

Substrate recognition by Arg/Pro-rich insert domain in calcium/calmodulin-dependent protein kinase kinase for target protein kinases

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Abbreviations: CaMKK, calcium/calmodulin-dependent protein kinase kinase; CaM, calmodulin; CaMK, calcium/calmodulin-dependent protein kinase; AMPK, AMP-activated protein kinase; LKB1, liver kinase B1; RP-domain, Arg/Pro-rich insert domain; KD, kinase dead; HA, hemagglutinin; TIM-063, 2-hydroxy-3-nitro-7*H*-benzo[de]benzo[4,5]-imidazo[2,1-*a*]isoquinolin-7-one.

Key words: calcium/calmodulin-dependent protein kinase, AMP-activated kinase, calcium/calmodulin-dependent protein kinase kinase, substrate recognition, Arg/Pro-rich insert domain (RP-domain)

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Abstract

Calcium/calmodulin-dependent protein kinase kinases (CaMKKs) activate CaMKI, CaMKIV, protein kinase B/Akt, AMP-activated protein kinase (AMPK) by phosphorylating Thr residues in activation loops to mediate various Ca^{2+} -signaling pathways. Mammalian cells expressing CaMKK α and CaMKK β lacking Arg/Pro-rich insert domain (RP-domain) sequences showed impaired phosphorylation of AMPK α , CaMKI α , and CaMKIV, whereas the autophosphorylation activities of CaMKK mutants remained intact and were similar to those of wild type CaMKKs. Liver kinase B1 (LKB1, an AMPK kinase) complexed with STRAD and MO25 and was unable to phosphorylate CaMKI α and CaMKIV; however, mutant LKB1 with the RP-domain sequences of CaMKK α and CaMKK β inserted between kinase subdomains II and III acquired CaMKI α and CaMKIV phosphorylating activity in vitro and in transfected cultured cells. Furthermore, ionomycin-induced phosphorylation of hemagglutinin (HA)–CaMKI α at Thr177, HA–CaMKIV at Thr196, and HA–AMPK α at Thr172 in transfected cells was significantly suppressed by cotransfection of kinase-dead mutants of CaMKK isoforms, but these dominant-negative effects were abrogated with RP-deletion mutants, suggesting that sequestration of substrate kinases by loss-of-function CaMKK mutants requires the RP-domain. This was confirmed by pulldown experiments that showed that dominant-negative mutants of CaMKK α and CaMKK β interact with target kinases but not RP-deletion mutants. Taken together, these results clearly indicate that both CaMKK isoforms require the RP-domain to recognize downstream kinases to interact with and phosphorylate Thr residues in their activation loops. Thus, the RP-domain may be a promising target for specific CaMKK inhibitors.

Introduction

Protein kinase cascades driven by multiple kinases cause systematic and effective amplification of intracellular signaling and are frequently observed in a wide variety of physiological pathways, including neuronal, metabolic, and pathological pathways, such as cancer [1-3]. The calcium/calmodulin-dependent protein kinase (CaMK) cascade was initially discovered in intracellular calcium signal transduction systems as activation cascades of CaMKI and CaMKIV by the upstream kinase, calcium/calmodulin-dependent protein kinase kinase (CaMKK) [4, 5]. CaMKK is composed of α and β isoforms in mammals [6-8] and is conserved in lower eukaryotes, such as *Caenorhabditis elegans* [9] and *Aspergillus nidulans* [10]. CaMKKs activate protein kinase B/Akt [11, 12] and AMP-activated protein kinase (AMPK) [13-15]. Several kinases can phosphorylate specific Thr residues in the activation loop of particular downstream protein kinases, including mitogen-activated protein kinase kinase [16], 3-phosphoinositide-dependent protein kinase 1 [17], and liver kinase B1 (LKB1) [18], with strict or redundant substrate specificity. For example, CaMKK can phosphorylate CaMKI α at Thr177, CaMKIV at Thr196, and AMPK α at Thr172, whereas LKB1 only phosphorylates AMPK family members [18], but not CaMKI α or CaMKIV, suggesting that CaMKK possesses specific mechanisms for recognizing CaMKI and CaMKIV. Compared with the primary structure of the catalytic core of other protein kinases, CaMKK isoforms possess a unique insert of 23 amino acids that is highly enriched with Arg/Pro residues (known as the RP-domain) between subdomains II and III [19] in the CaMKK catalytic domain. Deletion of the RP-domain in CaMKK α completely impairs its ability to phosphorylate and activate CaMKI α and CaMKIV, suggesting that the RP-domain plays an important role in specific substrate recognition [20]. However, the importance of the RP-domain

is not fully understood including a requirement of the domain for recognition of AMPK as a substrate. A previous loss-of-function study investigated RP-domain deletion and site-directed point mutants [20]. Clarification of the detailed mechanisms involved in substrate recognition of CaMKK would help in the development of specific enzyme inhibitors that could be applicable for CaMKK-related diseases, including prostate cancer [21, 22], lung cancer [23], hepatic cancer [24], gastric cancer [25], breast cancer [26], and metabolically-related hepatic diseases [27].

The present study analyzed CaMKK α/β mutants lacking RP-domain sequences and used a gain-of-function approach in LKB1, which is the activating kinase for AMPK family members [18], to gain insight into the substrate recognition mechanisms of CaMKK. Insertion of CaMKK RP-domains into the catalytic domain converted LKB1 into a kinase that could phosphorylate CaMKI α and CaMKIV in transfected cells as well as in vitro, suggesting a critical role of the RP-domain in the recognition of CaMKI α and CaMKIV. Furthermore, we showed that the RP-domain is involved in the interaction between CaMKK and target kinases, highlighting this domain as a potential target for novel CaMKK inhibitors.

Results

CaMKK activities, but not autophosphorylation activities of CaMKK α and β are impaired by deletion of RP-domain.

CaMKKs activate downstream protein kinases, such as CaMKI, CaMKIV, and AMPK, by phosphorylating Thr residues in their activation-loops to regulate a wide variety of physiological responses (2-5). We demonstrated that rat CaMKK α contains a unique RP-domain at residues 167–189, between subdomains II and III, in its catalytic core [19] (Fig. 1A and B) that plays an

important role in recognizing CaMKI α and CaMKIV as downstream substrates [20]. The RP-domain is conserved in both CaMKK isoforms (CaMKK α and CaMKK β , Fig. 1B) and the *C. elegans* CaMKK orthologue (Supporting Information Figure S1); however, the precise mechanisms and structure of the CaMKK RP-domain involved in substrate recognition remain unclear, since the crystal structure of human CaMKK β catalytic domain complexed with STO-609 revealed that the RP-domain was disordered (Fig. 1A)[28]. In addition, because of that the catalytic core of AMPK kinase, LKB1 does not possess the RP-domain sequence (Fig. 1B and Supporting Information Figure S1), an involvement of RP-domain of CaMKK in AMPK phosphorylation has not been elucidated. Therefore, His₆-tagged CaMKK isoforms of either wild type or RP-domain deletion (Δ RP) mutants were expressed in cultured mammalian COS-7 cells, followed by purification using Ni-sepharose chromatography to confirm the involvement of RP-domains of both CaMKK α and β in target kinase phosphorylation including AMPK phosphorylation. Immunoblot analyses using anti-CaMKK α (Fig. 1C, left panel) and anti-CaMKK β (Fig. 1C, right panel) antibodies showed that CaMKK α and β Δ RP mutants migrated slightly faster on SDS-PAGE gels than the corresponding wild type enzymes due to the lack of the 23 residues of the RP-domain. We confirmed catalytic activity in the mutants of both isoforms of the CaMKK Δ RP mutants as well as the wild type enzymes using an autophosphorylation activity assay (Fig. 1D). Incubation of purified wild type CaMKKs and CaMKK Δ RP mutants with Mg-ATP in the presence of calcium/calmodulin (Ca²⁺/CaM) for 15 and 30 min, followed by immunoblot analysis using an antibody against each CaMKK isoform revealed a decreased electrophoretic mobility of the immunoreactive bands of both wild type and Δ RP mutants compared with that of enzymes incubated under nonphosphorylation conditions (without ATP, Fig. 1 D, the first and fifth lanes

from left) due to the intramolecular autophosphorylation of CaMKKs [29]. Mobility shifts of the enzymes were completely abolished in the presence of the CaMKK inhibitor, TIM-063 (26 μ M) [30], indicating that Δ RP mutants of both CaMKK isoforms were catalytically active in a similar manner to each wild type CaMKK, which is consistent with our previous observation in recombinant CaMKK α expressed in *Escherichia coli* [20].

Next, we assessed the phosphorylation activities of Δ RP mutants toward target kinases, including CaMKI α , CaMKIV, and AMPK. In agreement with our previous findings [20], the Δ RP mutant of CaMKK α showed impaired phosphorylation of GST–CaMKI α at Thr177 (Fig. 2A, left panel) and GST–CaMKIV at Thr196 (Fig. 2B, left panel), whereas this was not seen with the wild type enzyme. We confirmed that the Δ RP mutant of CaMKK β isoform was unable to phosphorylate GST–CaMKI α and GST–CaMKIV (Fig. 2A and B, right panels). In addition, phosphorylation of AMPK α at Thr172 by both CaMKK isoforms was also impaired by deletion of the RP-domain (Fig. 2C). Taken together, these results clearly indicate that both CaMKK isoforms require the RP-domain to specifically recognize and phosphorylate AMPK α as well as CaMKI and CaMKIV.

LKB1 mutants with the RP-domain sequences of CaMKK α and CaMKK β inserted between kinase subdomains II and III acquire CaMKI α and CaMKIV phosphorylating activity in vitro and in transfected cells.

To test whether the short segment of the RP-domain was directly involved in the substrate recognition of CaMKK, we produced mutants of LKB1, a major AMPK kinase [18], inserted with the CaMKK RP-domain sequences. His₆-tagged mutants of LKB1 were constructed in which the

86Arg–89Pro sequences in kinase subdomains II and III were replaced by the RP-domain sequences of the CaMKK isoforms (165Leu–190Leu in rat CaMKK α and 201Ile–226Arg in rat CaMKK β) (Fig. 1B and Supporting Information Figure S1). Since it has been demonstrated that the LKB1 is activated by stable interactions with the pseudokinase STRAD (Ste20-related adaptor) [31] and the scaffolding protein MO25 α (mouse protein 25) [32] through an allosteric mechanism [33], we attempted to transiently express His–LKB1 with HA–MO25 and HA–STRAD in COS-7 cells, followed by purification with Ni–sepharose chromatography. Immunoblot analysis using anti-His tag and anti-HA antibodies clearly indicated that the Ni–sepharose-purified His–LKB1 including RP-insertion mutants were successfully purified as active complexes with the pseudokinase STRAD and the scaffolding protein MO25 (Fig. 3A). Although LKB1 and CaMKKs are able to phosphorylate a common substrate, AMPK α , in vitro (Fig. 2C and 3D), we confirmed that purified LKB1 wild type complexed with MO25 and STRAD was unable to phosphorylate GST–CaMKI α (Fig. 3B) and GST–CaMKIV (Fig. 3C), indicating that, unlike CaMKKs, LKB1 does not possess the substrate recognition mechanism for CaMKI α and CaMKIV. Interestingly, LKB1 mutants containing an inserted RP-domain derived from each CaMKK isoform were able to phosphorylate GST–CaMKI α (Fig. 3B) and GST–CaMKIV (Fig. 3C). These results clearly indicate that the RP-domains of CaMKK isoforms are directly involved in the recognition of CaMKI α and CaMKIV as substrates in vitro. Furthermore, the AMPK-phosphorylating activity of the LKB1 complex was not significantly affected by insertion of the RP-domain into the catalytic region of the enzyme (Fig. 3D). To test whether the LKB1 mutants with the RP-domain sequences of CaMKK α and CaMKK β acquire CaMKI α and CaMKIV phosphorylating activity in intact cells, we transfected His–LKB1 expression plasmid encoding

wild type (WT), a mutant with CaMKK α RP-domain sequence (+ α RP), or a mutant with CaMKK β RP-domain sequence (+ β RP) as used in Fig. 3 in the presence of HA-MO25 and HA-STRAD expression plasmids with HA-CaMKI α expression plasmid into COS-7 cells (Fig. 4A) or with HA-CaMKIV expression plasmid into HeLa cells (Fig. 4B). After incubation with 26 μ M a CaMKK inhibitor, TIM-063 [30] for 6 h to suppress endogenous CaMKK activity, the cells were stimulated with 1 μ M ionomycin for 5 min, followed by immunoblot analyses with either an anti-pCaMKI α at Thr177 (Fig. 4A, upper panel) or anti-pCaMKIV at Thr196 (Fig. 4B, upper panel) antibody. Whereas both HA-CaMKI α and HA-CaMKIV were not phosphorylated by endogenous CaMKK (–) or wild type LKB1 complex (WT) in the presence of TIM-063 as expected, expression of LKB1 mutants with the RP-domain sequences of CaMKK α (+ α RP) and CaMKK β (+ β RP) dramatically induced phosphorylation of HA-CaMKI α (Fig. 4A, upper panel) and HA-CaMKIV (Fig. 4B, upper panel). In addition, we performed an experiment similar to that shown in Fig. 4A. Instead of using ionomycin treatment to raise the concentration of intracellular Ca²⁺, we treated the HA-CaMKI α /His-LKB1 complex expressing COS-7 cells with 10 μ M ATP for 3 min. It has been shown that the treatment with extracellular ATP results in a Ca²⁺ release in COS-7 cells [34], which is probably due to stimulation of P2-purinergic receptor by the nucleotide [35]. We observed that the physiological stimulation (10 μ M ATP) induced phosphorylation of HA-CaMKI α by His-LKB1 mutants inserted with RP-domain sequences of CaMKK isoforms, but not by wild type His-LKB1 (Supporting Information Figure S2) in a similar manner to the results obtained with ionomycin treatment (Fig. 4A). In agreement with in vitro phosphorylation of CaMKI α and CaMKIV by the RP-domain insertion mutants of LKB1 shown in Fig. 3B and C, the RP-domains of both CaMKK isoforms have shown to be essential for recognition of downstream CaMKI and CaMKIV in intact

cells. Of note, TIM-063 has no ability to inhibit LKB1 activity up to 26 μ M (Supporting Information Figure S3).

Sequestration of substrate kinases by the dominant-negative CaMKK mutants requires the RP-domain in the cell.

The mechanisms involved in the RP-domain-mediated substrate recognition of CaMKK in intact cells was further examined by determining whether the RP-domain was required for the dominant-negative effect of the kinase-dead (KD) CaMKK mutant in transfected cultured cells. HA–CaMKI was cotransfected into COS-7 cells in the presence or absence of KD mutants of His₆-tagged CaMKK α (D293A) or CaMKK β (D329A). Cells were treated with 1 μ M ionomycin for 5 min, followed by immunoblot analyses of the phosphorylation of HA–CaMKI α at Thr177. Phosphorylation of Thr177 was readily observed in HA–CaMKI α , indicating that endogenous CaMKK was involved in HA–CaMKI α phosphorylation (Fig. 5A). Ionomycin-induced Thr177-phosphorylation of HA–CaMKI α was abolished by cotransfection of either His–CaMKK α KD (Fig. 5A, left panel) or His–CaMKK β KD (Fig. 5A, right panel) as a dominant-negative effect of the KD mutants. In contrast, cotransfection of His–CaMKK α KD, Δ RP or His–CaMKK β KD, Δ RP exhibited significantly reduced dominant-negative effects on the phosphorylation of HA–CaMKI α at Thr177. In agreement with the *in vitro* characterization of the RP-domain indicating that the domain of each CaMKK isoform is important for the interaction between CaMKK and downstream target kinases, interaction and sequestration of substrate kinases by the dominant-negative CaMKK mutants required the RP-domain in the cell. We also observed a similar impairment of the dominant-negative effects of His–CaMKK α KD and His–CaMKK β KD following

deletion of the RP-domain for ionomycin-induced phosphorylation of HA–CaMKIV at Thr196 in transfected HeLa cells (Fig. 5B) and HA–AMPK α at Thr172 in transfected COS-7 cells (Fig. 5C).

Direct interactions of the dominant-negative CaMKK mutants with substrate kinases require the RP-domain.

Deletion of the RP-domain significantly reduced the dominant-negative effect of His–CaMKK KD mutants on the phosphorylation of downstream target kinases by endogenous CaMKK in cultured cells, which may suggest that the RP-domain is directly involved in the interaction between CaMKK and target kinases in vivo. This was examined using a pulldown assay to evaluate the interaction between His–CaMKK KD mutants and target kinases. Using transfected COS-7 cell lysates, His₆-tagged CaMKK KD mutants of both α (Fig. 6, left panels) and β (Fig. 6, right panels) isoforms were shown to pull down using Ni–sepharose resin together with target kinases, including HA–CaMKI α (Fig. 6A, bottom panels), HA–CaMKIV (Fig. 6B, bottom panels), and HA–AMPK (Fig. 6C, bottom panels) in the presence of Ca²⁺/CaM, indicating a direct interaction between CaMKK KD mutants and target kinases. As expected, KD mutants of His–CaMKK α and β lacking an RP-domain (Δ RP) were unable to interact with all the target kinases (Fig. 6A–C, bottom panels).

Discussion

CaMKK-mediated calcium signaling pathways activate CaMKI, CaMKIV, PKB/Akt, and AMPK to regulate a wide variety of physiological responses under normal and pathophysiological conditions (2-5). Unlike other multifunctional CaMKs, CaMKKs possess strict substrate specificity

and preferably phosphorylate Thr residues in the activation loops of selected kinases. The RP-domain is a unique insertion region comprising 23 amino acids rich in Arg and Pro residues, which is located between subdomains II and III in the catalytic domain of CaMKK α and β (Fig. 1A and B). It has shown to be important for CaMKK activity and deletion of the RP-domain in CaMKK α impairs its phosphorylation and activation of CaMKI α and CaMKIV [20]. However, the molecular mechanisms involved in the RP-domain-mediated substrate recognition of CaMKK including AMPK recognition, remain unclear since X-ray crystallography revealed its structure is disordered [28]. The present study revealed that both CaMKK isoforms require the RP-domain for the phosphorylation of AMPK α as well as that of CaMKI and CaMKIV using a loss-of-function assay to delete the RP-domain in the two CaMKK isoforms. A recent biophysical study indicated that the autoinhibitory region of CaMKK2 (CaMKK β) blocks access to the RP-domain [36], that may also suggest that the autoinhibitory region interferes the CaMKK/substrate interaction. Gain-of-function analysis revealed that LKB1 mutants with an inserted RP-domain between subdomains II and III acquired CaMKI α and CaMKIV phosphorylating activity in transfected cultured cells as well as in vitro, whereas the LKB1 wild type was unable to phosphorylate CaMKI α and CaMKIV (Fig. 3 and 4). These results clearly indicate that the RP-domain is required for CaMKKs to recognize downstream kinases as phosphosubstrates. This was confirmed by the dominant-negative effect seen in living cultured cells and the impaired target kinase interaction between CaMKK KD mutants seen following deletion of the RP-domain. Of note, the CaMKK α KD mutant (D293A) functioned as a dominant-negative mutant for ionomycin-induced AMPK α phosphorylation in transfected cells (Fig. 5C) in a similar manner to the CaMKK β KD mutant (D329A) even though CaMKK β , but not CaMKK α , is known to be a bona fide activator of AMPK

[13-15]. Together with the findings that CaMKK α can interact with AMPK α in cells (Fig. 6C) and phosphorylate its Thr172 in vitro (Fig. 2C), CaMKK α may be involved, at least in part, in the calcium-dependent activation of AMPK in vivo. This is consistent with a previous report showing that the transfection of CaMKK α siRNA significantly suppressed 2-deoxyglucose induced AMPK activity and phosphorylation at Thr172 in HeLa cells [13-15], but this possibility still needs to be further investigation. The findings of our study clearly indicate that the AMPK α recognition mechanism of LKB1, which does not contain the RP-domain, is distinguished from that of CaMKK isoforms, which use the RP-domain for kinase/substrate interactions. Accumulating evidence indicates that CaMKK β is overexpressed and involved in various cancers, including prostate cancer [21, 22], hepatic cancer [24], gastric cancer [25], and breast cancer [26]; therefore, CaMKK may be a promising drug target for the treatment of these diseases. CaMKK inhibitors have been developed as ATP-competitive inhibitors, including STO-609 [37], TIM-063 [30], GSK650394 (also known as glucocorticoid-inducible kinase 1 inhibitor) [38, 39], and compounds based on scaffold hopping from GSK650394 [40]. Thus, our study highlights the RP-domain-mediated substrate recognition of CaMKK as a possible target for the development of a novel inhibitor of kinase–substrate interactions for CaMKK that may overcome the disadvantages of conventional ATP-competitive inhibitors that possess several off-target kinases. High-throughput methods for detection of interactions between CaMKK RP-domains and CaMKK-target kinases including CaMKI, CaMKIV, and AMPK, will be useful for screening novel CaMKK inhibitors. Investigation of such inhibitors is currently underway in our laboratory.

Materials and Methods

Materials: Recombinant GST–rat CaMKI α and GST–mouse CaMKIV were expressed in *E. coli* JM-109 as previously described [41] and purified using glutathione–sepharose (GE Healthcare Life Sciences, Buckinghamshire, England) chromatography. Recombinant rat AMPK was expressed in *E. coli* BL21–CodonPlus (DE3) (Stratagene, La Jolla, CA, USA) using a tricistronic py1 β 1His- α 1 plasmid (kindly provided by Dr. Dietbert Neumann, Swiss Federal Institute of Technology, Zurich, Switzerland), and purified as previously described [42]. Recombinant rat CaM was expressed in *E. coli* BL21 (DE3) using a pET–CaM plasmid (kindly provided by Dr. Nobuhiro Hayashi, Tokyo Institute of Technology, Yokohama, Japan)[43]. His₆-tagged mouse LKB1 cDNA were amplified by RT-PCR and subcloned into pME-18s expression vector followed by N-terminal His₆ (MGHHHHHH–) (pME–His–LKB1). Expression plasmids for HA-tagged mouse MO25 α (pME–HA–MO25) and HA-tagged human STRAD α (pME–HA–STRAD) were constructed by RT-PCR, followed by subcloning into pME–18s–HA vector. Template DNAs for mouse LKB1, mouse MO25 α , and human STRAD α were kindly provided by Dr. Hiroshi Takemori (National Institute of Biomedical Innovation, Osaka, Japan) [44]. Monoclonal antibodies against CaMKK α and CaMKK β were generated [45]. Anti-phosphoCaMKI α (at Thr177, clone 9H8), anti-phosphoCaMKIV (at Thr196, clone 8D4), and anti-phosphoAMPK α (at Thr172, clone 13E3) monoclonal antibodies were generated as described previously [41, 46]. Anti-His tag antibody (#66005-1-Ig) and anti-HA antibody (clone 12CA5, #1666606) were purchased from Proteintech (Rosemont, IL, USA) and Boehringer Mannheim (Germany), respectively. Anti-CaMKI antibody (#SC-1543) and anti-CaMKIV antibody (#610545) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and BD Transduction Laboratories (San Jose, CA, USA), respectively. TIM-063 (2-hydroxy-3-nitro-

7H-benzo[de]benzo[4,5]-imidazo[2,1-*a*]isoquinolin-7-one) was synthesized as previously described [30]. All other chemicals were obtained from standard commercial sources.

Construction of His₆-tagged CaMKK α , β and LKB1 mutants: Expression plasmids for His₆-tagged rat CaMKK α (His–CaMKK α) and His₆-tagged rat CaMKK β (His–CaMKK β) were constructed by subcloning of N-terminal His₆-tagged rat CaMKK α and β containing 5'GAATTCATGGGCCATCACCATCACCATCAC-2nd codon- encoded Met-Gly-His-His-His-His-His-2nd residue-) into EcoRI/NotI sites of pME18s vector. Expression plasmids for His₆-tagged CaMKK α lacking residues 167Q–189Q (His–CaMKK α Δ RP) and His₆-tagged CaMKK β lacking residues 203Q–225P (His–CaMKK β Δ RP) were constructed by inverse PCR using PrimeSTAR HS DNA polymerase (Takara, Japan), pME–His–rat CaMKK α and pME–His–rat CaMKK β as templates and the following phosphorylated primers: pME–His–CaMKK α Δ RP, 5'-CTGCTGCCCCTGGAGCGTGATACCAG-3' and 5'-CTTCAGTAACTTCTTTTGGAAAGAACTTTC-3' and pME–His–CaMKK β Δ RP, 5'-AGGGGCCCCATCGAGCAGGTGTATC-3' and 5'-TCGGATCAGCTTCTTTTGGACAGCAC-3'. Expression plasmids for His₆-tagged mouse LKB1 mutants in which residues 86R–89P was replaced by either CaMKK α RP-domain sequence (residues 165L–190L, pME–His–LKB1+ α RP) or CaMKK β RP-domain sequence (residues 201L–226R, pME–His–LKB1+ β RP) were constructed by inverse PCR using PrimeSTAR HS DNA polymerase and pME–His–LKB1 as a template and the following primers: pME–His–LKB1+ α RP, 5'-GGGTCCCAAGCTCCTCAGGGAGGGCCAGCCAAACAGCTGAATGGAGAGGCCAACGTCAAG-3' and 5'-TCTCGGGGGAGGACGGCGAGGAAAGCCATACTGCTTCAGCAGCTTTTCTTCTTGAGGAT-3' pME–His–LKB1+ β RP,

5'-GGTACTCGCCCAGCTCCAGGGGGCTGCATCCAGCCCAGGAATGGAGAGGCCAACGTCAAG-3',

and 5'-TCGAGGTGGGGGGCGACGTGGAAAGCCGGCCTGTCGGATCAGCTTTTCTTCTTGAGGAT-3',

where the RP-domain sequences of both CaMKK isoforms are underlined.

Expression plasmids for KD mutants (D293A) of rat CaMKK α (pME-His-CaMKK α D293A), and rat CaMKK α Δ RP (pME-His-CaMKK α D293A, Δ RP) were constructed by inverse PCR using PrimeSTAR HS DNA polymerase (Takara, Japan), with pME-His-CaMKK α and pME-His-CaMKK α Δ RP, respectively, as templates and the following phosphorylated primers: 5'-GCCGCCTTTGGTGTGTCAGCAACCAGTTTGAG-3' and 5'-GATCTTCAC GTGCCATCGTCCCCAAG-3'.

Expression plasmids for KD mutants (D329A) of rat CaMKK β (pME-His-CaMKK β D329A) and rat CaMKK β Δ RP (pME-His-CaMKK β D329A, Δ RP) were constructed by inverse PCR, with pME-His-CaMKK β and pME-His-CaMKK β Δ RP, respectively, as templates and the following phosphorylated primers: 5'-GCCGCCTTCGGCGTGAGCAATGAGTTC-3' and 5'-TATCTTGATGTG CCGTCCTCCCCAC-3'.

Expression and purification of His₆-tagged CaMKK α , CaMKK β , and LKB1 mutants in COS-7 cells:

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in two 10-cm dishes at 37 °C in 5% CO₂. His₆-tagged CaMKK isoforms and His₆-tagged LKB1, including various mutants, were expressed in COS-7 cells by transfection of expression plasmids (pME-His-rat CaMKK α 10 μ g, pME-His-rat CaMKK β 10 μ g, and pME-His-LKB1 3 μ g together with pME-HA-MO25 3 μ g and pME-HA-STRAD 3 μ g) using polyethylenimine "MAX" (Poly-sciences, Inc., Warrington, PA , USA) according to the manufacturer's protocol. After 48 h, cells were lysed with 1.2 mL of ice-cold lysis buffer (500 mM NaCl, 50 mM Tris-HCl

pH7.5, 50 mM Imidazole, and 0.1% Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan)) followed by purification with Ni-sepharose chromatography according to the manufacturer's protocol (GE Healthcare Life Sciences).

Protein kinase assay: Kinase assays were performed at 30 °C for the indicated time periods in 20 µL of a solution containing His-CaMKKα (~15 ng), His-CaMKKβ (~13 ng), and His-LKB1 complexed with HA-MO25 and HA-STRAD (400–500 ng), or their mutants, 50 mM HEPES pH 7.5, 10 mM Mg(CH₃COO)₂, 1 mM DTT, with or without (for phosphorylation of AMPK by His-LKB1 and His-LKB1 mutants, Fig.3 D) 2 mM CaCl₂/6 µM CaM, and 100 µM ATP in the presence of 10 µg GST-CaMKIα, GST-CaMKIV, or AMPK. Each reaction was initiated by the addition of ATP and terminated by the addition of 2× SDS-PAGE sample buffer followed by immunoblot analysis with antibodies against phosphoCaMKIα (at Thr177), phosphoCaMKIV (at Thr196), or phosphoAMPKα (at Thr172). Phosphorylation of each substrate was estimated by densitometric scanning of the immunoblots. Autophosphorylation of His-CaMKKα and β including ΔRP mutants were measured at 30 °C for 15 and 30 min in 20 µL of a solution containing 0.1–0.2 µg CaMKK isoforms, 50 mM HEPES pH 7.5, 10 mM Mg (CH₃COO)₂, 1 mM DTT, 2 mM CaCl₂, 6 µM CaM with or without 100 µM ATP in the absence or presence of 26 µM CaMKK inhibitor, TIM-063 [30]. The reaction was terminated by the addition of SDS-PAGE sample buffer followed by immunoblot analysis using an anti-CaMKKα or β antibody.

Ionomycin-induced phosphorylation of CaMKI, CaMKIV and AMPK in cultured cells: COS-7 cells and HeLa cells were cultured in 6-well dishes in DMEM supplemented with 10% fetal bovine

serum at 37 °C in 5% CO₂, transfected with 0.2 µg of HA–rat CaMKIα (pME–HA–CaMKIα), HA–mouse CaMKIV (pME–HA–CaMKIV), or HA–rat AMPKα (pME–HA–AMPKα) expression plasmid together with 1.8 µg of His–CaMKKα D293A (pME–His–CaMKKα D293A), His–CaMKKα D293A, ΔRP (pME–His–CaMKKα D293A, ΔRP), His–CaMKKβ D329A (pME–His–CaMKKβ D329A), His–CaMKKβ D329A, or ΔRP (pME–His–CaMKKβ D329A, ΔRP) expression plasmid or empty vector (pME18s) using polyethylenimine “MAX” (Poly-sciences, Inc.) according to the manufacturer’s protocol. For phosphorylation of HA–CaMKIα and HA–CaMKIV by LKB1 mutants in transfected cells (Fig. 4), COS-7 cells and HeLa cells (in 6-well dishes) were transfected with either 0.5 µg of HA–rat CaMKIα (pME–HA–CaMKIα) or HA–mouse CaMKIV (pME–HA–CaMKIV) expression plasmid together with or without 0.5 µg of His–LKB1 expression plasmid (pME–His–LKB1 wild type, pME–His–LKB1+αRP, or pME–His–LKB1+αRP) in the presence of pME–HA–MO25 (0.5 µg) and pME–HA–STRAD (0.5 µg). After 42 h, cells were cultured in serum-free medium for 6 h in the presence or absence of 26 µM TIM-063, followed by treatment with 1 µM ionomycin for 5 min. Then cells were lysed with 1× SDS–PAGE sample buffer (100 µL), followed by immunoblot analyses (15 µL of cell lysates) using corresponding anti-phosphoCaMKIα (at Thr177), anti-phosphoCaMKIV (at Thr196), or anti-phosphoAMPKα (at Thr172, #2535 from Cell Signaling Technology, Danvers, MA, USA).

Pulldown assay: COS-7 cells were cultured in 6-well plates in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂, and transfected with HA–rat CaMKIα (pME–HA–CaMKIα), HA–mouse CaMKIV (pME–HA–CaMKIV), or HA–rat AMPKα (pME–HA–AMPKα) expression plasmid with His–CaMKKα D293A (pME–His–CaMKKα D293A), His–CaMKKα D293A, ΔRP (pME–

His–CaMKK α D293A, Δ RP), His–CaMKK β D329A (pME–His–CaMKK β D329A), His–CaMKK β D329A, or Δ RP (pME–His–CaMKK β D329A, Δ RP) expression plasmids. After 48 h, cells were lysed with 500 μ L of ice-cold lysis buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.5, 1 mM DTT, 2 mM CaCl₂, 6 μ M CaM, and 0.1% Protease Inhibitor Cocktail), and the cell lysates were incubated with 50 μ L of Ni–sepharose (50% slurry) for 3 h at 4 °C. After extensive washing with ice-cold lysis buffer, Ni–sepharose resin was incubated with 40 μ L of ice-cold elution buffer (150 mM NaCl, 20 mM Tris–HCl pH7.5, 1 mM DTT, 2 mM CaCl₂, 6 μ M CaM, and 300 mM imidazole) followed by immunoblot analysis using 12- μ L sample volume.

Other methods: Immunoblot analysis was performed with the indicated primary antibodies and horseradish peroxidase-conjugated anti-mouse IgG (#NA931-1ML), anti-rabbit IgG (#NA934-1ML, GE Healthcare Life Sciences), or anti-goat IgG (#6420-05, SouthernBiotech, Birmingham, AL, USA) as the secondary antibody. A chemiluminescent reagent (PerkinElmer Life Sciences, Waltham, MA) was used for signal detection of the immunoblots, followed by quantification of the immunoreactivity with ImageJ software [47]. Protein concentrations in the samples were estimated by Coomassie brilliant blue G-250 dye (Bio-Rad Laboratories, Inc. Hercules, CA)-binding assay, using bovine serum albumin as a standard.

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Supporting Information:

Figure S1, Comparison of amino acid sequences of RP-domain in various CaMKKs.

Figure S2, Extracellular ATP-induced phosphorylation of CaMKI α by LKB1 mutants inserted with CaMKK RP-domain sequences in transfected COS-7 cells.

Figure S3, Effect of TIM-063 on LKB1 activity.

Figure legends

Figure 1. RP-deletion mutants of CaMKK isoforms are catalytically active. (A) Structure of the catalytic domain of human CaMKK β complexed with STO-609. Ribbon representation of the human CaMKK β structure was obtained from the Protein Data Bank (PDB) entry 2ZV2 [28] and visualized using the UCSF Chimera [48]. Disordered regions are indicated as dashed lines (red, RP-domain and blue, P-loop). (B) Schematic representation of rat CaMKK isoforms (α and β) and mouse LKB1 together with amino acid sequences of the RP-domains between subdomains II and III (red). Catalytic region of each kinase (Kinase Domain) and regulatory domain (Reg.) are indicated with the amino acid residue numbers. (C) Immunoblot analyses of purified His–CaMKK wild type and Δ RP mutant (left panel, CaMKK α ~1 μ g; right panel, CaMKK β ~3 μ g) using anti-CaMKK α (left panel) and anti-CaMKK β (right panel) antibodies. (D) Purified His–CaMKK wild type and Δ RP mutant (upper panel, CaMKK α ; lower panel, CaMKK β) were incubated without (-) or with 100 μ M ATP for the indicated time periods (15 and 30 min) in the absence (-) or presence (+) of 26 μ M TIM-063, followed by immunoblot analyses using anti-CaMKK α (upper panel) and anti-CaMKK β (lower panel) antibodies as described in “Materials and Methods.” The left lanes in panel C and D show the molecular mass markers.

Figure 2. Phosphorylation of GST–CaMKI α (A), GST–CaMKIV (B), and AMPK α (C) by His–CaMKK isoforms wild type (WT) and RP-deletion mutants (Δ RP). Wild type (WT, closed circle) and Δ RP mutant (Δ RP, open circles) of purified His–CaMKK α (left panels) or His–CaMKK β (right panels) as shown in Fig. 1C were incubated with corresponding substrates (GST–CaMKI α , GST–CaMKIV or

AMPK: 10 μ g) and 2 mM CaCl_2 /6 μ M CaM in the absence (0 min, – in insert panels) or presence of 100 μ M ATP for the indicated time periods (2–30 min) followed by immunoblot analyses (insert panels) using anti-phosphoCaMKI α (at Thr177) (A), anti-phosphoCaMKIV (at Thr196) (B), and anti-phosphoAMPK α (at Thr172) (C) antibodies as described in “Materials and Methods.” The phosphorylation level of each substrate was quantitated by densitometric scanning of the immunoblot (insert) and expressed as a percentage of the average value of the 10-min time point (panel A) or the 30-min time points (panel B and C) with wild type CaMKK (CaMKK α WT or CaMKK β WT) and plotted as duplicate experiments. Asterisks (insert) indicate phosphorylated CaMKK substrates. The left lanes in each panel (insert) show the molecular mass markers.

Figure 3. LKB1 mutants with the RP-domain sequences of CaMKKs inserted between kinase subdomains II and III acquire CaMKI α and CaMKIV phosphorylating activity in vitro. (A) His–LKB1 wild type (LKB1 WT), CaMKK α RP-insertion (LKB1+ α RP) and CaMKK β RP-insertion (LKB1+ β RP) mutants complexed with HA–STRAD and HA–MO25 were expressed in COS-7 cell and purified by Ni–sepharose chromatography as described in “Materials and Methods,” followed by an immunoblot analysis using anti-His tag and anti-HA antibodies. Phosphorylation of GST–CaMKI (B), GST–CaMKIV (C), and AMPK (D) by purified His–LKB1 wild type (closed triangle) and +RP-inserted mutants (red circle, LKB1+ α RP; blue circle, LKB1+ β RP) that were complexed with HA–STRAD and HA–MO25 as shown in panel A. LKB1 complex including wild type and RP-inserted mutants were incubated with corresponding substrates (10 μ g) and 2 mM CaCl_2 / 6 μ M CaM (omitted in AMPK phosphorylation, D) in the absence (0 min, – in insert panels)

or presence of 100 μ M ATP for the indicated time periods (2–30 min), followed by immunoblot analyses (insert panels) using anti-phosphoCaMKI α (at Thr177) (B), anti-phosphoCaMKIV (at Thr196) (C), and anti-phosphoAMPK α (at Thr172) (D) antibodies as described in “Materials and Methods.” The phosphorylation level of each substrate was quantitated by densitometric scanning of the immunoblot (insert) and expressed as a percentage of the average value of the 10-min time point (panel B) or the 30-min time point (panel D) with LKB1+ β RP or the 30-min time point with LKB1+ α RP (panel C) and plotted as duplicate experiments. Asterisks (insert) indicate phosphorylated CaMKK substrates. LKB1 WT, wild type; LKB1+ α RP, LKB-1 inserted with CaMKK α residues 165L–190L; LKB1+ β RP, LKB-1 inserted with CaMKK β residues 201I–226R. The left lanes in each panel show the molecular mass markers.

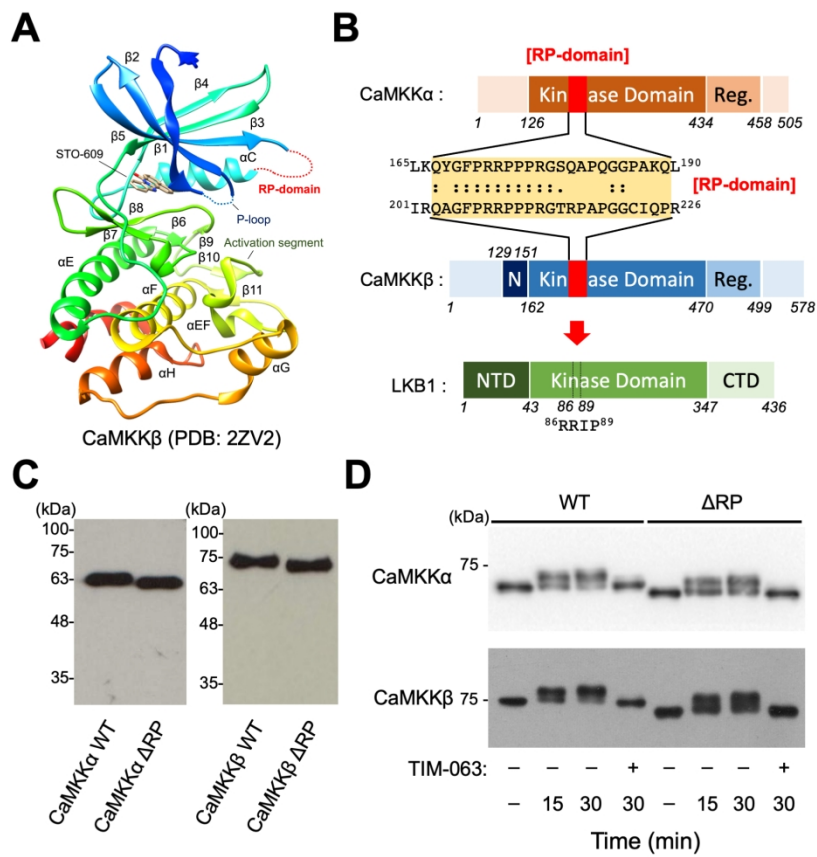
Figure 4. CaMKI α and CaMKIV phosphorylation in transfected cultured cells by LKB1 mutants with the RP-domain sequences of CaMKKs inserted between kinase subdomains II and III. COS-7 cells were transfected with expression plasmids for HA–CaMKI α (A) or HeLa cells were transfected with an expression plasmid for HA–CaMKIV (B), with or without (–) His–LKB1 wild type (WT), His–LKB1 mutant inserted with CaMKK α RP-domain sequence (+ α RP), or His–LKB1 mutant inserted with CaMKK β RP-domain sequence (+ β RP) as shown in Fig.3 in the presence of expression plasmids of HA–STRAD and HA–MO25 as described in “Materials and Methods.” Prior to ionomycin stimulation, the cells were cultured with 26 μ M TIM-063 in a serum-free medium for 6 h and then stimulated with 1 μ M ionomycin for 5 min and lysed, followed by immunoblot analyses using an anti-phosphoCaMKI α (at Thr177) (A, top panel), anti-phosphoCaMKIV (at Thr196) (B, top panel), anti-CaMKI (A, middle panel), anti-CaMKIV (B,

middle panel), or anti-His-tag antibody (A,B, bottom panels). The results represent duplicate experiments. The left lanes in panel A and B show the molecular mass markers.

Figure 5. Dominant-negative effect of CaMKK KD and Δ RP mutants on ionomycin-induced phosphorylation of CaMKK-target kinases in transfected cultured cells. COS-7 cells were transfected with expression plasmids for HA-CaMKI α (A), HA-AMPK α (C), or HeLa cells were transfected with an expression plasmid for HA-CaMKIV (B), without (-) or with His-CaMKK α KD mutant (α , left panels), His-CaMKK α KD mutant lacking RP-domain (α Δ RP, left panels), His-CaMKK β KD mutant (β , right panels), or His-CaMKK β KD mutant lacking RP-domain (β Δ RP, right panels) as described in “Materials and Methods.” The cells were then stimulated with 1 μ M ionomycin for 5 min and lysed, followed by immunoblot analyses using corresponding anti-phosphoCaMKI α (at Thr177) (A, top panels), anti-phosphoCaMKIV (at Thr196) (B, top panels), or anti-phosphoAMPK α (at Thr172) antibody (C, top panels). Cell lysates were also subjected to immunoblot analyses using either anti-HA (middle panels in A, B, and C) or anti-His tag antibody (lower panels in A, B, and C). The results represent duplicate experiments. The left lanes in each panel show the molecular mass markers. Asterisks indicate phosphorylated HA-CaMKI α at Thr177 (A), HA-CaMKIV at Thr196 (B), and HA-AMPK at Thr172 (C). KD, kinase dead.

Figure 6. Interaction of CaMKK KD mutants with CaMKI α , CaMKIV, and AMPK α . COS-7 cells were transfected with expression plasmids for HA-CaMKI α (A), HA-CaMKIV (B), or HA-AMPK α (C) with His-CaMKK α KD mutant (α , left panels), His-CaMKK α KD mutant lacking RP-domain (α Δ RP, left panels), His-CaMKK β KD mutant (β , right panels), or His-CaMKK β KD mutant lacking

RP-domain (β Δ RP, right panels) as described in Fig. 4. The cells were lysed and His–CaMKK mutants were pulled down with Ni–sepharose in the presence of 2 mM CaCl_2 , 6 μM CaM, followed by immunoblot analyses (*Pulldown*) with either anti-His tag (third panels in A, B, and C) or anti-HA antibody (bottom panels in A, B, and C) as described in “Materials and Methods.” Cell lysates (*Input*) were also subjected to immunoblot analyses using either anti-His tag (top panels in A, B, and C) or anti-HA antibody (second panels in A, B, and C). The results represent duplicate experiments. The left lanes in each panel indicate the molecular mass markers. KD, kinase dead.



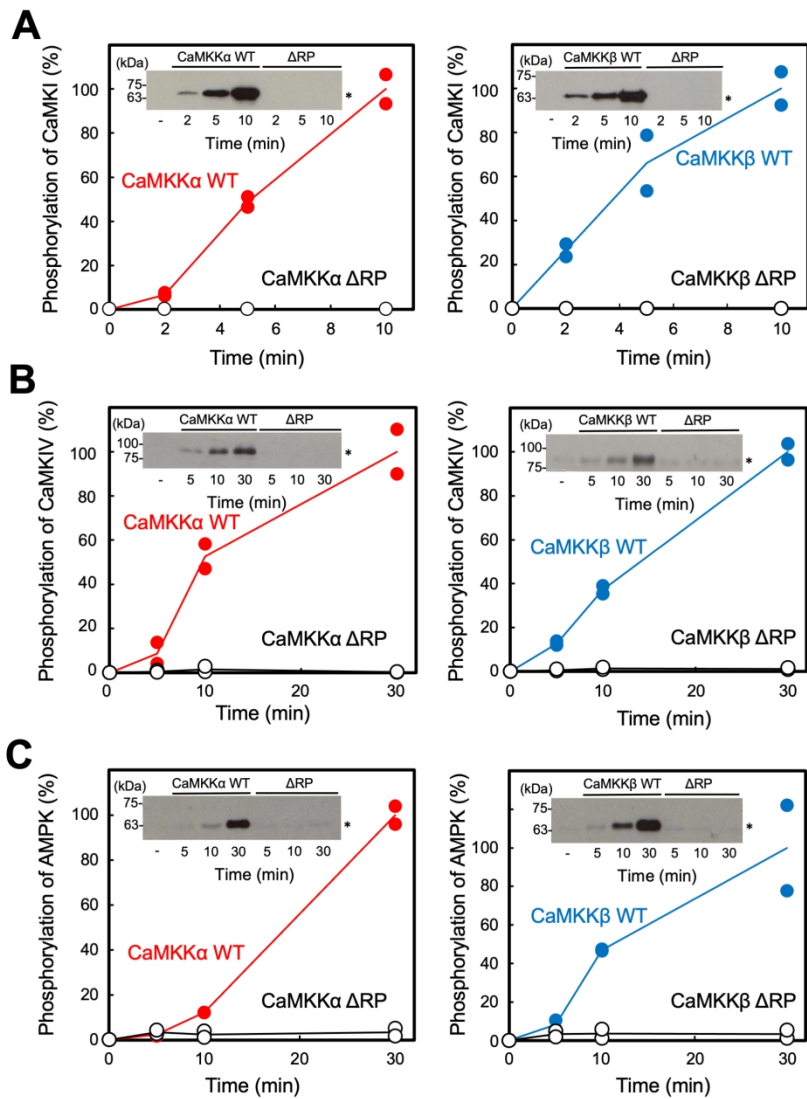


Figure 2

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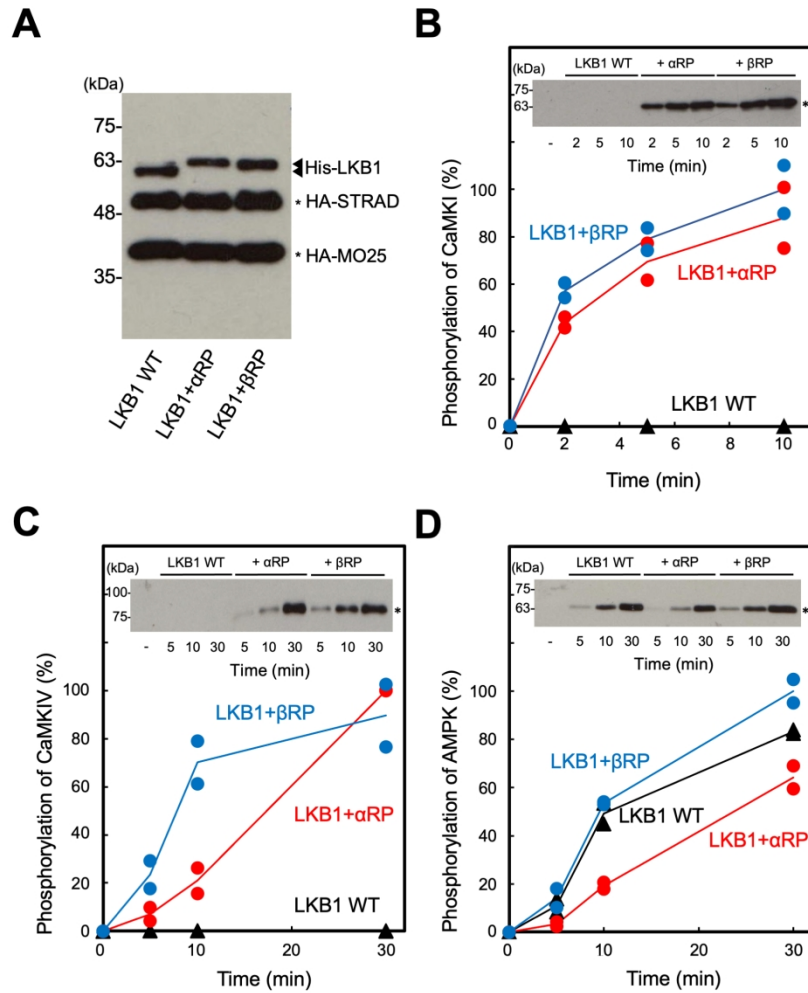
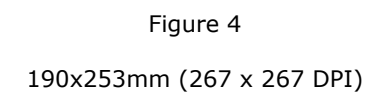


Figure 3

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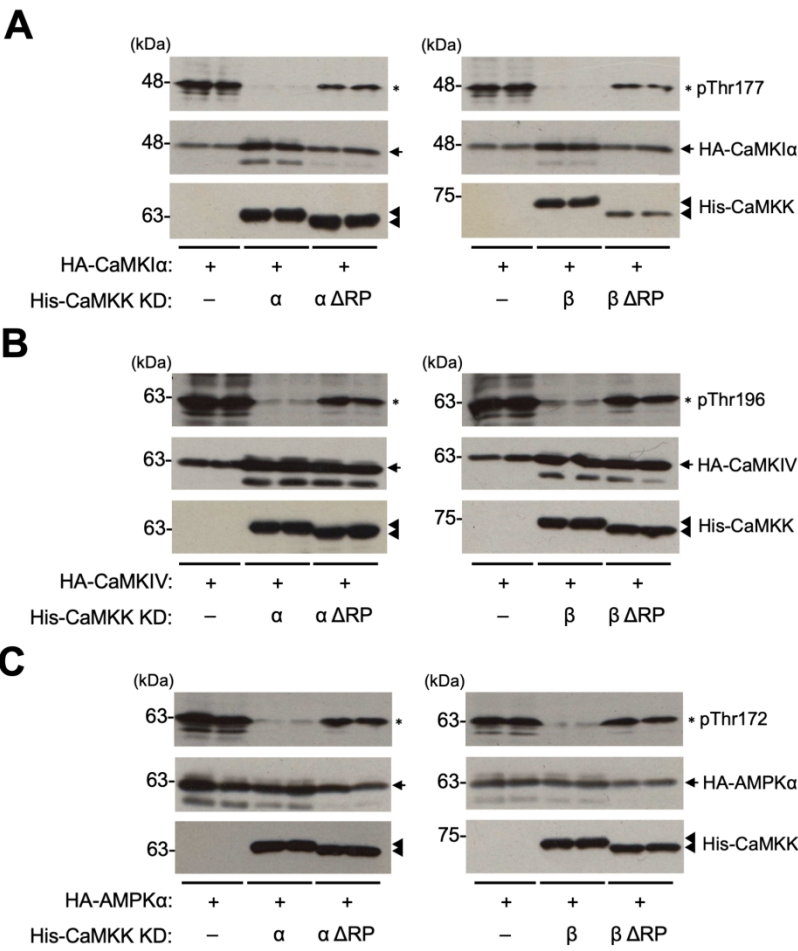


Figure 5

190x253mm (267 x 267 DPI)

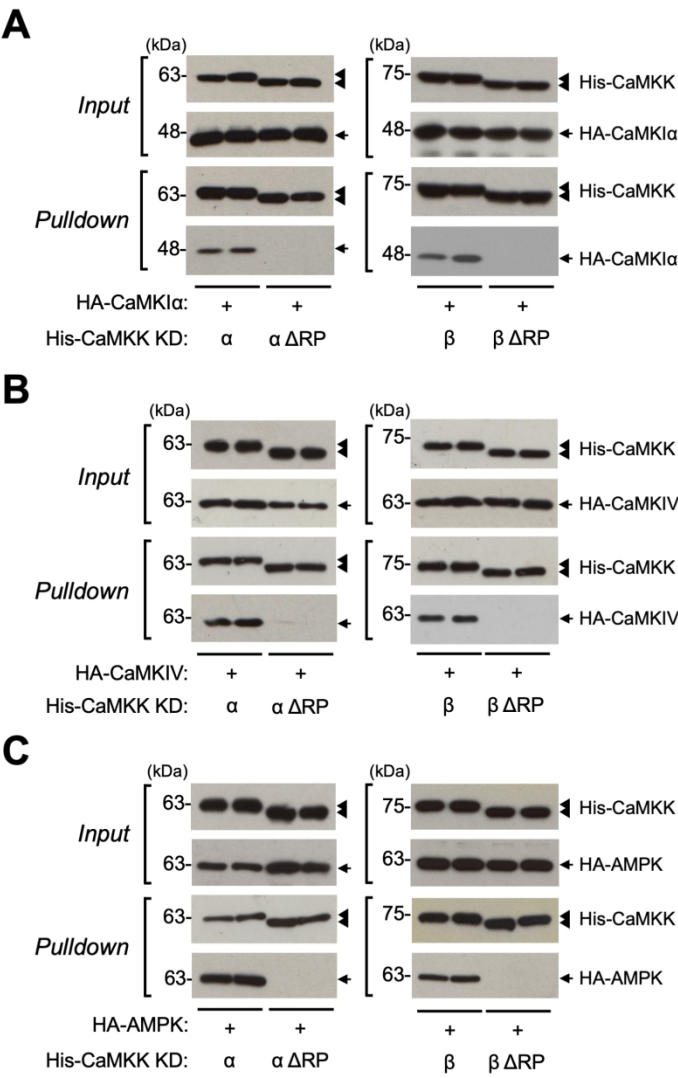


Figure 6

190x253mm (267 x 267 DPI)