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Generation of Autonomous Activity of Ca^{2+}/Calmodulin-Dependent Protein Kinase Kinase β by Autophosphorylation

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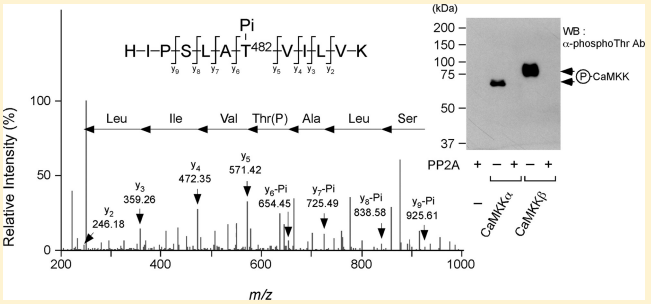
Supporting Information

ABSTRACT: Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKs) phosphorylate and activate specific downstream protein kinases, including CaMKI, CaMKIV, and 5′-AMP-activated protein kinase, which mediates a variety of Ca^{2+} signaling cascades. CaMKKs have been shown to undergo autophosphorylation, although their role in enzymatic regulation remains unclear. Here, we found that CaMKKα and β isoforms expressed in nonstimulated transfected COS-7 cells, as well as recombinant CaMKKs expressed in and purified from *Escherichia coli*, were phosphorylated at Thr residues. Introduction of a kinase-dead mutation completely impaired the Thr phosphorylation of these recombinant CaMKK isoforms. In addition, wild-type recombinant CaMKKs were unable to transphosphorylate the kinase-dead mutants, suggesting that CaMKK isoforms undergo Ca^{2+}/CaM-independent autophosphorylation in an intramolecular manner. Liquid chromatography−tandem mass spectrometry analysis identified Thr^{482} in the autoinhibitory domain as one of the autophosphorylation sites in CaMKKβ, but phosphorylation of the equivalent Thr residue (Thr^{446}) in the α isoform was not observed. Unlike CaMKKα that has high Ca^{2+}/CaM-dependent activity, wild-type CaMKKβ displays enhanced autonomous activity (Ca^{2+}/CaM-independent activity, 71% of total activity). This activity was significantly reduced (to 37%) by substitution of Thr^{482} with a nonphosphorylatable Ala, without significant changes in Ca^{2+}/CaM binding. In addition, a CaMKKα mutant containing the CaMKKβ regulatory domain was shown to be partially phosphorylated at Thr^{446}, resulting in a modest elevation of its autonomous activity. The combined results indicate that, in contrast to the α isoform, CaMKKβ exhibited increased autonomous activity, which was caused, at least in part, by autophosphorylation at Thr^{482}, resulting in partial disruption of the autoinhibitory mechanism.



Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) was originally identified as a novel member of the calmodulin kinase (CaMK) family. This kinase specifically phosphorylates the activation loop Thr residue in two multifunctional calmodulin (CaM) kinases, CaMKI (Thr^{177}) and CaMKIV (Thr^{196}), resulting in a large increase in catalytic efficiency.[1−3] Two CaMKK genes (CaMKKα and CaMKKβ) have been identified in mammals, both of which are strongly expressed in the brain. The α isoform is also expressed in various peripheral tissues such as thymus and spleen.[4,5] CaMKKβ contains three active splice variants (β1−β3), although their functional differences remain unclear.[6,7] An important role has been demonstrated for the CaMKK/CaMKIV cascade in the regulation of Ca^{2+}-dependent gene expression through phosphorylation of transcription factors such as cAMP-response element binding protein (CREB).[8] The CaMKK/CaMKI cascade has been shown to be involved in various neuronal functions, including spinogenesis,[9] dendritic arborization,[10] and cortical axon elongation.[11] Apart from its role in conventional CaMK cascades (CaMKK/CaMKI and CaMKK/CaMKIV), published biochemical evidence indicates that CaMKK also phosphorylates the Akt/protein kinase B[12] and 5′-AMP-activated protein kinase (AMPK) family members, including the catalytic subunit of AMPK (AMPKα) at Thr^{172},[13−16] and SAD-B (known as a brain-specific kinase, BRSK1) at Thr^{189},[17] resulting in significant catalytic activation, indicating that CaMKK confers Ca^{2+} dependence on other signaling pathways. It has also been demonstrated that CaMKK-mediated PKB/Akt activation might be involved in the anti-apoptotic effect of CaMKK in neurons.[12] Recent studies have shown that the Ca^{2+}-dependent phosphorylation and activation of AMPK that occur when T-cells are activated via the antigen receptor[18] or when HeLa cells are treated with a Ca^{2+} ionophore[19] are mediated by CaMKKβ.

Structure−function studies of CaMKKs have demonstrated that the CaMKK isoforms bind to Ca^{2+}/CaM complexes as well as to downstream CaMKI and CaMKIV.[5,20] Indeed, Ca^{2+}/CaM binding is absolutely required for the relief of CaMKKα autoinhibition,[21] which results in its activation, which is similar to those of other CaMKs. In contrast to CaMKKα, CaMKKβ

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exhibits significant autonomous activity (Ca^{2+}/CaM-independent activity), which involves the N-terminal region (residues 129−151).[22] Previous nuclear magnetic resonance and crystallographic studies of CaMKK have revealed a novel CaM-binding motif (1−16),[23,24] and the unique feature of this CaM-binding segment in CaMKK is required for an autoinhibitory mechanism that requires Ile^{441} of CaMKKα.[21] In addition to regulation by Ca^{2+}/CaM binding, CaMKKα has been shown to be phosphorylated by PKA *in vitro* and *in vivo*, resulting in a reduction in its activity, its level of CaM binding, and its extent of recruitment of 14-3-3 proteins.[25−29] This result demonstrates cross talk between two signal transduction systems that are mediated by intracellular Ca^{2+} and cAMP. In addition to PKA phosphorylation, CaMKK isoforms have been shown to undergo autophosphorylation *in vitro*.[5,22] However, the physiological role of autophosphorylation remains unclear.

Here we provide evidence of the autophosphorylation of both CaMKK isoforms. We further examined the autophosphorylation site in the regulatory domain of CaMKKβ and the role of this autophosphorylation in the increased autonomous activity of CaMKKβ.

EXPERIMENTAL PROCEDURES

Materials. Recombinant rat CaMKKα proteins, including point mutant proteins, were expressed in *Escherichia coli* BL21 Star (DE3) and purified using CaM-Sepharose chromatography. CaMKKβ proteins, including various point mutant proteins, CaMKKβ Δ129−151 and CaMKKβ residues 162−499, were expressed as GST fusion proteins in *E. coli* JM109 and purified using glutathione-Sepharose chromatography, followed by CaM-Sepharose chromatography. Purified GST fusion proteins were digested with PreScission protease (GE Healthcare UK, Ltd.) to cleave GST, followed by purification of the enzymes using glutathione-Sepharose chromatography.[22] GST-rat CaMKIα 1−293, $Lys^{49}Glu$ was expressed in *E. coli* JM109 and purified as described previously.[21] Expression plasmids for Flag-tagged CaMKKα and -β were constructed by introduction of corresponding PCR fragments into a pcDNA3 vector. Recombinant rat CaM was expressed in *E. coli* strain BL21(DE3) using plasmid pET-CaM (kindly provided by N. Hayashi, Fujita Health University, Toyoake, Japan) and purified using phenyl-Sepharose column chromatography.[30] Protein phosphatase 2A from human red blood cells was purchased from Millipore Corp. (Temecula, CA). The anti-CaMKIα antibody was kindly provided by H. Sakagami (Kitasato University, Kanagawa, Japan). The anti-phosphoThr (9381) and anti-CaMKK antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA) and BD Transduction Laboratories (San Diego, CA), respectively. The anti-Flag antibody (M2) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from standard commercial sources.

Site-Directed Mutagenesis. The following mutants were constructed by using PCR with PrimeSTAR HS DNA polymerase (Takara) and the following templates. Kinase-dead mutants of CaMKKα ($Asp^{293}Ala$) and CaMKKβ ($Asp^{329}Ala$) were created using pET-CaMKKα and pGEX-PreS-CaMKKβ templates, respectively. CaMKKβ mutants $Thr^{482}Ala$, $Thr^{488}Ala$, and $Thr^{482,\ 488}Ala$ were created using pGEX-PreS-CaMKKβ as a template. Two CaMKKα mutants [mt: $Leu^{440}His$, $Trp^{444}Leu$, and $Thr^{445}Ala$; mt (T446A): $Leu^{440}His$, $Trp^{444}Leu$, $Thr^{445}Ala$, and $Thr^{446}Ala$] were created using pET-CaMKKα as a template. The nucleotide sequences of all constructs used in this study were confirmed by sequencing using an ABI PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA).

Immunoprecipitation of Flag-Tagged CaMKK Isoforms. COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transfection of pcDNA-Flag-CaMKK isoforms (10 μg) into COS-7 cells (10 cm dishes) was conducted using the Lipofectamine reagent (Invitrogen, Carlsbad, CA). After being incubated for 48 h, the cells were collected with 1 mL of buffer B [150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 1% NP-40, 10% glycerol, 0.2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL trypsin inhibitor, and 0.5 μM okadaic acid] and sonicated. The cell extracts were centrifuged at 15000 rpm (20630*g*) for 15 min at 4 °C, and the supernatant was then precleared with 50 μL of Protein G-Sepharose (50% slurry, GE Healthcare UK, Ltd.), incubated with 5 μg of anti-Flag antibody for 1 h, and then incubated with 50 μL of Protein G-Sepharose (50% slurry) overnight. The immunoprecipitated samples were treated with PP2A after the immunoprecipitates had been extensively washed with buffer B, followed by washing with buffer C [150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and 0.2 mM $MnCl_2$].

PP2A Treatment of CaMKK Isoforms. Purified recombinant CaMKKs (1 μg) or immunoprecipitated CaMKKs were incubated with or without 0.12 unit of purified PP2A at 37 °C for 1 h in 40 μL of buffer C containing 50 mM 2-mercaptoethanol. The reaction was terminated by addition of 40 μL of 2× SDS−PAGE sample buffer, followed by Western blot analysis.

In Vitro Assay of CaMKK Activity. Purified recombinant CaMKKs, or their various mutants, were incubated with GST-CaMKIα (1−293, $Lys^{49}Glu$) or with kinase-dead mutants of CaMKK isoforms (1 μg) at 30 °C for various time periods in a solution containing 50 mM HEPES (pH 7.5), 10 mM $Mg(Ac)_2$, 1 mM DTT, 2 mM EGTA or 2 mM $CaCl_2$, and 7 μM CaM in the presence of 100 μM [γ-^{32}P]ATP or ATP. The reaction was initiated by the addition of [γ-^{32}P]ATP or ATP and was terminated either by spotting aliquots (15 μL) onto phosphocellulose paper (Whatman P-81) followed by washing with 75 mM phosphoric acid or by addition of an equal volume of 2× SDS−PAGE sample buffer, followed by Western blot analysis. Incorporation of ^{32}P into GST-CaMKIα (residues 1−293, $Lys^{49}Glu$) at Thr^{177} was quantified by scintillation counting.

Mass Spectrometry Analysis. Recombinant CaMKK isoforms, including various mutants (10 μg), were separated by SDS−PAGE (7.5 or 10%) and lightly stained with Coomassie Brilliant Blue, followed by in-gel digestion[31] with a protease or a protease cocktail such as trypsin, chymotrypsin (Roche Diagnostics, Penzberg, Germany), trypsin with chymotrypsin, trypsin with Glu-C (Roche Diagnostics), trypsin with Asp-N (Roche Diagnostics), or chymotrypsin with Asp-N. The following protease concentrations were used: 10 μg/mL trypsin, 17 μg/mL chymotrypsin, 10 μg/mL Glu-C, and 4 μg/mL Asp-N. Trypsin and Asp-N were incubated at 37 °C, and chymotrypsin and Glu-C were incubated at 25 °C. The first digestion was performed overnight, and the second digestion was incubated for 3 h. The digested peptides were eluted with 0.1% formic acid and subjected to liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis. LC−MS/MS analysis was performed on an LCMS-IT-TOF instrument (Shimadzu, Kyoto, Japan) interfaced with a nano reverse-phase

liquid chromatography system (Shimadzu). LC separation was performed using a Pico Frit Beta Basic C18 column (New Objective, Woburn, MA) at a rate of 300 nL/min. Peptides were eluted using gradients of 5 to 45% acetonitrile in 0.1% formic acid and sprayed directly into the mass spectrometer. The heated capillary temperature and electrospray voltage were set at 200 °C and 2.5 kV, respectively. MS/MS data were acquired in the datum-dependent mode by LCMS solution software (Shimadzu) and were converted to a single text file (containing the observed precursor peptide m/z, the fragment ion m/z, and intensity values) by Mascot Distiller (Matrix Science, London, U.K.). MS/MS data were obtained independently and merged for Mascot analysis. The search parameters were as follows: database, CaMKKα (505 amino acid residues) and CaMKKβ (577 amino acid residues for the full-length enzyme without the first Met + five residues at the N-terminus, G-P-I-L-D-); enzyme, all; variable modification, carbamidomethyl (C), oxidation (M), propionamid (C), and phospho (Ser/Thr).

Other Methods. Western blot analysis was performed using the secondary antibodies horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG (GE Healthcare UK, Ltd.) and a chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA) for detection. The level of Thr phosphorylation in CaMKKs was estimated by densitometry scanning of immunoreactive signals using the anti-phosphoThr antibody. CaM overlay analysis was conducted using 0.5 μg/mL biotinylated CaM (Calbiochem, San Diego, CA) as described previously.[32] Protein concentrations were estimated with the Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA) using bovine serum albumin as a standard.

Data Analysis. Average data are presented as the mean ± standard error. Student's t tests were used to calculate significance when two groups were compared. Probability (p) values of <0.05 were considered statistically significant.

RESULTS

Autophosphorylation of CaMKK Isoforms Expressed in COS-7 Cells and in *E. coli*. Previous studies have shown that CaMKKα is phosphorylated both in response to the elevation of the level of cAMP in cells and by cAMP-dependent protein kinase *in vitro*.[25,26] In addition, CaMKK isoforms have been shown to undergo autophosphorylation *in vitro*, although the physiological significance of this autophosphorylation remains unclear.[5,22] To examine the role of phosphorylation in CaMKK function, we first analyzed the phosphorylation of CaMKK isoforms in eukaryotic cells in the absence of stimulation. Flag-tagged CaMKK isoforms were immunoprecipitated from unstimulated transfected COS-7 cells and analyzed by Western blotting using a phosphoThr antibody. This assay showed that CaMKK isoforms are phosphorylated at Thr residues. Protein phosphatase 2A (PP2A) treatment completely abolished this phosphoThr signal (Figure 1A). These results indicate that CaMKK isoforms in cultured cells are either transphosphorylated by other endogenous cellular protein kinases or autophosphorylated. To examine the latter possibility, we checked the phosphorylation level of recombinant CaMKK isoforms. CaMKKα was expressed in *E. coli* and purified by CaM-Sepharose chromatography as previously described.[22] CaMKKβ was expressed as GST fusion proteins in *E. coli*, and the GST moiety was subsequently removed (Figure 1B). Interestingly, both α and β isoforms of purified CaMKKs expressed in *E. coli* are phosphorylated at Thr

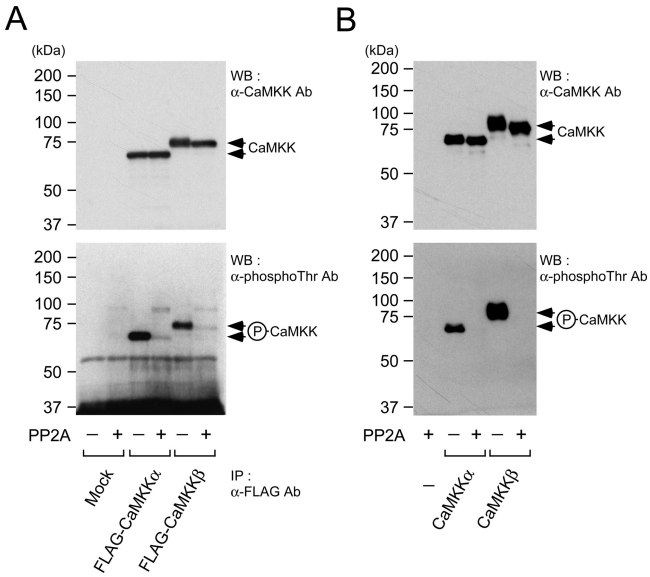

Figure 1. Phosphorylation of CaMKK isoforms expressed in COS-7 cells and in *E. coli*. Flag-tagged CaMKKα or -β, or empty vector (Mock), was transfected into COS-7 cells, followed by immunoprecipitation with an anti-Flag antibody. Flag immunoprecipitates (A) or purified recombinant CaMKKα or -β from *E. coli* (B) was incubated with (+) or without (−) 0.12 unit of PP2A at 37 °C for 1 h, followed by Western blot analysis using an anti-phosphoThr antibody (bottom). After the anti-phosphoThr antibody had been stripped, the blotting membranes were reprobed with an anti-CaMKK antibody (top). The arrows in the top panels indicate either Flag-tagged CaMKK isoforms (A) or recombinant CaMKK isoforms (B). The arrows in the bottom panels indicate either phosphorylated Flag-tagged CaMKK isoforms (A) or phosphorylated recombinant CaMKK isoforms (B). Results were representative of at least three independent experiments. The molecular masses in kilodaltons are indicated on the left-hand side. Abbreviations: WB, Western blot; IP, immunoprecipitation; Ab, antibody.

residues, and their phosphorylation levels were significantly reduced by PP2A treatment, consistent with the effect of PP2A on the enzymes immunoprecipitated from COS-7 cells. Introduction of kinase-dead mutations (Asp^{293}Ala in the α isoform and Asp^{329}Ala in the β isoform) into the recombinant CaMKK isoforms abolished the phosphorylation of both isoforms, whereas as expected, purified wild-type enzymes were phosphorylated (Figure 2A). This result clearly indicates that the phosphorylation of CaMKKs depends directly on their kinase activity, suggesting either intermolecular or intramolecular autophosphorylation. In addition, because the established activator of CaMKK, CaM, is not detected in *E. coli* (data not shown), the autophosphorylation of CaMKKs in *E. coli* apparently does not require Ca^{2+}/CaM, indicating that this phosphorylation represents Ca^{2+}/CaM-independent autophosphorylation of CaMKK isoforms. Next, to determine if the observed phosphorylation of CaMKK isoforms was a result of inter- or intramolecular autophosphorylation, kinase-dead mutants of the purified recombinant CaMKK isoforms were incubated with the purified recombinant wild-type enzyme in the presence of Mg-ATP and EGTA (Figure 2B). To confirm the CaMKK activities, we used a GST-CaMKIα (1−293, Lys^{49}Glu) mutant as a substrate that is kinase-dead and cannot undergo autophosphorylation. Thus, any phosphorylation of the CaMKI mutant is due to CaMKKs. Although the wild-type CaMKKs were capable of phosphorylating a GST-CaMKIα

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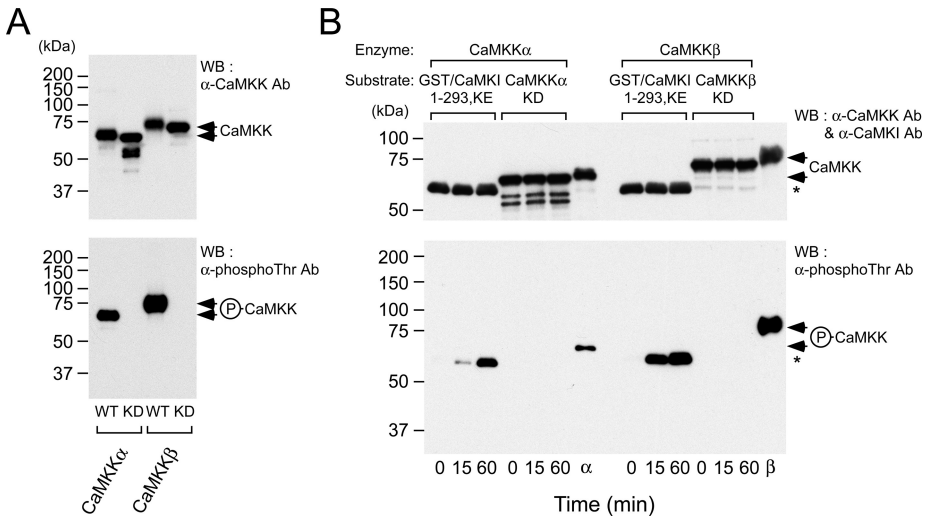

Figure 2. Autophosphorylation of recombinant CaMKK isoforms. (A) Wild-type (WT) or kinase-dead mutant (KD, Asp^{293}Ala in the α isoform and Asp^{329}Ala in the β isoform) CaMKK isoforms were analyzed by Western blotting using either an anti-phosphoThr antibody (bottom) or an anti-CaMKK antibody (top). (B) Wild-type CaMKKα or CaMKKβ (10 ng) was incubated without substrate, with GST-rat CaMKIα (1−293, Lys^{49}Glu) (1 μg), or with a kinase-dead mutant (KD, Asp^{293}Ala in the α isoform and Asp^{329}Ala in the β isoform, 1 μg) of its respective CaMKK isoform, in the presence of Ca^{2+}/CaM at 30 °C for the indicated time periods. After termination of the reaction, samples (100 ng of substrate) were analyzed by Western blotting using either an anti-phosphoThr antibody (bottom) or a mixture of anti-CaMKK and anti-CaMKI antibodies (top). Recombinant wild-type CaMKKα (lane α) and CaMKKβ (lane β) (100 ng of each) were also analyzed. The arrows in the top and bottom panels indicate recombinant CaMKKs and phosphorylated CaMKK isoforms, respectively. The asterisks in the top and bottom panels indicate GST-rat CaMKIα (1−293, Lys^{49}Glu) and phosphorylated GST-rat CaMKIα (1−293, Lys^{49}Glu), respectively. The purified CaMKKα KD mutant contains degradation products (doublet bands shown in lane 2 in panel A and in lanes 4−6 in panel B) that are detected with the anti-CaMKK antibody. The molecular masses in kilodaltons are indicated on the left-hand side. Abbreviations: WB, Western blot; Ab, antibody.

(1−293, Lys^{49}Glu) mutant substrate at Thr^{177}, they did not transphosphorylate the kinase-dead CaMKKs. Phosphorylation of the wild-type CaMKK isoforms was readily detected using the same amount (100 ng) of enzyme that was used for the phosphorylation assay of the mutant CaMKKs (Figure 2B, lane α and lane β). These results clearly suggest that the autophosphorylation of CaMKK isoforms was not an intermolecular process and probably occurred in an intramolecular manner.

Identification of the Autophosphorylation Sites in CaMKK Isoforms. To determine the autophosphorylation site(s) of CaMKK isoforms, we analyzed purified recombinant CaMKKs (10 μg) by in-gel digestion with a protease cocktail, followed by LC−MS/MS analysis. Among the peptides obtained from CaMKKα and -β, which spanned 83 and 89% of the entire amino acid sequence of the CaMKK isoforms, respectively, we detected two phosphorylation sites in the α isoform and four phosphorylation sites in the β isoform (Table 1 and Supplemental Data 1 of the Supporting Information). One of these autophosphorylation sites was identified as Thr^{482}, which was detected in a phosphopeptide corresponding to residues 476−487 of CaMKKβ that are located in its regulatory domain (Figure 3A).[21] LC−MS/MS analysis revealed that Thr^{482} was the only residue phosphorylated in this peptide. In contrast, the equivalent Thr residue (Thr^{446}) in the corresponding peptide (residues 440−451) of CaMKKα was detected as an unphosphorylated form (Figure 3B and Supplemental Data 2 of the Supporting Information).

Autophosphorylation at Thr^{482} Induces Autonomous Activity of CaMKKβ. Because Thr^{482} that was identified as one of the autophosphorylation sites is located in the regulatory domain of CaMKKβ [residues 471−499 (Figure 3B)], we next examined the role of autophosphorylation at Thr^{482} in CaMKKβ activity, using point mutants of CaMKKβ that were

Table 1. Identification of the Autophosphorylation Sites in CaMKK Isoforms[a]

CaMKK isoform	residues	peptide sequence	phospho-amino acid residue
α	86−102	AQVGPYS(p)T GPASHMSPR	Thr^{93}
α	173−188	RPPPRG(p)S QAPQGGPAK	Ser^{179}
β	18−27	DELG(p)SGGVSR	Ser^{22}
β	214−226	G(p)TRPAP GGCIQPR	Thr^{215}
β	476−487	HIPSLA(p)TVILVK	Thr^{482}
β	508−518	SLSAPGNLL(p)TK	Thr^{517}

[a]Recombinant CaMKK isoforms expressed in *E. coli* were subjected to LC−MS/MS analysis to identify autophosphorylation sites (see Supplemental Data 1 of the Supporting Information).

expressed in *E. coli* and subsequently purified. Substitution of Thr^{482} in CaMKKβ with the nonphosphorylatable residue Ala increased the electrophoretic mobility of CaMKKβ in SDS−PAGE gels compared to that of the wild-type enzyme, indicating that phosphorylation of the purified wild-type CaMKKβ occurs at Thr^{482} as described above (Figure 4A, left panel). This is consistent with a result of Western blot analysis using an anti-phosphoThr antibody showing that the Ala mutation significantly (~50%) reduced the extent of Thr phosphorylation of CaMKKβ (Figure 4A, middle panel). An assay of the kinase activity of the wild type and the Thr^{482}Ala CaMKKβ mutant, using GST-CaMKIα (1−293, Lys^{49}Glu) as a substrate in the presence or absence of Ca^{2+}/CaM (Figure 4B), revealed that the autonomous activity (Ca^{2+}/CaM-independent activity) of the Thr^{482}Ala mutant was significantly lower (37% of total activity) than that of the wild-type enzyme, which exhibited high autonomous activity (71% of total activity),

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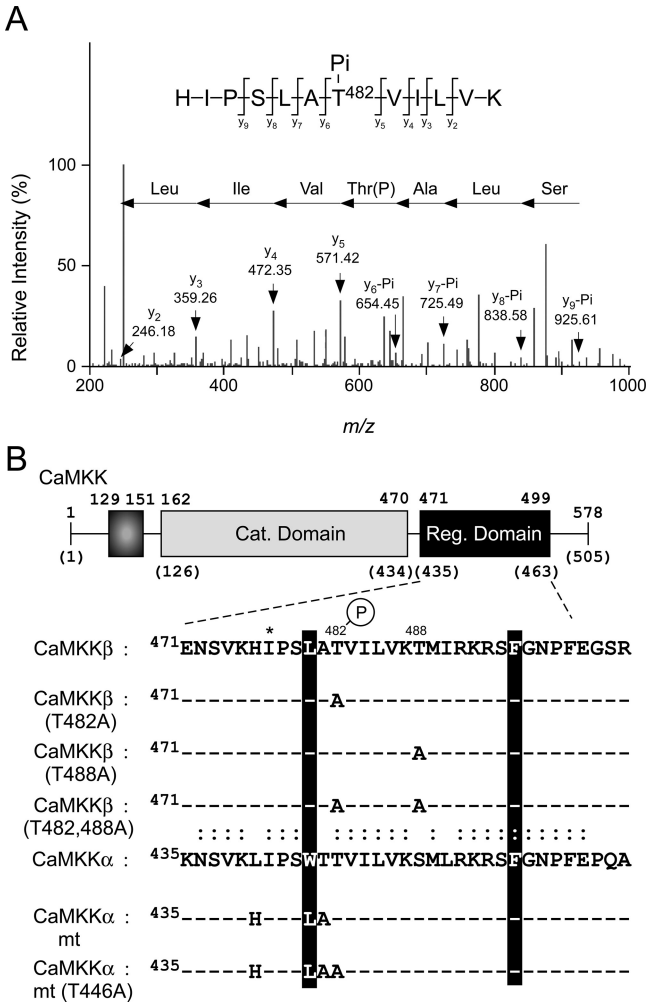

Figure 3. Phosphorylation of Thr[482] in the regulatory domain of CaMKK*β*. (A) Recombinant CaMKK*β* was subjected to SDS−PAGE, digested with a protease cocktail, and analyzed by LC−MS/MS, resulting in identification of phosphoThr[482]. The doubly charged ion of a peptide (residues 476−487) derived from CaMKK*β* was subjected to MS/MS analysis as described in Experimental Procedures. The observed y-ion fragment series generated by collision-induced dissociation is indicated by arrows. The observed fragment ions are indicated below the peptide sequence. (B) Schematic representation of CaMKK indicating the residue numbers of CaMKK*β* and CaMKK*α* (parentheses). Amino acid sequences of the regulatory domain of CaMKK*β* and CaMKK*α*, as well as those of the CaMKK mutants used in this study [CaMKK*β* Thr[482]Ala, CaMKK*β* Thr[488]Ala, CaMKK*β* Thr[482, 488]Ala, CaMKK*α* mt, and CaMKK*α* mt (Thr[446]Ala)], are given. PhosphoThr[482] is also shown. The N-terminal domain in CaMKK*β* corresponding to residues 129−151 is indicated by a shaded box. An asterisk indicates an Ile residue essential for the autoinhibition of CaMKKs.[21] Hydrophobic residues of CaMKKs for anchoring to CaM are indicated by black boxes.[23,24] Abbreviations: Cat., catalytic; Reg., regulatory.

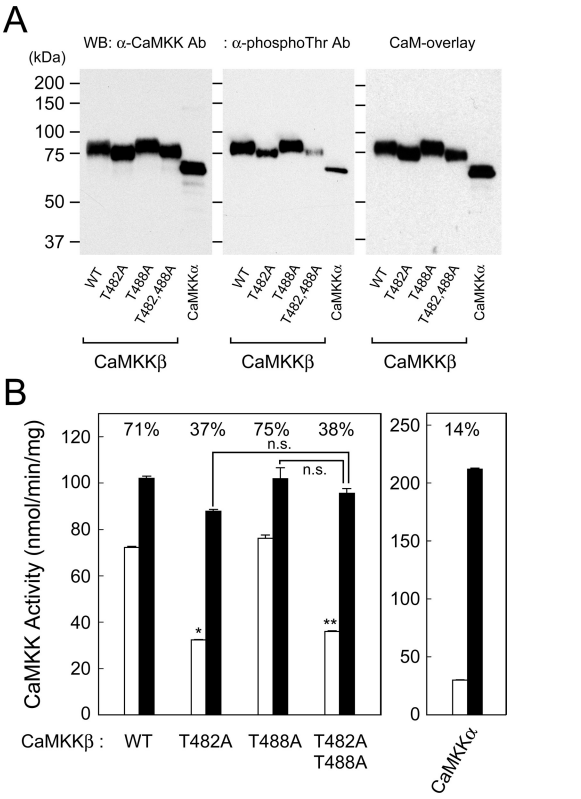

Figure 4. Involvement of Thr[482] autophosphorylation in the enhanced autonomous activity of CaMKK*β*. (A) Purified recombinant CaMKKs (20 ng), including CaMKK*β* wild type (WT), Thr[482]Ala (T482A), Thr[488]Ala (T488A), and Thr[482,488]Ala (T482,488A) and CaMKK*α* wild type (CaMKK*α*), were subjected to Western blot analysis using an anti-phosphoThr antibody (middle) or to CaM overlay analysis in the presence of 1 mM $CaCl_2$ (right). After the anti-phosphoThr antibody had been stripped, the blotting membrane was reprobed with an anti-CaMKK antibody (left). The molecular masses in kilodaltons are indicated on the left-hand side. Abbreviations: WB, Western blot; Ab, antibody. (B) CaMKK activities of the same purified recombinant CaMKKs as in panel A (10 ng) were measured at 30 °C for 10 min using 10 *μ*g of GST-rat CaMKI*α* (1−293, Lys[49]Glu) as a substrate in the presence of either 2 mM $CaCl_2$ and 7 *μ*M CaM (black bars) or 2 mM EGTA (white bars) as described in Experimental Procedures. The results represent the mean ± standard error of three experiments. Statistical differences are marked: $*p < 0.001$ vs CaMKK*β* wild type in the presence of EGTA; $**p < 0.001$ vs CaMKK*β* T488A in the presence of EGTA; n.s., not significant. The autonomous activity of each recombinant enzyme (percentage of the total activity) is indicated.

while the Ca^{2+}/CaM-dependent activity of the Thr[482]Ala mutant was apparently weakly reduced. This result is in good agreement with previous reports[5,22] and suggests that autophosphorylation of Thr[482] in CaMKK*β* partially disrupts its autoinhibitory activity, resulting in increased autonomous activity. This hypothesis is supported by the fact that CaMKK*α* is tightly regulated through an autoinhibitory mechanism in the absence of Ca^{2+}/CaM [autonomous activity of 14% (Figure 4 B, right panel)] and is not phosphorylated at the equivalent Thr residue (Thr[446]) in its autoinhibitory domain as described above (Supplemental Data 2 of the Supporting Information). To confirm this hypothesis, we introduced an Ala mutation at a neighboring unphosphorylated Thr residue (Thr[488]) in CaMKK*β* and then examined the activity of the purified mutant. The electrophoretic mobility, Thr phosphorylation level, and autonomous activity (75% of total activity) of the Thr[488]Ala mutant were indistinguishable from those of the wild-type enzyme (Figure 4A, B). Furthermore, introduction of an additional Ala mutation at Thr[482] into the Thr[488]Ala mutant resulted in significantly reduced autonomous activity (38% of total activity) of this double mutant, as well as increased mobility on SDS−PAGE gels and a significant reduction (~80%) in the magnitude of the phosphoThr signal, which was similar to that observed for the Thr[482]Ala mutant. It is

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noteworthy that the Ca^{2+}/CaM binding ability of these CaMKKβ mutants was apparently indistinguishable from that of the wild-type enzyme when the ability was examined using a CaM overlay assay (Figure 4A, right panel).

Requirement of Residues 129−151 for the Autophosphorylation of CaMKKβ. According to the mutagenesis study shown in Figure 4, the autophosphorylation of Thr^{482} is involved in generation of the autonomous activity of CaMKKβ; however, the Thr^{482}Ala mutant exhibited residual autonomous activity (~35% of total activity), indicating that the autophosphorylation at Thr^{482} is not the only mechanism for induction of the autonomous activity of the enzyme. A previous study has demonstrated that the N-terminal region (residues 129−151) of CaMKKβ plays an important role in generation of its autonomous activity.[22] Therefore, we then attempted to examine the role of the N-terminal region (residues 129−151) in the autophosphorylation of CaMKKβ by using CaMKKβ Δ129−151 lacking residues 129−151 and N-terminal and C-terminal truncation mutants (residues 162−499) (Figure 5). As

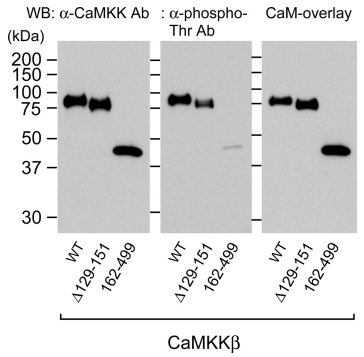

Figure 5. Autophosphorylaiton and Ca^{2+}/CaM binding of CaMKKβ mutants lacking residues 129−151. Purified recombinant CaMKKβ isoforms (50 ng), including the wild type (WT), a mutant lacking residues 129−151 (Δ129−151), and an N-terminal and C-terminal truncation mutant (residues 162−499) (162−499), were subjected either to Western blot analysis using an anti-CaMKK antibody (left) or an anti-phosphoThr antibody (middle) or to CaM overlay analysis in the presence of 1 mM $CaCl_2$ (right). The molecular masses in kilodaltons are indicated on the left-hand side. Abbreviations: WB, Western blot; Ab, antibody.

in previous work,[22] both CaMKKβ Δ129−151 and CaMKKβ residues 162−499 lacking residues 129−151 exhibited significantly reduced autonomous activities (6 and 8% of total activity, respectively) (data not shown). The Ca^{2+}/CaM binding ability of these CaMKKβ mutants was apparently indistinguishable from that of the wild-type enzyme (Figure 5, right panel). The phosphoThr signal of CaMKKβ Δ129−151, which contained all of the Thr autophosphorylation sites (Thr^{215}, Thr^{482}, and Thr^{517}) identified in this study (Table 1), was ~50% reduced (Figure 5, middle panel). In addition, the level of Thr phosphorylation of recombinant CaMKKβ residues 162−499, which lacked Thr^{517}, was >90% reduced compared to that of the wild-type enzyme. These results suggest that residues 129−151 are apparently required for the Thr autophosphorylation of CaMKKβ, including that at Thr^{482}.

Modest Elevation of the Autonomous Activity of a CaMKKα Mutant by Thr^{446} Phosphorylation. LC−MS/MS analysis of recombinant CaMKKα indicated that Thr^{446} in the autoinhibitory domain was not phosphorylated, which may have been the reason why this enzyme appeared to maintain low autonomous activity. If this is indeed the case, then phosphorylation of Thr^{446} should increase the level of the autonomous activity of CaMKKα. We therefore constructed and analyzed a CaMKKα mutant (CaMKKα mt), which was mutated so that its sequence corresponds to the N-terminal regulatory domain of CaMKKβ (Figure 3B). We first analyzed this recombinant CaMKKα mt using LC−MS/MS and obtained a phosphopeptide (see Supplemental Data 3 of the Supporting Information), as well as a nonphosphorylated peptide (data not shown), that corresponded to residues between His^{440} and Lys^{451}, indicating that Thr^{446} was partially phosphorylated in this recombinant CaMKKα mt. Because one of the key residues for anchoring to CaM $(Trp^{444})^{23}$ was mutated to Leu in this CaMKKα mt, we examined Ca^{2+}/CaM binding of the CaMKKα mt by CaM overlay analysis (Figure

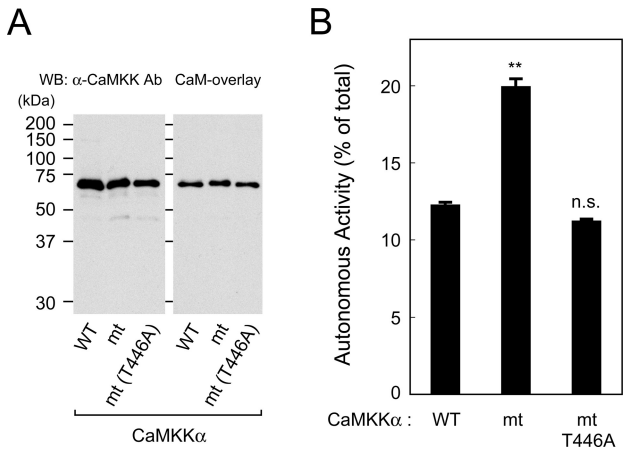

Figure 6. Modest elevation of the autonomous activity of a CaMKKα mutant. (A) Purified recombinant CaMKKα isoforms (50 ng), including the wild type (WT), the CaMKKα mutant (mt), and the CaMKKα mutant with a Thr^{446}Ala mutation [mt (T446A)], were subjected to either Western blot analysis using an anti-CaMKK antibody (left) or a CaM overlay analysis in the presence of 1 mM $CaCl_2$ (right). The molecular masses in kilodaltons are indicated on the left-hand side. Abbreviations: WB, Western blot; Ab, antibody. (B) Autonomous activity of the recombinant CaMKKα isoforms. The recombinant CaMKKα isoforms in panel A (10 ng) were incubated with GST-rat CaMKIα (1−293, Lys^{49}Glu) (10 μg) at 30 °C for 10 min in the presence of either 2 mM $CaCl_2$ and 7 μM CaM or 2 mM EGTA as described in Experimental Procedures. Autonomous activity is expressed as a percentage of total activity in the presence of Ca^{2+}/CaM. The results represent the mean ± standard error of three experiments. Statistical differences are marked: $^{**}p < 0.001$ vs CaMKKα wild type; n.s., not significant.

6A, right panel). Ca^{2+}/CaM binding of mutant CaMKKα was apparently indistinguishable from that of the wild-type enzyme. When we measured CaMKK activity in the absence or presence of Ca^{2+}/CaM (Figure 6B), a modest increase in the autonomous activity of the CaMKKα mt was observed compared to that of the wild-type enzyme. This modest elevation of the autonomous activity of the CaMKKα mt is likely due to autophosphorylation of Thr^{446}, because the autonomous activity of CaMKKα mt T446A, in which Thr^{446} was mutated to a nonphosphorylatable Ala, was indistinguishable from that of wild-type CaMKKα (Figure 6B).

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DISCUSSION

Previous results have shown that CaMKKβ differs from other CaMK family members, including the CaMKKα isoform, in that it exhibits activity that is enhanced by, but not completely dependent on, Ca^{2+}/CaM in the cell as well as *in vitro*.[5,22] This study shows that recombinant CaMKKβ undergoes autophosphorylation at Thr^{482} in the autoinhibitory domain that maintains the kinase in a partially active conformation in the absence of its activator, the Ca^{2+}/CaM complex. The autophosphorylation of CaMKKβ in the autoinhibitory domain, resulting in the generation of Ca^{2+}/CaM-independent activity (autonomous activity), is similar to the characteristic feature of the multifunctional CaMK, CaMKII.[33] By analogy to CaMKII, whose regulatory autophosphorylation at Thr^{286} (in the α isoform) in the autoinhibitory domain results in generation of Ca^{2+}/CaM-independent activity,[34,35] the autonomous activity of CaMKKβ is probably generated in part by disruption of the autoinhibitory mechanism by phosphorylation. Previous results have shown that Ile^{441} in CaMKKα (Ile^{477} in the β isoform), which is located in the proximity of the site that is autophosphorylated in CaMKKβ (Thr^{482}), is essential for autoinhibition of CaMKKα, because mutation of this conserved Ile to Asp resulted in generation of enhanced Ca^{2+}/CaM-independent activity (60% of total activity).[21] This result indicates that introduction of a negatively charged phosphate into the autoinhibitory domain might partially disrupt the interaction of the autoinhibitory domain with the catalytic core of CaMKKβ, resulting in generation of autonomous activity. Indeed, when we mutated Thr^{482} in CaMKKβ to Asp, the autonomous activity (~80% of total activity) of the mutant was comparable to that of the wild-type enzyme (data not shown). In contrast to that of CaMKII,[36,37] the autophosphorylation of CaMKKβ at Thr^{482} was suggested to be a Ca^{2+}/CaM-independent intramolecular process, because wild-type CaMKKβ was incapable of transphosphorylating the kinase-dead CaMKKβ mutant expressed in *E. coli*. Therefore, Thr^{482} phosphorylation of CaMKKβ is unlikely to be regulated by Ca^{2+}/CaM in a cellular context. Although we could not determine the stoichiometry of Thr^{482} phosphorylation in recombinant CaMKKβ, an increasing electrophoretic mobility and a significant reduction in the autophosphorylation level at Thr residues of the Thr^{482}Ala mutant (Figure 4) strongly suggest that the Thr^{482} is one of the major autophosphorylation sites in CaMKKβ. It is noteworthy that another isoform of CaMKK, CaMKKα, does not show Ca^{2+}/CaM-independent autophosphorylation at the equivalent Thr residue in the autoinhibitory domain. This result is consistent with the fact that the activity of CaMKKα is tightly suppressed by an autoinhibitory domain in the absence of the activator.[21] However, Thr^{482} autophosphorylation is not the only mechanism for induction of the autonomous activity of CaMKKβ, because residual autonomous activity of the Thr^{482}Ala mutant (~35% of total activity) was observed. In addition, a previous report suggested that the N-terminal region (residues 129−151) of CaMKKβ plays an important role in generation of its autonomous activity.[22] Here, we show that the N-terminal region (residues 129−151) is required for the Thr autophosphorylation in CaMKKβ as well as the enhanced autonomous activity.[22] This is probably due to the fact that the N-terminal region (residues 129−151) of CaMKKβ is also involved in maintaining the kinase in a partially active conformation without Ca^{2+}/CaM. These two different mechanisms appear to be important for the enhanced autonomous activity of CaMKKβ (~70% of total activity), which differs from the highly Ca^{2+}/CaM-dependent activity of the CaMKKα isoform. What is the physiological relevance of the autonomous activity of CaMKKβ? Accumulated evidence has demonstrated that AMPK is activated in cells by CaMKKβ phosphorylation, but not by CaMKKα phosphorylation, following the application of various stimuli, whereas both CaMKK isoforms are capable of phosphorylating and activating AMPK *in vitro*.[13−17] Except for the difference in autonomous activity, the biochemical properties, including substrate specificity, of the two CaMKK isoforms are similar. Therefore, enhanced autonomous activity of CaMKKβ might be essential for activation of AMPK in cells, because LKB1, an alternative activator of AMPK, is a constitutively active kinase.[38,39] It therefore remains to be determined what role CaMKKβ autonomous activity plays in a cellular context.

ASSOCIATED CONTENT

Supporting Information

LC−MS/MS analysis for identification of the autophosphorylation sites in CaMKK isoforms (Supplemental Data 1), LC−MS/MS analysis of residues 440−451 in CaMKKα (Supplemental Data 2), and LC−MS/MS analysis of residues 440−451 in CaMKKα mt (Supplemental Data 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; CaMKK, Ca^{2+}/CaM-dependent protein kinase kinase; GST, glutathione *S*-transferase; AMPK, 5′-AMP-activated protein kinase; PKA, cAMP-dependent protein kinase; PP2A, protein phosphatase 2A; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; LC, liquid chromatography; MS/MS, tandem mass spectrometry; DTT, dithiothreitol.

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