Differential AMP-activated Protein Kinase (AMPK) Recognition Mechanism of Ca^{2+}/Calmodulin-dependent Protein Kinase Isoforms*

Received for publication, March 18, 2016, and in revised form, May 4, 2016. Published, JBC Papers in Press, May 5, 2016, DOI 10.1074/jbc.M116.727867

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Ca^{2+}/calmodulin-dependent protein kinase β (CaMKKβ) is a known activating kinase for AMP-activated protein kinase (AMPK). In vitro, CaMKKβ phosphorylates Thr^{172} in the AMPKα subunit more efficiently than CaMKKα, with a lower $K_m$ ($\approx 2 \mu M$) for AMPK, whereas the CaMKIα phosphorylation efficiencies by both CaMKKs are indistinguishable. Here we found that subdomain VIII of CaMKK is involved in the discrimination of AMPK as a native substrate by measuring the activities of various CaMKKα/CaMKKβ chimeras mutants. Site-directed mutagenesis analysis revealed that Leu^{358} in CaMKKβ/Ile^{322} in CaMKKα confer, at least in part, a distinct recognition of AMPK but not of CaMKIα.

Ca^{2+}/calmodulin-dependent protein kinase isoforms (CaMKKS) were originally identified as members of the calmodulin (CaM) kinase family that phosphorylate and activate two multifunctional CaMKS, including CaMKI and CaMKIV, constituting Ca^{2+}-dependent kinase cascades named CaMK cascades (1–3). CaMK in mammals is derived from two genes (CaMKKα and CaMKKβ), with $\sim 70\%$ of amino acid sequence homology in the catalytic domains (4–6). The two genes (CaMKK) are known activating kinases for AMPK and regulation of autophagy by amino acid starvation (18). According to studies using either RNA interference or pharmacological inhibition of CaMKK in HeLa cells, which do not express LKB1, an alternative AMPK kinase, CaMKKβ has been shown to be responsible for Ca^{2+}−dependent activation of AMPK in vivo, whereas both CaMKK isoforms are capable of phosphorylating the AMPKα subunit at Thr^{172} in vitro (13–15). This was confirmed by the fact that STO-609 (19), a CaMKK inhibitor, suppressed ionomycin-induced AMPK phosphorylation in A549 cells (a human lung adenocarcinoma epithelial cell line) expressing STO-609-resistant CaMKKα but not STO-609-resistant CaMKKβ (20). These results indicate that the CaMKKβ isoform, rather than CaMKKα, preferably recognizes AMPK, suggesting that the recognition mechanism of AMPK by CaMKK isoforms as a substrate may differ from that of CaMKI. A previous report showed that CaMKKβ, but not CaMKKα, forms a stable complex with AMPK, which could explain why CaMKKβ is an AMPK kinase and CaMKKα is not (21). However, Fogarty et al. (22) reported that CaMKKβ activates AMPK without forming a stable complex with AMPK. Therefore, despite the well characterized regulatory mechanisms of CaMKK, including an autoinhibitory mechanism (23), and the role of Ca^{2+}/CaM-binding in the expression of its kinase activity (24), little is known about the molecular mechanism of substrate recognition of CaMKKS. To clarify the differential substrate specificity of CaMKK isoforms, especially for AMPK, we investigated enzymatic characterization of CaMKKS using various chimeras and site-directed mutants of CaMKK isoforms and identified a single residue in subdomain VIII that may be essential for the discrimination of AMPK as a substrate.

**Experimental Procedures**

**Materials**—Recombinant rat CaMKKα and β, including WT and mutants, were expressed in Escherichia coli BL21 Star (DE3) and purified using CaM-Sepharose and Q-Sepharose chromatographies. The GST−rat CaMKKβ catalytic domain (162–470) and the GST−rat CaMKKα catalytic domain (126–434) were constructed, and recombinant GST−fused CaMKKS, including chimera mutants, were expressed in E. coli JM109 and purified as described previously (25). GST−rat CaMKIα 1–293, K49E (GST−CaMKIα 1–293, KE) was expressed in E. coli JM109 and purified as described previously (23). Recom-
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binant kinase-dead AMPK (K45R) was expressed in E. coli strain BL21-CodonPlus (DE3) (Stratagene, La Jolla, CA) using the tricistronic pTrcHis-α plasmid (provided by Dr. Dietbert Neumann, Swiss Federal Institute of Technology, Zurich, Switzerland) and purified as described previously (26). Recombinant rat CaM was expressed in the E. coli BL21 (DE3) strain using the plasmid pET-CaM (provided by Dr. Nobuhiro Hayashi, Tokyo Institute of Technology, Yokohama, Japan) and purified as described previously (27). Antibodies against the AMPK α subunit (2532) and AMPK α subunit phosphorylated at Thr172 (2535) were purchased from Cell Signaling Technology (Danvers, MA). An anti-FLAG antibody (clone M2) was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from standard commercial sources.

Construction of CaMKKβ/α Chimera Mutants and CaMKKα Point Mutants—GST-CaMKKβ-(162–470), GST-CaMKKα-(126–434), and CaMKKβ/α-1; GST-CaMKKβ-(162–364)/CaMKKα-(329–434) and CaMKKβ/α-2; GST-CaMKKβ-(162–303)/CaMKKα-(268–434) were constructed using a pGEX-PreS vector as described previously (25). CaMKKβ/α-2, GST-CaMKKβ-(162–303)/CaMKKα-(268–326)/CaMKKβ-(363–470); CaMKKβ/α/β-3, GST-CaMKKβ-(162–303)/CaMKKα-(268–322)/CaMKKβ-(359–470); and CaMKKβ/α-β-4, GST-CaMKKβ-(162–303)/CaMKKα-(268–311)/CaMKKβ-(348–470) were constructed as follows. N-terminal fragments amplified by PCR using a sense primer (5′-GGTCTAGAGAATCAGAAGCTGCTG-3′) and various phosphorylated antisense primers (CaMKKβ/α-β-2, 5′-pGCCGGT-GTCAGAGATTGGCCTC-3′; CaMKKβ/α-β-3, 5′-pGATGT-GCCTCAGGGCCATGAA-3′; and CaMKKβ/α-β-4, 5′-pTGCGTTGTAGACTGGACAGCTG-3′) and CaMKKβ/α-2 as a template were digested by XbaI and C-terminal fragments amplified by PCR using an antisense primer (5′-CCGTCGCAG-TAGACCTCTCTTCTCGGT-3′) and the appropriate phosphorylated primers (CaMKKβ/α-β-2, 5′-pAAGATCTTCTTCTCCGG-AAGGCC-3′; CaMKKβ/α-β-3, 5′-pTCAGAAGACCCGGAGATCTTC-3′; and CaMKKβ/α-β-4, 5′-pGGCACGCTCTTCTTCAGGC-3′) were digested by SalI, and then both fragments were ligated into an XbaI/Sal-digested pGEX-PreS vector. CaMKKβ/α-β-1; GST-CaMKKβ-(162–303)/CaMKKα-(268–328)/CaMKKβ-(365–470) was constructed by overlapping PCR using CaMKKβ/α-β-2 as a template and PCR primers (5′-CCGTCGCAG-TAGACCTCTCTTCTCGGT-3′ and 5′-AGAAGCTCTGTGGCGGTCTGAGATGCGC-3′). Point mutants of CaMKKα were generated by inverse PCR using pET-CaMKKα as a template and PCR primers as follows: CaMKKα A321S, 5′-ATGCGCCCGAGTCCATTCTGACC-3′ and 5′-GAGATCTGGGTTCCTGCGGTAATGCGA-3′; CaMKKα I322L, 5′-ATGCGCCCGAGGCGCTTCTGACGACCC-3′ and 5′-GAGATCTGGGTTCCTGCGGTAATGCGA-3′.

The retroviral transfer vectors (pMSCV-MCS-IRES-EGFP) harboring the FLAG-CaMKKα triple mutant (A292T/L233F/I322L) into the GP2–293 packaging cell line. After 18 h of culture, the cells were cultured in the absence of FBS for 6 h and then treated with 1 μM ionomycin for 5 min. The cells were extracted with 1× SDS-PAGE sample buffer (100 μl), followed by immunoblot analysis using indicated antibodies.

Statistics—Student’s t tests were used to evaluate statistical significance when two groups were compared. p < 0.05 was considered to be statistically significant.

Other Methods—The CaM overlay method was performed using 0.5 μg/ml biotinylated CaM in the presence of 1 mM CaCl2, followed by detection of the CaM-binding signal using a chemiluminescence reagent (PerkinElmer Life Sciences) as described previously (28). Protein concentration was estimated by staining the samples with Coomassie Brilliant Blue (Bio-Rad) using bovine serum albumin as a standard.

Results and Discussion

CaMKKβ, but Not CaMKKα, Preferentially Phosphorylates AMPK in Vitro—It has been reported that the purified CaMKKβ from rat brain activated AMPK 7-fold more rapidly than purified CaMKKα in vitro (14), whereas both CaMKK isoforms equally phosphorylated CaM. To confirm a distinct substrate preference of CaMKK isoforms, we attempted to measure the direct phosphorylation of the activation-loop Thr (Thr172) of the α subunit of the AMPK heterotrimeric complex by purified recombinant CaMKK isoforms and compared this with the phosphorylation of the catalytic domain of rat CaMKIα (GST-CaMKIα 1–293, K49E) at Thr172. Recombinant CaMKK isoforms were expressed in the E. coli BL21 Star (DE3) strain and purified by CaM-coupled Sepharose column chromatography. The amounts of the enzymes for measuring sub-
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FIGURE 1. In vitro phosphorylation of CaMKI and AMPK by recombinant CaMKK isoforms. A, CaM overlay analysis of recombinant rat CaMKKs (20 ng), including wild-type CaMKKα (CaMKKα) and wild-type CaMKKβ (CaMKKβ) was performed as described under “Experimental Procedures.” The molecular masses in kilodaltons are indicated on the left. B and C, phosphorylation of either GST-CaMKI 1–293, K49E (B), or AMPKα K45R (C) by recombinant rat CaMKKα (closed circles) and CaMKKβ (open circles) was measured at 30 °C for various time points with 4 mM CaCl2/10 μM CaM and 200 μM [γ-32P]ATP. After termination of the reaction, the samples were subjected to 10% SDS-PAGE followed by Coomassie Brilliant Blue staining and autoradiography (insets). 32P incorporation into each substrate (arrows) was measured by Cerenkov counting of the excised gels. Results are expressed as the mean ± S.D. of three experiments.

strate phosphorylation were comparable, as judged by the CaM overlay method (Fig. 1A), because both CaMKK isoforms contain similar CaM-binding sequences (24). Both CaMKK substrates were mutated at a residue in the ATP-binding site (K45R in AMPKα and K49E in CaMKIα) to generate kinase-dead enzymes to avoid the feedback phosphorylation of CaMKKs by activated CaMKK target kinases (29). Time course experiments of phosphorylation of the catalytic domain of GST-CaMKIα 1–293, K49E (Fig. 1B), and AMPK (Fig. 1C) were performed with the same concentrations (1 μg/ml) of CaMKK isoforms in the presence of Ca2+/CaM. Although the phosphorylation profiles of CaMKI by both CaMKK isoforms were indistinguishable (Fig. 1B), CaMKKβ was shown to phosphorylate the Thr172 of AMPK 14-fold more rapidly than CaMKKα (Fig. 1C) under this experimental condition. When we compared the kinetic constant (Km) of CaMKK isoforms for AMPK based on the double reciprocal plots of phosphorylation data using various concentrations of AMPK (Fig. 2A), it was clear that the main reason for the distinct activities of CaMKK isoforms for AMPK phosphorylation was the significantly higher Kms (13.1 μM) of CaMKKβ for AMPK compared with that of CaMKKα (1.5 μM). These results are in good agreement with a previous report using purified CaMKKs from rat brain (14), suggesting that CaMKKβ is the dominant isoform with respect to the regulation of AMPK.

Subdomain VIII of CaMKK is involved in discrimination of AMPK as a substrate—To clarify the distinct substrate preference of CaMKK isoforms, we analyzed the activities of catalytic domain mutants of CaMKKβ (residues 162–470) and CaMKKα (residues 126–434) including the wild-type enzyme, which each lacks both the N-terminal extension domain and C-terminal domain containing an autoinhibitory segment and a CaM-binding segment (23). These catalytic domain mutants were expressed in E. coli JM109 and purified as GST fusion proteins (Fig. 3A). Thus, all of recombinant enzyme phosphorylated the protein substrates in a complete Ca2+/CaM-independent manner. When we compared the AMPK phosphorylation activity of GST-CaMKK catalytic domain mutants, including wild-type and CaMKKβ/α chimera mutants, we used the same concentrations (~1 μg/ml) of the enzymes as those used for measuring GST-CaMKIα 1–293 K49E phosphorylation activity, which were equalized (Fig. 3B, top panel). Despite the ~70% amino acid sequence homology of the catalytic domains between rat CaMKKα and β (5, 6), CaMKKβ phosphorylated AMPK ~6-fold more rapidly than CaMKKα (Fig. 3B, bottom panel), consistent with the result with full-length CaMKK isofoms, as shown in Fig. 1C. Although the activity of the CaMKKβ-(162–364)/CaMKKα-(329–434) mutant (CaMKKβ/α-1) was comparable with that of CaMKKβ-(162–470), the CaMKKβ-(162–303)/CaMKKα-(268–434) mutant (CaMKKβ/α-2) showed a significantly reduced activity compared with that of CaMKKβ-(162–470) (Fig. 3B, bottom panel). These data suggest that residues 304–364 in CaMKKβ are involved in the efficient phosphorylation of AMPKα. Indeed, we could confirm that the CaMKKβ mutant, in which residues 304–364 were replaced by an equivalent region (residues 268–328) in CaMKKα (CaMKKβ/α/β-1), exhibited a significantly lower activity toward the substrate AMPK than CaMKKβ-(162–470) (Fig. 3B, bottom panel) did, similar to CaMKKα-(126–434).

Involvement of Ser357-Leu358 of CaMKKβ in Efficient AMPK Phosphorylation—Among residues 304–364 in CaMKKβ and 268–328 in CaMKKα, 17 amino acid residues are different from their corresponding counterparts in these two kinases (Fig. 4A). To narrow down the primary sequence of CaMKKβ,
which is involved in the efficient phosphorylation of AMPK, we produced serial chimera mutants based on CaMKKβ/H9252/H9251/H9252 and measured their activities (Fig. 4A). We performed a phosphorylation assay against AMPK/H9251/K45R using the same concentration of CaMKKβ/H9251/H9252 chimera mutants as that used for GST-CaMKI1–293 K49E phosphorylation (Fig. 4B) as described in Fig. 3B. CaMKKβ/α-β-2 and CaMKKβ/α-β-3 were shown to phosphorylate AMPK with a lower efficiency similar to that of CaMKKβ/α-β-1, but CaMKKβ/α-β-4 exhibited significantly increased activity to phosphorylate AMPKα (Fig. 4B, bottom panel). These results indicate that Ser357-Leu358 in CaMKKβ apparently plays an important role for the efficient phosphorylation of AMPKα but not for CaMKIα as a substrate.

CaMKKβ Leu358 Plays Important Roles in Efficiently Phosphorylating AMPK—To identify the crucial amino acid(s) in CaMKKβ required for efficiently phosphorylating AMPKα, we...
produced a full-length CaMKKα mutant in which Ala321-Ile322 was replaced by the corresponding amino acid residues in CaMKKβ (Ser348-Leu358) (Fig. 5A) and measured the CaMKK activity toward CaMKI and AMPK as substrates. The amounts of the enzymes for measuring substrate phosphorylation were comparable, as judged by the CaM overlay method (Fig. 5B). The CaMKKα A321S mutant was shown to phosphorylate AMPK with a lower efficiency, in a similar manner as the CaMKKα wild type. In contrast, the CaMKKβ I322L mutant exhibited significantly enhanced kinase activity toward AMPK in a similar manner as the CaMKKβ wild type. We observed the AMPK phosphorylating activity of the CaMKKα I322L mutant in a complete Ca2+/CaM-dependent manner.
AMPK phosphorylation because CaMKKβ was thought to be responsible for AMPK phosphorylation in A549 cells but not CaMKKα. In contrast, we could observe significant ionomycin-induced AMPK phosphorylation in cells expressing a CaMKKα triple mutant (A292T/L233F/I322L, AT/LF/IL) even in the presence of STO-609, indicating that the CaMKKα triple mutant (AT/LF/IL) acquired an ability for phosphorylating AMPK in living cells. These results suggest that a single amino acid difference (Leu<sup>358</sup> in CaMKKβ/Ile<sup>322</sup> in CaMKKα) in the catalytic domain of CaMKK isoforms dictates the efficiency of the kinase to phosphorylate AMPK<sub>a</sub> in vivo as well as in vitro.

In conclusion, CaMKKβ, but not CaMKKα, was shown to be an upstream activating kinase for AMPK (13–15) because of its efficient phosphorylating activity of CaMKKβ with an ~9-fold higher affinity for AMPK than CaMKKα had. It is noteworthy that the kinase activities of both CaMKK isoforms toward CaMKI were indistinguishable. Our mutagenesis studies clearly indicated that a single amino acid (Leu<sup>358</sup> in CaMKKβ/Ile<sup>322</sup> in CaMKKα) in subdomain VIII plays a role, at least in part, in the discrimination of AMPK as a native substrate but not in the discrimination of CaMKI. This finding is in good agreement with a previous report demonstrating that subdomain VIII in MAPK/ERK kinase kinase 1 (MEKK1) was a contact site for its substrate, MKK4, thereby discriminating the substrate (30). In addition, Leu<sup>358</sup> in CaMKKβ is also conserved in mammalian CaMKKβs and in its counterpart (ckk-1) in the roundworm Caenorhabditis elegans (31) (Fig. 5A) but not in mammalian CaMKKα isoforms, suggesting that the CKK-1/AMPK pathway may be functional in nematodes.

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**Author Contributions**—Y. F., Y. K., and T. F. performed the experiments. N. K. and M. M. supervised the experiments and helped to edit the manuscript. H. T. designed the study and wrote the manuscript.

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**FIGURE 6. Phosphorylation of AMPK by the CaMKKα I322L mutant in A549 cells.** Either the FLAG-CaMKKα A292T, L233F mutant (FLAG-CaMKKα AT/LF) or the FLAG-CaMKKα A292T/L233F/I322L mutant (FLAG-CaMKKα AT/LF/IL) was expressed with a retrovirus expression system as described under "Experimental Procedures" and then stimulated without (+, open circles) or with 1 μM ionomycin for 5 min (+) in the absence (−, closed circles) or presence (+, open triangles) of 10 μg/ml STO-609. Stimulation was terminated, and then AMPK phosphorylation at Thr<sup>792</sup> was analyzed by immunoblotting with anti-phospho-AMPK antibody (top blot), anti-AMPK antibody (center blot), and anti-FLAG antibody (bottom blot), followed by quantification of the phosphorylation signal. The results are expressed as a percentage of the value in the absence of STO-609 (−) with ionomycin treatment (+) and displayed as scatterplots. The averages of three experiments are plotted as columns. *p < 0.01 versus control cells without stimulation in the absence of STO-609; **p < 0.01 versus ionomycin stimulated cells in the absence of STO-609; ***p < 0.05 versus control cells without stimulation in the absence of STO-609.

(supplemental Fig. 1), indicating that the enhanced kinase activity toward AMPKα as a substrate (Fig. 5C) was likely due to disruption of the autoinhibitory mechanism of CaMKKα (23) by this mutation. Therefore, we measured the kinetic constant (K<sub>cat</sub>) of the CaMKKα I322L mutant for AMPK based on the double reciprocal plots of phosphorylation data using various concentrations of AMPK (Fig. 2B) and obtained a K<sub>cat</sub> value (4.9 μM) that was significantly lower than that of CaMKKα (13.1 μM, Fig. 2A). Finally, to confirm the data obtained with *in vitro* experiments as described above by using living cells, we attempted to test the ionomycin-induced AMPK phosphorylation in A549 cells (a human lung adenocarcinoma epithelial cell line) in which FLAG-tagged CaMKKα mutants were exogenously expressed with a retrovirus expression system (Fig. 6). When we expressed a FLAG-tagged CaMKKα double mutant (A292T/L233F, AT/LF) in A549 cells, which was a CaMKK inhibitor (STO-609)-resistant mutant (20), 10 μg/ml STO-609 (19) treatment completely inhibited ionomycin-induced
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