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A new calcineurin inhibition domain in Cabin1

Hyonchol Jang [a], Eun-Jung Cho [b], Hong-Duk Youn [a,*]

[a] Department of Biochemistry and Molecular Biology, Cancer Research Institute, Interdisciplinary Program in Genetic Engineering, Seoul National University College of Medicine, 28 Yongon-dong, Chongro-gu, Seoul 110-799, Republic of Korea
[b] College of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea

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Abstract

Calcineurin (CN), a calcium-activated phosphatase, plays a critical role in various biological processes including T cell activation. Cabin1, a calcineurin binding protein 1, has been shown to bind directly to CN using its C-terminal region and inhibit CN activity. However, no increase in CN activity has been found in Cabin1ΔC T cells, which produce a truncated Cabin1 lacking the C-terminal CN binding region. Here, we report that Cabin1 has additional CN binding domain in its 701–900 amino acid residues. Cabin1 (701–900) blocked both CN-mediated dephosphorylation and nuclear import of NFAT and thus inhibited IL-2 production in response to PMA/ionomycin stimulation. This fact may explain why Cabin1ΔC mice previously showed no significant defect in CN-mediated signaling pathway.

Keywords: Calcineurin; Cabin1; Nuclear factor of activated T cells (NFAT); Calcineurin inhibition

Calcineurin (CN; also known as Protein phosphatase 3), a calcium and calmodulin-dependent protein serine/threonine phosphatase, is broadly distributed and its structure is highly conserved from yeast to human [1,2]. CN plays a critical role in various biological processes including cell proliferation, cardiovascular and skeletal muscle development, and apoptosis [3–5]. CN is best known for its role in the calcium dependent regulation of nuclear factor of activated T cells (NFAT) pathways that are involved in T cell activation [5–8]. CN dephosphorylates NFAT to promote its nuclear translocation and the subsequent NFAT-dependent gene expression [5–8]. Specifically, CN plays a pivotal role in the T cell receptor (TCR)-mediated signal transduction leading to the transcriptional activation of cytokines such as interleukin 2 (IL-2) and serves as a common target for the immunosuppressants FK506 and cyclosporin A [9,10].

Cabin1/cain, a calcineurin binding protein 1, was first identified as a CN binding and inhibiting protein [11,12]. Overexpression of full length Cabin1 or its C-terminal fragment in Jurkat T cells represses transcriptional activation of CN-responsive elements in the IL-2 promoter and blocks dephosphorylation of NFAT upon T cell activation [11]. In addition, to binding to CN, the C-terminal region of Cabin1 interacts with myocyte enhancer factor 2 (MEF2) and calmodulin in a mutually exclusive manner [13,14]. Overexpression of Cabin1 in a DO11.10 T hybridoma cells prevents induction of Nur77 by MEF2 and protects the cells from TCR-mediated apoptosis [13]. Cabin1 has also been implicated in regulation of neurotransmitter endocytosis in neuronal cells [15], and muscle cell development [16–19].

Transgenic mice that express activated form of CN in the heart develop cardiac hypertrophy and unexpected deaths within 24 week [20,21]. Homozygous Cabin1-null mice die in utero around embryonic day 12.5 [22]. However, Cabin1ΔC mice, which produce a truncated Cabin1 lacking the C-terminal CN and MEF2 binding region,

Abbreviations: Cabin1, calcineurin-binding protein-1; CN, calcineurin; MEF2, myocyte enhancer factor-2; NFAT, nuclear factor of activated T cells; IL-2, interleukin-2; TCR, T cell receptor; Cabin1ΔC, C-terminal Cabin1-truncated mutant.

* Corresponding author. Fax: +82 2 3668 7431.
E-mail address: hdyoun@snu.ac.kr (H.-D. Youn).

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are born at the expected Mendelian ratio, are healthy, reproduced normally and give no indication of major defect in the development and function of muscle cells or the nervous system [22]. In Cabin1ΔC T cells, no increase in nuclear translocation of NFAT is detected compared to wild-type T cells, although increased production of cytokines upon TCR stimulation is detected [22]. These reports imply that the deletion of its C-terminal CN binding fragment in Cabin1 is insufficient to impair its role severely in regulating CN activity.

In this study, we found that Cabin1 has an additional CN binding and inhibiting domain. Unlike other CN inhibitory proteins, Cabin1 binds to CN through two distinct domains and inhibits CN enzymatic activity.

Materials and methods

Cell culture and transient expression. Jurkat T cells were cultured at 37 °C and 5% CO_2 in RPMI medium 1640 supplemented with 1 mM glutamine, 10% fetal bovine serum and antibiotics and then transfected using electroporation (250 V, 975 μF). Human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics and then transfected using the calcium phosphate coprecipitation methods or Lipofectamine reagent (Invitrogen).

DNA constructs. Myc-tagged Cabin1 and its deletion mutants, HA-CNAβ2, NFAT-luc, ΔNFAT1 and pIL2-luc were described previously (11, 13, 14). Flag-CNAβ2 (FL) was made by subcloning of PCR products from full-length HA-CNAβ2 into pcDNA3.0-flag vector (Invitrogen). HA-tagged CN deletion mutants were generated by subcloning of PCR products from full-length HA-CNAβ2 into pSG5-HA vector (Stratagene). Flag-Cabin1 (FL), Flag-Cabin1 (ΔC/FL), Flag-Cabin1 (Δ701–900/FL), and Flag-Cabin1 (Δ701–900&ΔC/FL) were generated by subcloning of PCR products from full length Cabin1 into pcDNA5/FRT/DEST-Flag (Invitrogen).

Antibodies and reagents. Anti-Flag (M2) antibody was purchased from Sigma, anti-Myc (9E10) and anti-HA (16B12) antibodies from Covance, HRP-conjugated anti-mouse IgG from Pierce, Cy3-conjugated secondary anti-mouse IgG from Jackson Immunoresearch and DAPI from Calbiochem. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma.

Immunoprecipitation assay. Cells were harvested 36 h post-transfection, washed in phosphate-buffered saline, and lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitors). Cell lysates were immunoprecipitated with suitable antibodies along with protein-A/G beads (Santa Cruz). The immunoprecipitates were washed in lysis buffer, denatured in sodium dodecyl sulfate (SDS) loading buffer, and analyzed by Western blot.

Reporter assays. For the reporter gene assays using NFAT-luc, HEK293 cells were transfected with luciferase reporter plasmid, which contains multimerized NFAT binding site, along with CNAβ2 (N401) and various Cabin1 mutants. Cells were harvested 30 h after transfection and luciferase activities were measured with a Sirius luminometer (Berthold Detection Systems). For the reporter gene assays using pIL2-luc, Jurkat T cells were transfected with IL-2 promoter driven luciferase reporter plasmid and various Cabin1 mutants along with CNAβ2 (N401) if indicated. After 30 h of transfection, PMA (40 nM) and ionomycin (1 μM) or PMA (40 nM) alone were treated for 8 h and cells were harvested to assay.

Confocal microscopy. HEK293 cells grown on the cover glasses were transfected with mammalian expression vectors for GPF-fused NFAT4 (1–351), HA-CNAβ2 (N401) and various Myc-tagged Cabin1 mutants. Transfected cells were fixed with 4% (w/v) paraformaldehyde, then permeabilized with 0.5% Triton X-100. Ectopically expressed CNAβ2 (N401) and Cabin1 mutants were immunostained with anti-HA antibody or anti-Myc antibody followed by Cy3-conjugated secondary anti-mouse IgG antibody. Nucleus was stained with DAPI solutions. The protein localization was observed under a Zeiss LSM 510 laser scanning microscope.

NFAT mobility-shifting assay. HEK293 cells were transfected with mammalian expression vectors of HA tagged ΔNFAT1 along with CNAβ2 (N401) and various Cabin1 mutants. Cells were harvested and directly boiled in SDS sample buffer. Samples were subjected to 8% SDS–PAGE, transferred to nitrocellulose membrane, then immunoblotted with anti-HA monoclonal antibody.

RT-PCR. Jurkat T cells were transfected with expression vectors for HA-CNAβ2 (N401) and various Cabin 1 deletion mutants. After 24 h of transfection, cells were treated with PMA (40 nM) or DMSO as a control for 3 h. Total RNA isolation was performed using TRIzol® reagent following the manufacturer's instructions (Invitrogen). The cDNA was synthesized from 2 μg of RNA using RT-PCR. The PCR was performed using 5 μl of synthesized cDNA and specific IL-2 primers (sense: 5′-ATG TACAGGATGCAACTCCTGTCTT-3′, antisense: 5′-GTTAGTGTTGA GATGATGCTTTGAC-3′) or β-actin primers (sense: 5′-GGCATCCAC GAAACTACCTT-3′, antisense: 5′-CTGTGTGGACTTGGGAGAGG-3′).

Statistics. Data are presented as means ± standard deviations and P value was calculated using Student's t-test calculator (http://www.physics.csbsju.edu/stats/t-test.html). A value of $p < 0.05$ was considered statistically significant. All data presented are a representative of at least 3 separate experiments.

Results

Cabin1 has an additional CN binding domain

To investigate whether Cabin1 has additional CN binding domains, we used coimmunoprecipitation assay. Interestingly, Cabin 1 (N1800), which lacks the previously known C-terminal CN binding domain, was found to associate with CN. Further analysis revealed that Cabin1 (N900) but not Cabin1 (900–2116) could interact with CN, suggesting that the N-terminal 900 amino acid residues embrace CN interacting domain (Fig. 1A and B). Pull-down assay with bacterially purified His-CN [23] and *in vitro* translated various deletion mutants of Cabin1 revealed that Cabin1 (321–900), Cabin1 (501–900), and Cabin1 (701–900) as well as Cabin1 (2037C) could bind to CN (Fig. 1A and C). Thus Cabin 1 was found to have another CN binding domain in its N-terminal 701–900 amino acids region as well as the previously-known C-terminal 2117–2161 region. To determine whether the binding between newly found N-terminal domain of Cabin1 and CN was functional in the entire protein, we constructed Cabin1 deletion mutants which lack the amino acids 701–900 (Cabin1 (Δ701–900/FL)), the amino acids 2117–2220 (Cabin1 (ΔC/FL)), and both the amino acids 701–900 and 2117–2220 (Cabin1 (Δ701–900&ΔC/FL)). Coimmunoprecipitation assay using these Cabin1 deletion mutants and CN revealed that both Cabin1 (701–900) and Cabin1 (2117–2161) are functional in the entire protein (Fig. 1D).

Vice versa, we determined the domains in CN that are responsible for the association with Cabin1. The first 97 amino acid residues of CN were sufficient to bind to Cabin1 (701–900) (Supplementary Fig. 1A and C). In case of Cabin1 (2037C), 329–401 amino acid residues of CN were responsible for association with Cabin1 (2037C)

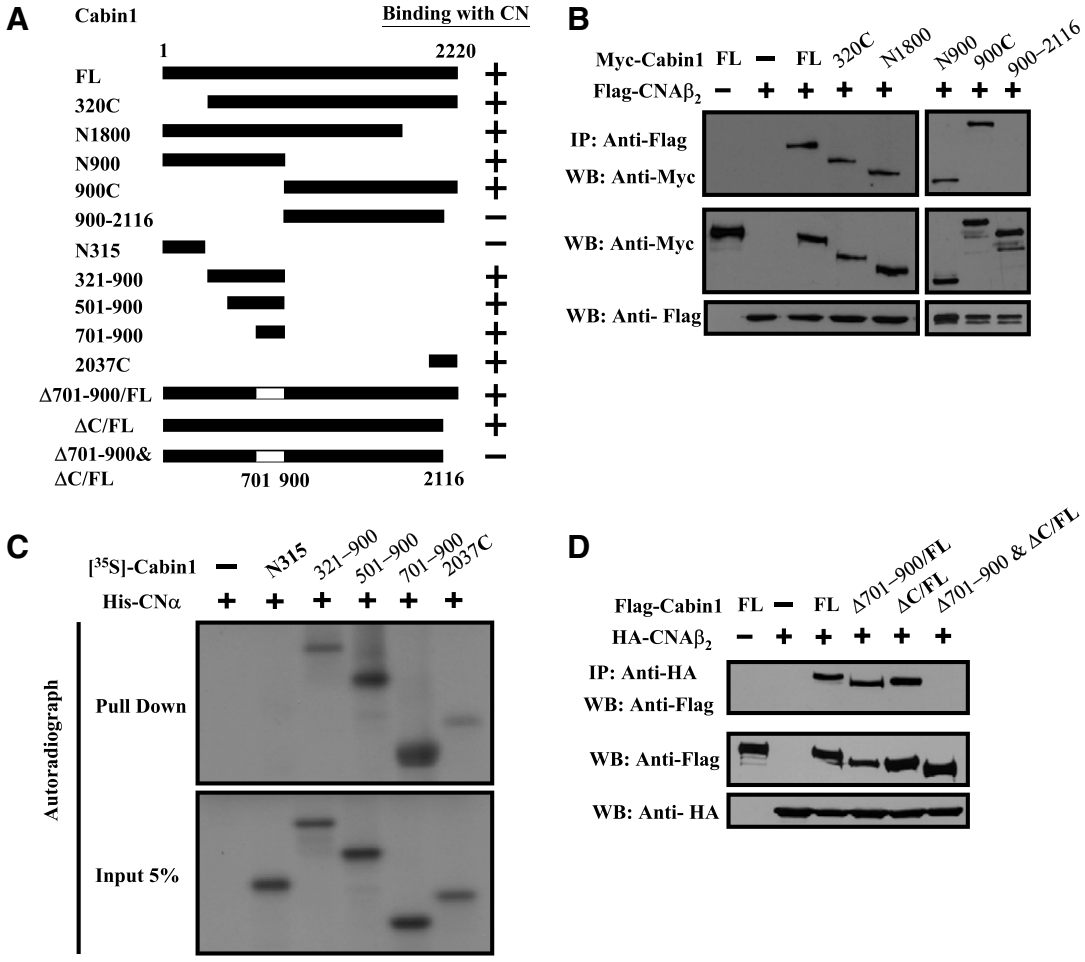

Fig. 1. Both N-terminal and C-terminal region of Cabin1 bind to CN. (A) Diagram of the full-length Cabin1 and its deletion mutants used in this study. (B) Whole cell lysates from HEK 293 cells transiently cotransfected with expression vectors for Myc-Cabin1 deletion mutants and Flag-CN were immunoprecipitated with anti-Flag antibody. Immunoprecipitates were then analyzed by immunoblotting with anti-Myc antibody. (C) *In vitro* transcribed/translated ^{35}S-labeled Cabin1 deletion mutants were incubated with hexa-His tagged CN and immobilized on TALON Metal Affinity Resin (BD Biosciences). Bound proteins were eluted and analyzed by SDS–PAGE and autoradiography. (D) Whole cell lysates from HEK293 cells transiently cotransfected with expression vectors for Flag-Cabin1 deletion mutants and HA-CN were immunoprecipitated with anti-HA antibody. Immunoprecipitates were analyzed by immunoblotting with anti-Flag antibody.

(Supplementary Fig. 1B and C). These results suggest that Cabin1 can bind to CN through two distinct regions (Supplementary Fig. 1D).

Cabin1 (701–900) inhibits CN activity in NFAT-driven reporter assay

Then, we analyzed whether the newly found CN binding domain of Cabin1 could inhibits CN activity. HEK293 cells were transfected with reporter gene under the control of multimerized NFAT binding site. NFAT-luc reporter gene alone didn't show any significant luciferase activity. However, cotransfection of CNA (N401), which lacks C-terminal autoinhibitory domain, enhanced the reporter gene about 70-folds and this enhanced activity was repressed by additional cotransfection of Cabin1 (FL) or Cabin1 (Δ701–900/FL) or Cabin1 (ΔC/FL). Whereas Cabin1 (Δ701–900&ΔC/FL), which lacks both previously and newly found CN binding domain, could not repress CN activity (Fig. 2A). Cabin1 (701–900) suppressed CN activity as efficiently as Cabin1 (2037C) whereas N-terminal 315 amino acid residues, which was found not to associate with CN (Fig. 1B), and Cabin1 (2037C(ΔCN)), which lacks previously known CN binding site [14], didn't suppress CN activity (Fig. 2B).

Both dephosphorylation and nuclear import of NFAT in response to CN activation are blocked by Cabin1 (701–900)

To determine whether the inhibition of NFAT-signaling by Cabin1 (701–900) is mediated through the inhibition of CN phosphatase activity, we examined the effect of Cabin1 (701–900) on the CN-mediated dephosphorylation of the N-terminal 460 amino acids fragment of NFAT1 (ΔNFAT1) upon stimulation with CNA (N401) (Fig. 3A). Transfection of ΔNFAT1 alone maintained it

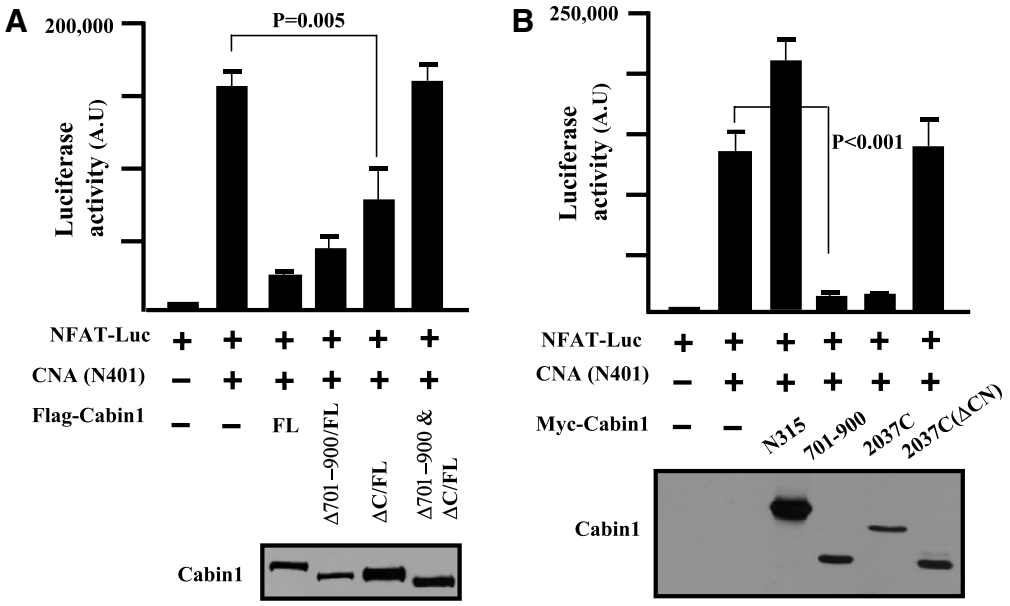

Fig. 2. Cabin1 (701–900) inhibits CN activity in NFAT-driven reporter assay. (A,B) HEK293 cells were transfected with NFAT-Luc reporter plasmid, expression vectors for a constitutively active CN mutant (CNA (N401)) and Cabin1 deletion mutants. Luciferase activity was measured after 30 h with a luminometer. The values represent arbitrary luciferase activity means ± standard errors ($n = 3$).

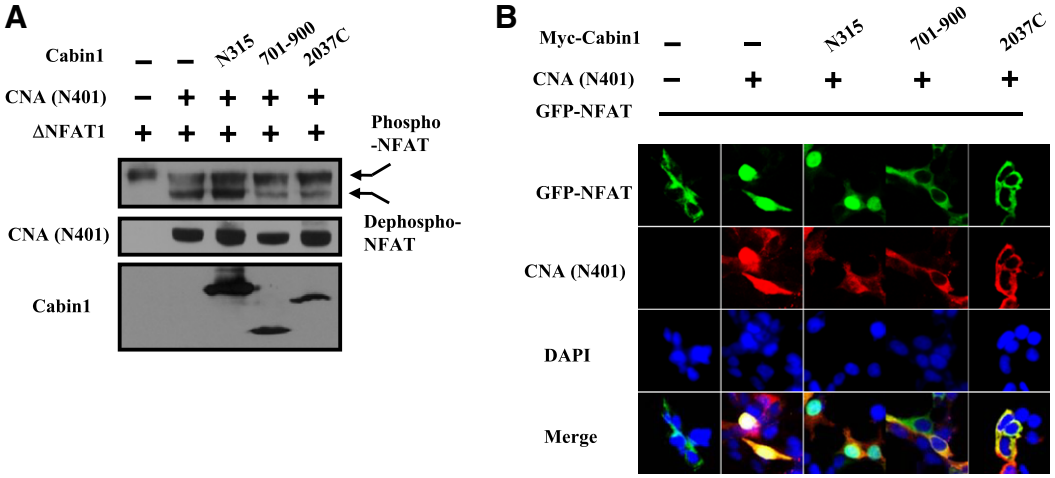

Fig. 3. Both dephosphorylation and nuclear import of NFAT in response to a constitutively active CN are inhibited by Cabin1 (701–900). (A) Whole cell lysates form HEK293 cells transfected with ΔNFAT1, CNA (N401) and various Cabin1 deletion mutants were analyzed by SDS–PAGE and Western blot. (B) HEK293 cells were transfected with expression vectors for GFP-NFAT, CNA (N401) and various Cabin1 deletion mutants. Cells were stained as described in Materials and methods and intracellular localization of NFAT and CN were observed in a microscope.

almost exclusively in the phosphorylated state. Cotransfection of CNA (N401) dephosphorylates more than half of the ΔNFAT1 and additional cotransfection of Cabin1 (701–900) or Cabin1 (2037C) inhibits the dephosphorylation of ΔNFAT1 by CNA (N401) significantly (Fig. 3A). Next, HEK293 cells were transfected with the expression vectors for GFP-NFAT4 along with CNA (N401) and various Cabin1 mutants. Overexpression of CNA (N401) was sufficient to trigger the nuclear translocation of GFP-NFAT4 (Fig. 3B). However, additional cotransfection of Cabin1 (701–900) blocked nuclear import of NFAT as efficiently as Cabin1 (2037) (Fig. 3B, Supplementary Fig. 2). Then we further investigate whether Cabin1 (701–900) blocks not only overexpressed CNA (N401) but also endogenous full-length CN. Jurkat T cells were transfected with ΔNFAT1 and various Cabin1 mutants. Upon stimulation with ionomycin, significant portion of ΔNFAT1 was dephosphorylated and this dephosphorylation was inhibited by transient overexpression of Cabin1 (701–900) or Cabin1 (2037C), whereas this dephosphorylation was not affected by transient overexpression of Cabin1 (N315) (Supplementary Fig. 3).

IL-2 production in response to PMA/ionomycin stimulation is inhibited by Cabin1 (701–900)

To investigate whether the newly found CN binding domain of Cabin1 can affect IL-2 production in T cells, Cabin1 deletion mutants were cotransfected into Jurkat T cells with a luciferase reporter gene under the control of the IL-2 promoter. Upon treatment with PMA and ionomycin, the IL-2 reporter gene was activated about 40-folds, and this activation was inhibited by Cabin1 (701–900) or Cabin1 (2037C) (Fig. 4A). It has been known that both PKCθ and calcium signaling is required in TCR signaling mediated induction of IL-2 [24]. Thus we dissect whether the inhibition of IL-2 reporter gene by Cabin1 (701–900) was caused by the inhibition of calcium signaling. Stimulation upon PMA alone or overexpression of CNA (N401) alone didn't activate IL-2 reporter gene (Fig. 4B). However, overexpression of CNA (N401) and stimulation upon PMA activated IL-2 reporter gene about 30-folds, and this activation was inhibited by Cabin1 (701–900) or Cabin1 (2037C) (Fig. 4B), suggesting that inhibition of IL-2 production by Cabin1 (701–900) is caused by the inhibition of CN activity. In order to reconfirm these results, we transfected Jurkat T cells with CNA (N401) along with various Cabin1 mutants and analyzed the change of the IL-2 mRNA upon stimulation with PMA. Stimulation upon PMA leads to induction of IL-2 mRNA, and cotransfection of Cabin1 (N315) didn't affect this induction, whereas cotransfection of Cabin1 (701–900) as well as Cabin1 (2037C) abrogated the induction of IL-2 mRNA (Fig. 4C). These findings demonstrate that Cabin1 has additional CN binding domain in its N-terminal 701–900 amino acid residues, which can sufficiently inhibit CN activity.

Dephosphorylation of R II phosphopeptide by CN is inhibited by Cabin1 (701–900)

Finally, we investigated the inhibition mode of CN by Cabin1 (701–900). we determined whether Cabin1 (701–900) could inhibit R II phosphopeptide dephosphorylation by CN. Whole cell lysates from HEK293 cells transiently transfected with HA-CN and various Cabin1 deletion mutants were immunoprecipitated with anti-HA antibody. The immunoprecipitates were activated by addition of calcium and calmodulin and then mixed with R II phosphopeptide. CN activity was measured by detecting free phosphates released using classic Malachite Green assay. The results showed that both Cabin1 (701–900) and Cabin1 (2037C) sufficiently inhibits dephosphorylation of R II

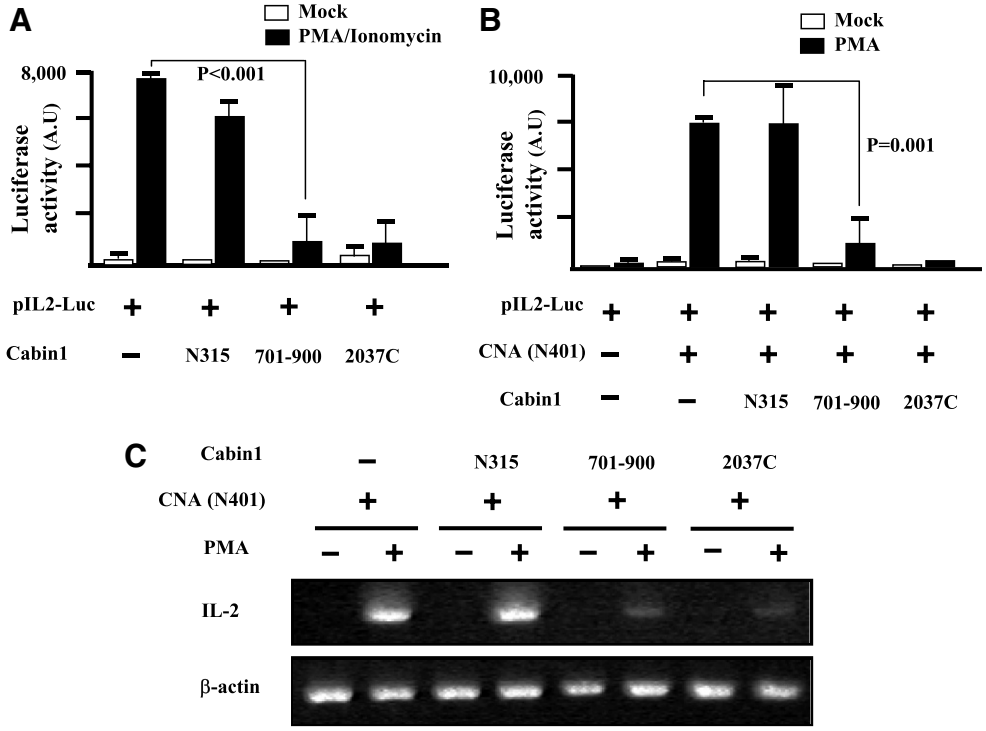

Fig. 4. Dominant inhibitory effect of overexpression of Cabin1 (701–900) on the transcriptional activation of CN responsive elements in the interleukin-2 promoter in response to PMA/ionomycin stimulation. (A) Jurkat T cells were transfected with IL-2 promoter driven luciferase reporter gene and various Cabin1 mutants. After 24 h of transfection, cells were treated with PMA (40 nM)/ionomycin (1 μM) or DMSO (control) for 8 h. Luciferase activity was measured with a luminometer. (B) Jurkat T cells were transfected with pIL-2-luc, CNA (N401) and various Cabin1 mutants. After 24 h of transfection cells were treated with PMA (40 nM) or DMSO (control) for 8 h. Luciferase activity was measured with a luminometer. The values represent arbitrary luciferase activity means ± standard errors ($n = 3$). (C) Jurkat T cells were transfected with CNA (N401) and various Cabin1 mutants. After 24 h of transfection, cells were treated with PMA (40 nM) or DMSO (control) for 3 h. Total RNA isolation and RT-PCR was carried out as described in Materials and methods.

phosphopeptide, whereas Cabin1 (N315) did not (Supplementary Fig. 4). Thus, Cabin1 (701–900) not only inhibits CN-NFAT signaling but also inhibits CN phosphatase activity.

Discussion

In this study, we found a previously unrecognized CN binding domain in N-terminal region of Cabin1. Previous studies on Cabin1 have mainly focused on the C-terminal region of Cabin1 presumably because of the initial recognition of the C-terminal fragment as a CN binding partner and the huge size of Cabin1. However, it has not been studied so far whether there were additional CN binding domains in the remaining regions [11–13]. Overexpression of full length Cabin1 in DO11.10 blocks TCR-mediated apoptosis [13]. Transgenic mouse which express C-terminal fragment of Cain in the heart demonstrate reduced cardiac calcineurin activity [17–21]. Considering these results, the C-terminal Cabin1-truncated mutant (Cabin1ΔC) expressing T cells were expected to be increased in CN activity. However, in Cabin1ΔC mice, Cabin1ΔC T cells showed no change in CN activity [22]. This discrepancy may be answered by our finding that Cabin1 has additional CN inhibition domain in its N-terminal region.

There are a number of CN-inhibiters, but their inhibition mode is different. The PxIxIT peptide analog, VIVIT competitively inhibits CN-NFAT binding, but not R II phosphopeptide dephosphorylation [25,26]. LxVP peptide inhibits both CN-NFAT binding and dephosphorylation of R II phosphopeptide [27]. Down Syndrom Critical Region 1 is a competitive inhibitor of CN [28]. The A kinase associated protein and the C-terminal fragment of Cabin1 are noncompetitive inhibitor of CN [12,29]. The newly found CN binding domain of Cabin1 binds to the N-terminal region of CN. The X-ray structure of the CN heterodimer [30,31] shows that 1–97 amino acid residues of CN is far from the phosphatase active site. Thus the binding of Cabin1 (701–900) may not block substrate from access to CN active site. Nevertheless, Cabin1 (701–900) inhibits both NFAT-signaling and dephosphorylation of R II phosphopeptide by CN. These facts imply that Cabin1 (701–900) has allosteric effect on CN and inhibits CN noncompetitive manner.

The fact that Cabin1-null mice die in utero [22] is surprising regarding the facts that transgenic mice that express activated form of CN in the heart or in the forebrain are born even though there are some defects in the heart or brain [20,32]. The dramatic difference between the phenotype of Cabin1ΔC mice and Cabin1-null mice [22] suggests that Cabin1ΔC region has unrevealed functions. In this study, we reveal that N-terminal 701–900 amino acid residues of Cabin1 are capable of binding and inhibiting CN. Additional study about the role of Cabin1ΔC region may increase the understanding of the physiological function of Cabin1.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.05.066.

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