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Biochemical and Biophysical Research Communications 316 (2004) 39–47
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Association of CPI-17 with protein kinase C and casein kinase I☆

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Received 25 November 2003

We dedicate this paper to the memory of our colleague Franz-Josef Johannes, whose contributions to protein kinase C research will be greatly missed.

Abstract

The protein kinase C-potentiated inhibitor protein of 17 kDa, called CPI-17, specifically inhibits myosin light chain phosphatase (MLCP). Phosphorylation of Thr-38 in vivo highly potentiates the ability of CPI-17 to inhibit MLCP. Thr-38 has been shown to be phosphorylated in vitro by a number of protein kinases including protein kinase C (PKC), Rho-associated coiled-coil kinase (ROCK), and protein kinase N (PKN). In this study we have focused on the association of protein kinases with CPI-17. Using affinity chromatography and Western blot analysis, we found interaction with all PKC isotypes and casein kinase I isoforms, CKIα and CKIε. By contrast, ROCK and PKN did not associate with CPI-17, suggesting that PKC may be the relevant kinase that phosphorylates Thr-38 in vivo. CPI-17 interacted with the cysteine-rich domain of PKC and was phosphorylated by all PKC isotypes. We previously found that CPI-17 co-purified with casein kinase I in brain suggesting they are part of a complex and we now show that CPI-17 associates with the kinase domain of CKI isoforms.

CPI-17 has been shown to inhibit myosin light chain phosphatase (MLCP) [1] which is a trimeric protein complex containing a phosphatase 1 catalytic subunit (PP1c). This phosphatase plays a pivotal role in smooth muscle contraction by regulating the Ca^{2+} sensitivity of MLC phosphorylation [2]. Protein phosphatase I (PP1) is a serine/threonine phosphatase involved in many cellular functions such as muscle contraction and cell division [3]. The activity of PP1 is negatively regulated by a number of other proteins that include inhibitor-1, inhibitor-2, DARPP-32, NIPP-1, RIPP-1 [4], and KEPI [5]. CPI-17 has also been shown to be involved in the regulation of cell morphology, re-organisation of microfilaments, and cell spreading [6]. Originally, CPI-17 was thought to be smooth muscle specific, but is also expressed in human platelets [7] and we and others found it in brain [8,9] where it may play a role in cerebellar long-term synaptic depression [9].

CPI-17 is phosphorylated on Thr-38 in vivo, thus increasing its ability to inhibit PP1c 1000-fold [1]; CPI-17 was originally shown to be phosphorylated by PKC in vitro. However, many other kinases phosphorylate CPI-17 on Thr-38, including protein kinase N (PKN) [10], Rho-associated coiled-coil kinase (ROCK) [11], HeLa zipper-interacting protein (ZIP)-like kinase [12], integrin-linked kinase (ILK) [13], and p21-activated protein kinase (PAK) [14].

Agonist stimulation of vascular smooth muscle leads to CPI-17 phosphorylation on Thr-38 resulting in increased vascular smooth muscle contractility [15]. By contrast, CPI-17 dephosphorylation in arterial smooth muscle occurs during nitric oxide induced relaxation

☆ *Abbreviations:* CKI, casein kinase I; CPI-17, protein kinase C-potentiated inhibitor of 17kDa; GST, glutathione *S*-transferase; HMG1, high-mobility group 1; MLCP, myosin light chain phosphatase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PKC, protein kinase C; PKN, protein kinase N; PP1, protein phosphatase 1; ROCK, Rho-associated coiled-coil kinase; VAMP, vesicle-associated membrane protein, C1, cysteine-rich domain.

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doi:10.1016/j.bbrc.2004.02.014

[16]. Recombinant CPI-17 phosphorylated on Thr-38 induced MLC phosphorylation and potentiated PKC-induced contraction of arterial smooth muscle strips [17,18]. In addition, Eto et al. [19], showed that histamine-induced vasoconstriction involves phosphorylation of CPI-17 by PKCα and δ, indicating that PKC may be the kinase that phosphorylates CPI-17 on Thr-38 in vivo. This is supported by the finding that this phosphorylation by PKC leads to myosin phosphatase inhibition in human platelets [7].

PKCs comprise a family of serine/threonine kinases classified into different groups according to activation parameters: conventional PKCs (PKC α, βI, βII, and γ), novel PKCs (PKCδ, ε, η, and θ), atypical PKCs (PKCζ and ι/λ), and PKD/PKCμ, which can be considered as a separate class [20]. Among many other functions, PKC isoforms are involved in the regulation of muscle contraction through inhibition of PP1c [3].

We have previously shown that CPI-17 co-purified with CKIα as part of a protein complex from mammalian brain [8]. CKIα is a member of the CKI family of serine/threonine kinases which have been shown to be involved in various biological functions such as membrane trafficking [21–23], RNA processing [24], and cell cycle progression [25]. We have now further characterised the interaction between CPI-17 and CKI.

In this report, we demonstrate that several PKCs and CKI isotypes from brain specifically bind to CPI-17. The CPI-17 binding sites were mapped to the C1 domain of PKC and to the kinase domain of CKI. We further demonstrate that CPI-17 serves as a substrate for all PKC classes.

Materials and methods

Plasmids. The GST-fusion construct of human CPI-17 was produced by cloning in pGEX4T1 [26]. GST-CPI-17 deletion mutant comprising residues 1–120 was created by amplifying the CPI-17 cDNA by polymerase chain reaction (PCR) using two oligonucleotides (5′-GCAGGATCCATGGCAGCTCAGCGGCTGGGC-3′) to create a 5′ *Bam*HI site and (5′-CACGAATTCTCACCTGTGGAGGCCTT GAAGCTTTC-3′) to create a 3′ *Eco*RI site (both are underlined in sequences). Amplified cDNA was inserted into pGEX4T1 vector (Pharmacia). CPI-17 point mutants were generated using the site-directed mutagenesis kit (Stratagene). PKCα (pCO2) was from Peter Parker and PKCλ (pcDNA3) was from Terje Johansen. The construction of PKCμ mutants (wt, 1–340, ΔAD, ΔPH) in pcDNA3 has been described [27] and PKCζ/pcDNA3.1 was obtained from Feng Liu. HA-CKIα/pcDNA3 was obtained from Frank McKeon, CKIδ, $CKI\delta_{\Delta 317}$, $CKI\delta_{KD}$, $CKI\gamma_1$, and $CKI\gamma_3$ (all cloned in pET8c) were from Peter Roach, $CKI\gamma_2$ (pSV2Zeo) and CKIε (pSP72) were from Louise Larose. CKIε (pV405) plasmid was provided by David Virshup and was sub-cloned in pSP72 as described previously [21].

Recombinant protein purification. GST-fusion proteins were purified as previously described [21,28].

Affinity chromatography on a CPI-17 column. Glutathione–Sepharose (GSH) affinity columns with ~25 mg GST and GST-CPI-17 were constructed. Sheep brain (~80 g) was homogenised in lysis buffer (50 mM Tris–HCl, pH 7.4, 10% glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) containing EDTA-free protease inhibitor tablets (Roche). Triton X-100 was then added to a final concentration of 0.5% and the mixture was incubated at 4 °C for 2 h with constant agitation and clarified by centrifugation at 20,000*g* for 30 min at 4 °C. This was followed by ultracentrifugation at 100,000*g* for 1 h at 4 °C. The supernatant was cleared through a 0.45 μm filter and incubated with GST for 2 h at 4 °C with constant agitation. Centrifugation at 4000 rpm for 5 min at 4 °C followed. The supernatant was batch-incubated with the GST or GST-CPI-17 column overnight at 4 °C. Beads were then left to settle and the columns were washed with lysis buffer containing 0.5% Triton. The columns were subsequently washed with 4 × 15 ml of lysis buffer. The proteins were eluted with lysis buffer containing 1 M NaCl and concentrated on Centricon 10 (Millipore). Protein concentration was determined using the Bio-Rad protein assay. Samples were analysed by 12.5% or 10% SDS–PAGE, transferred onto nitrocellulose, and Western blotted using CKIα and PKCμ antibodies (Santa Cruz, both 1:250), CamKII, CKIε, PKCα, PKCε, PKCλ, ROCK (ROKα), and PKN (PRK1), Transduction Laboratories (all 1:1000); PKCζ antibody (W.J. van Blitterswijk, 1:1000) and HMG1 antibody (Pharmingen, 1:1000).

In vitro binding between purified PKC isoforms and CPI-17. GST, GST-14-3-3ζ, and GST-CPI-17 (5 μg) were incubated with human recombinant PKCα, ε, μ, and ζ (1 U, Calbiochem) in binding buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM DTT, 1% Nonidet P-40 [NP40], and 0.1% BSA) for 2 h at 4 °C. GSH beads were then added and incubated for a further 1 h. Bead precipitates were then washed four times with binding buffer and bound proteins were eluted using SDS sample buffer. The samples were analysed by 10% SDS–PAGE, transferred onto nitrocellulose, and Western blotted using PKC antibodies.

In vitro binding between purified CKI and CPI-17. GST, GST-centaurin-α_1, and GST-CPI-17 (5 μg) were incubated with 1 U recombinant CKI (0.2 μg; *Schizosaccharomyces pombe ski1*, Upstate Biotechnology) in binding buffer for 2 h at 4 °C. GSH beads were then added and incubated for a further 1 h. Bead precipitates were then washed four times with binding buffer and once with kinase buffer (50 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM DTT). The washed beads were incubated with kinase buffer (without NaCl) containing 40 μM of a CKI-specific substrate phosphopeptide (KRRALS(p)VASLPGL, where S(p) is phosphoserine) and 50 μM ATP including [γ-^{32}P]ATP (Amersham) in a final volume of 60 μl. Samples were incubated at 30 °C for 30 min and centrifuged (13,000 rpm) and 20 μl of the reaction mixture was spotted in duplicate on P81 paper squares (Whatman). The papers were washed five times with 1% aqueous phosphoric acid and radioactivity retained on the papers was quantified by liquid scintillation counting.

Phosphorylation of 14-3-3ζ by a kinase eluted from the CPI-17 column. Five micrograms each of GST and GST-CPI-17 column eluates was incubated with 5 μg GST, GST-14-3-3ζ or GST-14-3-3ζ T233A mutant in kinase buffer (50 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, and 50 μM ATP including [γ-^{32}P]ATP, Amersham) in a final volume of 40 μl. Reactions were performed at 30 °C for 30 min. The reaction was stopped with 1 ml binding buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM DTT, 1% NP40, and 20 mM EDTA). GSH beads (20 μl) were added and the samples were incubated for 1 h at room temperature. The beads were washed three times with binding buffer and incubated with SDS sample buffer. Samples were analysed by 12.5% SDS–PAGE. Gels were stained with Coomassie blue and autoradiographed.

In vitro transcription and translation and GST pull-down assays. CKIα, CKIδ, CKIε, $CKI\gamma_1$, $CKI\gamma_2$, $CKI\gamma_3$, and PKCα, λ, μ, and ζ were expressed in vitro using a T7 TNT coupled transcription/translation reticulocyte lysate (Promega, Madison, WI). The reactions (50 μl) were performed following the manufacturer's instructions using [^{35}S]methionine (Amersham) for 90 min at 30 °C. Samples were then diluted threefold with binding buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM DTT, and 1% Nonidet P-40 [NP40]) and

incubated for 15 min at 30 °C with 5 μg GST, GST-CPI-17, GST-CPI-17 1–120, GST-14-3-3ζ or GST-VAMP. GSH beads and binding buffer (300 μl) were added to the reactions and incubated at room temperature for a further 1 h. The beads were washed five times with 1 ml of binding buffer and electrophoresed on 15% SDS–PAGE. After staining/destaining, the gels were incubated for 30 min with Amplify (Amersham Pharmacia), dried, and exposed to film.

Phosphorylation of CPI-17 by PKC and CKI. CPI-17 (5 μg) was incubated with 1 U each of PKCα, PKCε, PKCμ, and PKCζ in a buffer containing 40 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 2 mM EGTA (for PKCα, 3 mM $CaCl_2$ was included), 30 μg/ml phosphatidylserine, 8 μg/ml diacylglycerol, and 50 μM ATP including [γ-^{32}P]ATP. The reaction was performed at 30 °C for 15 min in a final volume of 40 μl. The reactions were stopped by the addition of SDS sample buffer and analysed on 15% SDS–PAGE. Gels were stained with Coomassie blue and autoradiographed. Histone HIII (Sigma) was phosphorylated under the same conditions to provide a measure of the relative incorporation of ^{32}P by each of the PKC forms. Between four and six separate autoradiographs of three different assays were scanned by "Aida"v2.11 software and the SEM was calculated.

Phosphorylation of CPI-17 (5 μg) by CKI was attempted as previously described [28].

Results

CPI-17 associates with all PKC sub-types

Since phosphorylation of Thr-38 plays a key role in regulating CPI-17 activity, we tested whether the kinases (PKC, Rho kinase, and PKN) that have been shown to phosphorylate CPI-17 in vitro also interacted with CPI-17. To test this, we passed a brain extract through a GST-CPI-17 column as described in "Materials and methods." For the brain lysate approximately 225 mg of total protein was loaded on the affinity column and 22.4 μg binding proteins were eluted. An aliquot of the proteins that eluted was separated on a 10% SDS–PAGE gel, transferred onto nitrocellulose, and Western blotted with anti-PKCα, ε, λ, and ζ antibodies. Fig. 1A shows that PKCα (conventional PKC, cPKC), PKCε (novel PKC, nPKC), and PKCλ and ζ (atypical PKCs, aPKCs) specifically associated with CPI-17, since no PKCs were eluted from the GST control column. Fig. 1A also shows that a high level of PKCμ/PKD was present in the eluate. In contrast to PKC, the kinases ROCK and PKN did not interact with CPI-17 (data not shown). This suggests that PKC may represent the physiological kinase. In addition, if we compare the amount of kinase eluted from the column to that present in the brain extract, a higher level of aPKCs (PKCλ and ζ) was recovered from the CPI-17 affinity chromatography compared to cPKC (PKCα) and nPKC (PKCε). This indicated that atypical PKCs might have a higher affinity for CPI-17.

The association of PKCs with CPI-17 was verified in an independent system using in vitro transcription/translation assay. PKCα, λ, μ, and ζ were expressed and labelled with [^{35}S]methionine in a reticulocyte lysate, and incubated with GST, GST-14-3-3ζ, and GST-CPI-17. The pull-down assays (Fig. 2A) show that all these isoforms associated with GST-CPI-17.

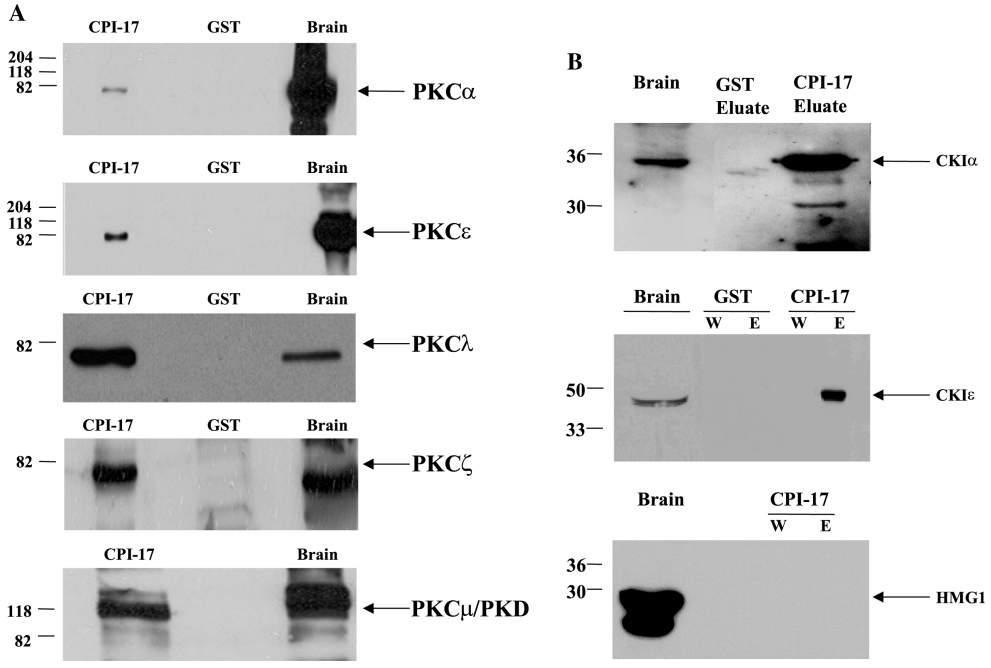

Fig. 1. Isoforms of PKC and CKI elute from the GST-CPI-17 column. (A) All PKC classes elute from the GST-CPI-17 column. Proteins eluted from the GST-CPI-17 (labelled "CPI-17") and the GST column ("GST") were separated on 10% SDS–PAGE, transferred onto nitrocellulose, and Western blotted with PKCα, ε, λ, μ, and ζ antibodies. A brain extract was used as a positive control for the antibodies. The positions of the molecular weight markers (kDa) are indicated. (B) CKIα and CKIε elute from the GST-CPI-17 column. GST-CPI-17 column eluate ("CPI-17") and a brain lysate extract ("Brain") were run on 12.5% SDS–PAGE, transferred onto nitrocellulose, and Western blotted with anti-CKIα, ε, and HMG1 antibodies as indicated. The positions of the molecular weight markers (kDa) are indicated, as are the positions of CKIα, CKIε, and HMG1.

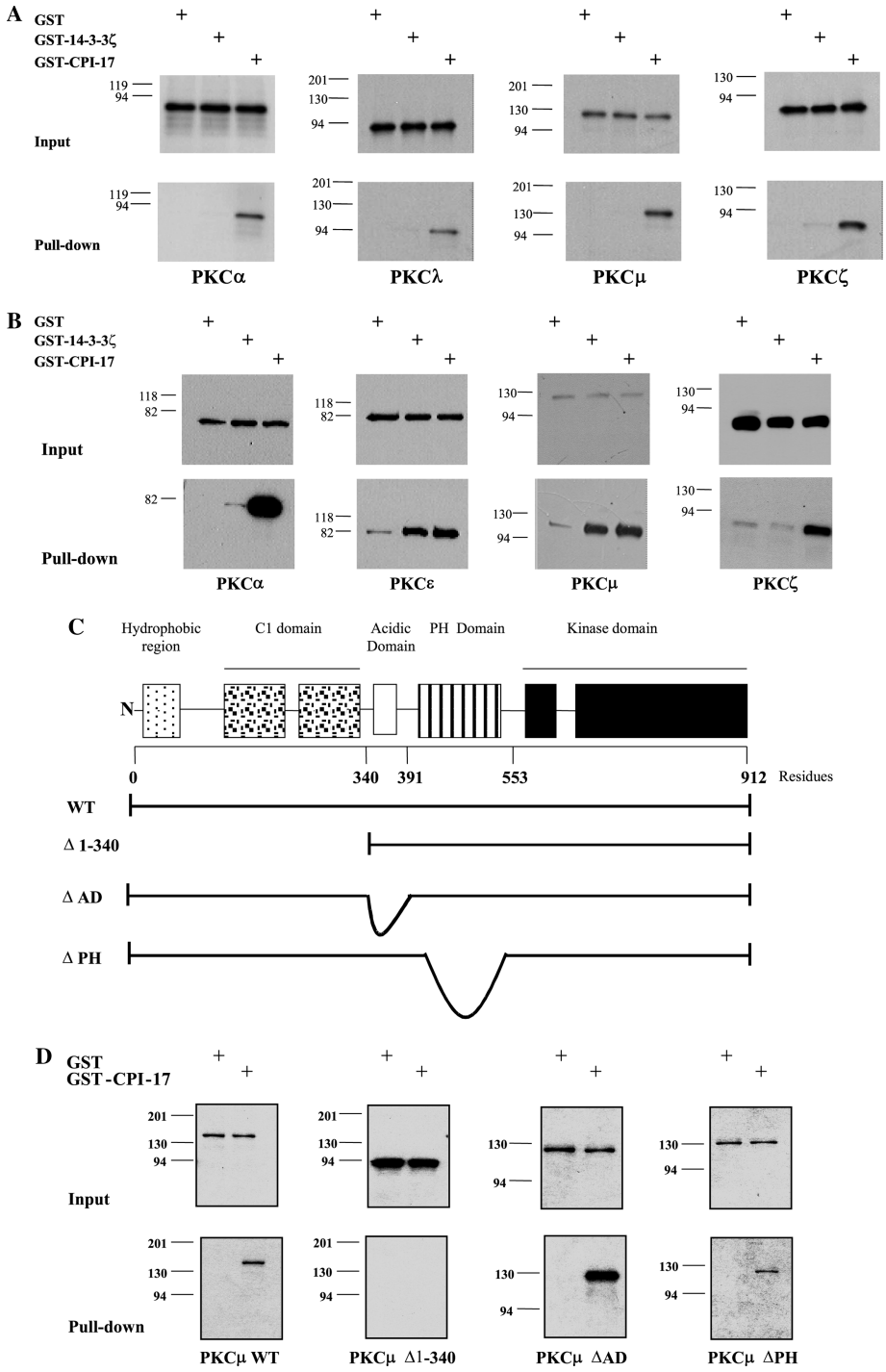

Fig. 2. Direct association of CPI-17 with PKC isoforms. (A) PKCα, λ, μ, and ζ were expressed and labelled with [^{35}S]methionine in a reticulocyte lysate and incubated for 15 min at 30 °C with GST, GST-14-3-3ζ, and GST-CPI-17 in the presence of 1% Nonidet P-40. GSH beads were added and incubated at room temperature for 1 h. Beads were washed and samples were analysed by 10% SDS–PAGE followed by autoradiography (panel Pull-down). A 2% aliquot of the lysate was loaded on the gel to visualise expressed PKCs (panel Input). The positions of the molecular weight markers (kDa) are indicated. (B) CPI-17 directly associates with PKC isoforms from all sub-families. GST, GST-14-3-3ζ, and GST-CPI-17 were incubated with human recombinant PKCα, PKCε, PKCμ, and PKCζ. A GST-pull-down assay was performed and the samples were analysed on 10% SDS–PAGE, transferred onto nitrocellulose, and Western blotted with PKCα, ε, μ, and ζ antibodies as indicated. Top panels represent a 2% aliquot of the assays to visualise the PKCs (panel "Input"). Bottom panels represent the GST-pull-down assays (panel "Pull-down"). The positions of the molecular weight markers (kDa) are indicated. (C) Functional domains of the PKCμ constructs. The deletion constructs were as follows: Δ1–340, deletion of residues 1–340; ΔAD, deletion of acidic domain and ΔPH, deletion of PH domain. (D) Association of CPI-17 with the C1 domain of PKC. PKCμ wt and PKCμ deletion mutants (Δ1–340, Δ336–391, and Δ417–553) were expressed in a reticulocyte lysate and incubated with GST and GST-CPI-17 as described in (A). GST-pull-down assays were analysed by SDS–PAGE and autoradiographed for ^{35}S (panel Pull-down). A 2% aliquot of the lysate was loaded on the gel (panel Input). The positions of the molecular weight markers (kDa) are indicated.

In order to determine whether the binding between CPI-17 and PKC was direct, GST, GST-CPI-17, and GST-14-3-3ζ were incubated with recombinant PKCα, PKCε, PKCμ, and PKCζ, and then pulled down with GSH beads (Fig. 2B). Western blot analysis was performed with the respective PKC antibodies because the small amount of PKC used in the experiment did not allow us to follow the association by Coomassie blue staining. The results showed that GST-CPI-17 directly bound to all PKC isoforms tested. In addition, 14-3-3ζ directly associated with PKCε and PKCμ, but not with PKCα nor PKCζ (Fig. 2B). Our results are in agreement with a reported interaction between 14-3-3ζ and PKCμ [27]. These data demonstrated direct binding between CPI-17 and PKC and also between 14-3-3 and PKCε and μ, although we could not detect binding between 14-3-3ζ and PKC in the IVTT experiments shown in Fig. 2A. The discrepancy between binding of PKCμ to 14-3-3 in Figs. 2A and B may be due to a lower sensitivity with the radioactive proteins than with the Western blots. In summary, using three different methods, we have shown that CPI-17 associates specifically and directly with all PKC isotypes. This suggested that CPI-17 associated with a domain common to all PKC isoforms, i.e., the cysteine-rich domain (C1) or the kinase domain.

CPI-17 binds to the C1 domain of PKCμ

We used the PKCμ isoform to map the CPI-17 binding site since a large amount of this kinase was recovered with CPI-17 and it appears to bind with a high affinity. PKCμ wt and PKCμ deletion mutants Δ1–340 (ΔC1), Δ336–391 (ΔAD), and Δ417–553 (ΔPH) (Fig. 2C) were expressed using [^{35}S]methionine and incubated with GST and GST-CPI-17. As shown in Fig. 2D, PKCμ wt bound to GST-CPI-17. Deletion of the acidic domain or the PH domain of PKCμ did not affect the binding to CPI-17. However, no binding was observed between CPI-17 and PKCμ Δ1–340 (Fig. 2D). According to these data amino acids 1–340 of PKCμ were necessary for the association to CPI-17, indicating that the C1 domain of PKCμ represented the target region for binding with CPI-17. This is not unexpected as several proteins have been described to be associated with the C1 region of PKC [27,29]. Since all PKC isoforms have at least one C1 domain [20], these results complement those in Figs. 2A and B showing that all PKC isoforms associated in vitro with CPI-17.

Specific and direct association of CPI-17 with CKI isoforms

CKIα phosphorylates 14-3-3ζ and τ on residue 233 [28] and also co-purifies with CPI-17 [8]. To confirm that CPI-17 associates with CKI, the fractions eluted from the CPI-17 affinity chromatography were incubated with 14-3-3ζ wt and 14-3-3ζ T233A mutant and subjected to an in vitro kinase assay. Only 14-3-3ζ wt, but not 14-3-3ζ T233A, was phosphorylated by a kinase present in the GST-CPI-17 eluate (Fig. 3A). As a control, 14-3-3ζ was not phosphorylated when incubated with the proteins eluted from the GST column. This result suggested that a CKI-like activity was eluted from the CPI-17 column. The elution of CKIα and CKIε/δ isoforms from the GST-CPI-17 column was then demonstrated by Western blot analysis (Fig. 1B).

To further investigate the association of CKI with CPI-17, CKIα, δ, ε, and γ_1, γ_2, and γ_3 were expressed and labelled with [^{35}S]methionine, and then incubated with GST and with GST-CPI-17. All the CKI isoforms tested bound to CPI-17. However, the association was stronger with CKIα, δ, and ε than with CKIγ_{1-3} (Fig. 3B). Therefore, it appeared that CPI-17 interacted preferentially with some CKI isoforms.

To investigate binding specificity between CPI-17 and CKI isoforms, CKIα and 14-3-3ζ were expressed and labelled with [^{35}S]methionine and incubated with GST and GST-CPI-17. GST-CPI-17 bound to CKIα but not 14-3-3 (data not shown). In addition, CKIδ was expressed and labelled with [^{35}S]methionine and incubated with GST, GST-CPI-17, GST-14-3-3ζ or GST-VAMP (vesicle-associated membrane protein). These results confirmed the selectivity of interaction between CKI and CPI-17. Interestingly, GST-14-3-3ζ also bound to CKIδ (Fig. 3C). This is the first demonstration that CKI isoforms bind to 14-3-3 although we have previously shown that 14-3-3ζ is phosphorylated by CKIα [28].

To determine whether the binding between CKI and CPI-17 was direct, recombinant yeast CKI (*ski1*) was incubated with GST, GST-CPI-17, and GST-centaurin-α_1 (as a positive control [21]). The GST-fusion proteins were pulled down with GSH beads and a kinase assay was performed using the CKI phosphopeptide substrate. Results showed that the yeast CKI directly associated with GST-centaurin-α_1 and GST-CPI-17 (Fig. 3D).

CPI-17 binds to the kinase domain of CKI

Since CPI-17 bound to all CKI isoforms tested (α, δ, ε, γ_1, γ_2, and γ_3), one can postulate that CPI-17 interacted with a region common to all members of the CKI family. CKI isoforms are characterised by a conserved kinase domain and by variable amino- and carboxyl-terminal tails. CKIα wt, CKI 17–325 (without the N-terminal tail), CKI 17–287 (KD, containing only the kinase domain), and CKIδ (Δ317) (containing the kinase domain) were expressed and labelled with [^{35}S]methionine and incubated with GST and GST-CPI-17. CKIα wt and the mutants CKIα 17–287 (KD) and CKIδ (Δ317), which contained only the kinase domain of CKI, all bound to CPI-17 (Fig. 3E). Our data

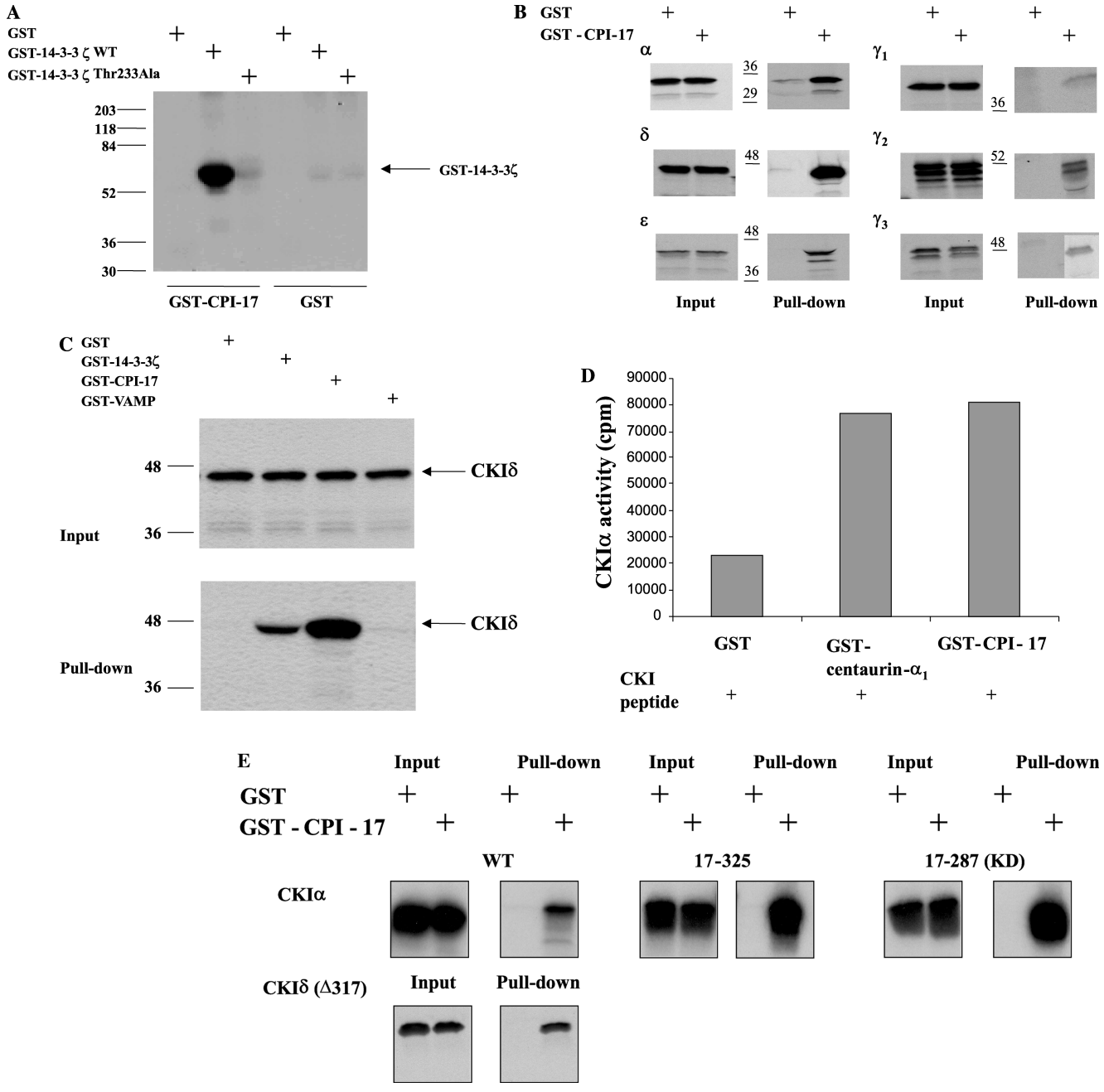

Fig. 3. Direct interaction between CPI-17 and CKI. (A) A CKI-like activity associates with CPI-17. Proteins eluted from the GST-CPI-17 column (3 first lanes) or from the GST column (3 right-hand lanes) were incubated for 15 min at 30 °C with GST, GST-14-3-3ζ wt, and GST-14-3-3ζ T233A in the presence of 50 μM [γ-^{32}P]ATP and kinase buffer. A GST-pull-down assay was performed and the samples were analysed by 12.5% SDS–PAGE and autoradiography. The positions of the molecular weight markers (kDa) are indicated, as is the position of the phosphorylated GST-14-3-3ζ. (B) CPI-17 associates with all CKI isoforms. CKIα, δ, ε, and γ_{1-3} isoforms (as indicated) were expressed in a reticulocyte lysate and incubated with GST and GST-CPI-17 as described in Fig. 2A. GST-pull-down assays were analysed by 12.5% SDS–PAGE and autoradiography ("Pull-down"). A 2% aliquot of the lysate was analysed ("Input"). The positions of the molecular weight markers (kDa) are indicated. (C) CKIδ associates specifically with CPI-17. CKIδ, expressed in a reticulocyte lysate, was incubated with GST, GST-14-3-3ζ, GST-CPI-17, and GST-VAMP as described in Fig. 2A. An aliquot of the lysate ("Input") and the GST-pull-down assays ("Pull-down") were analysed as in Fig. 2A. The position of the molecular weight markers (kDa) is indicated on the left and the position of CKIδ is shown on the right. (D) CPI-17 directly binds to CKI. Recombinant yeast CKI was incubated with GST, GST-centaurin-α_1, and GST-CPI-17 for 2 h at 4 °C. GSH beads were then added. Beads were washed and subjected to an in vitro kinase assay using the CKI-specific phosphopeptide substrate. The presence of ^{32}P incorporated into the peptide (cpm) was quantified by liquid scintillation counting and represents CKI activity associated with the beads. The results are representative of three different experiments. (E) Association of CPI-17 with the kinase domain of CKI isoforms. CKIα wt and CKIα mutants 17–325 (deleted of the specific N-terminus of CKIα), 17–287 (KD, comprising only the kinase domain), and CKIδ (Δ317) (kinase domain) were expressed in a reticulocyte lysate and incubated with GST or GST-CPI-17 as in Fig. 2A. An aliquot of the lysate ("Input") and the GST-pull-down assays ("Pull-down") were analysed as in Fig. 2A.

Table 1
Phosphorylation of CPI-17 by all PKC classes

PKC isoform	CPI-17 (average ± SEM)	Histone (average ± SEM)	Ratio of CPI-17/histone phosphorylation
α	6296 ± 386	5134 ± 307	1.23:1
ε	5402 ± 228	2948 ± 312	1.83:1
ζ	2782 ± 238	800 ± 329	3.48:1
μ/PKD	428 ± 83	115 ± 30	3.72:1

PKCα, ε, μ, and ζ were incubated with CPI-17 in the presence of 50 μM [γ-^{32}P]ATP and kinase buffer for 15 min at 30 °C. The samples were analysed on 15% SDS–PAGE followed by autoradiography. The equivalent region of the gel after incubation with PKC in the absence of CPI-17 was blank. The data are presented as an average of four to six determinations ± SEM from different exposures of autoradiographs from three different assays.

demonstrated that the kinase domain of CKI is sufficient for binding to CPI-17, complementing our data that all CKI isoforms associated directly with CPI-17.

Phosphorylation of CPI-17 by PKC isotypes

Since both PKC and CKI bind to CPI-17, we determined whether CPI-17 served as a substrate for these kinases. For that purpose, PKCα, ε, μ, and ζ were incubated with CPI-17 in the presence of 50 μM [γ-^{32}P]ATP and kinase buffer. The results indicated that all PKC isoforms were able to phosphorylate CPI-17 in vitro (Table 1). PKCα phosphorylated CPI-17 to a similar extent to PKCε and to a much greater extent than ζ and μ. By contrast, CPI-17 was not phosphorylated by CKI to any significant extent (data not shown).

Discussion

CPI-17 is phosphorylated by several kinases on Thr-38 in vitro, which increases the inhibitory potency of CPI-17 on PP1 by 1000-fold [1]. Among the kinases that we tested, which are known to phosphorylate CPI-17 in vitro on Thr-38, only PKC family members associated with CPI-17. This suggests that PKC may be the relevant kinase phosphorylating this site in vivo. A previous study has shown that CPI-17 is phosphorylated by c and n PKC isotypes (PKCα and δ) but not significantly by ROCK and PKN [19]. ROCK and PKN were not eluted from the CPI-17 column. In agreement with the affinity chromatography results, showing that PKC isoforms from all classes associated with CPI-17, this interaction was confirmed by GST-pull-down assays.

We show that the association between CPI-17 and PKC is direct, and our data suggest it occurs via the C1 domain of PKC. All PKC isoforms have at least one conserved C1 domain. The cysteine fingers in the C1 region of PKC provide a binding site for lipid second messengers as well as for regulatory proteins affecting kinase activity [20]. In atypical PKCs, this domain does not bind lipid but rather serves as a protein interaction domain [30]. Other proteins have been found to interact with the C1 domain of PKCμ, including 14-3-3τ [29] and Bruton's tyrosine kinase [27]. We also show an interaction between 14-3-3ζ with PKCμ and with PKCε in agreement with previous reports [29,31]. Having demonstrated that PKC isoforms from different sub-families bound to CPI-17 in vitro, we also show that isoforms (PKCα, ε, μ, and ζ) from the different PKC classes phosphorylated CPI-17 in vitro. Another study has shown that c and n PKC isoforms were both able to phosphorylate CPI-17 and that the latter was a more efficient kinase [19]. These authors used the same cPKC (PKCα) and another nPKC, PKCδ. However, in our study, there was a similar level of phosphorylation of CPI-17 by c and n PKC. The Table shows that PKCα and ε phosphorylated CPI-17 to a similar and greater extent than the other kinases, using 1 U of fresh PKC according to the vendor's specifications.

As well as distinct co-factor requirements, every class of conventional, novel, and atypical PKC has a different specificity for different substrates. For example, protamine sulphate is phosphorylated to a relatively similar extent by PKCα, ε, and ζ and has been used as "control" substrate [32] however it is a poor substrate for PKCμ/PKD [33]. We therefore used histone as a measure of the relative phosphorylation by the four forms of kinase, although it is known that phosphorylation of histone H III by PKCε, ζ [32], and μ [33] is relatively poor compared to phosphorylation by PKCα. When the level of phosphorylation of CPI-17 and histone is compared for each enzyme, it is seen that CPI-17 is phosphorylated to a relatively better extent than histone by PKCε, ζ, and μ.

We have previously found that CPI-17 co-purified with CKI from brain [8]. In this report, we confirmed the interaction between CPI-17 and CKI isoforms. We identified CKIα and ε in the eluate of the CPI-17 affinity column. GST-pull-down assays revealed that all six CKI isoforms (CKIα, CKIδ, CKIε, and CKIγ_1, CKIγ_2, and CKIγ_3) bound to CPI-17, suggesting that the association must be via a common binding site. CKI isoforms are characterised by a conserved kinase domain and by variable amino- and carboxyl-terminal tails [34] and we show in this report that CPI-17 binds to the kinase domain of CKI isoforms. However, we found that CPI-17 associated preferentially with CKIε/δ > CKIα > CKIγ_{1-3}. We have shown that centaurin-α_1 also

binds to the kinase domain of CKI [21] but in contrast to CPI-17, centaurin-α_1 interacted to a similar extent with all CKI isoforms [21]. Although we have shown a direct interaction between CPI-17 and CKI, CPI-17 (like centaurin-α1 [21]) is not a significant substrate for this kinase. It has been shown that CKI is a dual specificity protein kinase [35] but since CPI-17, even when subjected to prior phosphorylation on Thr-38 (data not shown), was not a significant substrate for CKIα, we can rule out tyrosine 41 as a site for this kinase.

CPI-17 is composed of a novel domain also found in LIMK-2 and in another PP1 inhibitor PHI-1 and of a unique C-terminal region (residues 121–147) [26]. This unique region is not important for binding either to CKI or to PKC (data not shown). Therefore, the similarity in the conserved domain in CPI-17, PHI-1 and LIMK-2 would suggest that PHI-1 and LIMK-2 might also bind CKI and PKC. Indeed, PHI-1 has been shown to be phosphorylated by PKC [36].

We have also identified by mass spectrometry a number of novel potential CPI-17-interacting proteins that elute from the CPI-17 column (unpublished data). These proteins include cytoskeletal proteins (β-actin, myosin heavy chain, microtubule-associated protein 1A, and dynactin I); the adaptor protein, beta-adaptin (involved in the formation of intracellular transport vesicles); the assembly protein AP50 (associated with clathrin coated vesicles); and the chaperone heat shock protein HSP70 (confirmed by Western blotting, unpublished data); and a RING finger protein, HAC1, indicating that CPI-17 might be involved in a large number of cellular processes such as cytoskeletal assembly, vesicular trafficking, and endocytosis and/or exocytosis in brain and that its role might not be limited to regulating MLCP activity during smooth muscle contraction. This is in agreement with a report showing that CPI-17 is involved in the regulation of cell morphology, the re-organisation of microfilaments, cell spreading, and platelet secretion [6,7].

In conclusion, CPI-17 associates directly with all PKC isoforms probably via their C1 domain, and with the kinase domain of CKI isoforms. CPI-17 was phosphorylated by all PKCs tested, but not by CKI.

Acknowledgments

We thank the following for providing constructs. Peter Parker (Cancer Research UK, Lincoln's Inn Fields); Terje Johansen (Biochemistry Department, University of Tromso), Feng Liu (Department of Pharmacology, UTHSCSA, San Antonio, TX), Frank McKeon (Department of Cell Biology, Harvard Medical School, Boston), Peter Roach (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis), Louise Larose (Department of Experimental Medicine, McGill University, Montreal), and David Virshup (Department of Oncological Sciences, University of Utah, Salt Lake City) for providing us with PKCα (pCO2) (P.P.), PKCλ (pcDNA3) (T.J.), PKCζ (pcDNA3.1) (F.L.), HA-CKIα (pcDNA3) (F.M.), CKIδ (pET8c), CKIδ Δ317 (CKIδ KD) (pET8c), $CKI\gamma_1$ (pET8c) and $CKI\gamma_3$ (pET8c) (P.R.), $CKI\gamma_2$ (pSV2Zeo) (L.L), and CKIε (pV405) (D.V.). We would also like to thank Cali Hyde (UCL) for CKI peptide synthesis and W.J. Blitterswijk for the PKCζ antibody. The authors are very grateful to Preeti Kerai and Alex Peden for critical reading of the manuscript. This work was supported by the Medical Research Council (to A.A. and T.D.) and the Faculty of Medicine Scholarship, University of Edinburgh (to E.Z.).

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