The Regulator of Calcineurin 1 (RCAN1/DSCR1) Activates the cAMP Response Element-binding Protein (CREB) Pathway*

Seon Sook Kim and Su Ryeon Seo

From the Department of Molecular Bioscience, College of Biomedical Science, Kangwon National University, Chuncheon 200-701, Republic of Korea

Background: The regulator of the calcineurin 1 (RCAN1) gene is located on human chromosome 21, the trisomy of which causes Down syndrome (DS).

Results: RCAN1 increases the phosphorylation of transcription factor CREB.

Conclusion: RCAN1 activates CREB signaling by inhibition of calcineurin activity.

Significance: Identification of RCAN1 as a CREB signaling regulator could contribute to the understanding of DS pathogenesis.

The regulator of calcineurin 1 (RCAN1; also called DSCR1, Adapt78, and MCIP) gene is located in the region of the Down syndrome critical region (DSCR) on human chromosome 21, the trisomy of which causes Down syndrome (DS) (10–13). The RCAN1 gene consists of seven exons and can be alternatively spliced (14). The predominant isoforms of the RCAN1 protein are RCAN1-1 encoded by exon 1 and RCAN1-4 encoded by exon 4 (14). The mRNA of RCAN1-1 is translated into RCAN1-1S and RCAN1-1L (14). Elevated expression of RCAN1 has been reported in DS, brains of Alzheimer’s disease patients, and in the peri-infarct cortex after experimental stroke (14–16). The RCAN1 protein binds and inhibits calcineurin activity (15).

Calcineurin (PP2B) is a Ca\(^{2+}\)/calmodulin-dependent serine/threonine phosphatase that is ubiquitously expressed throughout the body (17). The calcineurin heterodimer consists of a catalytic A subunit and a regulatory B subunit. The binding of Ca\(^{2+}\)/calmodulin to the catalytic A subunit displaces an autoinhibitory B subunit, which allows the access of protein substrates to the catalytic A subunit. In addition to its direct effects on several substrates, including the nuclear factor of activated T cells (NFAT) family of transcription factors, calcium channels, DRAPP-32, GABA receptors, and glutamate receptors by promoting dephosphorylation, calcineurin also indirectly regulates a wide range of proteins in neurons by releasing repression of phosphatase 1 (PP1) (18, 19). Calcineurin has been implicated in many cellular signaling processes, such as neuronal synaptic plasticity and memory formation (20, 21). Dysregulation of its activity has also been associated with brain disease and injury, such as Alzheimer’s disease and ischemia (22, 23).

In this study, we examined the possible role of RCAN1 in the regulation of CREB activity, which is important in neuronal activity. We found that the expression of RCAN1 increased the phosphorylation of CREB and CRE-mediated gene transcription in response to the activation of the intracellular cAMP pathway. Furthermore, we found that the ability of RCAN1 to increase the phosphorylation of CREB depended on the inhibition of calcineurin activity. Our data reveal a novel function of cAMP response element-binding protein (CREB)2 is a major transcriptional regulator in the expression of a variety of genes necessary for the development and function of the nervous system through interactions with cAMP response elements (CRE) within their promoter sequence (1). In the brain, CREB is a key factor that regulates neural functions in learning, memory, and synaptic plasticity (1, 2). Although CREB is known to be activated by multiple stimuli such as the Ca\(^{2+}\)/calmodulin-dependent protein kinase IV (CaMKIV) (3, 4), ribosomal S6 kinase (RSK), and mitogen/stress-activated kinase (MSK) families (5), the protein kinase A (PKA)-mediated phosphorylation of Ser133 in CREB is essential for the activation of transcription in response to cAMP (6). Multiple studies have suggested that regulation of CREB phosphorylation is critical for maintaining a homeostasis under many pathological conditions by regulating the expression of several genes either directly or indirectly (7–9).

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RCAN1 in the regulation of CREB activity through the negative regulation of the calcineurin signaling pathway.

EXPERIMENTAL PROCEDURES

Materials and Expression Vectors—Anti-HA, anti-Myc, and anti-FLAG antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-phospho-CREB (Ser-133), anti-CREB, and anti-CBP antibodies were purchased from Cell Signaling Technology, Inc. Anti-calcineurin antibody was purchased from BD Biosciences. Anti-RCAN1 antibody was purchased from ECM Biosciences (Versailles, KY). Forskolin, CPT-cAMP, and 38-amino acid pituitary adenylate cyclase activating polypeptide (PACAP38) were purchased from Sigma-Aldrich. The ChIP assay kit was purchased from Millipore. The siRNAs targeting the calcineurin were purchased from Thermo Scientific. The sequences for the calcineurin and control siRNAs were 5′-GGUGAAAGCUGUUCUUU-3′ and 5′-UUAAGGUC-GGCUUAGGUUU-3′, respectively. The expression vector for triple HA-RCAN1 (1-1S) was kindly provided by S. de la Luna. The expression vector for HA-calcineurin A was a gift from B. A. Rothermel. The expression vectors for pCG-CREB and CREB (S133A) were gifts from K. Saeki. The expression vectors for pRS-RCAN1 shRNA and CREB (S133A) were purchased from Origene (Rockville, MD).

Cell Culture—PC12 cells were maintained in DMEM supplemented with 5% FBS, 10% horse serum, penicillin, and streptomycin. For the stable transfectant, cells were transfected with the HA-RCAN1 plasmid by the Lipofectamine 2000 method (Invitrogen) and selected in G418-containing growth medium.

Western Blot Analysis—Cells were transfected with the expression vectors by the Lipofectamine 2000 method (Invitrogen). After 24 h, cells were lysed in lysis buffer (20 mM Tris-Cl (pH 7.9), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride). For immunoblot analysis, total cell lysates were separated by 10% SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked in TBST buffer (20 mM Tris-Cl (pH 7.6), 137 mM NaCl, 0.05% Tween 20, 3% nonfat dried milk) for 30 min and then incubated overnight at 4 °C in TBST buffer containing the appropriate antibodies.

ChIP Assay—ChIP analysis was performed according to the manufacturer’s instructions (Millipore). Cells were cross-linked in 1% formaldehyde solution for 10 min at 37 °C. After washing with PBS, cells were resuspended in lysis buffer (10 mM Tris-Cl, (pH 8.0), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride). Cell lysates were sonicated 8 times for 5 s on ice. After centrifugation, the supernatant was precleared by incubation with 40 μl of 50% (v/v) protein A/G-agarose beads for 30 min at 4 °C. Precleared supernatant was incubated with either anti-phospho-CREB or anti-CBP antibodies overnight. ChIP samples were then washed and eluted. Immunoprecipitated DNA was recovered by phenol/chloroform/isoamyl alcohol extraction. DNA was amplified by PCR with primers specific to the c-fos promoter region containing CRE. The sequence of primer pairs were 5′-TTCTCTGTTCGCTCATGACG-3′ (forward) and 5′-CTTCTCAGTTGCTAGCTGCAA-3′ (reverse).

Reporter Gene Assays—PC12 cells were transfected with the indicated expression vectors by the Lipofectamine2000 method (Invitrogen). Luciferase activity was measured using the luciferase assay system (Promega) and was normalized for transfection efficiency using a β-galactosidase-expressing vector (pCMV5.LacZ) and the Galacto-Star system (PerkinElmer Life Sciences).

RT-PCR—RNA preparation, reverse transcription, and PCR were performed as described previously (24). The primers were as follows: β-actin, 5′-CATGTTTGAAGCTCTCA-ACACCCC-3′ (forward) and 5′-GCCATCTTGTGCTGAGTCTAG-3′ (reverse); c-fos, 5′-TGGTGAAACCCCTACGCTAC-3′ (forward) and 5′-TTCTTTTCTTGGAT-TCT3′ (reverse).

Statistics—Densitometric scans of Western blot analyses were quantified using ImageJ software. Values are expressed as mean ± S.D. of three independent experiments. Statistical significance was performed using Student’s t test. Differences between two means with p < 0.05 were considered as significant.

RESULTS

RCAN1 Increases the Cyclic AMP and PKA-dependent Phosphorylation of CREB—To investigate whether RCAN1 affected the cAMP-activated CREB phosphorylation in neuronal PC12 cells, we ectopically expressed HA-tagged RCAN1 in neuronal PC12 cells and treated the cells with forskolin, an adenylate cyclase activator (Fig. 1A). We observed that RCAN1 increased the forskolin-induced phosphorylation of CREB (Ser-133) (Fig. 1A). This increased phosphorylation of CREB was caused by the augmentation of intracellular cAMP levels because the membrane-permeable cAMP analog 8CPT-cAMP increased the phosphorylation of CREB to the same extent as forskolin (Fig. 1B).

To determine whether the ability of RCAN1 to increase the phosphorylation of CREB was specifically targeted to the Ser-133 residue on CREB, we expressed a mutant CREB in which the Ser-133 residue on CREB was replaced by Ala (S133A). As shown in Fig. 1C, we did not detect any significant increase in the phosphorylation of the mutant CREB (S133A) by RCAN1, indicating that the increased phosphorylation of CREB by RCAN1 was specifically targeted to the Ser-133 residue of CREB.

Because cAMP binds to the regulatory subunit of PKA and releases the active catalytic subunit from PKA, we examined whether the ability of RCAN1 to increase cAMP-dependent phosphorylation of CREB may also occur in PKA-dependent phosphorylation of CREB. As shown in Fig. 1D, coexpression of RCAN1 with the catalytic subunit of PKA caused the enhanced phosphorylation of the Ser-133 residue of CREB.

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To further demonstrate that the ability of RCAN1 to increase cAMP-dependent phosphorylation of CREB occurs through PKA-dependent intracellular signaling mechanism, PC12 cells were preincubated with H-89, a pharmacological inhibitor of PKA (Fig. 1E). Consistently, inhibition of PKA with H-89 prevented the RCAN1-enhanced phosphorylation of CREB by forskolin.

To investigate the physiological relevance of RCAN1 on cAMP-dependent phosphorylation of CREB, PC12 cells were transfected with pRS-RCAN1 shRNA expression vectors to knock down the endogenous RCAN1. A Western blot analysis showed that the expression of endogenous RCAN1 was significantly decreased by the transfection of pRS-RCAN1 expression vectors (Fig. 1F). The knockdown of endogenous RCAN1 significantly inhibited the phosphorylation of CREB in response to the forskolin, indicating that RCAN1 acts as a positive regulator of CREB phosphorylation (Fig. 1G). Taken together, these results indicate that RCAN1 enhances the cAMP and PKA-dependent phosphorylation of CREB.

RCAN1 Enhances CREB-mediated Gene Transcription—Phosphorylation of CREB has been shown in many cellular systems to activate the CRE-driven gene transcription (6). To determine whether RCAN1 exerted an enhanced effect on CRE-driven gene transcription, PC12 cells were transfected with the CRE-reporter construct. As shown in Fig. 2A, the expression of RCAN1 resulted in increased CRE gene transcription induced by forskolin in a dose-dependent manner. The enhanced CRE-driven gene transcription by RCAN1 was abrogated by the expression of the inactive CREB mutant (S133A), indicating that the ability of RCAN1 to enhance CRE-driven gene expression was mediated by CREB activation (Fig. 2B).

Because the activation of PKA is involved in the phosphorylation of CREB and subsequent CRE-driven gene expression, we...
assessed the ability of RCAN1 to enhance the PKA-dependent CRE-reporter activity. As shown in Fig. 2C, coexpression of RCAN1 with the catalytic subunit of PKA enhanced the PKA-dependent CRE-luciferase activity. Furthermore, this increased CRE-driven gene transcription was suppressed by either inactive CREB (S133A) or dominant negative A-CREB, which selectively inhibits the DNA-binding activity of wild-type CREB (25). These results verify that the ability of RCAN1 to increase CRE-driven gene transcription is mediated by CREB transcriptional activation. Collectively, these results indicate that RCAN1 enhanced cAMP- and PKA-dependent CRE-driven gene transcription through CREB activation.

Cyclic AMP-induced Activity of CREB Is Increased in the Cells Stably Overexpressing RCAN1—To further examine the role of RCAN1 in the regulation of CREB activity, we established a RCAN1 stable clone that had a mild increase in expression (Fig. 3A). As shown in Fig. 3B, the phosphorylation of CREB in response to forskolin was significantly increased in the stable RCAN1-overexpressing cells, which was similar to the levels from the transient transfection. Furthermore, the increased basal level of CREB phosphorylation was observed in stable RCAN1-overexpressing cells compared with control cells, thus increasing the physiological relevance of these findings.
PACAP38 is an important neuropeptide that was initially isolated from ovine hypothalamic extracts on the basis of its potent ability to activate adenylate cyclase in rat anterior pituitary cells (26, 27). Because PACAP38 is one of the external ligands that increase in the concentration of intracellular cAMP, we determined if the phosphorylation of CREB by PACAP38 was enhanced in cells stably overexpressing RCAN1. As shown in Fig. 3C, the PACAP38-stimulated CREB phosphorylation was significantly enhanced in cells stably overexpressing RCAN1 when compared with control cells, indicating that RCAN1 regulates the activation of CREB by extracellular neuropeptides. These results support the notion that the expression of RCAN1 enhances the phosphorylation of CREB.

**RCAN1-enhanced Phosphorylation of CREB Depends on the Inhibition of Calcineurin**—The Ca$^{2+}$/calmodulin-dependent phosphatase calcineurin has been reported to exert either a stimulatory or inhibitory effect on the regulation of CREB activity in several experimental conditions (28–30). Because RCAN1 was identified as an inhibitor of calcineurin, we investigated the involvement of calcineurin in the RCAN1-mediated CREB activation pathway.

To elucidate the possible mechanism underlying the enhancement of CREB phosphorylation by RCAN1, we first examined if the inhibition of calcineurin contributed to the phosphorylation of CREB (Fig. 4A). As shown in Fig. 4A, a significant accumulation of CREB phosphorylation was observed by the presence of well known calcineurin inhibitors, such as FK506 and cyclosporin A, implying that calcineurin may serve as a negative modulator for CREB activation.

To determine whether RCAN1 altered calcineurin signaling in our experimental conditions, PC12 cells were transfected with a calcineurin-responsive reporter construct from the IL-2 gene (31, 32). Consistent with a previous report (13), expression of the catalytically active subunit of calcineurin (CnA) stimulated IL-2 gene transcription (Fig. 4B). Coexpression of RCAN1 with CnA resulted in a significant decrease of calcineurin-dependent IL-2 gene transcription, indicating that RCAN1 inhibited calcineurin activity (Fig. 4B).

To determine whether the ability of RCAN1 to activate CREB was due to the inhibition of calcineurin activity, we examined if the expression of CnA reversed CREB activation in cells stably overexpressing RCAN1 (Fig. 4C). As shown in Fig. 4C, the phosphorylation of CREB in cells stably overexpressing RCAN1 in the presence or absence of forskolin was significantly suppressed by the expression of CnA, suggesting that the inhibition of calcineurin by RCAN1 may contribute to the increased phosphorylation of CREB.

To provide further evidence that the ability of RCAN1 to enhance CREB activation depended on the calcineurin inhibition, we analyzed forskolin-induced CRE-reporter activity (Fig. 4D). The coexpression of CnA with RCAN1 resulted in the suppression of RCAN1, which enhanced CRE-luciferase activity in response to forskolin (Fig. 4D).

To further assess that the ability of RCAN1 to enhance CREB activation depended on the calcineurin, we utilized siRNAs targeting to the calcineurin. Efficient knockdown of endogenous calcineurin was observed by Western blot analysis (Fig. 4E). As shown in Fig. 4E, RCAN1 could not enhance the phosphorylation of CREB by forskolin in calcineurin knockdown cells, suggesting that calcineurin was required for the enhancement of CREB activity by RCAN1 (Fig. 4E).

Collectively, these results suggest that the inhibition of calcineurin signaling by RCAN1 enhances CREB activation and CRE-mediated gene transcription.

**RCAN1 Induces the Transcription of c-fos by a Calcineurin-inhibitory Mechanism**—We next investigated if the ability of RCAN1 to increase CREB activity contributed to the endogenous CRE-target gene expression. We examined the transcription of c-fos, which is known to have a CRE promoter site and is induced by forskolin (25). As shown in Fig. 5A, the expression of c-fos mRNA was induced by forskolin. Furthermore, the induction of c-fos mRNA by forskolin was significantly enhanced in cells stably overexpressing RCAN1, suggesting that RCAN1 induced the endogenous CRE-target gene expression in response to forskolin (Fig. 5A).

To verify if the enhanced expression of c-fos mRNA by RCAN1 was correlated with a physical binding of CREB to the c-fos promoter in vivo, we performed a ChIP assay. The immunoprecipitated chromatin with anti-phospho-CREB antibodies were amplified by PCR using c-fos promoter-specific primers covering the CRE site from −58 bp to −51 bp. As shown in Fig. 5B, the extent of CRE bound with phospho-CREB was significantly enhanced in cells stably overexpressing RCAN1 compared with that of control cells. Furthermore, the binding of CREB-binding protein (CBP), a coactivator protein that is known to be recruited upon binding of CREB on CRE element, was also increased in RCAN1-overexpressing cells. These results suggest that RCAN1 enhances CREB binding on the c-fos promoter in vivo.

To determine whether the ability of RCAN1 to enhance expression of c-fos mRNA in response to forskolin was related to the inhibition of calcineurin activity, we examined if the coexpression of CnA with RCAN1 reversed the increased c-fos expression by RCAN1. As shown in Fig. 5C, the coexpression of CnA with RCAN1 suppressed the RCAN1-mediated increased expression of c-fos mRNA, suggesting that the increased expression of c-fos mRNA depended on the inhibition of calcineurin activation by RCAN1. Taken together, these results suggest that RCAN1 induces the expression of the endogenous CRE-containing c-fos gene through calcineurin-inhibitory mechanism (Fig. 6).

**DISCUSSION**

In this study, we have reported the first evidence that RCAN1 expression increase the phosphorylation of CREB in response to the cAMP-dependent intracellular pathway. We have also suggested that the ability of RCAN1 to increase the phosphorylation of CREB may result from an inhibition of a calcineurin-dependent intracellular signaling mechanism.

Several lines of evidence suggest that calcineurin may control the phosphorylation state of CREB (Bito et al. (29), Hagiwara et al. (33, and King et al. (34)). For example, in cultured rat hippocampal neurons, the inactivation of calcineurin results in CREB phosphorylation for extended durations and robust CRE-dependent transcription following stronger stimulations (29). The phosphorylated CREB is quickly...
dephosphorylated by a calcineurin-mediated disinhibition of PP1 following mild synaptic stimulation (18, 33, 34). Consistent with these reports, we observed that inhibition of calcineurin with well known calcineurin inhibitors, such as FK506 and CsA, exhibited a significant accumulation of CREB phosphorylation.

**FIGURE 4. Inhibition of calcineurin increases CREB phosphorylation.** A, PC12 cells were incubated with FK506 (100 nM) or cyclosporine A (CsA) (0.5 nM) for the indicated times. The cell extracts were immunoblotted with anti-pCREB and anti-CREB antibodies. B, PC12 cells were transfected with either an IL2-luciferase reporter construct alone or together with the indicated expression vectors. After 24 h, cells were analyzed for luciferase activity. The results were normalized with β-galactosidase activity. C, PC12 cells stably overexpressing either an empty vector or HA-RCAN1 were treated with forskolin for 10 min. The cell extracts were immunoblotted with anti-phospho-CREB and anti-CREB antibodies. D, PC12 cells were transfected with either a CRE-luciferase reporter construct alone or together with the indicated expression vectors. After 24 h, cells were treated with forskolin for 6 h and were analyzed for luciferase activity. The results were normalized with β-galactosidase activity. E, PC12 cells were transfected with either calcineurin-targeting siRNA or control siRNA together with the HA-RCAN1 expression vector. After 24 h, cells were treated with or without forskolin for 10 min. The cell extracts were immunoblotted with the indicated antibodies. The graphs are the mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01; †, p < 0.05.
It was unclear whether the effect of RCAN1 in the activation of CREB was direct, indirect, or a combination of the two. However, it is likely that the effects of RCAN1 are mediated by the inhibition of calcineurin because transfection studies have demonstrated that the expression of CnA suppresses RCAN1-induced CREB and CRE target gene activation. Consistently, expression of CnA abrogated the ability of RCAN1 to trigger the induction of endogenous c-fos mRNA expression, which contains a CRE promoter.

The increased basal level of CREB phosphorylation in the cells stably overexpressing RCAN1 increased the physiological relevance of this study in DS pathogenesis. Consistent with our observation, disturbance of CREB activity in overexpression mutants of Nebula, the Drosophila homolog of human RCAN1, have been reported (35). Moreover, elevated levels of phosphorylated CREB have been reported in fetal DS brains (36). On the basis of our observations, we speculate that induction of a prolonged activation of CREB by RCAN1 in DS pathogenesis may influence the abnormal regulation of a wide range of CREB-dependent target gene expressions, which is implicated in the development of the nervous system, in the process of learning and memory, and in the progression of neurodegenerative diseases.

There is a growing interest in the ability of RCAN1 to contribute to the phenotypes related with DS and Alzheimer’s disease pathogenesis through its association with calcineurin. Decreased calcineurin activity by overexpression of RCAN1 has been proposed to result in increased hyperphosphorylation of tau, leading to the formation of neurofibrillary tangles (14, 37). Because calcineurin activity regulates the rate of inactivation of critical effector such as CREB, it seems likely that overexpression of RCAN1, as observed in DS and AD, may enhance CREB signaling by blocking calcineurin activity.

The RCAN1 gene is located on the DSCR of human chromosome 21. It has been speculated that a number of genes in the DSCR locus contribute to the characteristics of DS individuals. Recently, overexpression of the dual-specificity tyrosine (Y) phosphorylation-regulated kinase 1A (DYRK1A) gene, which maps to the region of the DSCR, has been shown to lead to the neurodevelopmental delay and cognitive defects (38, 39). It has been reported that DYRK1A is involved in the neuronal differ-
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...entiation through the phosphorylation of CREB (40). These findings raise the possibility that RCAN1 might cooperate with other genes on the DSCR in generating pathogenic features associated with DS.

Our studies suggest that RCAN1 is an important component of the CREB regulatory circuit and acts by placing a constraint on calcineurin activity. Consistent with this notion, the RCAN1 knockout mice exhibited significant defects in spatial learning and memory, reduced associative cued memory, and impaired late-phase long-term potentiation (L-LTP), a form of synaptic plasticity dependent on PKA (37). This report suggests that RCAN1 provides a restriction on calcineurin activity during memory formation. These defects were similarly observed in mice with inducible overexpression of constitutively active calcineurin (41, 42). Thus, perturbation of CREB regulatory circuit by an increased dosage of RCAN1 may be sufficient to disrupt optimal neuronal function. To enhance our findings that RCAN1 regulates CREB function in DS pathogenesis, identification of direct endogenous CREB target genes may have important insight into the causes and treatments of DS pathogenesis.

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