_2$ (Fig. 3A). ASK1(K709R)-transfected cells expressed the recombinant protein in similar amounts. SAPK or p38 MAPK signaling cascade or both may participate in signaling pathways leading to apoptosis (*25–28*). To investigate the effects of ASK1 on cellular growth or viability, we measured [^{3}H]thymidine incorporation in the stable transfectants. Inhibition of [^{3}H]thymidine incorporation was observed in the cells transfected with ASK1 but not in cells transfected with the vector alone or ASK1(K709R) (Fig. 3B). The dose-depen-

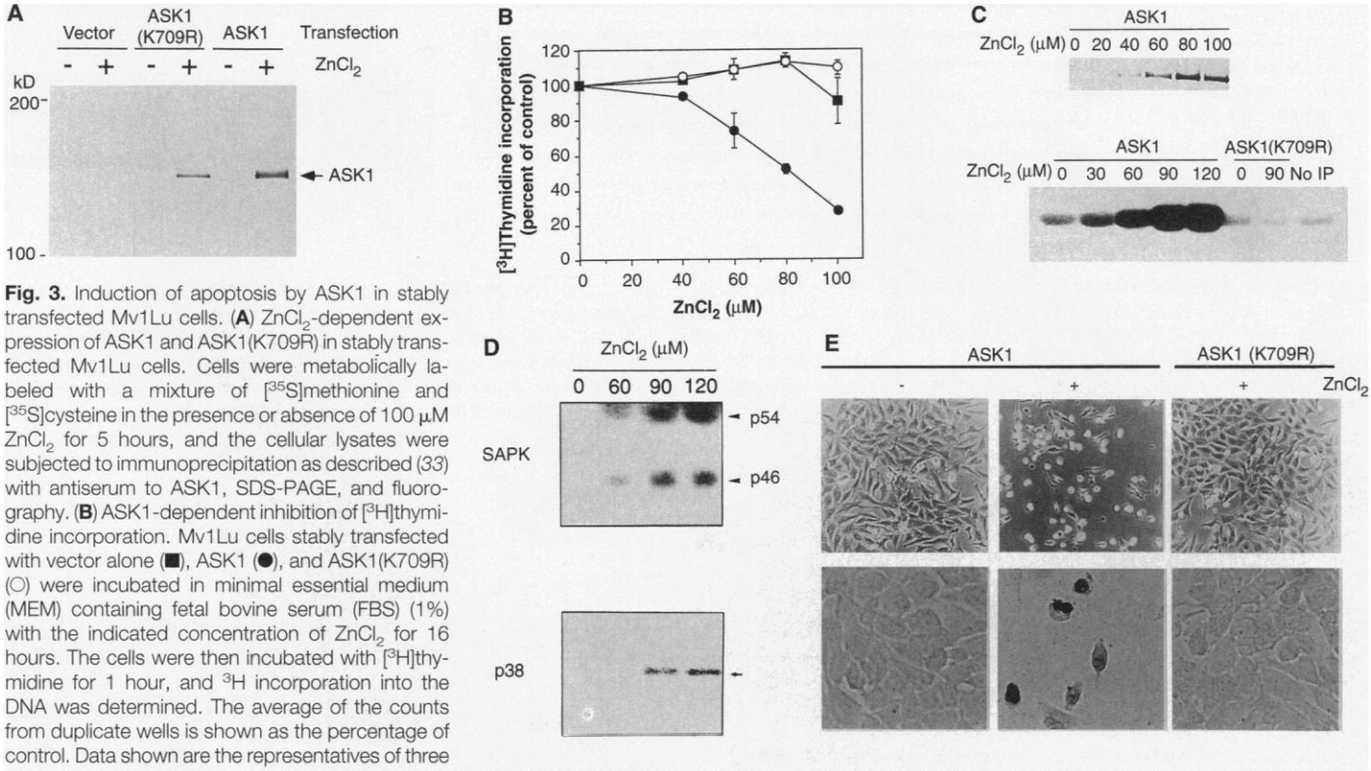

Fig. 3. Induction of apoptosis by ASK1 in stably transfected Mv1Lu cells. (**A**) $ZnCl_2$-dependent expression of ASK1 and ASK1(K709R) in stably transfected Mv1Lu cells. Cells were metabolically labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine in the presence or absence of 100 μM $ZnCl_2$ for 5 hours, and the cellular lysates were subjected to immunoprecipitation as described (*33*) with antiserum to ASK1, SDS-PAGE, and fluorography. (**B**) ASK1-dependent inhibition of [^{3}H]thymidine incorporation. Mv1Lu cells stably transfected with vector alone (■), ASK1 (●), and ASK1(K709R) (○) were incubated in minimal essential medium (MEM) containing fetal bovine serum (FBS) (1%) with the indicated concentration of $ZnCl_2$ for 16 hours. The cells were then incubated with [^{3}H]thymidine for 1 hour, and ^{3}H incorporation into the DNA was determined. The average of the counts from duplicate wells is shown as the percentage of control. Data shown are the representatives of three independent experiments. Error bars represent the SD. (**C**) Dose-dependent expression (top) and activation (bottom) of ASK1 by $ZnCl_2$ induction. (Top) ASK1 was immunoprecipitated as described in (A) after the ASK1-transfected Mv1Lu cells were induced by the indicated amount of $ZnCl_2$ for 5 hours. (Bottom) Immunoprecipitated ASK1 was subjected to the MKK3-p38 coupled kinase assay. (**D**) ASK1-dependent activation of endogenous SAPK (top) and p38 (bottom). Mv1Lu cells stably transfected with ASK1 were incubated with the indicated concentration of $ZnCl_2$ for 5 hours. The cell extracts were assayed for SAPK activity (*29*) with gel-immobilized c-Jun as a substrate (top), or for p38 activity in an immune complex kinase assay (*29*) with a polyclonal antibody to p38 with His-ATF2 as a substrate (bottom). The positions of p54 and p46 SAPKs (top) and ATF2 (bottom) are indicated by arrowheads and an arrow, respectively. (**E**) ASK1-dependent cell death. (Top) Cells were incubated with MEM containing FBS (1%) in the presence (+) or absence (−) of 100 μM $ZnCl_2$ for 26 hours. Representative cell morphology was determined by phase-contrast microscopy (original magnification, ×33). (Bottom) Cells were incubated in MEM without FBS in the presence or absence of 100 μM $ZnCl_2$ for 25 hours and stained by the TUNEL method with an in situ cell death detection kit (Boehringer Mannheim). Apoptotic cells are shown by dark brown staining. Photographs were taken at a higher magnification (×82.5) than in the top panel.

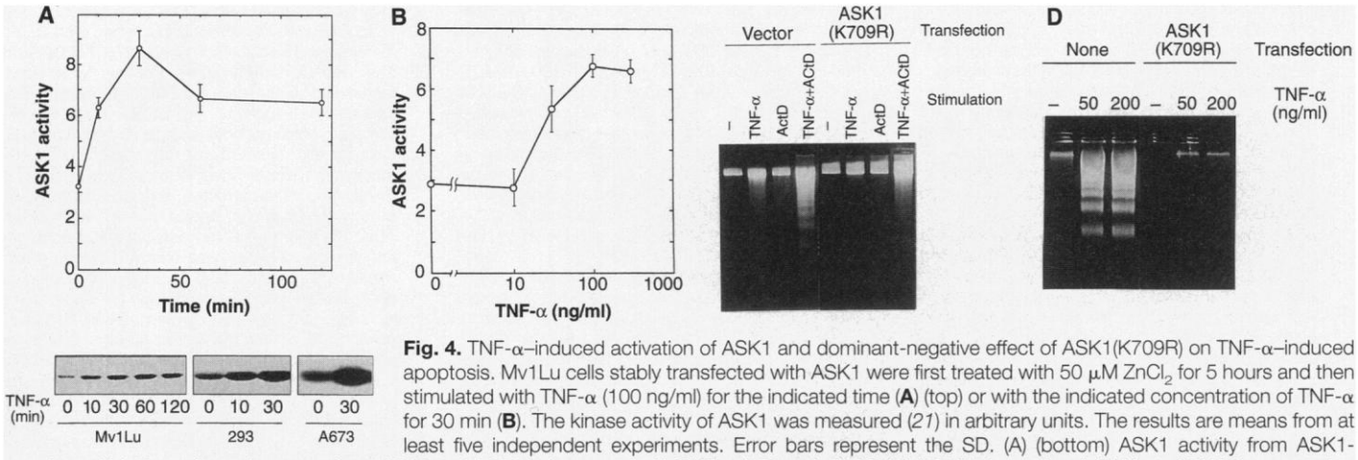

Fig. 4. TNF-α–induced activation of ASK1 and dominant-negative effect of ASK1(K709R) on TNF-α–induced apoptosis. Mv1Lu cells stably transfected with ASK1 were first treated with 50 μM $ZnCl_2$ for 5 hours and then stimulated with TNF-α (100 ng/ml) for the indicated time (**A**) (top) or with the indicated concentration of TNF-α for 30 min (**B**). The kinase activity of ASK1 was measured (*21*) in arbitrary units. The results are means from at least five independent experiments. Error bars represent the SD. (A) (bottom) ASK1 activity from ASK1-transfected Mv1Lu cells and nontransfected 293 and A673 cells treated with TNF-α (100 ng/ml). ASK1(K709R)-dependent inhibition of DNA fragmentation in TNF-α–treated 293 (**C**) and Jurkat (**D**) cells. 293 cells (2×10^6) were transiently transfected with 2 μg of pcDNA3 control vector or pcDNA3-ASK1(K709R) by the use of Tfx-50 (Promega) according to the manufacturer's protocol. Eight hours after transfection, cells were treated with TNF-α (100 ng/ml) with or without 300 nM actinomycin D (ActD, which enhanced TNF-α–induced apoptosis) for 16 hours. Apoptotic cells detached from culture plate were collected, and total DNA was isolated and analyzed by 2% agarose gel electrophoresis. This experiment was repeated more than five times with similar results. In (D), 3×10^6 nontransfected Jurkat cells or selectively isolated populations of ASK1(K709R)-expressing Jurkat cells (*32*) were treated with the indicated concentrations of TNF-α for 5.5 hours. Cytoplasmic DNA was extracted (*35*) and analyzed by 2% agarose gel electrophoresis.

dent inhibition of [^{3}H]thymidine incorporation by $ZnCl_2$ correlated well with the dose-dependent expression and activation of ASK1 (Fig. 3C). Furthermore, endogenous SAPK and p38 were activated (*29*) in parallel with the ASK1 activities (Fig. 3D).

Induction of expression of ASK1 but not ASK1(K709R) induced cytoplasmic shrinkage and nuclear condensation, which became apparent within 6 hours after addition of $ZnCl_2$ (*30*). The typical morphological properties of apoptotic cells became most evident after more than 24 hours (Fig. 3E). The apoptotic cell death induced by ASK1 was confirmed by an in situ staining of cells with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method (Fig. 3E) as well as by genomic DNA fragmentation (*30*). Taken together, these observations indicate that overexpression of ASK1 results in apoptosis.

Tumor necrosis factor–α (TNF-α) causes apoptosis and activates both SAPK and p38 signaling systems (*4*, *31*). We therefore examined whether treatment of cells with TNF-α resulted in the activation of ASK1. ASK1 immunoprecipitates from TNF-α–treated cells were subjected to a coupled kinase assay with MKK3 and catalytically inactive p38 (Fig. 4, A and B). Treatment of cells with TNF-α activated the ASK1 in ASK1-transfected Mv1Lu cells within 5 min (Fig. 4A) in a dose-dependent manner (Fig. 4B). In addition, endogenous ASK1 was also activated by TNF-α in other cell types in which apoptosis is induced by TNF-α, including human 293 embryonal kidney cells, A673 rhabdomyosarcoma cells (Fig. 4A), Jurkat T cells, and KB epidermal carcinoma cells (*22*). Furthermore, when ASK1(K709R) was transiently transfected into 293 cells (transfection efficiency was greater than 60% as determined by β-galactosidase staining) (Fig. 4C) or Jurkat cells (*32*) (Fig. 4D), DNA fragmentation induced by TNF-α was reduced, suggesting that ASK1(K709R) acts as a dominant-negative mutant, and that ASK1 is necessary for the TNF-α–induced apoptotic response.

Our results suggest that proinflammatory cytokines may activate ASK1, which contributes to cellular apoptosis, possibly by activating SEK1-SAPK and MKK3/MAPKK6-p38 signaling cascades. SAPK and p38 signaling pathways are both indispensable for apoptosis triggered by nerve growth factor withdrawal in differentiated neuronal cells (*25*). However, a possibility that ASK1-induced apoptosis in our system is mediated by effector pathways other than SAPK and p38 is not excluded. ASK1 might be a constitutively active kinase because ASK1 that was transiently expressed in COS7 cells or induced by $ZnCl_2$ in Mv1Lu cells was active in vitro and in vivo. However, overexpression of ASK1 might result in activation, or the transfection procedure or the treatment with a heavy metal may subject the cells to stress, and thereby activate ASK1. Thus, identification of regulatory mechanisms of ASK1 should provide insights into signaling pathways leading to apoptosis.

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11. An amplified oligo(dT)-primed λgt11 cDNA library from human erythroleukemia (HEL) cells [M. Poncz *et al.*, *Blood* **69**, 219 (1987)] was screened with a ^{32}P-labeled PCR fragment. Hybridization and purification of positive bacteriophage were performed as described (*33*). Nucleotide sequencing was done on both strands with a Sequenase DNA sequencing kit (U.S. Biochemical). Among 18 clones obtained, the 3 longest clones, termed clone 20, clone 27, and clone 72, were entirely sequenced. The sequence of clone 72 started from the middle of the open reading frame and ended by a stretch of polyadenylate. The sequences of clones 20 and 27 covered the 5′ part of ASK1 cDNA, and the overlapping parts with clone 72 were identical in sequence. The ASK1 cDNA sequence, combining the clone 20 and clone 72, yielded a 4533–base pair (bp) sequence with an ATG codon starting at position 268 followed by a 4175-bp open reading frame encoding 1375 amino acids.
12. Clone 27 contained a 4-bp deletion at position 805 to 808 with in-frame upstream stop codons, resulting in an NH_2-terminally truncated protein product of ASK1 (Fig. 1A). Although the functional importance of this truncated form of ASK1 is unknown, the longer form of ASK1 derived from the overlapping clones 20 and 72 was used for the functional studies throughout this report.
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14. ASK1 cDNA was introduced into a yeast expression plasmid pNV11 [H. Shibuya *et al.*, *Nature* **357**, 700 (1992)]. SHO1 is an SH3 domain–containing transmembrane osmosensor that constitutes another signaling pathway leading to hyperosmolarity responses by way of HOG1 activation independently of SSK2 or SSK22 (*13*). Single or double mutant strains of SHO1, SSK2, or SSK22 are resistant to hyperosmotic medium; however, strains with defects in SHO1, SSK2, and SSK22 are unable to grow in hyperosmotic medium.
15. ASK1 could not restore the osmotic response in a PBS2 [downstream target of SHO1, SSK2, and SSK22 (*13*)]–defective yeast strain (K. Irie and K. Matsumoto, unpublished data), which indicates that ASK1 activity observed in TM257-H1 was mediated by the PBS2-HOG1 signaling pathway.
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19. *Xenopus* MAPK [Y. Gotoh *et al.*, *EMBO J.* **10**, 2661 (1991)] and *Xenopus* MAPKK (*34*) were cloned as described. Coding regions for rat SAPKα (*4*), human p38 [J. Han, B. Richter, Z. Li, V. V. Kravchenko, R. J. Ulevitch, *Biochim. Biophys. Acta* **1265**, 224 (1995)], mouse SEK1 (*5*), and human MKK3 (*6*) were amplified by PCR. An HA tag was introduced into the Bgl II and Eco RI sites of a mammalian expression vector pSRα456 [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)], yielding pSRα-HA1. The cDNAs encoding MAPK, SAPKα, p38, MAPKK, SEK1, and MKK3 were subcloned into the Bgl II site of pSRα-HA1. ASK1 cDNA was introduced into another mammalian expression vector, pcDNA3 (Invitrogen). For transient expression, COS7 cells were transfected with lipofectamine (Life Technologies) according to the manufacturer's instructions. For preparing extracts, cells were lysed in a buffer solution containing 20 mM tris-HCl (pH 7.5), 12 mM β-glycerophosphate, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 3 mM dithiothreitol (DTT), 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and aprotinin (20 μg/ml). Cell extracts were clarified by centrifugation at 15,000*g* for 10 min. For immunoprecipitation, the supernatants were incubated with polyclonal antiserum to ASK1 (*24*) or monoclonal antibody to HA (12CA5) for 1 hour at 4°C. After the addition of protein A–Sepharose (Pharmacia Biotech), the lysates were incubated for an additional 1 hour. The beads were washed twice with a solution containing 500 mM NaCl, 20 mM tris-HCl (pH 7.5), 5 mM EGTA, 1% Triton X-100, 2 mM DTT, and 1 mM PMSF, then twice with a solution containing 150 mM NaCl, 20 mM tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, and 1 mM PMSF and subjected to kinase assays.
20. Myelin basic protein was from Sigma. ATF2 was provided by S. J. Baker and T. Curran (St. Jude Children's Research Hospital). Hexahistidine (His)-tagged c-Jun (*7*) and glutathione-S-transferase (GST)–catalytically inactive (K57D) *Xenopus* MAPK (*34*) were prepared as described. MPK2 (*16*), a *Xenopus* counterpart of mammalian p38, was used as a substrate for SEK1 and MKK3. SEK1 phosphorylates and activates p38 as well as SAPK at least in vitro (*6*). His-tagged catalytically inactive (K54R) p38 was prepared as described [T. Moriguchi *et al.*, *J. Biol. Chem.* **270**, 12969 (1995)]. To measure the activity to phosphorylate MBP, c-Jun, ATF2, or catalytically inactive MAPK or p38, we incubated the immune complex for 30 min at 30°C with 3 μg of each substrate in a final volume of 25 μl of a solution containing 20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP (adenosine triphosphate) (0.3 μCi). The reaction was stopped by addition of Laemmli's sample buffer and boiling. After SDS–polyacrylamide gel electrophoresis (PAGE), phosphorylation of these proteins was quantified with an image analyzer (Fujix BAS2000).
21. His-tagged *Xenopus* MAPKK and SEK1 (XMEK2) and human MKK3 and MAPKK6 were bacterially expressed and purified as described [Y. Gotoh *et al.*, *Oncogene* **9**, 1891 (1994)]. To measure the activity of an immune complex, we first incubated 0.2 μg of His-MAPKK, His-SEK1, His-MKK3, or His-MAPKK6 with the immune complex for 15 min at 30°C in a final volume of 25 μl of a solution containing 20 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 100 μM ATP and subsequently for 7 min at 25°C with 0.3 μCi of [γ-^{32}P]ATP and 3 μg of GST–catalytically inactive MAPK (for MAPKK) or His-tagged catalytically inactive p38 (for SEK1, MKK3, and MAPKK6) in the same solution (final volume, 35 μl). To measure the kinase activity of wild-type p38, we used His-tagged wild-type p38 and ATF2 instead of catalytically inactive p38. Samples were analyzed by SDS-PAGE and image analyzer.
22. Y. Gotoh and E. Nishida, unpublished data.
23. To avoid the possibility that constitutively expressed ASK1 might induce cell death, resulting in a failure to obtain stable transformants, we used a metallothionein-inducible promoter system. ASK1 and ASK1(K709R) cDNAs were subcloned into pMEP4 vector (Invitrogen) at convenient enzyme cleavage sites. Transfection of cDNAs was done with Transfectam (Promega) according to the manufacturer's instructions, and selection by hygromycin B was done as described [M. Saitoh *et al.*, *J. Biol. Chem.* **271**, 2769 (1996)]. Several independent colonies were cloned, and the expression of ASK1 protein was determined by immunoprecipitation (*33*) with antiserum to ASK1 (*24*). Two independent positive clones were used for the assays with essentially the same results.
24. Antiserum to ASK1 was raised against the peptide sequence TEEKGRSTEEGDCESD (amino acids 554 to 669) that was coupled to keyhole limpet hemocyanin by a glutaraldehyde method, mixed with Freund's adjuvant, and used to immunize rabbits as described (*33*).
25. Z. Xia, M. Dickens, J. Raingeaud, R. J. Davis, M. E. Greenberg, *Science* **270**, 1326 (1995).
26. Y.-R. Chen, C. F. Meyer, T.-H. Tan, *J. Biol. Chem.* **271**, 631 (1996).
27. N. L. Johnson *et al.*, *ibid.*, p. 3229.
28. M. Verheij *et al.*, *Nature* **380**, 75 (1996).
29. To measure the activity of SAPK, we subjected each cell extract to a kinase detection assay within a polyacrylamide gel (in-gel kinase assay) containing c-Jun as a substrate, as described (*7*). To examine the activity of p38, we immunoprecipitated p38 with polyclonal antibody to p38 (C-20, Santa Cruz) as described (*19*) except for the presence of 0.1% SDS during the immunoprecipitation, and the kinase activity was detected with ATF2 as a substrate.
30. H. Ichijo and K. Miyazono, unpublished data.
31. J. Raingeaud *et al.*, *J. Biol. Chem.* **270**, 7420 (1995).
32. The pcDNA3-ASK1(K709R) plasmid was transfected into Jurkat cells by DMRIE-C reagent (Life Technologies) together with pHook-1 plasmid (Invitrogen), which encodes a single-chain antibody fusion protein directed to the hapten phOx (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) and thereby allows the selective isolation of transfected cells with magnetic beads coated with phOx. ASK1(K709R)-transfected populations of cells (co-transfection efficiency was nearly 100% as determined by β-galactosidase staining) were isolated on phOx-coated magnetic beads with the Capture-Tec kit (Invitrogen), allowed to grow, counted, and treated with TNF-α. Nontransfected Jurkat cells and isolated Jurkat cells that were transfected with pHook-1, and control pcDNA3 plasmids were similarly sensitive to TNF-α in the DNA fragmentation assay (*30*).
33. H. Ichijo *et al.*, *J. Biol. Chem.* **268**, 14505 (1993).
34. H. Kosako, E. Nishida, Y. Gotoh, *EMBO J.* **12**, 787 (1993).
35. Cytoplasmic small fragmented DNA was isolated as described [K. S. Selins and J. J. Cohen, *J. Immunol.* **139**, 3199 (1987)] with minor modifications. Briefly, 3×10^6 cells were lysed with 200 μl of a buffer containing 20 mM tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% Triton X-100. Cell extracts were clarified by centrifugation at 15,000*g* for 5 min. The lysates were incubated with proteinase K (0.2 mg/ml) and ribonuclease A (0.1 mg/ml) at 42°C for 1 hour. DNA was then purified by ethanol precipitation after phenol-chloroform extraction.
36. We thank S. J. Baker and T. Curran for ATF2; T. Maeda for TM257-H1; M. Poncz for HEL cDNA library; H. Okazaki and T. Sudo (Kirin Brewery, Japan) for oligonucleotides and advice; T. Kitagawa and C.-H. Heldin for valuable comments; A. Hanyu for technical assistance; U. Engström for preparing the synthetic peptide; and K. Saeki, T. Inage, K. Takeda, H. Nishitoh, and K. Tobiume for discussion. Supported by Grants-in-Aid for scientific research from the Ministry of Education, Science, and Culture of Japan. H.I. and K.M. are supported by grants from Mochida Memorial Foundation for Medical and Pharmaceutical Research and Toray Scientific Foundation.

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TECHNICAL COMMENTS

Detecting Phases of Iron

C. S. Yoo *et al.* (*1*) conducted in situ heating experiments on Fe at pressures (*P*) up to 130 GPa and conclude that (i) the β phase recognized by Saxena *et al.* (*2*) and Boehler (*3*) does not exist, but instead that there is an ε′ phase which occupies part of the stability field of the γ phase (FCC); (ii) only the ε (HCP) phase is stable above 50 GPa; and (iii) there may be a new phase appearing above *P* of 110 GPa and a temperature (*T*) of 3000 K. None of these conclusions is warranted on the basis of the x-ray data as presented by Yoo *et al.* (*1*). We have used the same experimental facilities (*4*) and obtained results that show evidence of transformation of ε (HCP) to a DHCP structure. On the basis of our further work (*5*), we confirm that the DHCP phase is indeed the β phase and that its stability extends beyond *P* of 50 GPa.

Our difficulty in accepting the results of Yoo *et al.* (*1*) is apparent from the data in figure 4 of their report: There is a striped area representing an ε′ phase of Fe in the middle of the γ phase field; no x-ray pattern of this ε′ phase is given in the report. The *PT* of the γ phase field is established from the results of several different workers on the basis of different techniques, including resistance-wire heating performed by one of the co-authors of the Yoo *et al.* (*1*) report. The triple point γ(or β)-ε-melt was located at 76.5 ± 4 GPa and 2950 ± 100 K by Saxena *et al.* (*2*) after consideration of all the available experimental data. There is no discussion of why all this data should be abandoned in favor of a study based on the use of an unstabilized laser.