Biochemical and Biophysical Research Communications 410 (2011) 45–51

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of a novel CaMKK substrate

Tomohito Fujimoto [a], Naoya Hatano [b], Naohito Nozaki [c], Saki Yurimoto [a], Ryoji Kobayashi [a], Hiroshi Tokumitsu [a,*]

[a] Department of Signal Transduction Sciences, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan
[b] The Integrated Center for Mass Spectrometry, Graduate School of Medicine, Kobe University, Hyogo 650-0017, Japan
[c] Bio-Frontier Research Center, Tokyo Institute of Technology, Kanagawa 226-8503, Japan

ARTICLE INFO

Article history:
Received 10 May 2011
Available online 24 May 2011

Keywords:
CaMKK
Syndapin I
Phosphorylation
Ca^{2+}-signaling

ABSTRACT

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) phosphorylates and activates specific downstream protein kinases including CaMKI, CaMKIV and 5′-AMP-activated protein kinase. In order to examine the variety of CaMKK-mediated signaling pathways, we searched for novel CaMKK substrate(s) using N^6-(1-methylbutyl)-ATP and genetically engineered CaMKKα mutant, CaMKKα ($Phe^{230}Gly$), that was capable of utilizing this ATP analogue as a phosphate donor. Incubation of rat brain extracts with recombinant CaMKKα ($Phe^{230}Gly$), but not with wild-type kinase, in the presence of N^6-(1-methylbutyl)-ATP and Ca^{2+}/CaM, induced significant threonine phosphorylation of a 50 kDa protein as well as CaMKI phosphorylation at Thr^{177}. The 50 kDa CaMKK substrate was partially purified by using serial column chromatography, and was identified as Syndapin I by LC-MS/MS analysis. We confirmed that recombinant Syndapin I was phosphorylated by CaMKKα and β isoforms at Thr^{355} *in vitro*. Phosphorylation of HA-Syndapin I at Thr^{355} in transfected HeLa cells was significantly induced by co-expression of constitutively active mutants of CaMKK isoforms. This is the first report that CaMKK is capable of phosphorylating a non-kinase substrate suggesting the possibility of CaMKK-mediated novel Ca^{2+}-signaling pathways that are independent of downstream protein kinases.

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) has been classified as a novel member of the calmodulin kinase (CaMK) family. CaMKK specifically phosphorylates the activation loop Thr residue (Thr^{177} and Thr^{196}, respectively) in two multifunctional CaMKs, CaMKI and CaMKIV, resulting in a large increase in their catalytic efficiency [1,2]. In mammals, two CaMKK genes (CaMKKα and CaMKKβ) have been identified, both of which are highly expressed in the brain [3,4]. Accumulated biochemical evidence indicates that CaMKK also phosphorylates Akt/Protein kinase B [5] and AMPK family members including the catalytic subunit of AMPK at Thr^{172} [6–8] and SAD-B at Thr^{189} [9], resulting in significant catalytic activation.

One important role that has been demonstrated for the CaMKK/CaMKIV cascade is in the regulation of Ca^{2+}-dependent gene expression, where it phosphorylates transcription factors such as CREB [10]. The CaMKK/CaMKI cascade has been shown to be involved in various neuronal functions including spinogenesis [11], dendritic arborization [12] and cortical axon elongation [13]. It has also been suggested that CaMKK-mediated PKB/Akt activation might be involved in anti-apoptotic effects in neurons [5]. Recent studies have shown that Ca^{2+}-dependent phosphorylation and activation of AMPK is mediated by CaMKKβ when T-cells are activated via the antigen receptor [14] or when HeLa cells are treated with a Ca^{2+} ionophore [15]. Based on these studies, it has been predicted that the physiological role of CaMKKs is to act as regulatory enzymes for downstream protein kinases in various Ca^{2+}-dependent cellular processes. The question remains if there are any target substrates of CaMKK that are not protein kinases. In order to address this question, we searched for novel CaMKK substrates by using an ATP analogue (N^6-(1-methylbutyl)-ATP) and a CaMKKα mutant that was capable of using this ATP analogue as a phosphate donor.

2. Materials and methods

2.1. Materials

Recombinant CaMKKα, β, GST-CaMKKα 126–434 and GST-CaMKIα (1–293, $Lys^{49}Glu$) were expressed in *Escherichia coli* and purified as described previously [16,17]. Expression plasmids for truncated CaMKKα (pME-CaMKKα (1–434)) and β (pME-CaMKKβ

* Corresponding author. Address: Department of Signal Transduction Sciences, Faculty of Medicine, Kagawa University, 1750-1 Miki-cho, Kita-gun, Kagawa 761-0793, Japan. Fax: +81 87 891 2368.
E-mail address: tokumit@med.kagawa-u.ac.jp (H. Tokumitsu).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.05.102

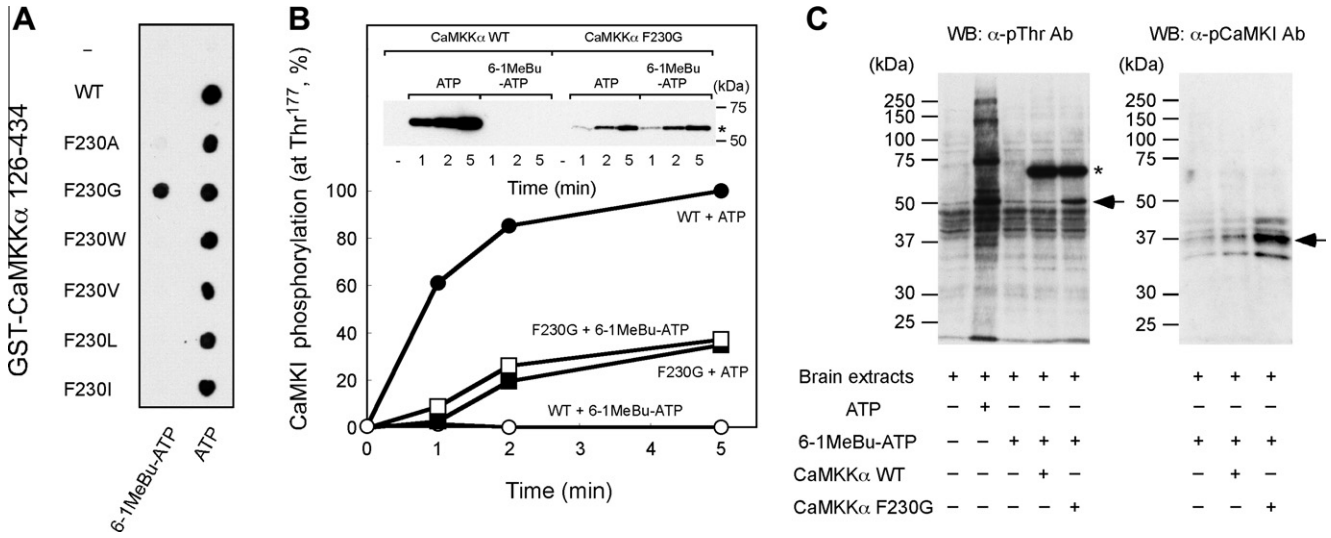

Fig. 1. *In vitro* phosphorylation of CaMKK substrates in rat brain by CaMKKα mutant (Phe^{230}Gly) with N^6-(1-methylbutyl)-ATP. *A*, GST-CaMKIα (1–293, Lys^{49}Glu) was incubated without (–) or with 20 ng of GST-CaMKKα (126–434, WT) or its various point mutants (Phe^{230}Ala; F230A, Phe^{230}Gly; F230G, Phe^{230}Trp; F230 W, Phe^{230}Val; F230V, Phe^{230}Leu; F230L, Phe^{230}Ile; F230I) at 30 °C for 15 min in the presence of 1 mM EGTA with either 100 μM ATP or 6-1MeBu-ATP, followed by dot blotting using anti-phosphoCaMKI antibody. *B*, recombinant CaMKKα wild-type (WT) (5 ng, *circles*) or CaMKKα Phe^{230}Gly (F230G) (5 ng, *squares*) was incubated with GST-CaMKIα (1–293, Lys^{49}Glu) at 30 °C for the indicated time periods with 1 mM $CaCl_2$/7 μM CaM, in the presence of either 100 μM ATP (*closed symbols*) or 6-1MeBu-ATP (*open symbols*). After termination of the reaction, samples were subjected to Western blot analysis (*insert*) with anti-phosphoCaMKI antibody, followed by quantification of the immunoreactive signals by densitometric scanning. Phosphate incorporation into GST-CaMKIα (1–293, Lys^{49}Glu indicated by an asterisk) is expressed as a percentage of the value of the reaction for the 5-min time point in the presence of CaMKKα wild-type and ATP. *C*, Rat brain extract (180 μg) was incubated in the absence (–) or presence (+) of 100 μM ATP or 6-1MeBu-ATP at 30 °C for 30 min in the presence of 2 mM $CaCl_2$/3 μM CaM, 0.5 μM okadaic acid and 1 μM microcystin LR without (–), or with (+) either CaMKKα wild-type (0.3 μM) or CaMKKα Phe^{230}Gly (0.3 μM) followed by Western blot analysis with either anti-phosphoThr antibody (*left panel*) or anti-phosphoCaMKI antibody (*right panel*). The asterisk indicates autophosphorylated CaMKKα. Arrows indicate the 50 kDa CaMKK substrate (*left panel*) and phosphorylated CaMKI (*right panel*), respectively.

(1–470)), including their kinase dead mutants (pME-CaMKKα (1–434, Asp^{293}Ala) and pME-CaMKKβ (1–470, Asp^{329}Ala)), were constructed by introduction of PCR fragments into a pME18s vector. Anti-phosphoCaMKI (at Thr^{177}) antibody was generated as described previously [18]. Anti-phosphoThr and anti-GST antibodies were obtained from Cell Signaling Technology, Inc and GE Healthcare UK Ltd., respectively. N^6-(1-methylbutyl)-ATP (6-1MeBu-ATP) was purchased from the BIOLOG Life Science Institute.

2.2. Partial purification of rat Syndapin I

Three rat brains were homogenized with 30 mL of homogenization buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 10 μg/mL trypsin inhibitor) and then centrifuged at 30,190 *g* at 4 °C for 30 min. Rat brain extract was mixed with two volumes of the homogenization buffer without NaCl and was then subjected to Q Sepharose column chromatography (1 mL gel volume). Step-wise elution was carried out using various concentrations of NaCl (100–800 mM). The eluted fraction containing the 50 kDa CaMKK substrate (the 300 mM NaCl step) was concentrated and then subjected to Superose 6 gel filtration (HR10/30) using a buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 2 mM EGTA and 2 mM EDTA. The eluted fraction containing the 50 kDa CaMKK substrate was applied onto a MonoQ column (HR5/10) equipped with an FPLC system. Elution was carried out using a linear gradient of NaCl (150–600 mM).

2.3. In vitro assay of CaMKK activity

Recombinant CaMKKs including various mutants, were incubated with GST-CaMKIα (1–293, Lys^{49}Glu, 10 μg) at 30 °C for various time periods in a solution containing 50 mM HEPES, pH 7.5, 10 mM $Mg(Ac)_2$, 1 mM DTT, 1 mM EGTA or 1 mM $CaCl_2$ and 7 μM CaM in the presence of either 100 μM ATP or 100 μM 6-1MeBu-ATP. The reaction was terminated either by spotting aliquots (2 μL) onto a nitrocellulose membrane or by addition of an equal volume of 2X SDS–PAGE sample buffer followed by Western blot analysis. Phosphate incorporation into GST-CaMKIα (1–293, Lys^{49}Glu) was visualized on the dot blotted or Western blotted membrane by immunodetection using an anti-phosphoCaMKI antibody.

2.4. Mass spectrometry analysis

A 30-μL sample of the eluate from the MonoQ column (fraction 22) was separated by SDS–7.5% PAGE and lightly stained with Coomassie Brilliant Blue. Next, two gel slices were excised in the region of the 50 kDa band, which were in-gel digested with 10 μg/mL trypsin (Promega) overnight at 37 °C [19]. The digested peptides were eluted with 0.1% formic acid and were subjected to LC-MS/MS analysis as described previously [9], except for using an LCMS-IT-TOF (Shimadzu) interfaced with a nano reverse-phase liquid chromatography system (Shimadzu).

LC-MS/MS was used to identify the phosphorylation site of Syndapin I by CaMKKs. Phosphorylated Syndapin I (10 μg) was separated by SDS–7.5% PAGE followed by in-gel digestion with protease (Roche Diagnostics) cocktail such as trypsin, chymotrypsin, trypsin plus chymotrypsin, trypsin plus Glu-C, trypsin plus Asp-N, chymotrypsin plus Asp-N. Protease concentrations were used at 10 μg/mL trypsin, 17 μg/mL chymotrypsin, 10 μg/mL Glu-C, and 4 μg/mL Asp-N, respectively. The following steps were then performed as described above.

2.5. cDNA cloning, expression and purification of rat Syndapin I

Rat Syndapin I cDNA (AF104402) was obtained by RT-PCR with PrimeSTAR HS DNA polymerase (Takara) using rat brain cDNA

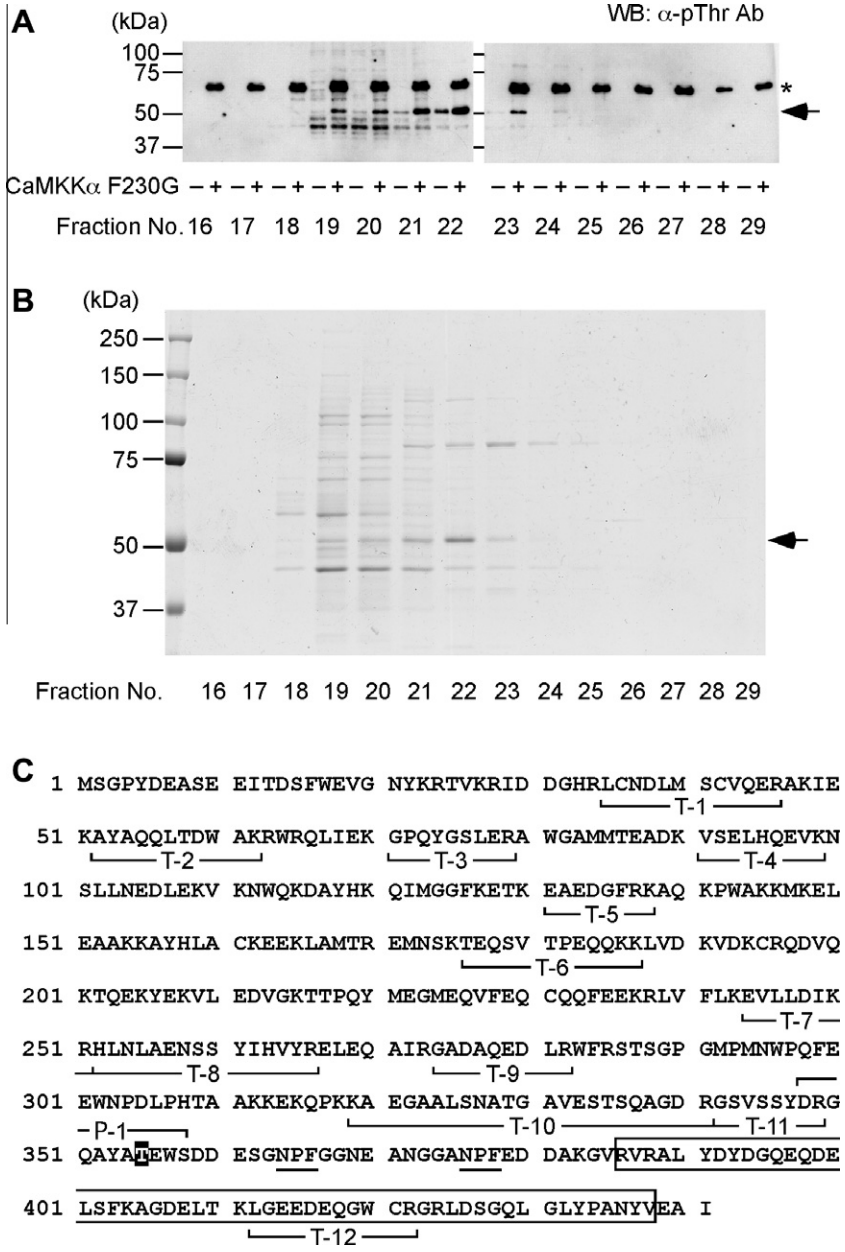

Fig. 2. Purification and identification of the 50 kDa CaMKK substrate from rat brain extract. Rat brain extract was subjected to Q Sepharose column chromatography followed by Superose 6 gel filtration and was then subjected to MonoQ column chromatography. Fractions 16–29 eluted from the MonoQ column were incubated in the presence of 100 μM 6-1MeBu-ATP at 30 °C for 30 min in a solution containing 2 mM $CaCl_2$/3 μM CaM, 0.5 μM okadaic acid, 1 μM microcystin LR without (–) or with (+) CaMKKα $Phe^{230}Gly$ (0.3 μM), followed by Western blot analysis with anti-phosphoThr antibody (*panel A*). Fractions 16–29 eluted from MonoQ column were also subjected to SDS–PAGE and stained with Coomassie Brilliant Blue (*panel B*). The asterisk in *panel A* indicates autophosphorylated CaMKKα $Phe^{230}Gly$, and the arrow in *panel A* and *B* indicates the 50 kDa CaMKK substrate. *C*, The amino acid sequences of the peptides (T-1∼T-12, see "Supplemental Data") obtained by LC-MS/MS analysis of the 50 kDa protein in fraction 22 as shown in *panel B* were aligned with those of rat Syndapin I deduced from its cDNA (AF104402). The phosphorylation site of rat Syndapin I (Thr^{355}) in the phosphopeptide (residues 348–358, P-1) determined in this study is indicated by a black box solid bar. NPF motifs are underlined and the SH3 domain is boxed.

(QUICK-Clone; Clontech Laboratories, Inc.) as a template. The PCR fragment was subcloned into the pGEX-KG-PreS vector to insert the PreScission protease cleavage site (Leu-Glu-Val-Leu-Phe-Gln/Gly-Pro) between GST and Syndapin I. The vector was introduced into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene), and expression of the recombinant protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Then GST-Syndapin I was purified by glutathione-Sepharose (GE Healthcare UK Ltd.). To obtain recombinant Syndapin I, purified GST-Syndapin I was digested with PreScission protease followed by removal of cleaved GST and the protease by glutathione-Sepharose chromatography. An expression plasmid for HA-Syndapin I (pME-HA-Syndapin I) was constructed by inserting the PCR fragment encoding Ser^{2} to Ile^{441} of rat Syndapin I into the pME18s-HA vector. The $Thr^{355}Ala$ mutation was introduced by PCR.

2.6. In vitro phosphorylation of Syndapin I by CaMKK isoforms

Either recombinant Syndapin I (10 μg) or GST-Syndapin I (10 μg) was incubated without or with recombinant CaMKKα or β isoform (30 ng) at 30 °C for the indicated time periods in a solution containing 50 mM HEPES (pH 7.5), 10 mM $Mg(Ac)_2$, 1 mM DTT and 1 mM ATP in the presence of 2 mM $CaCl_2$/10 μM CaM. After

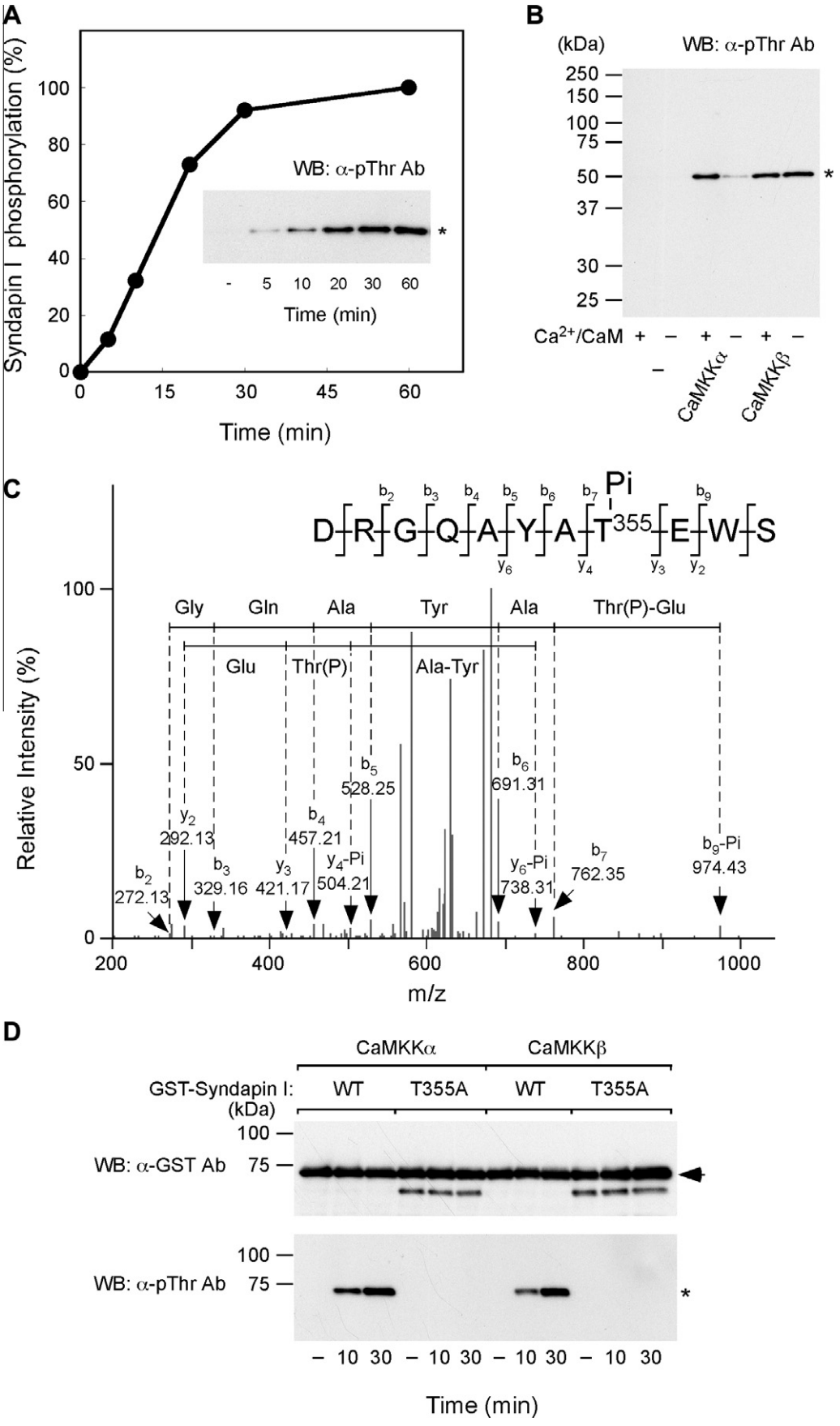
A
Syndapin I phosphorylation (%)
WB: α-pThr Ab
- 5 10 20 30 60
Time (min)
Time (min)
B
(kDa)
WB: α-pThr Ab
250
150
100
75
50
37
30
25
Ca2+/CaM + – + – + –
– CaMKKα CaMKKβ
C
D[R][G][Q][A][Y][A][T355]Pi[E][W][S]
Relative Intensity (%)
Gly Gln Ala Tyr Ala Thr(P)-Glu
Glu Thr(P) Ala-Tyr
b2 272.13
y2 292.13
b3 329.16
y3 421.17
b4 457.21
y4-Pi 504.21
b5 528.25
b6 691.31
y6-Pi 738.31
b7 762.35
b9-Pi 974.43
m/z
D
CaMKKα CaMKKβ
GST-Syndapin I: WT T355A WT T355A
(kDa)
WB: α-GST Ab
WB: α-pThr Ab
– 10 30 – 10 30 – 10 30 – 10 30
Time (min)

Fig. 3. Phosphorylation of recombinant Syndapin I at Thr^{355} by CaMKK isoforms *in vitro*. *A*, CaMKKα wild-type was incubated with recombinant Syndapin I at 30 °C for the indicated time periods with 2 mM $CaCl_2$/10 μM CaM and 1 mM ATP. After termination of the reaction, samples were subjected to Western blot analysis (*insert*) with anti-phosphoThr antibody, followed by quantification of the immunoreactive signals by densitometric scanning. Phosphate incorporation into Syndapin I is expressed as a percentage of the value of the reaction at the 60-min time point. *B*, either CaMKKα or β isoform was incubated with Syndapin I and 1 mM ATP at 30 °C for 30 min in the presence (+) or absence (−) of 2 mM $CaCl_2$/10 μM CaM, followed by Western blot analysis with anti-phosphoThr antibody. The asterisk in *panel A and B* indicates phosphorylated Syndapin I. *C*, identification of Thr^{355} in rat Syndapin I as a site phosphorylated by CaMKKα. Syndapin I, which was phosphorylated by CaMKKα for 60 min, was subjected to in-gel digestion, followed by LC-MS/MS analysis. The singly charged ion of a peptide (residues 348–358) derived from phosphorylated Syndapin I was subjected to MS/MS analysis as described under Section 2. The observed b-ion and y-ion fragment series generated by collision-induced dissociation are indicated. The observed fragment ions are indicated above and below the peptide sequence. *D*, either the wild-type (WT) or the Thr^{355}Ala mutant (T355A) of GST-Syndapin I was incubated without (−) or with CaMKKα or CaMKKβ at 30 °C for 10 and 30 min in the presence of 2 mM $CaCl_2$/10 μM CaM and 1 mM ATP, followed by Western blot analysis with anti-phosphoThr antibody (*lower panel*). After stripping of the anti-phosphoThr antibody, the blotting membrane was reprobed with anti-GST antibody (*upper panel*). The arrow and asterisk indicate GST-Syndapin I and phosphorylated GST-Syndapin I, respectively.

termination of the reaction, the samples (100 ng protein) were subjected to Western blot analysis.

2.7. Immunoprecipitation of HA-Syndapin I

HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS. Transfection of pME-HA-Syndapin I wild-type or Thr^{355}Ala (5 μg) into HeLa cells (10 cm dishes) was carried out using Lipofectamine reagent (Invitrogen) with or without 1 μg of pME-CaMKKα (1–434), pME-CaMKKα (1–434, Asp^{293}Ala), pME-CaMKKβ (1–470) or pME-CaMKKβ (1–470, Asp^{329}Ala). After 48 h of culture, cells were lysed as described previously [9], followed by immunoprecipitation with 5 μg of anti-HA antibody (Roche Applied Science). Thirty μL of SDS–PAGE sample buffer was added to the immunoprecipitates, followed by Western blot analysis using 10 μL of each sample.

3. Results and discussion

3.1. Engineered CaMKKα is capable of utilizing an ATP analogue as a phosphate donor

We aimed to identify novel CaMKK targets by using a method developed by Shokat and coworkers, which has been used for identification of substrates of a number of other kinases including Src [20], JNK [21] and ERK2 [22]. This method involves mutating the ATP-binding site of a protein kinase, which allows the kinase to utilize ATP analogues as phosphate donors. We first constructed a CaMKK mutant that was capable of utilizing an ATP analogue that has bulky side groups at the N-6 position, N^6-(1-methylbutyl)-ATP (6-1MeBu-ATP). Based on comparison of the amino acid sequence of the CaMKKα catalytic domain with that of other protein kinases, the Phe^{230} was identified as one of the conserved residues that come into close contact with the N-6 position of ATP. We therefore replaced this Phe^{230} in a GST-fused CaMKKα catalytic domain (residues 126–434) with one of various residues and purified these recombinant enzymes to test their ability to utilize 6-1MeBu-ATP, as well as ATP, as a phosphate donor (Fig. 1A). For this purpose, we used a dot blot assay in which a kinase-dead CaMKIα mutant, GST-CaMKIα (1–293 Lys^{49}Glu), was used as a substrate [16]. Site-specific phosphorylation of the substrate was detected by anti-phosphoCaMKI (at Thr^{177}) antibody. The dot blot assay revealed that the most of the CaMKKα mutants including the wild-type enzyme could utilize ATP but not 6-1MeBu-ATP as a phosphate donor (Fig. 1A). Only the Phe^{230}Gly mutant could utilize 6-1MeBu-ATP. This result is consistent with previous studies that mutation of one or two conserved amino acids at the ATP binding site to smaller amino acid residues allows the mutant kinase to utilize ATP analogues containing a bulky substituent at the N-6 position. Then we compared the kinase activity of recombinant full-length wild-type CaMKKα with that of the full-length Phe^{230}Gly mutant. A time course analysis of the substrate phosphorylation confirmed that the CaMKKα Phe^{230}Gly is capable of using both ATP and 6-1MeBu-ATP as a phosphate donor with a similar efficiency and wild-type enzyme is incapable of utilizing the ATP analogue (Fig. 1B), which is consistent with the result of Fig. 1A. The result showed that the activity of the mutant CaMKKα is ~6 times lower than that of the wild-type enzyme.

3.2. Phosphorylation of rat brain extracts using a CaMKKα mutant and an ATP analogue

In order to identify novel CaMKK targets, we next incubated rat brain extracts with or without purified wild-type CaMKKα or its Phe^{230}Gly mutant in the absence or presence of either ATP or 6-1MeBu-ATP, followed by detection of Thr phosphorylation of brain proteins by Western blotting with anti-phosphoThr antibody (Fig. 1C, *left panel*). The number of proteins phosphorylated by endogenous protein kinases in the rat brain extract was greatly increased when the extract was incubated with ATP. However, neither the activity of endogenous kinases nor that of added recombinant wild-type CaMKKα was significantly enhanced by incubation with 6-1MeBu-ATP, as judged by Western blotting of phosphorylated proteins. This result suggested that endogenous brain kinases as well as wild-type CaMKKα were apparently incapable of using 6-1MeBu-ATP as a phosphate donor. In contrast, when the rat brain extract was incubated with the Phe^{230}Gly mutant of CaMKKα in the presence of 6-1MeBu-ATP, Thr phosphorylation of a 50 kDa protein was significantly induced. Phosphorylation of this protein was not observed with wild-type CaMKKα that is incapable of using the ATP analogue. In addition, significant phosphorylation at the activation Thr (Thr^{177}) of the endogenous CaMKI was only induced when the rat brain extract was incubated with mutant, but not with wild-type, CaMKKα together with the ATP analogue (Fig. 1C, *right panel*). These results clearly indicated that the observed 50 kDa protein was a direct substrate of CaMKKα.

3.3. Purification and identification of the 50 kDa CaMKKα substrate as Syndapin I

To identify the 50 kDa protein that was phosphorylated by the CaMKKα mutant in the presence of the ATP analogue as shown in Fig. 1C, we performed serial column chromatography of rat brain extract (see Section 2). The 50 kDa protein was detected by incubation of the fractions eluted from each column with 6-1MeBu-ATP in either the absence or the presence of the CaMKKα Phe^{230}Gly followed by Western blotting with the anti-phosphoThr antibody. Using this assay, fractions 19–23 eluted from the MonoQ column were shown to contain a 50 kDa protein that was phosphorylated by the CaMKKα mutant, with the highest amount of this protein being detected in fraction 22 (Fig. 2A). This result was in good agreement with the elution profile of a 50 kDa protein that was detected using Coomassie blue staining (indicated by an *arrow* in Fig. 2B), indicating that the 50 kDa CaMKK substrate was partially purified. We then subjected fraction 22 to LC-MS/MS analysis to identify the 50 kDa CaMKK substrate. Based on the LC-MS/MS analysis of

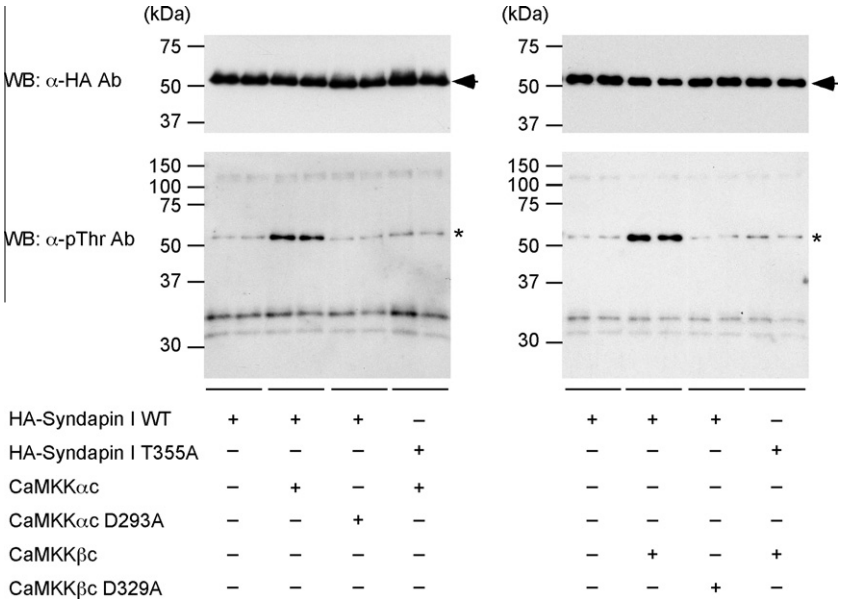

Fig. 4. Phosphorylation of Syndapin I by constitutively active mutants of CaMKK isoforms in transfected HeLa cells. HeLa cells were transfected with an expression vector encoding either HA-Syndapin I wild-type (WT) or Thr^{355}Ala mutant (T355A) without (–) or with (+) CaMKKα 1–434 (CaMKKαc); CaMKKα 1–434, Asp^{293}Ala (CaMKKαc D293A); CaMKKβ 1–470 (CaMKKβc) or pME-CaMKKβ 1–470, Asp^{329}Ala (CaMKKβc D329A) expression vectors. After 48 h of culture, duplicate cell lysates were immunoprecipitated with anti-HA antibody followed by Western blot analysis using anti-phosphoThr antibody (*lower panels*). After stripping of the anti-phosphoThr antibody, the blotting membranes were reprobed with anti-HA-antibody (*upper panels*). The arrows and the asterisks indicate immunoprecipitated HA-Syndapin I and phosphorylated HA-Syndapin I, respectively.

the 12 peptides (T-1 to T-12) generated from the 50 kDa protein band, the sequence of all of the peptides corresponded to sequences of rat Syndapin I (Fig. 2C). This result identified the 50 kDa CaMKK substrate as Syndapin I.

3.4. Phosphorylation of rat Syndapin I by CaMKK isoforms at Thr^{355} in vitro

We next confirmed that CaMKKα was capable of phosphorylating recombinant Syndapin I in the presence of Ca^{2+}/CaM *in vitro* (Fig. 3A). Whereas CaMKKα phosphorylated Syndapin I in a Ca^{2+}/CaM-dependent manner, the β isoform was shown to phosphorylate Syndapin I both in the presence and absence of Ca^{2+}/CaM (Fig. 3B), which was in good agreement with the significant autonomous activity of CaMKKβ [4,17]. To determine the site(s) of Syndapin I that are phosphorylated by CaMKKs, we analyzed phosphorylated Syndapin I with CaMKKα by LC-MS/MS analysis as described under Section 2. We detected a phosphopeptide corresponding to residues 348–358 of rat Syndapin I (Fig. 2C, P-1). LC-MS/MS analysis identified a single phosphorylation site at Thr^{355} in the peptide (Fig. 3C). Phosphorylation of Thr^{355} was also detected following analysis of CaMKKβ-phosphorylated Syndapin I (data not shown). The presence of only one phosphorylation site was confirmed by the fact that substitution of Thr^{355} by Ala completely impaired GST-Syndapin I phosphorylation by CaMKK isoforms (Fig. 3D).

3.5. Phosphorylation of rat Syndapin I at Thr^{355} by CaMKK isoforms in transfected cells

Finally, in order to test whether CaMKK was capable of phosphorylating Syndapin I in cultured cells (Fig. 4), we transfected HA-tagged-Syndapin I into unstimulated HeLa cells that were co-transfected with or without constitutively active CaMKKs lacking autoinhibitory and Ca^{2+}/CaM-binding domains [16]. Immunoprecipitated HA-Syndapin I was weakly phosphorylated at Thr residue(s) in cells without co-transfected CaMKKs. Thr phosphorylation of a HA-Syndapin I was significantly increased by co-expression of either constitutively active CaMKKα 1–434 (Fig. 4, *left panel*) or constitutively active CaMKKβ 1–470 (Fig. 4, *right panel*) but not by their kinase-dead mutants (CaMKKα 1–434, Asp^{293}Ala and CaMKKβ 1–470, Asp^{329}Ala). We also confirmed that the co-transfected active CaMKKs could not induce the Thr phosphorylation of a HA-Syndapin I Thr^{355}Ala mutant, indicating the phosphorylation of Syndapin I at Thr^{355} by CaMKK isoforms in cultured cells.

In conclusion, we identified Syndapin I as the first non-kinase substrate for CaMKK by using an ATP analogue (6-1MeBu-ATP) and a genetically engineered CaMKKα (Phe^{230}Gly) that is capable of utilizing the ATP analogue as a phosphate donor. Syndapin has been demonstrated to be involved in synaptic vesicle endocytosis through the interaction of its SH3 domain with proline-rich domains of Dynamin I, Synapsin, Synaptojanin and N-WASP (neural Wiskott–Aldrich syndrome protein) [23–25]. In addition, Syndapin I has two NPF motifs (residues 364–366 and 376–378) to which the EH (Eps15 homology) protein domain binds [26]. These NPF motifs are in close proximity to the CaMKK phosphorylation site (Thr^{355}). It will be of interest to examine whether CaMKK-induced Thr^{355} phosphorylation of Syndapin affects the association of Syndapin I with EHD proteins. Therefore, determination of the potential role of CaMKK in Syndapin I-mediated synaptic transmission remains an interesting avenue for further investigation. Finally, the present study also suggests the possibility that CaMKK mediates novel Ca^{2+}-signaling pathway(s), which are independent of downstream protein kinases.

Acknowledgments

We thank Momoko Nitta (Kagawa University, Kagawa, Japan) for excellent technical assistance. This work was supported in part by Grants-in-aid for Scientific Research to H.T. (21570143) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.102.

References

[1] T.R. Soderling, The Ca-calmodulin-dependent protein kinase cascade, Trends Biochem. Sci. 24 (1999) 232–236.

[2] A.R. Means, The year in basic science: calmodulin kinase cascades, Mol. Endocrinol. 22 (2008) 2759–2765.

[3] H. Tokumitsu, H. Enslen, T.R. Soderling, Characterization of a Ca^{2+}/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase, J. Biol. Chem. 270 (1995) 19320–19324.

[4] K.A. Anderson, R.L. Means, Q.H. Huang, B.E. Kemp, E.G. Goldstein, M.A. Selbert, A.M. Edelman, R.T. Fremeau, A.R. Means, Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca^{2+}/calmodulin-dependent protein kinase kinase beta, J. Biol. Chem. 273 (1998) 31880–31889.

[5] S. Yano, H. Tokumitsu, T.R. Soderling, Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway, Nature 396 (1998) 584–587.

[6] S.A. Hawley, M.A. Selbert, E.G. Goldstein, A.M. Edelman, D. Carling, D.G. Hardie, 5′-AMP activates the AMP-activated protein kinase cascade and Ca^{2+}/calmodulin activates the calmodulin-dependent protein kinase I cascade via three independent mechanisms, J. Biol. Chem. 270 (1995) 27186–27191.

[7] A. Woods, K. Dickerson, R. Heath, S.P. Hong, M. Momcilovic, S.R. Johnstone, M. Carlson, D. Carling, Ca^{2+}/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells, Cell Metab. 2 (2005) 21–33.

[8] R.L. Hurley, K.A. Anderson, J.M. Franzone, B.E. Kemp, A.R. Means, L.A. Witters, The Ca^{2+}/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases, J. Biol. Chem. 280 (2005) 29060–29066.

[9] T. Fujimoto, S. Yurimoto, N. Hatano, N. Nozaki, N. Sueyoshi, I. Kameshita, A. Mizutani, K. Mikoshiba, R. Kobayashi, H. Tokumitsu, Activation of SAD kinase by Ca^{2+}/calmodulin-dependent protein kinase kinase, Biochemistry 47 (2008) 4151–4159.

[10] M. Sheng, M.A. Thompson, M.E. Greenberg, CREB: a Ca^{2+}-regulated transcriptional factor phosphorylated by calmodulin-dependent protein kinases, Science 252 (1991) 1427–1430.

[11] T. Saneyoshi, G. Wayman, D. Fortin, M. Davare, N. Hoshi, N. Nozaki, T. Natsume, T.R. Soderling, Activity-dependent synaptogenesis: regulation by a CaM-kinase kinase/CaM-kinase I/betaPIX signaling complex, Neuron 57 (2008) 94–107.

[12] G.A. Wayman, S. Impey, D. Marks, T. Saneyoshi, W.F. Grant, V. Derkach, T.R. Soderling, Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2, Neuron 50 (2006) 897–909.

[13] N. Ageta-Ishihara, S. Takemoto-Kimura, M. Nonaka, A. Adachi-Morishima, K. Suzuki, S. Kamijo, H. Fujii, T. Mano, F. Blaeser, T.A. Chatila, H. Mizuno, T. Hirano, Y. Tagawa, H. Okuno, H. Bito, Control of cortical axon elongation by a GABA-driven Ca^{2+}/calmodulin-dependent protein kinase cascade, J. Neurosci. 29 (2009) 13720–13729.

[14] P. Tamas, S.A. Hawley, R.G. Clarke, K.J. Mustard, K. Green, D.G. Hardie, D.A. Cantrell, Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and Ca^{2+} in T lymphocytes, J. Exp. Med. 203 (2006) 1665–1670.

[15] S.A. Hawley, D.A. Pan, K.J. Mustard, L. Ross, J. Bain, A.M. Edelman, B.G. Frenguelli, D.G. Hardie, Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase, Cell Metab. 2 (2005) 9–19.

[16] H. Tokumitsu, M. Muramatsu, M. Ikura, R. Kobayashi, Regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase, J. Biol. Chem. 275 (2000) 20090–20095.

[17] H. Tokumitsu, M. Iwabu, Y. Ishikawa, R. Kobayashi, Differential regulatory mechanism of Ca^{2+}/calmodulin-dependent protein kinase kinase isoforms, Biochemistry 40 (2001) 13925–13932.

[18] H. Tokumitsu, N. Hatano, H. Inuzuka, S. Yokokura, N. Nozaki, R. Kobayashi, Mechanism of the generation of autonomous activity of Ca^{2+}/calmodulin-dependent protein kinase IV, J. Biol. Chem. 279 (2004) 40296–40302.

[19] N. Hatano, T. Hamada, Proteome analysis of pitcher fluid of the carnivorous plant *Nepenthes alata*, J. Proteome Res. 7 (2008) 809–816.

[20] K. Shah, Y. Liu, C. Deirmengian, K.M. Shokat, Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates, Proc. Natl. Acad. Sci. USA 94 (1997) 3565–3570.

[21] H. Habelhah, K. Shah, L. Huang, A.L. Burlingame, K.M. Shokat, Z. Ronai, Identification of new JNK substrate using ATP pocket mutant JNK and a corresponding ATP analogue, J. Biol. Chem. 276 (2001) 18090–18095.

[22] S.T. Eblen, N.V. Kumar, K. Shah, M.J. Henderson, C.K. Watts, K.M. Shokat, M.J. Weber, Identification of novel ERK2 substrates through use of an engineered kinase and ATP analogs, J. Biol. Chem. 278 (2003) 14926–14935.

[23] V. Anggono, K.J. Smillie, M.E. Graham, V.A. Valova, M.A. Cousin, P.J. Robinson, Syndapin I is the phosphorylation-regulated dynamin I partner in synaptic vesicle endocytosis, Nat. Neurosci. 9 (2006) 752–760.

[24] J. Modregger, B. Ritter, B. Witter, M. Paulsson, M. Plomann, All three PACSIN isoforms bind to endocytic proteins and inhibit endocytosis, J. Cell Sci. 113 (Pt. 24) (2000) 4511–4521.

[25] B. Qualmann, R.B. Kelly, Syndapin isoforms participate in receptor-mediated endocytosis and actin organization, J. Cell Biol. 148 (2000) 1047–1062.

[26] A. Braun, R. Pinyol, R. Dahlhaus, D. Koch, P. Fonarev, B.D. Grant, M.M. Kessels, B. Qualmann, EHD proteins associate with syndapin I and II and such interactions play a crucial role in endosomal recycling, Mol. Biol. Cell 16 (2005) 3642–3658.