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A functional interaction between CPI-17 and RACK1 proteins in bronchial smooth muscle cells

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Abstract

CPI-17 is a phosphorylation-dependent inhibitor of smooth muscle myosin light chain. Using yeast two-hybrid system, we have identified the receptor for activated C kinase 1 (RACK1) as a novel interaction partner of CPI-17. The direct interaction and co-localization of CPI-17 with RACK1 were confirmed by immunoprecipitation and confocal microscopy analysis, respectively. An *in vitro* assay system using recombinant/purified proteins revealed that the PKC-mediated phosphorylation of CPI-17 was augmented in the presence of RACK1. These results suggest that RACK1 may play a role in PKC/CPI-17 signaling pathway.

Keywords

Yeast two-hybrid system; CPI-17; RACK1; Ca^{2+} sensitization

1. Introduction

The 17 kDa PKC-potentiated protein phosphatase 1 inhibitor protein (CPI-17), also called as protein phosphatase 1, regulatory (inhibitor) subunit 14A (PPP1R14A), is a phosphorylation-dependent inhibitor of smooth muscle myosin light chain phosphatase (MLCP). CPI-17 was originally discovered in porcine aorta smooth muscle extract [1], and the expression is highly restricted to smooth muscle tissues [2,3]. Its inhibitory effect on MLCP leads to an increased myosin light chain (MLC) phosphorylation, resulting in an enhancement of smooth muscle contraction under the constant intracellular Ca^{2+} concentration [3,4], so called Ca^{2+} sensitization of smooth muscle contraction.

It has been established that phosphorylation of CPI-17 is involved in the protein kinase C (PKC)-mediated Ca^{2+} sensitization of smooth muscle contraction [4,5]. The PKC/CPI-17-mediated inhibition of MLCP is now considered as one of the pathways for Ca^{2+} sensitization of smooth muscle contraction induced by an activation of G-protein coupled receptor [6]. On the other hand, agonist stimulation also caused a translocation of CPI-17 to plasma membrane in bronchial smooth muscle [7]. Interestingly, the agonist-induced translocation of CPI-17 occurred prior to its phosphorylation [7]. The results suggest that an unknown protein(s) might be involved in the interaction between PKC and CPI-17. The

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Conflict of interest statement

None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

current study was carried out to identify an interaction partner(s) of CPI-17 using yeast two-hybrid system.

2. Materials and methods

2.1. Yeast two-hybrid screening

Yeast two-hybrid analysis was carried out using the Clontech Matchmaker two-hybrid system 3 (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. For bait construction, full-length of mouse CPI-17 cDNA (GenBank Accession No. NM_026731) was obtained by RT-PCR using RNAs prepared from mouse lungs and following primer pairs: 5′-GCGGGTGACGGTCA-TATGGCTGCGCAGCGG-3′ as 5′-primer and 5′-GCGGGATCCGGGTG GGGCAGTGTGGGCCCGGT-3′ as 3′-primer. The PCR fragments were cloned into *NdeI* and *Bam*HI sites of pGBKT7 vector in frame with the GAL4 DNA-binding domain, and the resultant plasmid was named as pGBKT7-mCPI-17. The rat lung Matchmaker cDNA library cloned in frame with the GAL4 activation domain in the pACT2 vector was used as a prey. The bait pGBKT7-mCPI-17 was transformed into yeast strain AH109. The bait construct did not show any toxic effect and autonomous transcriptional activation on the host strain. The prey pACT2-library was then transformed into the bait-transformed AH109 cells, and the cells were incubated on minimal synthetic dropout medium for yeast (SD)/-His/-Leu/-Trp at 30 °C. The colonies grew on the SD/-His/-Leu/-Trp were then plated on SD/-Ade/-His/-Leu/-Trp/X-α-Gal and incubated at 30 °C. The plasmids of positive clones were isolated and transformed into *Escherichia coli* DH5α, and prey plasmids were recovered by ampicillin selection. The positive prey clones were identified by DNA sequencing using 5′-sequencing primer, 5′-CTATTCGATGATGAAGATACCCCACCAAACCC-3′, and 3′-sequencing primer, 5′-GTGAACTTGCGGGGTTTTTCAGTATCTACGAT-3′.

2.2. Immunofluorescence and confocal microscopy

Normal human bronchial smooth muscle cells (hBSMCs; Cambrex Bio Science Walkersville, Inc., Walkersville, MD) were maintained in SmBM medium (Cambrex) supplemented with 5% fetal bovine serum, 0.5 ng/mL human epidermal growth factor (hEGF), 5 μg/mL insulin, 2 ng/mL human fibroblast growth factor-basic (hFGF-β), 50 μg/mL gentamicin and 50 ng/mL amphotericin B. Cells were maintained at 37 °C in a humidified atmosphere (5% CO_2), fed every 48–72 h, and passaged when cells reached 90–95% confluence. The hBSMCs at passages 7–9 were seeded in 8-well Lab-Tek chamber slide glass (Nunc, Scotts Valley, CA) at a density of 3500 cells/cm^2 and, when 80–85% confluence was observed, cells were fixed with 10% formaldehyde in PBS (10 min) and permeabilized by incubation with 0.5% Triton X-100 in PBS for 10 min. After blocking with 5% skim milk in PBS for 1 h, the cells were incubated with goat anti-CPI-17 antibody and rabbit anti-RACK1 antibody (1:200 dilution in 1% skim milk–PBS, respectively) for 2 h. After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated anti-goat IgG antibody and Alexa Fluor 568-conjugated anti-rabbit IgG antibody (Life Technologies Corp., Carlsbad, CA) for 1 h. Confocal scanning analysis of the stained cells mounted with DABCO mounting medium (Sigma–Aldrich, St. Louis, MO) was carried out with RADIANCE 2100 confocal imaging system (Bio-Rad, Hercules, CA).

2.3. Co-immunoprecipitation and immunoblotting

Since the expressions of both CPI-17 and RACK1 were confirmed in the brain [8,9], the mouse whole brain was used to test for an association between endogenous CPI-17 and RACK1. In brief, tissues were homogenized in lysis buffer (10 mM Tris–HCl, pH7.5, 1 × protease inhibitor cocktail) in the presence or absence of detergent mixture (0.1% Nonidet P40 and 0.05% sodium deoxycholate). The tissue debris was removed by centrifugation at

16,100 × *g* for 10 min at 4 °C, and the resultant supernatant was incubated with protein G-agarose (Roche, Basel, Switzerland) to remove non-spe-cific absorption. After centrifugation (12,000 × *g*, 20 s at 4 °C), the resultant supernatant was incubated with anti-CPI-17 antibody (1:100) or its control IgG for 1 h at 4 °C on a rotary shaker. After addition of protein G-agarose, the mixture was then incubated overnight at 4 °C on a rotary shaker. Subsequently, immune complexes were washed with lysis buffer, boiled for 4 min in 2 × SDS sample buffer, and subjected to 15% SDS–PAGE. The proteins were then transferred to PVDF membrane, and RACK1 and CPI-17 were visualized using specific antibodies to each protein.

2.4. Functional association assay

To test for a functional association between CPI-17 and RACK1 proteins, the effect of RACK1 on the PKC-mediated phosphoryla-tion of CPI-17 was measured using the CycLex PKC super family assay kit (Cyclex, Co., Ltd., Nagano, Japan) according to the manufacturer's instructions with modification. In brief, the 96-well microtitre plates pre-coated with CPI-17 were incubated with 50 mU/mL of rat brain PKCs (Merck, Darmstadt, Germany) in the absence or presence of 4 μg/mL of recombinant RACK1 (Abnova, Walnut, CA) for 20 min at 30 °C. After a frequent washing, the wells were incubated with anti-phospho-Thr38-CPI-17 antibody for 1 h at room temperature. Subsequently, plates were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody for 1 h at room temperature. After a further washing, all plates were developed with tetramethylbenzidine (TMB) substrate solution (100 μL/well), stopped with 0.5 N H_2SO_4 (100 μL/well) and read at 450 nm using a Bio-Rad microplate reader (Bio-Rad).

2.5. Statistical analyses

All data were expressed as the mean with S.E. Statistical significance of difference was determined by unpaired Student's *t*-test (StatView for Macintosh ver. 5.0, SAS Institute, Inc., NC). A value of $P < 0.05$ was considered significant.

3. Results and discussion

To identify a novel protein(s) interacting with CPI-17, a rat lung cDNA library was screened using yeast two-hybrid system, since relatively high levels of CPI-17 mRNA are expressed in the airways of rats [10] and functional expression of CPI-17 protein has been suggested in these tissues [7,10–13]. As a result, 37 positive cDNAs were isolated that potentially bind to CPI-17. Among them, five cDNA clones had unannotated sequences. While an association of CPI-17 with PKCs has been suggested [14], none of the PKC iso-forms were found in the positive clones. DNA sequencing and basic alignment searches for the NCBI database revealed that one of the positive clones corresponded to Gnb2l1 (GenBank Accession No. NM_130734), also known as receptor for activated C kinase 1 (RACK1), a homolog of the β-subunit of heterotrimeric GTP-binding proteins [15]. RACK1 was originally identified on the basis of its ability to bind the activated form of protein kinase C (PKC), stabilize this protein and facilitate its trafficking within the cell [15]. Previous studies have shown that RACK1 plays a role in complex protein–protein interactions between signaling molecules such as integrins, phosphodiesterase 4D5, Src tyrosine kinase, RhoA small GTPase, as well as PKC [16,17]. RACK1 might thus be a multi-functional protein that plays regulatory roles in diverse signal trans-duction pathway.

To confirm the specific interaction between CPI-17 and RACK1 in mammals, the co-immunoprecipitation experiments were performed with proteins extracted from mouse whole brain. As shown in Fig. 1, both CPI-17 and RACK1 proteins were expressed in the brain lysates. Western blot analysis of the co-precipitant with anti-CPI-17 antibody revealed

no detectable band for RACK1 in the presence of detergent mixture (Fig. 1). However, a weak but distinct band for RACK1 was detected when the tissue homogenate was prepared in the absence of detergent mixture (Fig. 1). These findings indicate that CPI-17 interacts with RACK1 in mammalian cells, but the association might be weak and detergent-sensitive.

To further confirm the interaction between CPI-17 and RACK1 proteins, cultured human bronchial smooth muscle cells (hBSMCs) were immunostained with anti-CPI-17 and anti-RACK1 antibodies, and localization of these proteins was evaluated under a confocal microscope. The hBSMCs were used since functional expression of CPI-17 protein has been suggested in bronchial smooth muscle cells [7,10–13]. Consistent with previous our report using bronchial smooth muscle tissue [7], CPI-17 protein was expressed in the cytosol of hBSMCs (Fig. 2, left). Similarly, as reported in airway epithelial cells [18], RACK1 protein is also expressed mainly in the cytosol of hBSMCs (Fig. 2, middle). The merged image (Fig. 2, right) revealed that CPI-17 and RACK1 are colocalized in hBSMCs, indicating that CPI-17 interacts with RACK1 in hBSMCs.

To demonstrate the functional association between CPI-17 and RACK1 proteins, the effect of RACK1 on PKC-mediated phosphory-lation of CPI-17 was determined in the *in vitro* system using recombinant CPI-17 fragment, recombinant RACK1, and rat brain PKC proteins. As shown in Fig. 3, the system used detected the phos-phorylation of CPI-17 induced by PKC. The PKC-induced phosphor-ylation of CPI-17 was sensitive to GF109203X, an inhibitor for PKCs. The PKC-mediated phosphorylation of CPI-17 was augmented with statistical significance ($P < 0.05$) in the presence of recombinant RACK1 protein (Fig. 3), indicating that RACK1 facilitates the phosphorylation of CPI-17 mediated by PKC.

In conclusion, the current study revealed for the first time that RACK1 is one of the binding partners of CPI-17. The results also indicate that association of RACK1 with CPI-17 facilitates the phos-phorylation of CPI-17 mediated by PKC.

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Abbreviations

CPI-17	17 kDa PKC-potentiated protein phosphatase 1 inhibitor protein
PPP1R14A	protein phosphatase 1, regulatory (inhibitor) subunit 14A
RACK1	receptor for activated C kinase 1
MLC	myosin light chain
MLCP	MLC phosphatase

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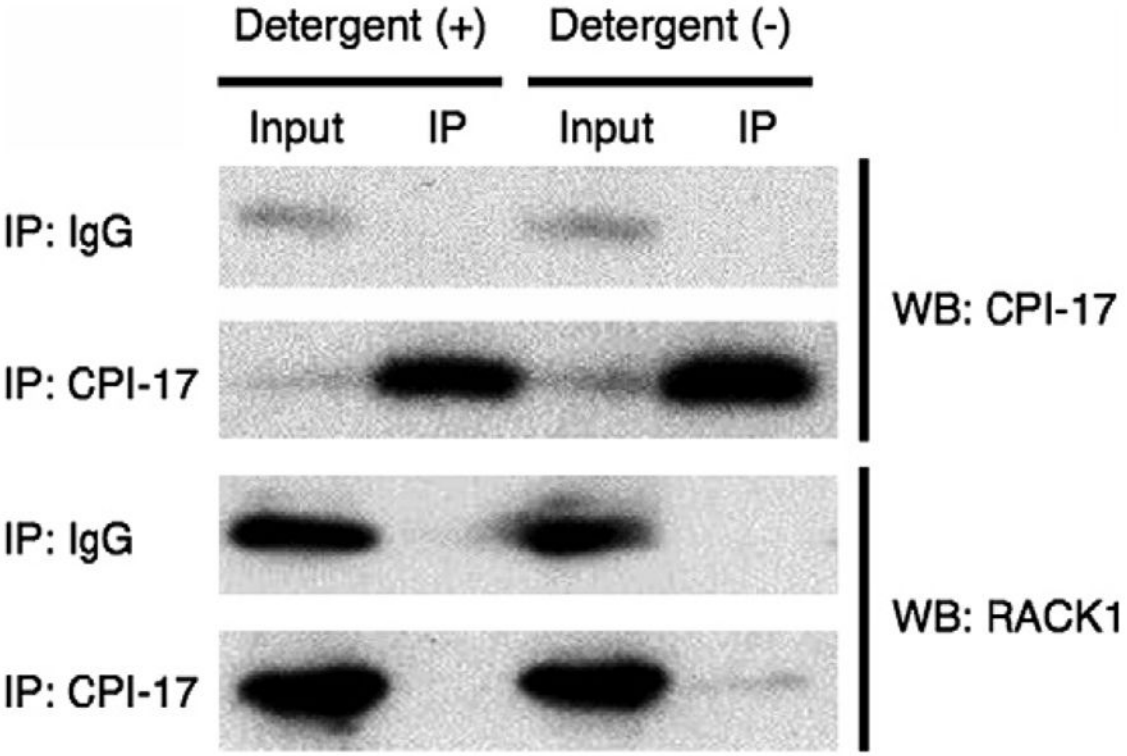

Fig. 1.

RACK1 co-immunoprecipitates with CPI-17. Mouse brain tissues were homogenized in lysis buffer in the presence (+) or absence (−) of detergent mixture (see Section 2.3). Then the tissue lysates were incubated with anti-CPI-17 antibodies for immunoprecipitation. Respective immunoprecipitants (IP) were divided into two aliquots and analyzed by Western blot (WB) with anti-CPI-17 or anti-RACK1 antibodies. The total lysates (input) were also analyzed. The blots are representative of at least four independent experiments.

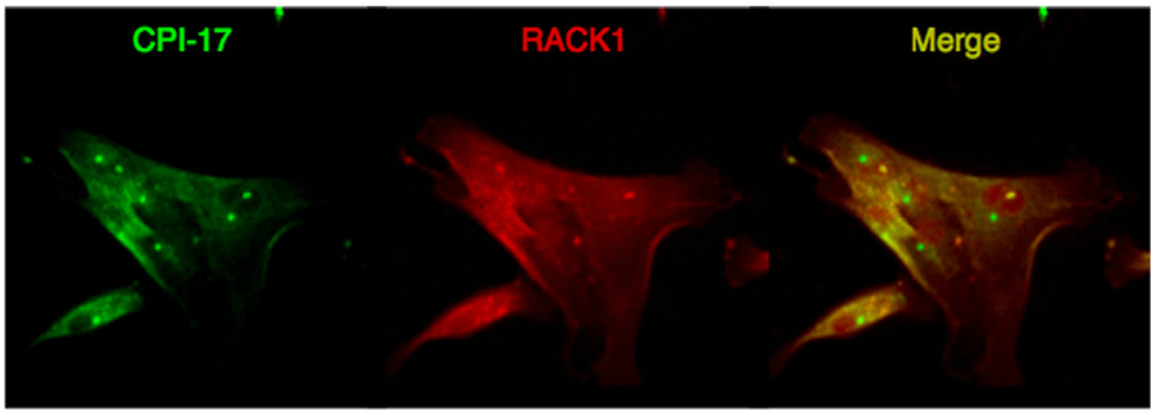

Fig. 2.

Confocal images of immunostaining in cultured human bronchial smooth muscle cells (hBSMCs). CPI-17 (left; green) and RACK1 (middle; red) proteins were visualized by immunostaining with anti-CPI-17 and anti-RACK1 antibodies, respectively. A merged image (right) shows regions of signal overlap appeared in yellow. The photos are representative of five independent experiments.

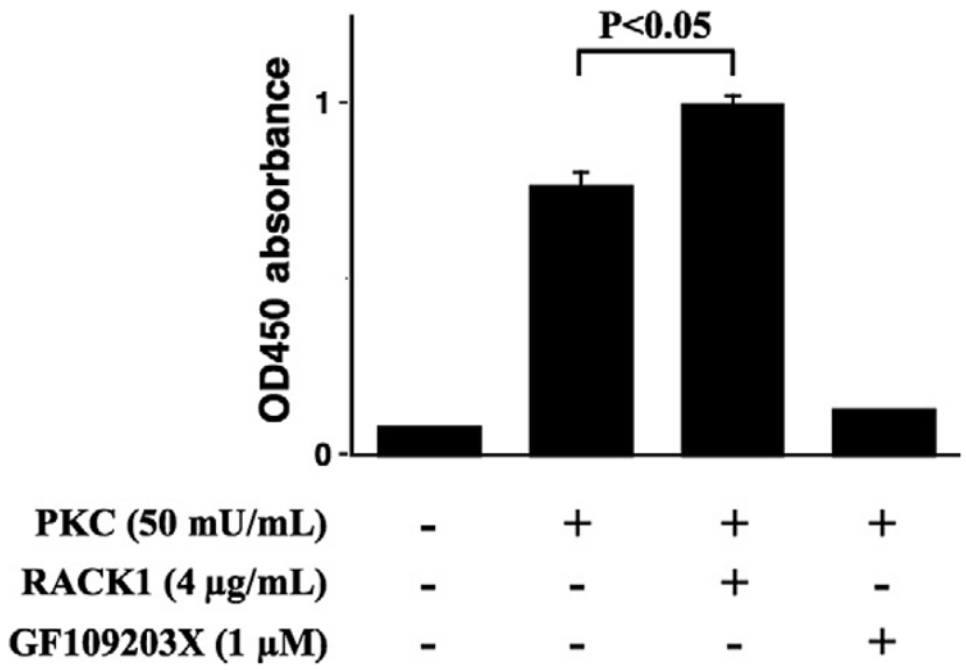

Fig. 3.

Augmentation of PKC-induced activation of CPI-17 by RACK1. The PKC-induced phosphorylation of CPI-17 was determined *in vitro* in the absence and presence of recombinant RACK1 protein using an assay system as described in Section 2.4. Each column represents the mean ± SEM from at least four independent experiments. Note that the PKC-induced phosphorylation of CPI-17, that is sensitive to GF109203X, was significantly augmented in the presence of RACK1 ($P < 0.05$ by Bonferroni/Dunn's test).