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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).

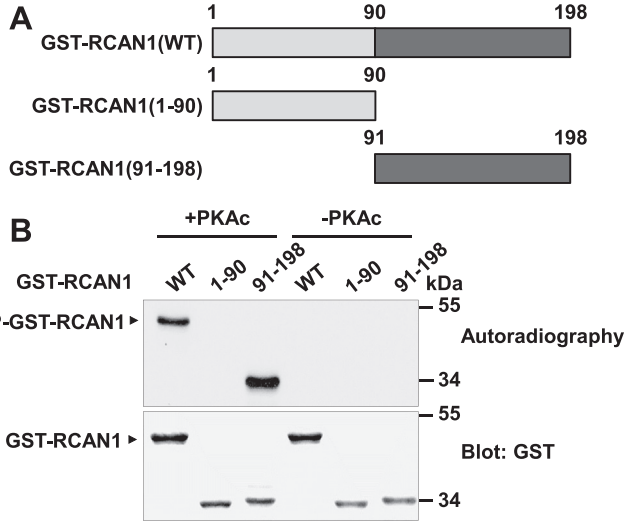

Fig. 1. PKA phosphorylates the C-terminal region RCAN1 *in vitro*. (A) Schematic diagram of GFP-RCAN1 constructs. (B) *In vitro* kinase assay was performed using purified GST-RCAN1 proteins in the presence or absence of the catalytic subunit of PKA. The upper panel shows the autoradiography result, and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