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Identification of a Member of the MAPKKK Family as a Potential Mediator of TGF-β Signal Transduction

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The mitogen-activated protein kinase (MAPK) pathway is a conserved eukaryotic signaling module that converts receptor signals into various outputs. MAPK is activated through phosphorylation by MAPK kinase (MAPKK), which is first activated by MAPKK kinase (MAPKKK). A genetic selection based on a MAPK pathway in yeast was used to identify a mouse protein kinase (TAK1) distinct from other members of the MAPKKK family. TAK1 was shown to participate in regulation of transcription by transforming growth factor–β (TGF-β). Furthermore, kinase activity of TAK1 was stimulated in response to TGF-β and bone morphogenetic protein. These results suggest that TAK1 functions as a mediator in the signaling pathway of TGF-β superfamily members.

Activation of MAPKs after ligand binding to various receptors has been correlated with numerous cellular responses, including proliferation, differentiation, and regulation of specific metabolic pathways in differentiated cell types. The MAPK signal transduction pathways include three protein kinases, MAPKKK, MAPKK, and MAPK; MAPKKK phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK (*1*). Thus, MAPK cascades constitute functional units that couple upstream input signals to a variety of outputs. Several MAPK cascades have been identified and characterized in organisms as diverse as yeasts and mammals (*1*). In the budding yeast *Saccharomyces cerevisiae*, at least six MAPK pathways have been identified and individual MAPK cascades regulate distinct responses (*2*). This marked reiteration in yeast suggests that a similar reiteration of signal transduction modules may exist in mammalian cells to mediate responses to different extracellular stimuli.

One of the MAPK pathways in *S. cerevisiae* controls the response to mating pheromone (*2*). This signaling cascade consists of the Ste11p, Ste7p, and Fus3p or Kss1p kinases, which correspond to MAPKKK, MAPKK, and MAPK, respectively. These yeast proteins act sequentially to transmit a signal to the transcription factor Ste12p, which activates transcription of mating-specific genes such as *FUS1* (Fig. 1A) (*2*). We developed a genetic approach for the assay of mammalian MAPKKK activity that relies on a *STE7*^{P368} mutation in the yeast pheromone–induced MAPK pathway (*3*); an activated form of mammalian Raf (RafΔN) or MEKK1 (MEKK1ΔN) can substitute for Ste11p activity in a Ste7p^{P368}-dependent manner as monitored by the histidine phenotype (His) conferred by the mating pathway–responsive reporter gene *FUS1p::HIS3* (Fig. 1B). We used this approach to screen a complementary DNA (cDNA) expression library (*4*) from a murine cell line, BAF-B03, for MAPKKKs that might suppress the transcriptional defect of *ste11Δ STE7*^{P368} cells. One cDNA clone was isolated that activated the *FUS1p::HIS3* reporter gene in a Ste7p^{P368}-dependent manner (Fig. 1B). This cDNA encodes a protein kinase, which we designated TAK1 for TGF-β–activated kinase.

To obtain a full-length clone, we screened the same cDNA library with the TAK1 cDNA insert as a probe and five clones were identified. Four clones contained cDNA corresponding to an additional ~230 base pairs of sequence in the 5′ region (*5*). The full-length TAK1 cDNA encodes a protein of 579 amino acids (Fig. 2A). The primary sequence of the TAK1 protein contains a putative NH_2-terminal protein kinase catalytic domain and a 300-residue COOH-terminal domain (Fig. 2B). The catalytic domain contains consensus sequences that correspond to protein kinase

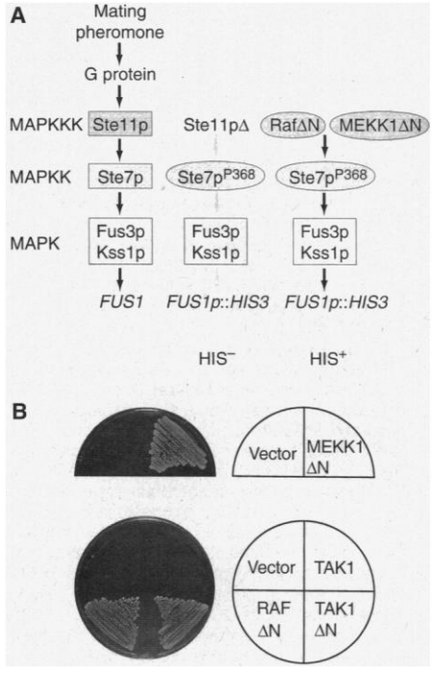

Fig. 1. Screening for mammalian MAPKKK family members in yeast. (**A**) Model for the yeast pheromone–stimulated MAPK pathway. The pheromone-stimulated MAPK pathway induces transcription of mating-specific genes such as *FUS1*. The *FUS1p::HIS3* reporter gene comprises the *FUS1* upstream activation sequence joined to the *HIS3* open reading frame, and allows signal activity in a *his3Δ FUS1p::HIS3* strain to be monitored by the ability of cells to grow on medium lacking exogenous histidine (His phenotype). A *his3Δ ste11Δ FUS1p::HIS3 STE7*^{P368} (proline substitution at serine-368) strain has a His$^-$ phenotype because the activity of Ste7p^{P368} is dependent on the presence of the upstream Ste11p MAPKKK (*3*). Expression of a mammalian MAPKKK such as RafΔN or MEKK1ΔN in this strain confers a His$^+$ phenotype (*27*). (**B**) Suppression of the *ste11Δ* mutation by mammalian genes. Strain SY1984-P (*his3Δ ste11Δ FUS1p::HIS3 STE7*^{P368}) was transformed with various plasmids (*27*), and each transformant was streaked onto SC-His plates and incubated at 30°C. Plasmids were as follows: (upper panel) YCplac22 (vector) and pRS314PGKMEKKCAT (MEKK1ΔN); (lower panel) pNV11 (vector), pNV11-HU11F (TAK1), pADU-RafΔN (RafΔN), and pNV11-HU11 (TAK1ΔN).

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subdomains I to XI (6). The catalytic domain shows ~30% identity with the amino acid sequences of the catalytic domains of Raf-1 (7) and MEKK1 (8) (Fig. 2C). The COOH-terminal 300 amino acids downstream of the kinase domain show no marked similarity to other proteins.

The protein encoded by the TAK1 cDNA insert (TAK1ΔN) identified by genetic selection in yeast lacks the NH_2-terminal 22 amino acids of the full-length protein (Fig. 2A). In contrast to TAK1ΔN, expression of the full-length TAK1 cDNA failed to suppress the *ste11Δ* mutation (Fig. 1B), suggesting that deletion of the NH_2-terminal 22 amino acids may activate TAK1 kinase. Thus, this NH_2-terminal segment appears to inhibit the function of TAK1. Eleven of the

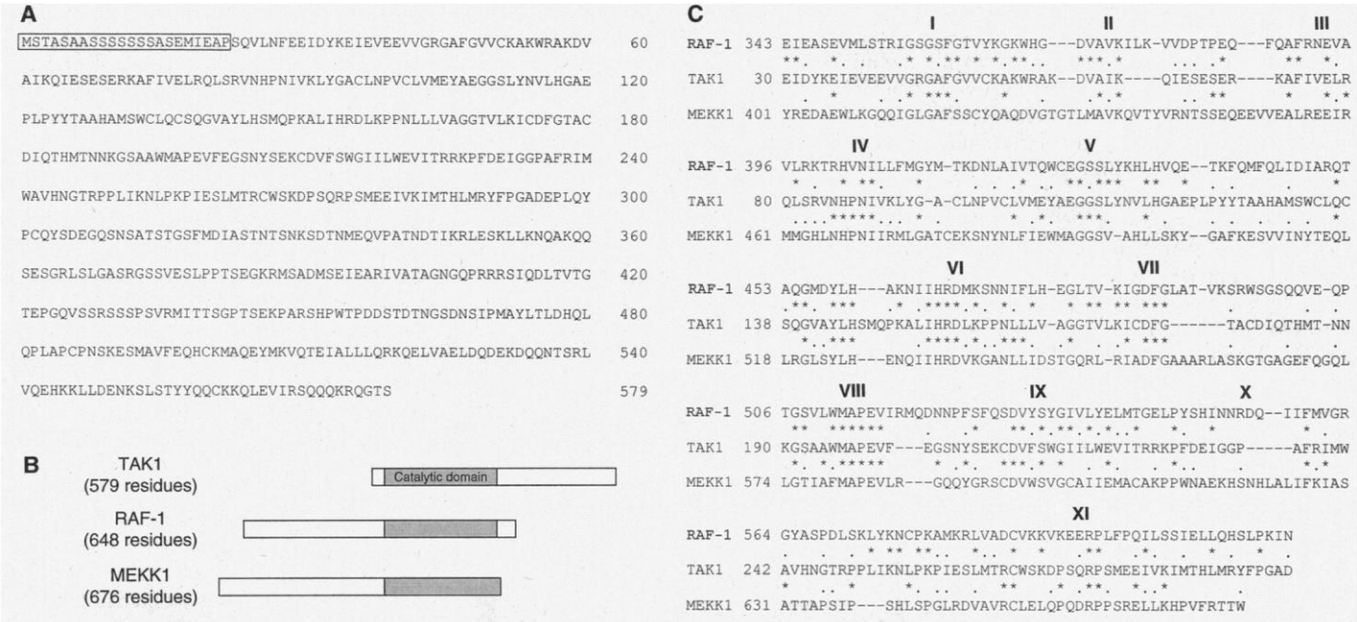
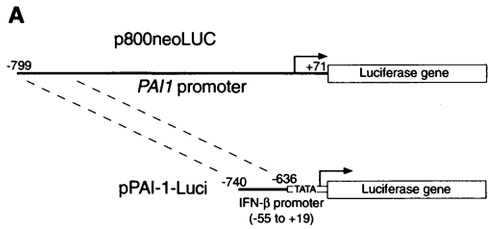


Fig. 2. (**A**) Predicted amino acid sequence of the TAK1 protein. The TAK1 nucleotide sequence was determined by the dideoxynucleotide chain termination and Maxam-Gilbert methods. The amino acid sequence deduced from the nucleotide sequence of full-length TAK1 cDNA is shown (*28*). The accession number for the TAK1 sequence is D76446. The first 22 amino acids missing from TAK1ΔN isolated with the yeast system are boxed. (**B**) Organization of domains of the predicted TAK1, RAF-1 (*7*), and MEKK1 (*8*) proteins. Numbers in parentheses indicate the number of amino acids in each protein. Shaded and open bars represent the catalytic domains and the kinase-unrelated domains, respectively. (**C**) Comparison of amino acid sequences among catalytic domains. Identical and conserved amino acids relative to those of TAK1 are indicated by asterisks and dots, respectively. Numbers on the left indicate amino acid positions relative to the initiator methionine of each protein. Consensus sequences corresponding to conserved kinase subdomains I to XI (*6*) are indicated by roman numerals. The groups of amino acids considered to be similar are Ser, Thr, Pro, Ala, and Gly; Asn, Glu, Asp, and Gln; His, Arg, and Lys; Met, Ile, Leu, and Val; and Phe, Tyr, and Trp. (**D**) TAK1 mRNA abundance in various mouse tissues. Total RNA (5 μg) was prepared from various mouse tissues, probed in RNA blots with ^{32}P-labeled TAK1 and actin cDNAs, and subjected to autoradiography (*9*). The positions of 28*S* and 18*S* ribosomal RNAs are indicated on the right.



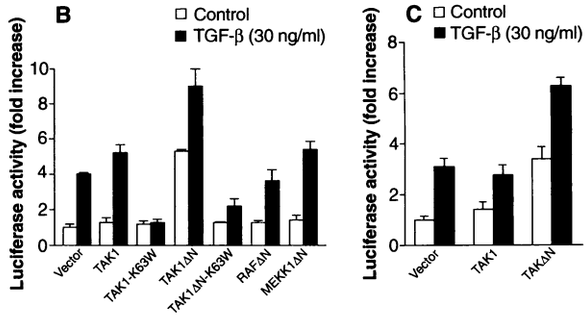


Fig. 3. Effect of TAK1 expression on TGF-β–inducible gene expression. (**A**) Structures of reporter plasmids. Plasmid p800neoLUC contains the human *PAI1* promoter sequences from nucleotides −799 to +71 (*13*). Plasmid pPAI-1–Luci contains, from 5′ to 3′, a TGF-β–responsive element of the *PAI1* promoter (nucleotides −740 to −636) and the promoter region of the human interferon β (IFN-β) gene from nucleotides −55 to +19 (*16*). These promoter sequences were fused to a luciferase gene. The direction of transcription is indicated by arrows, and TATA represents the TATA box. (**B**) Effect of TAK1 expression on activation of the *PAI1* promoter. MV1Lu cells were transiently transfected with the reporter plasmid p800neoLUC and the indicated plasmids (*11*) by the calcium phosphate method as described previously (*10*). Cells were subsequently incubated with or without human TGF-β1 (30 ng/ml) for 20 hours, extracts were prepared, and luciferase assays were performed as described (*29*). Luciferase activity is expressed as fold increase relative to unstimulated cells transfected with vector. All transfections and luciferase assays were performed at least five times, with triplicates in each experiment. Data are means ± SEM of luciferase activity from triplicates in a representative experiment. (**C**) Effect of TAK1 expression on activation of a TGF-β–responsive element. Mv1Lu cells transiently transfected with the reporter plasmid pPAI-1–Luci and the indicated plasmids were incubated with or without TGF-β1 (30 ng/ml) for 20 hours, and cell lysates were assayed for luciferase activity. All transfections and luciferase assays were performed at least three times, with triplicates in each experiment. Data are means ± SEM of triplicates from a representative experiment.

first 22 amino acids at the NH_2-terminus are serine or threonine residues that, through phosphorylation, may recruit a signaling molecule or engage the catalytic domain to inhibit kinase activity.

To analyze the expression pattern of the *TAK1* gene, we performed Northern (RNA) blot analysis (Fig. 2D). Total cellular RNA was isolated from various mouse tissues and hybridized with ^{32}P-labeled TAK1 cDNA (9). The major TAK1 mRNA was ~2.5 kb. In some cell lines, a second, larger RNA was detected. TAK1 mRNA was present in all tissues examined, and was particularly abundant in thymus and brain, with a lesser amount in liver.

To define the function of TAK1 in mammalian cells, we cloned the cDNA into the mammalian expression vector pEF (*10*), in which expression is controlled by the human elongation factor–1α (EF-1α) gene promoter (*11*). The TAK1 expression vector was then cotransfected with various reporter plasmids whose expression is influenced by distinct ligands. As detailed below, we observed an effect of TAK1 on gene induction by TGF-β. Early cellular responses to TGF-β include an increase in the amount of mRNA encoding plasminogen activator inhibitor–1 (PAI-1) (*12*). To investigate the effect of TAK1 on this response, we performed a transient transfection assay with a reporter plasmid (p800neoLUC) (Fig. 3A) (*13*) containing the luciferase gene under the control of the TGF-β–inducible *PAI1* gene promoter. Mink lung epithelial (Mv1Lu) cells transiently transfected with p800neoLUC responded to TGF-β with a four- to fivefold increase in reporter gene activity (Fig. 3B). TAK1 and TAK1ΔN expression plasmids were each cotransfected with p800neoLUC into Mv1Lu cells. Overexpression of TAK1ΔN constitutively activated luciferase expression (Fig. 3B) to an extent similar to that observed in vector transfectants treated with TGF-β. Thus, an activated TAK1 is able to signal in the absence of TGF-β. Addition of TGF-β to cells transfected with TAK1ΔN cDNA further increased luciferase expression. We also examined the effect of activated forms of other MAPKKKs—RafΔN and MEKK1ΔN (*11*)—on luciferase expression in the same transient transfection assay. RafΔN and MEKK1ΔN had little effect on constitutive luciferase activity (Fig. 3B). Thus, the effect of TAK1ΔN on TGF-β signaling is not a result of a nonspecific effect of increased MAPKKK activity.

To confirm that the effect observed was mediated by the kinase activity of TAK1, we constructed catalytically inactive TAK1ΔN-K63W, in which the lysine of the adenosine triphosphate (ATP)–binding site at position 63 was replaced by tryptophan (*14*). This mutation is expected to destroy the kinase and signaling activities of TAK1ΔN. Cotransfection of TAK1ΔN-K63W cDNA with p800neoLUC had no effect on constitutive luciferase activity (Fig. 3B). These results suggest that the kinase activity of TAK1ΔN is required for TGF-β–independent expression of *PAI1*. Furthermore, the kinase-negative TAK1ΔN partially inhibited TGF-β–induced luciferase expression. Overexpression of the full-length TAK1-K63W also inhibited TGF-β–stimulated luciferase activity (Fig. 3B), presumably by sequestering essential elements in the pathway. Our results suggest that TAK1 may function as a mediator of the TGF-β signaling pathway.

The TGF-β–responsive element in the *PAI1* promoter is located between nucleotides −740 and −636 (*12*, *15*). We therefore used a luciferase reporter containing this region, pPAI-1–Luci (Fig. 3A) (*16*), to examine the effect of TAK1 on the activation of gene expression by TGF-β. TGF-β consistently induced a two- to threefold increase in luciferase activity in Mv1Lu cells transfected with pPAI-1–Luci. Cotransfection of this reporter with TAK1ΔN resulted in constitutive activation of gene expression (Fig. 3C). Thus, the sequence between nucleotides −740 and −636 of the *PAI1* promoter contributes to the response to TGF-β and TAK1.

We investigated whether the kinase activity of TAK1 could be activated by treatment of cells with TGF-β. To identify a suitable exogenous substrate, we performed in vitro kinase reactions with TAK1 immunoprecipitated from yeast cells expressing hemagglutinin (HA) epitope–tagged TAK1 (*17*). Active TAK1 phosphorylated and activated the XMEK2 (SEK1) subfamily (*18*) of MAPKKs. In contrast, no phosphorylation of the MAPKK MEK1 (*1*), histone, or myelin basic protein was detected (*19*). An expression construct encoding HA epitope–tagged TAK1 (HA-TAK1) (*20*) was transiently transfected into MC3T3-E1 murine osteoblastic cells (*21*). After treatment of the cells with TGF-β, HA-TAK1 was isolated by immunoprecipitation and its activity was determined in a coupled kinase assay (*22*). TAK1 activity increased within 5 min

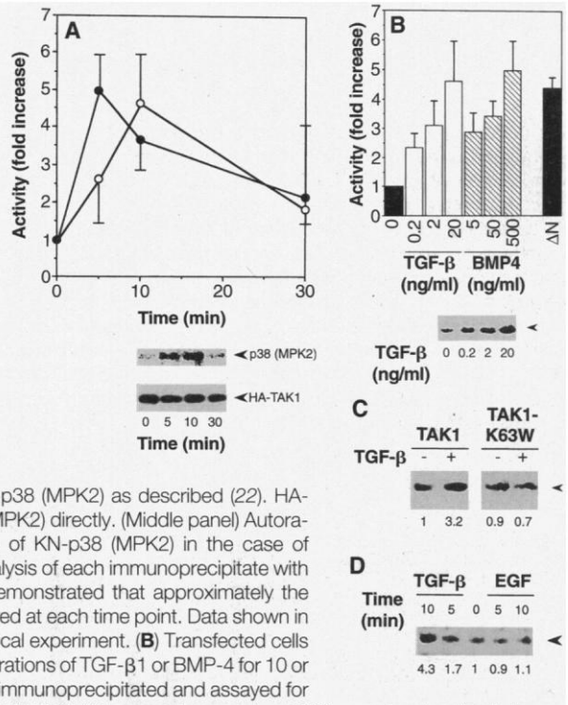


Fig. 4. Effects of TGF-β and BMP on the activity of TAK1 in MC3T3-E1 cells. (**A**) An HA-TAK1 expression construct (*20*) was transiently transfected into MC3T3-E1 murine osteoblastic cells. The cells were treated with TGF-β (20 ng/ml) (○) or BMP-4 (100 ng/ml) (●) for 0 to 30 min, after which HA-TAK1 was immunoprecipitated (*30*) and assayed for kinase activity. Activity was expressed as fold increase relative to that of HA-TAK1 from unstimulated cells and is presented as means ± SEM from at least three experiments (upper panel). The activity of immunoprecipitated TAK1 was assayed by its ability to activate recombinant XMEK2 (SEK1), whose activity was assayed by its ability to phosphorylate recombinant kinase–negative (KN) p38 (MPK2) as described (*22*). HA-TAK1 did not phosphorylate KN-p38 (MPK2) directly. (Middle panel) Autoradiogram showing the phosphorylation of KN-p38 (MPK2) in the case of TGF-β1. (Lower panel) Immunoblot analysis of each immunoprecipitate with monoclonal antibody 12CA5 to HA demonstrated that approximately the same amount of HA-TAK1 was recovered at each time point. Data shown in middle and lower panels are from a typical experiment. (**B**) Transfected cells were treated with the indicated concentrations of TGF-β1 or BMP-4 for 10 or 5 min, respectively, and HA-TAK1 was immunoprecipitated and assayed for kinase activity as described in (A). An HA-TAK1ΔN expression construct (*20*) was also transiently transfected into MC3T3-E1 cells, and HA-TAK1ΔN was immunoprecipitated and assayed for its activity (ΔN) as above. Data are means ± SEM from at least three experiments and are expressed as fold increase relative to the activity of HA-TAK1 from unstimulated cells (upper panel). (Lower panel) Representative autoradiogram showing the phosphorylation of KN-p38 (MPK2), indicated by the arrowhead, in the case of TGF-β1 treatment. (**C**) MC3T3-E1 cells transiently transfected with HA-TAK1 or HA-TAK1-K63W constructs were incubated with (+) or without (−) TGF-β1 (20 ng/ml) for 10 min. HA-TAK1 or HA-TAK1-K63W was immunoprecipitated and subjected to in vitro kinase assays as described in (A). Arrowhead indicates KN-p38 (MPK2). The fold stimulation of kinase activity as quantitated by densitometry is indicated below each lane. (**D**) MC3T3-E1 cells were exposed to TGF-β1 (20 ng/ml) or EGF (30 nM) for the indicated times. Cell lysates were subjected to immunoprecipitation with antibodies to TAK1 (*31*), and immunoprecipitates were subjected to in vitro kinase assays as described in (A). Arrowhead indicates KN-p38 (MPK2). The fold stimulation of kinase activity as quantitated by densitometry is indicated below each lane. Experiments in (C) and (D) were repeated at least twice with similar results.

of exposure of cells to TGF-β, was maximal after 10 min, and decreased to nearly basal values within 30 min (Fig. 4A). TGF-β stimulated TAK1 activity in a dose-dependent manner (Fig. 4B). The TGF-β–induced kinase activity could have been attributable to the activation of TAK1 or to the activation of a coprecipitating protein kinase. To distinguish between these possibilities, we also performed the experiment with the HA epitope–tagged version of a kinase-defective TAK1 mutant (HA-TAK1-K63W). The wild-type and mutant TAK1 proteins were immunoprecipitated from transiently transfected cells before and after TGF-β stimulation. TGF-β activated only the wild-type TAK1 (Fig. 4C). These results confirmed that the TGF-β–induced protein kinase activity associated with the TAK1 immunoprecipitates was attributable to the activation of TAK1. We also examined whether TAK1 was activated by bone morphogenetic protein (BMP), a member of the TGF-β superfamily (*23*), or by epidermal growth factor (EGF). BMP-4 also stimulated TAK1 activity in a time- and dose-dependent manner (Fig. 4, A and B), whereas TAK1 activation was not observed in cells treated with EGF (*24*). The failure of EGF to induce activation of TAK1 was not the result of failure of MC3T3-E1 cells to respond to EGF, because EGF induced expression of *Fos* in these cells (*25*).

TAK1ΔN activates *PAI1* expression independently of TGF-β in Mv1Lu (Fig. 3) and MC3T3-E1 (*25*) cells, suggesting that the kinase activity of the TAK1ΔN protein is increased in the absence of TGF-β stimulation of cells. This possibility was investigated by expressing the HA epitope–tagged version of TAK1ΔN (HA-TAK1ΔN) (*20*) in MC3T3-E1 cells and examining TAK1ΔN activity in the immunoprecipitation kinase assay. The TAK1ΔN protein exhibited higher basal kinase activity than TAK1 (Fig. 4B), consistent with the notion that TAK1ΔN is constitutively active.

Finally, we investigated whether the kinase activity of endogenous TAK1 could be activated by TGF-β in MC3T3-E1 cells. The endogenous TAK1 was activated by treatment of these cells with TGF-β, but not by EGF treatment (Fig. 4D). Together, these data establish that TAK1 is specifically activated by members of the TGF-β superfamily of ligands.

We have identified TAK1, a member of the MAPKKK family, as a candidate for a positive mediator of TGF-β signal transduction. TGF-β signals through a heteromeric complex of the type I and type II TGF-β receptors (*26*), which are transmembrane proteins that contain cytoplasmic serine- and threonine-specific kinase domains. However, little is known at the molecular level of the signaling mechanism downstream of the TGF-β receptors. Our observation that TAK1 activates XMEK2 (SEK1) in vitro raises the possibility that a SAPK (stress-activated protein kinase; also known as JNK, or c-Jun NH_2-terminal kinase) type of MAPK (*1*, *18*) may be activated by TGF-β receptors. Exposure of MC3T3-E1 cells to TGF-β1 induced a moderate increase in endogenous SAPK activity (*24*). Alternatively, a previously uncharacterized MAPKK-MAPK cascade may exist downstream of TAK1 in the TGF-β signaling pathway.

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5. With the TAK1 cDNA insert as a probe, a mouse cDNA library generated from BAF-B03 cell mRNA was screened. The positive clone was subcloned into the Eco RI site of the pBS vector to generate pBS-TAK1-5′. The clone extended ~230 base pairs further to the 5′ end and contained the initiator ATG. Primer extension analysis confirmed that the obtained clone contains the entire 5′ region of the cDNA.
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28. 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.
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30. Untreated or treated cells were scraped into a buffer solution (*22*), and the cell extracts were centrifuged for 10 min at 15,000*g*. The supernatant was subjected to immunoprecipitation with antibody to HA. Briefly, a portion (300 μl) of the supernatant was mixed with 20 μl of the antibody and 20 μl of protein A–Sepharose (Pharmacia), and the immune complex was separated, washed twice with phosphate-buffered saline, and used for the kinase assay (*22*).
31. Polyclonal antibodies to TAK1 were generated in a rabbit immunized with a glutathione-S-transferase fusion protein containing full-length TAK1.

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