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The Phosphatase-Resistant Isoform of CaMKI, Ca^{2+}/Calmodulin-Dependent Protein Kinase Iδ (CaMKIδ), Remains in Its "Primed" Form without Ca^{2+} Stimulation

Yukako Senga,[†,‡] Atsuhiko Ishida,[*,§] Yasushi Shigeri,[∥] Isamu Kameshita,[†] and Noriyuki Sueyoshi[*,†]

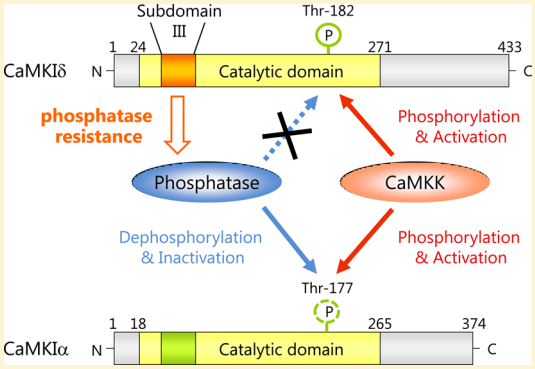
[†]Department of Life Sciences, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

[‡]National Institute of Advanced Industrial Science and Technology, Amagasaki, Hyogo 661-0974, Japan

[§]Laboratory of Molecular Brain Science, Graduate School of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan

[∥]National Institute of Advanced Industrial Science and Technology, Ikeda, Osaka 563-8577, Japan

ABSTRACT: Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI) is known to play pivotal roles in Ca^{2+} signaling pathways. Four isoforms of CaMKI (α, β, γ, and δ) have been reported so far. CaMKI is activated through phosphorylation by the upstream kinase, CaMK kinase (CaMKK), and phosphorylates downstream targets. When CaMKI was transiently expressed in 293T cells, CaMKIα was not phosphorylated at all under low-Ca^{2+} conditions in the cells. In contrast, we found that CaMKIδ was significantly phosphorylated and activated to phosphorylate cAMP response element-binding protein (CREB) under the same conditions. Herein, we report that the sustained activation of CaMKIδ is ascribed to its phosphatase resistance resulting from the structure of its N-terminal region. First, we examined whether CaMKIδ is more readily phosphorylated by CaMKK than CaMKIα, but no significant difference was observed. Next, to compare the phosphatase resistance between CaMKIα and CaMKIδ, we assessed the dephosphorylation of the phosphorylated CaMKIs by CaMK phosphatase (CaMKP/PPM1F). Surprisingly, CaMKIδ was hardly dephosphorylated by CaMKP, whereas CaMKIα was significantly dephosphorylated under the same conditions. To date, there have been no detailed reports concerning dephosphorylation of CaMKI. Through extensive analysis of CaMKP-catalyzed dephosphorylation of various chimeric and point mutants of CaMKIδ and CaMKIα, we identified the amino acid residues responsible for the phosphatase resistance of CaMKIδ (Pro-57, Lys-62, Ser-66, Ile-68, and Arg-76). These results also indicate that the phosphatase resistance of CaMKI is largely affected by only several amino acids in its N-terminal region. The phosphatase-resistant CaMKI isoform may play a physiological role under low-Ca^{2+} conditions in the cells.



Protein kinases regulate a wide variety of cellular processes such as cell proliferation, development, differentiation, and apoptosis.[1] Protein kinases not only phosphorylate their substrate proteins but also undergo autophosphorylation or are phosphorylated by other protein kinases. In many cases, the phosphorylation reactions of protein kinases are important steps in kinase regulation.[2] Consequently, protein phosphatases that dephosphorylate protein kinases are also responsible for the regulation of protein kinases.[3] Thus, intracellular signal transduction is constructed on the basis of the subtle balance between phosphorylation and dephosphorylation.

Multifunctional Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), including CaMKI, CaMKII, and CaMKIV, are Ser/Thr protein kinases that are abundantly expressed in the central nervous system. They are thought to act as central downstream effectors of the calcium signal by phosphorylating a variety of neuronal substrates.[4] CaMKII is regulated by Ca^{2+}/CaM-dependent autophosphorylation,[5] while CaMKI and CaMKIV are activated by phosphorylation of the Thr residue in the activation loop by the upstream kinase, CaMKK. CaMKI has been shown to comprise a family of four isoforms (α, β, γ, and δ) encoded by separate genes. CaMKI requires Ca^{2+}/CaM and CaMKK, an upstream CaMK that is also activated by Ca^{2+}/CaM, for its full activation.[6−8] In previous studies, to investigate the biological significance of CaMKIδ during zebrafish embryogenesis, we have isolated cDNA clones of CaMKIδ isoforms (CaMKIδ-S, CaMKIδ-L, and CaMKIδ-LL).[9,10] Knockdown of CaMKIδ-S/L by morpholino-based antisense oligonucleotides resulted in an increase in the number of abnormal embryos with a short trunk, kinked tail, and small head. In contrast, knockdown of CaMKIδ-LL resulted in an increase in the number of abnormal embryos with small fins and underdeveloped cartilage. These phenotypes were rescued by co-injection with recombinant CaMKIδ, but not with their

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kinase-dead mutants. These results suggest that the kinase activity of CaMKIδ isoforms plays a crucial role in the early stages of the embryogenesis of zebrafish. To date, much about the mechanisms of positive regulation of CaMKs by (auto)-phosphorylation has been published. There is also an increasing amount of evidence of mechanisms of negative regulation of CaMKII and CaMKIV by protein phosphatases.[11−16] However, less attention has been paid to the negative regulation of CaMKI.[17−19]

CaMKI regulates extracellular signal-regulated kinase (ERK)-dependent long-term potentiation,[20] recruitment of calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors,[21] dendritic arborization,[22] neurite outgrowth,[23] filopodia motility, and axon formation.[24,25] It is thought that these phenomena are subtly modulated by dephosphorylation of CaMKI as well as its phosphorylation, like other CaMKs. However, little is known about the mechanisms of CaMKI dephosphorylation. To address this question, we focused on the cellular dephosphorylation of CaMKI in this study.

In a previous study, CaMKI was shown to be activated by phosphorylation by the upstream kinase, CaMKK, in response to stimuli that trigger intracellular Ca^{2+} influx.[26] When CaMKI was transiently expressed in 293T cells, CaMKIα was not phosphorylated at all under low-Ca^{2+} conditions in the cells. In contrast, CaMKIδ was significantly phosphorylated and activated to phosphorylate cyclic AMP-responsive element-binding protein (CREB) under the same conditions. These data suggest that CaMKIδ has unique mechanisms for regulating its kinase activity. We speculated that CaMKK phosphorylates CaMKIδ as well as CaMKIα, but CaMKIδ can remain in the phosphorylated form (the potentially active, primed form) because of its phosphatase resistance, even in the absence of Ca^{2+} stimulation. In this study, to compare phosphatase resistance between CaMKIα and CaMKIδ, we explored the mechanism of CaMKIδ dephosphorylation by CaMK phosphatase (CaMKP/PPM1F),[18,27] which dephosphorylates and regulates multifunctional CaMKs.

MATERIALS AND METHODS

Materials. ATP, bovine serum albumin, anti-CREB antibody, anti-phospho-CREB (pSer133) antibody, and anti-β-actin antibody were purchased from Sigma-Aldrich Chemicals. Anti-myc antibody was purchased from Invitrogen. Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies were obtained from ICN Pharmaceuticals. An anti-His_6 antibody and Silver Stain II Kit were purchased from Wako. Anti-CaMKIα antibody was obtained from Abcam, and anti-CaMKIδ antibody was obtained from Funakoshi. Recombinant rat CaM[28] and mouse CaMKKα[29] were expressed in *Escherichia coli* and purified as described previously. Antibody against phospho-CaMKI (Thr177) was produced by immunizing BALB/c mice with an antigenic phosphopeptide.[30] Multi-PK antibody (M8C) was prepared as described previously.[31]

Construction of Plasmids. pETzCaMKIδ-LL(WT), pETrCaMKIα(WT), pETrCREB, and pETzCaMKP were prepared as described previously.[10,32−34] pETzCaMKIδ(1−327) was generated using primers (5′-AGT AGT ATG GGA AGC AGC ATG GAT C-3′ and 5′-GCC CAA CTG CAG TCT CCT CAT G-3′) and pETzCaMKIδ(WT). pETrCaMKIα(1−322) was generated using primers (5′-ACC AGC CAG GAG GGA CAG GG-3′ and 5′-GCC CAG CTG CAG CTT CCT CA-3′) and pETrCaMKIα(WT).

Plasmids for hPPM1A, hPPM1B, and hPPM1D were generated using sense primers (5′-TTT TCT AGA ATG GGA GCA TTT TTA GAC AAG CCA-3′ for hPPM1A, 5′-TTT TCT AGA ATG GGT GCA TTT TTG GAT AAA CCC-3′ for hPPM1B, and 5′-TTT GCT AGC ATG GCG GGG CTG GTA CTC GC-3′ for hPPM1D), antisense primers (5′-AAA **CTC GAG** CCA CAT ATC ATC TGT TGA TGT AGA GTC AG-3′ for hPPM1A, 5′-AAA **CTC GAG** TAT TTT TTC ACC ACT CAT CTT TGT CCC-3′ for hPPM1B, and 5′-AAA ***GTC GAC*** ATA GCA AAC ACA AAC AGT TTT CCT GTG-3′ for hPPM1D), and KIBB5611, KIBB8150, and KIEE4537 (Kazusa DNA Research Institute) as a template, respectively. The XbaI (underlined)−XhoI (bold underline) or NheI (italic underline)−SalI (bold and italic underline) fragments were inserted into the XbaI−XhoI or NheI−SalI sites of pET-23a(+), respectively (Novagen).

Plasmids for rCaMKIα/zCaMKIδ chimera mutants were constructed by the inverse polymerase chain reaction (PCR) method[35] with sense primers (5′-CCT AAA AAA GCT CTG AAG GGG AAG-3′ for chimera 1, 5′-ACT ATG GGC ATC GTC CAT CGA G-3′ for chimera 2, 5′-GCT CAG AAA CCG TAC AGT AAA GCT GT-3′ for chimera 3, 5′-GAG AAC ATA GTG GCT CTT GAA GAC ATC T-3′ for chimera 1-1, 5′-ATG CAG CTT GTG TCT GGA GGA GA-3′ for chimera 1-2, and 5′-AAA GAC GCC AGC ACA CTC ATC AG-3′ for chimera 1-3), antisense primers (5′-AAT GCA TTT GAT GGC CAC CAG T-3′ for chimera 1, 5′-GTG CAG GTA CTT GAC AGC ATC CAG-3′ for chimera 2, 5′-CAG GAC CTC AGG GGC CAC AT-3′ for chimera 3, 5′-GTG CTT GAT CTT GTG TAA GAC GGC-3′ for chimera 1-1, 5′-GAT GAG GTA GAG GTG GCC CCC-3′ for chimera 1-2, and 5′-TTC TGT GTA GAA TCC TTT CTC CAC AAT T-3′ for chimera 1-3), and pETrCaMKIα(WT)/zCaMKIδ(WT) as a template. The 5′-ends of the PCR fragments were then phosphorylated by T4 polynucleotide kinase (Nippon Gene) and self-ligated by T4 DNA ligase (Nippon Gene). The various zCaMKIδ and rCaMKIα mutants containing an NheI site at the 5′-ends and an XhoI site at the 3′-ends were inserted into the pET-23a(+) vector (Novagen).

Mutagenesis of Pro-57, Lys-62, Ser-66, Ile-68, and Arg-76 was performed by the inverse PCR method with sense primers (5′-GCT AAA AAA GCT CTG AAG GGG AAG-3′ for P57A, 5′-GAG GGG AAG GAG AGC AGC ATC G-3′ for K62E, 5′-GGC AGC ATC GAG AAC GAG ATC G-3′ for S66G, 5′-AGC AGC ATG GAG AAC GAG ATC G-3′ for I68M, and 5′-CAC AAA ATT AAG CAT GAG AAC ATA GTG-3′ for R76H; underlines show the mutation sites), antisense primers (5′-GAT GCA TTT CAC AGC GTA CAT CTT T-3′ for P57A, 5′-CAG AGC TTT TTT AGG GAT GCA TTT CA-3′ for K62E, 5′-CTC CTT CCC CTT CAG AGC TTT TTT-3′ for S66G and I68M, and 5′-CAG AAC GGC GAT CTC GTT CTC G-3′ for R76H). and pETzCaMKIδ(WT) as a template. The 5′-ends of the PCR fragments were then phosphorylated by T4 polynucleotide kinase and self-ligated by T4 DNA ligase. The various zCaMKIδ and rCaMKIα mutants containing an NheI site at the 5′-ends and XhoI site on the 3′-ends were inserted into the pET-23a(+) vector.

For mammalian cells, the following plasmids were prepared. pczCaMKIδ, pcrCaMKIα, pcmCaMKIδ, and pczCaMKP were prepared as described previously.[10,30,32] pcrCREB was generated by PCR using specific primers (5′-GCT AGC GTT ATG ACC ATG GAC TCT GGA GC-3′ and 5′-**CTC GAG** ATC TGA CTT GTG GCA GTA AAG G-3′) and

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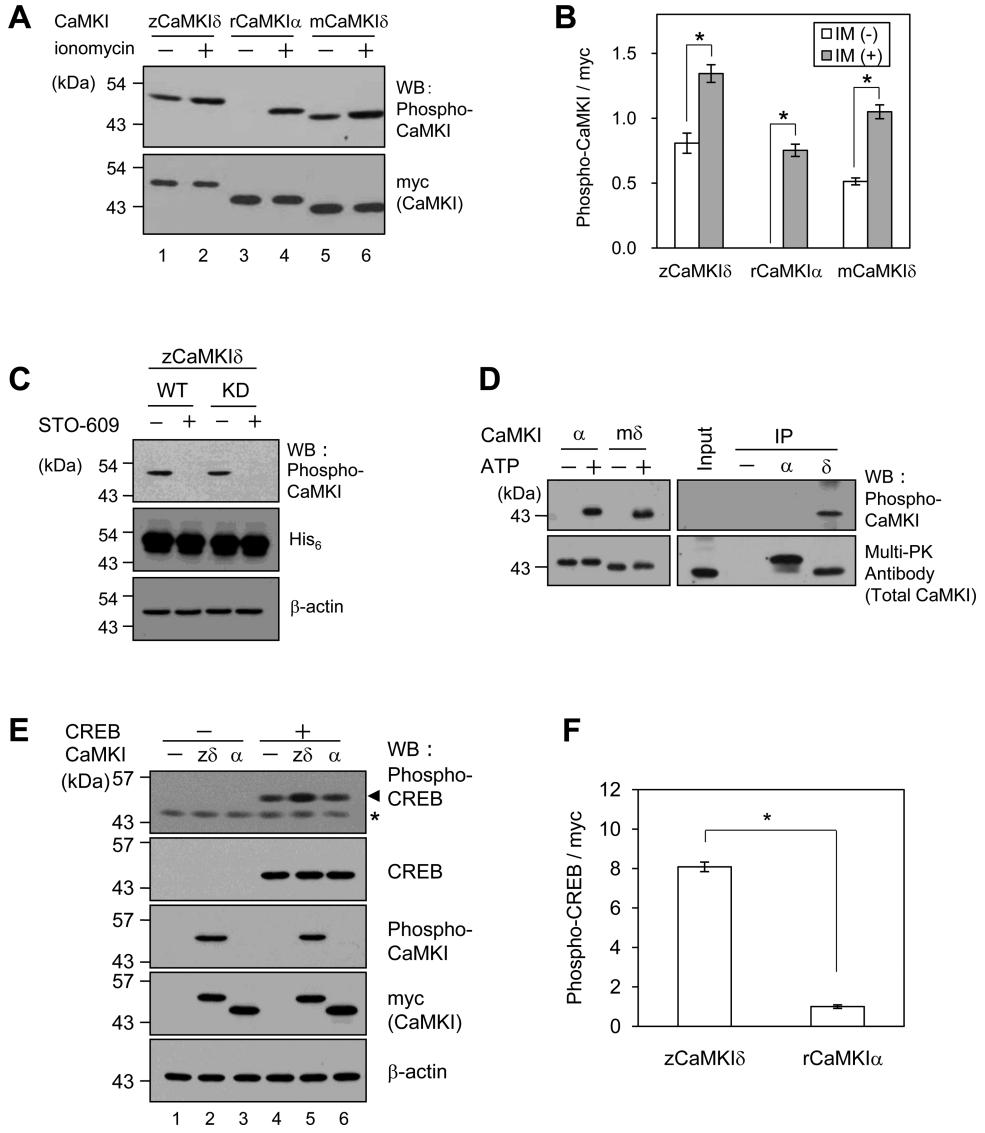

Figure 1. Phosphorylation and activation of CaMKI in mammalian cells. (A) pcDNA-CaMKIs were transfected into 293T cells. Cells expressing the indicated myc-tagged proteins were stimulated with 1 μM ionomycin for 5 min and lysed with SDS sample buffer. The cell lysates (20 μg of protein) were subjected to SDS−PAGE and then analyzed by Western blotting with an anti-phospho-CaMKI antibody (top panel). The lysates were also analyzed by Western blotting using anti-myc antibodies to detect myc-tagged CaMKI (bottom panel). (B) The Western blot using the phospho-CaMKI antibody was quantified by Scion Image software. Data are shown as means ± SD of three independent experiments. (C) pcDNA-CaMKIδ(WT) or the kinase-dead (KD) mutant was transfected into 293T cells and then cultured for 20 h either with (+) or without (−) 10 μg/mL STO-609. Cells expressing the indicated myc-tagged proteins without prior stimulation were lysed with SDS sample buffer. The cell lysates were subjected to SDS−PAGE and then analyzed by Western blotting with anti-phospho-CaMKI, anti-His_6 (for CaMKI detection), and anti-β-actin antibodies. (D) Purified His_6-tagged CaMKIα and CaMKIδ (20 ng) were phosphorylated by CaMKK. They were subjected to 10% SDS−PAGE and detected by Western blotting with anti-phospho-CaMKI and M8C Multi-PK antibodies (left panel). Endogenous CaMKIα or CaMKIδ proteins were immunoprecipitated from 293T cell lysates with anti-CaMKIα or anti-CaMKIδ antibodies and detected by Western blotting using anti-phospho-CaMKI and M8C antibodies (right panel). (E) pcDNA-CaMKIs were cotransfected with pcDNA-rCREB into 293T cells. Cells expressing the indicated myc-tagged proteins without prior stimulation were lysed with SDS sample buffer. The cell lysates (20 μg of protein) were subjected to SDS−PAGE and then analyzed by Western blotting with anti-phospho-CREB(Ser133), anti-CREB, anti-phospho-CaMKI, anti-myc (for CaMKI detection), and anti-β-actin antibodies. The arrowhead and the asterisk indicate the phospho-CREB and the nonspecific band, respectively. (F) CREB phosphorylation mediated by the transfected CaMKIs shown in panel E was quantified by Scion Image software. The band intensity of the background phosphorylation (lane 4) was subtracted from that of the zCaMKIδ-induced phosphorylation (lane 5) or rCaMKIα-induced phosophorylation (lane 6) to yield quantitation of phosphorylation due to the transfected CaMKIs. The values obtained were normalized to the respective CaMKI protein detected by Western blotting with an anti-myc antibody. Data are shown as means ± SD of three independent experiments. The asterisks denote significant differences as determined by a Student's t test ($p < 0.005$).

pETrCREB[34] as a template. pczCaMKIδ (P57A, K62E, S66G, I68M, R76H) was generated by PCR using specific primers (5′-AAA AAG CTT GTT ATG GCT CGG GAG AAC GGA GA-3′ and 5′-TTT **CTC GAG** CGC TTG GTC CCA GTT ACC ACT GTG C-3′) and pETzCaMKIδ (P57A, K62E, S66G, I68M, R76H) as a template. The HindIII (underline)−XhoI (bold underline) fragment was inserted into the HindIII−XhoI sites of pcDNA3.1(+)/*myc*-His B (Invitrogen).

Cell Culture and Transfection. 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako)

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containing 10% heat-inactivated fetal calf serum (FCS). Cells were grown at 37 °C in a humidified incubator with a 5% CO_2/95% air atmosphere. Transfections of 293T cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 293T cells (6×10^5) were plated in a 35 mm dish in 2 mL of DMEM containing 10% FCS. After being cultured for 24 h, the cells were incubated for 24 h in 1 mL of DMEM containing 5% FCS, 6 μL of Lipofectamine 2000, and 3 μg of plasmid DNA for transfection. Transfected cells were starved in serum-free DMEM for 6 h and then stimulated by addition of 1 μM ionomycin at 37 °C. After stimulation, the medium was removed and 150 μL of sodium dodecyl sulfate (SDS) sample buffer was added to stop the reaction. The samples were boiled for 5 min, electrophoresed on a SDS−polyacrylamide gel, and analyzed by Western blotting.

Immunoprecipitation. 293T cells were lysed in an immunoprecipitation buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol (DTT), 10 μg/mL chymostatin, 10 μg/mL pepstatin, 10 μg/mL leupeptin, and 10 μg/mL antipain]. The supernatant was incubated with an anti-CaMKIα antibody or anti-CaMKIδ antibody coupled to protein G-Sepharose at 4 °C for 2 h in the immunoprecipitation buffer. After being washed with the immunoprecipitation buffer to remove unbound proteins, the Sepharose gel was boiled with 2× SDS−polyacrylamide gel electrophoresis (PAGE) sample buffer. The resulting supernatant was subjected to SDS−PAGE and analyzed by Western blotting with the indicated antibodies.

Expression and Purification of Recombinant Proteins. pETCaMKIs, pETrCREB, and pETCaMK phosphatases were introduced into *E. coli* strain BL21(DE3). For recombinant CaMKIs, the transformed bacteria were grown at 37 °C to an OD_{600} of 0.6−0.8, and then isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. After 24 h at 25 °C, the bacteria were harvested by centrifugation (2300*g*) at 2 °C for 10 min and suspended in buffer A [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 40, and 1 mM phenylmethanesulfonyl fluoride]. For recombinant zCaMKP, hCaMKP, hPPM1A, and hPPM1B, the transformed bacteria were cultured at 25 °C for 24 h without IPTG induction and collected as described above. For recombinant hPPM1D, the transformed bacteria were cultured at 18 °C for 6 h via IPTG induction and collected as described above. After the harvest, the bacterial cells were sonicated and cell debris was removed by centrifugation (20000*g*) at 4 °C for 10 min, and the obtained supernatant was loaded onto a HiTrap Chelating HP column (GE Healthcare Bio-Sciences) pre-equilibrated with buffer A. The column was washed with buffer A, buffer A containing 20 mM imidazole, or buffer A containing 50 mM imidazole and then eluted with buffer A containing 200 mM imidazole. The purified fractions were pooled, dialyzed against 20 mM Tris-HCl (pH 7.5) containing 0.05% Tween 40 and 1 mM 2-mercaptoethanol, and used for the characterization of the enzyme.

SDS−PAGE and Western Blotting. SDS−PAGE was performed essentially according to the method of Laemmli[36] on slab gels consisting of a 10 or 12% acrylamide separation gel and a 3% stacking gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare Bio-Sciences), and immunoreactive protein bands were detected essentially according to a method described previously.[29]

Phosphorylation of CaMKI by CaMKK. Phosphorylation of CaMKI was conducted at 30 °C for the indicated time in a reaction mixture (20 μL) consisting of 40 mM Hepes-NaOH (pH 8.0), 5 mM $Mg(CH_3COO)_2$, 2 mM DTT, 0.5 mM $CaCl_2$, 1 μM CaM, 2.5 μg of CaMKI, 125 ng of CaMKK, and 100 μM ATP. The reaction was initiated by the addition of CaMKI and stopped by the addition of 20 μL of 2× SDS−PAGE sample buffer, followed by Western blotting.

Protein Phosphatase Assay Using Phosphoproteins as Substrates. The protein phosphatase assay was conducted using CaMKI as a phosphoprotein substrate. Recombinant CaMKI (2.5 μg) was phosphorylated by CaMKK (125 ng) at 30 °C for 30 min in a reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 10 mM $Mg(CH_3COO)_2$, 0.1 mM EGTA, 1 μM CaM, 0.5 mM $CaCl_2$, and 100 μM ATP. The reaction was stopped with termination buffer (80 μL) containing ice-cold 50 mM Tris-HCl (pH 7.5), 0.05% Tween 40, 2 mM DTT, and 2.5 mM EGTA. Dephosphorylation of phospho-CaMKI was conducted at 30 °C for 10 min in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM $MnCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 2 mM DTT, 100 ng of phospho-CaMKI, and 2.5, 5, or 10 ng of CaMKP. The reaction was started by addition of CaMKP and terminated by addition of an equal volume of 2× SDS−PAGE sample buffer. The sample was then subjected to SDS−PAGE and analyzed by Western blotting using phospho-CaMKI antibody.

Other Methods. Protein concentrations were determined by the method of Bensadoun and Weinstein using bovine serum albumin as a standard.[37] Nucleotide sequences were determined by the dideoxynucleotide chain termination method with BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems) and a DNA sequencer (model 3100, Applied Biosystems).

RESULTS

Phosphorylation and Activation of CaMKIδ. Our studies of the biochemical properties of CaMKIs revealed that the zebrafish CaMKIδ isoform was significantly phosphorylated under low-Ca^{2+} conditions (resting state) in the cells. In an effort to clarify the molecular mechanisms underlying the anomalous phosphorylation of zCaMKIδ, we examined the phosphorylation of exogenously expressed zCaMKIδ in 293T cells, as well as that of mouse CaMKIδ. Because zCaMKIα had not been examined in detail, we used rat CaMKIα, of which the subcellular localization has been shown to be very similar to that of zCaMKIδ,[9] as a control. In living cells, CaMK was activated by stimulation with ionomycin, a calcium ionophore (Figure 1A, lanes 2, 4, and 6, and Figure 1B). When CaMKI isoforms were transiently expressed in 293T cells, CaMKIα was not phosphorylated at all under low-Ca^{2+} conditions in the cells (Figure 1A, lane 3, and Figure 1B). In contrast, both zCaMKIδ and mCaMKIδ were significantly phosphorylated under the same conditions (Figure 1A, lanes 1 and 5, and Figure 1B), suggesting that the observed sustained phosphorylation was not characteristic of the zebrafish enzyme, but a common property of CaMKIδ conserved in vertebrates. Next, to confirm that CaMKIδ is phosphorylated by CaMKK in 293T cells, we examined whether phosphorylation of zCaMKIδ by endogenous CaMKK is inhibited by the selective CaMKK inhibitor, STO-609. Under low-Ca^{2+} conditions in the cells, the level of phosphorylation of zCaMKIδ(WT) and the kinase-dead

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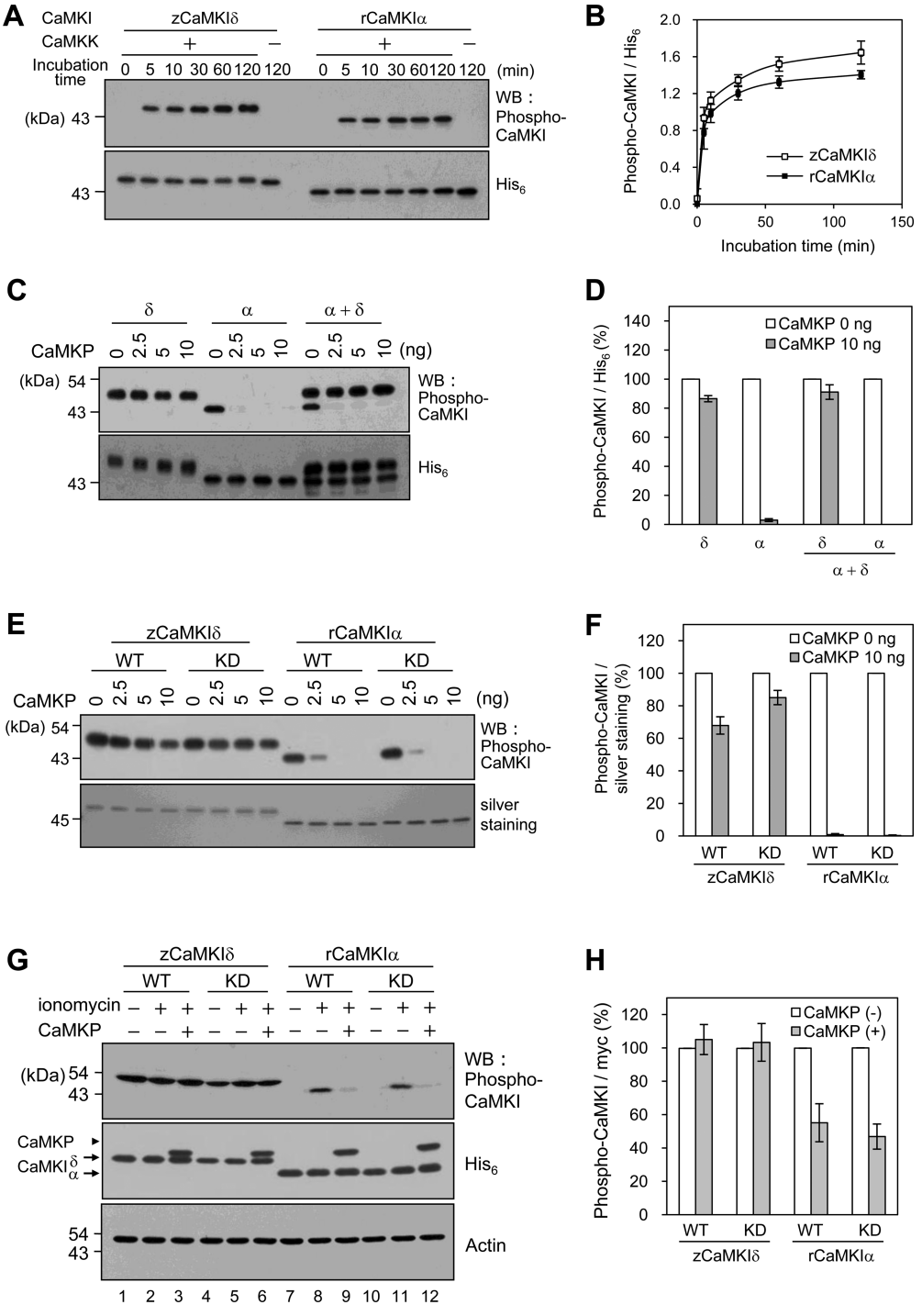

Figure 2. Dephosphorylation of CaMKIs by CaMKP *in vitro* and in mammalian cells. (A) Time course of phosphorylation of CaMKIs by mouse CaMKK*α*. Purified CaMKIs were incubated with Ca^{2+}/CaM in the presence or absence of CaMKK*α* in a reaction mixture. After incubation at 30 °C, the reactions were stopped by addition of 2× SDS−PAGE sample buffer, and the mixtures were subjected to 10% SDS−PAGE and then analyzed by Western blotting with anti-phospho-CaMKI and anti-His_6 antibodies (for CaMKI detection). (B) Phospho-CaMKIs (A, top panel) and total CaMKIs (A, bottom panel) were quantified by Scion Image software, and the ratios of phospho-CaMKI to total CaMKI (His_6) were calculated. Data are means ± SD of three independent determinations. (C) The phosphatase activity of CaMKP was determined using phospho-CaMKI as a substrate. Dephosphorylation of CaMKI (CaMKI*δ*, CaMKI*α*, and their combination) was monitored by Western blotting with an anti-phospho-CaMKI antibody (top panel). The total CaMKI level was examined by Western blotting with an anti-His_6 antibody (bottom panel). (D) Phospho-CaMKI (C, top panel) and total CaMKI (C, bottom panel) were quantified by Scion Image software, and they are shown as phospho-CaMKI/total CaMKI (His_6). Data are means ± SD of three independent determinations. (E) The phosphatase activity of CaMKP was determined using phospho-CaMKI as a substrate. Dephosphorylation of CaMKI was monitored by Western blotting with an anti-phospho-CaMKI antibody (top panel), and the total CaMKI level was examined by silver staining (bottom panel). (F) Phospho-CaMKI (E, top panel) and total CaMKI (E, bottom panel) were quantified by Scion Image software, and they are shown as phospho-CaMKI/total CaMKI. Data are means ± SD of three independent determinations. (G) 293T cells transfected with pcDNA-CaMKIs alone or together with pcDNA-CaMKP were cultured for 24 h. Subsequently, the cells were cultured in serum-free medium for 6 h and stimulated with 1 *μ*M ionomycin for 5 min. The cells were lysed with SDS−PAGE sample

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Figure 2. continued

buffer and analyzed by Western blotting with anti-phospho-CaMKI antibody (top panel). The lysates were also analyzed by Western blotting using anti-myc antibody to detect myc-tagged CaMKIs (arrows) and myc-tagged CaMKP (arrowhead) (middle panel). β-Actin was also examined for loading control (bottom panel). (H) Quantification of phospho-CaMKI (G, top panel) and CaMKI (G, middle panel, arrows) by Scion Image software is shown as phospho-CaMKI/total CaMKI (myc). Data are means ± SD of three independent determinations.

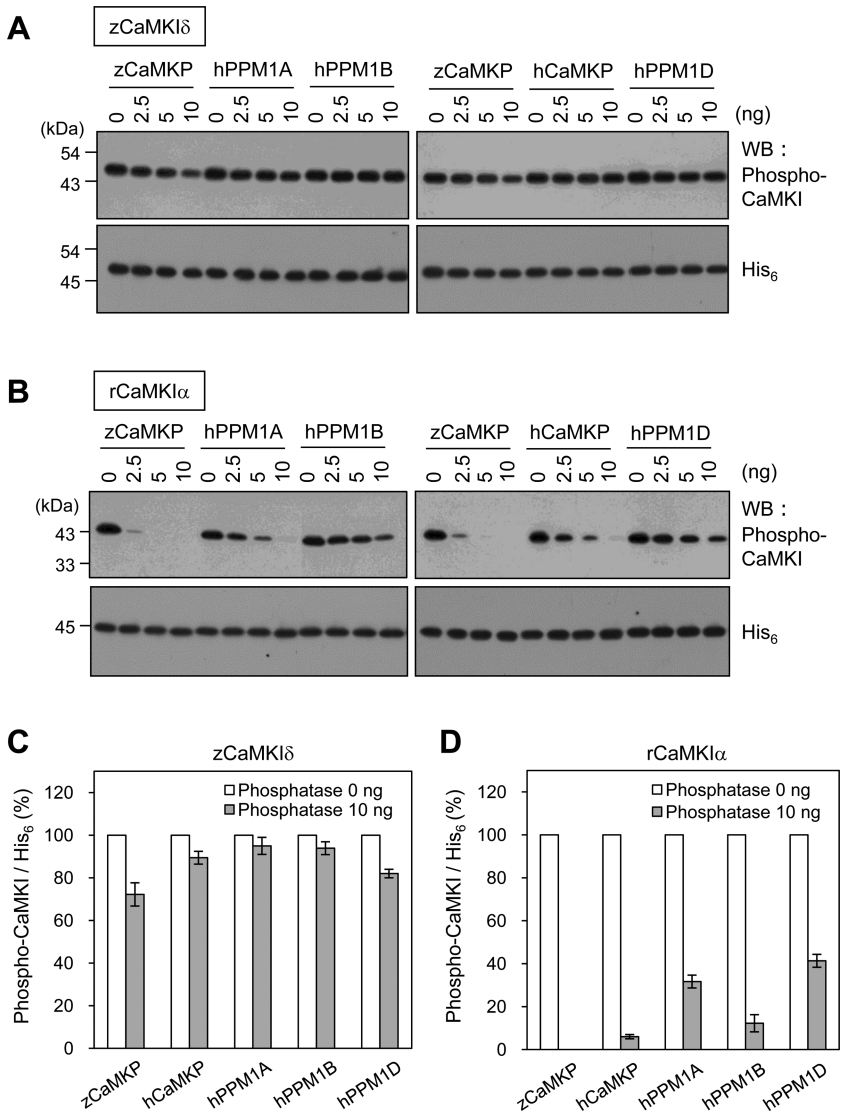

Figure 3. Dephosphorylation of CaMKIs by other Ser/Thr phosphatases such as PPM1A, PPM1B, and PPM1D *in vitro*. (A and B) The phosphatase activity was determined using phospho-CaMKIδ and phospho-CaMKIα as substrates, respectively. Dephosphorylation of CaMKIs was monitored by Western blotting with an anti-phospho-CaMKI antibody (top panel), and the total CaMKI level was examined by Western blotting with an anti-His_6 antibody (bottom panel). (C and D) Phospho-CaMKI (A and B, top panel) and CaMKI (A and B, bottom panel) were quantified by Scion Image software, and they are shown as phospho-CaMKI/total CaMKI (His_6). Data are means ± SD of three independent determinations.

mutant (KD) was significantly reduced after treatment with STO-609 (Figure 1C). Therefore, we used the zebrafish enzyme in the following characterization of CaMKIδ.

Furthermore, to examine the phosphorylation of endogenous CaMKI, the cell lysates were immunoprecipitated with an anti-CaMKIα or anti-CaMKIδ antibody (Figure 1D). In this experiment, endogenous CaMKIs were detected by Western blotting with Multi-PK antibody. The Multi-PK antibody was produced to detect a wide variety of Ser/Thr protein kinases[31] and has been used for the detection of various protein kinases. When Multi-PK antibody was used in the Western blotting analysis, similar immunoreactive bands of CaMKIα and CaMKIδ were detected with and without ATP. That is, the Multi-PK antibody detected nonphosphorylated CaMKI as well as phosphorylated CaMKI. When the cell lysates were immunoprecipitated with an anti-CaMKIδ antibody, the CaMKIδ protein was phosphorylated under low-Ca^{2+} conditions in the cells (Figure 1D, right panel). In contrast, CaMKIα was not phosphorylated at all under the same conditions.

Next, to examine whether CaMKIδ is phosphorylated and activated to phosphorylate CREB under low-Ca^{2+} conditions in the cells, we co-expressed CaMKI and CREB in 293T cells. The phosphorylation level of CaMKI and CREB was analyzed by

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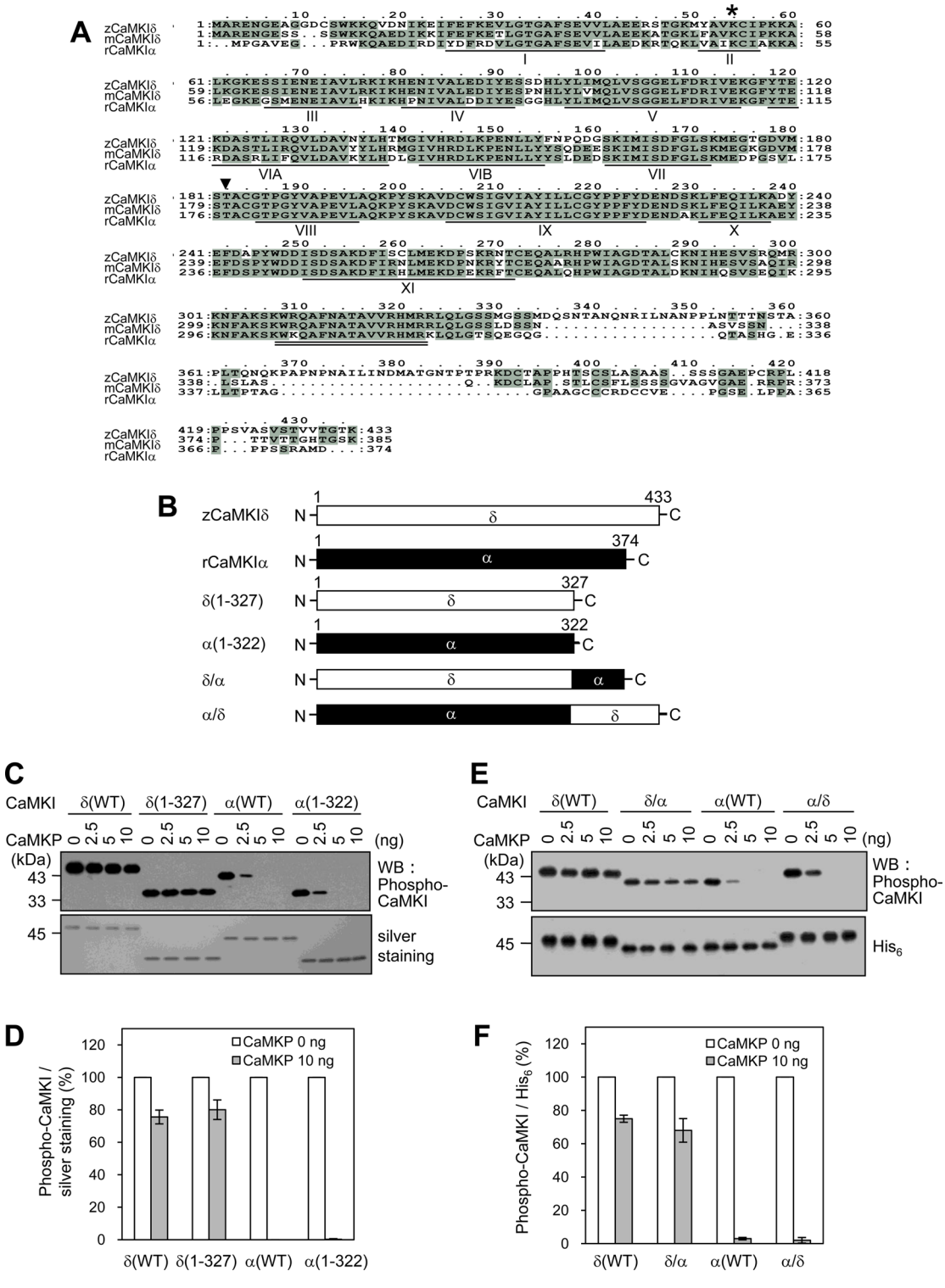

Figure 4. Dephosphorylation of CaMKI C-terminal deletion mutants and chimera mutants by CaMKP. (A) Alignment of amino acid sequences of zCaMKIδ (GenBank accession number BC160632), mCaMKIδ (GenBank accession number BC141413), and rCaMKIα (GenBank accession number NP598687). The identical amino acids are highlighted in gray. Twelve subdomains specific to protein kinases are shown with solid underlines. The ATP-binding site and the CaMKK phosphorylation site are shown with an asterisk and an arrowhead, respectively. The putative CaM-binding domains are double-underlined. (B) Schematic illustration of the primary structures of zCaMKIδ(WT), rCaMKIα(WT), zCaMKIδ(1−327), rCaMKIα(1−322), zCaMKIδ(1−327)/rCaMKIα(323−374) (δ/α), and rCaMKIα(1−322)/zCaMKIδ(328−433) (α/δ) chimeric mutants. zCaMKIδ and rCaMKIα are shown by a white box and a black box, respectively. (C and E) The phosphatase activity of CaMKP was determined using the indicated phospho-CaMKI mutant as a substrate. Dephosphorylation of the WT and mutant CaMKI was monitored by Western blotting with an anti-phospho-CaMKI antibody (top panel). The total CaMKI level was examined by silver staining (C, bottom panel) or by Western blotting using anti-His_6 antibody (E, bottom panel). (D and F) Relative phosphorylation levels of CaMKIs were quantified by Scion Image software and indicated as phospho-CaMKI/total CaMKI. Data are means ± SD of three independent determinations.

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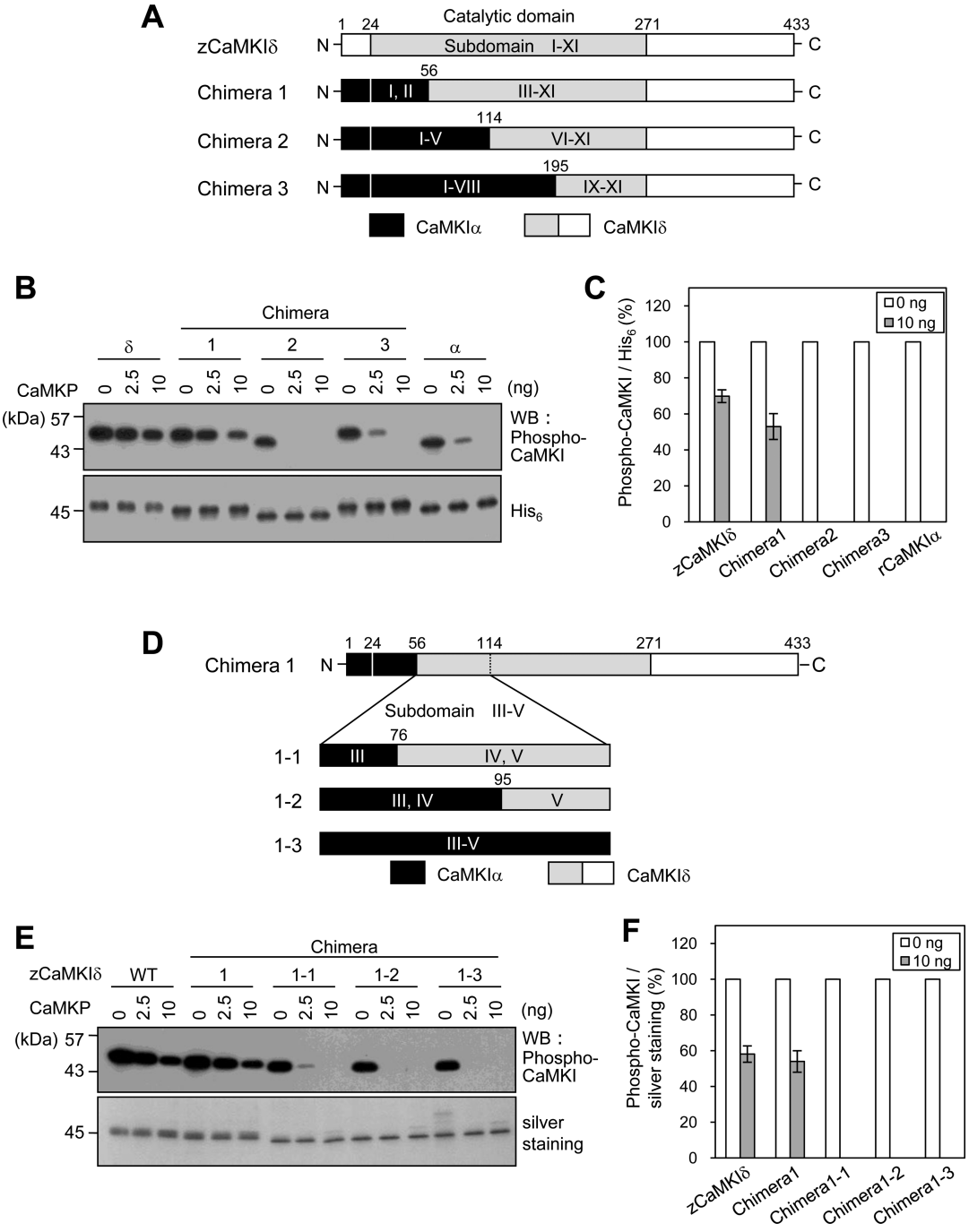

Figure 5. Dephosphorylation of zCaMKI*δ* N-terminal chimera mutants by CaMKP. (A) Schematic illustration of the primary structures of zCaMKI*δ* and N-terminal chimera mutants. The catalytic domains of zCaMKI*δ* and rCaMKI*α* are shown by gray boxes and black boxes, respectively. (B) The phosphatase activity of CaMKP was determined using the indicated phospho-CaMKI chimera as a substrate. Dephosphorylation of the CaMKI chimeras was monitored by Western blotting with an anti-phospho-CaMKI antibody (top panel), and the total CaMKI level was examined by Western blotting using an anti-His_6 antibody (bottom panel). (C) Relative phosphorylation levels of CaMKI chimeras were quantified by Scion Image software and indicated as phospho-CaMKI/total CaMKI. Data are means ± SD of three independent determinations. (D) Schematic illustration of the primary structures of the Chimera 1 mutant and the additional N-terminal chimera mutants (chimeras 1-1, 1-2, and 1-3). The catalytic domains of zCaMKI*δ* and rCaMKI*α* are shown by gray boxes and black boxes, respectively. (E) The phosphatase activity of CaMKP was determined using the indicated phospho-CaMKI chimeras as a substrate. Dephosphorylation of CaMKI was monitored by Western blotting with an anti-phospho-CaMKI antibody (top panel), and the total CaMKI levels were examined by silver staining (bottom panel). (F) Relative phosphorylation levels of CaMKI chimeras were quantified by Scion Image software and indicated as phospho-CaMKI/total CaMKI. Data are means ± SD of three independent determinations.

Western blotting using a phospho-CaMKI-specific antibody and a phospho-CREB-specific antibody. As shown in Figure 1E, CaMKI*δ*, but not CaMKI*α*, was significantly phosphorylated and activated to phosphorylate CREB under low-Ca^{2+} conditions in the cells (Figure 1E, lanes 5 and 6, and Figure 1F).

Dephosphorylation of CaMKI by CaMKP *in Vitro* and *in Vivo*. The significant phosphorylation and activation of

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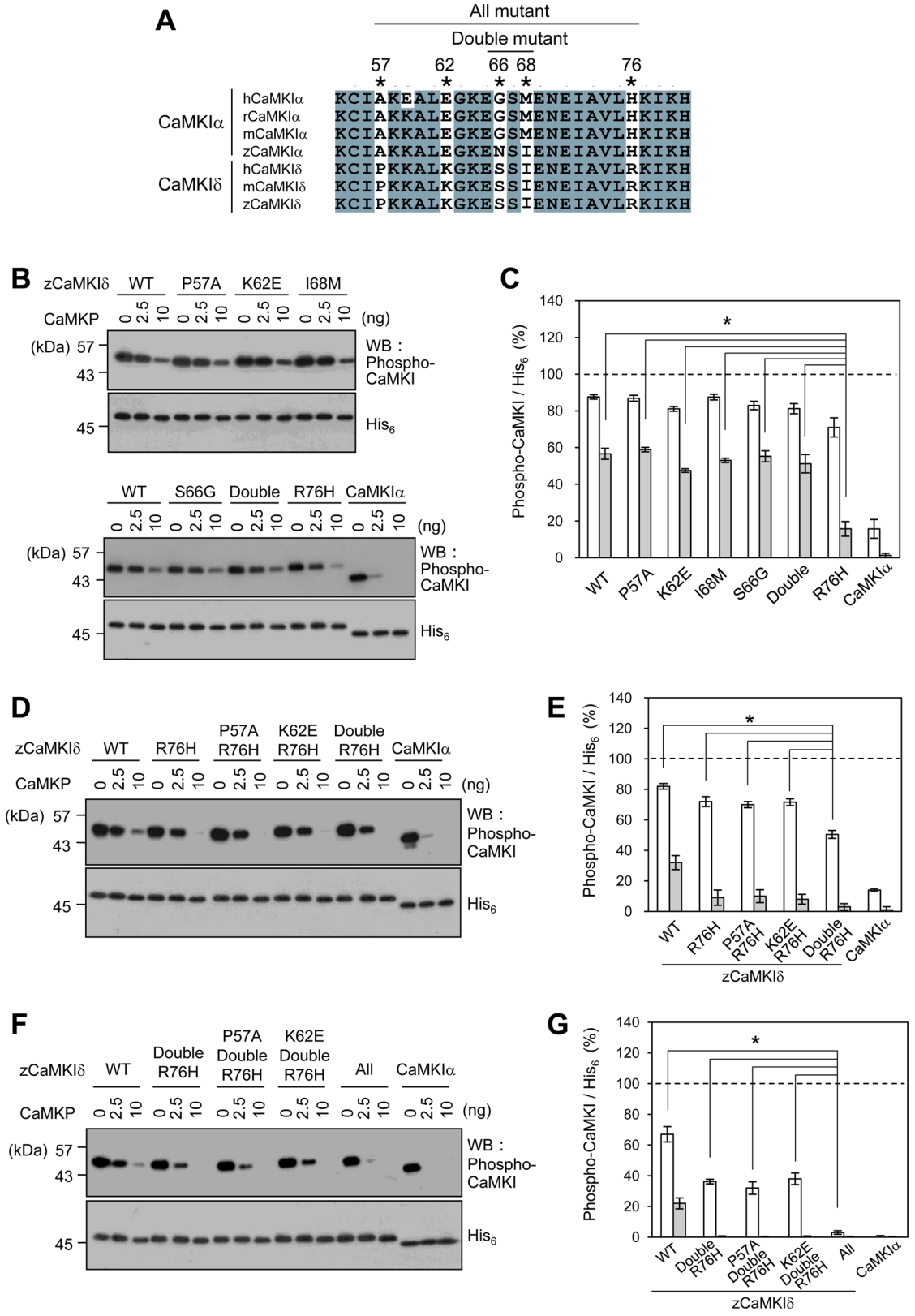

Figure 6. Effects of subdomain III of zCaMKIδ. (A) Comparison of the amino acid sequences of subdomain III of CaMKIs. Asterisks show the different amino acids between CaMKIα and CaMKIδ. These residues in CaMKIδ were mutated to those in CaMKIα in the following experiments. (B, D, and F) The phosphatase activity of CaMKP was determined using the indicated mutant of phospho-CaMKI as a substrate. Dephosphorylation of CaMKI was monitored by Western blotting with anti-phospho-CaMKI antibody (top panel), and the total CaMKI was detected by an anti-His_6 antibody (bottom panel). (C, E, and G) Relative phosphorylation levels of CaMKI were quantified by Scion Image software and indicated as phospho-CaMKI/total CaMKI. The values are expressed as a percentage of the control reaction (CaMKP 0 ng, shown as a dotted line). White bars and gray bars show dephosphorylation by 2.5 and 10 ng of CaMKP, respectively. Data are means ± SD of three independent determinations. Asterisks denote significant differences as determined by a Student's t test ($p < 0.005$).

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CaMKIδ under low-Ca^{2+} conditions in the cells may exist because of enhanced phosphorylation by CaMKK. To examine whether CaMKIδ is more readily phosphorylated by CaMKK than CaMKIα, we prepared recombinant CaM, CaMKK, and CaMKI expressed in *E. coli*. When CaMKIs were phosphorylated by CaMKK in the presence of Ca^{2+}/CaM and CaMKK, only a modest difference in phosphorylatability was observed between them (Figure 2A,B). Next, to compare the phosphatase resistance of CaMKIα and CaMKIδ, we conducted phosphatase assays using CaMKP, which dephosphorylates and regulates multifunctional CaMKs. Surprisingly, CaMKIδ was highly resistant to dephosphorylation by CaMKP, whereas CaMKIα was significantly dephosphorylated under the same conditions (Figure 2C,D). To examine whether the phosphatase resistance of CaMKIδ depends on kinase activity, we performed phosphatase assays using kinase-dead (KD) mutants of CaMKIδ and CaMKIα (K54R and K49R, respectively). No significant changes in the phosphatase resistance of the kinases were observed between the WT and KD mutant (Figure 2E,F). Furthermore, we examined whether phospho-CaMKI is dephosphorylated in living cells. CaMKI was transiently transfected into 293T cells alone or in combination with CaMKP. The transfected cells were stimulated with ionomycin, and the phosphorylation level of CaMKI was analyzed by Western blotting using a phospho-CaMKI-specific antibody. As shown in panels G and H of Figure 2, the phosphorylation level of CaMKIα(WT) and KD mutants markedly increased after stimulation with ionomycin (Figure 2G, lanes 8 and 11). Conversely, under the same conditions, the phosphorylation was significantly attenuated when the cells were cotransfected with CaMKP and CaMKIα (Figure 2G, lanes 9 and 12). In contrast, CaMKIδ in the cells cotransfected with CaMKP and CaMKIδ was hardly dephosphorylated by CaMKP (Figure 2G, lanes 3 and 6). Next, we conducted phosphatase assays using other Ser/Thr phosphatases such as PPM1A, PPM1B, and PPM1D. As shown in Figure 3, CaMKIδ was also highly resistant to dephosphorylation by phosphatases other than CaMKP, whereas CaMKIα was significantly dephosphorylated under the same conditions. These data suggest that CaMKIδ is phosphatase-resistant.

The N-Terminal Region around Subdomain III of CaMKIδ Is the Region Responsible for the Phosphatase Resistance. In this study, we showed that CaMKIδ was hardly dephosphorylated by CaMKP compared with CaMKIα. When we compared the primary sequences of CaMKIδ and CaMKIα, we noticed that the C-terminal sequence of CaMKIδ was quite different from that of CaMKIα (Figure 4A). To identify the region responsible for the phosphatase resistance, we prepared various deletion mutants and chimeric proteins of CaMKIδ and CaMKIα: CaMKIδ(1−327), CaMKIα(1−322), CaMKIδ(1−327)/CaMKIα(323−374) (δ/α), and CaMKIα(1−322)/CaMKIδ(328−433) (α/δ), with His_6 tags on their C-termini (Figure 4B). To compare the phosphatase resistance among these mutants, we conducted *in vitro* phosphatase assays using CaMKP. As shown in Figure 4C−F, the phosphatase resistance of the deletion mutants and chimeric mutants of CaMKIδ and CaMKIα was the same as that of their wild-type counterparts. These results indicate that the C-terminal region of the CaMKI family enzymes (CaMKIα, residues 323−374; and CaMKIδ, residues 328−433) is not essential for the phosphatase resistance of CaMKI.

Next, to identify the region responsible for the phosphatase resistance, we prepared more chimeric mutants (chimeras 1−3), which were fusion proteins of the N-terminal domain of CaMKIα and the C-terminal domain of CaMKIδ (Figure 5A). When we conducted the phosphatase assays using these chimera mutants, the chimera 1 mutant was hardly dephosphorylated by CaMKP, but chimera 2 and 3 mutants were significantly dephosphorylated under the same conditions (Figure 5B,C). The chimera 1 mutant contained subdomains III−XI of CaMKIδ, and the chimera 2 mutant contained subdomains VI−XI of CaMKIδ. These results suggest that subdomains III−V of CaMKIδ are responsible for the phosphatase resistance. Furthermore, we prepared additional chimeric mutants (chimeras 1-1, 1-2, and 1-3), which were fusion proteins of the N-terminal domain of CaMKIα and the C-terminal domain of CaMKIδ (Figure 5D). When we conducted the phosphatase assays using these chimeric mutants, they were significantly dephosphorylated as in the case of the chimera 2 mutant (Figure 5E,F). These results suggest that the N-terminal region around subdomain III of CaMKIδ plays an important role in phosphatase resistance.

In an attempt to identify the key sites responsible for the phosphatase resistance in CaMKIδ, we focused on the five amino acid residues, Pro-57, Lys-62, Ser-66, Ile-68, and Arg-76, located in subdomain III of CaMKIδ, as they were different between CaMKIδ and CaMKIα in various species (Figure 6A). To explore which residues in CaMKIδ are essential for phosphatase resistance, we prepared various point mutants of CaMKIδ: CaMKIδ(P57A), CaMKIδ(K62E), CaMKIδ(S66G), CaMKIδ(I68M), CaMKIδ(S66G,I68M:Double), and CaMKIδ(R76H). When we conducted the phosphatase assays using these mutants as substrates, CaMKIδ(R76H) was more readily dephosphorylated by CaMKP than the other point mutants (Figure 6B,C). Therefore, in the next experiment, we constructed various combination mutants of CaMKIδ containing the R76H mutation: CaMKIδ(P57A,R76H), CaMKIδ(K62E,R76H), and CaMKIδ(Double,R76H). In these mutants, CaMKIδ(Double,R76H) was more readily dephosphorylated by CaMKP than the other mutants (Figure 6D,E). Moreover, we constructed various combination mutants of CaMKIδ(Double,R76H): CaMKIδ(P57A,Double,R76H), CaMKIδ(K62E,Double,R76H), and CaMKIδ(P57A,K62E,Double,R76H;All). Surprisingly, although the "All" mutant was markedly dephosphorylated by CaMKP, CaMKIδ(P57A,Double,R76H) and CaMKIδ(K62E,Double,R76H) were less significantly dephosphorylated than the "All" mutant under the same conditions (Figure 6F,G). To examine whether this can be applied to other protein phosphatases, CaMKIδ(WT), CaMKIδ(All), and CaMKIα(WT) were dephosphorylated by PPM1A, PPM1B, and PPM1D. Figure 7 shows that CaMKIδ(All) was significantly dephosphorylated to an extent similar to that of CaMKIα(WT) by these phosphatases. These results strongly suggest that all five residues of CaMKIδ, Pro-57, Lys-62, Ser-66, Ile-68, and Arg-76, are critical for phosphatase resistance, irrespective of the protein phosphatases responsible for its dephosphorylation, though the contribution of each residue is unclear.

The N-Terminal Region of CaMKIδ Is Essential for Its Phosphatase Resistance in Cells. In this study, we revealed that the N-terminal region of CaMKIδ is important for its phosphatase resistance *in vitro*. Next, we examined whether like CaMKIα(WT), the CaMKIδ "All" mutant is significantly dephosphorylated by CaMKP in mammalian cells. CaMKI was transiently transfected into 293T cells alone or in combination with CaMKP. The transfected cells were

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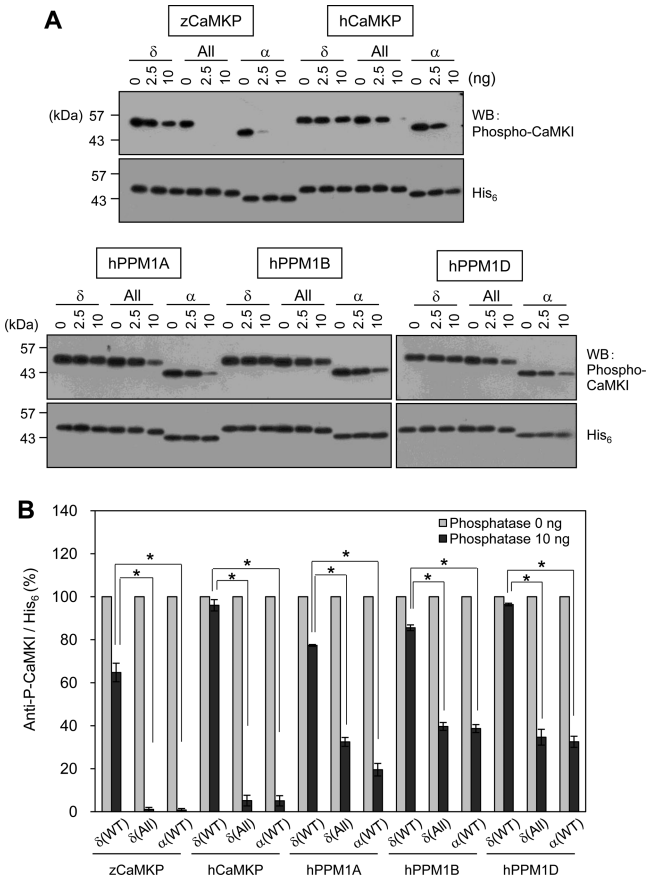

Figure 7. Dephosphorylation of the "All" mutant of CaMKIδ by other Ser/Thr phosphatases such as PPM1A, PPM1B, and PPM1D *in vitro*. (A) The indicated phosphatase activities were assessed using phosphorylated CaMKIα, CaMKIδ, or CaMKIδ "All" mutant as a substrate. Dephosphorylation of the CaMKIs was monitored by Western blotting with an anti-phospho-CaMKI antibody (top panel), and total CaMKI was detected by an anti-His_6 antibody (bottom panel). (B) Phospho-CaMKI (A, top panel) and total CaMKI (A, bottom panel) were quantified by Scion Image software and are indicated as phospho-CaMKI/total CaMKI. Data are means ± SD of three independent determinations. Asterisks denote significant differences as determined by a Student's *t* test ($p < 0.005$).

stimulated with ionomycin, followed by Western blotting analysis using a phospho-CaMKI-specific antibody, to examine the phosphorylation level of CaMKI in the cells. As shown in Figure 8A, the phosphorylation level of CaMKI markedly increased when the CaMKI-transfected cells were treated with ionomycin (Figure 8A, lanes 2, 5, and 8). As expected, in contrast to wild-type CaMKIδ, the "All" mutant of CaMKIδ (P57A, K62E, S66G, I68M, R76H) was dephosphorylated by CaMKP (Figure 8A, lanes 3 and 6). The immunoreactive bands were quantitated using Scion Image software (Figure 8B). These results indicate that the phosphatase resistance of CaMKIδ is ascribed to the difference in the primary structure of its subdomain III compared with the primary structure of that of CaMKIα.

DISCUSSION

In this study, we show that CaMKIδ has unusual phosphatase resistance, because of its unique structure in the N-terminal domain; thereby, it was anomalously phosphorylated and primed in 293T cells. The primed form of CaMKIδ was more readily activated than other isoforms of CaMKI such as CaMKIα, leading to enhancement of CREB phosphorylation even at low Ca^{2+} levels.

CaMKI has an autoinhibitory domain and a CaM-binding domain in the C-terminal region to regulate its kinase activity. In the presence of Ca^{2+}, CaM binds to the CaM-binding domain of CaMKI to relieve the autoinhibition, which in turn results in phosphorylation by CaMKK of Thr-177 in the activation loop of CaMKI to fully activate it.[6,7] Therefore, in the resting state, cellular CaMKI should be in the inactive state, because of the low Ca^{2+} levels in the cell. However, we found that Thr-182 (the equivalent of Thr-177 of rCaMKIα) in the activation loop of CaMKIδ was highly phosphorylated even in the absence of stimulation that elevates the intracellular Ca^{2+} level. Moreover, the constitutively phosphorylated CaMKIδ appeared to be active as evidenced by enhanced phosphorylation of the physiological CaMKI substrate, CREB, which was not observed with CaMKIα (Figure 1E,F). This observation led us to investigate the mechanism of the sustained phosphorylation and/or activation of CaMKIδ in the cells.

Ishikawa et al.[38] have reported that CaMKIδ was phosphorylated and/or activated in the resting state in HeLa cells, because of Ca^{2+}-independent autonomous activity of CaMKKβ3. It has also been reported that CaMKKα shows strictly Ca^{2+}/CaM-dependent kinase activity, whereas CaMKKβ shows significant kinase activity even in the absence of Ca^{2+}/CaM.[39] Therefore, we examined the *in vitro* phosphorylation of CaMKIδ and CaMKIα by CaMKKα. No significant difference in the phosphorylation time course was observed between them (Figure 2A,B). Similarly, no significant difference in the time course was observed between the CaMKKβ-catalyzed phosphorylation of CaMKIδ and CaMKIα (data not shown). Therefore, it is unlikely that CaMKIδ is more readily phosphorylated by CaMKK than CaMKIα, leading to the observed sustained phosphorylation of CaMKIδ.

Because CaMKI activity is supposed to be regulated not only by CaMKK but also by protein phosphatases responsible for dephosphorylation of Thr-177, we next examined the dephosphorylation of CaMKIδ and CaMKIα by CaMKP. Surprisingly, CaMKIδ was highly resistant to dephosphorylation by CaMKP, whereas CaMKIα was readily dephosphorylated by CaMKP. This was also the case with other Ser/Thr phosphatases such as PPM1A, PPM1B, and PPM1D, as CaMKIδ was also resistant to dephosphorylation by these phosphatases (Figures 3 and 7). On the basis of these lines of evidence, we concluded that the observed anomalous phosphorylation of CaMKIδ in the cells exists because of its resistance to dephosphorylation.

So far, little attention has been given to the regulation of CaMKI functions by dephosphorylation. Therefore, we conducted a detailed investigation of the dephosphorylation of CaMKIδ by CaMKP and found that the characteristic resistance of CaMKIδ to protein phosphatases can be ascribed to the structure of the N-terminal region of CaMKIδ. Using various CaMKIδ mutants, we clarified that only several amino acid residues located around subdomain III were critical for dephosphorylation of the Thr residue within the activation loop (Figures 5 and 6). In these experiments, however, we estimated the phosphatase resistance by changing the amounts of CaMKP and using a fixed concentration of the substrate, phospho-CaMKIδ, because of difficulties in performing detailed kinetic analysis. Therefore, we cannot exclude the possibility that CaMKIδ mutants in which we could not detect phosphatase

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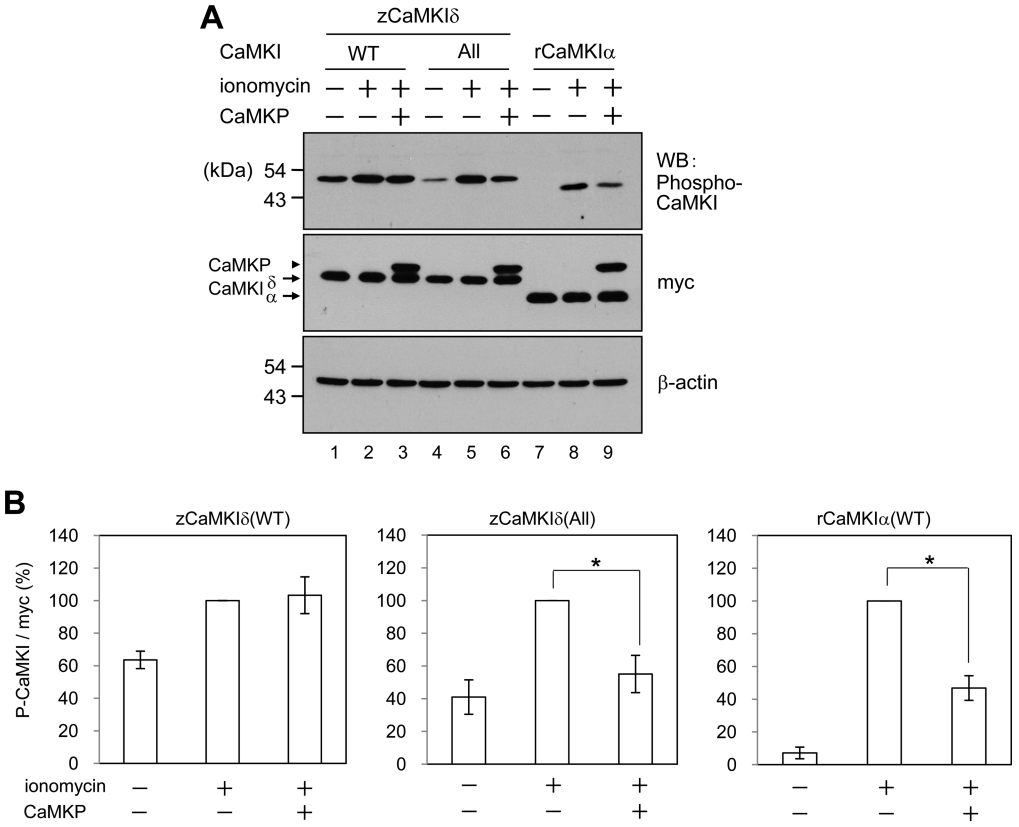

Figure 8. Dephosphorylation of the CaMKIδ "All" mutant by CaMKP in mammalian cells. (A) 293T cells transfected with pcDNA-CaMKIs alone or together with pcDNA-CaMKP were cultured for 24 h. Subsequently, the cells were cultured in serum-free medium for 6 h and stimulated with 1 μM ionomycin for 5 min. The cells were lysed with SDS−PAGE sample buffer and analyzed by Western blotting with an anti-phospho-CaMKI antibody (top panel). The lysates were also analyzed by Western blotting using an anti-myc antibody to detect myc-tagged CaMKI (arrows) and myc-tagged CaMKP (arrowhead) (middle panel). β-Actin was examined by Western blotting with an anti-β-actin antibody for loading control (bottom panel). (B) Phospho-CaMKI (A, top panel) and CaMKI (A, middle panel, arrows) were quantified by Scion Image software and are indicated as phospho-CaMKI/total CaMKI. The values are expressed as a percentage of the control (ionomycin +, CaMKP −). Data are means ± SD of three independent determinations. Asterisks denote significant differences as determined by a Student's t test ($p < 0.005$).

resistance showed altered K_m values compared with those of wild-type CaMKI.

In subdomain III, there is an α-helix called αC, which is known to be important for ATP binding prior to conformational changes by phosphorylation of the Thr residue in the activation loop of various protein kinases.[40−42] In a proposed model of CaMKIα, the conformational change induced by ATP binding and CaM binding is supposed to be stabilized by ionic interactions between Glu-66 in αC and Lys-49 within the ATP-binding region.[42,43] In CaMKIδ, however, several residues around Glu-66 in CaMKIα are replaced with other amino acids (Ala-57 → Pro, Glu-62 → Lys, and Met-68 → Ile). These replacements may induce a conformation of the αC helix different from that of CaMKIα. Because the αC helix is located near the activation loop in the tertiary structure of various protein kinases, it is possible that the difference in the conformation of the αC helix affects the accessibility of the Thr residue to protein phosphatases, causing the characteristic phosphatase resistance of CaMKIδ.

Dephosphorylation of the Thr residue is likely to be independent of the kinase activity of CaMKI, because the kinase-dead mutants of CaMKIδ and CaMKIα (K54R and K49R, respectively) did not show altered kinetics of dephosphorylation of the Thr residue compared with those of the respective wild-type enzymes (Figure 2E−H). In this study, we found that as in the case of the zebrafish CaMKIδ, the Thr residue in the activation loop of the mouse CaMKIδ was significantly phosphorylated by CaMKK without Ca^{2+} stimulation, suggesting that the observed phosphatase resistance of CaMKIδ is conserved among vertebrates. Once CaMKIδ is phosphorylated and/or activated by the upstream kinase CaMKK, it becomes resistant to protein phosphatases because of its unique structure in the N-terminal region. The constitutively phosphorylated CaMKIδ would be in a primed state, which can more readily be activated in response to Ca^{2+} signals than ordinary CaMKI such as CaMKIα, leading to an increased level of phosphorylation of CaMKI substrates such as CREB. This may contribute to acceleration or potentiation of Ca^{2+} signaling in a variety of vertebrate cells.

CREB is thought to be a physiological substrate of CaMKI.[44] It has been reported that CaMKIδ expressed in PC12 cells phosphorylates Ser-133 of CREB upon ionomycin stimulation, enhancing the transcriptional activity of CREB.[26] Phosphorylation of Ser-133 of CREB by CaMKIα and CaMKIγ has also been reported to be involved in dendrite formation via upregulation of Wnt.[22] Furthermore, it has been reported that CaMKIα-catalyzed phosphorylation of CREB positively regulates neuroprotective signaling pathways induced by brain ischemia through activation of synaptic *N*-methyl-D-aspartate (NMDA) receptors and upregulation of brain-derived neurotrophic factor (BDNF).[45] Sakagami et al.[26] have shown that CaMKIδ expressed in hippocampal neurons was mostly

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localized in the cytosol but rapidly translocated into the nucleus upon KCl stimulation. On the other hand, CaMKIα expressed in the neuron hardly altered its localization upon KCl stimulation. In our recent study,[10] we have found that the endogenous CaMKIδ in zebrafish fibroblasts was present in both the cytosol and the nucleus. Although the regulatory mechanism of the intracellular localization of CaMKIδ is currently unknown, it is likely that the priming of CaMKIδ by robust phosphorylation of Thr-182 is involved in the regulation of its localization. If this is the case, the endogenous CaMKIδ in zebrafish fibroblasts is probably, at least in part, in the primed state under low-Ca^{2+} conditions.

Activation of CaMKI by an increase in the intracellular Ca^{2+} level is involved in a variety of signal transduction pathways in the cell. Among CaMKI isoforms, both CaMKIα and CaMKIδ are cytosolic enzymes that show similar substrate specificity. The former is susceptible to protein phosphatases, whereas the latter is resistant to them. This suggests different physiological roles of CaMKI isoforms. Constitutive priming of CaMKIδ by resistance to protein phosphatases, reported here, may be a molecular mechanism for ensuring neuronal signaling by efficient phosphorylation of CaMKI substrate proteins, even under low-Ca^{2+} conditions or with enhanced phosphatase activity.

AUTHOR INFORMATION

Corresponding Authors

*Laboratory of Molecular Brain Science, Graduate School of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan. Telephone: +81-82-424-6526. Fax: +81-82-424-0759. E-mail: aishida@hiroshima-u.ac.jp.

*Department of Life Sciences, Faculty of Agriculture, Kagawa University, Ikenobe 2393, Miki-cho, Kagawa 761-0795, Japan. Telephone and fax: +81-87-891-3114. E-mail: sueyoshi@ag.kagawa-u.ac.jp.

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Notes

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ABBREVIATIONS

CaM, calmodulin; CaMKI, Ca^{2+}/calmodulin-dependent protein kinase I; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; CaMKP, Ca^{2+}/calmodulin-dependent protein kinase phosphatase; CREB, cyclic AMP-responsive element-binding protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FCS, fetal calf serum; IPTG, isopropyl β-D-thiogalactopyranoside; SD, standard deviation.

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