was either unaffected (Fig. 3, A and B) or only partially inhibited (50%) (Fig. 3C).

Thus, like calnexin and calreticulin, ERp57 binding required glucose trimming of the N-linked carbohydrate side chains. We propose that ERp57 functions in combination with calnexin and calreticulin as a molecular chaperone of glycoprotein biosynthesis. We observed a time-dependent decrease in the amount of the PL62.CHO cross-linking products with calnexin, calreticulin, and the associated ERp57 (*18*). This suggested that the interaction between ERp57 and nascent glycoproteins was transient, like other molecular chaperone-substrate interactions (*3*, *5*, *19*). We believe a specific modulation of glycoprotein folding could be achieved by coupling the lectin-like properties of calnexin and calreticulin (*2*) with the thiol-dependent reductase activity of ERp57 (*14*).

REFERENCES AND NOTES

1. M.-J. Gething and J. Sambrook, *Nature* **355**, 33 (1992).
2. C. Hammond and A. Helenius, *Curr. Opin. Cell Biol.* **7**, 523 (1995).
3. W.-J. Ou, P. H. Cameron, D. Y. Thomas, J. J. M. Bergeron, *Nature* **364**, 771 (1993); J. R. Peterson, A. Ora, P. N. Van, A. Helenius, *Mol. Biol. Cell* **6**, 1173 (1995); W. M. Nauseef, S. J. McCormick, R. A. Clark, *J. Biol. Chem.* **270**, 4741 (1995).
4. C. Hammond, I. Braakman, A. Helenius, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 913 (1994).
5. D. N. Hebert, B. Foellmer, A. Helenius, *Cell* **81**, 425 (1995).
6. J. D. Oliver, R. C. Hresko, M. Mueckler, S. High, *J. Biol. Chem.* **271**, 13691 (1996).
7. C. Labriola, J. J. Cazzulo, A. J. Parodi, *J. Cell Biol.* **130**, 771 (1995); C. G. Parker, L. I. Fessler, R. E. Nelson, J. H. Fessler, *EMBO J.* **14**, 1294 (1995).
8. J. J. M. Bergeron, M. B. Brenner, D. Y. Thomas, D. B. Williams, *Trends Biochem. Sci.* **19**, 124 (1994).
9. A. Helenius, *Mol. Biol. Cell* **5**, 253 (1994).
10. S. M. Hurtley and A. Helenius, *Annu. Rev. Cell Biol.* **5**, 277 (1989).
11. PPL92.Con encodes the NH_2-terminal 92 amino acids of the wild-type bovine preprolactin sequence followed by a stop codon. PPL92.CHO encodes the NH_2-terminal 88 amino acids of the wild-type sequence, followed by 4 amino acids incorporating a consensus site for N-linked glycosylation, and finishing with a stop codon. PPL92.Con was prepared by means of the polymerase chain reaction; PPL92.CHO was prepared by the ligation of a linker to the 3′ end of Pvu II–cut PPL cDNA. The amino acid sequence of the COOH-terminal 10 residues of each polypeptide is as follows: PPL92.Con: Met-Ala-Leu-Asn-Ser-Cys-His-Thr-Ser-Ser, PPL92.CHO: Met-Ala-Leu-Asn-Ser-Cys-Asn*-Ser-Thr-Ser (where * is the glycosylation site). After import and cleavage, the resulting polypeptides, PL62.Con (apparent molecular mass 7 kD) and PL62.CHO (apparent molecular mass 9 kD), have potential SMCC reactive groups at residues 4, 11, and 58 (Cys) and residues 42 and 48 (Lys), in addition to the free amino group at the NH_2-terminus.
12. Antisera recognizing ERp57, the heavy chain–binding protein (BiP), and a 55-kD ER calcium-binding protein (ERC-55) were tested. Of these, only ERp57 was found to be cross-linked to PL62.CHO and no cross-linking products with PL62.Con were detected.
13. ERp57 is also known as GRP58, ERp61, ER-60, Q-2, and HIP-70 [C. F. Bennett, J. M. Balcarek, A. Varrichio, S. T. Crooke, *Nature* **334**, 268 (1988); S. P. Srivastava, N. Chen, Y. Liu, J. L. Holtzman, *J. Biol. Chem.* **266**, 20337 (1991); N. Hirano *et al.*, *Biochem. Biophys. Res. Commun.* **204**, 375 (1994)].
14. S. P. Srivastava, J. A. Fuchs, J. L. Holtzman, *Biochem. Biophys. Res. Commun.* **192**, 971 (1993); N. Hirano *et al.*, *Eur. J. Biochem.* **234**, 336 (1995).
15. R. Urade, M. Nasu, T. Moriyama, K. Wada, M. Kito, *J. Biol. Chem.* **267**, 15152 (1992); M. Otsu, R. Urade, M. Kito, F. Omura, M. Kikuchi, *ibid.* **270**, 14958 (1995).
16. Dog pancreas microsomes were solubilized in 1% (w/v) saponin and centrifuged at 100,000*g*, and the supernatant was applied to a concanavalin A–Sepharose column to remove glycoproteins. The unbound material was precipitated with $(NH_4)_2SO_4$, dialyzed against 10 mM tris-HCl (pH 7.5), and applied to a Resource Q column (Pharmacia). Bound proteins were eluted with a 0 to 1 M NaCl gradient; ERp57 eluted as a single protein and was identified by its molecular weight and by NH_2-terminal amino acid sequencing. The purified protein was then used to raise a rabbit polyclonal antibody. The resulting anti-ERp57 only functions for immunoprecipitation after SDS denaturation (Fig. 1B); thus, a reciprocal coimmunoprecipitation experiment (Fig. 2B) using anti-ERp57 in the first round was not possible.
17. After import of *S. cerevisiae* prepro–α factor cDNA (in pGEM) into microsomes, the resulting protein has three sites for N-linked glycosylation and nine lysine residues from which cross-linking may occur. Human IFN-γ has two sites for N-linked glycosylation, one cysteine residue, and 20 lysine residues. The transcription vector used was as described [N. J. Bulleid, E. Curling, R.B. Freedman, N. Jenkins, *Biochem. J.* **268**, 777 (1990)].
18. When the addition of SMCC to isolated microsomes was delayed for increasing lengths of time (*19*), the efficiency of cross-linking between PL62.CHO and ER components decreased. No loss of nascent PL62.CHO was observed over the same period. The estimated half-life for the PL62.CHO cross-linking products was 60 min for adducts with calnexin, calreticulin, and associated ERp57, and >120 min for the adduct with PDI.
19. P. Klappa, R. B. Freedman, R. Zimmermann, *Eur. J. Biochem.* **232**, 755 (1995).
20. D. Stueber, I. Ibrahimi, D. Cutler, B. Dobberstein, H. Bujard, *EMBO J.* **3**, 3143 (1984).
21. S. High *et al.*, *J. Cell Biol.* **121**, 743 (1993).
22. Cell-free translation was carried out under reducing conditions, protein synthesis was inhibited by the addition of emetine to a final concentration of 2 mM, and the membranes were isolated by centrifugation. The membranes were resuspended in LS buffer [250 mM sucrose, 100 mM KOAc, 5 mM $Mg(OAc)_2$, and 50 mM Hepes-KOH (pH 7.9)], which permits the oxidation of full-length prolactin, and the samples were treated with puromycin for 5 min. SMCC was added to a final concentration of 1 mM from a 50 mM stock in DMSO; samples were incubated for 10 min and then quenched by addition of 0.1 volumes of 50 mM 2-mercaptoethanol and 500 mM glycine. All reactions were carried out at the translation temperature of 26°C.
23. Rabbit antibodies to canine ERp57 were purified from crude serum by affinity purification using protein immobilized on nitrocellulose [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)].
24. All of the glycosylated forms indicated in Fig. 3D were sensitive to digestion with endoglycosidase H. After import, signal sequence cleavage, and glycosylation, the major forms of PPα and IFN-γ had apparent molecular masses of 29 and 25 kD, respectively.

25. Supported by grants from the UK Biotechnology and Biological Sciences Research Council (BBSRC), the Human Frontier Science Program Organization (HFSPO), and the UK Medical Research Council. S.H. is a BBSRC Advanced Research Fellow. N.J.B. is a Royal Society Research Fellow. We thank C. Iwahashi for assistance with construct preparation, A. Helenius and members of his laboratory for advice and reagents, C. Stirling for *S. cerevisiae* prepro–α factor cDNA, J. L. Holtzman for anti-ERp57 (Q-2) serum, and several other groups that provided antisera to ER proteins.

5 June 1996; accepted 31 October 1996

Early Onset of Reproductive Function in Normal Female Mice Treated with Leptin

Farid F. Chehab,* Khalid Mounzih, Ronghua Lu, Mary E. Lim

Numerous studies have revealed an association between nutritional status, adiposity, and reproductive maturity. The role of leptin, a hormone secreted from adipose tissue, in the onset of reproductive function was investigated. Normal prepubertal female mice injected with leptin grew at a slower rate than controls as a result of the hormone's thinning effects, but they reproduced up to 9 days earlier than controls and showed earlier maturation of the reproductive tract. These results suggest that leptin acts as a signal triggering puberty, thus supporting the hypothesis that fat accumulation enhances maturation of the reproductive tract.

A link between body fat content and the onset of puberty in females was first proposed over 30 years ago (*1*, *2*). More recent studies documenting delayed puberty in lean female ballet dancers (*3*, *4*) and accelerated puberty in obese females (*5*) support the concept that a metabolic signal produced by adipose tissue may control the onset of reproductive function (*6*). The ability of leptin, a hormone secreted by adipose tissue, to restore fertility to mice that are genetically deficient in leptin (*7*) suggests that this hormone may be a signal triggering the onset of reproductive function.

To explore this possibility, we injected human recombinant leptin into normal prepubertal female mice and monitored its circulatory levels over time (*8*). Leptin had a

Department of Laboratory Medicine, University of California, 505 Parnassus Avenue, San Francisco, CA 94143–0134, USA.

*To whom correspondence should be addressed.

half-life ($T_{1/2}$) of 60 min and was undetectable 7 hours after injection. Because of its short $T_{1/2}$, in subsequent experiments we administered leptin daily between 5:00 and 7:00 p.m. near the onset of the dark period, when it was most likely to exert its metabolic effects. Leptin slowed growth of the mice and caused a significant decrease in body weight compared with mice treated with phosphate-buffered saline (PBS) from day 3 ($P = 0.003$) and throughout the treatment (9) (Fig. 1A). This effect was associated with a significant decrease in food intake ($P < 0.001$) in the leptin group compared with PBS controls (Fig. 1B).

If leptin is involved in signaling puberty and the onset of reproductive function, then leptin-treated mice should attain reproductive maturity earlier than control mice (*10*). Copulatory plugs were detected in leptin-treated mice at an earlier age than in control mice (Fig. 1C). Between the ages of 30 and 39 days, 85% (11 of 13) of leptin-treated mice and 17% (2 of 12) of PBS-treated mice had a copulatory plug ($P = 0.001$, Fisher's exact test). Leptin treatment thus appeared to accelerate behavioral estrus and mating. The body weight of the leptin-treated mice at the time the copulatory plug was detected was 13% less than that of controls (15.9 ± 0.2 versus 18.3 ± 0.3 g, respectively; $P < 0.0001$). Furthermore, successful pregnancies and deliveries occurred in 46 and 42% of leptin- and PBS-treated groups, respectively, showing that leptin treatment did not interfere with successful ovulation, pregnancy, or delivery of pups.

To assess whether leptin affected maturation of the reproductive tract in prepubertal mice, we determined (i) the timing of vaginal opening, (ii) the progress toward the first estrous cycle, and (iii) the weights of uteri, ovaries, and oviducts (*11*). These parameters are reliable indices for the target actions of reproductive hormones. Vaginal opening was advanced by 1 to 4 days in leptin-treated mice compared with PBS-treated controls (Fig. 2A) (*12*). As a result, progression toward the first estrus was initiated earlier in the leptin-treated mice. By day 29, 5 of 12 leptin-treated mice had passed through estrus and progressed to metestrus (Fig. 2B), thereby completing their first estrous cycle. In contrast, none of 12 control mice had reached this point at day 29.

We evaluated gonadal steroid action by assessing the weights of target reproductive organs. For example, estradiol was assayed by its growth-stimulatory effects on the uterus. In leptin-treated prepubertal mice, weights of the uteri, ovaries, and oviducts were, respectively, 53, 37.5, and 43.8% greater than those of controls (Fig. 2C). We also determined the levels of the gonadotropin luteinizing hormone (LH) and the gonadal steroid 17β-estradiol (*13*) on day 29 when the mice were killed. LH concentrations were lower in leptin-treated mice than in controls (5.2 ± 0.4 versus 6.9 ± 0.4 ng/ml, respectively; $P = 0.007$). In addition, there was a trend toward lower concentrations of 17β-estradiol in leptin-treated mice compared with PBS controls, although the difference was not statistically significant (9.5 ± 1.1 versus 10.3 ± 1.1 pg/ml, respectively). The lower concentrations of LH and 17β-estradiol in leptin-treated mice at the time of death are consistent with their earlier progression through estrus compared with PBS-treated mice.

Finally, we assessed the levels of endogenous leptin in PBS- and leptin-treated mice at 30, 35, and 39 days of age (*14*). In the PBS group, plasma leptin concentrations increased by 61% between 30 and 39 days of age, whereas in the leptin group, endogenous leptin did not increase over time. This result suggests that exogenous leptin interferes with the normal age-related increase in leptin (Table 1).

Our findings suggest that leptin acts as a signal for puberty, as evidenced by its ability to accelerate reproduction, vaginal opening, onset of the first estrous cycle, and maturation of reproductive tissues concomitant with changes in LH and 17β-estradiol levels. The involvement of leptin in initiation of reproductive function supports pre-

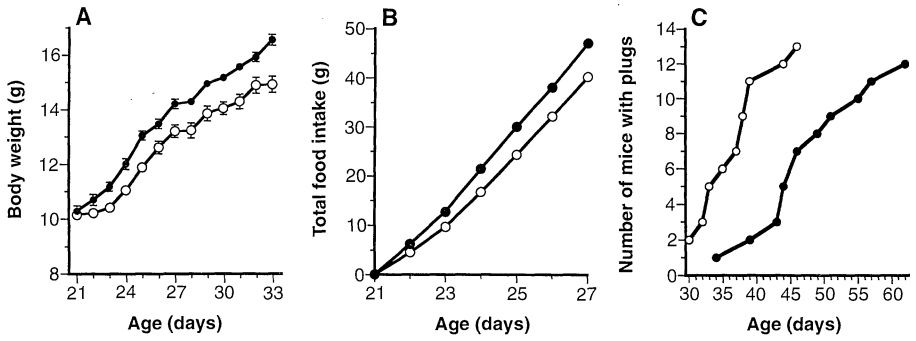

Fig. 1. Effect of leptin treatment on body weight (**A**), food intake (**B**), and presence of copulatory plugs (**C**) in prepubertal C57BL/6J mice treated with PBS (●) and leptin (○). Each value represents the mean ± SEM of 12 (PBS) or 13 (leptin) animals. Error bars are too small to be shown on the scale in (B) for PBS and leptin groups (SEM ranges, 0.1 to 1.2 and 0.3 to 0.7, respectively). Pairwise comparison of values from day 22 and onward is statistically significant ($P < 0.001$) by Student's *t* test. The age distribution curve of the leptin-treated mice in (**C**) is shifted to the left, reflecting mating at an earlier age than in controls.

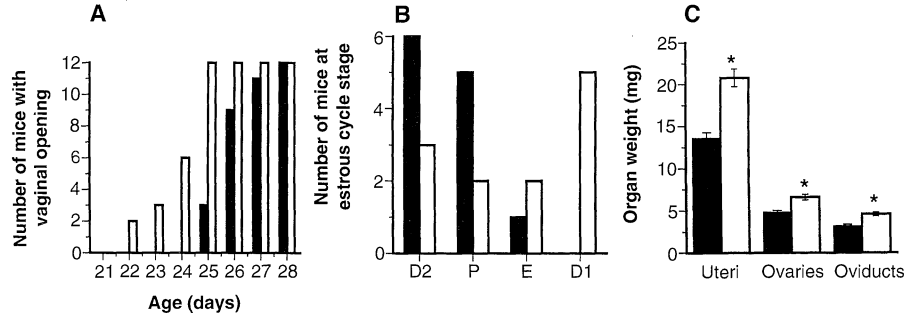

Fig. 2. Reproductive function in mice treated with leptin (□) or PBS (■). (**A**) Number of mice with vaginal opening after initiation of treatment; $P = 0.014$ at day 24 and $P < 0.001$ at day 25 (Fisher's exact test). (**B**) Number of mice at different stages of the estrous cycle at day 29. Diestrus, proestrus, estrus, and metestrus are denoted by D2, P, E, and D1, respectively. Metestrus, $P = 0.037$ (Fisher's exact test). (**C**) Weights of reproductive organs at day 29. Statistical significance by Student's *t* test is indicated by an asterisk. Uteri, $P < 0.004$; ovaries, $P < 0.0001$; oviducts, $P = 0.001$.

Table 1. Endogenous leptin concentrations in PBS- and leptin-treated prepubertal mice during growth. Data are means ± SEM. *P* value represents statistical significance in mice between 30 and 39 days of age by analysis of variance and Student-Newman-Keuls tests. There are four mice per time point. NS, not significant.

Treatment	Leptin (ng/ml)			
	30 days	35 days	39 days	*P*
PBS	2.3 ± 0.1	2.9 ± 1.4	3.7 ± 0.4	<0.05
Leptin	2.9 ± 0.1	3.1 ± 0.1	3.2 ± 0.2	NS

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.

REFERENCES AND NOTES

1. G. C. Kennedy and J. Mitra, *J. Physiol. (London)* **166**, 408 (1963).
2. ———, *ibid.*, p. 395.
3. R. E. Frisch and J. W. McArthur, *Science* **185**, 949 (1974).
4. R. E. Frisch, G. Wyshak, L. Vincent, *N. Engl. J. Med.* **303**, 17 (1980).
5. L. Zacharias, R. J. Wurtman, M. Schatzoff, *Am. J. Obstetr. Gynecol.* **108**, 833 (1970).
6. R. E. Frisch and R. Revelle, *Science* **109**, 397 (1970).
7. F. F. Chehab, M. E. Lim, R. Lu, *Nature Genet.* **12**, 318 (1996).
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).
15. G. C. Kennedy, *J. Endocrinol.* **16**, 9 (1957).
16. R. E. Frisch, *Fed. Proc.* **39**, 2395 (1980).
17. ———, D. M. Hegsted, K. Yoshinaga, *Proc. Natl. Acd. Sci. U.S.A.* **72**, 4172 (1975).
18. F. E Johnston, R. M. Malina, M. A. Galbraith, *Science* **174**, 1148 (1971).

19. We thank R. Weiner and M. Grumbach for suggestions and comments. Supported by NIH grant HLS 53762 (F.F.C.) and NIH training grant DK 07636 (K.M.).

10 September 1996; accepted 18 November 1996

Induction of Apoptosis by ASK1, a Mammalian MAPKKK That Activates SAPK/JNK and p38 Signaling Pathways

Hidenori Ichijo,* Eisuke Nishida, Kenji Irie, Peter ten Dijke, Masao Saitoh, Tetsuo Moriguchi, Minoru Takagi, Kunihiro Matsumoto, Kohei Miyazono, Yukiko Gotoh

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

H. Ichijo, M. Saitoh, K. Miyazono, Department of Biochemistry, The Cancer Institute, Tokyo, Japanese Foundation for Cancer Research, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan.
E. Nishida, T. Moriguchi, Y. Gotoh, Department of Genetics and Molecular Biology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan.
K. Irie and K. Matsumoto, Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan.
P. ten Dijke, Ludwig Institute for Cancer Research, Box 595, Biomedical Center, S-751 24, Uppsala, Sweden.
M. Takagi, Department of Oral Pathology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan.

*To whom correspondence should be addressed.