Activation of SAD Kinase by Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase†

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ABSTRACT: To search for the downstream target protein kinases of Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK), we performed affinity chromatography purification of a rat brain extract using a GST-fused CaMKKα catalytic domain (residues 126–434) as the affinity ligand. Proteomic analysis was then carried out to identify the CaMKK-interacting protein kinases. In addition to identifying the catalytic subunit of 5′-AMP-activated protein kinase, we identified SAD-B as interacting. A phosphorylation assay and mass spectrometry analysis revealed that SAD-B was phosphorylated *in vitro* by CaMKK at Thr^{189} in the activation loop. Phosphorylation of Thr^{189} by CaMKKα induced SAD-B kinase activity by over 60-fold. In transfected COS-7 cells, kinase activity and Thr^{189} phosphorylation of overexpressed SAD-B were significantly enhanced by coexpression of constitutively active CaMKKα (residues 1–434) in a manner similar to that observed with coexpression of LKB1, STRAD, and MO25. Taken together, these results indicate that CaMKKα is capable of activating SAD-B through phosphorylation of Thr^{189} both *in vitro* and *in vivo* and demonstrate for the first time that CaMKK may be an alternative activating kinase for SAD-B.

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK)[1] was originally identified and cloned as an activating protein kinase for two multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), CaMKI and CaMKIV (reviewed in refs *1* and *2*). CaMKK phosphorylates a Thr residue in the activation loop of downstream CaMKs (Thr^{177} in CaMKIα and Thr^{196} in CaMKIV), resulting in a large increase in their catalytic efficiency (*3–5*). In mammals, CaMKK consists of two isoforms (α, *β*) derived from different genes (*3, 6*). CaMKKα is abundant in brain and thymus (*3*), and CaMKK*β* is broadly expressed (*6*). CaMKK has been identified from various nonmammalian species as well, including *Caenorhabditis elegans* and *Aspergillus nidulans*, as has downstream CaMK, indicating that the CaMKK/CaMK pathway (termed the CaMK cascade) is evolutionally conserved from lower eukaryotes to mammals (*7–9*). Recent studies using *camkk* mutant mice indicated that CaMKKα is required for hippocampus-dependent contextual fear memory formation and that CaMKK*β* is required for spatial, but not contextual fear, memory formation; however, the detailed molecular mechanisms accounting for these physiological roles of CaMKK remain unclear (*10–12*).

CaMKK is a Ca^{2+}/CaM-dependent enzyme that has an N-terminal catalytic domain (CD) and a regulatory domain containing an autoinhibitory and a CaM-binding segment at its C-terminus (*3, 13, 14*). Structure/function studies indicated that Ca^{2+}/CaM binding is required for the release of the autoinhibitory domain from the catalytic core, resulting in generation of CaMKK catalytic activity in a manner similar to other CaMKs (*15*). Interestingly, the solution structure of CaM in complex with a rat CaMKKα peptide as well as the 1.9 Å crystal structure of CaM bound to *C. elegans* CaMKK peptide demonstrated that CaMKK possess a novel CaM-binding motif (1–16 subclass), in which the two key hydrophobic residues, Trp^{444} and Phe^{459}, are placed 16 residues apart, interacting with the N-terminal and C-terminal domain of CaM, respectively, in the opposite direction to that observed for other CaMKs such as CaMKII (1–10 subclass) and myosin light chain kinase (1–14 subclass) (*16, 17*). The CD contains a unique Arg/Pro-rich 22-residue insert that plays an important role for recognition of downstream CaMKs, though the detailed molecular mechanism remains unclear (*7*).

In addition to the activation of CaMKI and CaMKIV, CaMKK has recently been demonstrated to activate other protein kinases including PKB (*18*) and 5′-AMP-activated

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[1] Abbreviations: CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; CaMKK, CaMK kinase; AMPK, 5′-AMP-activated protein kinase; GST, glutathione *S*-transferase; CD, catalytic domain; LC, liquid chromatography; MS/MS, tandem mass spectrometry; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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protein kinase (AMPK) through phosphorylation of activation loop Thr residues (*19–22*). Indeed, ionomycin-induced AMPK activation in HeLa cells was inhibited by either a CaMKK inhibitor, STO-609 (*23*), or RNAi treatment to reduce CaMKKβ expression (*21, 22*). These results suggest the possibility that the elevation of intracellular Ca^{2+} triggers and activates Ca^{2+}-independent protein kinase pathways via CaMKK activation.

Here, in order to search for novel CaMKK targets, we performed a functional proteomic method using affinity chromatography with the CaMKK CD as ligand to partially purify CaMKK-interacting proteins; we combined this approach with liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis for the identification of CaMKK-interacting kinases. Using this method, we identified both the catalytic subunit of AMPK and SAD-B as CaMKK targets and demonstrated that SAD kinase is strongly activated by CaMKK through activation loop phosphorylation *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials. GST-CaMKKα (126–434) was recently constructed (*24*), and the recombinant protein was expressed in *Escherichia coli* JM-109, followed by purification by glutathione-Sepharose chromatography. Recombinant rat CaMKK isoforms were expressed in *E. coli* BL21(DE3) as previously described (*25*). A recombinant heterotrimeric complex of rat AMPK was expressed in *E. coli* BL21-CodonPlus (DE3) (Stratagene, La Jolla, CA) using the tricistronic plasmid pγ1β1His-α1 (kindly provided by Dr. Dietbert Neumann, Swiss Federal Institute of Technology, Zurich, Switzerland) and purified by Ni-NTA agarose column chromatography (Qiagen, Hilden, Germany) (*26*). Recombinant rat CaM was expressed in *E. coli* BL21(DE3) using the pET-CaM vector (kindly provided by Dr. Nobuhiro Hayashi, Fujita Health University, Toyoake, Japan) and was purified by phenyl-Sepharose column chromatography. Antibodies to the catalytic subunit of AMPK and phospho-AMPK α (at Thr^{172}) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Anti-SAD-B antibody was obtained from ProSci, Inc. (Poway, CA). Expression plasmids for LKB1, STRAD, and MO25 were kindly provided by Dr. Hiroshi Takemori (National Institute of Biomedical Innovation, Osaka, Japan). Anti-phospho-SAD-B (at Thr^{189}) monoclonal antibody (9A4) was generated against the synthetic phosphopeptide corresponding to residues 183–197 [GDSLLET(p)SCGSPHYA] of rat SAD-B. The phosphopeptide was conjugated with keyhole limpet hemocyanin via the N-terminal cysteine and was injected into Balb/c mice as described previously (*27*). The antibody was purified using MEP HyperCel (Pall Life Sciences, East Hills, NY) column chromatography according to the manufacturer's protocol. All other chemicals were obtained from standard commercial sources.

Affinity Chromatography. GST and GST-CaMKKα (126–434) (750 μg) were applied to separate glutathione-Sepharose columns (200 μL bed volume; GE Healthcare UK Ltd., Buckinghamshire, U.K.), and then the columns were washed with 10 mL of buffer A [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EGTA, 1 mM EDTA]. Three fresh whole rat brains were homogenized with 30 mL of buffer B (buffer A containing 0.2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL trypsin inhibitor), followed by centrifugation at 43380*g* for 30 min. The supernatant (10 mL) was applied to each Sepharose column as described above, followed by washing with 50 mL of buffer B followed by 10 mL of buffer A. The proteins that interacted with GST-CaMKK (126–434) were eluted by adding 300 μL of buffer A containing 30 units of PreScission protease (GE Healthcare UK Ltd.) to each Sepharose resin, followed by incubation at 4 °C overnight. Thirty microliters of SDS−PAGE sample buffer was added to the eluate, and then each sample was stored at −30 °C until analysis by mass spectrometry or Western blotting.

Mass Spectrometry Analysis. A 150 μL sample of the eluate from the GST-CaMKK (126–434)-coupled glutathione-Sepharose column described above was concentrated and then separated by SDS−10% PAGE and lightly stained with Coomassie Brilliant Blue. Next, eight gel slices were excised from each sample lane in the ~50–100 kDa range, followed by in-gel digestion with 10 μg/mL trypsin (Promega, Madison, WI) overnight at 37 °C (*28*). The digested peptides were eluted with 0.1% formic acid and were subjected to LC-MS/MS analysis. LC-MS/MS analysis was performed on a Q-Tof 2 quadrupole/time-of-flight hybrid mass spectrometer (Micromass, Manchester, U.K.) interfaced with a CapLC capillary reverse-phase liquid chromatography system (Micromass). A 90 min linear gradient from 5% to 45% acetonitrile in 0.1% formic acid was produced and was split at a 1:20 ratio; the gradient solution was then injected into a NanoLC column (PepMap C18, 75 μm × 150 mm; LC Packings, Sunnyvale, CA) at 100 nL/min. The eluted peptides were sprayed directly into the mass spectrometer. The MS/MS data were acquired by MassLynx software (Micromass) and converted to a single text file (containing the observed precursor peptide *m/z*, the fragment ion *m/z*, and intensity values) by ProteinLynx software (Micromass). The file was analyzed using the Matrix Science Mascot MS/MS Ion Search (http://www.matrixscience.com) to search and assign the obtained peptides to the NCBI nonredundant database. We set the search parameters as follows: dDatabase, NCBInr; taxonomy, *all*; enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide tol, ±0.2 Da; MS/MS tol, ±0.2 Da.

LC-MS/MS was used to identify the phosphorylation site of GST-SAD-B. Two micrograms of phosphorylated recombinant GST-SAD-B was separated by SDS−10% PAGE. The following steps were then performed as described above, with two exceptions: first, in-gel digestion was performed with 17 μg/mL chymotrypsin (Roche Applied Sciences, Switzerland) overnight at 25 °C; second, the search parameters were as follows: database, GST-SAD-B-Hisx6 (1022 amino acid residues); enzyme, *all*; variable modification, oxidation (M) and phospho (Ser/Thr); peptide tol, ±0.1 Da; MS/MS tol, ±0.1 Da.

Cloning and Construction of Rat SAD-B cDNAs. Rat SAD-B cDNA (accession number AB365521) was obtained by reverse transcriptase-mediated PCR (RT-PCR) with PrimeSTAR HS DNA polymerase (Takara, Tokyo, Japan) using rat brain cDNA (QUICK-Clone; Clontech Laboratories, Inc., Mountain View, CA) as template with the sense primer (5′-CCGGGGGACCGGTCGGGCCGGGACCAAGGG-3′) and antisense primer (5′-CCTATCCCTGCAGGGCAAGGCCAAGTTTTG-3′) derived from the genomic sequence

in rat chromosome 1. The initial PCR was followed by a second PCR, using sense primer 5′-GGTCTAGACATGTCGTCCGGGTCCAAGGAA-3′ and antisense primer 5′-TGTCCCCCCCTCCCAACATACTCCTTGGGC-3′. The PCR fragments were subcloned into the *Xba*I/*Sma*I site of the pGEX-KG-PreS-His_6 vector. For expression of GST-SAD-B, a PCR fragment encoding Met^1−Pro^{1015} of SAD-B (sense primer 5′-GGTCTAGACATGTCGTCCGGGTCCAAGGAA-3′ and antisense primer 5′-GGGTAGAGGGGTTCCATTGGTGGCCAGGAG-3′) was ligated into the pGEX-KG-PreS-His_6 vector (*Xba*I/*Sma*I sites), resulting in the addition of GHHHHHH at the C-terminal end (after Pro^{1015}) of GST-SAD-B (pGEX-KG-PreS-SAD-B-His_6). A Thr^{189}Ala mutant of pGEX-KG-PreS-SAD-B-His_6 was created by PCR with PrimeSTAR HS DNA polymerase using pGEX-KG-PreS-SAD-B-His_6 as template. Constructs for expression of GST-SAD-B in COS-7 cells were produced by PCR amplification of GST-SAD-B without the His_6 tag, using either wild type or the Thr^{189}Ala mutant cDNA (pGEX-KG-PreS-SAD-B-His_6) as template, followed by introduction of the PCR fragments into the pME18s vector. The nucleotide sequences of all constructs used in this study were confirmed by an ABI PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA).

Purification of Recombinant GST-SAD-B. GST-fused rat SAD-B wild type and Thr^{189}Ala mutant cDNAs (pGEX-KG-PreS-SAD-B-His_6) were introduced into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene), and expression of the recombinant proteins was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside. An *E. coli* pellet containing GST fusion protein was lysed with PBS followed by purification using glutathione-Sepharose column chromatography, as described in the manufacturer's protocol. Further purification was then carried out by using Ni-NTA agarose column chromatography to obtain GST-fused proteins containing the full-length SAD-B.

Phosphorylation and Activation of GST-SAD-B and AMPK in Vitro. Purified recombinant GST-SAD-B (1 μg) or a heterotrimeric complex of AMPK (3 μg) was incubated with or without recombinant CaMKK (0.3 μg) at 30 °C for various time periods in a reaction solution containing 50 mM HEPES (pH 7.5), 10 mM $Mg(OAc)_2$, 1 mM DTT, 2 mM $CaCl_2$, 5 μM CaM, and either 200 μM [γ-^{32}P]ATP (~500 cpm/pmol) or 200 μM ATP for SAD-B kinase activity assays, mass spectrometry analysis, or Western blotting. The reaction was terminated by the addition of SDS−PAGE sample buffer. The samples were then subjected to SDS−7.5% PAGE followed by Western blot analysis, autoradiography, or mass spectrometry analysis. For SAD-B kinase activity measurement, the reaction was initiated by the addition of ATP and terminated by 10-fold dilution with ice-cold stop buffer [50 mM HEPES (pH 7.5), 2 mg/mL bovine serum albumin, 10% ethylene glycol, and 2 mM EDTA], and then 5 μL of diluted sample was incubated in a solution (25 μL) containing 50 mM HEPES (pH 7.5), 10 mM $Mg(OAc)_2$, 1 mM DTT, 1 mM EGTA, 200 μM [γ-^{32}P]ATP (~2000 cpm/pmol), and 100 μM AMARA peptide (Upstate Biotechnology, Inc., Lake Placid, NY) at 30 °C for 10 min. The reaction was terminated by spotting aliquots (20 μL) onto phosphocellulose paper (Whatman P81) followed by washing with 75 mM phosphoric acid. Phosphate incorporation into AMARA peptide was quantitated by liquid scintillation counting of the filters.

Activation and Phosphorylation of SAD-B in COS-7 Cells. COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transfection of pME-GST-SAD-B wild type or Thr^{189}Ala (10 μg) into COS-7 cells was carried out using Lipofectamine reagent (Invitrogen, Carlsbad, CA) with or without 2 μg of CaMKKα (1–434) expression vector, or with 0.7 μg each of LKB1, STRAD, and MO25 expression vectors. After 40 h of incubation, the cells were extracted with buffer C [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 0.2 mM phenylmethanesulfonyl fluoride, 10 mg/L leupeptin, 10 mg/L trypsin inhibitor, 1 μM microcystin LR, 0.5 μM okadaic acid], and then the cell extract was incubated with 50 μL of glutathione-Sepharose (50% slurry) for 16 h at 4 °C followed by washing extensively with buffer C. GST-SAD-B was eluted from the resin by addition of 30 μL of elution buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM reduced glutathione, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 1 μM microcystin LR, 0.5 μM okadaic acid], followed by SAD-B kinase activity measurement or Western blot analysis using 5 μL samples as described above.

Other Methods. Western blot analysis was performed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (GE Healthcare UK Ltd.) as secondary antibody, and a chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA) was used for detection. The protein concentration was estimated by staining the samples with Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc., Hercules, CA) using bovine serum albumin as a standard.

Data Analysis. Average data are presented as the mean and SE. Student's *t* tests were used to calculate significance when two groups were compared. Probability (*p*) values of less than 0.05 were considered statistically significant.

RESULTS

Identification of Target Protein Kinases for CaMKK Using a Functional Proteomic Approach. In order to elucidate the CaMKK-mediated signaling pathway, the identification of the target protein kinases for this enzyme is thought to be extremely important. CaMKK has been reported to phosphorylate a Thr residue in the activation loop of a limited number of protein kinases including CaMKI, CaMKIV, PKB, and AMPK (*3–5, 13, 18–22*). This indicates that the substrate specificity of CaMKK is relatively strict, suggesting that the interaction of the CaMKK CD and its target protein kinases is specific. Previous studies have also detected direct binding of CaMKK with a target protein kinase, CaMKIV (*7, 29*). Therefore, we performed affinity purification of CaMKK targets using the CD of CaMKKα (residues 126–434) as an affinity ligand, followed by mass spectrometry analysis for identification of CaMKK-interacting proteins. We deleted the C-terminal segment of CaMKKα (residues 435–505) including the regulatory domain in order to disrupt the autoinhibitory function of the segment, resulting in generation of a constitutively active kinase domain (*13*). As shown in Figure 1A, we constructed and purified a GST-fused CaMKKα CD (residues 126–434) for the affinity ligand. This GST-CaMKKα CD had a cleavage site (Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro) for PreScission protease between GST and the CaMKKα CD, which led to the specific elution of the

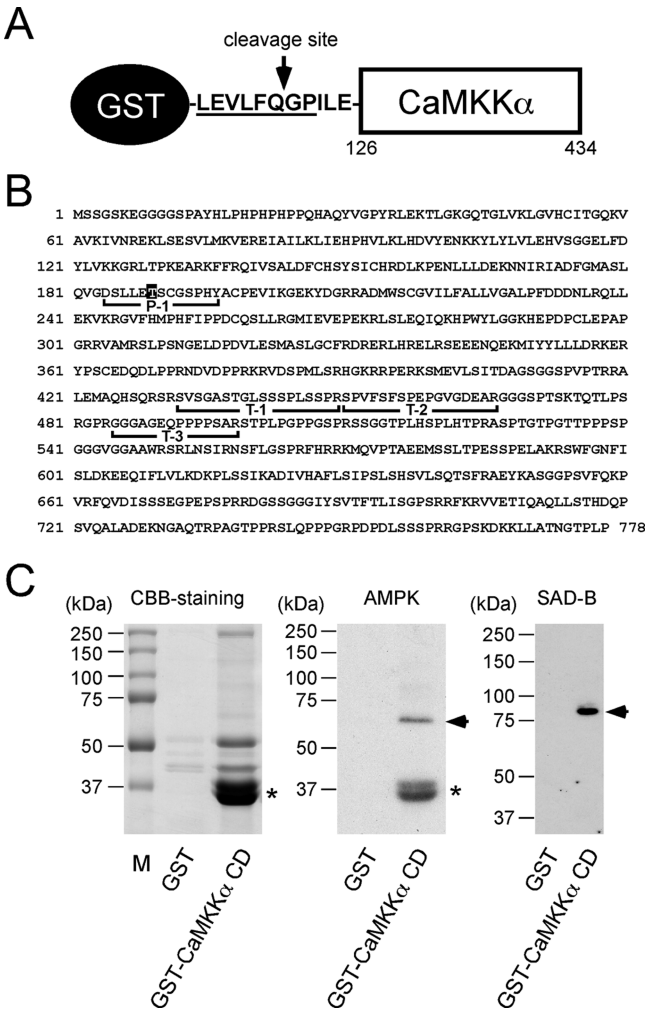

FIGURE 1: Identification of rat SAD-B as a novel target for CaMKK by functional proteomics. (A) Schematic representation of the GST-fused CD of rat CaMKKα (residues 126–434) as an affinity ligand for purification of CaMKK-interacting proteins. Glutathione-Sepharose was coupled with GST or GST-CaMKKα CD followed by purification and identification of CaMKK-interacting proteins from rat brain extracts as described under Experimental Procedures. The cleavage site indicates the location of the recognition sequence (Leu-Glu-Val-Leu-Phe-Gln↓Gly-Pro) for PreScission protease. (B) Amino acid sequence for rat SAD-B as deduced from cDNA isolated in this study (accession number AB365521). The amino acid sequences of the peptides (T-1, T-2, and T-3) from rat SAD-B, eluted from GST-CaMKKα CD-coupled glutathione-Sepharose, were determined by LC-MS/MS. The phosphorylation site of SAD-B (Thr^{189}) determined in this study is indicated by a solid bar (P-1). (C) Rat brain extract was subjected to functional proteomic analysis as described in the legend to (A). Thirty microliters of eluted samples from glutathione-Sepharose coupled with GST (GST) or GST-CaMKKα CD (GST-CaMKKα CD) was subjected to SDS−PAGE, followed by either Coomassie Brilliant Blue (CBB) staining (left panel) or Western blotting of 5 μL samples using anti-AMPK α antibody (middle panel) or anti-SAD-B antibody (right panel). The arrows indicate AMPK α (middle panel) and SAD-B (right panel). The asterisks indicate cleaved CaMKKα CD (left panel), which was nonspecifically bound to the secondary antibody (middle panel). Lane M in the left panel shows the molecular mass marker.

CaMKKα CD-interacting proteins from the affinity matrix by protease treatment. GST-fused CaMKKα CD was coupled with glutathione-Sepharose resin (Figure 1A). As a control, we prepared a glutathione-Sepharose column coupled with GST alone. An equal volume of rat brain extract was applied to each column, followed by extensive washing of each column. CaMKKα CD-interacting proteins were eluted together with cleaved CaMKKα CD by incubation with PreScission protease. The eluate from the GST-CaMKKα CD-coupled column was subjected to SDS−PAGE, followed by protein staining (Figure 1C, left panel). The proteins with a molecular mass range of ~40–250 kDa that eluted from the columns together with the cleaved CaMKKα CD appeared to specifically interact with CaMKKα CD, since we did not detect any proteins with a molecular mass higher than 60 kDa in the eluate from the GST-coupled glutathione-Sepharose (Figure 1C, left panel, lane GST). As shown in Figure 1C (left panel), a protein band with a molecular mass of ~50 kDa was observed in the eluate from the GST-CaMKKα CD-coupled glutathione-Sepharose. Identification and characterization of this protein will be performed elsewhere. Next, to identify the CaMKKα CD-interacting proteins, we excised eight gel slices in the ~50–100 kDa range from the SDS−PAGE gel, in which the eluates from CaMKKα CD-coupled glutathione-Sepharose were separated. The slices were then subjected to in-gel digestion with trypsin. The digested peptides eluted from each slice were subjected to LC-MS/MS analysis in order to identify the proteins by searching the NCBI database. A set of 808 unique peptides, which were assigned to 189 mammalian proteins, was obtained from eluates from CaMKKα CD-coupled glutathione-Sepharose (data not shown). Among the proteins we detected from GST- and GST-CaMKKα CD-coupled glutathione-Sepharose samples, 89 proteins were found to occur in both samples. Among the proteins eluted from CaMKKα CD-coupled glutathione-Sepharose, we detected one peptide (^{364}VPFLVAETPR^{373}) derived from the catalytic subunit (α1) of AMPK, which has been recently shown to be phosphorylated *in vitro* and *in vivo* by CaMKK at Thr^{172} (*19–22*). Western blot analysis with an anti-AMPK α subunit antibody clearly indicated that the catalytic subunit of AMPK specifically interacted with CaMKKα, not with GST (Figure 1C, middle panel).

Identification of SAD-B from Rat Brain as a CaMKK Substrate. In addition to identifying a known substrate for CaMKK (AMPK α1) with this functional proteomic approach, in the eluate from the CaMKKα CD-coupled affinity resin we obtained two peptides (Figure 1B, T-1 and T-3) that completely matched and one peptide (Figure 1B, T-2) with a single amino acid difference (12th Val residue) from the corresponding amino acid sequences of mouse SAD-B. Specific interaction of SAD-B with CaMKKα CD was also confirmed by Western blot analysis with anti-SAD-B antibody (Figure 1C, right panel); this result was similar to results observed with the AMPK α1 subunit (Figure 1C, middle panel). Because rat SAD-B cDNA had not yet been cloned, we prepared its cDNA (accession number AB365521) by an RT-PCR method as described in Experimental Procedures. The deduced amino acid sequence of rat SAD-B has 97% homology with its mouse counterpart and includes all three peptide sequences (T-1, T-2, and T-3) identified in this study (Figure 1B). SAD-B is also known as brain-specific kinase 1 (BRSK1), which is a member of the AMPK family. It has been shown that SAD-B is required for neuronal polarization (*30, 31*) and regulates neurotransmitter release presynaptically (*32*).

Activation of SAD-B by CaMKK-Mediated Phosphorylation. A recent study demonstrated that SAD-B is activated

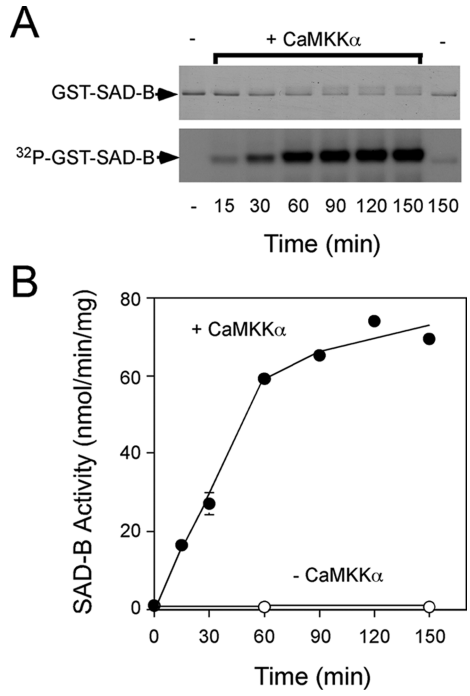

FIGURE 2: Phosphorylation and activation of rat SAD-B by CaMKKα *in vitro*. (A) Time course of SAD-B phosphorylation. GST-SAD-B wild type (1 μg) was incubated without (−) or with 0.3 μg of CaMKKα (+CaMKKα) using 200 μM [γ-^{32}P]ATP at 30 °C for the indicated periods of time in a reaction solution as described under Experimental Procedures. Phosphorylated GST-SAD-B was subjected to SDS−7.5% PAGE, followed by protein staining (top panel) or autoradiography (bottom panel). (B) Activation of SAD-B by CaMKKα. GST-SAD-B (1 μg) was incubated without (−CaMKKα, open circles) or with CaMKKα (+CaMKKα, closed circles) using 200 μM ATP at 30 °C for the indicated periods of time under the same conditions described in (A), and then 5 μL of diluted sample was subjected to SAD-B activity assay as described under Experimental Procedures. Results represent the mean and SE of three experiments.

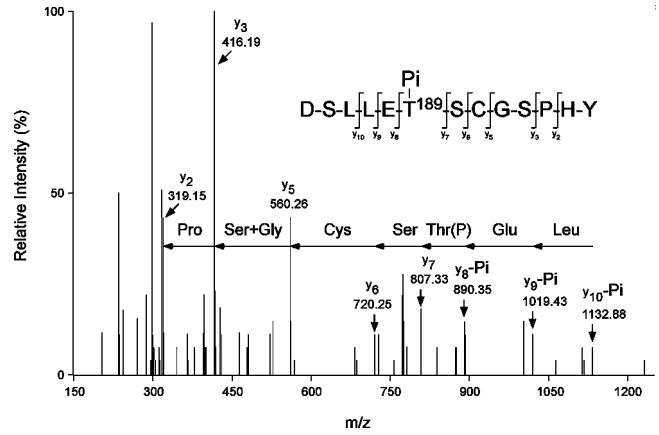

FIGURE 3: Identification of Thr^{189} in rat SAD-B as a site phosphorylated by CaMKK. GST-SAD-B, which was phosphorylated by CaMKKα for 60 min (as shown in Figure 2A), was subjected to SDS−PAGE, digested with chymotrypsin, and analyzed by LC-MS/MS to identify phospho-Thr^{189}. The singly charged ion of a peptide (residues 184–196) derived from phosphorylated GST-SAD-B was subjected to MS/MS analysis as described under Experimental Procedures. The observed y-ion fragment series generated by collision-induced dissociation is indicated by arrows. The observed fragment ions are indicated above and below the peptide sequence.

by kinase LKB1 through phosphorylation of an activation loop Thr residue (*33*). Therefore, we first examined whether rat SAD-B could be phosphorylated and activated by CaMKK *in vitro*. For this purpose, we constructed and purified GST-SAD-B, in which a His_6 tag was inserted at the C-terminal end, to obtain a GST-fused protein containing full-length SAD-B. When GST-SAD-B was incubated in the presence of Ca^{2+}/CaM and [γ-^{32}P]ATP, significant ^{32}P incorporation into GST-SAD-B was observed only in the presence of CaMKKα (Figure 2A), indicating that CaMKKα is capable of phosphorylating SAD-B. We also measured SAD-B kinase activity after incubating with or without CaMKKα for various time periods under the same conditions as the phosphorylation experiment shown in Figure 2A (Figure 2B). We observed very low or insignificant basal SAD-B kinase activity without CaMKK phosphorylation (time point, 0 min). Incubation of SAD-B with CaMKKα resulted in significant induction of SAD-B kinase activity, by over 60-fold (Figure 2B, closed circles). We confirmed that CaMKK was not capable of phosphorylating AMARA peptide under the same conditions shown in Figure 2B (data not shown). The increase in activity of SAD-B by incubation with CaMKKα was saturated around 60 min under these conditions, which was similar to what we observed in ^{32}P incorporation into SAD-B, shown in Figure 2A. These results strongly suggest that CaMKKα phosphorylates SAD-B, resulting in a large induction of its kinase activity. It is noteworthy that SAD-B underwent very low autophosphorylation within 150 min (Figure 2A, right lanes) with no significant increase in activity (Figure 2B, open circles).

*Identification of Phosphorylation Site of SAD-B by CaMKK*α. To determine the site(s) of SAD-B that are phosphorylated by CaMKKα, we analyzed phosphorylated GST-SAD-B (1 μg) as described above by in-gel digestion with chymotrypsin, followed by LC-MS/MS analysis. We analyzed GST-SAD-B phosphorylated by CaMKKα for 60 min, since ^{32}P incorporation and induction of activity are well correlated and saturated at that time point (Figure 2). We detected a phosphopeptide corresponding to residues 184–196 in rat SAD-B (Figure 1B, P-1). LC-MS/MS analysis also revealed a single phosphorylation site at Thr^{189} in the peptide (Figure 3), which is an activation loop Thr residue. To confirm the phosphorylation of SAD-B at Thr^{189} by CaMKK, resulting in increasing activity, we used a GST-SAD-B Thr^{189}Ala mutant (TA) in a phosphorylation assay as well as an activity assay (Figure 4A,B). Isoform specificity of CaMKK as an activator for SAD-B was also examined using a recombinant CaMKKβ isoform since it was shown that CaMKKβ activates AMPK 7-fold more rapidly than CaMKKα *in vitro* (*34*). The mutation at Thr^{189} to Ala in SAD-B completely abolished ^{32}P incorporation into GST-SAD-B and activation of kinase activity by both the α and β isoforms of CaMKK, indicating that direct phosphorylation of SAD-B at Thr^{189} by CaMKK isoforms induces its kinase activity. We also showed that CaMKKα was apparently preferable to CaMKKβ as a SAD-B activator (Figure 4A,B), whereas the phosphorylation activities of both CaMKK isoforms toward the catalytic subunit of AMPK (α1) at Thr^{172} were indistinguishable under these assay conditions (Figure 4C). These results demonstrated that CaMKKα is capable of phosphorylating SAD-B at Thr^{189}, resulting in a large induction of its kinase activity *in vitro*.

*Activation of SAD-B by Phosphorylation with CaMKK*α *in Transfected Cells.* Next, we examined whether CaMKK

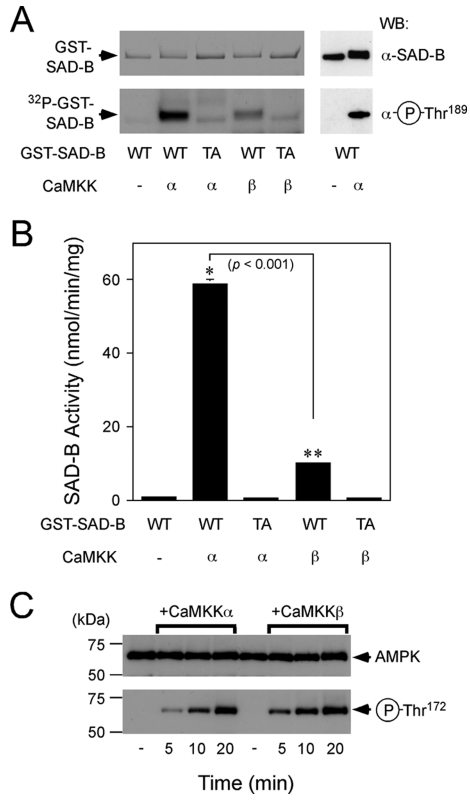

FIGURE 4: Activation of SAD-B through phosphorylation at Thr[189] by CaMKK isoforms. (A, B) GST-SAD-B wild type (WT, 1 μg) or the Thr[189]Ala mutant enzyme (TA, 1 μg) was phosphorylated without (−) or with either the α isoform (α) or β isoform (β) of CaMKK (0.3 μg) at 30 °C for 60 min in a reaction solution containing either 200 μM [γ-^{32}P]ATP (A, left panels) or 200 μM ATP (A, right panels, and B) as described under Experimental Procedures. The reaction was terminated by the addition of SDS−PAGE sample buffer (A). The samples were then subjected to SDS−7.5% PAGE (100 ng for Western blots) followed by protein staining (A, upper left panel), autoradiography (A, lower left panel), and Western blotting using either anti-SAD-B antibody (A, upper right panel) or anti-phospho-SAD-B antibody (A, lower right panel). For SAD-B activity assay (B), the phosphorylation reaction with or without CaMKK was terminated by 10-fold dilution with ice-cold stop buffer. Next, 5 μL of diluted sample was subjected to a SAD-B activity assay using 100 μM AMARA peptide as described under Experimental Procedures. Results represent the mean and SE of three experiments. Statistical differences are marked with asterisks: *, $p < 0.001$ versus GST-SAD-B wild type alone; **, $p < 0.01$ versus GST-SAD-B wild type alone; Student's t test. (C) The recombinant heterotrimeric complex of rat AMPK (3 μg) was incubated without (−) or with either the α isoform (+CaMKKα) or β isoform (+CaMKKβ) of CaMKK (0.3 μg) at 30 °C for the indicated periods of time under the same conditions shown in (A). The reaction was terminated by the addition of SDS−PAGE sample buffer. The samples were then subjected to SDS−PAGE followed by Western blotting using anti-AMPK α subunit antibody (upper panel) or anti-phospho-AMPK α subunit (phospho-Thr[172]) antibody (lower panel). Results are representative of at least three independent experiments.

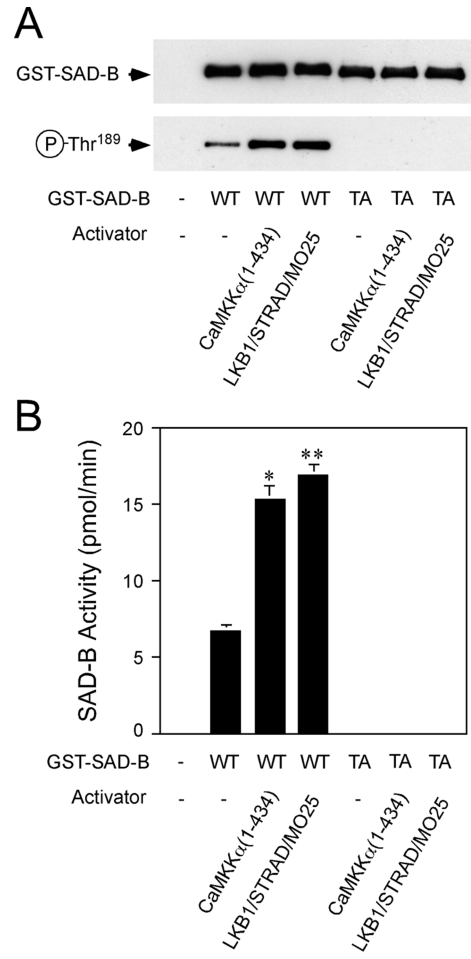

FIGURE 5: Phosphorylation and activation of rat SAD-B by constitutively active CaMKK in transfected COS-7 cells. COS-7 cells were transfected with 10 μg of an empty plasmid (−), 10 μg of GST-SAD-B wild type (WT), or 10 μg of Thr[189]Ala mutant (TA) expression vector with 2 μg of empty vector (−), 2 μg of CaMKKα (1–434) expression vector [CaMKKα (1–434)], or 0.7 μg each of LKB1, STRAD, and MO25 expression vectors (LKB1/STRAD/MO25). After 40 h of culture, cells were lysed, and GST-SAD-B was partially purified with glutathione-Sepharose resin as described under Experimental Procedures. Five microliters of partially purified samples was subjected to Western blot analysis using anti-SAD-B antibody (A, upper panel) or anti-phospho-SAD-B (Thr[189]) antibody (A, lower panel) and then subjected to a SAD-B activity assay using 100 μM AMARA peptide as described under Experimental Procedures (B). Results represent the mean and SE of three experiments. Statistical differences are marked with asterisks: *, $p < 0.05$ versus GST-SAD-B wild type alone; **, $p < 0.005$ versus GST-SAD-B wild type alone; Student's t test. Similar results were obtained for at least three independent experiments.

phosphorylates and activates SAD-B *in vivo* using transfected COS-7 cells (Figure 5). In order to evaluate the phosphorylation level at Thr[189] in SAD-B, we generated an anti-phospho-Thr[189] monoclonal antibody that specifically recognized phosphorylated SAD-B by CaMKK (Figure 4A, right panels). GST-SAD-B cDNA was transfected into COS-7 cells together with either a constitutively active form of CaMKKα cDNA (1–434) or combined LKB1/STRAD/MO25 cDNAs. Partially purified GST-SAD-B from transfected COS-7 cells was analyzed by Western blotting using an anti-SAD-B antibody to confirm the amount of the enzyme assayed (Figure 5A, upper panel) and then subjected to Western blot analysis using an anti-phospho-SAD-B (Thr[189]) antibody (Figure 5A, lower panel) and a SAD-B kinase assay (Figure 5B). As shown in Figure 5A, GST-SAD-B was phosphorylated at Thr[189] even without cotransfection of activating protein kinases. This observation is in good agreement with GST-SAD-B from transfected COS-7 cells exhibiting significant protein kinase activity even without cotransfection of activating kinases (Figure 5B), although the basal activity of recombinant GST-SAD-B expressed in *E. coli* was very low or undetectable without phosphorylation of the activation loop Thr[189] (Figures 2 and 4). These results indicate that a certain population of GST-

SAD-B in COS-7 cells has already been phosphorylated and activated by endogenous activating kinase(s), which is supported by the undetectable activity of the $Thr^{189}Ala$ mutant enzyme when transfected in COS-7 cells, whether in the presence or absence of exogenously expressed activating kinases, including CaMKKα (1–434) and LKB1 (Figure 5). Cotransfection of the constitutively active form of CaMKKα (1–434) enhanced SAD-B activity by ~2.3-fold and was accompanied by an increasing level of phosphorylation at Thr^{189} (~2.7-fold). This enhancement of SAD-B activity and the activation loop phosphorylation by constitutively active CaMKKα were similar to what we observed with cotransfection of LKB1/STRAD/MO25. We also confirmed that Ala substitution at Thr^{189} completely impaired the induction of SAD-B activity by either CaMKKα or LKB1, indicating that CaMKK can phosphorylate Thr^{189} of SAD-B, resulting in induction of its kinase activity in cultured cells.

DISCUSSION

In this report, we describe the development of a proteomic approach using kinase−substrate interaction to identify target kinases for CaMKK. In addition to detection of a known substrate for CaMKK, the catalytic subunit of AMPK, SAD-B kinase, known as a member of the AMPK family, has been shown to specifically interact with the CaMKK CD. However, we could not detect conventional CaMKK targets including CaMKI and CaMKIV by this method, since the interaction of both CaMKs with the CD of CaMKK requires Ca^{2+}/CaM binding to downstream CaMKs, and the affinity purification of CaMKK CD-interacting proteins in this study was performed in the presence of the Ca^{2+}-chelator EGTA. AMPK is now known to be regulated by a family of upstream AMPK kinases, including the CaMKKs and the tumor suppressor kinase LKB1 (*19–22, 35, 36*). Indeed, we demonstrated that SAD-B was activated by CaMKK-mediated phosphorylation at Thr^{189} in a manner similar to the activation by LKB1 in complex with two accessory subunits, STRAD and MO25, *in vitro* and in transfected cells (*40*). By using the anti-phospho-SAD-B (Thr^{189}) antibody, we also observed *in vitro* phosphorylation of another isoform of SAD kinase (SAD-A) by CaMKKα (data not shown). Thus, SAD kinases have become the second member of the AMPK family to be a potential downstream target for CaMKK. It would be of interest to know whether other members of the AMPK family can be activated by CaMKK-mediated phosphorylation. In contrast to the conventional targets for CaMKK, including CaMKI and CaMKIV, which exhibit basal Ca^{2+}/CaM-dependent kinase activity and show that activation loop phosphorylation provides catalytic activation through the modulation of substrate binding, i.e., a decreasing apparent K_m for the substrate (*14, 37*), SAD-B has almost no basal kinase activity without activation loop phosphorylation. This may indicate that the activation loop phosphorylation of SAD-B alters structural components in the kinase domain, resulting in modulating the phosphoryl transfer step rather than affecting substrate binding (*38*). In addition, the rate of activation of SAD-B by CaMKK (Figure 2) is apparently lower than that of CaMKI or CaMKIV but similar to that of PKB (*7, 18*), indicating that the apparent affinity of SAD-B for CaMKK may be lower than that of CaMKI or CaMKIV. This interpretation is supported by the observation that SAD-B was not coimmunoprecipitated with CaMKK from rat brain extract in either the presence or absence of Ca^{2+}/CaM (data not shown).

AMPK has been shown to regulate many aspects of cellular metabolism, especially in response to metabolic stress (*39*). More recent studies have identified a broader role for AMPK in cellular homeostasis and signaling (*40–43*). In particular, CaMKK-mediated activation of AMPK has been shown to be involved in Ca^{2+}-dependent macroautophagy (*44*) and in contraction-stimulated glucose uptake in skeletal muscle (*45*). Unlike ubiquitously expressed AMPK, SAD-B is known as a brain-specific kinase (BRSK1) (*30, 32*), which correlates well with the tissue distribution of α and β isoforms of CaMKK, which are relatively abundant in the brain (*46*). Therefore, the activation of SAD-B by CaMKK in neurons, where the two kinases are expressed endogenously, will be a focus for future studies in this area. In addition, mammalian SAD kinases have been shown to be required for neuronal polarization (*30*), and recent studies have also demonstrated that the involvement of LKB1 in neuronal polarization requires phosphorylation and activation of SAD kinases (*31, 47*). This does not preclude CaMKK as a *bona fide* activating kinase for SAD-B. SAD-B is associated with synaptic vesicles and presynaptically regulates neurotransmitter release (*32*). Whereas SAD-B definitely requires Thr^{189} phosphorylation by upstream kinases for kinase activity as described in this study and elsewhere (*33*), and SAD-B kinase activity is required for neurotransmitter release in neurons (*32*), synaptic activity-dependent regulation of SAD-B phosphorylation and activation remains unclear. It is widely accepted that modulation of the presynaptic Ca^{2+} influx is one of the main mechanisms by which neurotransmitter release can be controlled (*48*). CaMKK has been demonstrated to be a Ca^{2+}/CaM-dependent protein kinase (*3, 6, 13*), although LKB1 shows constitutive activity with pseudokinase STRAD and MO25 (*35, 49, 50*). Therefore, the present finding that SAD-B is activated by CaMKK may shed light on the dynamic regulation of the SAD-B kinase pathway by means of increasing the Ca^{2+} concentration in neurons, resulting in control of neurotransmitter release. Future studies specifically designed to explore this issue will be necessary to understand the physiological significance and regulatory mechanisms of the CaMKK/SAD kinase pathway.

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REFERENCES

1. Soderling, T. R., and Stull, J. T. (2001) Structure and Regulation of Calcium/Calmodulin-Dependent Protein Kinases. *Chem. Rev. 101*, 2341–2352.
2. Hook, S. S., and Means, A. R. (2001) Ca^{2+}/CaM-dependent kinases: from activation to function. *Annu. Rev. Pharmacol. Toxicol. 41*, 471–505.
3. Tokumitsu, H., Enslen, H., and Soderling, T. R. (1995) Characterization of a Ca^{2+}/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase. *J. Biol. Chem. 270*, 19320–19324.
4. Selbert, M. A., Anderson, K. A., Huang, Q.-H., Goldstein, E. G., Means, A. R., and Edelman, A. M. (1995) Phosphorylation and activation of Ca^{2+}-calmodulin-dependent protein kinase IV by Ca^{2+}-calmodulin-dependent protein kinase Ia kinase. Phosphory-

lation of threonine 196 is essential for activation. *J. Biol. Chem. 270*, 17616–17621.
5. Haribabu, B., Hook, S. S., Selbert, M. A., Goldstein, E. G., Tomhave, E. D., Edelman, A. M., Snyderman, R., and Means, A. R. (1995) Human calcium-calmodulin dependent protein kinase I: cDNA cloning, domain structure and activation by phosphorylation at threonine-177 by calcium-calmodulin dependent protein kinase I kinase. *EMBO J. 14*, 3679–3686.
6. Anderson, K. A., Means, R. L., Huang, Q. H., Kemp, B. E., Goldstein, E. G., Selbert, M. A., Edelman, A. M., Fremeau, R. T., and Means, A. R. (1998) Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca^{2+}/calmodulin-dependent protein kinase kinase beta. *J. Biol. Chem. 273*, 31880–31889.
7. Tokumitsu, H., Takahashi, N., Eto, K., Yano, S., Soderling, T. R., and Muramatsu, M. (1999) Substrate recognition by Ca^{2+}/Calmodulin-dependent protein kinase kinase. Role of the arg-pro-rich insert domain. *J. Biol. Chem. 274*, 15803–15810.
8. Eto, K., Takahashi, N., Kimura, Y., Masuho, Y., Arai, K., Muramatsu, M. A., and Tokumitsu, H. (1999) Ca^{2+}/Calmodulin-dependent protein kinase cascade in *Caenorhabditis elegans*. Implication in transcriptional activation. *J. Biol. Chem. 274*, 22556–22562.
9. Joseph, J. D., and Means, A. R. (2000) Identification and characterization of two Ca^{2+}/CaM-dependent protein kinases required for normal nuclear division in *Aspergillus nidulans*. *J. Biol. Chem. 275*, 38230–38238.
10. Mizuno, K., Ris, L., Sanchez-Capelo, A., Godaux, E., and Giese, K. P. (2006) Ca^{2+}/calmodulin kinase kinase alpha is dispensable for brain development but is required for distinct memories in male, though not in female, mice. *Mol. Cell. Biol. 26*, 9094–9104.
11. Blaeser, F., Sanders, M. J., Truong, N., Ko, S., Wu, L. J., Wozniak, D. F., Fanselow, M. S., Zhuo, M., and Chatila, T. A. (2006) Long-term memory deficits in Pavlovian fear conditioning in Ca^{2+}/calmodulin kinase kinase alpha-deficient mice. *Mol. Cell. Biol. 26*, 9105–9115.
12. Mizuno, K., Antunes-Martins, A., Ris, L., Peters, M., Godaux, E., and Giese, K. P. (2007) Calcium/calmodulin kinase kinase beta has a male-specific role in memory formation. *Neuroscience 145*, 393–402.
13. Tokumitsu, H., and Soderling, T. R. (1996) Requirements for calcium and calmodulin in the calmodulin kinase activation cascade. *J. Biol. Chem. 271*, 5617–5622.
14. Matsushita, M., and Nairn, A. C. (1998) Characterization of the mechanism of regulation of Ca^{2+}/ calmodulin-dependent protein kinase I by calmodulin and by Ca^{2+}/calmodulin-dependent protein kinase kinase. *J. Biol. Chem. 273*, 21473–21481.
15. Tokumitsu, H., Muramatsu, M., Ikura, M., and Kobayashi, R. (2000) Regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase. *J. Biol. Chem. 275*, 20090–20095.
16. Osawa, M., Tokumitsu, H., Swindells, M. B., Kurihara, H., Orita, M., Shibanuma, T., Furuya, T., and Ikura, M. (1999) A novel target recognition revealed by calmodulin in complex with Ca^{2+}-calmodulin-dependent kinase kinase. *Nat. Struct. Biol. 6*, 819–824.
17. Kurokawa, H., Osawa, M., Kurihara, H., Katayama, N., Tokumitsu, H., Swindells, M. B., Kainosho, M., and Ikura, M. (2001) Target-induced conformational adaptation of calmodulin revealed by the crystal structure of a complex with nematode Ca^{2+}/calmodulin-dependent kinase kinase peptide. *J. Mol. Biol. 312*, 59–68.
18. Yano, S., Tokumitsu, H., and Soderling, T. R. (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature 396*, 584–587.
19. Hawley, S. A., Selbert, M. A., Goldstein, E. G., Edelman, A. M., Carling, D., and Hardie, D. G. (1995) 5′-AMP activates the AMP-activated protein kinase cascade, and Ca^{2+}/calmodulin activates the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. *J. Biol. Chem. 270*, 27186–27191.
20. Hong, S. P., Momcilovic, M., and Carlson, M. (2005) Function of mammalian LKB1 and Ca^{2+}/calmodulin-dependent protein kinase kinase alpha as Snf1-activating kinases in yeast. *J. Biol. Chem. 280*, 21804–21809.
21. Woods, A., Dickerson, K., Heath, R., Hong, S. P., Momcilovic, M., Johnstone, S. R., Carlson, M., and Carling, D. (2005) Ca^{2+}/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab. 2*, 21–33.
22. Hurley, R. L., Anderson, K. A., Franzone, J. M., Kemp, B. E., Means, A. R., and Witters, L. A. (2005) The Ca^{2+}/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *J. Biol. Chem. 280*, 29060–29066.
23. Tokumitsu, H., Inuzuka, H., Ishikawa, Y., Ikeda, M., Saji, I., and Kobayashi, R. (2002) STO-609, a specific inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase kinase. *J. Biol. Chem. 277*, 15813–15818.
24. Tokumitsu, H., Inuzuka, H., Ishikawa, Y., and Kobayashi, R. (2003) A single amino acid difference between alpha and beta Ca^{2+}/calmodulin-dependent protein kinase kinase dictates sensitivity to the specific inhibitor, STO-609. *J. Biol. Chem. 278*, 10908–10913.
25. Tokumitsu, H., Iwabu, M., Ishikawa, Y., and Kobayashi, R. (2001) Differential regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase isoforms. *Biochemistry 40*, 13925–13932.
26. Neumann, D., Woods, A., Carling, D., Wallimann, T., and Schlattner, U. (2003) Mammalian AMP-activated protein kinase: functional, heterotrimeric complexes by co-expression of subunits in *Escherichia coli*. *Protein Expression Purif. 30*, 230–237.
27. Kimura, K., Nozaki, N., Saijo, M., Kikuchi, A., Ui, M., and Enomoto, T. (1994) Identification of the nature of modification that causes the shift of DNA topoisomerase II beta to apparent higher molecular weight forms in the M phase. *J. Biol. Chem. 269*, 24523–24526.
28. Kikuchi, M., Hatano, N., Yokota, S., Shimozawa, N., Imanaka, T., and Taniguchi, H. (2004) Proteomic analysis of rat liver peroxisome: presence of peroxisome-specific isozyme of Lon protease. *J. Biol. Chem. 279*, 421–428.
29. Lemrow, S. M., Anderson, K. A., Joseph, J. D., Ribar, T. J., Noeldner, P. K., and Means, A. R. (2004) Catalytic activity is required for calcium/calmodulin-dependent protein kinase IV to enter the nucleus. *J. Biol. Chem. 279*, 11664–11671.
30. Kishi, M., Pan, Y. A., Crump, J. G., and Sanes, J. R. (2005) Mammalian SAD kinases are required for neuronal polarization. *Science 307*, 929–932.
31. Barnes, A. P., Lilley, B. N., Pan, Y. A., Plummer, L. J., Powell, A. W., Raines, A. N., Sanes, J. R., and Polleux, F. (2007) LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. *Cell 129*, 549–563.
32. Inoue, E., Mochida, S., Takagi, H., Higa, S., Deguchi-Tawarada, M., Takao-Rikitsu, E., Inoue, M., Yao, I., Takeuchi, K., Kitajima, I., Setou, M., Ohtsuka, T., and Takai, Y. (2006) SAD: a presynaptic kinase associated with synaptic vesicles and the active zone cytomatrix that regulates neurotransmitter release. *Neuron 50*, 261–275.
33. Lizcano, J. M., Goransson, O., Toth, R., Deak, M., Morrice, N. A., Boudeau, J., Hawley, S. A., Udd, L., Makela, T. P., Hardie, D. G., and Alessi, D. R. (2004) LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J. 23*, 833–843.
34. Hawley, S. A., Pan, D. A., Mustard, K. J., Ross, L., Bain, J., Edelman, A. M., Frenguelli, B. G., and Hardie, D. G. (2005) Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab. 2*, 9–19.
35. Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R., and Hardie, D. G. (2003) Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol. 2*, 28.
36. Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D. (2003) LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr. Biol. 13*, 2004–2008.
37. Tokumitsu, H., Brickey, D. A., Glod, J., Hidaka, H., Sikela, J., and Soderling, T. R. (1994) Activation mechanisms for Ca^{2+}/calmodulin-dependent protein kinase IV. Identification of a brain CaM-kinase IV kinase. *J. Biol. Chem. 269*, 28640–28647.
38. Adams, J. A. (2003) Activation loop phosphorylation and catalysis in protein kinases: is there functional evidence for the autoinhibitor model? *Biochemistry 42*, 601–607.
39. Hardie, D. G., Scott, J. W., Pan, D. A., and Hudson, E. R. (2003) Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett. 546*, 113–120.
40. Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D., and Kahn, B. B. (2002) Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature 415*, 339–343.
41. 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., Foufelle, F., Ferre, P., Carling, D., Kimura, S.,

Nagai, R., Kahn, B. B., and Kadowaki, T. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med. 8*, 1288–1295.

42. Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y. B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferre, P., Birnbaum, M. J., Stuck, B. J., and Kahn, B. B. (2004) AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature 428*, 569–574.
43. Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab. 1*, 15–25.
44. Hoyer-Hansen, M., Bastholm, L., Szyniarowski, P., Campanella, M., Szabadkai, G., Farkas, T., Bianchi, K., Fehrenbacher, N., Elling, F., Rizzuto, R., Mathiasen, I. S., and Jaattela, M. (2007) Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. *Mol. Cell 25*, 193–205.
45. Jensen, T. E., Rose, A. J., Jorgensen, S. B., Brandt, N., Schjerling, P., Wojtaszewski, J. F., and Richter, E. A. (2007) Possible CaMKK-dependent regulation of AMPK phosphorylation and glucose uptake at the onset of mild tetanic skeletal muscle contraction. *Am. J. Physiol. Endocrinol. Metab. 292*, E1308–E1317.
46. Sakagami, H., Saito, S., Kitani, T., Okuno, S., Fujisawa, H., and Kondo, H. (1998) Localization of the mRNAs for two isoforms of Ca^{2+}/calmodulin-dependent protein kinase kinases in the adult rat brain. *Brain Res. Mol. Brain Res. 54*, 311–315.
47. Shelly, M., Cancedda, L., Heilshorn, S., Sumbre, G., and Poo, M. M. (2007) LKB1/STRAD promotes axon initiation during neuronal polarization. *Cell 129*, 565–577.
48. Augustine, G. J., Santamaria, F., and Tanaka, K. (2003) Local calcium signaling in neurons. *Neuron 40*, 331–346.
49. Baas, A. F., Boudeau, J., Sapkota, G. P., Smit, L., Medema, R., Morrice, N. A., Alessi, D. R., and Clevers, H. C. (2003) Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD. *EMBO J. 22*, 3062–3072.
50. Boudeau, J., Baas, A. F., Deak, M., Morrice, N. A., Kieloch, A., Schutkowski, M., Prescott, A. R., Clevers, H. C., and Alessi, D. R. (2003) MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. *EMBO J. 22*, 5102–5114.

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