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PKA regulates calcineurin function through the phosphorylation of RCAN1: Identification of a novel phosphorylation site

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ABSTRACT

Calcineurin is a calcium/calmodulin-dependent phosphatase that has been implicated in T cell activation through the induction of nuclear factors of activated T cells (NFAT). We have previously suggested that endogenous regulator of calcineurin (RCAN1, also known as DSCR1) is targeted by protein kinase A (PKA) for the control of calcineurin activity. In the present study, we characterized the PKA-mediated phosphorylation site in RCAN1 by mass spectrometric analysis and revealed that PKA directly phosphorylated RCAN1 at the Ser 93. PKA-induced phosphorylation and the increase in the half-life of the RCAN1 protein were prevented by the substitution of Ser 93 with Ala (S93A). Furthermore, the PKA-mediated phosphorylation of RCAN1 at Ser 93 potentiated the inhibition of calcineurin-dependent pro-inflammatory cytokine gene expression by RCAN1. Our results suggest the presence of a novel phosphorylation site in RCAN1 and that its phosphorylation influences calcineurin-dependent inflammatory target gene expression.

1. Introduction

Calcineurin (PP2B) is a calcium (Ca^{2+})/calmodulin (CaM)-dependent serine/threonine phosphatase expressed in most mammalian tissues. It consists of a catalytic subunit A (60 kDa) and a regulatory subunit B (19 kDa) [1]. It can be found in the nucleus and cytosol and is involved in a variety of cellular signaling processes, such as T cell activation, muscle growth and differentiation, cardiac functions, neuronal synaptic plasticity and memory formation [1,2]. Calcineurin is known to play a role in the immune response through the activation of nuclear factors of activated T cells (NFAT). NFAT was first identified as an inducible nuclear factor that binds to the interleukin-2 (IL-2) promoter in T cells [3,4]. In the resting state, it is sequestered in the cytosol, with its nuclear localization signal masked by hyperphosphorylation. Dephosphorylation of NFAT by calcineurin leads to the exposure of the nuclear translocation signal and its translocation into the nucleus [5]. Once it reaches the nucleus, NFAT interacts with distinct DNA-binding elements to induce the expression of multiple cytokines that control the expansion of T cells [6]. Through actions on NFAT-dependent transcriptional regulation, calcineurin appears to be involved in many inflammatory signaling pathways.

Recently, regulator of calcineurin 1 (RCAN1) has been identified as an endogenous calcineurin binding partner, and it has been reported to act as a modulator in calcineurin-mediated signaling pathways [7]. The RCAN1 gene consists of seven exons and is expressed as three isoforms due to alternative splicing [8]. Each isoform is expressed at different levels in diverse tissues, including the brain, heart, liver and skeletal muscle tissues [8]. Several studies have shown that the function of RCAN1 in modulating calcineurin activity is dependent on its phosphorylation state [9,10]. For example, extracellular signal-regulated kinase (ERK), glycogen synthase kinase-3 (GSK-3), Dyrk1A and NF-κB inducing kinase (NIK) have been suggested to phosphorylate RCAN1 [9–12]. The phosphorylation of RCAN1 by these kinases can either positively or

Abbreviations: RCAN1, regulator of calcineurin 1; PKA, protein kinase A; NFAT, nuclear factors of activated T cells.

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negatively modulate calcineurin function. Dyrk1A and NIK have been shown to enhance the inhibition of calcineurin by RCAN1 [9,10]. In contrast, the stimulatory effects of Rcn1 (*yeast* homolog of RCAN1) involves phosphorylation by Mck1, a member of GSK-3 family [12]. Phosphorylation can cause an increase or decrease in the half-life of the RCAN1 protein [9,10,13]. Once it is phosphorylated, its stability is dynamically regulated by the ubiquitin-proteasome and chaperone-mediated autophagy (CMA) pathways [9,14,15].

In accordance with these reports, we have previously found that protein kinase A (PKA) induces the phosphorylation of RCAN1 [16]. However, the direct phosphorylation site in RCAN1 has not been determined. In the present study, we performed mass spectrometric analysis to map the PKA-dependent phosphorylation site in RCAN1 and suggest its potential role in calcineurin-mediated inflammatory signaling.

2. Materials and methods

2.1. Materials

Anti-GST, anti-HA and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology. Catalytic subunit of protein kinase A (PKAc), phorbol 12-myristate 13-acetate (PMA), ionomycin and cycloheximide (CHX) were purchased from Sigma–Aldrich. An expression vector for pCMV-PKAc was purchased from Clontech and was subcloned into p3xFLAG-CMV (Sigma). An NFAT-driven reporter plasmid (pGL-IL2-Luc) was kindly provided by G. R. Crabtree. An expression vector for HA-RCAN1 was kindly provided by S. de la Luna. HA-RCAN1 (S93A) was generated from HA-RCAN1 with a QuikChange XL Site-Directed Mutagenesis kit (Agilent Technology). GST-RCAN1 deletion constructs were produced using a PCR-based method.

2.2. Cell culture

HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. The cells were transfected with the indicated expression vectors by the Lipofectamine method (Invitrogen), according to the manufacturer's instruction.

2.3. Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-Cl, pH 7.9, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0.2 mM phenylmethylsulfonyl fluoride). Total cell lysates were separated by 10% SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat dried milk with TBST buffer (20 mM Tris-Cl, pH 7.6, 137 mM NaCl and 0.05% Tween-20) for 30 min and were then incubated overnight at 4 °C in TBST buffer containing the appropriate antibody.

2.4. In vitro kinase assays

GST-RCAN1, GST-RCAN1(1–90) and GST-RCAN1(91–198) recombinant proteins were purified using glutathione-Sepharose beads according to the manufacturer's instructions (GE Healthcare). The eluted proteins were phosphorylated in the presence or absence of the PKAc (5 units) in a reaction buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM DTT, 100 μM ATP and 5 μCi [γ-^{32}P]-ATP at 30 °C for 30 min. After phosphorylation, the samples were subjected to SDS-PAGE, and the proteins were visualized by autoradiography.

2.5. Reporter gene assays

HEK293 cells were transfected with the indicated expression vectors using Lipofectamine (Invitrogen). Luciferase activity was measured using a Dual luciferase assay system (Promega). *Firefly* luciferase activity was normalized with *Renilla* luciferase activity. The results are presented as the mean ± SD of three independent experiments.

2.6. RT-PCR

The RNA preparation, reverse transcription and PCR were performed as previously described [16]. The primer sequences were as follows: β-actin, 5′-CATGTTTGAGACCTTCAACACCCC-3′ (forward) and 5′-GCCATCTCTTGCTCGAAGTCTAG-3′ (reverse); TNF-α, 5′-TTCCTGATCGTGGCAGGCGC-3′ (forward) and 5′-CAGCTCCACGC-CATTGGCCA-3′ (reverse); and Cox-2, 5′-TGCCCGACTCCCTTGGTGT-3′ (forward) and 5′-CCCGCAGCCAGATTGTGGCA-3′ (reverse).

2.7. Liquid chromatography-mass spectrometry (LC-MS)/MS analysis

The RCAN1 protein that reacted in the presence or absence of the PKAc was excised from a Coomassie Brilliant Blue (CBB)-stained gel and digested with trypsin for 18 h at 37 °C. The peptides were separated with a C18 reverse-phase column and analyzed with a nano electrospray ionization mass spectrometer (nESI-LC-MS/MS). UltiMate Nano LC Systems were used with an FAMOS autosampler and a Switchos-column switching (LC-Packings, Amsterdam, the Netherlands). The peptides were eluted over a 90-min gradient of 3–45% acetonitrile with 0.1% formic acid at 0.2 μl per min through a 15 cm analytical column (Zorbax 300SB-C18, Agilent Technologies). The column outlet was coupled to a high-voltage ESI source, which was interfaced to a QSTAR mass spectrometer (Applied Biosystems). AnalystQS software (version 1.1, Applied Biosystems) was used to generate peak lists. The acquired data were searched in the National Center for Biotechnology Information (NCBI) nonredundant whole protein database (nrdb90) using the MASCOT software package (Version 2.1, Matrix Sciences, UK).

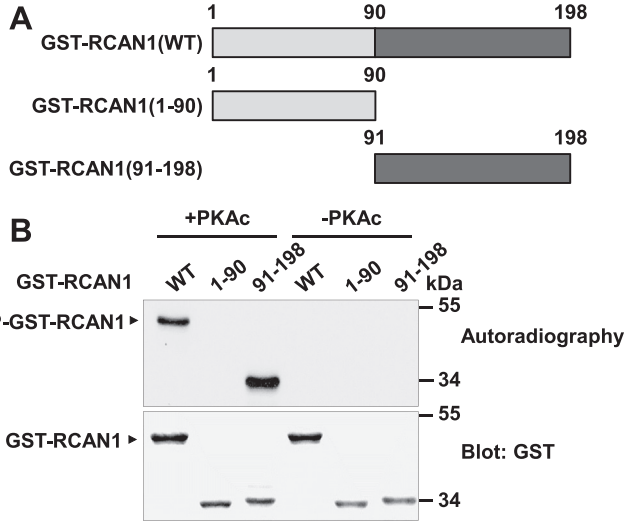

Fig. 1. PKA phosphorylates the C-terminal region RCAN1 *in vitro*. (A) Schematic diagram of GFP-RCAN1 constructs. (B) *In vitro* kinase assay was performed using purified GST-RCAN1 proteins in the presence or absence of the catalytic subunit of PKA. The upper panel shows the autoradiography result, and the bottom panel shows the Western blot result using an anti-GST antibody.

3. Results

3.1. PKA phosphorylates RCAN1 in its C-terminal region

In our previous study, we observed that PKA may be able to induce RCAN1 phosphorylation [16]. To determine the region of the RCAN1 protein that is specifically targeted for PKA-induced phosphorylation, we generated a GST-tagged deletion construct encoding either the N-terminal (1–90) or C-terminal (91–198) half of RCAN1, respectively (Fig. 1A). GST-tagged RCAN1 protein fragments were purified, and an *in vitro* kinase assay was performed (Fig. 1B). We observed that the catalytic subunit of PKA (PKAc) induced the phosphorylation of RCAN1 in its C-terminal region as well as that of full-length RCAN1 (Fig. 1B). However, it had no effect on the RCAN1 fragment containing the N-terminal region, suggesting that the RCAN1 region critical for PKA-induced phosphorylation is located within the C-terminal amino acids.

3.2. PKA directly phosphorylates Ser 93 in RCAN1

To identify the PKA-dependent phosphorylation site in RCAN1, the RCAN1 protein in the reaction mixture was purified and

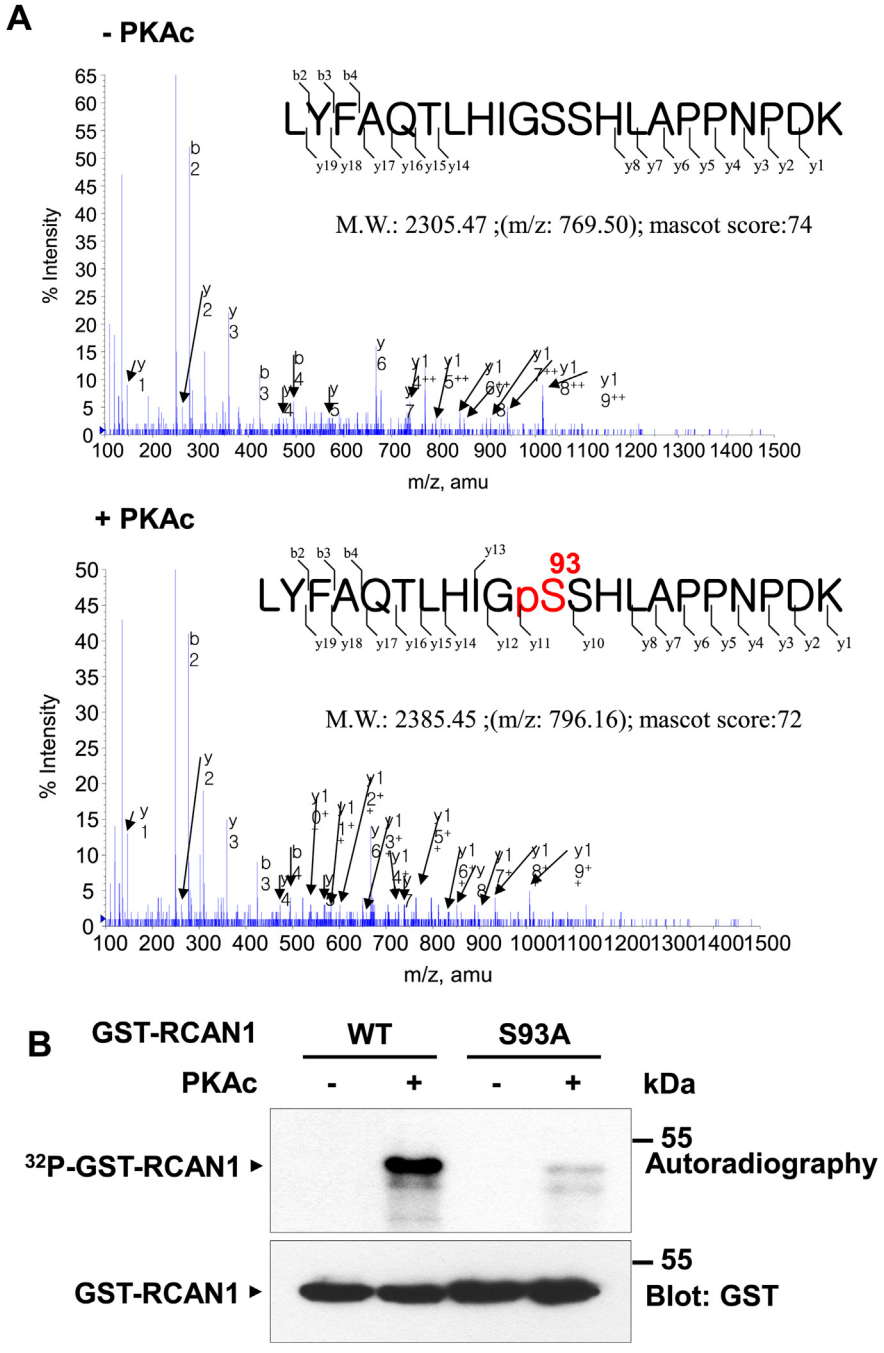

Fig. 2. Identification of PKA phosphorylation site in RCAN1. (A) Mass spectrometric analysis of RCAN1 that reacted in the presence or absence of the catalytic subunit of PKA. The unmodified peptide is shown in the upper panel (−PKAc), and the modified peptide (+PKAc) is shown in the bottom panel. The labeled peaks correspond to the masses of the b and y ions of the mono-phosphorylated peptide LYFAQTLHIGSSHLAPPNPDK, indicating that the Ser 93 was phosphorylated. (B) *In vitro* kinase assay was performed using purified GST-RCAN1 (WT) and GST-RCAN1 (S93A) proteins in the presence or absence of the catalytic subunit of PKA, and the results were visualized by autoradiography.

analyzed by mass spectrometry (Fig. 2A). As shown in Fig. 2A, we found that Ser 93 in RCAN1 was modified by PKA. To confirm phosphorylation of RCAN1 at this site, we created point mutant whose Ser 93 was substituted with Ala (S93A) (Fig. 2B). Consistent with the mass spectrometric data, RCAN1 phosphorylation was significantly inhibited in the S93A mutant, indicating that the Ser 93 was targeted for phosphorylation by PKA.

3.3. PKA increases RCAN1 protein stability through phosphorylation of Ser 93

Several previous reports have suggested that RCAN1 protein stability *in vivo* is dynamically regulated by phosphorylation [9,10,13]. To determine the ability of PKA to phosphorylate RCAN1 at Ser 93 *in vivo*, we first expressed HA-RCAN1 in the presence or absence of the PKA in HEK293 cells (Fig. 3A). RCAN1 expression was significantly enhanced by the coexpression of PKA, which may be indicative of RCAN1 phosphorylation. However, the expression of the RCAN1 mutant (S93A) was not changed by PKA coexpression, indicating that Ser 93 is important for the PKA-mediated protein expression (Fig. 3A).

We next investigated whether the ability of PKA to increase RCAN1 expression is due to a phosphorylation-dependent increase in protein stability (Fig. 3B and C). The stability of this protein was chased using cycloheximide, an inhibitor of *de novo* protein synthesis, revealing that PKA significantly prolonged the half-life of wild-type RCAN1 (Fig. 3B and C). In contrast, the half-life of the Ser 93 mutant RCAN1 (S93A) was not significantly altered by the coexpression of the PKA. Taken together, these results suggest that PKA induces the phosphorylation of RCAN1 at Ser 93 and that is subsequently increases the half-life of this protein *in vivo*.

3.4. PKA-induced Ser 93 phosphorylation of RCAN1 inhibits calcineurin-mediated target gene expression

To elucidate the physiological function of PKA-dependent RCAN1 phosphorylation, we investigated whether the phosphorylation of Ser 93 is critical for the regulation of the calcineurin-NFAT signaling pathway. Because RCAN1 was identified to be a negative modulator of the calcineurin signaling pathway, we first examined whether the PKA-mediated Ser 93 phosphorylation of RCAN1 affects calcineurin-dependent target gene transcription (Fig. 4A). For this experiment, HEK293 cells were transfected with an NFAT-luciferase reporter construct that was derived from interleukin-2 (IL-2), which is a target of calcineurin. Consistent with previous reports, the activation of calcineurin by the combined treatment with Ca^{2+}-ionophore (ionomycin) and phorbol ester (PMA) led to the significant induction of NFAT-dependent IL-2 transcription (Fig. 4A) [5,17]. The induction of calcineurin-dependent NFAT reporter activity was inhibited by RCAN1 expression. Furthermore, the coexpression of PKA enhanced the inhibition of calcineurin-dependent NFAT reporter activity by RCAN1. However, this PKA-dependent enhanced function of RCAN1 was not observed in the Ser 93 mutant RCAN1 (S93A) (Fig. 4A).

We next investigated whether the PKA-dependent Ser 93 phosphorylation of RCAN1 affects the ability of this protein to inhibit calcineurin-mediated endogenous target gene expression. We examined the transcription of tumor necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (Cox-2), which are known to have NFAT binding sites in their promoters and to be induced by calcineurin [18–20]. RCAN1 caused the inhibition of TNF-α mRNA expression in response to the ionomycin and PMA treatments, and this inhibitory effect was enhanced by PKA coexpression (Fig. 4B). However, the PKA-dependent enhancement of the inhibition of TNF-α expression by RCAN1 was not observed in the S93A mutant (Fig. 4). The enhanced effect of PKA on RCAN1 function in calcineurin-NFAT signaling was consistently observed for Cox-2 transcription, but this effect was not observed in the S93A mutant (Fig. 4B). Taken together, these results indicate that the PKA-dependent Ser 93 phosphorylation of RCAN1 is important for the inhibition of calcineurin-NFAT signaling *in vivo*.

4. Discussion

NFAT, a major calcineurin substrate, was first identified as an inducible nuclear factor that binds to the antigen receptor response

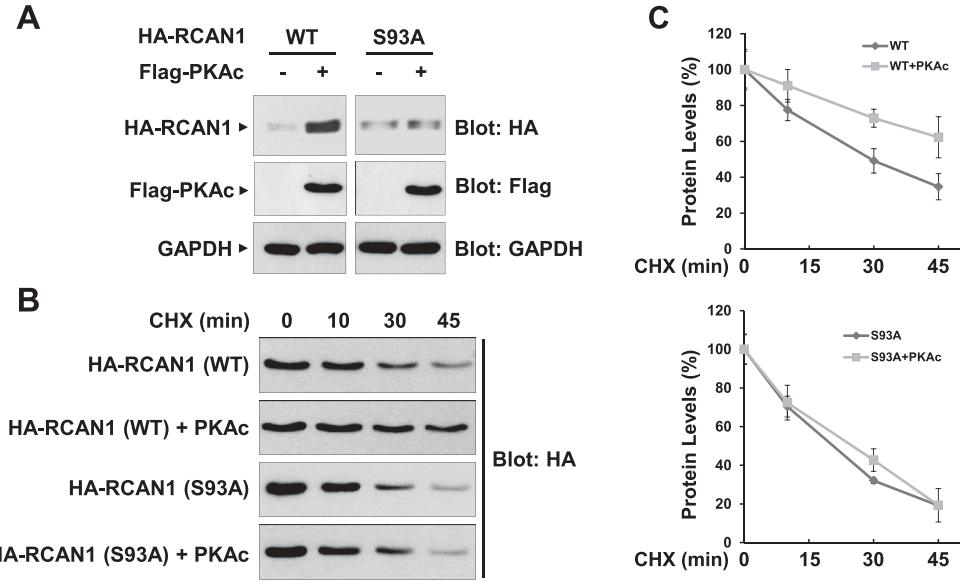

Fig. 3. PKA increases the stability of the RCAN1 protein. (A) HEK293 cells were transfected with the indicated expression vectors. After 24 h, the cell extracts were immunoblotted with the indicated antibodies. (B) HEK293 cells were transfected with the indicated expression vectors. After 24 h, the cells were treated with cycloheximide (CHX, 50 μM) for the indicated times. The cell extracts were immunoblotted with an anti-HA antibody. (C) The relative levels of RCAN1 (WT) and RCAN1 (S93A) mutant in (B) were quantified and plotted ($n = 3$).

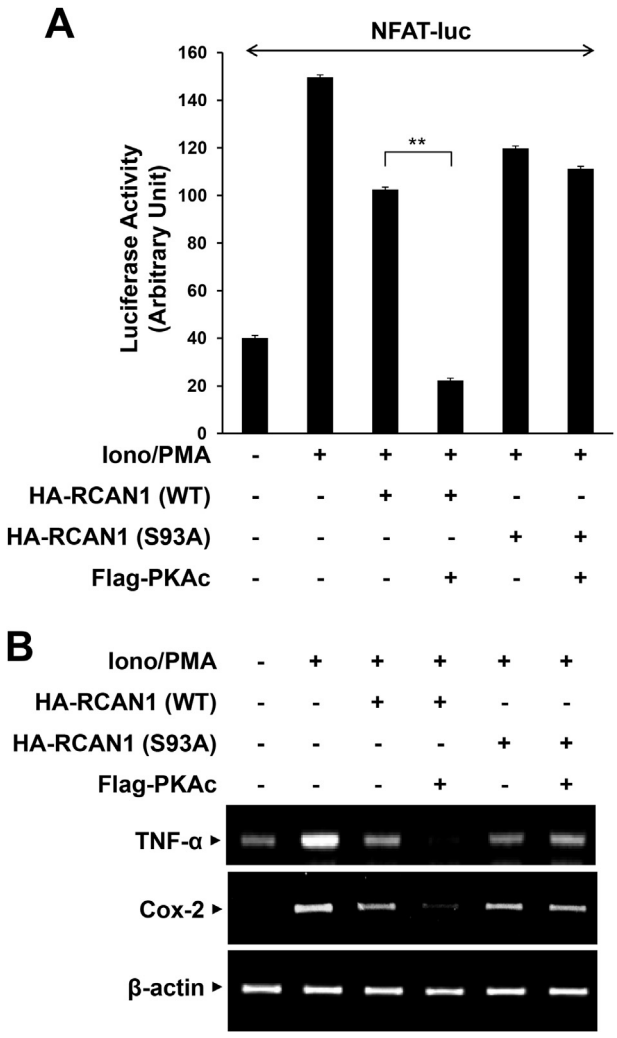

Fig. 4. Phosphorylation of the Ser 93 of RCAN1 by PKA enhances the inhibition of calcineurin activity. (A) HEK293 cells were transfected with the NFAT reporter construct alone or together with the indicated expression vectors. After 24 h, the cells were treated with ionomycin (500 nM) and PMA (10 ng/ml) for 6 h. *Renilla* luciferase activity was used to normalize transfection efficiency. The results are represented as the mean ± SD of three independent experiments. **, $P < 0.01$. (B) HEK293 cells were transfected with the indicated expression vectors and incubated with or without ionomycin (500 nM) and PMA (10 ng/ml) for 6 h. The mRNA levels of TNF-α, Cox-2 and β-actin were measured by RT-PCR (B).

element-2 (ARRE-2) in the IL-2 promoter in T cells [3,4]. Subsequent studies have revealed that NFAT is ubiquitously expressed in immune and nonimmune cells in vertebrates and is involved in a broad range of cellular signaling processes, including inflammatory T cell responses, the development of the nervous system, cardiac functions and angiogenesis, through inducing the expression of diverse target genes [6].

Regulators of calcineurin activity may be used as modulators in the treatment of calcineurin-related pathogeneses. For example, cyclosporine (CsA) and FK506 are widely used in the treatment of inflammatory disorders and act by inhibiting NFAT activation in T cells [21]. Along with these pharmacological inhibitors, an endogenous regulator of calcineurin, RCAN1 has been identified. RCAN1 proteins are highly conserved from yeasts to humans and in vertebrates, and this protein can bind directly to calcineurin [22]. Previous reports have suggested that the effect of the RCAN1 protein on calcineurin activity can be altered by post-translational modifications, such as phosphorylation. The phosphorylation of RCAN1 in the regulation of calcineurin activity has been described to have both stimulatory and inhibitory effects. For instance, GSK-3β- and ERK-mediated phosphorylations at the Ser 108 and Ser 112 of RCAN1 activate calcineurin signaling [12]. Similarly, the MEKK3-mediated activation of calcineurin-NFAT signaling is associated with phosphorylation of RCAN1 at Ser 108 and Ser 112 [23]. TGF-β-activated kinase 1 (TAK1) phosphorylates RCAN1 at the Ser 94 and Ser 136, converting it from an inhibitor to a facilitator of calcineurin-NFAT signaling [24]. Moreover, multiple phosphorylations of this protein at Ser 93, Ser 108, Ser 112, Thr 124 and Thr 153 by p38α MAPK kinase decrease its binding affinity for calcineurin *in vitro* [25]. In contrast, the Dyrk1A-dependent phosphorylation of RCAN1 at Ser 112 and Thr 192 enhances its inhibitory effects on calcineurin [10]. Additionally, the NIK-mediated phosphorylation of RCAN1 at an unknown site potentiates its inhibition of calcineurin activity [9]. In accordance with these reports, our studies show evidence that the Ser 93 of RCAN1 is phosphorylated by PKA. Moreover, the PKA-dependent phosphorylation of RCAN1 at Ser 93 improves its the negative regulatory effect on calcineurin-mediated pro-inflammatory cytokine expression.

Our data are in support of the immunosuppressive functions of the PKA-RCAN1 pathway in many calcineurin-mediated pathogeneses, such as immune dysfunction, brain dysfunction, heart disease and cancer. In accordance with this notion, a recent study has shown that RCAN1-deficent mice display greatly increased systemic levels of pro-inflammatory cytokines in respiratory tract infections [26]. In addition, RCAN1-defecient bone marrow-derived mast cells (BMMCs) show increased calcineurin activity and the enhanced transcriptional activation of NF-κB and NFAT following stem cell factor (SCF) stimulation [27]. Thus, considering the broad biological and pathological roles of calcineurin, the identification of the PKA-RCAN1 mediated calcineurin-NFAT signaling pathway in this study may facilitate the development of effective treatments for many immunological disorders.

Conflict of interest

None declared.

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