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Interaction of PKCα with the armadillo repeats facilitates the N-terminal phosphorylation of β-catenin

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ABSTRACT

Protein kinase Cα (PKCα) phosphorylates the Ser33/37/Thr41 residues of β-catenin, which lacks a typical PKCα canonical sequence, but little is known about its underlying mechanism. Here we showed that Ser33/Ser37/Thr41 of β-catenin fragments encompassing the armadillo repeats 1–5 (β-catenin$_{1-781}$, β-catenin$_{1-682}$, and β-catenin$_{1-422}$) are phosphorylated by PKCα whereas β-catenin$_{1-138}$ lacking these repeats is not phosphorylated. Binding-site analysis revealed that PKCα directly interacts with β-catenin through the sites on the armadillo repeats 1–5. In addition, axin fragments (365–500), which interacts with β-catenin through armadillo repeats 3–5, disrupted PKCα/β-catenin association and inhibited β-catenin phosphorylation by PKCα. In HEK293 cells, the levels of β-catenin$_{1-781}$ and β-catenin$_{1-422}$ were decreased whereas the amount of β-catenin$_{1-138}$ was unchanged by pharmacological stimulation of PKCα. Our results suggest that the association of PKCα with the armadillo repeats of β-catenin placed the Ser33/37/Thr41 residues of β-catenin in close proximity to PKCα, thereby facilitating PKCα-mediated β-catenin phosphorylation.

1. Introduction

Protein kinase Cα (PKCα), a serine/threonine protein kinase, plays important roles in the regulation of fundamental cellular processes, including cell growth and differentiation [1–3]. Increasing evidences suggest the involvement of PKCα-mediated signaling pathways in the regulation of the Wnt/β-catenin pathway. Wnt5a is involved in the mobilization of intracellular Ca^{2+} followed by the activation of PKCα and antagonizes the Wnt/β-catenin pathway [4,5]. Orford and colleagues reported that PKCα inhibitors cause β-catenin accumulation in human breast cancer cells [6]. Recently, we demonstrated that PKCα catalyzes the phosphorylation of β-catenin at Ser33/Ser37/Thr41, which marks it for ubiquitin-dependent proteasomal degradation [7], and showed that the activation of PKCα suppresses the proliferation of colon cancer cells by promoting β-catenin phosphorylation and degradation [8,9].

β-Catenin is involved in the regulation of cell–cell adhesion and in the control of the Wnt/β-catenin pathway [10,11]. The central region of β-catenin, which is composed of 12 highly ordered armadillo repeats, mediates the interaction with a variety of proteins including axin, adenomatous polyposis coli (APC), and T cell factor (TCF) family transcription factors. The C-terminal domain (CTD) of β-catenin is implicated in transcriptional functions, whereas the N-terminal domain (NTD) contains the Ser33/Ser37/Thr41 residues that regulate β-catenin protein stability through their phosphorylation [12]. Ser33/Ser37/ Thr41 residues of β-catenin do not display the typical features of canonical PKCα phosphoacceptor sites, specified by Ser/Thr either upstream or downstream positively charged residues [13]. Nevertheless, phosphorylation of these noncanonical sequences has been well established using bacterially expressed β-catenin and purified PKCα [7,9]. These results allow us to hypothesize that β-catenin may possess a functional element committed to its recognition by PKCα independent of the sequence encompassing the phosphoacceptor site (Ser33/Ser37/Thr41).

2. Materials and methods

2.1. Cell culture, transfection, and chemicals

HEK293 cell was obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 120 μg/ml

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penicillin, and 200 μg/ml streptomycin. Transfection was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A23187 was purchased from Sigma (St. Louis, MO) and dissolved in Me_2SO. CGK062 was synthesized as previously described [9] and dissolved in Me_2SO.

2.2. Plasmid and recombinant proteins

β-Catenin and its deletion mutant constructs were inserted into pGEX-4T-1 vector (Amersham Biosciences). GST-fusion proteins were expressed in BL-21 cell. The bacteria were grown in LB medium supplemented with 50 μg/ml ampicillin and induced with 0.4 mM IPTG for 4hr at 30 °C at an *A*600 of 0.7. The bacteria were pelleted resuspended in PBS containing protease inhibitor cocktail (Roche) and Sonicate at 30% setting for 10 min. Cell debris was removed by centrifugation and then GST-fusion proteins were isolated by affinity chromatography on glutathione-agarose (Sigma), eluted with 10 mM glutathione in PBS and stored at −20 °C.

2.3. In vitro kinase assay

Kinase assays were performed with purified GST-β-catenin$_{wt}$ and β-catenin deletion mutants were incubated with PKCα (Promega) at 30 °C for 30 min in kinase assay buffer (20 mM Mops, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, PKA/CaMK inhibitor cocktail, PKC lipid activator, magnesium/ATP cocktail). The proteins were analyzed by Western blotting.

2.4. GST pull-down assay

Purified β-catenin$_{wt}$ and β-catenin deletion mutants were mixed with PKCα (sigma) in 500 μl of ADBII (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 mM DTT, 1 mM $CaCl_2$) containing pre-equilibrated glutathione–Sepharose 4B beads, followed by incubation at 4 °C with gentle rotation. In competition assay, purified β-catenin$_{wt}$ were mixed with Axin (362–500) in 500 μl of ADBII containing pre-equilibrated glutathi-

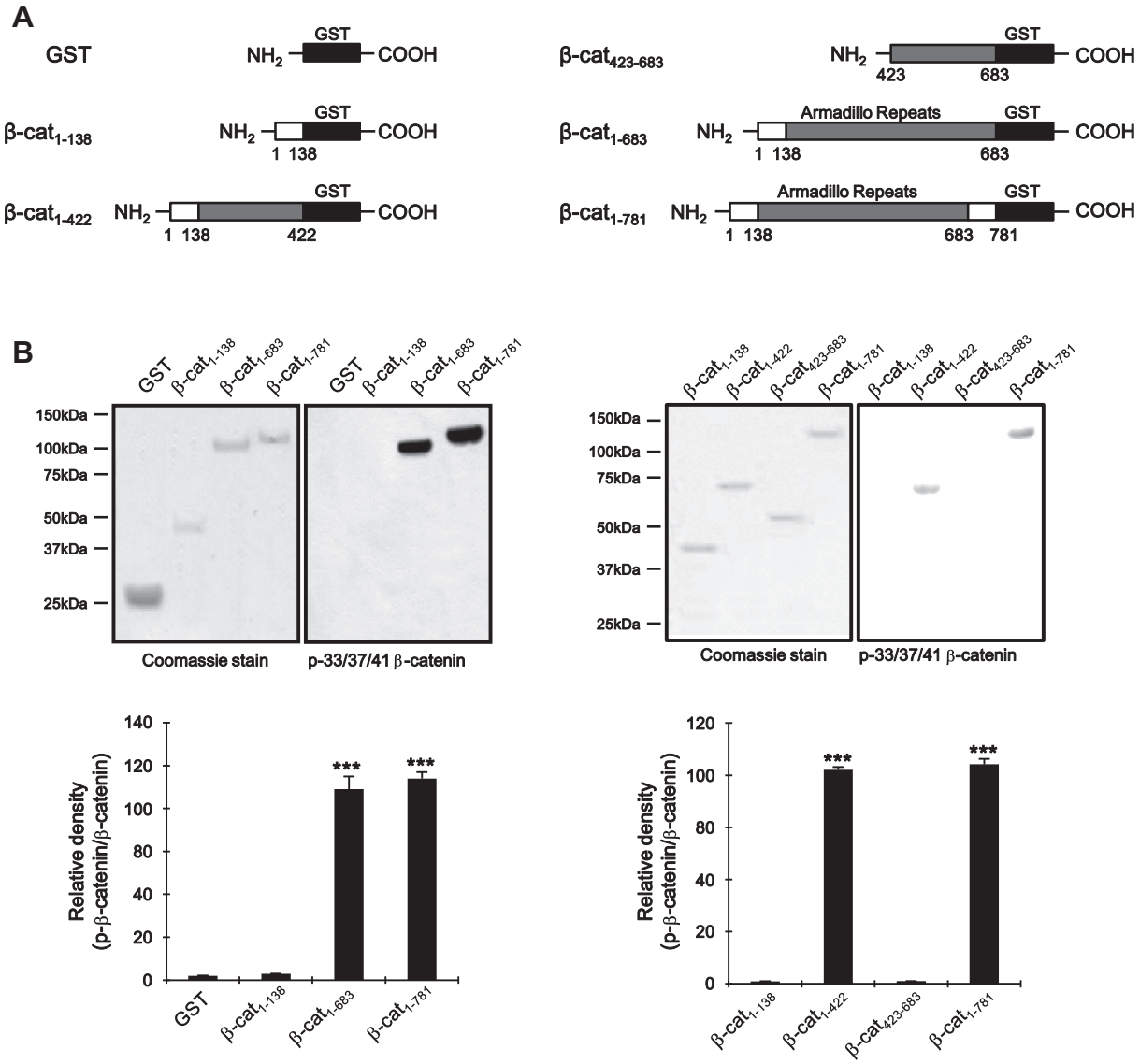

Fig. 1. The armadillo repeats 1–5 of β-catenin is required for PKCα-mediated β-catenin phosphorylation at Ser33/Ser37/Thr41. (A) Schematic representation of full-length β-catenin and deletion mutants that were used in this study. (B) GST-β-catenin$_{1-781}$ and β-catenin deletion mutants were incubated with purified PKCα, and then the samples were analyzed by Western blotting with anti-phospho-p33/37/41-β-catenin antibodies. The histogram shows the average volume density of three experiments, and the bars indicated standard deviations. $^{***}P < 0.001$, compared with the β-catenin$_{1-138}$ group.

one-Sepharose 4B beads, followed by incubation at 4 °C with gentle rotation. After 1 h, 100 ng of PKCα were added to the mixture and incubated 2 h at 4 °C. Beads were then centrifuged at 600×*g* for 1 min and washed five times with 1 ml of PBS. Proteins bound to the beads were resuspended in 50 μl 2 × LDS loading buffer and analyzed by Western blotting for anti-β-catenin, anti-PKCα antibodies and Coomassie staining.

2.5. Western blot analysis

Proteins were separated using 4–12% gradient of SDS–PAGE (Invitrogen) and transferred to PVDF membranes (GE Healthcare Life Sciences). The membranes were blocked with 5% nonfat milk and probed with anti-β-catenin, anti-phospho-β-catenin (Ser33/37/Thr41), anti-PKCα and anti-GFP antibodies (Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology) and visualized using the ECL system (Santa Cruz Biotechnology).

2.6. Statistical analysis

The student's *t*-test was used compare means between control and experimental groups. All experiments were performed three times. Statistical significance was set at $P < 0.001$, $P < 0.01$ or $P < 0.05$. Results presented as the mean ± SD.

3. Results and discussion

3.1. Armadillo repeats are required for PKCα-mediated β-catenin phosphorylation

To identify the functional element for PKCα-mediated β-catenin phosphorylation, we performed an *in vitro* kinase assay by using recombinant β-catenin and C-terminally truncated β-catenin fragments as substrates (Fig. 1A), and then analyzed the phosphorylation at Ser33/Ser37/Thr/41 by using a phospho-specific β-catenin antibody. As shown in Fig. 1B, PKCα readily phosphorylated Ser33/Ser37/ Thr41 on full-length β-catenin (β-catenin$_{1-781}$) and CTD deletion mutant (β-catenin$_{1-682}$). In addition, Ser33/Ser37/Thr41 phosphorylation of β-catenin$_{1-422}$, which lacks the entire CTD and the armadillo repeats 6–12, was efficiently catalyzed by PKCα (Fig. 1B, right panel). However, further deletion of the segment 132–423 containing the armadillo repeats 1–5 (β-catenin$_{1-138}$) resulted in the suppression of PKCα-mediated Ser33/Ser37/Thr41 phosphorylation (Fig. 1B), suggesting that this phosphorylation depends on the presence of the armadillo repeats 1–5.

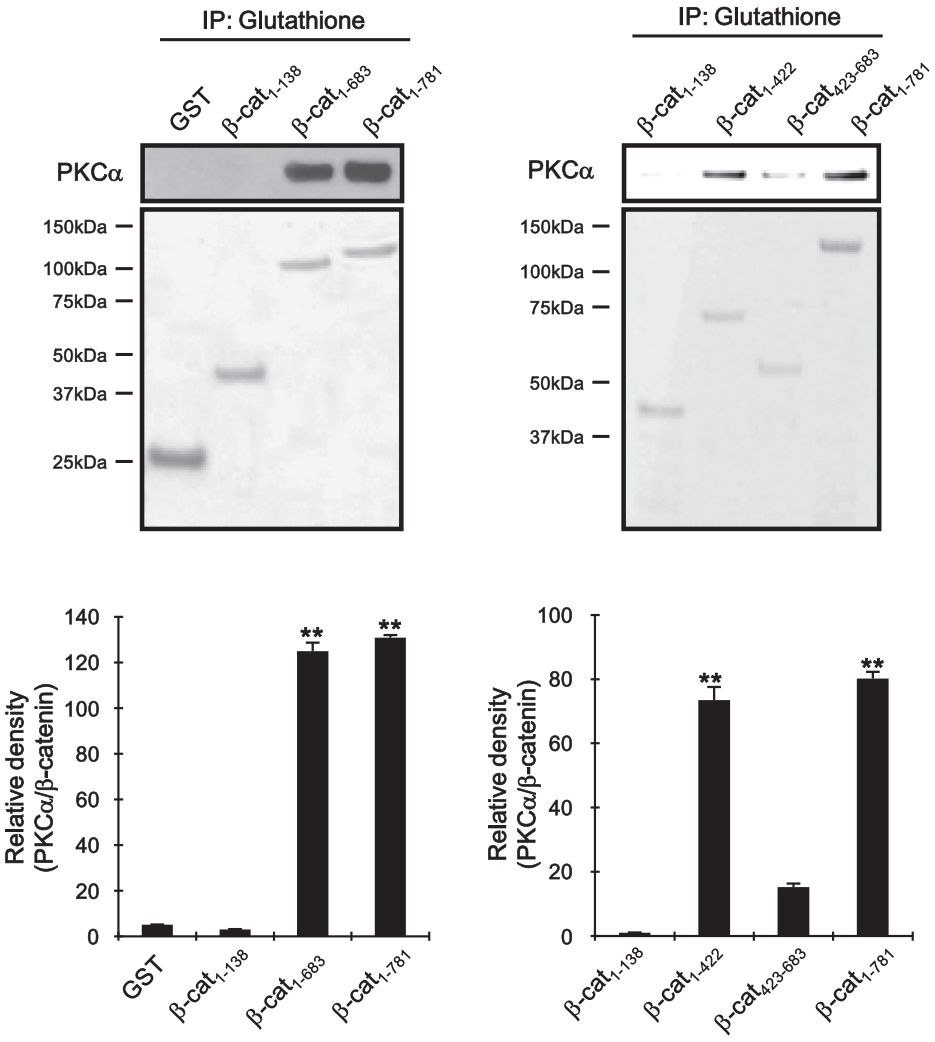

Fig. 2. PKCα interacts with the armadillo repeats 1–5 of β-catenin. GST-β-catenin$_{wt}$ and β-catenin deletion mutants were incubated with purified PKCα and then pull-down with glutathione–Sepharose bead. Unbound proteins were washed away, and the complexes were analyzed by Western blotting with anti-PKCα antibodies. The histogram shows the average volume density of three experiments, and the bars indicated standard deviations. $^{**}P < 0.01$, compared with the β-catenin$_{1-138}$ group.

3.2. PKCα interacts with armadillo repeats of β-catenin

It has been demonstrated that the formation of a kinase/substrate complex facilitates the phosphorylation of the substrate at noncanonical sites by the kinase [14]. Given that the high-affinity phosphorylation of the Ser33/Ser37/Thr41 residues of β-catenin by PKCα relies on the integrity of the NTD including the armadillo repeats 1–5, we tested weather these repeats directly contribute to the binding of PKCα. Glutathione S-transferase (GST) pull-down assay showed that β-catenin$_{1-781}$, β-catenin$_{1-682}$, and β-catenin$_{1-422}$, which all encompass the armadillo repeats 1–5, established stable interactions with PKCα (Fig. 2). In contrast, both β-catenin$_{1-138}$ and β-catenin$_{423-683}$, which lack the armadillo repeats 1–5, did not form a complex with PKCα (Fig. 2), indicating that PKCα interacts with the armadillo repeats 1–5 of β-catenin, but not with the NTD, which contains the phosphorylation sites for PKCα. These results indicate that the armadillo repeats 1–5 directly contribute to the binding of PKCα to β-catenin

3.3. Axin disrupts PKCα/β-catenin interaction

To confirm these observations with the binding-site mapping of PKCα on β-catenin, we examined whether recombinant axin (365–500), which binds to β-catenin at the armadillo repeats 3–5 [15], could compete with PKCα for binding to β-catenin. As expected, PKCα associated with β-catenin$_{wt}$, but this interaction was disrupted by the addition of axin (Fig. 3A), which also attenuated the phosphorylation of Ser33/Ser37/Thr41 in a concentration-dependent manner (Fig. 3B). Therefore, these results suggest that PKCα interacts with β-catenin through the armadillo repeats 1–5 and this interaction is essential for PKCα-mediated β-catenin phosphorylation at Ser33/Ser37/Thr41.

3.4. Armadillo repeats are required for PKCα-mediated β-catenin degradation

Previous studies have demonstrated that activated PKCα promotes β-catenin degradation by catalyzing its Ser33/Ser37 phosphorylation, which is recognized by F-box β-transducin repeat-containing protein (β-TrCP), a component of the ubiquitin ligase complex [16]. To examine the effect of the armadillo repeats 1–5 on PKCα-mediated β-catenin degradation, DNA constructs expressing β-catenin$_{1-781}$ and β-catenin deletion mutants (β-catenin$_{1-422}$ and β-catenin$_{1-138}$) were transfected into HEK293 cells, followed by treatment with increasing concentrations of A23187 and CGK062, which are known activators of PKCα. Western blot analysis showed that activation of PKCα with both A23187 and CGK062 consistently resulted in the downregulation of β-catenin$_{1-781}$ (Fig. 4A and B, upper panel). Notably, the amount of β-catenin$_{1-422}$ was decreased, whereas the level of β-catenin$_{1-138}$, which lacks the armadillo repeats 1–5, was unchanged by treatment of A23187 and CGK062 (Fig. 4A and B, middle and lower panel). These results suggest that the armadillo repeats 1–5 of β-catenin are required for PKCα-mediated β-catenin degradation.

Several kinases such as CK1/GSK-3β, PKA/GSK-3β and PKCα, catalyze the N-terminal phosphorylation of β-catenin, which does not exhibit typical phosphorylation sequences. Previous studies

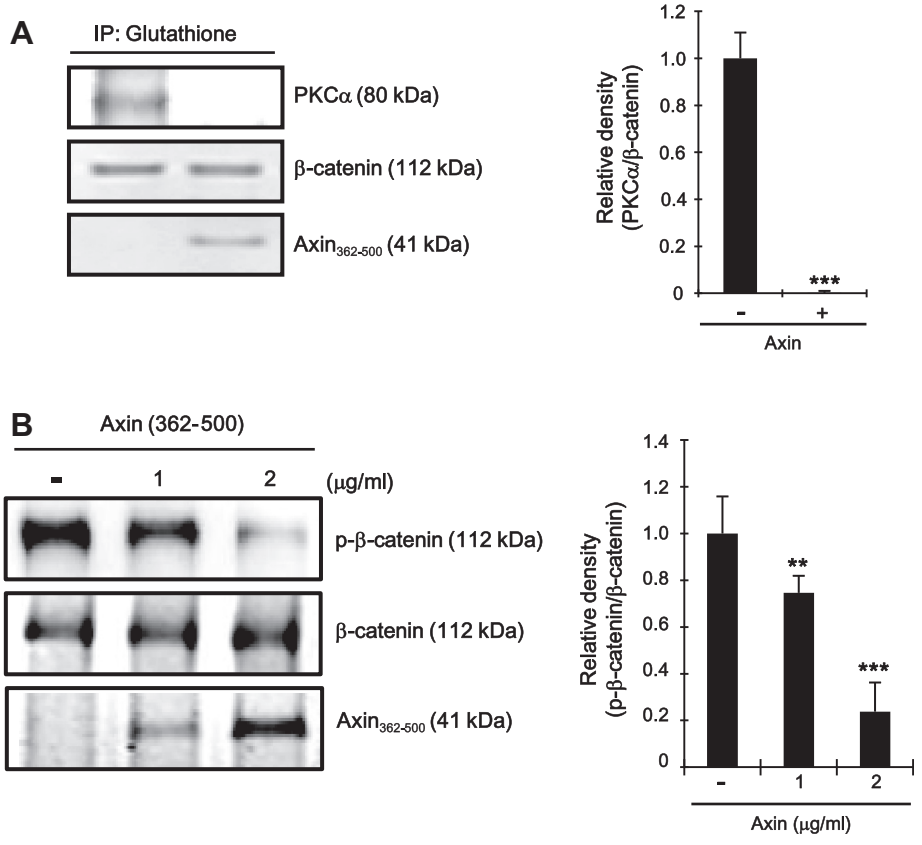

Fig. 3. Axin competes with PKCα for binding to β-catenin. (A) Axin disrupts with PKCα for binding to β-catenin. Axin fragment (362–500) was added to GST-β-catenin with PKCα and then pull-down with glutathione–Sepharose bead, and then the complexes were visualized by Western blotting with anti-PKCα and anti-β-catenin antibodies. The histogram shows the average volume density of three experiments, and the bars indicated standard deviations. $^{***}P < 0.001$, compared with the Axin-untransfected group. (B) The indicated amounts of Axin fragment (362–500) were added to GST-β-catenin$_{wt}$ with a constant amount of PKCα and then the samples were analyzed by Western blotting with anti-phospho- p33/37/41-β-catenin and β-catenin antibodies. The histogram shows the average volume density of three experiments, and the bars indicated standard deviations. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the axin-untreated group.

suggested that the formation of supramolecular complexes of the substrate and kinases forces the phosphorylation of noncanonical sequences. For instance, axin functions as a scaffold connecting CK1/GSK-3β to β-catenin, thereby facilitating the phosphorylation of β-catenin at the N-terminal Ser/Thr residues in the Wnt/β-catenin pathway (Fig. 4C, left panel) [17]. Similarly, presenilin bridges PKA/GSK-3β to β-catenin for its N-terminal phosphorylation [18]. In addition, a recent study demonstrated that the first armadillo repeat, which is recognized by CK1, is essential for CK1-mediated phosphorylation of β-catenin Ser45, lacking a typical CK1 consensus sequence [19]. In this study, in the absence of scaffolding proteins, the direct association of PKCα with β-catenin through the armadillo repeats 1–5 placed the Ser33/Ser37/Thr41 residues of β-catenin in close proximity to PKCα, thereby facilitating PKCα-mediated β-catenin phosphorylation and subsequent degradation (Fig. 4C, right panel). Taken together, our results provide new

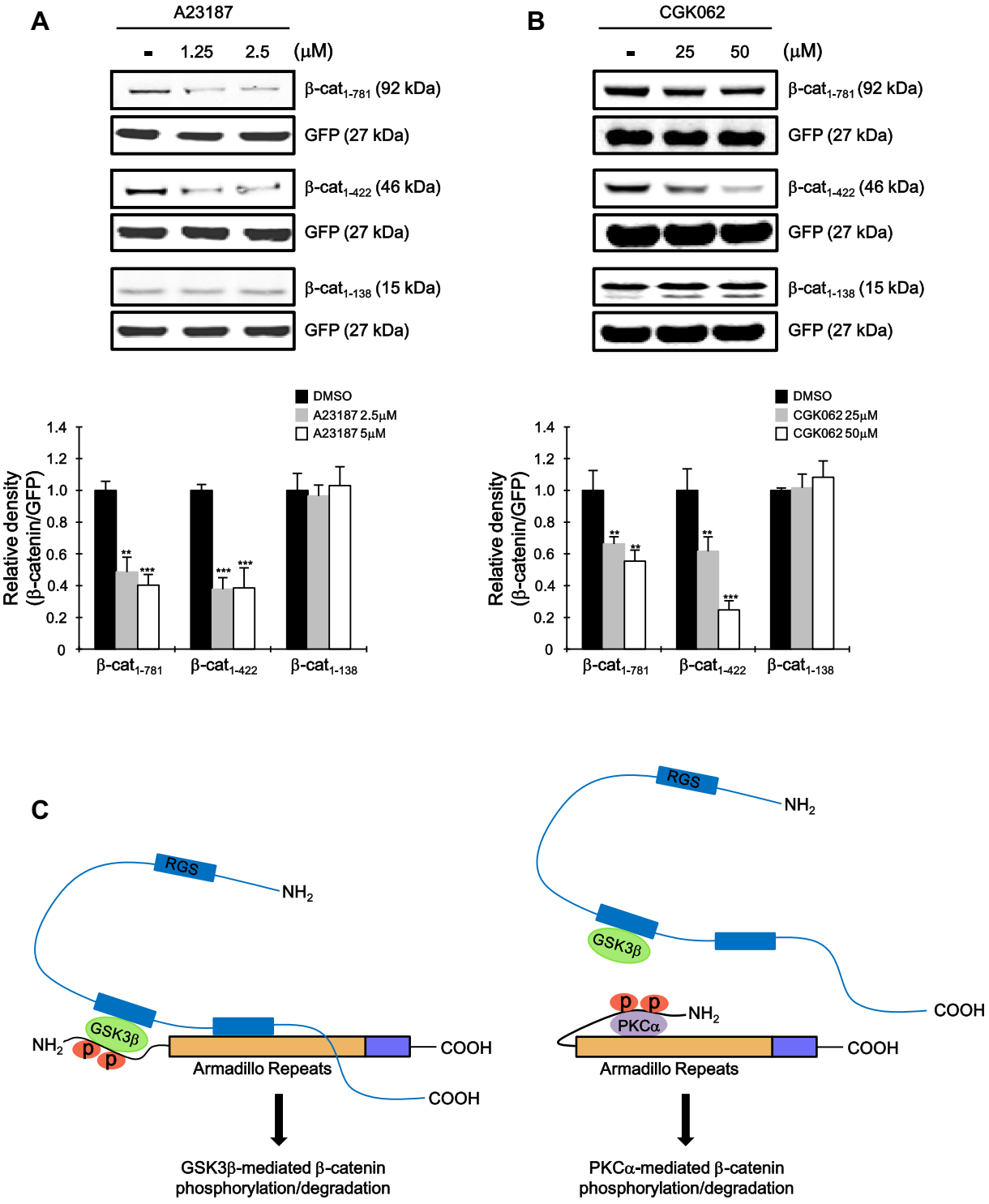

Fig. 4. The armadillo repeats 1–5 of β-catenin is essential for PKCα-mediated β-catenin degradation. (A), (B) HEK293 cells were transfected with β-catenin$_{1-781}$, β-catenin$_{1-422}$ or β-catenin$_{1-138}$ expression plasmids for 30 h and then incubated with A23187 (A) or CGK062 (B) for 15 h. Cytosol extracts were analyzed by Western blotting with anti-β-catenin and anti-GFP antibodies. The histogram shows the average volume density of three experiments, and the bars indicated standard deviations. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with the DMSO-treated group. (C) Proposed model for PKCα-mediated β-catenin degradation and phosphorylation.

insights into the mechanism of β-catenin phosphorylation by PKCα.

Acknowledgments

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