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# Identification and characterization of novel components of a $Ca^{2+}$/calmodulin-dependent protein kinase cascade in HeLa cells

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**Abstract In this report, we cloned a novel calmodulin-kinase (CaM-KIδ) from HeLa cells and characterized its activation mechanism. CaM-KIδ exhibits $Ca^{2+}$/CaM-dependent activity that is enhanced (~30-fold) in vitro by phosphorylation of its Thr180 by CaM-K kinase (CaM-KK)α, consistent with detection of CaM-KIδ-activating activity in HeLa cells. We also identified a novel CaM-KKβ isoform (CaM-KKβ-3) in HeLa cells whose activity was highly $Ca^{2+}$/CaM-independent. Transiently expressed CaM-KIδ exhibited enhanced protein kinase activity in HeLa cells without ionomycin stimulation. This sustained activation of CaM-KIδ was completely abolished by Thr180Ala mutation and inhibited by CaM-KK inhibitor, STO-609, indicating a functional CaM-KK/CaM-KIδ cascade in HeLa cells.**


*Key words:* $Ca^{2+}$; CaM-kinase cascade; CaM-kinaseδ; CaM-K kinase

## 1. Introduction

$Ca^{2+}$/calmodulin-dependent protein kinases (CaM-Ks) are implicated in the regulation of wide variety of biological events that are mediated by intracellular $Ca^{2+}$ including muscle contraction, neurotransmitter release, and gene expression. Recent studies have demonstrated that two multifunctional CaM-Ks, CaM-KI and IV are themselves activated by the phosphorylation of a Thr residue in their activation loop by an upstream CaM-K kinase (CaM-KK). This constitutes a novel $Ca^{2+}$-signal transduction pathway, CaM-K cascade (reviewed in [1,2]). Two CaM-KK genes, CaM-KKα and CaM-KKβ, have been cloned in mammals and homologues have been found in *Caenorhabditis elegans* and *Aspergillus nidulans* [3–8].

It has been shown that CaM-KI is activated in response to elevation of intracellular $Ca^{2+}$ mediated by membrane depolarization in PC12 cells through phosphorylation by CaM-KK [9]. We have also demonstrated that the CaM-KK mediated activation of CaM-KI in *C. elegans* results in induction of CREB-dependent transcription in transfected cells and in living nematode [10,11]. Recently, Suizu et al. reported that the activation of CaM-KK/CaM-KI cascade induces myosin II phosphorylation at Ser19, resulting in regulation of actin filament organization in mammalian cells [12]. However, the various physiological function(s) mediated by the mammalian CaM-KK/CaM-KI cascade and the CaM-KK involved in regulating CaM-KI activity in vivo are not well understood.

In further characterizing CaM-KK/CaM-K cascade, we report here the cloning of a novel human CaM-KI isozyme (CaM-KIδ) as well as a novel isoform of CaM-KKβ in HeLa cells. Furthermore, we characterized the activation of CaM-KIδ through phosphorylation by CaM-KK isoforms in vitro and in transfected cells.

## 2. Materials and methods

### 2.1. Cloning of CaM-KIδ and CaM-KKβ-3

The method used to clone of CaM-KIδ has been previously reported [13]. Briefly, a HeLa cDNA library in the λZAPII vector was screened with a 1.0 kbp *Sal*I/*Hin*dIII fragment derived from bovine stomach MLCK (pSL17) under low stringency conditions, and then the positive clone was subcloned into pBluescript SK(−). Cloning of CaM-KKβ-3 was performed by RT-PCR using random hexamer-primed cDNA prepared from HeLa cells as a template and specific primers for human CaM-KKβ-1 (sense primer, 5′-CCAGTGTGCTGGATGAAGCTGGCGCATGC-3′; antisense primer, 5′-CACACGTGCTGGGTTTCCGTAGGACATGCT-3′; nested primer, 5′-CTATGGAAACGCGGTGCAGCAGCCCCCCAC-3′, [15]). The first PCR was carried out using the sense and the antisense primers followed by the second PCR using the nested antisense primer. A 1.8 kbp PCR fragment contained a 1827 bp clone (CaM-KKβ-3) and a 1698 bp clone (CaM-KKβ-3x). The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AB081726 (CaM-KIδ), AB081336 (CaM-KKβ-3) and AB081337 (CaM-KKβ-3x).

### 2.2. Construction and purification of recombinant CaM-KIδ and CaM-KKβ-3

CaM-KIδ cDNA was amplified by PCR using the sense primer, 5′-GCTCTAGAGATGGCCCGGGAGAACGGCGAG-3′ and the antisense primer, 5′-CCGCTCGAGTCAGCTGAGGGATTCTGG-3′ and then ligated into the *Xba*I/*Xho*I sites of the pGEX-KG-PreS vector [14]. The Thr180Ala mutant of CaM-KIδ was created by site-directed mutagenesis using a mutagenic oligonucleotide (5′-AGTTCCACAGGCAGCGGACATCACATCTCC-3′). GST–CaM-KIδs were expressed in the *Escherichia coli* JM-109 by induction with 1 mM IPTG and purified by glutathione–Sepharose column chromatography. The cDNA of CaM-KKβ-3 was introduced into pET-16b (Novagen). Recombinant CaM-KKβ-3 was expressed in the *E. coli* strain BL-21 (DE3) and purified by ProBond™ nickel-chelating resin (Invi-

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*Abbreviations:* CaM-K, $Ca^{2+}$/calmodulin-dependent protein kinase; HA, hemagglutinin

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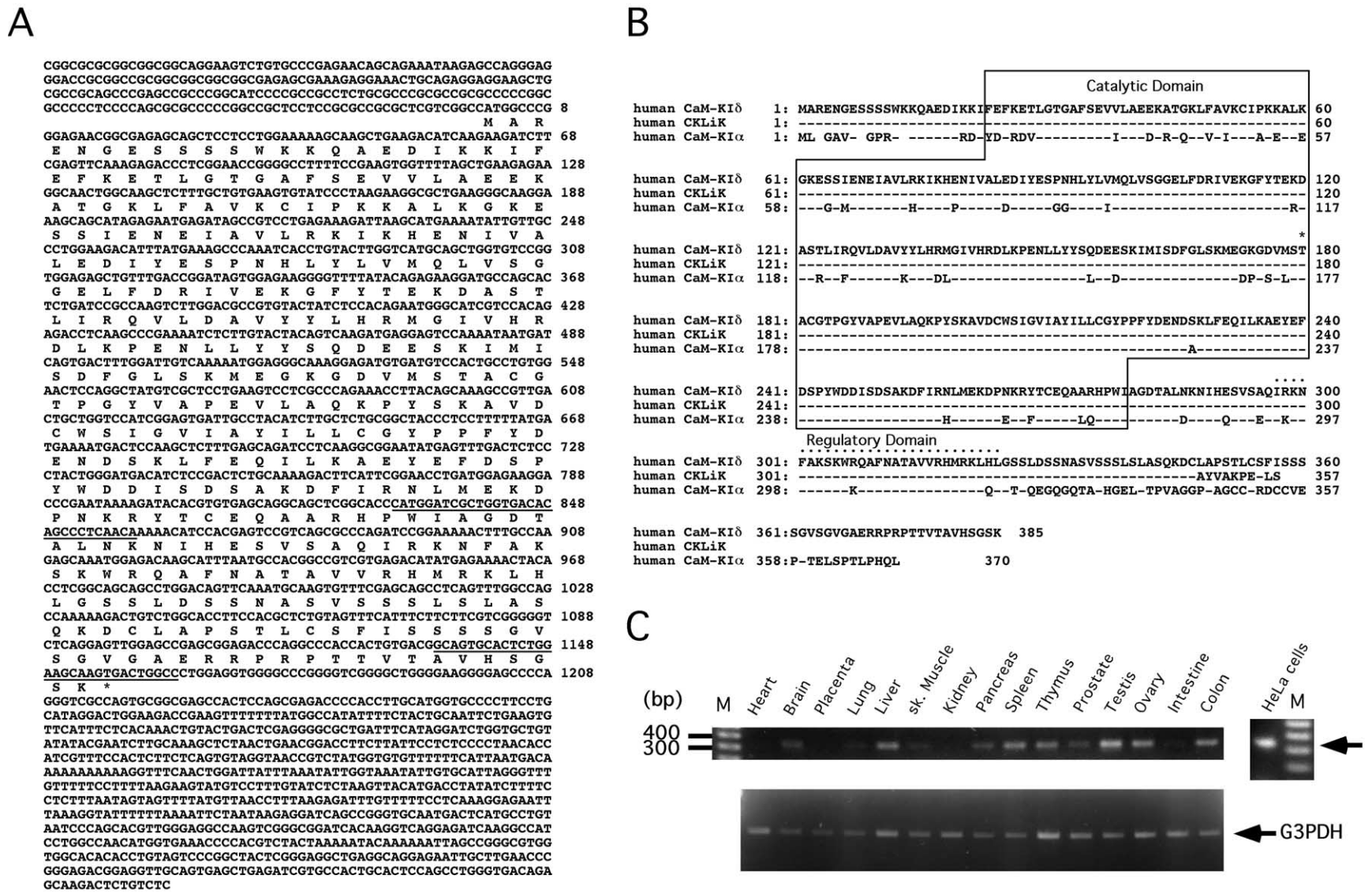


Fig. 1. Cloning and mRNA expression pattern of human CaM-KIδ. A: Nucleotide and deduced amino acid sequence of human CaM-KIδ. B: Comparison of amino acid sequences of human CaM-KIδ, human CKLiK [20] and human CaM-KIα. Residues identical to human CaM-KIδ are indicated with a dash and gaps in the alignment are indicated as a space. An asterisk indicates the Thr180 residue in the activation loop. A catalytic domain is indicated with a box. A putative regulatory domain including autoinhibitory and CaM-binding regions is indicated with a dot. C: RT-PCR analysis for human CaM-KIδ was performed using specific primers (underlined sequences in panel A) with cDNAs from HeLa cells (right panel) and human tissues (left panel). RT-PCR for G3PDH (lower panel) was performed as a control experiment. M, 100 bp DNA ladder marker.

trogen) followed by CaM–Sepharose chromatography according to the manufacturer's protocol.

*2.3. RT-PCR analysis*

RT-PCR analysis of CaM-KIδ was performed with multiple human tissue cDNA panels (Clontech) using 5′-CATGGATCGCTGGTGA-CACAGCCCTCAACA-3′, and 5′-GGCCAGTCACTTGCTTCCA-GAGTGCACTGC-3′ as primers (Fig. 1A). PCR reaction was carried out according to the manufacturer's protocol using TITANIUM™ Taq DNA polymerase (Clontech).

*2.4. Activation and phosphorylation of CaM-KIδ in vitro*

Either purified recombinant CaM-KKα (2.8 μg/ml [14]) or HeLa cell extract (0.5 mg/ml) was incubated with either 1.8 μg GST–CaM-KIδ or the Thr180Ala mutant at 30°C for 10 min in a solution (10 μl) containing 50 mM HEPES (pH 7.5), 10 mM $Mg(Ac)_2$, 1 mM DTT, either 200 μM ATP or 100 μM [$\gamma$-$^{32}$P]ATP in the presence of either 1 mM EGTA or 1 mM $CaCl_2$/10 μM CaM with 1 μM microcystin LR (for treatment with HeLa cell extract). The reaction was initiated by the addition of ATP and terminated by addition of SDS–PAGE sample buffer and then analyzed by SDS–PAGE followed by autoradiography or terminated by dilution with 200 μl of 50 mM HEPES, pH 7.5, 2 mg/ml bovine serum albumin, 10% ethylene glycol, and 1 mM EDTA. Five μl of the diluted sample (43 ng of CaM-KIδ) was then subjected to the protein kinase assay. CaM-KIδ activity was measured at 30°C for 5 min using 40 μM syntide-2 as a substrate and 100 μM [$\gamma$-$^{32}$P]ATP (1000–2000 cpm/pmol) in the presence of either 1 mM EGTA or 1 mM $CaCl_2$/2 μM CaM as previously described [16]. Results represent the mean and standard error of the mean (S.E.M.) of three experiments.

*2.5. Transient expression and immunoprecipitation of hemagglutinin (HA)-CaM-KIδ and HA-CaM-KIV*

Either HA-CaM-KIδ or HA-CaM-KIV (2 μg) was transfected into HeLa and COS-7 cells with or without 1.5 μg CaM-KKβ-3-expressing plasmid DNA and 20 μg LipofectAMINE Reagent (Invitrogen) in 2.5 ml medium as previously described [17]. After 20 h incubation, the cells were further cultured in serum-free medium for 6 h and then treated with or without 1 μM ionomycin for 5 min. Stimulation was terminated by the addition of 1 ml of lysis buffer and then HA-tagged kinases were immunoprecipitated with 4 μg of anti-HA antibody (clone 12CA5, Roche Molecular Biochemicals) followed by measurement of protein kinase activity in the presence of 100 μM [$\gamma$-$^{32}$P]ATP, 1 mM $CaCl_2$/2 μM CaM (or 1 mM EGTA for HA-CaM-KIδ1-296) and 10 μg/ml STO-609 [17] using 40 μM syntide-2 as a substrate at 30°C for 5 min [18]. Results represent the mean and S.E.M. of three independent transfections.

*2.6. CaM-KK activity assay*

Activity of recombinant human CaM-KKβ-3, CaM-KKβ-3x or rat CaM-KKα was measured by using 10 μg GST–CaM-KI1-293, K49E as a substrate and 100 μM [$\gamma$-$^{32}$P]ATP in the presence of either 1 mM EGTA or 1 mM $CaCl_2$/2 μM CaM at 30°C for 5 min [19].

## 3. Results and discussion

A HeLa cDNA library was screened under low stringency conditions with a partial cDNA fragment derived from bovine MLCK that contains both the catalytic and regulatory domains [13]. This allowed us to isolate a 2235 bp clone encoding a stretch of 385 amino acid residues (Fig. 1A) that shares considerable homology with human CaM-KI (80% identity) and that is strikingly similar to a CaM-KI like kinase (CKLiK) that was originally identified as a granulocyte-specific kinase [20] (Fig. 1B). It has been suggested that CKLiK may play a role in transducing chemokine-induced signals regulating human granulocyte functions [20]. Alignment of the deduced amino acid sequences and the nucleotide sequences (data not shown) of both protein kinases reveals that their catalytic domains and their putative regulatory domains, including the CaM-binding site, are completely identical (Fig. 1B). However, their C-terminal tail regions after Cys347 differed. This sequence homology suggests that the CaM-dependent kinase clone identified by our screening is likely a C-terminal alternative spliced isoform of CKLiK. Based on the structural similarity to CaM-KI family, we tentatively termed the isolated clone as CaM-KIδ. RT-PCR analysis reveals that in addition to HeLa cells, CaM-KIδ possesses a widespread tissue distribution but expressed very weak or undetectable levels in the heart, placenta and kidney (Fig. 1C).

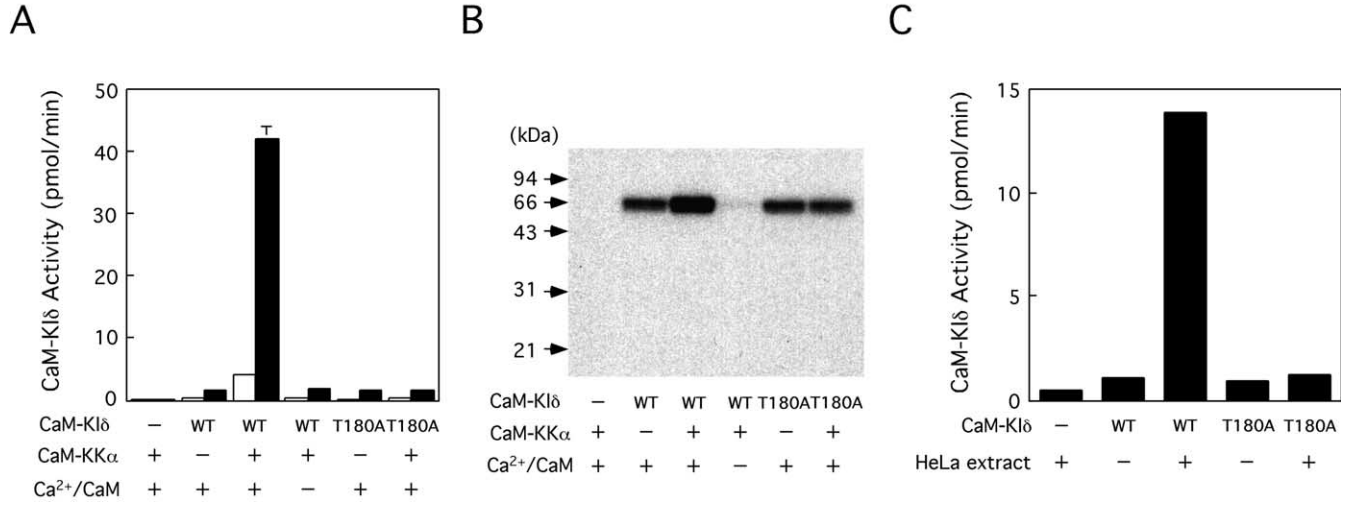


Fig. 2. Activation and phosphorylation of CaM-KIδ in vitro. A: Activation of CaM-KIδ by CaM-KKα. Either GST–CaM-KIδ wild-type (WT) or Thr180Ala mutant (T180A) was incubated with (+) or without (−) purified recombinant CaM-KKα at 30°C for 10 min with 200 μM ATP in the presence of either 1 mM EGTA (−) or 1 mM $CaCl_2$/10 μM CaM (+). After terminating the reaction, GST–CaM-KIδ was subjected to the protein kinase assay as described in Section 2 in the either presence of 1 mM EGTA (open bar) or 1 mM $CaCl_2$/2 μM CaM (closed bar). B: Phosphorylation of either GST–CaM-KIδ wild-type (WT) or Thr180Ala mutant (T180A) by CaM-KKα was analyzed by SDS–15% PAGE followed by autoradiography. C: Activation of CaM-KIδ by HeLa cell extract. Either GST–CaM-KIδ wild-type (WT) or Thr180Ala mutant (T180A) was incubated with (+) or without (−) HeLa cell extract (0.5 mg/ml) and then subjected to the protein kinase assay.

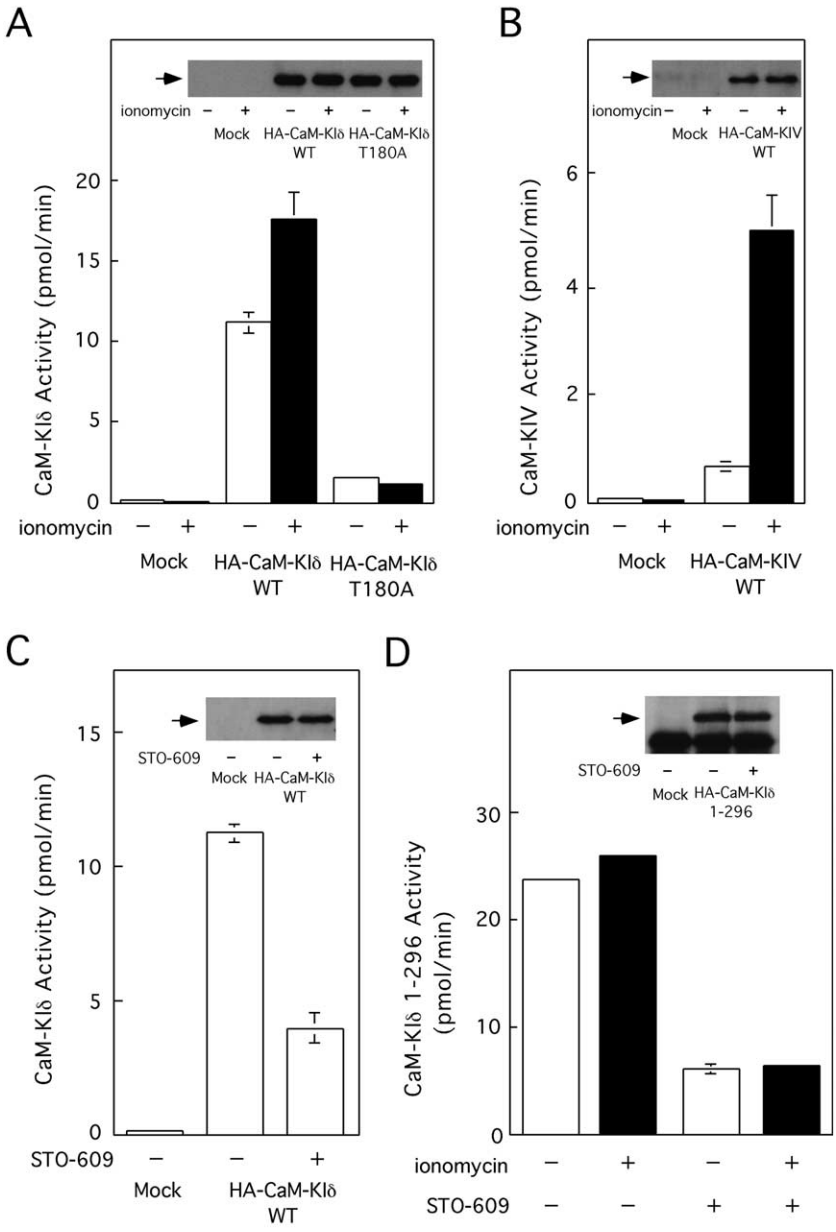


Fig. 3. Activation of CaM-KIδ in transfected HeLa cells. A,B: HeLa cells were transfected with either empty vector (Mock), HA-CaM-KIδ-expressing vector (A) or HA-CaM-KIV-expressing vector (B). After 20 h incubation, the cells were further cultured in serum-free medium for 6 h and then treated with (+) or without (−) 1 μM ionomycin for 5 min and then immunoprecipitated samples were subjected to protein kinase assay in the presence of 1 mM $CaCl_2$/2 μM CaM and 10 μg/ml STO-609 [17] as described in Section 2. Immunoprecipitated samples (1/5 vol.) were subjected to CaM-overlay in the presence of 1 mM $CaCl_2$ (inset). Arrows indicate HA-CaM-KIδ (A) and HA-CaM-KIV (B). C,D: Effect of CaM-KK inhibitor, STO-609 on the activation of CaM-KIδ in HeLa cells. HeLa cells were transfected with either empty vector (Mock), HA-CaM-KIδ-expressing vector (C) or HA-CaM-KIδ 1-296-expressing vector (D) and then cultured for 20 h either with (+) or without (−) 10 μg/ml STO-609 followed by culturing in serum-free medium for 6 h either with (+) or without (−) 10 μg/ml STO-609. After treatment with (+) or without (−) 1 μM ionomycin for 5 min (D) or no treatment (C), the cells were lysed and immunoprecipitated samples were subjected to protein kinase assay as described above. Immunoprecipitated samples (1/5 vol.) were subjected to either CaM-overlay as described above (C inset) or Western blotting analysis using anti-HA antibody (D inset).

This result indicates that CaM-KIδ may play a role in $Ca^{2+}$-mediated regulation of the functions of various cells.

Since the activation loop of CaM-KIδ contains the Thr180 residue involved in kinase activation (Fig. 1B), we assessed whether the kinase activity of CaM-KIδ could be activated by a CaM-KK in vitro by using purified GST-fused CaM-KIδ. In the absence of CaM-KKα, CaM-KIδ exhibited $Ca^{2+}$/CaM-dependent kinase activity (34.5 ± 2.0 nmol/min/mg) (Fig. 2A). This activity was elevated approximately 30-fold (979.2 ± 43.4 nmol/min/mg) by treatment with CaM-KKα in a $Ca^{2+}$/CaM-dependent manner (Fig. 2A), which was associated with Thr180 phosphorylation by CaM-KKα (Fig. 2B). Thus CaM-KKα can phosphorylate Thr180 in CaM-KIδ, resulting in a large induction of its catalytic efficiency. Whereas we observed $Ca^{2+}$/CaM-dependent autophosphorylation of CaM-KIδ, it had no impact on CaM-KIδ activity (Fig. 2A,B).

In agreement with in vitro activation of CaM-KIδ as shown in Fig. 2A, we found that HeLa cell extract can activate CaM-

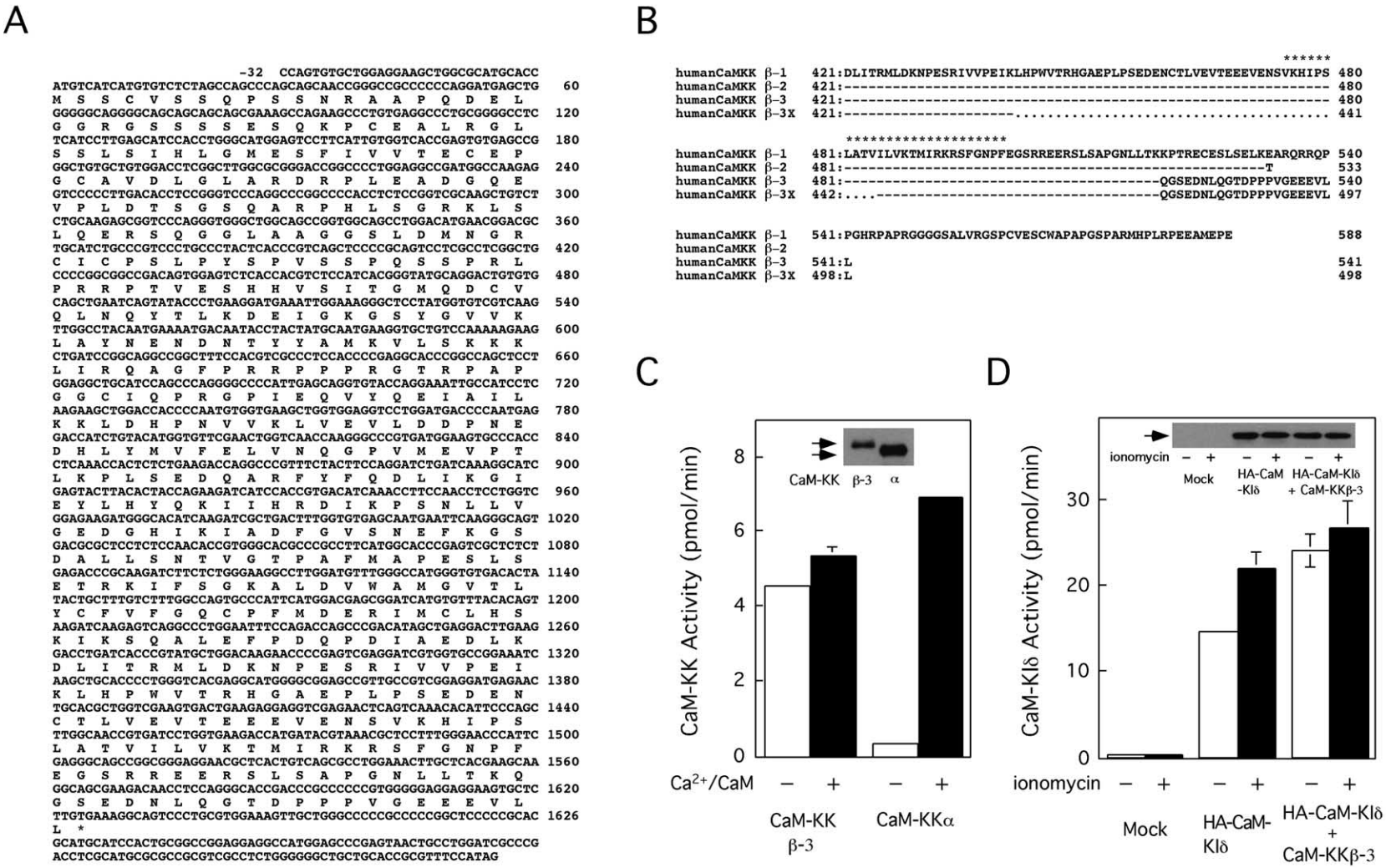


Fig. 4. Cloning and characterization of human CaM-KKβ-3. A: Nucleotide and deduced amino acid sequence of human CaM-KKβ-3. B: Comparison of C-terminal amino acid sequences of human CaM-KKβ-3, CaM-KKβ-3x, CaM-KKβ-1 and CaM-KKβ-2 [15]. Residues identical to the human CaM-KKβ-1 are indicated with a dash and gaps in the alignment are indicated as a dot. Asterisks indicate a putative regulatory domain including autoinhibitory and CaM-binding regions. C: CaM-KK activity of either recombinant CaM-KKβ-3 (0.32 μg/ml) or rat CaM-KKα (1.1 μg/ml) was measured in the presence of either 1 mM EGTA (−) or 1 mM $CaCl_2$/2 μM CaM (+) at 30°C for 5 min. Recombinant CaM-KKβ-3 (83 ng) and rat CaM-KKα (280 ng) were analyzed by CaM-overlay as described in Fig. 3 (inset). D: Activation of CaM-KIδ by CaM-KKβ-3 in transfected COS-7 cells. COS-7 cells were transfected with either 3 μg of empty vector (Mock) or 1.5 μg HA-CaM-KIδ-expressing vector with 1.5 μg empty pME18s vector or 1.5 μg CaM-KKβ-3-expressing vector. After 20 h incubation, the cells were further cultured in serum-free medium for 6 h and then treated with (+) or without (−) 1 μM ionomycin for 5 min. HA-CaM-KIδ was immunoprecipitated and then subjected to the protein kinase assay as described in Fig. 3B. Immunoprecipitated samples (1/5 vol.) were analyzed by CaM-overlay (inset). Arrow indicates HA–CaM-KIδ.

KIδ wild-type but not Thr180Ala mutant (Fig. 2C), indicating the presence of CaM-KIδ-activating kinase in the cells. We then further characterized the activation of CaM-KIδ in intact cells by transient transfection of HA-tagged CaM-KIδ into HeLa cells. In contrast to the parallel experiment using HA-CaM-KIV, whose kinase activity was lowered by the serum starvation and whose $Ca^{2+}$/CaM-dependent activity was significantly (8-fold) enhanced by ionomycin stimulation (Fig. 3B), CaM-KIδ exhibited enhanced protein kinase activity even without ionomycin stimulation (Fig. 3A). This suggests that the CaM-KIδ pool in HeLa cells is maintained in a partially active form during the cell growth; however, ionomycin treatment further enhanced CaM-KIδ activity (1.5-fold). That its sustained activation involves the phosphorylation of its Thr180 residue is revealed by the assay with the Thr180Ala mutant, which exhibited a reduced protein kinase activity that was no longer induced by ionomycin stimulation (Fig. 3A). Furthermore, enhanced activity of HA-tagged CaM-KIδ in resting cells was significantly reduced by treatment of the cells with CaM-KK inhibitor, STO-609 after transfection (60–70% inhibitory rate, Fig. 3C). Next we examined the mechanism of this sustained activation of CaM-KIδ by using a $Ca^{2+}$/CaM-independent mutant (residues 1–296) which lacks putative autoinhibitory and CaM-binding regions (Fig. 1B). Recombinant CaM-KIδ 1–296 phosphorylates the peptide substrate (syntide-2) in a complete $Ca^{2+}$/CaM-independent manner (data not shown). Immunoprecipitated HA-CaM-KIδ 1-296 from transfected HeLa cells exhibited significantly increased autonomous activity (Fig. 3D) that was no longer induced by ionomycin stimulation unlike wild-type CaM-KIδ (Fig. 3A). This mutant CaM-KIδ was apparently fully activated in resting HeLa cells by endogenous CaM-KK which is active without $Ca^{2+}$-mobilization or is activated at resting $Ca^{2+}$ levels, since the increased autonomous activity of HA-CaM-KIδ 1-296 was inhibited by STO-609 treatment (75% inhibitory rate) either with or without ionomycin stimulation. Taken together these results, HeLa cells contain CaM-KK(s) that activate CaM-KIδ in both presence and absence of ionomycin stimulation and the sustained activation of CaM-KIδ is contributed at least in part by $Ca^{2+}$-independent CaM-KK.

In order to identify the CaM-KK that activates CaM-KIδ in HeLa cells, we performed an RT-PCR that specifically amplifies CaM-KK isoforms using cDNA prepared from HeLa cells as a template. As a result, we could detect CaM-KKα in HeLa cells but two recently cloned isoforms of CaM-KKβ, CaM-KKβ-1 and β-2 [15] have not been detected (data not shown). However, we have isolated a novel CaM-KKβ isoforms from HeLa cells by RT-PCR using specific 5′-UTR and 3′-UTR sequences in CaM-KKβ-1 as the PCR primers and obtained two full-length clones. The 1.8 kb clone, as shown in Fig. 4A,B, encodes a 541 amino acid protein that lacks exon 16 [15] and therefore has a C-terminal sequence that is distinct from that of CaM-KK β-1 and β-2 isoforms. We therefore termed this novel splice isoform of CaM-KKβ as CaM-KKβ-3. The nucleotide sequence of 1.7 kb clone revealed that the clone is identical with CaM-KKβ-3 except in that it also lacks exon 14 [15], which encodes the C-terminus of the catalytic domain as well as N-terminal half of the regulatory domain (Leu442–Val484, [14]) (Fig. 4B). We denoted this splice isoform as CaM-KKβ-3x. Isolation of the full-length cDNAs of CaM-KKβ-3 and CaM-KKβ-3x in this study is in good agreement with a recent finding based on RT-PCR amplification of partial cDNA fragments of CaM-KKβ, suggesting that the additional forms of CaM-KKβ transcripts are generated through alternative splicing of the internal exons 14 and/or 16 [15]. RT-PCR analysis indicated that both are expressed in all human peripheral tissues we tested (data not shown) indicating that CaM-KKβ-3 is a non-neuronal CaM-KK and could be a candidate of CaM-KIδ activator.

Next we examined the activity and the $Ca^{2+}$/CaM-dependency of CaM-KKβ-3 by using GST–CaM-KI 1-293 K49E as a substrate (Fig. 4C). Whereas CaM-KK activity of rat CaM-KKα is completely dependent on $Ca^{2+}$/CaM, recombinant CaM-KKβ-3 showed enhanced autonomous activity (80% of total activity). This is consistent with a previous observation indicating an enhanced autonomous activity of rat CaM-KKβ [14]. Although CaM-KKβ-3 exhibited an increased autonomous activity, activation of CaM-KIδ by CaM-KKβ-3 in vitro was in a completely $Ca^{2+}$/CaM-dependent manner (data not shown) as well as CaM-KKα (Fig. 2A), suggesting that the Thr180 in CaM-KIδ can not be accessed and phosphorylated by CaM-KKβ-3 without binding of $Ca^{2+}$/CaM to CaM-KIδ, which is analogous to CaM-KI activation in vitro [21,22].

Finally, we attempted to reconstitute CaM-KKβ-3/CaM-KIδ cascade in transfected COS-7 cells (Fig. 4D). Similar to what observed in transfected HeLa cells, HA-CaM-KIδ is maintained as an active form in transfected COS-7 cells even without ionomycin stimulation. The addition of ionomycin raised (∼1.5-fold) CaM-KIδ activity indicating that COS-7 cells contain CaM-KIδ-activating activity. When the COS-7 cells were cotransfected with vectors expressing HA-CaM-KIδ and CaM-KKβ-3, CaM-KIδ activity was raised to the level of the ionomycin-induced CaM-KIδ activity in single transfected cells. This indicates that CaM-KKβ-3 is capable of phosphorylating and activating CaM-KIδ in intact cells.

In summary, we have identified and cloned a novel human member of the CaM-KI subfamily that we have denoted as CaM-KIδ. Here we have shown that CaM-KIδ is activated by CaM-KK through phosphorylation of Thr180 both in vitro and in vivo. We observed the sustained activation of CaM-KIδ in intact cells, which is quite unlike the CaM-KIV activation. Based on our data, a part of CaM-KIδ could be constitutively activated at resting $Ca^{2+}$ levels in the cells through phosphorylation of Thr180 by CaM-KK. This is also consistent with our finding that resting HeLa cells contain CaM-KIδ-activating activity and CaM-KKβ-3 exhibited enhanced $Ca^{2+}$/CaM-independent activity. Taken together, CaM-KK/CaM-KIδ cascade is functionally operated in HeLa cells and apparently involved in the sustained activation of CaM-KIδ.

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