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Characterization of the CaMKK β -AMPK Signaling Complex

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Abstract

The AMP-activated protein kinase (AMPK) is a critical regulator of energy homeostasis, and is a potential target for treatment of metabolic diseases as well as cancer. AMPK can be phosphorylated and activated by the tumor suppressor LKB1 or the Ca²⁺/CaM-dependent protein kinase kinase β (CaMKK β). We previously identified a physical complex between CaMKK β and AMPK (Anderson, K. A., Ribar, T. J., Lin, F., Noeldner, P. K., Green, M. F., Muehlbauer, M. J., Witters, L. A., Kemp, B. E., and Means, A. R. (2008) *Cell Metabolism* 7, 377–388). Here we expand our analysis of the CaMKK β -AMPK signaling complex and show that whereas CaMKK β can form a complex with and activate AMPK, CaMKK α cannot. In addition, we show that CaMKK β and AMPK associate through their kinase domains, and CaMKK β must be in an active conformation in order to bind AMPK but not to associate with an alternative substrate, Ca²⁺/Calmodulin-dependent protein kinase IV (CaMKIV). Our results demonstrate that CaMKK β and AMPK form a unique signaling complex. This raises the possibility that the CaMKK β -AMPK complex can be specifically targeted by small molecule drugs to treat disease.

Keywords

AMPK; CaMKK β Ca²⁺; Calmodulin

1.0. Introduction

The AMP-activated protein kinase (AMPK) is a critical regulator of cellular energy homeostasis that is activated following periods of cellular stress during which the ATP to AMP ratio decreases. AMPK activation down-regulates pathways which consume energy such as protein synthesis, glycogen production and glucose-regulated gene transcription while simultaneously up-regulating pathways which produce ATP such as fatty acid oxidation, glycolysis and glucose transport [1]. AMPK also plays an important role in energy homeostasis at the level of the whole organism [2]. The actions of AMPK in the central nervous system in response to hormones from the periphery have been implicated in the regulation of feeding behavior, body weight, and systematic glucose and lipid metabolism [3]. Due to its important role in regulation of energy homeostasis, AMPK is a promising drug target for the treatment of type II diabetes and other diseases of the metabolic syndrome [4]. In addition, AMPK has recently been implicated in the regulation

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of cell growth, protein synthesis and cell cycle regulation which are all processes important for tumor formation making AMPK a potential target for cancer therapy [5].

AMPK functions as a heterotrimer consisting of a catalytic α subunit and regulatory β and γ subunits. In mammals there are multiple isoforms of each AMPK subunit ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, $\gamma 3$) encoded by separate genes. AMPK activation requires AMP binding as well as phosphorylation of the activation loop T172 in the catalytic α subunit. AMP binding has several effects on AMPK activation. First, binding of AMP to the γ subunit of the AMPK heterotrimer allosterically activates the enzyme, presumably through release of auto-inhibition [6]. Much progress has been made in understanding this mechanism as several partial crystal structures of AMPK have been solved recently [7–10]. In addition, binding of AMP to AMPK protects the enzyme from dephosphorylation by PP2C [11]. Recent work has also demonstrated that AMP directly stimulates AMPK phosphorylation provided that the AMPK β subunit is myristoylated [12]. To date two AMPK kinases have been characterized, the first is the tumor suppressor LKB1 [13, 14] and the second is Ca^{2+} /Calmodulin-dependent protein kinase kinase β (CaMKK β) [15–17]. The active form of LKB1 is a heterotrimer consisting of LKB1, the inactive pseudokinase STRAD and the scaffold-like MO25. The functions of STRAD and MO25 are to control the activity and localization of LKB1 [18]. LKB1 is ubiquitously expressed and appears to be the kinase most important for activation of AMPK in response to cellular stress in liver and skeletal muscle. In contrast to LKB1, the activation of AMPK by CaMKK β does not require an alteration of the ATP:AMP ratio, but rather occurs in response to an increase in intracellular Ca^{2+} . The expression pattern of CaMKK β in cells and tissues is more limited than that of LKB1 and is highest in multiple regions of the brain [19]. Previous work from our lab has demonstrated that a CaMKK β -AMPK signaling pathway in the hypothalamus is important in the regulation of appetite [20]. This led us to propose that activation of AMPK by LKB1 may be most critical at the cellular level while regulation of AMPK by CaMKK β may be more important at the whole body level [21].

Our previous work also revealed the presence of a stable complex which forms between AMPK and CaMKK β by demonstrating that the two enzymes co-immunoprecipitate from brain and cultured human cells [20]. Here we perform a more detailed examination of the CaMKK β -AMPK signaling complex. We show that CaMKK β , but not CaMKK α , forms a complex with and activates AMPK. We find that CaMKK β and AMPK associate through their kinase domains, and that CaMKK β must be in an active conformation in order to bind AMPK but not to associate with an alternative substrate, Ca^{2+} /Calmodulin-dependent protein kinase IV (CaMKIV). Thus, CaMKK β and AMPK form a unique signaling complex which raises the possibility that this complex could be specifically targeted by small molecule drugs to inhibit CaMKK β -AMPK signaling pathways.

2.0. Materials and methods

2.1. Purification of CaMKKs

Full length rat FLAG-CaMKK α and FLAG-CaMKK β were expressed from the pSG5 vector in HEK 293a cells using Lipofectamine 2000 (Invitrogen, 11668–019). For each transfection, 20 μg of DNA and 40 μl of Lipofectamine 2000 were used per 15 cm dish following the manufacturer's suggested protocol. HEK 293a cells were maintained in DMEM (Mediatech, 10-013-CV) with 10% FBS (Gemini Bio-Products, 900–108) and cells were about 75% confluent at the time of transfection. Cells were lysed 24–36 h after transfection in 1 ml/plate of NP-40 lysis buffer (25 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.5% Nonident P-40, 25 mM NaH_2PO_4 , 2 mM EGTA, 2 mM EDTA, 40 mM NaF, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ pepabloc and 100 nM okadaic acid). Lysate was clarified by centrifugation at $18,000 \times g$ for 30 min and then incubated for 2 h with constant rocking at

4°C with 20 µl of anti-FLAG M2-agarose (Sigma, A2220) per ml of lysate. The resin was washed twice with lysis buffer and once with TBS prior to elution for 1 h on ice with 40 µl of TBS containing 300 ng/µl FLAG peptide (Sigma, F3290). Protein concentrations were measured by fractionating aliquots of each preparation on a 10% SDS-PAGE gel along with BSA standards followed by quantification of Coomassie blue stained bands.

2.2. CaMKK Kinase Assays

CaMKK catalytic activity was assessed by its ability to phosphorylate a bacterially expressed, catalytically-inactive AMPK $\alpha 1$ (D139A) $\beta 1\gamma 1$ heterotrimer. The catalytic aspartic acid (D139) was mutated in the AMPK $\alpha 1$ subunit to eliminate background caused by auto-phosphorylation. Purification of AMPK $\alpha 1\beta 1\gamma 1$ from bacteria has been described previously [22]. For each 25 µl assay, approximately 50 ng of partially purified CaMKK β was incubated in a buffer containing 1.5 µM AMPK $\alpha 1$ (D139A) $\beta 1\gamma 1$, 0.1% Tween-20, 50 mM HEPES pH 7.0, 150 mM NaCl, 6.25 mM MgCl₂, 0.5 mM DTT, 200 µM ATP and 2 µCi EasyTides adenosine 5'-triphosphate γ -³²P (ATP γ -³²P, Perkin Elmer). Assays measuring Ca²⁺/CaM autonomous activity also contained 1 mM EGTA, while assays measuring Ca²⁺/CaM-dependent activity contained 1 mM CaCl₂ and 1 µM bovine testis CaM. Reactions were terminated at the times indicated in the figure legends by the addition of SDS-loading buffer followed by boiling of the samples. The samples were resolved on 10% SDS-PAGE gels and stained with Coomassie blue. Gels were dried and subjected to autoradiography. Bands corresponding to the α -subunit of AMPK were cut from the gel, and the amount of ³²P incorporated was measured using scintillation counting.

CaMKK activity was also assayed against full length, catalytically inactive GST-CaMKI (K49A). GST-CaMKI (K49A) was purified as previously described [23, 24]. For these assays varying amounts of GST-CaMKI were incubated with approximately 50 ng of purified CaMKK in a buffer containing 0.1% Tween-20, 50 mM HEPES pH 7.0, 150 mM NaCl, 6.25 mM MgCl₂, 0.5 mM DTT, 1 mM CaCl₂, 1 mM bovine testis CaM, 200 µM ATP and 2 µCi EasyTides adenosine 5'-triphosphate γ -³²P (ATP γ -³²P, Perkin Elmer). Reactions were terminated after 20 min incubation by the addition of SDS-loading buffer followed by boiling of the samples. Samples were then analyzed as above for assays using AMPK as a substrate.

2.3. Generation of CaMKK mutants

All point mutants used were generated using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene, 200521). Primers were site specific and flanked the mutation for 13–15 base pairs on each side. Mutations were confirmed by sequencing the entire coding region of mutated constructs at the Duke University DNA Analysis Facility.

CaMKK chimeras were constructed using two sequential rounds of PCR. The first round of PCR amplified the desired fragments of CaMKK α and CaMKK β using primers containing overhangs corresponding to the region of attachment to CaMKK β or CaMKK α , respectively. For the second round of PCR the products from the first round were combined along with primers to amplify the entire construct. The primers used to amplify the CaMKK chimeras also contained BamHI sites which were used to ligate the final product into the pSG5 vector. All constructs were sequenced for accuracy at the Duke University DNA Analysis Facility.

2.4. Immuno-blotting

Extracts were resolved on 8% or 10% SDS-PAGE gels and transferred to Immobilon-FL PVDF membranes (Millipore, IPFL00010). Membranes were blocked in a buffer containing 0.2 × PBS, 0.1% casein, 0.5% fish gelatin and 0.04% sodium azide for 1 h at room

temperature. Primary antibodies were diluted in blocking buffer with 0.1% Tween-20 at the following concentrations: FLAG (Sigma, F1804) 1:1000, pT172-AMPK α (Cell Signaling, 2531) 1:1000, total AMPK α (Epitomics, 1596-1) 1:1000, CaMKIV (BD Transduction Labs, 610275) 1:1000, GST (Santa Cruz, sc-138) 1:200, Calreticulin (BD Transduction Labs, 612136) 1:1000, ACL (Cell Signaling, 4332) 1:1000, and β -actin (Sigma, A5441) 1:10,000. After incubation with primary antibody membranes were washed with 1 $\times$ PBS containing 0.1% Tween-20. Membranes were then incubated with appropriate fluorescently-conjugated secondary antibodies, either IRDye800 goat anti-mouse IgG (Rockland, 610-132-121) or Alexa Fluor 680 goat anti-rabbit IgG (Molecular Probes, A21109), at a 1:5000 dilution in blocking buffer containing 0.1% Tween-20 and 0.02% SDS. Membranes were washed with 1 $\times$ PBS containing 0.1% Tween-20. Membranes were then scanned with an Odyssey Infrared imager (Li-Cor Biosciences).

2.5. Co-Immunoprecipitations

For immunoprecipitation of CaMKKs from HEK 293a cells, 10 μ g of each FLAG-CaMKK construct was transiently transfected using 20 μ l of Lipofectamine 2000 per 100 mM dish according to the manufacture's protocol. For co-immunoprecipitation of FLAG-CaMKK β and GST-AMPK constructs, 7 μ g of FLAG-CaMKK β and 7 μ g of GST-AMPK were co-transfected using 28 μ l of Lipofectamine 2000 per 100 mM dish according to the manufacture's protocol. When indicated, cells were treated with 1 μ M ionomycin or DMSO control for 5 min prior to lysis. Lysates were made from cells 36–48 h after transfection in NP-40 lysis buffer containing 2mM EGTA and 2mM EDTA as indicated. Lysates (approximately 1 mg of total protein) were clarified by centrifugation at 18,000 $\times$ g for 30 min, and then incubated with 10 μ l of anti-FLAG M2 agarose (Sigma, A2220) for 2 h at 4°C with constant rocking. Beads were then washed twice in NP-40 lysis buffer, once in TBS, and eluted with 1 $\times$ TBS containing 300 ng/ μ l FLAG peptide (Sigma, F3290). Immunoprecipitates were then subjected to immuno-blot analysis.

2.6. *In vitro* binding assay

FLAG-CaMKK β beads were purified as described above up until the elution step. Prior to elution, FLAG-CaMKK β beads were incubated with bacterially purified trimeric AMPK α 1 β 1 γ 1 which has been previously described [22] in a buffer containing 25 mM Tris-HCl pH7.4, 50 mM NaCl, and 0.5% Nonident P-40. Reactions also contained 1mM CaCl₂ and 1 μ M CaM or 10 mM MgCl₂ and 400 μ M ATP as indicated. After 2 h incubation with constant rocking at 4°C, samples were washed extensively in the same buffer as above. FLAG-CaMKK β and associated AMPK was then eluted off the anti-FLAG M2 agarose using 300 ng/ μ l FLAG peptide diluted in TBS. Eluted samples were then subjected to immuno-blot analysis as described above.

2.7. Immunofluorescent staining of 293a cells

HEK 293a cells were cultured on fibronectin (20 μ g/ml) coated coverslips placed in 6-well dishes. Cells were transfected with 2 μ g per well of pSG5-HA-CaMKK β using Lipofectamine 2000 according to the manufactures protocol. Following transfection cells were cultured for an additional 24 h before being fixed in 4% formaldehyde diluted in PBS for 15 minutes at room temperature. Following fixation, cells were permeabilized and blocked in a solution containing 1% normal donkey serum and 0.2% Triton X-100 diluted in PBS for 45 min at room temperature. Cells were then incubated overnight with an anti-HA antibody directly conjugated to Alexa Fluor 594 (Molecular Probes, A-21288) diluted 1:100 in PBS at 4°C in a humidified chamber. The next day coverslips were washed with PBS, incubated with Oregon Green 514 phalloidin (Molecular Probes, O-7465) diluted 1:1000 and 1 μ g/ml DAPI diluted in PBS for 10 minutes, and washed again with PBS. Coverslips were mounted onto slides using ProLong Gold antifade mounting media (Invitrogen,

P36934). Stained HEK 293a cells were visualized on a Zeiss LSM 510 inverted confocal microscope using a 63× oil immersion objective.

2.8. Analysis of CaMKK β crystal structure

The CaMKK β structure used is contained in PDB file 2ZV2. Images were generated using PyMol software [25].

2.9. AMPK kinase assay

CaMKK β -AMPK complexes were isolated as described above. To measure activity of AMPK co-immunoprecipitated with CaMKK β , 100 ng of the purified complex was incubated in a buffer containing 20 μ M SAMS peptide, 0.1% Tween-20, 50 mM HEPES pH 7.0, 150 mM NaCl, 0.5 mM DTT, 1 mM CaCl₂, 1 μ M CaM, 10 mM MgCl₂, 2 μ Ci EasyTides adenosine 5'-triphosphate γ -³²P (ATP γ -³²P, Perkin Elmer), and 200 μ M ATP. Reactions also contained 100 μ M AMP as indicated. Reactions were incubated at 30°C for 20 min before being spotted onto P81 filters (Whatman) and washed extensively in 75 mM phosphoric acid. The amount of radioactivity on filters was quantified by scintillation counting.

2.10. Detergent Fractionation

Cytoplasm and membrane fractions from HEK 293a cells transfected with FLAG-CaMKK β were isolated following a previously published procedure [26].

3.0 Results

3.1. CaMKK β , but not CaMKK α , is an AMPK kinase

Previous reports have demonstrated that CaMKK α can phosphorylate AMPK, but at a much lower rate than CaMKK β [15–17]. We decided to examine the regulation of AMPK by CaMKK α more carefully *in vitro* and in our cell based system. First, we purified both FLAG-CaMKK α and FLAG-CaMKK β which had been expressed in the human HEK 293a cell line. We then used these partially purified kinases in an *in vitro* kinase assay to examine their ability to phosphorylate the bacterially purified, catalytically inactive AMPK mutant AMPK α 1(D139A) β 1 γ 1 (Fig. 1A). We found that CaMKK α activity against AMPK was much lower than CaMKK β activity in either the presence or absence of calmodulin (CaM). As a control we tested the ability of CaMKK α and CaMKK β to phosphorylate CaMKI, and found their activities against this substrate to be identical (Fig. 1B). We then examined the ability of CaMKK α to phosphorylate AMPK on the activating residue Thr-172 in response to a Ca²⁺ signal in cells. For this experiment we transfected HEK 293a cells with vector control, CaMKK α or CaMKK β before stimulating them with the Ca²⁺ ionophore, ionomycin. As seen in Fig. 1 C, cells expressing CaMKK β have higher levels of both basal and ionomycin stimulated p-Thr172 AMPK α levels compared to vector control. However, cells expressing CaMKK α have p-Thr172 AMPK α levels similar to vector control. Finally, we tested the ability of CaMKK α to co-immunoprecipitate with AMPK. For this experiment we expressed FLAG-CaMKK α and FLAG-CaMKK β in HEK 293a cells and immunoprecipitated the CaMKKs with anti-FLAG M2-agarose. We immuno-blotted the precipitates for total AMPK α and found that while CaMKK β was able to form a complex with AMPK, CaMKK α was not (Fig. 1D). Thus, we confirm previous reports that CaMKK α has limited activity against AMPK either *in vitro* or in a cell based system and show for the first time that CaMKK α does not form a complex with AMPK whereas CaMKK β does.

3.2. The CaMKK β -AMPK complex is regulated by the activity of CaMKK β

Previous studies of the CaMKK β -AMPK complex show that it is disrupted in the presence of the metal chelators EDTA and EGTA ([20] and Fig. 1C). Since CaMKK β binds Mg²⁺/ATP and Ca²⁺/CaM we wanted to examine the contribution of each to formation of the CaMKK β -AMPK complex. To test the requirements for Mg²⁺/ATP and Ca²⁺/CaM, we developed an *in vitro* binding assay in which we incubated purified FLAG-CaMKK β attached to anti-FLAG M2-agarose with bacterially purified heterotrimeric AMPK $\alpha 1\beta 1\gamma 1$. Using this system the concentrations of Mg²⁺/ATP and Ca²⁺/CaM were easily controlled. We found that only samples where Mg²⁺/ATP and Ca²⁺/CaM were present allowed for an interaction between CaMKK β and AMPK (Fig. 2A). Since Ca²⁺/CaM binding to CaMKK β may be required prior to ATP binding, as is the case for CaMKI [27], we tested the requirement for Mg²⁺/ATP vs. Ca²⁺/CaM binding more thoroughly. To accomplish this, a series of CaMKK β mutants was expressed in HEK 293a cells and tested for ability to co-immunoprecipitate with endogenous AMPK. First, we used a truncated form of CaMKK β (CaMKK β 1-460) which lacks the autoinhibitory and CaM-binding domains and is active in the absence of Ca²⁺/CaM. We found this mutant able to form a complex with AMPK (Fig. 2B). The ATP-binding lysine (K193) and the catalytic aspartic acid (D311) were then mutated in the context of this constitutively active truncation mutant. These mutants were unable to form a complex with AMPK (Fig. 2B). Interestingly, while the truncated, inactive mutants were unable to form a complex with AMPK, they did form a complex with CaMKIV, another well characterized target of CaMKK β (Fig. 2C). In fact, interaction with CaMKIV was considerably enhanced with the inactive mutants of CaMKK β . These data indicate that Ca²⁺/CaM binding is required for full length CaMKK β to bind AMPK, but that the autoregulatory and C-terminal domains are not required for the interaction. In addition, there is a requirement for Mg²⁺/ATP binding to CaMKK β for interaction with AMPK, but not CaMKIV.

3.3. CaMKK β and AMPK associate through their kinase domains

We next designed a series of experiments to determine the domains of CaMKK β and AMPK required for their interaction. Since the truncated CaMKK β 1-460 can immunoprecipitate AMPK, we concluded that the autoregulatory and C-terminal domains of CaMKK β are not critical for complex formation. We then examined the requirement for the N-terminal and kinase domains of CaMKK β for interaction with AMPK. Since N-terminal truncation mutants of CaMKK β are unstable in HEK293a cells (unpublished observations), we made a series of CaMKK α /CaMKK β chimeras. The CaMKKs share a similar domain structure with N and C terminal domains of unknown function, a kinase domain and an autoregulatory domain consisting of overlapping autoinhibitory and CaM-binding domains (Fig. 2D). Since CaMKK α does not bind AMPK we were interested in identifying chimeras where replacing a domain of CaMKK α with the equivalent domain of CaMKK β leads to complex formation with AMPK, or chimeras where switching a CaMKK β domain with the equivalent CaMKK α domain leads to loss of AMPK complex formation. First, we examined if the N-terminal domain was involved in CaMKK β complex formation with AMPK by generating a CaMKK α construct with the CaMKK β N-terminal domain and a CaMKK β construct with a CaMKK α N-terminal domain (chimeras 1 and 2, Fig. 2D). Our data reveal that only the construct containing the CaMKK α N-terminus fused to CaMKK β co-immunoprecipitated with AMPK (chimera 2, Fig. 2E). This indicates that the N-terminus is not involved in binding to AMPK. Next we examined the need for the RP domain. The RP domain is an arginine and proline rich domain inserted into the kinase domain of both CaMKKs. Previous reports have shown that the RP domain of CaMKK α is critical for interaction with CaMKIV [28]. Our previous work demonstrated a need for the RP domain of CaMKK β for its interaction with AMPK, but not CaMKIV [20]. We thus make CaMKK α and CaMKK β constructs where the RP domains had been switched for that of CaMKK β and CaMKK α ,

respectively (chimeras 3 and 4, Fig. 2D). When these constructs were tested in our co-immunoprecipitation assay, only the CaMKK β with the CaMKK α RP domain formed a complex with AMPK (chimera 4, Fig. 2E). This indicates that the RP domain of CaMKK β is not sufficient to confer AMPK binding to CaMKK α . From these collective data we conclude that the kinase domain of CaMKK β is responsible for binding to AMPK, as only constructs containing this domain were able to co-immunoprecipitate with AMPK.

We then examined the domains of AMPK necessary for interaction with CaMKK β . To this end we expressed full length FLAG-CaMKK β with GST fusions of the AMPK α 1 subunit, and examined their ability to co-immunoprecipitate. We found that AMPK α 1_{T172A} as well as truncated AMPK α 1₁₋₃₁₂, which contains only the AMPK kinase domain, co-immunoprecipitated with CaMKK β to the same extent as wild type (Fig. 3A). Thus, the kinase domain of AMPK α 1 is sufficient to interact with CaMKK β .

We next performed an experiment to examine the effects of AMP on the CaMKK β -AMPK complex. Our previous work demonstrated that the CaMKK β -AMPK complex lacks the γ subunit of AMPK necessary for AMP binding [20]. Here, we purified CaMKK β -AMPK complexes by co-immunoprecipitation from HEK 293a cells and measured their activity against the AMPK substrate SAMS peptide in the presence or absence of AMP. Consistent with our previous data, we found that AMP had no effect on AMPK activity when the AMPK is associated with CaMKK β (Fig. 3B). As a positive control we immunoprecipitated endogenous AMPK from HEK 293a cells and tested its activity in the same assay. Here we did show a statistically significant increase in AMPK kinase activity in the presence of AMP (Fig. 3B). From these data we conclude that the CaMKK β -AMPK signaling complex is not allosterically activated by AMP.

3.4. The CaMKK β -AMPK complex is cytoplasmic

Recent work with an AMPK activity reporter indicates that LKB1 activation of AMPK activity is limited to the cytoplasm, while AMPK activated downstream of CaMKK β is present in both the cytoplasm and the nucleus [29]. We thus examined the localization of CaMKK β in HEK 293a cells transfected with HA-CaMKK β and stained with an antibody against HA. Using confocal microscopy, we generated a series of z-stacked images to examine co-localization of the HA-CaMKK β signal and the nucleus stained with DAPI. We found the majority of the CaMKK β signal to be present in the cytoplasm, but were unable to detect any signal in the nucleus (Fig. 4A). However, we also observed CaMKK β at the plasma membrane where it co-localized with actin (Fig. 4B). Recent work has demonstrated that AMP and nutrient stress lead to membrane association of AMPK [12] so we questioned where the CaMKK β -AMPK complex localized by transfecting HEK 293a cells with FLAG-CaMKK β followed by detergent fractionation to isolate cytoplasmic and membrane fractions. To determine purity of our fractions we immuno-blotted for the ER resident calreticulin and the cytoplasmic metabolic enzyme ATP-citrate lyase (ACL) which we found enriched in the membrane and cytoplasmic fractions, respectively. We detected FLAG-CaMKK β in the cytoplasmic and membrane fractions in accordance with our immunofluorescence data (Fig. 4C). We next immunoprecipitated CaMKK β from these cell fractions and then immuno-blotted for AMPK. We found that while CaMKK β was present in both cytoplasmic and membrane fractions, only the CaMKK β present in the cytoplasm formed a complex with AMPK (Fig 4C). We also examined localization of the CaMKK β -AMPK complex following Ca²⁺-stimulation. For these experiments, the cells were treated for 5 min with the Ca²⁺ ionophore, ionomycin prior to fractionation and immunoprecipitation. Following Ca²⁺-stimulation we were unable to observe any change in the localization of the CaMKK β -AMPK complex (Fig. 4C). From these experiments we conclude that interaction between CaMKK β and AMPK predominantly occurs in the cytoplasm.

4.0 Discussion

In this study we further characterize the CaMKK β -AMPK signaling complex. From our experiments we conclude that CaMKK β is an AMPK kinase while CaMKK α is not, most likely due to its inability to form a complex with AMPK. We show here that CaMKK β and AMPK associate through their kinase domains and formation of the CaMKK β -AMPK complex requires active CaMKK β which is bound to Mg²⁺/ATP and Ca²⁺/CaM. In addition, an RP domain is required for interaction of CaMKK β and AMPK. These requirements are unique to the CaMKK β -AMPK complex, and are not required for interaction of CaMKK β with CaMKIV. To better understand the CaMKK β /AMPK interaction we examined the crystal structure of the kinase domain of CaMKK β bound to its inhibitor STO-609 (Sup. Fig. 1 and [31]). In the structure, K193 forms contacts with both STO-609 and E234 located on the α C helix (Sup. Fig. 1). In structures of active kinases this K193 to E234 contact is required for ATP phosphate orientation and Mg²⁺ coordination which is critical for catalysis [32, 33]. In addition, this contact is important in stabilizing the change in orientation of the α C helix in several kinases from the “out” conformation of the inactive kinase to the “in” conformation of the active kinase [34, 35]. Furthermore, in the case of cyclin A binding to CDK2, the “in” conformation of the α C helix in CDK2 generates a large part of the surface to which cyclin A binds [34]. Thus, the K193A mutation could both destabilize the conformation of the active, closed ATP binding pocket and disrupt orientation of the α C helix. Since the K193A mutation in CaMKK β disrupts binding to AMPK, but not CaMKIV we conclude that an active, closed ATP binding pocket and properly orientated α C helix could be required for CaMKK β association with AMPK, but not CaMKIV.

Interestingly, in most kinases the β 3 sheet, which contains the ATP coordinating lysine, is connected to the α C helix, which contains the conserved glutamic acid, through a short loop. In the CaMKKs the RP domain is inserted between the β 3 sheet and α C helix, and in the structure of CaMKK β with STO-609 the RP domain is unordered. Again, our data show that removal of the RP domain from CaMKK β disrupts interaction with AMPK but not CaMKIV. However, replacing the RP domain of CaMKK β with that of CaMKK α maintains the interaction with AMPK. These data imply that it is not the sequence of the RP domain that is required to interact with AMPK. Rather, one possible explanation for these observations is that the RP domain may contribute to the appropriate alignment of the β 3 sheet and the α C helix in the active, closed conformation of the ATP binding pocket. At any rate, we conclude that CaMKK β interacts with its substrates AMPK and CaMKIV through different mechanisms. In addition, we suggest that, based on examination of the CaMKK β crystal structure, proper alignment of the ATP binding pocket and α C helix is required for CaMKK β interaction with AMPK, but not CaMKIV. These observations are provocative as they imply that the CaMKK β -AMPK complex could be targeted with a small molecule independently of the CaMKK β -CaMKIV complex, which could be helpful for the treatment of disease. Certainly this idea is worthy of future research efforts.

In a recent work, Fogarty et al failed to isolate a CaMKK β -AMPK complex [30], and there are several possible explanations why this is the case. First, our data suggest that the pool of CaMKK β bound to AMPK is small compared to the total amount of CaMKK β in the cell. Thus, in our studies we use a FLAG-tagged version of CaMKK β and are able to immunoprecipitate to near completion the CaMKK β over-expressed in HEK 293a cells whereas Fogarty et al performed a similar experiment but used an antibody specific to CaMKK β . We have tested several commercially available CaMKK β antibodies as well as antibodies generated in our lab, but have yet to identify an antibody able to immunoprecipitate CaMKK β with the efficiency of the FLAG antibody. Our hypothesis is that Fogarty et al failed to immunoprecipitate CaMKK β efficiently enough to detect its association with AMPK. In addition, Fogarty et al failed to perform experiments similar to

ours where endogenous AMPK was immunoprecipitated from brain extracts and found to be associated with endogenous CaMKK β (20). We believe these experiments were successful due to the greater efficiency of the AMPK antibody to immunoprecipitate endogenous protein when compared to CaMKK β antibodies.

Finally, we show herein that the CaMKK β -AMPK complex is present in the cytoplasm in resting cells as well as following Ca²⁺ stimulation. Recent work with a genetically encoded AMPK sensor shows that LKB1 can activate AMPK in the cytoplasm, but not the nucleus following energy stress. In contrast, activation of AMPK following a Ca²⁺ stimulation occurs first in the cytoplasm, followed by activation in the nucleus [29]. While we are unable to detect CaMKK β in the nucleus, it is possible that phosphorylation of AMPK by CaMKK β is required for shuttling AMPK into the nucleus. Such a situation would be analogous to the requirement of CaMKK β phosphorylation of CaMKIV for entry of CaMKIV into the nucleus [36].

5.0. Conclusions

From our work we conclude that CaMKK β and AMPK form a unique signaling complex which differs from the CaMKK β -CaMKIV signaling complex. We demonstrate the kinase domains of CaMKK β and AMPK are necessary for their interaction and that CaMKK β must be active. In addition, we show that the CaMKK β -AMPK complex localizes to the cytoplasm. These observations indicate that the CaMKK β -AMPK signaling complex could be specifically targeted which may provide for the specific targeting of CaMKK β -dependent AMPK signaling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AMP	adenosine 5'-monophosphate
AMPK	AMP-activated protein kinase
ACL	ATP-citrate lyase
ATP	adenosine 5'-triphosphate
CaM	calmodulin
CaMKIV	Ca ²⁺ /Calmodulin-dependent protein kinase IV
CaMKK	Ca ²⁺ /Calmodulin-dependent protein kinase kinase
DAPI	4',6-diamidino-2-phenylindole
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)- <i>N,N,N',N'</i> -tetraacetic acid

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Figure 1. CaMKK β , but not CaMKK α , is an AMPK kinase

(A) *In vitro* kinase assay measuring the ability of CaMKK α or CaMKK β to phosphorylate AMPK. CaMKK preparations were incubated with bacterially produced AMPK α 1(D139A) β 1 γ 1 in the presence of γ ³²P-ATP and Ca²⁺/CaM or EGTA for the indicated amounts of time. Reactions were then subjected to SDS-PAGE and staining with Coomassie brilliant blue. Bands corresponding to the AMPK α subunit were excised and amount of ³²P incorporated was measured by scintillation counting. Data shown are from a single experiment and results are representative of 3 independent experiments.

(B) *In vitro* kinase assay measuring the ability of CaMKK α and CaMKK β to phosphorylate CaMKI. CaMKK preparations were incubated with increasing amounts of bacterially expressed GST-CaMKI (K49A) in the presence of Ca²⁺/CaM and γ ³²P-ATP as described in Materials and Methods. Reactions were then subjected to SDS-PAGE and staining with Coomassie brilliant blue. Bands corresponding to CaMKI were excised and amount of ³²P incorporated was measured by scintillation counting. Data shown are from a single experiment and results are representative of 3 independent experiments.

(C) HEK 293a cells transfected with pSG5 vector control, FLAG-CaMKK β or FLAG-CaMKK α were treated with DMSO or 1 μ M ionomycin for 5 minutes as indicated prior to lysis and immuno-blot analysis with the antibodies indicated on the right. In each lane 25 μ g of total protein were analyzed. Blots shown are from a single experiment and results are representative of 3 independent experiments.

(D) FLAG-CaMKK β and FLAG-CaMKK α were transfected into HEK 293a cells and immunoprecipitated with anti-FLAG M2 agarose in the presence or absence of the metal chelators EGTA and EDTA as indicated. Immunoprecipitates were analyzed by immuno-blot for the presence of AMPK. Membranes were stained with Coomassie brilliant blue (CBB) as a loading control. Blots shown are from a single experiment and results are representative of 3 independent experiments.

Figure 2. The CaMKK β -AMPK complex is regulated by the activity of CaMKK β

(A) *In vitro* binding assay to examine association of FLAG-CaMKK β and bacterially produced AMPK α 1 β 1 γ 1 in buffers containing Ca²⁺/CaM and Mg²⁺/ATP as indicated.

(B) HEK 293a cells were transfected with the indicated FLAG-CaMKK constructs. FLAG-CaMKK was then immunoprecipitated with anti-FLAG M2 agarose, and immunoprecipitates were blotted for the presence of endogenous AMPK α . Membranes were stained with Coomassie brilliant blue and a loading control. The blot shown is representative of 3 independent experiments.

(C) HEK 293a cells were transfected with the indicated FLAG-CaMKK constructs and untagged CaMKIV. FLAG-CaMKK was then immunoprecipitated with anti-FLAG M2 agarose, and immunoprecipitates were blotted for the presence of CaMKIV. Membranes were stained with Coomassie brilliant blue as a loading control. The blot shown is representative of 3 independent experiments.

(D) Diagram showing CaMKK chimeras. N indicates N-terminal domain, AR indicates auto-regulatory domain and C indicates C-terminal domain. Chimeras are labeled from 1 to 4.

(E) HEK 293a cells were transfected with the indicated FLAG-CaMKK constructs. Numbers 1–4 correspond to chimeras illustrated in (D). FLAG-CaMKK was then immunoprecipitated with anti-FLAG M2 agarose, and immunoprecipitates were blotted for the presence of AMPK. Membranes were also blotted with FLAG antibody as a loading control.

Figure 3. The kinase domain of AMPK interacts with CaMKKβ

(A) HEK 293a cells were transfected with FLAG-CaMKKβ and the indicated GST-AMPK constructs. FLAG-CaMKK was then immunoprecipitated with anti-FLAG M2 agarose, and immunoprecipitates were blotted for the presence of GST-AMPK. Membranes were blotted with FLAG as a loading control. The blot shown is representative of 3 independent experiments.

(B) FLAG-CaMKKβ-AMPK complexes were isolated from HEK 293a cells and activity of AMPK was measured in the presence or absence of AMP against SAMS peptide. As a control, total AMPK was immunoprecipitated from HEK 293a cells and assayed under the same conditions. Data shown are the average of 3 independent experiments. Error bars represent standard error and p-values were calculated using an unpaired Student's t-test.

Figure 4. The CaMKKβ-AMPK complex is cytoplasmic

(A) HEK 293a cells expressing HA-CaMKKβ were stained with HA antibody to visualize CaMKKβ (red), Oregon Green phalloidin to stain actin (green) and DAPI (blue) to visualize nucleus. Confocal microscopy was used to generate a series of Z-stacked images. The panel on top shows the Z-plane through the green bar while the panel on right shows the Z-plane through the red bar.

(B) HEK 293a cells expressing HA-CaMKKβ were stained with Oregon green phalloidin to visualize actin (left), HA antibody to visualize CaMKKβ (middle) and DAPI to visualize the nucleus. The right panel shows an overlay of all three signals.

(C) Subcellular fractionation of HEK 293a cells expressing FLAG-CaMKKβ shows CaMKKβ in both membrane and cytoplasm fractions. Cells were treated with DMSO or 1μM ionomycin for 5 min prior to fractionation as indicated. The membrane marker calreticulin and the cytoplasm marker ACL were used to determine purity of fractions (left). Immunoprecipitation of FLAG-CaMKKβ from membrane and cytoplasm fractions shows that the CaMKKβ-AMPK complex is cytoplasmic (right). As a control CaMKKβ was also immunoprecipitated from unfractionated cells.