Mammalian TAK1 Activates Snf1 Protein Kinase in Yeast and Phosphorylates AMP-activated Protein Kinase in Vitro*

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Milica Momcilovic1, Seung-Pyo Hong2, and Marian Carlson§1
From the Departments of 1Biochemistry and Molecular Biophysics and 2Genetics and Development and Microbiology, Columbia University, New York, New York 10032

The Snf1/AMP-activated protein kinase (AMPK) family is important for metabolic regulation and is highly conserved from yeast to mammals. The upstream kinases are also functionally conserved, and the AMPK kinases LKB1 and Ca2+/calmodulin-dependent protein kinase kinase activate Snf1 in mutant yeast cells lacking the native Snf1-activating kinases, Sak1, Tos3, and Elm1. Here, we exploited the yeast genetic system to identify members of the mammalian AMPK kinase family by their function as Snf1-activating kinases. A mouse embryo cDNA library in a yeast expression vector was used to transform sak1Δ tos3Δelm1Δ yeast cells. Selection for a Snf1+ growth phenotype yielded cDNA plasmids expressing LKB1, Ca2+/calmodulin-dependent protein kinase kinase, and transforming growth factor-β-activated kinase (TAK1), a member of the mitogen-activated protein kinase kinase kinase family. We present genetic and biochemical evidence that TAK1 activates Snf1 protein kinase in vivo and in vitro. We further show that recombinant TAK1, fused to the activation domain of its binding partner TAB1, phosphorylates Thr-172 in the activation loop of the AMPK catalytic domain. Finally, expression of TAK1 and TAB1 in HeLa cells or treatment of cells with cytokines stimulated phosphorylation of Thr-172 of AMPK. These findings indicate that TAK1 is a functional member of the Snf1/AMPK kinase family and support TAK1 as a candidate for an authentic AMPK kinase in mammalian cells.

The Snf1/AMP-activated protein kinase (AMPK) family has major roles in regulation of glucose and lipid metabolism, maintenance of cellular energy homeostasis, and cellular stress responses (reviewed in Refs. 1 and 2). In mammalian cells, reduced energy availability (high cellular AMP:ATP ratio) causes activation of AMPK, which promotes glucose transport and ATP-generating metabolic processes, inhibits ATP-consuming processes, and regulates transcription. AMPK is also regulated by leptin, adiponectin, and ghrelin (3–5) and has a role in controlling appetite and food intake (5, 6). In humans, AMPK is an important therapeutic target for type 2 diabetes (2, 7).

In the yeast Saccharomyces cerevisiae, Snf1 protein kinase (8) is the ortholog of AMPK (9, 10). Snf1 protein kinase, like AMPK, is heterotrimeric, comprising a catalytic subunit (Snf1/α), and two regulatory subunits (β and Snf4/γ). Mutation of SFN1 causes the Snf− (sucrose-nonfermenting) phenotype, which is characterized by inability to utilize carbon sources that are less preferred than glucose. Like AMPK, Snf1 protein kinase regulates transcription, metabolic enzymes, and transporters in response to stress, particularly carbon stress (reviewed in Refs. 11 and 12).

The Snf1/AMPK-activating kinases in the kinase cascade are also highly conserved between yeast and mammals (Fig. 1). Yeast contains three homologous kinases that phosphorylate the activation loop Thr-210 of the Snf1 catalytic subunit: Sak1 (Snf1-activating kinase, previously Pak1), Tos3, and Elm1 (13–15). Mutant yeast cells lacking these three kinases (sak1Δ tos3Δ elm1Δ cells) cannot activate Snf1 and exhibit a Snf− phenotype (13, 15). Their mammalian sequence homologs, LKB1 and Ca2+/calmodulin-dependent protein kinase kinase (CaM KK), phosphorylate Thr-172 in the activation loop of the AMPK catalytic subunit and activate AMPK in vitro and in vivo (13, 16–22). The yeast and mammalian kinases exhibit striking functional interchangeability. Tos3 and Elm1 phosphorylate and activate AMPK in vitro (13, 15). LKB1, in complex with its partners STRADα and MO25α, and CaM KK phosphorylate Snf1 in vitro and activate Snf1 in sak1Δ tos3Δ elm1Δ mutant yeast, conferring a Snf− growth phenotype (16, 20).

Here, we took advantage of this conservation of the Snf1/AMPK pathway and exploited the yeast genetic system in an effort to identify new members of the AMPK kinase family. Given that yeast, a simple unicellular organism, has three Snf1 protein kinase kinases, it seems likely that mammals have multiple AMPK kinases. The heterologous function of LKB1 and CaM KK in yeast provides the basis for a convenient and powerful genetic selection for mammalian AMPK kinases: the restoration of the Snf− growth phenotype in sak1Δ tos3Δ elm1Δ mutant yeast. The power of this selection lies not only in its simplicity but also in its sensitivity. The Snf1 pathway is robust, and very little activity is required for growth; for example, expression of LKB1 alone restores growth despite causing only...
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EXPERIMENTAL PROCEDURES

Yeast Strains—*S. cerevisiae* strains were W303-1A (MATa ura3 trp1 ade2 his3 can1 leu2), MCY4908 (W303-1A *snf1Δ10*), MCY5138 (MATa sak1Δ::kanMX4 tos3Δ::kanMX4 elm1Δ::ADE2 ura3 trp1 ade2 his3 can1 leu2), MCY5115 (MATa sak1Δ::kanMX4 ura3 trp1 ade2 his3 can1 leu2), and MCY5125 (W303-1A *elm1Δ::kanMX4*). Synthetic complete (SC) medium lacking appropriate supplements was used to select for plasmids.

Selection for Mammalian Snf1-activating Kinases in Yeast—DNA of a two-hybrid library prepared from mouse 17-day embryo cDNAs in a yeast expression plasmid vector carrying the *LEU2* marker (Clontech catalog number 638846) was used to transform (36) yeast strain MCY5138 (see Fig. 2). A total of $5 \times 10^6$ transformants were selected on 500 plates of SC solid medium containing 2% glucose and lacking leucine. Colonies from each plate were resuspended in SC medium and transferred to a fresh plate of SC-leucine solid medium containing 2% raffinose plus the respiratory inhibitor antimycin A (1 μg/ml). Growth on this medium requires activation of Snf1 protein kinase; in control experiments, colonies expressing LKB1 appeared in 3–7 days. After 5–7 days, two colonies from each plate were picked and retested for growth. Plasmid DNAs were rescued by passage through bacteria, retested by transformation of MCY5138, and sequenced. One plasmid was saved from each plate.

Plasmids—pK98, expressing GAD-TAK1, was recovered above. pRH104 (37), pRH105 (16), and pRH123 (16) express HA-Sak1, HA-Tos3, and HA-CaMKKα, respectively, from vector pWS93 (38). GAD-Snf1 was expressed from pSG1 (39). pMM25 and pMM29 express HA-TAK1 and LexA-TAB1, respectively, from mouse cDNAs (Open Biosystems) cloned into pWS93 (38) and pBTM116 (40). pMM26, expressing TAK1K63W with Lys-63 altered to Trp, was constructed from pMM25 by using the QuikChange site-directed mutagenesis kit (Stratagene); three independent mutant plasmids behaved similarly. cDNAs encoding residues 1–318 of the wild-type (WT) and mutant kinase domain of AMPK, AMPK-KD-KDWT, and AMPK-KD-T172A (gifts of L. Witters; see Ref. 41), were transferred to vector pET32a (Novagen) to yield pMM45 and pMM57, respectively, expressing His-tagged proteins. pMM33, pMM35, and pMM37 express TAK1, TAK1K63W, and TAB1, respectively, from vector pCMV-FLAG2 (Invitrogen); TAK1 proteins were not recognized by anti-FLAG, although sequence analysis confirmed the FLAG tag. Snf1T210A and Gal83, tagged with green fluorescent protein (GFP), were expressed from their native promoters on pKH43 (37) and pRT13 (42).

Analysis of Proteins—Proteins were separated by SDS-PAGE in 8% polyacrylamide. Immunoblot analysis was carried out with anti-Snf1 (8), monoclonal anti-HA (12CA5), anti-LexA (Invitrogen), anti-FLAG (Sigma), anti-TAK1 (Upstate), anti-Phospho-Thr-172-AMPK and anti-AMPKα (Cell Signaling Technologies), and anti-His-6-peroxidase (Roche Diagnostics). Antibodies were detected with chemiluminescence using ECL Plus or ECL Advance (Amersham Biosciences). Blots were incubated in 0.2 M glycine, pH 2, for 5 min and washed before reprobing.

Assay of Snf1 Activity by Phosphorylation of SAMS Peptide—Yeast cells were grown to mid-log phase in SC medium containing 2% glucose, collected by filtration, incubated in SC with 0.05% glucose for 15 min, and collected by filtration. Extracts were prepared from two independent cultures, Snf1 was partially purified, and phosphorylation of the synthetic peptide HMRSAMSGLHLVKRR (SAMS peptide; Ref. 43) was assayed as described (9, 37). Each preparation was assayed twice, with dilutions to confirm linearity. Kinase activity is expressed as nanomoles of phosphate incorporated into the peptide per minute per milligram of protein (43).
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Assay of Phosphorylation of Recombinant Snf1 and AMPK Catalytic Domains—Glutathione S-transferase (GST) fusions to the mutant Snf1 catalytic domains Snf1KD-K84R and Snf1KD-T210A were expressed in bacteria and purified as described (16). His-tagged AMPK-KD-WT and AMPK-KD-T172A catalytic domains were expressed in bacteria and purified using AKTA fast protein liquid chromatography on chelating HiTrap resin (Amersham Biosciences). Bound proteins were eluted with a linear gradient as described by the manufacturer. Cultures of MCY5138 expressing HA-TAK1 and/or LexA-TAB1 were grown in SC with 2% glucose, followed by filtration. HA-tagged proteins were immunoprecipitated using anti-HA antibody as described (13). The immunoprecipitated proteins were then eluted with a linear gradient as described by the manufacturer (Amersham Biosciences). Bound proteins were eluted with a linear gradient as described by the manufacturer. Cultures of MCY5138 expressing HA-TAK1 and/or LexA-TAB1 were grown in SC with 2% glucose, collected by filtration, and incubated in 0.05% glucose for 30 min, and collected by filtration. HA-tagged proteins were immunoprecipitated from extracts (200 μg) with anti-HA antibody as described (13).

Kinases were assayed for phosphorylation of GST-Snf1KD (3 μg) or AMPK-KD (0.5 μg) substrates using [γ-32P]ATP as described (16). His-tagged recombinant human TAK1-TAB1 fusion protein (100 ng; Upstate catalog number 14-600) was incubated with substrates and cold ATP.

Analysis of Phosphorylation of AMPK in HeLa Cells—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mm L-glutamine. Cells were transfected with DNAs (8 μg/6-cm dish) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. TNF-α and IL-1β were purchased from R&D Systems. Cells were lysed by the addition of ice-cold lysis buffer as described (20), except without prior rinsing. Lysates were collected immediately and clarified by brief centrifugation in the cold.

RESULTS

Genetic Selection for Mammalian Snf1-activating Kinases in Yeast—The sak1Δ tos3Δ elm1Δ mutant yeast strain lacks all three native Snf1 protein kinase kinases and therefore exhibits the Snf− (sucrose-nonfermenting) phenotype, which is characterized by the ability to utilize glucose but not alternative carbon sources. To identify mammalian Snf1-activating kinases, and thus candidates for AMPK kinases, we selected mammalian cDNAs that allow sak1Δ tos3Δ elm1Δ cells to grow on raffinose, as shown schematically in Fig. 2. We used a library of mouse 17-day embryo cDNAs fused to the Gal4 activation domain (GAD) in a yeast expression vector to transform sak1Δ tos3Δ elm1Δ yeast cells and then selected for growth on raffinose. We recovered the cDNA plasmid from Snf− colonies by passage through Escherichia coli, retransformed sak1Δ tos3Δ elm1Δ yeast cells to confirm that the cDNA conferred a Snf− phenotype, and identified the cDNA by sequencing. In a screen of 5 × 10⁶ transformants, we recovered 49 cDNA clones expressing AMPK kinase to confer a Snf− phenotype. We confirmed this approach. TAK1 is thus a candidate for a Snf1-activating kinase and potentially an AMPK kinase.

Growth Phenotype Conferred by TAK1 Requires Snf1 Protein Kinase—We first sought to confirm that the ability of TAK1 to confer growth on raffinose requires Snf1 protein kinase. A cDNA plasmid expressing GAD-TAK1 was used to transform sak1Δ mutant cells. The transformants did not grow on raffinose (Fig. 3A), indicating that TAK1 requires Snf1 protein kinase to confer a Snf+ phenotype and does not function by
Snf1 catalytic activity in sak1Δ tos3Δ elm1Δ mutant cells expressing HA-TAK1. Cells were grown to mid-log phase in glucose and then shifted to medium containing 0.05% glucose for 30 min, a condition that results in activation of Snf1 in wild-type cells. Cell extracts were prepared, and phosphorylation of a synthetic peptide substrate, the SAMS peptide, by partially purified Snf1 protein kinase was determined. The presence of HA-TAK1 in the mutant cells resulted in the activation of Snf1 to levels similar to those caused by CaMKKα (Fig. 4A), which is roughly 2-fold reduced relative to wild type (16). Coexpression of LexA-TAB1 with HA-TAK1 did not substantially increase activation of Snf1 (Fig. 4A), consistent with the growth phenotypes (Fig. 3). Amounts of Snf1 protein were similar in all assays, and coexpression of TAB1 did not result in elevated levels of TAK1, although TAK1 appeared to stabilize TAB1, as judged by immunoblot analysis (Fig. 4C). Together with growth assays, these data suggest that in yeast cells, TAK1 functions as a Snf1-activating kinase and does so largely independently of TAB1. We cannot exclude the possibility that a native yeast protein functionally substitutes for TAB1, but no yeast sequence homolog is evident.

**TAK1 Catalytic Activity Is Required for Activation of Snf1 Protein Kinase**—To determine whether the effects of TAK1 in yeast cells were due to the catalytic activity of TAK1, we introduced a mutation altering Lys-63 to Trp, which was previously shown to abolish catalytic activity of TAK1 (23). The kinase-dead mutant protein, TAK1K63W, was expressed (Fig. 4D) but did not confer growth on raffinose, indicating that catalytic activity and not some other property of the protein is required (Fig. 3C). In accord with this result, TAK1K63W did not activate Snf1 protein kinase activity in vivo, as judged by phosphorylation of the SAMS peptide (Fig. 4B). Thus, the function of TAK1 as a Snf1-activating kinase in yeast requires its catalytic activity.

**TAK1-TAB1 Phosphorylates the Activation Loop Thr-210 of Recombinant Snf1 Catalytic Domain**—We next assayed the ability of TAK1 purified from yeast cells to phosphorylate the kinase domain of Snf1 (Snf1KD) in vitro. We used as substrates two bacterially expressed, inactive forms of the Snf1 catalytic domain, GST-Snf1KD-K84R, which has a substitution of the conserved Lys of the ATP-binding site, and GST-Snf1KD-T210A, which is mutant for the activation loop Thr-210. HA-TAK1 was expressed in sak1Δ tos3Δ elm1Δ cells in combination with LexA-TAB1 or LexA, immunoprecipitated with anti-HA antibody, and incubated with different substrates and [γ-32P]ATP. TAB1 has been reported to stimulate the autophosphorylation of TAK1 (45, 46). The presence of TAB1 increased the phosphorylation of Snf1KD substrates (Fig. 5A) but did not increase the recovery of TAK1 (Fig. 5C); longer exposure revealed very weak phosphorylation of Snf1KD by TAK1 in the absence of TAB1 (Fig. 5A, lower panel). These results stand in contrast to the minimal effect of TAB1 on activation of Snf1 by TAK1 in yeast cells; however, the substrate in vivo was full-length Snf1 protein, presumably in the context of the heterotrimeric Snf1 protein kinase complex.

Both Snf1KD substrates were phosphorylated by TAK1 with TAB1, although Snf1KD-T210A was phosphorylated less strongly than was Snf1KD-K84R (Fig. 5A), and Coomas-
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**FIGURE 5. Phosphorylation of recombinant Snf1 catalytic domain.** A–C, sak1Δ tosΔ elm1Δ cells expressed HA-TAK1 (+), LexA-TAB1 (+), HA (−), or LexA (−), as indicated. Cell extracts were prepared, and proteins were immunoprecipitated with anti-HA antibody and incubated with bacterially expressed GST-Snf1-KD-K84R, GST-Snf1-KD-T210A, or no substrate (None) and [γ-32P]ATP. A, products were separated by SDS-PAGE and detected by autoradiography. The lower panel shows a longer exposure. Molecular size markers (in kDa) are indicated. Arrowheads, GST-Snf1KD substrate; asterisks, HA-TAK1. B, Coomassie Blue staining of gel shown in panel A. C, samples of the immunoprecipitated proteins used in panel A were immunoblotted with anti-phospho-Thr-172-AMPK antibody to detect phosphorylated Thr-210 (pT210) of Snf1 and with anti-HA. D, recombinant His-tagged TAK1-TAB1 fusion protein was incubated with substrates, as in panel A, and with cold ATP. Mock incubations with no added TAK1-TAB1 (−) were carried out as controls for specificity of the antibody. Proteins were immunoblotted with anti-phospho-Thr-172-AMPK and anti-His antibodies; Snf1 has a stretch of His residues.

sie Blue staining confirmed that both substrates were present at similar levels (Fig. 5B). These findings suggest that TAK1 recognizes Thr-210, as well as other residues. To directly assess the phosphorylation of Thr-210, we carried out immunoblot analysis with anti-phospho-Thr-172-AMPK-specific antibody, which cross-reacts with phospho-Thr-210 of Snf1. This antibody detected Snf1KD-K84R, but not Snf1KD-T210A, indicating that TAK1 phosphorylates Thr-210 in the activation loop (Fig. 5C).

To confirm that TAK1, and not a coprecipitating kinase, is responsible for phosphorylation of Thr-210, we incubated recombinant human TAK1-TAB1 fusion protein (TAK1 residues 1–303 fused to TAB1 residues 437-end) (47) with the Snf1KD substrates and cold ATP. Immunoblot analysis with phospho-Thr-172-AMPK antibody detected Snf1KD-K84R but not Snf1KD-T210A (Fig. 5D). These biochemical studies indicate that TAK1 phosphorylates the activation loop Thr-210 of Snf1 in vitro and, together with genetic evidence, suggest that TAK1 functions directly as a Snf1-activating kinase in yeast cells in vivo.

**FIGURE 6. Effects of TAK1 in elm1Δ and sak1Δ yeast cells.** A, elm1Δ cells expressing HA-TAK1, HA-TAK1K63W, or HA (vector) and LexA-TAB1 or LexA (vector) were grown on selective SC + 2% glucose and were imaged by differential interference contrast (DIC). B, sak1Δ cells expressing Gal83-GFP, HA-TAK1, and LexA-TAB1 were grown in selective SC + 2% glucose and shifted to 0.05% glucose for 10 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). GFP fluorescence, 4′,6-diamidino-2-phenylindole staining, and differential interference contrast are shown. Cells were viewed using a Nikon Eclipse E800 fluorescence microscope, and images were taken with an Orca100 (Hamamatsu) camera by using Open Lab (Improvision) software.

TAK1 Restores Normal Cell Morphology in elm1Δ Cells—The Snf1 protein kinase kinases Elm1 and Sak1 have other roles in the cell besides activation of Snf1, and we tested TAK1 for the ability to provide these distinct functions. The elm1 mutation is named for the elongated morphology of the mutant cells, which results from defects in cell cycle progression and has no apparent relationship to Snf1 (48–51). Expression of TAK1, with or without TAB1, in elm1Δ cells restored normal cell morphology, whereas expression of TAK1K63W with TAB1 had no effect (Fig. 6A). Thus, TAK1 functionally substitutes for Elm1 with respect to this phenotype. These findings suggest that TAK1 and Elm1 phosphorylate substrate(s) that are not efficiently recognized by Sak1 or Tos3. Neither LKB1 nor CaMKK substituted for Elm1 in this regard (16).

The sak1Δ mutation prevents the nuclear enrichment of Snf1 protein kinase containing the Gal83 β subunit (Snf1-Gal83) in response to glucose limitation (37). Although activation of Snf1 is required for this nuclear enrichment (37), evidence suggests that Sak1 also functions in another capacity, besides activating Snf1, to promote the nuclear enrichment of Snf1-Gal83 (16). To test whether TAK1 provides this function, we expressed TAK1 and TAB1 in sak1Δ cells carrying a centromeric plasmid expressing Gal83-GFP from its native promoter. Cells were grown to mid-log phase in medium containing 2% glucose and shifted to 0.05% glucose for 10 min. Microscopic observation revealed no nuclear enrichment of Gal83-GFP (Fig.
Together, these findings suggest that with respect to function, TAK1 is more closely related to Elm1 than to Sak1.

Recombinant TAK1-TAB1 Fusion Protein Phosphorylates Thr-172 of AMPK Catalytic Domain—The above evidence that TAK1 functions in vivo and in vitro as a Snf1-activating kinase supports TAK1 as a candidate for an AMPK kinase. To examine whether TAK1 phosphorylates AMPK on the activation loop Thr-172 in vitro, we expressed in bacteria the wild-type AMPK catalytic domain, AMPK-KD-WT, and a mutant version containing a replacement of Thr-172 with Ala, AMPK-KD-T172A.

We tested the purified proteins as substrates for Tos3, which phosphorylates AMPK on Thr-172 (13). HA-Tos3 was expressed in sak1Δ tos3Δ elm1Δ cells, immunoprecipitated with anti-HA, and incubated with the substrates and [γ-32P]ATP. As expected, AMPK-KD-WT, but not AMPK-KD-T172A, was phosphorylated (data not shown).

We then assayed phosphorylation of AMPK-KD substrates by TAK1. HA-TAK1 was expressed in sak1Δ tos3Δ elm1Δ cells in combination with LexA or LexA-TAB1, immunoprecipitated, and incubated with both versions of AMPK-KD and [γ-32P]ATP. The presence of TAB1 increased the phosphorylation of both substrates (Fig. 7A), without increasing the recovery of TAK1 (Fig. 7B). A mock immunoprecipitation with no cell extract gave results similar to the control sample with HA and LexA-TAB1 (Fig. 7A and data not shown). TAK1, when coexpressed with TAB1, phosphorylated AMPK-KD-WT more strongly than AMPK-KD-T172A, and close inspection revealed a doublet in the case of the wild-type substrate, suggesting that Thr-172 is one of the sites recognized. We were unable to assay phosphorylation using phospho-Thr-172-AMPK antibody, however, because AMPK-KD migrated close to immunoglobulin.

To determine whether TAK1 recognizes Thr-172, we incubated recombinant human TAK1-TAB1 fusion protein with AMPK-KD-WT and AMPK-KD-T172A and cold ATP. Immunoblot analysis with phospho-Thr-172-AMPK antibody detected AMPK-KD-WT but not the mutant protein lacking Thr-172 (Fig. 7B). Thus, TAK1 phosphorylates the activation loop Thr-172 of AMPK in vitro.

Coexpression of TAK1 and TAB1 in HeLa Cells Increases AMPK Phosphorylation—These findings suggest TAK1 as a candidate for an AMPK kinase in mammalian cells. To explore this possibility, we transfected HeLa cells, which do not express the major AMPK kinase LKB1 (52), with combinations of plasmids for transient expression of TAK1, TAK1K63W, and TAB1 from the vector pCMV-FLAG2. At 30 h after transfection, cells were subjected to serum-free medium for 4 h. Cell lysates, prepared by a rapid lysis procedure, were subjected to immunoblot analysis with anti-phospho-Thr-172-AMPK-specific antibody, and blots were reprobed with anti-AMPKα, anti-TAK1, and anti-FLAG antibodies (Fig. 8A). Expression of TAK1 alone had little or no effect, but coexpression of TAK1 and TAB1 led to increased phosphorylation of Thr-172, whereas levels of AMPK catalytic subunit remained constant. Kinase-dead TAK1K63W, with TAB1, did not increase phosphorylation of Thr-172. TAK1K63W was expressed at lower levels than TAK1, which was not the case in yeast (Fig. 4D), suggesting that in HeLa cells, the kinase-dead protein is either less stable or deleterious. Similar results were observed when cells were transfected to serum-free medium for 14 h at 12, 18, or 24 h after transfection (Fig. 8A). Coexpression of TAK1 and TAB1 also increased...
phosphorylation of AMPK in HeLa cells treated with 0.5 M sorbitol or 1 mM hydrogen peroxide for 15 min (Fig. 8B). Both treatments are known to activate AMPK, but hyperosmotic stress does not alter the AMP:ATP ratio (7) and has been shown to stimulate TAK1 activity (25, 53). Together, these findings indicate that coexpression of TAK1 and TAB1 stimulates phosphorylation of AMPK in HeLa cells. Although TAK1 is capable of phosphorylating AMPK in vitro, it remains possible that the effects of TAK1 on AMPK in vivo are indirect.

Several cytokines stimulate TAK1 activity, including TGF-β, TNF-α, and IL-1 (23, 25, 26, 53). Exposure to TGF-β induces phosphorylation of AMPK on Thr-172 in HepG2 cells (54, 55). To test the effects of TNF-α and IL-1, we subjected HeLa cells to serum-free medium for 14 h, treated with cytokine, and analyzed cell lysates by immunoblotting. In both cases, we detected a modest increase in Thr-172 phosphorylation between 2 and 10 min (Fig. 8C), consistent with the possibility that native TAK1 phosphorylates AMPK.

**DISCUSSION**

Taking advantage of the conservation of the Snf1/AMPK pathway, we have used the yeast system to identify putative AMPK kinases by their function as Snf1-activating kinases. This genetic selection yielded two authentic AMPK kinases, LKB1 and CaMKKβ, and a new candidate, TAK1. The utility of this genetic approach is that it is based on function. Although LKB1 and CaMKK are homologous to the three yeast Snf1 protein kinase kinases, TAK1 was not identified as a candidate AMPK kinase on the basis of sequence similarity. In this study, we used a mouse embryo cDNA library, which may not represent the entire repertoire of AMPK kinases. Different libraries from other developmental stages or from specific tissues may yield additional AMPK kinases. Such kinases are potentially useful therapeutic targets in the AMPK pathway.

We present genetic and biochemical evidence that validates TAK1 as a Snf1-activating kinase. We further show that recombinant TAK1-TAB1 phosphorylates AMPK on Thr-172 in vitro and that overexpression of TAK1 and TAB1 stimulates phosphorylation of AMPK in HeLa cells. The stimulatory effects of TGF-β (54), TNF-α, and IL-1 on phosphorylation of AMPK in HeLa cells are also in accord with the possibility that TAK1 phosphorylates AMPK. Together, these findings support TAK1 as a candidate for an authentic AMPK kinase in mammalian cells.

We also note that other work has connected TAB1 with AMPK. TAB1 interacts with the Ω2 isof orm of the catalytic subunit of AMPK in mouse heart, and activation of AMPK promoted the association of β38 MAPK with TAB1 in ischemic heart (56); however, TAK1 was not implicated, and evidence suggests that TAK1 and TAB1 have some independent roles (44).

Further studies are required to validate TAK1 as an AMPK kinase in mammalian cells, and the regulation of AMPK by cytokines warrants further investigation in different cell types. It will also be interesting to address the possibility that TAK1 phosphorylates other members of the AMPK-related protein kinase family, as does LKB1 (57).

**REFERENCES**

1. Kemp, B. E., Stapleton, D., Campbell, D. J., Chen, Z. P., Murthy, S., Walter, M., Gupta, A., Adams, J. J., Katis, F., Van Denderen, B., Jennings, I. G., Iseli, T., Michell, B. J., and Witters, L. A. (2003) *Biochem. Soc. Trans.* **31**, 162–168.

2. Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005) *Cell Metab.* **1**, 15–25.

3. Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D., and Kahn, B. B. (2002) *Nature* **415**, 339–343.

4. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foulfelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and Kodawski, T. (2002) *Nat. Med.* **8**, 1288–1295.

5. Andersson, U., Filipsson, K., Abbott, C. R., Woods, A., Smith, K., Bloom, S. R., Carling, D., and Small, C. J. (2004) *J. Biol. Chem.* **279**, 12005–12008.

6. Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y. B., Lee, A., Xue, B., Mu, I., Foulfelle, F., Ferre, P., Birnbaum, M. J., Stuck, B. J., and Kahn, B. B. (2004) *Nature* **428**, 569–574.

7. Fryer, L. G., Parbu-Patel, A., and Carling, D. (2002) *J. Biol. Chem.* **277**, 25226–25232.

8. Celenza, J. L., and Carlson, M. (1986) *Science* **233**, 1175–1180.

9. Woods, A., Munday, M. R., Scott, J., Yang, X., Carlson, M., and Carling, D. (1994) *J. Biol. Chem.* **269**, 19509–19516.

10. Mitchellhill, K. I., Stapleton, D., Gao, G., House, C., Michell, B., Katis, F., Witters, L. A., and Kemp, B. E. (1994) *J. Biol. Chem.* **269**, 2361–2364.

11. Carlson, M. (1999) *Curr. Opin. Microbiol.* **2**, 202–207.

12. Hardie, D. G., Carling, D., and Carlson, M. (1998) *Annu. Rev. Biochem.* **67**, 821–855.

13. Hong, S.-P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 8839–8843.

14. Nath, N., McCartney, R. R., and Schmidt, M. C. (2003) *Mol. Cell. Biol.* **23**, 3909–3917.

15. Sutherland, C. M., Hawley, S. A., McCartney, R. R., Leech, A., Stark, M. J., Schmidt, M. C., and Hardie, D. G. (2003) *Curr. Biol.* **13**, 1299–1305.

16. Hong, S. P., Momcilovic, M., and Carlson, M. (2005) *J. Biol. Chem.* **280**, 21804–21809.

17. Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G., Neumann, D., Schlaßner, U., Wallmann, T., Carlson, M., and Carling, D. (2003) *Curr. Biol.* **13**, 2004–2008.

18. Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 3329–3335.

19. Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R., and Hardie, D. G. (2003) *J. Biol. (Bronx N. Y.)* **2**, 28.

20. Woods, A., Dickerson, K., Heath, R. H., Hong, S. P., Momcilovic, M., Johnstone, S. R., Carlson, M., and Carling, D. (2005) *Cell Metab.* **2**, 21–33.

21. Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., and Hardie, D. G. (2005) *Cell Metab.* **2**, 9–19.

22. Hurley, R. L., Anderson, K. A., Franzon, J. M., Kemp, B. E., Means, A. R., and Witters, L. A. (2005) *J. Biol. Chem.* **280**, 29060–29066.

23. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, L., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995) *Science* **270**, 2008–2011.

24. Shibuya, H., Iwata, H., Masuyama, Y., Gotoh, Y., Yamaguchi, K., Irie, K., Matsumoto, K., Nishida, E., and Ueno, N. (1998) *EMBO J.* **17**, 1019–1028.

25. Shirakabe, K., Yamaguchi, K., Shibuya, H., Irie, K., Matsuda, S., Moriguchi, T., Gotoh, Y., Matsumoto, K., and Nishida, E. (1997) *J. Biol. Chem.* **272**, 8141–8144.

26. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) *Nature* **398**, 252–256.

27. Lee, J., Mira-Aribbe, L., and Ulevitch, R. J. (2000) *J. Leukocyte Biol.* **68**, 909–915.

28. Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K.,...
Ninomiya-Tsuji, J., and Matsumoto, K. (2000) Mol. Cell 5, 649–658
29. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) Nature 412, 346–351
30. Takaesu, G., Surabhi, R. M., Park, K. J., Ninomiya-Tsuji, J., Matsumoto, K., and Gaynor, R. B. (2003) J. Mol. Biol. 326, 105–115
31. Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagiwara, M. (1996) J. Biol. Chem. 271, 13675–13679
32. Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagiwara, M. (1996) J. Biol. Chem. 271, 13675–13679
33. Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E., and Matsumoto, K. (1996) Science 272, 1179–1182
34. Ishitani, T., Takaesu, G., Ninomiya-Tsuji, J., Shibuya, H., Gaynor, R. B., and Matsumoto, K. (2003) EMBO J. 22, 6277–6288
35. Cheung, P. C., Nebreda, A. R., and Cohen, P. (2004) Biochem. J. 378, 27–34
36. Gietz, R. D., and Woods, R. A. (2002) Methods Enzymol. 350, 87–96
37. Hedbacker, K., Hong, S. P., and Carlson, M. (2004) Mol. Cell. Biol. 24, 8255–8263
38. Song, W., and Carlson, M. (1998) EMBO J. 17, 5757–5765
39. Jiang, R., and Carlson, M. (1996) Genes Dev. 10, 3105–3115
40. Fields, S., and Song, O. (1989) Nature 340, 245–246
41. Crute, B. E., Seefeld, K., Gamble, J., Kemp, B. E., and Witters, L. A. (1998) J. Biol. Chem. 273, 35347–35354
42. Hedbacker, K., Townley, R., and Carlson, M. (2004) Mol. Cell. Biol. 24, 1836–1843
43. Davies, S. P., Carling, D., and Hardie, D. G. (1989) Eur. J. Biochem. 186, 123–128
44. Shim, J. H., Xiao, C., Paschal, A. E., Bailey, S. T., Rao, P., Hayden, M. S., Lee, K. Y., Bussey, C., Steckel, M., Tanaka, N., Yamada, G., Akira, S., Matsumoto, K., and Ghosh, S. (2005) Genes Dev. 19, 2668–2681
45. Kishimoto, K., Matsumoto, K., and Ninomiya-Tsuji, J. (2002) J. Biol. Chem. 275, 7359–7364
46. Sakurai, H., Miyoshi, H., Mizukami, J., and Sugita, T. (2000) FEBS Lett. 474, 141–145
47. Sakurai, H., Nishi, A., Sato, N., Nizukami, J., Miyoshi, H., and Sugita, T. (2002) Biochem. Biophys. Res. Comm. 297, 1277–1281
48. Blacketer, M., Koehler, C., Coats, S., Myers, A., and Madaule, P. (1993) Mol. Cell. Biol. 13, 5567–5581
49. Bouquin, N., Barral, Y., Courbeyrette, R., Blondel, M., Snyder, M., and Mann, C. (2000) J. Cell Sci. 113, 1435–1445
50. Sreenivasan, A., Bishop, A. C., Shokat, K. M., and Kellogg, D. R. (2003) Mol. Cell. Biol. 23, 6327–6337
51. Sreenivasan, A., and Kellogg, D. (1999) Mol. Cell. Biol. 19, 7983–7994
52. Tiainen, M., Yliskokala, A., and Makela, T. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9248–9251
53. Cheung, P. C., Campell, D. G., Nebreda, A. R., and Cohen, P. (2003) EMBO J. 22, 5793–5805
54. Suzuki, A., Kusakai, G., Kishimoto, A., Shimojo, Y., Ogura, T., Lavin, M. F., and Esumi, H. (2004) Biochem. Biophys. Res. Commun. 324, 986–992
55. Suzuki, A., Kusakai, G., Shimojo, Y., Chen, J., Ogura, T., Kobayashi, M., and Esumi, H. (2005) J. Biol. Chem. 280, 31557–31563
56. Li, J., Miller, E. J., Ninomiya-Tsuji, J., Russell, R. R., III, and Young, L. H. (2005) Circ. Res. 97, 872–879
57. Lizcano, J. M., Goransson, O., Toth, R., Deak, M., Morrice, N. A., Boudreau, J., Hawley, S. A., Udd, L., Makela, T. P., Hardie, D. G., and Alessi, D. R. (2004) EMBO J. 23, 833–843