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Original

Possible roles of N- and C-terminal unstructured tails of CPI-17 in regulating Ca^{2+} sensitization force of smooth muscle

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Abstract

CPI-17 regulates the myosin phosphatase and mediates the agonist-induced contraction of smooth muscle. PKC and ROCK phosphorylate CPI-17 at Thr38 leading to a conformational change of the central inhibitory domain (PHIN domain). The N- and C-terminal tails of CPI-17 are predicted as unstructured loops and their sequences are conserved among mammals. Here we characterized CPI-17 N- and C-terminal unstructured tails using recombinant proteins that lack the potions. Recombinant CPI-17 proteins at a physiologic level (10 μM) were doped into beta-escin-permeabilized smooth muscle strips for Ca^{2+} sensitization force measurement. The ectopic full-length CPI-17 augmented the PDBu-induced Ca^{2+} sensitization force at pCa6.3, indicating myosin phosphatase inhibition. Deletion of N- and C-terminal tails of CPI-17 attenuated the extent of PDBu-induced Ca^{2+}-sensitization force. The N-terminal deletion dampened phosphorylation at Thr38 by protein kinase C (PKC), and the C-terminal truncation lowered the affinity to the myosin phosphatase. Under the physiologic conditions, PKC and myosin phosphatase may recognize CPI-17 N-/C-terminal unstructured tails inducing Ca^{2+} sensitization force in smooth muscle cells.

Key words: PP1, protein kinase C (PKC), force development, myosin, evolution

Introduction

Dysmotility of smooth muscle cells has been linked to pathological conditions, such as hypertension, vascular spasm, asthma and gastroparesis (1–5). Smooth muscle contraction is triggered in response to an elevation of cytoplasmic [Ca^{2+}] that activates the myosin light chain kinase (6). Plus, agonist stimulation suppresses

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the myosin phosphatase, inducing the Ca^{2+}-sensitization force (7–10). In addition to the Ca^{2+} regulatory circuit through ion channels and transporters, the Ca^{2+}-sensitization pathways contribute to conferring diversity in the agonist-induced responses of smooth muscle tissues (11). Accumulating lines of evidence strongly suggest that disturbances in the Ca^{2+}-sensitization signaling are associated with hyper- and hypo-responsiveness of smooth muscles that occur under pathologic conditions (2–5, 11, 12). To fully understand smooth muscle dysmotility, we need deeper insights into the molecular basis of the Ca^{2+} sensitization signaling.

CPI-17, a smooth muscle specific inhibitor of the myosin phosphatase contributes to the Ca^{2+} sensitization pathways in smooth muscles (11–13). In a current model, stimulation of G protein coupled receptors induces a sequential activation of PKC and ROCK, phosphorylating CPI-17 at Thr38, suppressing the myosin phosphatase and inducing the Ca^{2+} sensitization force of smooth muscles (11–13). CPI-17-mediated Ca^{2+} sensitization is ceased by the slow dephosphorylation of the phospho-CPI-17 by the myosin phosphatase (14–17). In addition to the protein expression levels, fluctuations in the level of CPI-17 phosphorylation are associated with pathological conditions (4, 12, 18, 19). In experimental models, upregulation of CPI-17 expression causes hypertensive phenotype (20) and ablation of CPI-17 gene results in a hypotensive phenotype in the blood pressure (2, 21), suggesting pathophysiologic roles CPI-17 phosphorylation in regulating smooth muscle contraction.

CPI-17 protein consists of three parts of structural domains, an inhibitory global domain with four helices and two tail domains at N- and C-terminus where are predicted as unstructured (13, 22). Phosphorylation of CPI-17 at Thr38 induces a series of conformational changes, resulting in re-alignment of the four alpha-helices in the inhibitory domain (23, 24). Structure-function analyses using recombinant CPI-17 proteins revealed the central domain of CPI-17 between 22–120 that is sufficient for the potent inhibition of a purified myosin phosphatase (22). Curiously, the amino acid sequences in N- and C-terminal portions of CPI-17 are almost identical among mammals and partially similar to zebrafish paralogs (25). To fully understand the molecular basis of CPI-17 signaling in mediating Ca^{2+}-sensitization force, we examined physiologic roles of the unstructured domains of CPI-17 using beta-escin-permeabilized stomach fundus smooth muscle strips and found new structural elements driving the Ca^{2+} sensitization pathways.

Methods

Myography — The protocol of the mouse experiments was approved by IACUC at Thomas Jefferson University and Okayama University of Science. Swiss Webster or ICR mouse (female, retired) was euthanized using CO_2 gas followed by cervical dislocation. Longitudinal layers of stomach fundus were cleaned under a stereomicroscope and strips (1 × 3 mm) were excised. Loops were made at both ends of strips using rayon monofilaments and the strips were set to an SI-H KG7B force transducer, whose voltage outputs were digitized with Powerlab (AD Instruments, Colorado Springs, CO, USA) via the loops. Strips were dipped in the normal external solution (150 mM NaCl in 5 mM HEPES, 5.6 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 4 mM KCl, pH 7.3) and then stimulated for 5 min with high K^+ buffer (124 mM KCl in 5 mM HEPES, 5.6 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 27 mM NaCl, pH 7.3). The relaxation-contraction cycle was repeated until the extent of the force became stable. Permeabilization was conducted for 30 min with 40 μM beta escin (Sigma Aldrich, St. Louis, MO, USA) at 25 °C in the intracellular G1 relaxing solution (1 mM EGTA in the cytoplasmic solution with 30 mM PIPES-KOH (pH 7.1), 74 mM potassium methanesulfonate, 2 mM Mg^{2+}, 4.5 mM MgATP and 10 mM phospho-creatine) including 1 μM A23187. The permeabilized strips were then incubated for 15 min with 10 μM CPI-17 and 1 μM CaM. The Ca^{2+} sensitization force was produced by stimulating the strips with the pCa6.3 solution buffered with 10 mM EGTA in the cytoplasmic solution supple-

mented with 3 μM PDBu, 10 μM CPI-17 and 1 μM CaM. Maximum force was produced by switching [Ca^{2+}] from pCa6.3 to pCa4.5 using the Ca^{2+}-buffered cytoplasmic solution supplemented with 1 μM microcystin LR (MCLR). The force development was monitored and determined at desired time points using LabChart software (AD Instruments) (26).

Phosphorylation assay — Phosphorylation of recombinant CPI-17 proteins was determined by phospho-dot blotting as described previously (27). Fundus smooth muscle strips were homogenized with the lysis buffer including 50 mM MOPS-NaOH pH7.0, 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% Tween-20, 4 mM 4-benzenesulfonyl fluoride (AEBSF) and 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), incubated for 30 min on ice and then cleared by centrifugation for 20 min at 4 °C. Phosphorylation of CPI-17 protein (0.2 mg/ml) was carried out for 15 min with the lysates in the presence of 1 mM ATP, 3 μM PDBu, 1 μM MCLR, 5 mM $MgCl_2$, 25 mM MOPS-NaCl (pH7.0), 4 mM AEBSF and 0.5 mM TCEP at 25 °C. The reaction was terminated with 2x RIPA buffer (0.4 M NaCl, 2 mM EDTA, 2% Triton X-100, 0.2% sodium deoxycholate, 0.2% SDS, and 0.1 M Tris-HCl, pH 8.0) and triplicate aliquot (each 2 μl) of the mixture was spotted onto nitrocellulose membrane. Phospho-CPI-17 was detected using anti-P-CPI-17(T38) antibody (28) and quantified by densitometry using ImageQuant™ software of Amersham Imager 680 (GE Healthcare, Boston, MA, USA).

Pulldown assay — Recombinant CPI-17 proteins were thio-phosphorylated for 90 min at 37 °C using 1 mM ATPγS and active PKC fragment prepared from human erythrocyte (24). Human leiomyosarcoma cell culture (SKN, obtained from JCBB Cell Bank, Osaka, Japan) were homogenized with the lysis buffer and the cleared extracts were used as a source of intact myosin phosphatase complex. The thio-phosphorylated CPI-17 was mixed with the cell extracts and S-protein agarose beads (Merck-Millipore, Burlington, MA, USA) and rocked for 30 min at 4 °C. After washing three times with the lysis buffer, the myosin phosphatase complex bound to the CPI-17 on beads was eluted using 2x Laemmuli buffer (Nacarai Tesque Inc, Kyoto, Japan) and analyzed by immunoblotting using anti-PP1 delta (29) and anti-6xHis antibodies (Qiagen, Germantown, MD, USA). Densitometry was conducted to quantify the extent of bound PP1 and 6xHis-tagged CPI-17.

Bioinformatic analysis — cDNA sequences of CPI-17 (PPP1R14A) and its analogs PHI-1 (PPP1R14B), KEPI (PPP1R14C) and GBPI (PPP1R14D) were obtained from GenBank at NCBI (https://www.ncbi.nlm.nih.gov/). Homologs were detected by BLASTP search at NCBI site using human CPI-17 sequence. Based on the amino acid sequences of each hit, candidates with over 80% identity and E value of >6e-52 vs human sequences were considered as avian CPI-17 homologs. Multiple sequence alignment was conducted using Cluster Omega program at EMBL-EBI (30) (https://www.ebi.ac.uk/Tools/msa/clustalo/) with a default setting. Venn diagrams of avian homologs of each CPI-17 family member in the hit list were generated using the online tool of Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Others — Recombinant CPI-17 proteins including N-terminal 6xHis and S-tag were purified from the bacterial lysates using HisPur Cobalt™ affinity resins (Pierce, Pittsburgh, PA, USA) as described previously (22). Recombinant protein solutions were dialyzed against 5 mM MOPS-NaOH, pH7.0, 4 mM AEBSF and 0.5 mM TCEP at 4 °C, and diluted with the pCa6.3 solution to 100 μM. ANOVA with Dunnett's test was conducted using Kaleidagraph software (Synergy Software).

Results

Originally, CPI-17 protein and cDNA were isolated from pig aorta smooth muscle (31, 32). Next generation sequencing approaches have uncovered CPI-17-like genes, at least in part, in most vertebrates. Figure 1A shows amino acid sequence alignment of CPI-17 homologs of mammals (human, mouse, wombat), birds (small

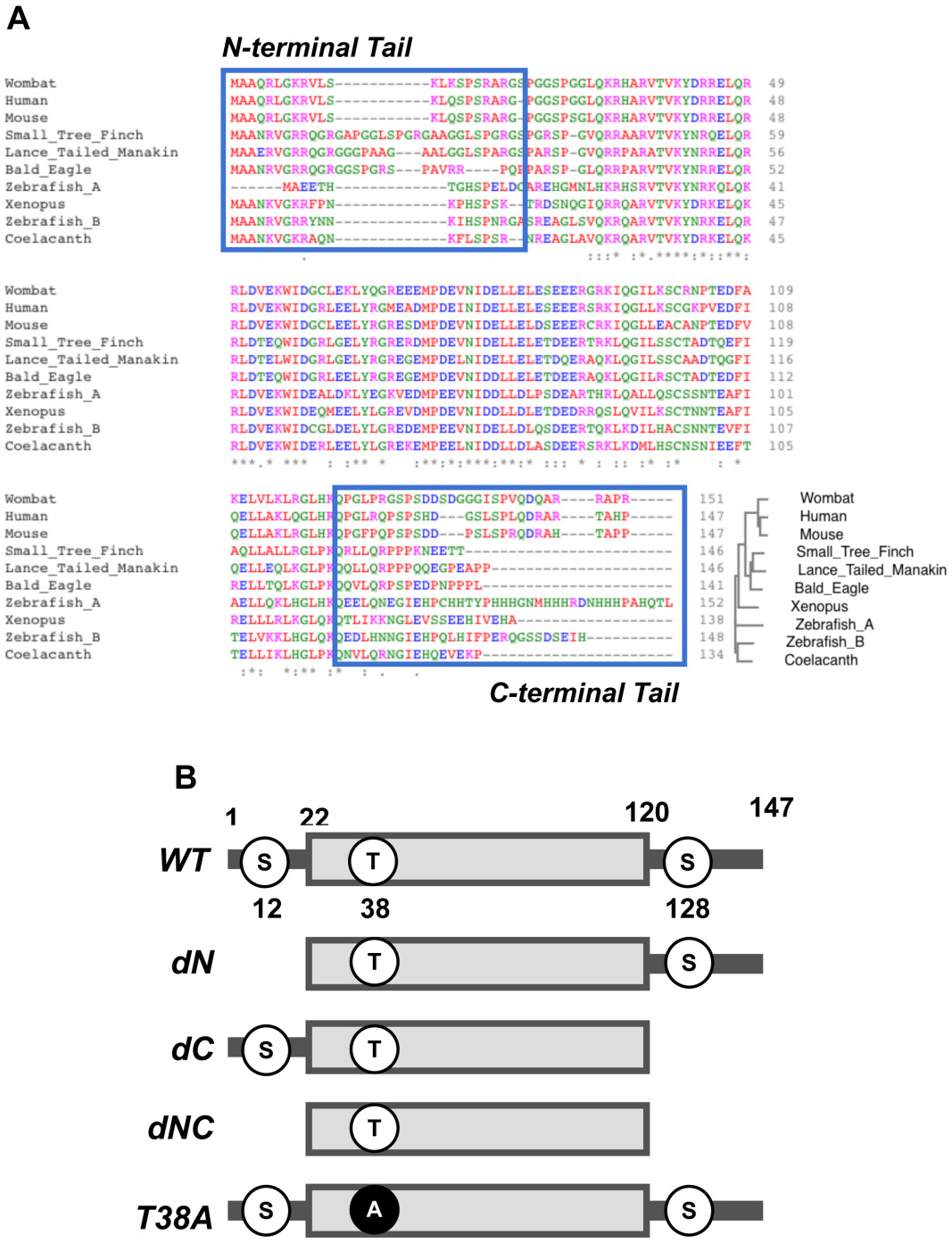

Fig. 1. Structure of CPI-17 and the recombinant proteins used.
(A) Cluster Omega program (EMBL-EBI) was used for alignment and phylogenic tree generation of amino acid sequences of CPI-17 (PPP1R14A). Blue lines indicate the unstructured regions at N-and C-termini. An asterisk indicates positions fully conserved, a colon and period indicate positions in which chemical properties of residues are conserved. (B) Schematic illustration of recombinant CPI-17 proteins used for the assays. Box indicates the central inhibitory domain (PHIN domain). Circles indicate Ser12, Thr38 and Ser128, whose phosphorylation occurs in cells.

tree finch, lance tailed manakin, bald eagle), an amphibian (xenopus) and fishes (zebrafish, coelacanth). The overall sequences are almost identical among mammals including a marsupial, wombat. On the other hand, a less similarity was found in the amino acid sequences of N-terminal and C-terminal domains of birds, xenopus

and fishes, compared with those of mammals, that reflects the shape of the phylogenetic tree (Fig. 1A, bottom right). The N-terminal tail of avian CPI-17 homologs includes an 8–11-residue insert that distinguish from other species.

To understand functions of CPI-17 N-/C-terminal domains in the Ca^{2+} sensitization signaling, recombinant CPI-17 proteins, WT, dN (22–147), dC (1–120), and dNC (22–120) were prepared from bacteria lysates (Fig. 1B) and subjected to myography assay using skinned smooth muscle strips (Fig. 2). CPI-17 T38A substitute was used as a negative control that does not inhibit the myosin phosphatase. Longitudinal layers of mouse fundus smooth muscle strips were permeabilized with beta escin, and the intracellular Ca^{2+} stores were abolished with A23187. Permeabilized strips were stimulated with a PKC activator, 3 μM PDBu, in pCa6.3 solution to produce the Ca^{2+} sensitization force. Without addition of ectopic CPI-17, PDBu stimulation induced the force to 25% of maximum force induced by 1 μM MCLR, a myosin phosphatase inhibitor (Fig. 2B, red line), suggesting phosphorylation of the endogenous CPI-17. When CPI-17 WT protein at a physiologic level (10 μM) (33) was doped into the permeabilized strips at 15 min prior to the stimulation, the PDBu-induced force reached to double of control (51% of maximum; Fig. 2A and B, blue lines), indicating the Ca^{2+} sensitization force induced by the ectopic CPI-17. On the other hand, the inactive version of the T38A protein failed to increase the PDBu-induced force (Fig. 2, gray), suggesting that the ectopic CPI-17 protein (Thr38) is phosphorylated by endogenous kinase(s) and inhibits myosin phosphatase in the skinned strips. None of dN (green), dC (orange) and dNC (black) versions of CPI-17 produced the Ca^{2+} sensitization force. Clearly, the N-/C-terminal unstructured loops are required for a full potency of CPI-17.

What is the role of the N-/C-terminal unstructured domains in the Ca^{2+} sensitization? We tested whether the truncations affect CPI-17 phosphorylation at Thr38. Recombinant CPI-17 proteins were phosphorylated using the extracts of fundus smooth muscle as a mixture of endogenous kinases (Fig. 3). To mimic the permeabilized strips used in Fig. 2, 3 μM PDBu was added to the reaction mixture. Less extent of the phosphorylation at Thr38 occurred with the CPI-17 proteins including N-terminal truncations, such as dN and dNC version, compared with WT (Fig. 3A). On the other hand, the C-terminal truncation resulted in a moderate reduction in Thr38 phosphorylation (Fig. 3A). Thr38 phosphorylation of the WT protein was suppressed with a PKC inhibitor (3 μM GF109203x (GFx)), but not by a ROCK inhibitor (1 μM H1152) (Fig. 3B), suggesting that the major kinase in the lysates is PKC, which is affected by the N-terminal truncation. Phosphorylation without MCLR did not alter the outcomes, suggesting that the N-/C-terminal tails are independent from the stability of the phosphorylation.

Binding affinities of the truncated CPI-17 proteins with myosin phosphatase were assessed by the pull-down assay (Fig. 4). 6xHis-/S-tagged CPI-17 protein was pre-thiophosphorylated using excess amount of PKC. Thiophosphorylated CPI-17 was mixed with cell lysates containing the myosin phosphatase. The complex of 6xHis-/S-tagged CPI-17 protein and myosin phosphatase was captured using S-protein agarose beads and the bound myosin phosphatase was detected by immunoblotting using anti-PP1 delta antibody (Fig. 4A). Densitometry was conducted to quantify the extents of PP1 and CPI-17 co-precipitated with the beads (Fig. 4B). Compared with CPI-17 WT, lesser extent of PP1 was co-precipitated with the thiophosphorylated dN, dC, and dNC proteins. Blank (BLK) indicates the beads without CPI-17. Deletion of either the N- or C-terminal portion may decrease the affinity to myosin phosphatase.

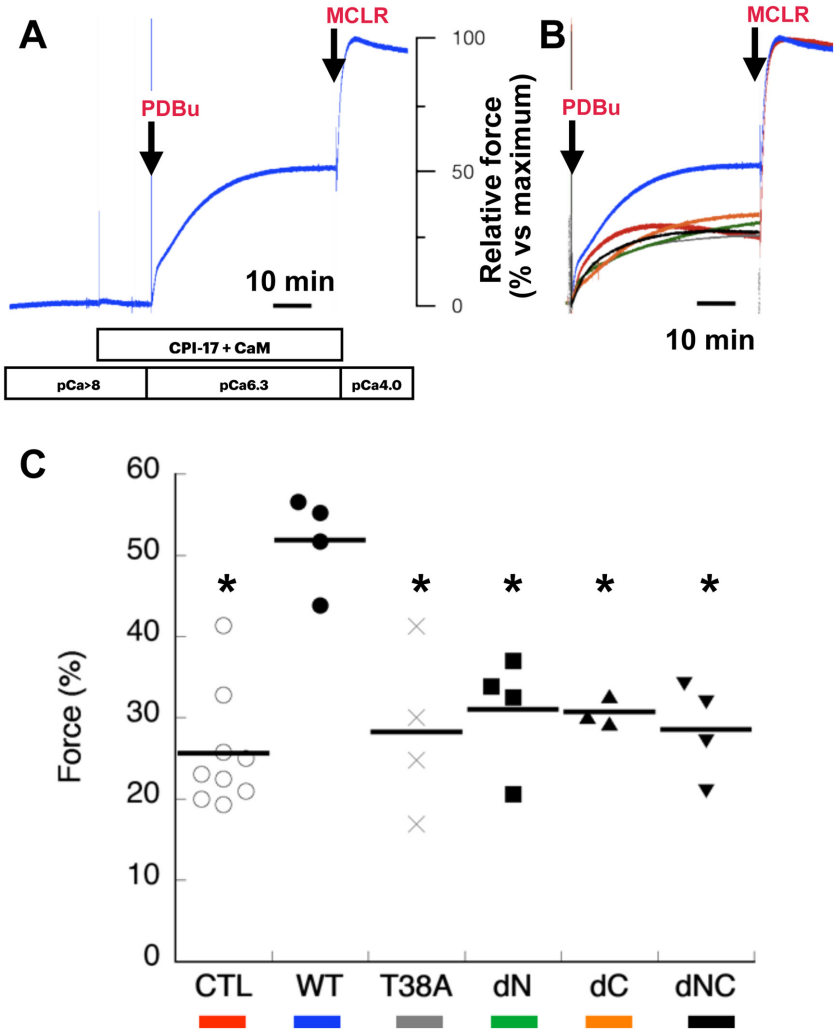

Fig. 2. Force production of permeabilized strips induced by recombinant CPI-17 proteins.
(A) Representative relative force trace of the permeabilized smooth muscle strips. Permeabilized smooth muscle strips was doped with recombinant CPI-17 (10 μM) in the G1 solution, and then stimulated with the pCa6.3 solution including 3 μM PDBu. After the force reached to plateau, the maximum extent of force was induced with the pCa4.5 solution including 1 μM MCLR. Bar indicates 10 min. (B) Representative force traces in the presence of WT (blue), dN (green), dC (orange), dNC (black) and T38A (gray). Red line indicates force trace without recombinant CPI-17. (C) Dot plot of the Ca^{2+} sensitization force induced by recombinant CPI-17. Mean values of relative extent of force development are shown in horizontal lines. * indicates $p<0.05$ by Dunnett's test vs. WT.

Discussion

In this work we showed that the unstructured N- and C-terminal tails of CPI-17 are required for inducing the Ca^{2+} sensitization force. Our previous data and a recent report of inhibition assay using purified myosin phosphatase suggested that these segments at both ends were negligible for the inhibitory potency (25, 33). This discrepancy suggests multiple factors that contribute to the full potency of CPI-17 producing the Ca^{2+} sensitization signaling in smooth muscle cells.

N-terminal deletion of CPI-17 diminished phosphorylation at Thr38 (Fig. 3) and the binding to myosin phosphatase (Fig. 4), both of which likely cause the decreased potency inducing Ca^{2+} sensitization force. Zem-

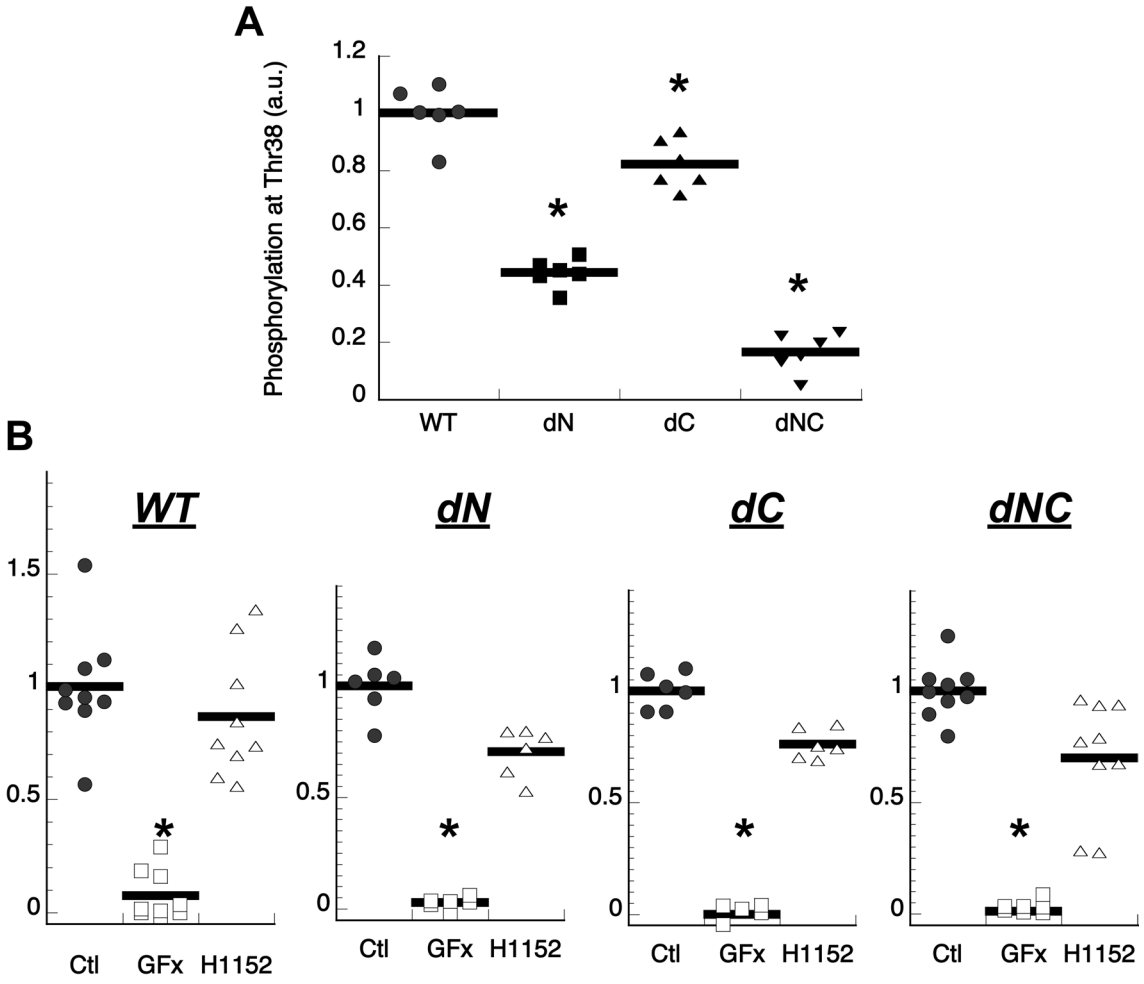

Fig. 3. Phosphorylation of recombinant CPI-17 by kinases in smooth muscle extracts.
(A) Recombinant CPI-17 proteins were phosphorylated in the presence of fundus smooth muscle extracts and subjected to phospho-dot blot assay. Densitometric data of each dot were normalized against the values of the WT protein. Mean values of relative extent of phosphorylation are shown in horizontal lines. * indicates $p<0.05$ by Dunnett's test vs. WT. (B) Phosphorylation of each protein was conducted in the presence of 3 μM GFx and 1 μM H1152. * indicates $p<0.05$ by Dunnett's test vs. control without antagonist (Ctl).

lickova et al. reported that CPI-17 directly binds to the C1 regulatory domain of PKC isoforms (34). Although the docking site of CPI-17 has yet to be identified, CPI-17 N-terminal tail possibly functions as a scaffold against PKC and facilitates phosphorylation at Thr38. The N-terminal loop of CPI-17 is also required for binding to myosin phosphatase. We have shown that the phosphorylated Thr38 directly docks at the active site of PP1 in the myosin phosphatase complex (16, 35), and a sulphonyl group failed to mimic the phosphate group (11). In addition, based on the computer simulation model of the myosin phosphatase and CPI-17 complex, the segment between amino acid 22–30 of CPI-17 is close proximity to MYPT1, the regulatory subunit of myosin phosphatase, and indeed the segment is capable of binding to MYPT1 (24). We presume that the N-terminal 1–21 tail likely provides further contact sites and/or stabilizes the binding surface of CPI-17 to myosin phosphatase in smooth muscle.

We are puzzled about functions of the C-terminal tail of CPI-17. The CPI-17-mediated Ca^{2+} sensitization force clearly requires an intact C-terminal tail (Fig. 2). PKC phosphorylated dC-CPI-17 as well as WT. Thus, the loss of affinity to myosin phosphatase (Fig. 4) seems to be the main factor for the decreased potency to

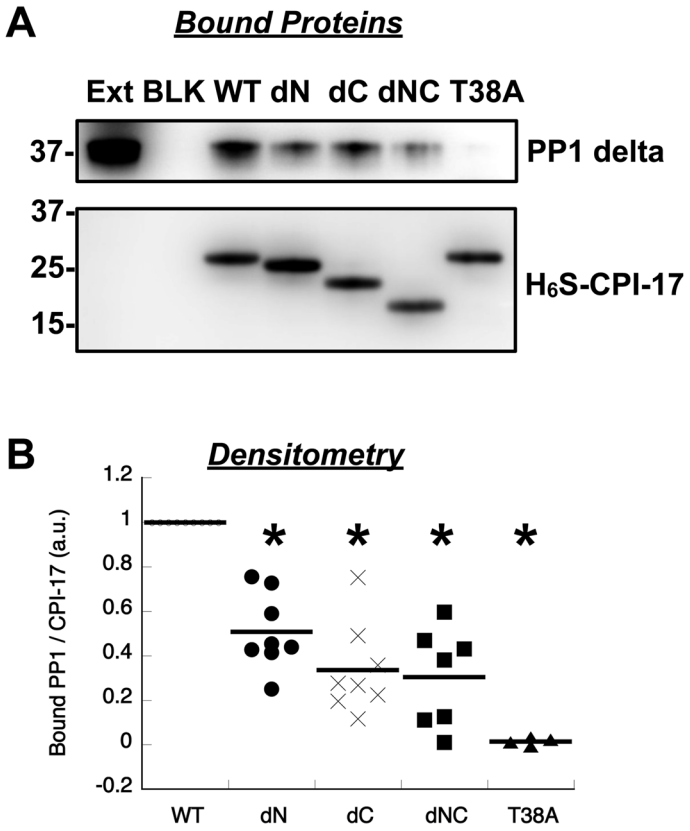

Fig. 4. Binding of CPI-17 with myosin phosphatase in smooth muscle extracts.
Pulldown assay with recombinant CPI-17 proteins were conducted using smooth muscle cell extracts as a source of the myosin phosphatase. Myosin phosphatase bound to the resins coupled with CPI-17 proteins was detected by immunoblotting using anti-PP1 delta antibody (A). Each densitometric value of the bound PP1 delta was normalized by that of the extent CPI-17 on the beads. Mean values of relative binding of PP1 are shown in horizontal lines (B). Ext; an input sample subjected to the pulldown assay, BLK; blank beads without protein. Numbers indicate molecular weight in kDa. * indicates $p<0.05$ by Dunnett's test vs. WT.

induce the Ca^{2+} sensitization force. However, our previous data shows that phosphorylated dC-CPI-17 inhibits purified myosin phosphatase as well as WT, suggesting that the binding of phospho-Thr38 to the active site of myosin phosphatase is independent of the C-terminal tail (22). The intact inhibitory potency of dC-version agrees with the 3D structural model of the phospho-CPI-17-myosin phosphatase complex, in which the C-terminal tail is distal to the contact site. It should be noted that both the inhibition assay and the 3D structural model were conducted using the myosin phosphatase that lacks the C-terminal domain of MYPT1. CPI-17 C-terminal tail is possibly needed for inhibition of the physiologic myosin phosphatase complex with intact MYPT1 and M20 accessary subunit that binds to the MYPT1 C-terminal region. Multiple structural elements of CPI-17 including the unstructured N-/C-terminal tails are apparently required for the physiologic interaction with the intact myosin phosphatase inducing the Ca^{2+} sensitization force of smooth muscle. Interaction of CPI-17 with the intact myosin phosphatase complex may define the potency of CPI-17 in smooth muscle and it deserves further investigation. We should also note that CPI-17 N-/C-terminal tails include multiple phosphorylation sites. Although known phosphorylation sites, Ser12 (36) and Ser128 (37), are not conserved among species, the functions of the N-/C-terminal tails are possibly regulated through phosphorylation at conserved Ser/Thr residues in these regions.

Amino acid sequences of the CPI-17 central four-helix bundle, named PHIN domain, as well as the N-/C-terminal tails are conserved among mammals (Fig. 1). On the other hand, the sequence of the N- and C-terminal tails of human CPI-17 seems to be distinguishable from the closest homolog of birds and fishes, such as small tree finch (Camarhynchus parvulus) and zebrafish (Fig. 1). The N-/C-terminal unstructured tails of CPI-17 will provide evolutionary insights into the Ca^{2+} sensitization signaling of smooth muscle. Lang et al. reported that the recombinant zebrafish CPI-17 paralogs lacking N-/C-terminal tails inhibit the myosin phosphatase in HeLa cells (25). We should note differences in the conditions of the assays. The level of the ectopic CPI-17 transiently expressed in HeLa cells is probably higher, compared with our myograph experiments in which a physiologic concentration of CPI-17 (10 μM) (33) was doped into permeabilized smooth muscle.

Because CPI-17 N-/C-terminal tails are required for producing the Ca^{2+} sensitization in smooth muscle, other members of the CPI-17 family, PHI-1 (PPP1R14B), KEPI (PPP1R14C), and GBPI (PPP1R14D), whose N-/C-terminal tails are significantly different each other (Fig. 5, top), unlikely mimic the role. For example, in current genomic database with 83 avian species (Fig. 5), we found only a few avian homologs of human CPI-17 (PPP1R14A) and PHI-1, whereas KEPI-like genes are detected in 71, and GBPI-like genes are found in 34 species (Fig. 5, bottom). The data strongly suggest that CPI-17 pathways are limited in a few species of birds, unlike KEPI and GBPI. Indeed, no CPI-17 homologs are detected in arterial smooth muscle of farm chicken that failed to respond to PDBu stimulation (38). CPI-17 signaling seems to be compensated in most avian species

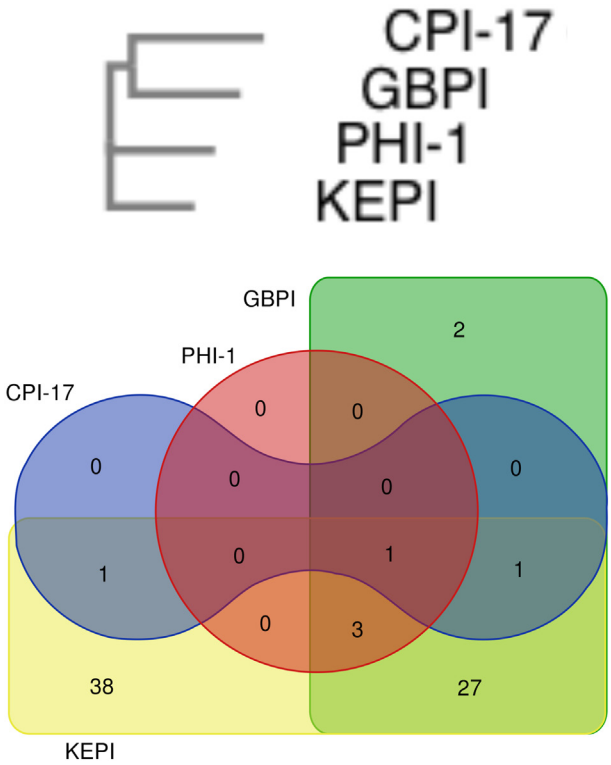

Fig. 5. Homologs of the CPI-17 family in avian species.
BLASTP searches for avian homologs of CPI-17, PHI-1, KEPI and GBPI in GenBank were conducted using each human sequence as a bait. For CPI-17, sequences with over 80% identity and E value of >6e-52 were considered as avian CPI-17 homologs that include small tree finch (Camarhynchus parvenus), lance-tailed manakin (Chiroxiphia lanceolate) bald eagle (Haliaeetus leucocephalus). Phylogenic tree (top) indicates similarity of four CPI-17 family members of small tree finch. Venn diagram (bottom) indicates distribution of the homologs of avian species.

through unknown mechanisms. On the other hand, a CPI-17 homolog was detected in pigeon smooth muscle and it mediates PDBu stimulation, indicating CPI-17-mediated Ca^{2+} sensitization pathways in smooth muscle (38). Thus, none of PHI-1, KEPI or GBPI can compensate the function of CPI-17. The N-/C-terminal tails of the CPI-17 family will provide new clues for dissecting regulatory circuits of cellular PP1 pathways.

Conflict of Interest

No conflict of interest needs to be disclosed.

Acknowlegdement

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