Oligomerization of Ca^{2+}/calmodulin-dependent protein kinase kinase

Yusei Fukumoto[a,*], Yuhei Harada[a,*], Satomi Ohtsuka[a], Naoki Kanayama[a], Masaki Magari[a], Naoya Hatano[a], Hiroyuki Sakagami[b] and Hiroshi Tokumitsu[a]

[a]Applied Cell Biology, Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama 700-8530 Japan

[b]Department of Anatomy, Kitasato University School of Medicine, Kanagawa 252-0374, Japan

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**Corresponding author: Hiroshi Tokumitsu

Applied Cell Biology, Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama 700-8530 Japan

TEL/FAX: +81-86-251-8197, E-mail: tokumit@okayama-u.ac.jp

*Y. F. and Y. H. contributed equally to this work.

Abbreviations

CaMKK, Ca^{2+}/CaM-dependent protein kinase; CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; AMPK, AMP-activated protein kinase; PKB, protein kinase B, GST, glutathione-*S*-transferase; PFA, paraformaldehyde; DSS, disuccinimidyl suberate

Abstract

Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKα and β) are regulatory kinases for multiple downstream kinases, including CaMKI, CaMKIV, PKB/Akt, and AMP-activated protein kinase (AMPK) through phosphorylation of each activation-loop Thr residue. In this report, we biochemically characterize the oligomeric structure of CaMKK isoforms through a heterologous expression system using COS-7 cells. Oligomerization of CaMKK isoforms was readily observed by treating CaMKK transfected cells with cell membrane permeable crosslinkers. In addition, His-tagged CaMKKα (His–CaMKKα) pulled down with FLAG-tagged CaMKKα (FLAG–CaMKKα) in transfected cells. The oligomerization of CaMKKα was confirmed by the fact that GST–CaMKKα/His–CaMKKα complex from transiently expressed COS-7 cells extracts was purified to near homogeneity by the sequential chromatography using glutathione-sepharose/Ni-sepharose and was observed in a Ca^{2+}/CaM-independent manner by reciprocal pulldown assay, suggesting the direct interaction between monomeric CaMKKα. Furthermore, the His-CaMKKα kinase-dead mutant (D293A) complexed with FLAG–CaMKKα exhibited significant CaMKK activity, indicating the active CaMKKα multimeric complex. Collectively, these results suggest that CaMKKα can self-associate in the cells, constituting a catalytically active oligomer that might be important for the efficient activation of CaMKK-mediated intracellular signaling.

Key words; CaMKK, oligomerization, Ca^{2+}-signaling, phosphorylation, CaM kinase cascade

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase (CaMKK) was originally identified as an activator of CaMKIα and CaMKIV by phosphorylating their activation loop Thr residue (Thr177 in CaMKIα and Thr196 in CaMKIV) [1,2]. It was then identified as a protein kinase B(PKB)/Akt activator [3]. In mammals, CaMKK consists of two isoforms (α and β) and is expressed in lower eukaryotes, including *Caenorhabditis elegans* and *Aspergillus nidulans* [4-8]. Recently, accumulated evidence indicated that CaMKKβ activates AMP-activated protein kinase (AMPK) through phosphorylation of Thr172 in AMPKα, resulting in various metabolic and pathophysiological responses, including hepatic steatosis and cancer cell growth [9-14]. CaMKK is a functional member of the CaMK family, activated by Ca^{2+}/CaM-binding. A previous study demonstrated that rat CaMKKα catalytic domain mutant (residues 84–434) exhibited constitutive kinase activity in the absence of Ca^{2+}/CaM, suppressed by adding a synthetic peptide corresponding to the regulatory region (residues 438–463), indicating that CaMKK is also subject to similar intrasteric autoinhibitory mechanisms to the other CaMK family [15]. Suppression of the kinase activity of CaMKKα by the regulatory peptide was canceled by adding Ca^{2+}/CaM, indicating that the residues 438–463 contain autoinhibitory and calmodulin-binding regions. Indeed, NMR spectroscopy analysis of Ca^{2+}/CaM complexed with the autoinhibitory peptide (residues 438–463) revealed a novel 1–16 CaM-binding motif [16] that was also confirmed by X-ray crystallography with CaM–*C. elegans* CaMKK peptide (residue 331–357) complex at 1.8 Å resolution [17]. In addition to Ca^{2+}/CaM-binding, CaMKK is regulated by phosphorylation, including autophosphorylation [18,19] and trans-phosphorylation by multiple protein kinases, including cAMP-dependent protein kinase, cyclin-dependent kinase 5, glycogen synthase kinase

3, and AMPK in cultured cells [20-25] and by 14-3-3 protein binding [23,26-28]. Recently, Xy Ling N *et al*. reported that FLAG-tagged CaMKKβ and HA-tagged CaMKKβ mutant (Arg311Cys) might form a dimer or larger oligomer based on the immunoprecipitation assay from exogenously expressed cell lysate [29]. However, the detailed mechanism of CaMKK oligomerization remains unclear.

In this study, we biochemically demonstrated and characterized the multimeric complex of CaMKKα, indicating that CaMKKα can form catalytically active oligomers in cultured cells by self-association.

2. Materials and Methods

2.1. Materials

GST–rat CaMKIα 1–293, Lys49Glu (GST–CaMKIα 1–293, K49E) was expressed in *E. coli* JM109 and purified as previously described [15]. Recombinant rat CaM was expressed in *E. coli* BL21 (DE3) using the plasmid pET–CaM (kindly provided by Dr. Nobuhiro Hayashi, Tokyo Institute of Technology, Yokohama, Japan) [30]. The anti-His tag antibody and anti-FLAG antibody were obtained from Proteintech and FUJIFILM Wako Pure Chemical Corporation, respectively. Anti-GST antibody (27457701V) was purchased from GE Healthcare. The anti-phosphoCaMKIα at Thr177 (clone 9H8) monoclonal antibody was generated as previously described [31]. The anti-CaMKKα and anti-CaMKKβ monoclonal antibodies were generated as previously described [32]. All other reagents were obtained from standard commercial sources.

2.2. CaMKK and CaMKI expression plasmids

Expression plasmids for the CaMKKα/β wild type (pME–CaMKKα/β) and the FLAG-tagged-rat CaMKKα wild type (pME–FLAG–CaMKKα) were constructed using the pME18s vector as previously described [33]. FLAG-tagged-rat CaMKKβ wild type expression plasmid (pcDNA–FLAG–CaMKKβ) was constructed using pcDNA3 vector [18]. Expression plasmids for GST-rat CaMKKα (pME–GST–CaMKKα) were constructed by subcloning a PCR fragment encoding GST-rat CaMKKα into EcoRI/XhoI site of pME18s vector. N-terminal Hisx6-tagged rat CaMKKα containing 5'GAATTCATGGGCCATCACCATCACCATCAC-2nd codon– encoding Met-Gly-His-His-His-His-His-His-2nd residue– were inserted into EcoRI/NotI sites of pME18s vector (pME–His–CaMKKα). An expression plasmid for CaMKKα Asp293Ala (D293A) was constructed by inverse PCR using primers (5'GCCGCCTTTGGTGTCAGCAACCAGTTTGAG3'/5'GATCTTCACGTGCCCATCGTCCCCAAG3') and pME–His–CaMKKα as templates. FLAG–rat CaMKIα expression plasmid (pME–FLAG–CaMKIα) was constructed by PCR using pGEX–rat CaMKIα as a template and primers (5' GGGAATTCCCAGGGGCAGTGGAAGG3'/5'GGCTCGAGTCAGTCCATGGCCCTAG3'), followed by inserting the PCR product into EcoRI/XhoI sites of pME–FLAG vector.

2.3. Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5 % CO_2. COS-7 cells placed in 6-well dishes were transfected with 1 μg of His–CaMKKα (pME–His–CaMKKα) with or without 1 μg of FLAG–CaMKKα(pME–FLAG–CaMKKα), FLAG–CaMKKβ (pcDNA–FLAG–CaMKKβ), FLAG–CaMKIα (pME–FLAG–CaMKIα) or GST–CaMKKα (pME–GST–CaMKKα) expression plasmid using polyethyleneimine "Max"

(Polysciences, Inc.) according to the manufacturer's protocol. After a 48-h culture, transfected COS-7 cells were subjected to chemical crosslinking or pulldown assay as described below.

2.4. Chemical crosslinking of CaMKKs in transfected cells

COS-7 cells were transfected with 2 μg of either rat CaMKKα or rat CaMKKβ expression plasmid (pME–CaMKKα/β) as described above. In addition, cells were treated with either 2% paraformaldehyde (PFA, Sigma-Aldrich) or 1 mM disuccinimidyl suberate (DSS, Tokyo Chemical Industry Co., Ltd.) for 10 min at room temperature. Subsequently, the reaction was quenched with 500 mM Tris-HCl pH 8.0 for 15 min at room temperature. After washing with PBS twice, the cells were lysed with 200 μL of lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.05% Tween 20, 0.1% protease inhibitor cocktail (Nakalai tesque), followed by immunoblot analysis (5 μL sample).

2.5. Ni-pulldown assay

COS-7 cells (6-well dishes) transiently expressing His–CaMKKα together with FLAG–CaMKKα, FLAG–CaMKKβ, FLAG–CaMKIα or GST–CaMKKα were lysed with 500 μL of lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 7.5, 30mM imidazole), followed by centrifugation at 19,060 x g for 10 min. The supernatant was incubated with 50 μL of Ni-sepharose (50 % slurry, GE-Healthcare) in the absence or presence of 2 mM $CaCl_2$/ 5 μM CaM at 4^{o}C overnight, followed by washing with 1 mL of lysis buffer in the absence or presence of 2 mM $CaCl_2$ for five times with gentle end-over-end mixing. Pulldown samples were eluted with 30–100 μL of elution buffer (500 mM NaCl,

50 mM Tris-HCl pH 7.5, 500 mM imidazole), followed by adding an equivolume of 2 x SDS-PAGE buffer and then analyzed by immunoblotting.

2.6.GST-pulldown assay

COS-7 cells (6-well dishes) transiently expressing GST–CaMKKα together with or without FLAG–CaMKKα were lysed with 500 µL of lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.05% Tween 20), followed by centrifugation at 19,060 x g for 10 min at 4°C. The supernatant was incubated with 50 µL of glutathione-sepharose (50% slurry, GE-Healthcare) in the presence of 2 mM EGTA or 2 mM $CaCl_2$/ 5 µM CaM at 4^{o}C overnight, followed by washing 5 times with 1 mL of lysis buffer in the presence of 2 mM EGTA or 2 mM $CaCl_2$ with gentle end-over-end mixing. Pulldown samples were eluted with 50 µL of 1 x SDS-PAGE buffer and then analyzed by immunoblotting.

2.7. Purification of GST–CaMKKα/His–CaMKKα oligomeric complex.

GST–CaMKKα expression plasmids (pME–GST–CaMKKα, 5 µg) were transfected into COS-7 cells (10 cm dish) together with His–CaMKKα expression plasmids (pME–His–CaMKKα, 5 µg) as described above. Transfected COS-7 cells (3 dishes) were lysed with 3 mL of lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1mM DTT, and 0.1% protease inhibitor cocktail). The cell lysate was applied onto 200 µL of a glutathione-sepharose column at 4^{o}C, followed by extensive washing with 10 mL of lysis buffer (3-times). GST–CaMKKα complex was eluted from glutathione-sepharose resin by adding lysis buffer containing 10 mM glutathione and 10 mM imidazole (1 mL of fraction volume). The eluate was applied onto 200 µL of Ni-NTA agarose

(Qiagen) column, followed by extensive washing with 10 mL of lysis buffer containing 10 mM imidazole (3-times), GST–CaMKKα/His–CaMKKα complex was eluted by adding lysis buffer (200 μL of fraction volume) containing 300 mM imidazole. Eluted samples (10 μL) were subjected to SDS-7.5% PAGE, followed by CBB staining.

2.8. In vitro CaMKK activity assay

CaMKK activity of pulldown samples (3 μL) by Ni-sepharose as described above, was measured at 30°C for the indicated time points in a solution 20 μL containing 50 mM HEPES pH 7.5, 10 mM $Mg(Ac)_2$, 2 mM DTT, 100 μM ATP and 30 μg GST–CaMKIα 1–293, K49E in the presence of 2 mM $CaCl_2$/6 μM CaM. Each reaction was initiated by the adding ATP. The reaction was terminated by adding SDS-PAGE sample buffer, followed by immunoblot analysis of GST–CaMKIα 1–293, K49E (0.6 μg) using either an anti-phosphoCaMKIα (at Thr177) antibody or anti-GST antibody.

2.9. Other methods

Immunoblot analysis was performed using the indicated primary antibodies and horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare) as the secondary antibody. A chemiluminescent reagent (PerkinElmer Life Sciences) was used for signal detection. Protein concentrations in the samples were estimated using Coomassie Brilliant Blue (Bio-Rad Laboratories, Inc.) and bovine serum albumin as a standard.

3. Results and Discussion

3.1. Oligomerization of CaMKKα and β in transfected COS-7 cells

CaMKKα and β are monomeric kinases that regulate multiple Ser/Thr protein kinases, including CaMKI, CaMKIV, PKB, and AMPK, constituting multiple Ca^{2+}-signaling cascades. We treated CaMKKα and β-transfected COS-7 cells with cell membrane permeable crosslinkers, paraformaldehyde (PFA), and disuccinimidyl suberate (DSS), followed by immunoblot analysis with either an anti-CaMKKα or anti-CaMKKβ antibody to examine the conformational species of CaMKKs in cultured cells (Fig. 1). Whereas CaMKKα migrates on SDS-PAGE gel at a monomeric position of ~65 kDa without crosslinker treatment, multiple immunoreactive bands detected by anti-CaMKKα antibody at ~130, ~180, and >200 kDa were observed with treatment of either PFA or DSS (Fig. 1A). This indicates that CaMKKα may form multimeric complexes or complexes with endogenous cellular proteins. We also observed similar results with CaMKKβ in transfected COS-7 cells (Fig. 1B), consistent with a recent report showing that crosslinked CaMKK2 (β) with PFA in HEK293 cells appeared as a major band at ~135–180 kDa [34].

3.2. Characterization of CaMKKα oligomerization

We attempted to perform the pulldown assay using Ni-Sepharose from the lysates of COS-7 cells exogenously expressing His–CaMKKα together with FLAG–CaMKKα or β to characterize the multimeric complex formation of CaMKK in intact cells. As shown in Fig. 2A, FLAG–CaMKKα but not CaMKKβ was pulled down with His–CaMKKα, indicating that CaMKKα can form a homomeric but not heteromeric complex. We performed a pulldown assay of His–CaMKKα with FLAG-CaMKIα, one of CaMKK target kinase in the absence of Ca^{2+}/CaM to assess the specificity of the interaction between His–CaMKKα and FLAG–CaMKKα, (Fig. 2B). Unlike FLAG–CaMKKα, FLAG–CaMKIα is incapable of stably interacting with His–CaMKKα, suggesting a specific interaction of

CaMKKα oligomers. We next examined Ca^{2+}/CaM-dependency of the oligomer formation of CaMKKα by reciprocal pulldown assay using COS-7 cell lysates expressing GST–CaMKKα and His–CaMKKα in the absence or presence of 2 mM $CaCl_2$/5 μM CaM (Fig. 3A). Glutathione-sepharose pulldown assays demonstrated that GST–CaMKKα was pulled down together with His–CaMKKα regardless of the presence or absence of Ca^{2+}/CaM although His–CaMKKα alone was not pulled down with the resin. The reciprocal pulldown of His–CaMKKα with GST–CaMKKα by Ni-Sepharose indicated that the oligomerization of CaMKKα occurred in a Ca^{2+}/CaM-independent manner, similar to the results with the GST-pulldown assay. We performed a sequential purification of GST–CaMKKα/His–CaMKKα oligomeric complex using glutathione-sepharose and Ni-sepharose chromatographies from transfected COS-7 cell lysates in the absence of Ca^{2+}/CaM to exclude the possibility that the oligomerization of CaMKKα in transfected cells occurs indirectly, such as mediated by scaffold proteins or unknown cellular proteins (Fig. 3B). Coomassie Brilliant Blue staining of the eluted fractions from each purification step on SDS-PAGE revealed that the initial purification step by glutathione-sepharose resin enriched GST-CaMKKα together with a small amount of His–CaMKKα and subsequent column chromatography by Ni-sepharose successfully purified the GST–CaMKKα/His–CaMKKα multimeric complex to near homogeneity with comparable amounts of both CaMKKs. This result may rule out the possibility that cellular scaffold proteins mediate CaMKKα oligomeric structure.

3.3. CaMKKα oligomer is catalytically active.

We constructed expression vectors for a kinase-dead mutant of His–CaMKKα (D293A) to characterize the enzymatic activity of CaMKKα oligomeric complex, followed by expressing with

or without FLAG–CaMKKα in COS-7 cells. Then, the CaMKKα oligomeric complex was pulled down with Ni-sepharose resin to measure the CaMKK activity toward GST–CaMKIα 1–293, K49E as a substrate in the presence of Ca^{2+}/CaM. FLAG–CaMKKα was not pulled down with Ni-sepharose (Fig.4A); therefore, no CaMKK activity was detected (Fig. 4B). As expected, a pulldown sample from transfected cells with a kinase-dead His–CaMKKα mutant (D293A) alone exhibited undetectable CaMKK activity. However, His–CaMKKα (D293A) complexed with FLAG–CaMKKα can phosphorylate GST–CaMKIα 1–293, K49E at Thr177 in a similar manner to His–CaMKKα wild type alone. This result indicates that His–CaMKKα interacts with catalytically active FLAG–CaMKKα to form an active CaMKKα oligomer.

In this report, the homo-oligomerization of CaMKKα was biochemically demonstrated in transfected cultured cells by crosslinking, pulldown assay, and sequential purification. Crosslinked CaMKKα in transfected COS-7 cells with multiple molecular weights from 130 to >200 kDa may indicate that the existence of homo-dimeric, -trimeric, and -tetrameric complex as well as monomeric CaMKKα in cultured cells. The pulldown assay revealed a specific interaction of CaMKKα homomeric oligomer that is a catalytically active complex. Our result of the crosslinking experiment using CaMKKβ (Fig. 1B) also agrees with the observation that FLAG–CaMKK2 was co-immunoprecipitated with an HA–CaMKK2 (R311C) mutant from transfected CaMKK2 null HAP1 cells, suggesting the functional CaMKK2 oligomer [29]. Among Ca^{2+}/CaM-dependent protein kinases, CaMKII holoenzymes are known to be multimers of 8–10 subunits through the interaction of the associated domain of all subunits [35,36]. Oligomerization of the active form of CaMKK might be important for efficient phosphorylation and activation of downstream kinases including CaMKI, IV, PKB/Akt, and AMPK to generate Ca^{2+}-dependent

kinase activation cascade. Further studies elucidating the molecular mechanisms of oligomerization, including the stoichiometry of the CaMKK multimeric complex and identifying the self-association region in the enzyme, are necessary to clarify the important role(s) of the multimeric structure of CaMKK in the CaMK cascade.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Author contributions: Y.F. and Y.H. performed the experiments. S.O., M.M., N.K., N.H. supervised the experiments. H.S. generated antibodies against CaMKK isoforms and contributed to drafting the manuscript. H.T. conceived, designed the study, and prepared the final version of the manuscript. All authors contributed to the analysis and interpretation of the data.

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Figure legends

Fig. 1. Oligomerization of CaMKK isoforms. COS-7 cells were transfected with either rat CaMKKα (A) or rat CaMKKβ (B) expression plasmid, followed by treatment without (-) or with paraformaldehyde (PFA) or disuccinimidyl suberate (DSS) as described in "Materials and Methods." After quenching the crosslinking reaction, the cell lysates were subjected to immunoblot analysis using an anti-CaMKKα antibody (A) or anti-CaMKKβ antibody (B) as described in "Materials and Methods.". Molecular mass markers are indicated on the left lane in each panel. Arrowheads indicate a multimeric complex of CaMKK isoforms. The results were represented as duplicate experiments.

Fig. 2. Pulldown of His–CaMKKα/FLAG–CaMKKα complex from transfected COS-7 cells. COS-7 cells were transfected with (+) or without (-) an expression plasmid of Hisx6 tagged-CaMKKα (His–CaMKKα) together with (+) or without (-) either FLAG-tagged CaMKKα (FLAG–CaMKKα), FLAG-tagged CaMKKβ (FLAG–CaMKKβ) or FLAG-tagged rat CaMKIα (FLAG–CaMKIα, B) expression plasmid as described in "Materials and Methods." After lysis of the transfected cells, pulldown assay was performed with Ni-sepharose resin without $CaCl_2$, followed by immunoblot analysis using an anti-His tag antibody (1st and 3rd panels in A and B) or anti-FLAG tag antibody (2nd and 4th panels in A and B). Immunoblot analyses of cell lysates (*Cell lysate*, 1st and 2nd panels in A and B) and pulldown samples (*Pulldown*, 3rd and 4th panels in A and B) are indicated. Molecular mass markers are indicated on the left lane in each panel.

Fig. 3. Pulldown and purification of GST–CaMKKα/His–CaMKKα complex from transfected COS-7 cells. A. COS-7 cells were transfected without (-) or with (+) an expression plasmid of GST–CaMKKα and/or His-CaMKKα and lysed, followed by pulldown assay using either Ni-sepharose resin (Ni-sepharose) or glutathione-sepharose resin (Glutathione-sepharose) in the absence (-) or presence (+) of 2 mM $CaCl_2$/5μM CaM as described in "Materials and Methods." Cell lysates (*Cell lysate*) and pulldown samples (*Pulldown*) were subjected to immunoblot analyses using an anti-CaMKKα antibody. B. Sequential purification of GST–CaMKKα/His–CaMKKα complex from transfected COS-7 cells. COS-7 cells were transfected with expression plasmids of GST–CaMKKα together with His–CaMKKα, and then the cell lysates were purified by glutathione-sepharose chromatography (Glutathione-sepharose), followed by Ni-NTA agarose chromatography (Ni-NTA Agarose) as described in "Materials and Methods.". In each purification step, eluted fractions (fraction number 1–4) were collected and subjected to SDS-PAGE analysis with Coomassie Brilliant Blue staining. Cell lysate (Cell lysate) and flow-through fractions (FT) were also analyzed. Molecular mass markers are indicated on the left lane in each panel.

Fig. 4. CaMKK activity of His–CaMKKα/FLAG–CaMKKα multimeric complex. COS-7 cells were transfected without (-) or with (+) an expression plasmid of either His–CaMKKα wild type (WT) or His–CaMKKα kinase-dead mutant (D293A) together without (-) or with FLAG–CaMKKα wild type (WT) expression plasmid as described in "Materials and Methods." After lysis of the transfected cells, a pulldown assay was performed with Ni-sepharose resin without $CaCl_2$, followed by performing a CaMKK activity assay in the absence (time point; 0) or presence of 100 μM ATP, 30 μg GST–CaMKIα 1–293, K49E and 2 mM $CaCl_2$/6 μM CaM for 5 and 10 min as

described in “Materials and Methods.” A. Pulldown samples (2.5 μL) were subjected to immunoblot analysis using an anti-FLAG tag antibody (*upper panel*) or anti-His tag antibody (*lower panel*). B. Phosphorylation reaction samples were subjected to immunoblot analysis using an anti-CaMKIα phoshoThr177 antibody (*upper panel*) or anti-GST antibody (*lower panel*). A molecular mass marker is indicated on the left lane in each panel.

Figure 1

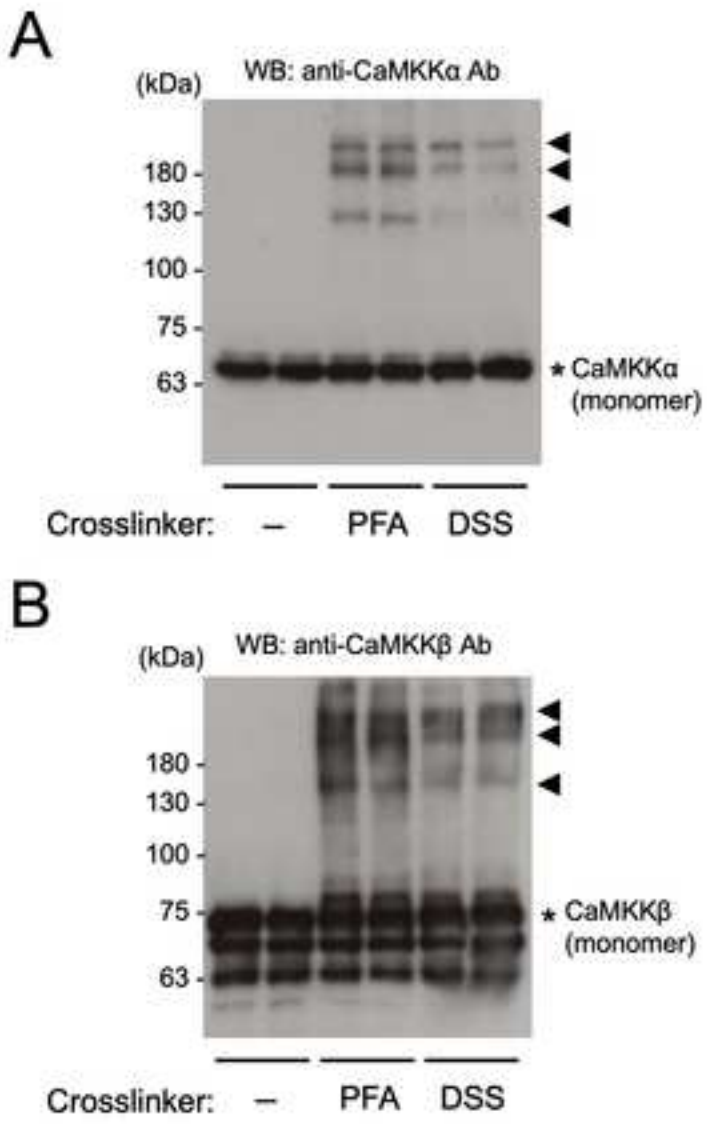

Figure 2

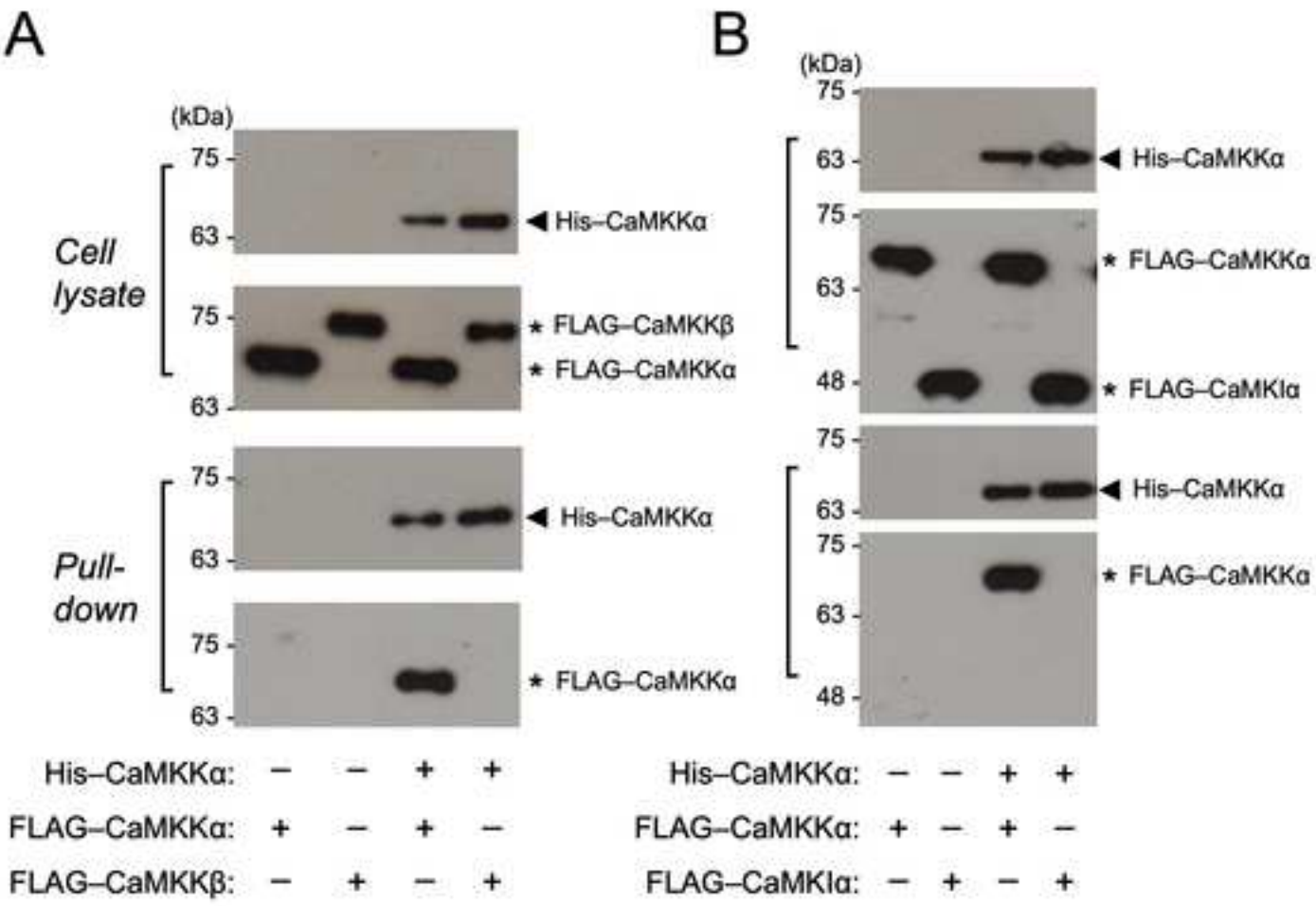

Figure 3

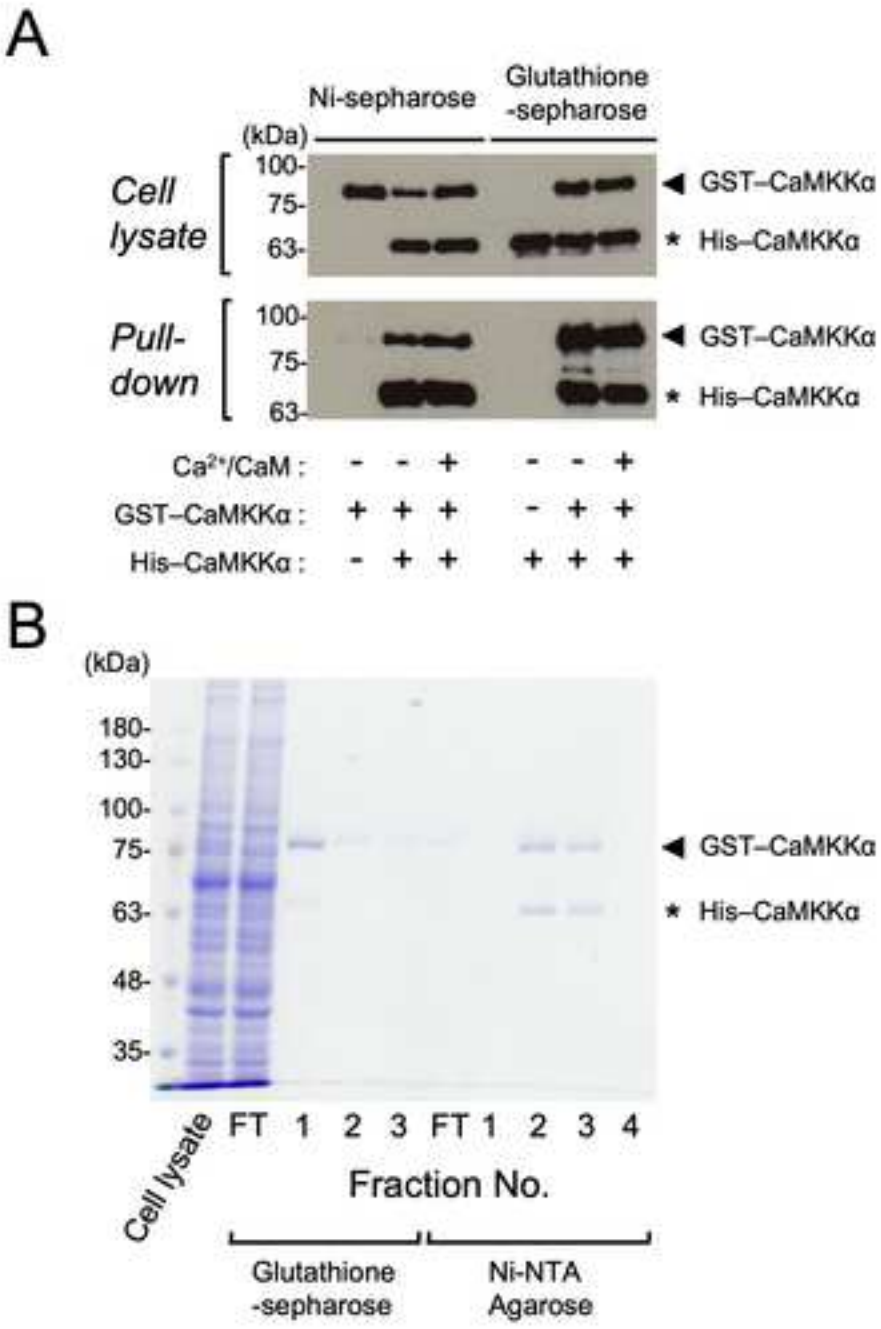

Figure 4