Inhibition of Bcl10-mediated activation of NF-κB by BinCARD, a Bcl10-interacting CARD protein

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Abstract **We have identified a novel CARD-containing protein from EST database, BinCARD (Bcl10-interacting protein with CARD). BinCARD was ubiquitously expressed. Co-immunoprecipitation, In vitro binding, mammalian two-hybrid, and immunostaining assays revealed that BinCARD interacted with Bcl10 through CARD. BinCARD potently suppressed NF-κB activation induced by Bcl10 and decreased the amounts of phosphorylated Bcl10. Mutations at the residue Leu17 or Leu65, which is highly conserved in CARD, abolished the inhibitory effects of BinCARD on both Bcl10-induced activation of NF-κB and phosphorylation of Bcl10. Further, expression of BinCARD inhibited Bcl10 phosphorylation induced by T cell activation signal. These results suggest that BinCARD interacts with Bcl10 to inhibit Bcl10-mediated activation of NF-κB and to suppress Bcl10 phosphorylation.**

Keywords: CARD; BinCARD; NF-κB activation; Bcl10; Phosphorylation

1. Introduction

Apoptosis plays a role not only in the development and homeostasis of multicellular organism but also in the pathogenesis [1]. Apoptotic signals are mediated by the association of proteins containing homologous domains, such as death domain (DD), death effector domain (DED), and caspase recruitment domain (CARD) [2]. The CARD was first identified in caspases and their adaptor molecules. Recently, several CARD-containing proteins have been identified (Bcl10, CARD8/TUCAN, CARD10, CARD11, and CARD14), the function of which appears to be directed primarily toward regulation of nuclear factor-κB (NF-κB) [3–6]. Mutations in the CARD-containing molecules are associated with diseases; mutation in Bcl10 is related with lymphoma of mucosa-associated lymphoid tissue [7]. ASC, apoptosis-associated speck-like protein-containing CARD, is also associated with familiar Mediterranean fever [8].

Bcl10 (also known as CIPER/c-CARMEN/CLAP/c-E10) has found to be essential for NF-κB activation in T or B cells [3,9]. Bcl10 (−/−) lymphocytes fail to activate NF-κB in response to CD3, CD3/CD28, and IgM ligation [10]. Several CARD family proteins also have been described to drive NF-κB activation through association with Bcl10 [5]. Thus, Bcl10 is an important convergence for CARD-containing proteins that regulate NF-κB activation. Here, we describe a novel protein that contains an N-terminal CARD. It interacts with Bcl10 through CARD and downregulates NF-κB activation induced by Bcl10, named BinCARD (Bcl10-interacting protein with CARD).

2. Materials and methods

2.1. Database search

A BinCARD cDNA (GenBank Accession No. AK057716) was identified by searching the expressed sequence tag (EST) databases of GenBank™ using the nucleotide sequence encoding a CARD as a query with the blastn program.

2.2. Antibodies and reagents

Polyclonal anti-BinCARD antibody was generated against purified glutathione *S*-transferase (GST)–CARD fusion proteins of BinCARD. Anti-Bcl10 antibody (SC-331.3) was purchased from Santa Cruz Biotechnology, Inc. PMA with ionomycin (Sigma) and anti-CD3 with anti-CD28 antibodies (BD Biosciences) were used for stimulation of Jurkat cells.

2.3. Plasmid construction

The plasmids were generated with standard polymerase chain reaction (PCR) using the following primer pairs: pBinCARD, 5′-CCGGAATTCATGACAG ATCAGACCTATTGTG-3′, 5′-GCTCTAGACTAGGAGCGCCATGCAGC-3′; pHA-BinCARD-N, 5′-CCGGAATTCATGACAGATCAGACCTATTGTG-3′, 5′-CCTAGTCTAGAATCTGAGTTCTGCAGAGC-3′; and pHA-BinCARD-C, 5′-GCGAATTCCGTAAGTTCCACATCACC-3′, 5′-GCTCTAGATAGGAGCGCCATGCAGC-3′. Site-directed mutagenesis was performed using the following primer pairs: pBinCARD-L17A, 5′-CAGGACACGCCTTTCCCGACAGGCCATGGGCGC-3′, 5′-GCGCCCATGGCCTGTCGGGAAAGGCGTGTCCTG-3′; pBinCARD-L65A, 5′-GTGCGGCTCTGTGACGCCCTGAGCCACCTGCAG-3′, 5′-CTGCAGGTGGCTCAGGGCGTCACAGAGCCGCAC-3′. pNF-κB-luc and pβact-lacZ were previously described [11].

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Abbreviations: CARD, caspase recruitment domain; BinCARD, Bcl10-interacting protein with CARD; GST, glutathione *S*-transferase; NF-κB, nuclear factor-κB; HA, hemagglutinin

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2.4. Northern blot analysis

Human MTN blot (Cat.#7780-1) was obtained from CLONTECH. CARD of BinCARD cDNA was radiolabeled with [α-^{32}P]dCTP, hybridized, washed according to the manufacturer's instructions, and exposed to autoradiography film.

2.5. Cell culture and transfection

HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) containing 10% fetal bovine serum (Equi-bio Tech, Inc.). Jurkat cells were maintained in RPMI1640 medium (Life Technologies, Inc.). Transfection was performed as described earlier [11]. Jurkat cells were transfected with control or pbabe-BinCARD plasmid and selected with puromycin (1 μg/ml) for stable single clone.

2.6. Luciferase and β-galactosidase assays

Luciferase activities of cell extracts were determined using a Luciferase Assay System (Promega). β-Galactosidase activity was examined after mixing with β-galactosidase assay buffer (2×) containing 200 mM sodium phosphate (pH 7.3), 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml *o*-nitrophenyl-β-D-galactopyranoside. The absorbance at 420 nm was measured using ELISA reader (Molecular Device).

2.7. Mammalian two-hybrid assay

Cells were transfected with the following plasmids: pCMV-BD/BinCARD, pCMV/AD fused to Bcl10 mutants, pFR-Luc firefly reporter (Stratagene), and pβact-LacZ. The panel of CARD domains used for the screen was described previously [6]. After 24 h, luciferase and β-galactosidase activities were determined.

2.8. Immunoprecipitation

Cells were lyzed with RIPA buffer [50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF]. Immunoprecipitation was performed with anti-hemagglutinin (HA) and protein-A-coupled Sepharose CL-4B (Amersham–Pharmacia Biotech).

2.9. Immunostaining

Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 3 min, and incubated with primary antibodies overnight at 4 °C. Cells were incubated with FITC or TRITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc.) and bisbenzamide (Hoechst 33258). Fluorescent images were observed under fluorescence microscopy (LEICA).

2.10. Phosphatase assay

Cells were lyzed in phosphatase assay buffer [10 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF]. Cell lysates (20 μg) were incubated with calf intestine phosphatase (New England Biolabs.) for 1 h at 37 °C.

3. Results

3.1. Identification and expression of BinCARD

To identify CARD-containing proteins, we screened public database on the analysis of domain architectures (http://SMART.embl-heidelberg.de). This search identified an EST clone (GenBank Accession No. AK057716) encoding a 228-amino acids protein with homology to a consensus CARD (Fig. 1A). We termed this protein as BinCARD. Comparisons of the cDNA sequence with the genomic sequences indicated that BinCARD consists of four exons, spanning 17.14 kb on chromosome 9q22.32. Alignment analyses revealed that the N-terminal region of BinCARD (residues 8–100) shares significant sequence similarity with the CARD motifs of ced-3, caspase-1, caspase-2, caspase-4, caspase-5, and RAIDD (Fig. 1B and C).

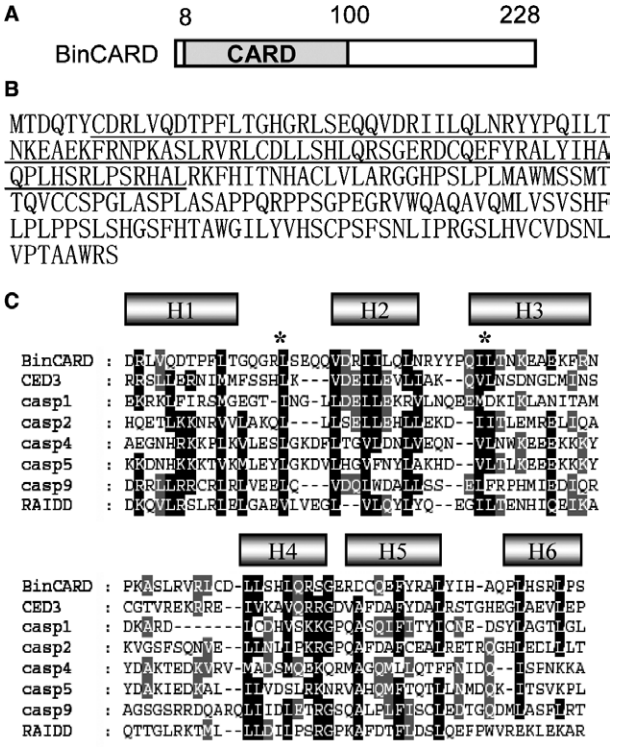

Fig. 1. Sequence analysis of BinCARD. (A) Schematic representation of the domain structure of BinCARD. (B) The predicted amino acid sequence of BinCARD. The region encompassing the CARD motif is underlined. (C) Alignment of the CARD of BinCARD with those of the CARD-containing proteins. The conserved sequences are darkly shaded. Predicted α-helixes (H) are indicated and asterisks represent the residues mutated in the mutation analysis of BinCARD.

Northern blot analysis was performed to determine the distribution of BinCARD transcripts in human tissues using the CARD of BinCARD as a probe. BinCARD transcript was detected in all tissues examined: BinCARD was highly expressed

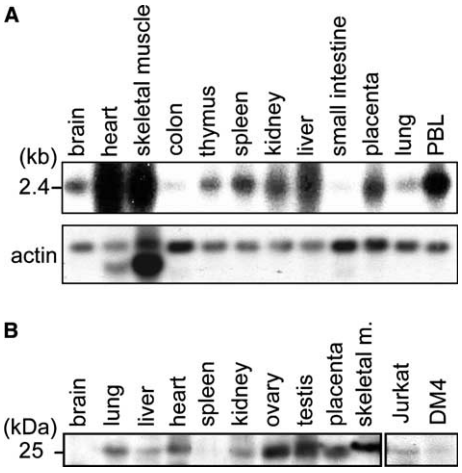

Fig. 2. Expression of BinCARD mRNA and protein. (A) BinCARD mRNA of human adult tissues was examined by Northern blot analysis. (B) BinCARD protein of human adult tissues and cell lines was examined by Western blot analysis using anti-BinCARD antibody.

in heart, skeletal muscle, and peripheral blood leukocyte (Fig. 2A). BinCARD protein was observed in a variety of human tissues, Jurkat T cells, and DM4 melanoma cells examined with Western blot analysis using anti-BinCARD antibody (Fig. 2B).

3.2. BinCARD binds to Bcl10 through CARD–CARD interaction

The CARD mediates specific homophilic interaction with other CARD-containing molecules. To identify the binding partner of BinCARD, we performed a mammalian two-hybrid screening of several CARD-containing proteins for their abilities to interact with the CARD of BinCARD. CARD of BinCARD specifically interacted with the CARD of Bcl10 and did not interact with CARD of BinCARD (Fig. 3A). To determine the interaction motif of Bcl10, deletion mutants of Bcl10 were generated. BinCARD interacted with Bcl10 through CARD (Fig. 3B). A physical association between BinCARD and Bcl10 was examined with in vitro assay (Fig. 3C). Pull-down assay revealed that BinCARD directly associated with Bcl10 in vitro. However, introduction of the mutation in the highly conserved leucine residue at 65 (L65A) of BinCARD abolished its ability to bind to Bcl10 (Fig. 3C).

To examine an intracellular interaction of BinCARD with Bcl10, HA-tagged Bcl10 and BinCARD expression plasmids were transfected into HEK293T cells. Cell lysates were immunoprecipitated with the anti-HA antibody (Fig. 3D, upper panel). Western blot analysis of the immunoprecipitates showed that BinCARD and Bcl10 associated with each other.

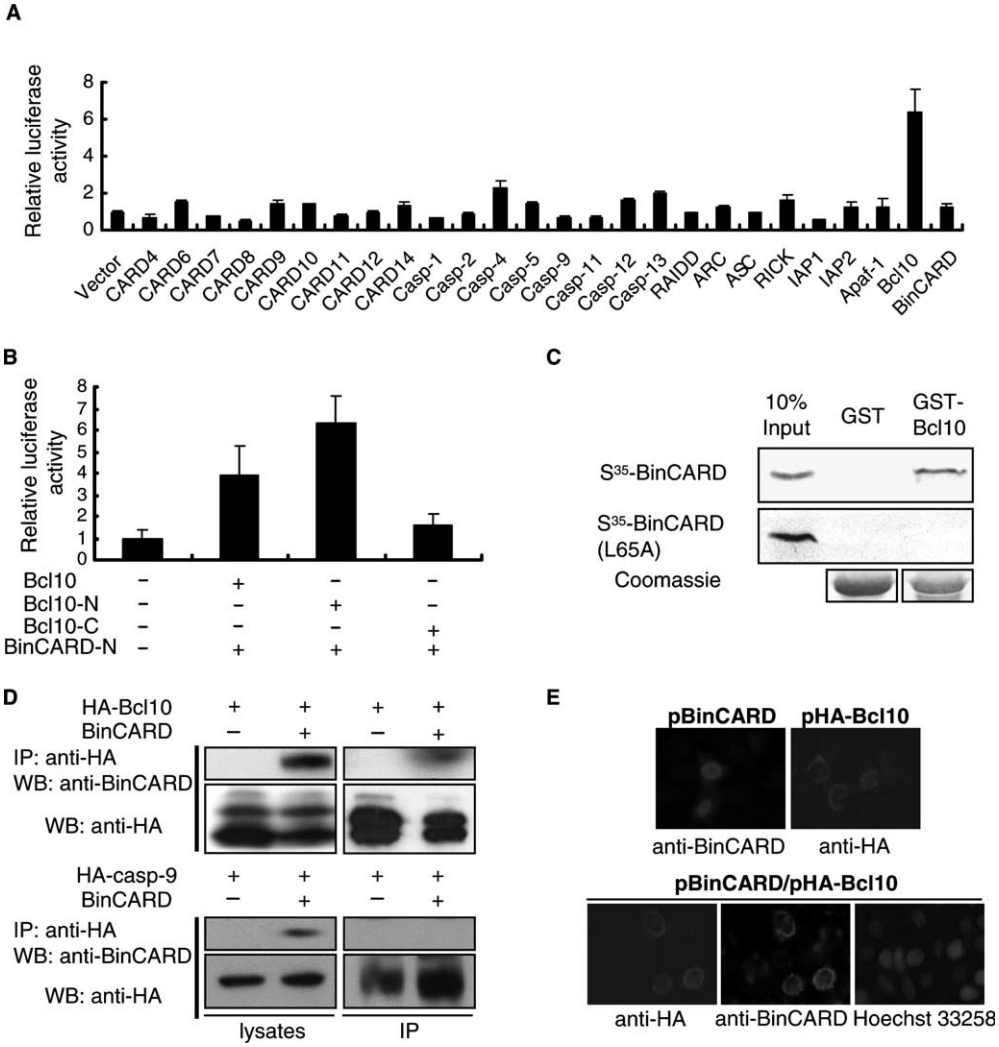

Fig. 3. Interaction of BinCARD with Bcl10 via CARD. (A) CARD of BinCARD interacts with CARD of Bcl10 examined by mammalian two-hybrid analysis. HEK293T cells were transfected with the reporter construct in combination with pCMV-BD/BinCARD and CARD of CARD-containing proteins of pCMV-AD [9]. After 36 h, cells were collected and assayed for relative luciferase activity as a measure of intracellular protein–protein interaction. (B) HEK293T cells were transfected with pCMV-BD/BinCARD and either wild type, N-terminal (Bcl10-C), or C-terminal (Bcl10-N) deletion mutant of pCMV-AD/Bcl10 and then assayed for relative luciferase activity. (C) In vitro interaction of BinCARD and Bcl10. GST or GST-Bcl10 proteins bound to glutathione–Sepharose beads were incubated with ^{35}S-labeled BinCARD or L65A mutant. (D) HEK293T cells were co-transfected with pBinCARD and either pHA-Bcl10 (upper panel) or pHA-caspase-9 (lower panel). Immunuprecipitation was performed with anti-HA antibody and the immunoprecipitates and cell lysates were analyzed by Western blotting with anti-BinCARD antibody. (E) HeLa cells were transfected with pBincard and/or pHA-Bcl10. After 36 h, cells were stained by anti-Bincard and anti-HA antibodies. In lower panel, different color of fluorescence was observed for Bcl10 (TRITC) and BinCARD (FITC). Nuclei were stained by Hoechst 33258.

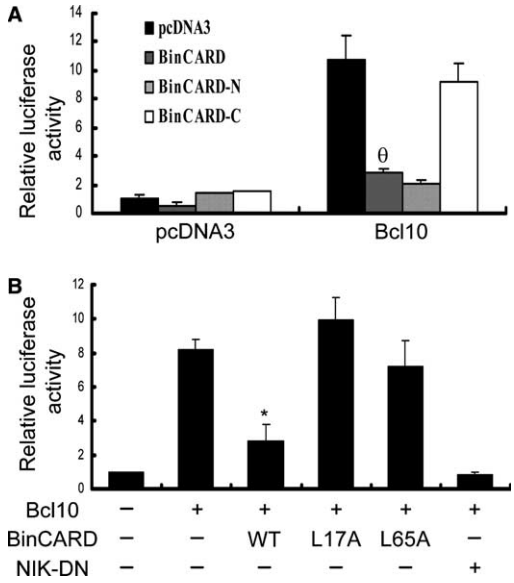

Fig. 4. Inhibition of Bcl10-induced activation of NF-κB by BinCARD. (A) HEK293T cells were transfected with pNF-κB-luc and either pcDNA3 or pBcl10, along with either wild type or deletion mutants of BinCARD. pβact-lacZ was included as an internal control. After 48 h, cell lysates were prepared and luciferase activities were measured and normalized by β-galactosidase activity. Bars depict means ± S.D. θ indicates the statistical difference with respect to Bcl10 alone ($p < 0.01$). (B) Point mutants of L17A and L65A and DN mutant of NIK (NIK-DN) were expressed with Bcl10 and NF-κB reporter gene, and relative luciferase activities were determined. The asterisk indicates the statistical significance with respect to Bcl10 alone ($P < 0.03$).

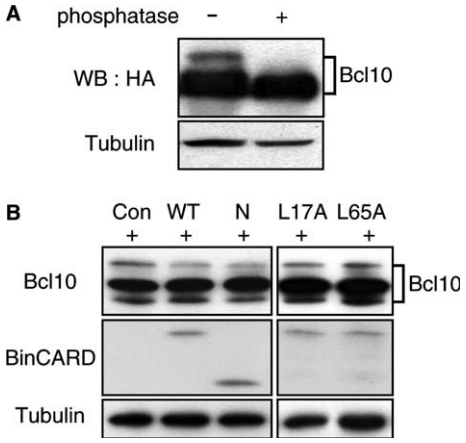

Fig. 5. Modulation of Bcl10 phosphorylation by BinCARD. (A) Cell extracts were prepared from HEK293T cells expressing HA-Bcl10, incubated with (+) or without (−) calf intestine phosphatase for 1 h at 37 °C. (B) HEK293T cells were co-transfected with pHA-Bcl10 and either pcDNA3 (Con), pBinCARD (WT), pBinCARD-N (N), pBinCARD-L17A (L17A), or pBinCARD-L65A (L65A). Cell extracts were analyzed using anti-HA (upper panel), anti-BinCARD (middle panel), or anti-Tubulin antibody (lower panel).

However, caspase-9, which contains CARD at its N-terminus, did not interact with BinCARD (Fig. 3D, lower panel).

Further, co-expression of Bcl10 and BinCARD induced translocation of BinCARD from nucleus to cytosol, while Bincard and Bcl10 were detected predominantly in nucleus and cytoplasmic filaments, respectively (Fig. 3E). Similar change in the subcellular localization of BinCARD-N (CARD of Bincard) was observed in the cells overexpressing Bcl10 (data not shown). These results support that BinCARD interacts with Bcl10 in cells.

3.3. BinCARD inhibits Bcl10-mediated activation of NF-κB

It has been found that CARD proteins can function as components of signaling pathways that lead to activation of the NF-κB [12]. We tested whether BinCARD could modulate Bcl10-mediated activation of NF-κB using luciferase reporter assay. As shown in Fig. 4A, expression of Bcl10 increased NF-κB activity 10-fold compared with control. Interestingly, expression of BinCARD or BinCARD-N exhibited inhibitory effects on Bcl10-induced activation of NF-κB. It has been reported that point mutation and deletion of amino acids in the CARD domain significantly influenced the binding activity and function of CARD proteins [3]. To characterize the CARD of BinCARD in the inhibition of NF-κB activity, we generated two point mutants of BinCARD replacing conserved leucine residue with alanine (L17A and L65A) (Fig. 1C). Point mutations at 17 or 65 abolished the NF-κB inhibitory activity of BinCARD (Fig. 4B). The dominant repressor mutant of NIK (NIK-DN) efficiently blocked NF-κB activation induced by Bcl10 [9]. Similar expression levels of the mutants of BinCARD were confirmed by immunoblotting (data not shown).

3.4. BinCARD reduces the phosphorylation of Bcl10

Bcl10 migrates on SDS–polyacrylamide gels as multiple bands in size due to phosphorylation [9]. We observed that slow migrating bands appeared to be phosphorylated forms of Bcl10, since treatment of the cell lysates with phosphatase eliminated the slowest migrating form (Fig. 5A). To examine whether BinCARD can affect on the phosphorylation of Bcl10, Bcl10 was co-transfected with BinCARD. When expressed alone, the phosphorylated forms of Bcl10 were increased by the treatment with phosphatase inhibitor, okadaic acid (data not shown). Interestingly, co-expression of BinCARD or BinCARD-N decreased the phosphorylated form of Bcl10, the slow migrating band (Fig. 5B). Disappearance of phosphorylated Bcl10 by BinCARD correlated with the NF-κB inhibitory activity of BinCARD. L17A and L65A mutants lacking NF-κB inhibitory activity could not reduce the amounts of the phosphorylated Bcl10 (Fig. 5B, upper panel). Western blot analysis revealed that the expression level of BinCARD mutants was similar to wild type (Fig. 5B, middle panel). These data suggest that BinCARD inhibits the phosphorylation of Bcl10 in a CARD-dependent manner.

3.5. BinCARD inhibits Bcl10 phosphorylation induced by T cell activation signal

Bcl10 knockout (−/−) lymphocytes failed to activate NF-κB in response to CD3, CD3/CD28, and IgM ligation [10] and Bcl10 was phosphorylated and translocated to the lipid-raft after T cell receptor activation [13]. To examine whether BinCARD can function in T cell signaling, we generated stable lines overexpressing BinCARD in Jurkat T cells. RT-PCR was performed as described earlier [11] (Fig. 6A). Consistent with the previous reports [16], co-stimulation of Jurkat-puro control cells with anti-CD3/CD28 antibodies or with a combi-

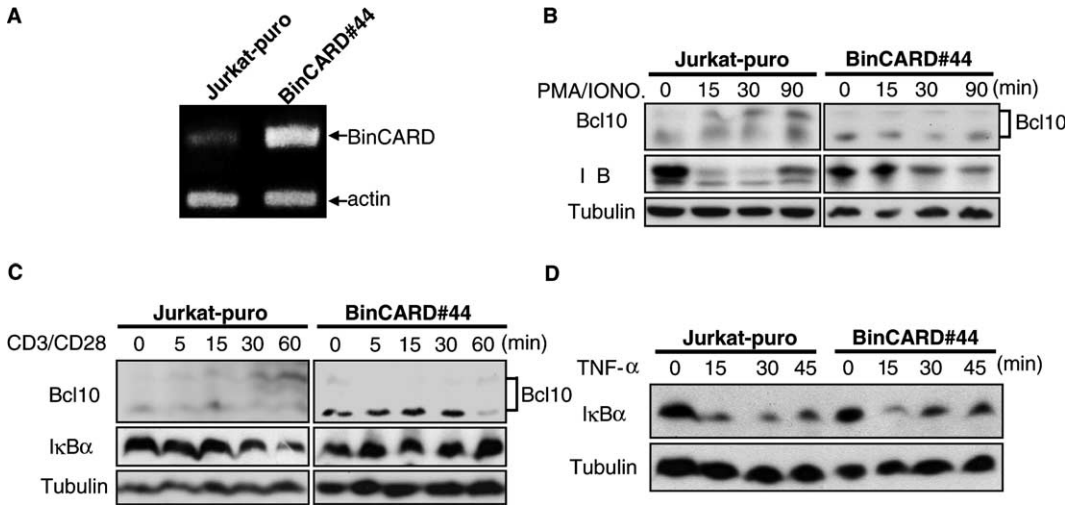

Fig. 6. Suppression of Bcl10 phosphorylation by BinCARD during T cell activation signaling. (A) RT-PCR was performed using cDNAs prepared from Jurkat-puro and Jurkat-BinCARD#44 cells. Actin was shown as control. (B) Cells were stimulated with PMA (20 ng/ml) and ionomycin (IONO., 1 μM) for the indicated times. Cell extracts were analyzed with immunoblotting using anti-Bcl10, anti-BinCARD, anti-IκBα, and anti-Tubulin antibodies. (C, D) Jurkat-puro and Jurkat-BinCARD#44 cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) antibodies or TNF-α (20 ng/ml) for the indicated times. Bcl10 phosphorylation and IκBα degradation were examined by Western blot analysis.

nation of phorbol 12-myristate 13-acetate (PMA) and ionomycin, which mimics the downstream events of TCR activation, induced Bcl10 phosphorylation (Fig. 6B and C). However, expression of BinCARD (BinCARD#44) suppressed Bcl10 phosphorylation and IκBα degradation induced by T cell activation signal (Fig. 6B and C). TNF-α-mediated degradation of IκBα was normal in BinCARD#44 cells (Fig. 6D). These results suggest that BinCARD inhibits Bcl10 phosphorylation by being recruited into the Bcl10 complexes during TCR activation signaling.

4. Discussion

Knockout and transgenic studies reveal that the Bcl10-mediated activation pathway of NF-κB plays an essential role in antigen receptor signaling in B and T cells [10]. Our finding is that BinCARD binds to Bcl10 and inhibits NF-κB activation through its CARD. The inhibitory effects of BinCARD on NF-κB activity correlate with its binding ability to Bcl10. CARD proteins that assemble with Bcl10, such as CARD9, CARD10, CARD11, and CARD14, transduce upstream stimuli to the activation of NF-κB [5,6]. BinCARD may engage in this signaling complex through interaction with Bcl10, which deserves to be further elucidated.

Bcl10 is modified by phosphorylation [9,14,15] and the phosphorylation of Bcl10 might play a distinct role in the regulation of NF-κB activation induced by T cell activation signal. Our observations that the expression of BinCARD decreased the phosphorylated form of Bcl10 suggest that BinCARD might prevent the phosphorylation of Bcl10 by binding to it. Co-immunoprecipitation and in vitro binding assays imply that BinCARD may interact better with the unphosphorylated form of Bcl10, leading us to propose that BinCARD may secure Bcl10 inactive.

As a negative regulator of NF-κB activity, BinCARD might also be recruited into the Bcl10 complexes to downregulate the NF-κB signaling during T cell activation. Recent report has demonstrated that Carma1 is a component of Bcl10-mediated NF-κB activation complexes [16]. However, our in vitro binding assays indicate that though Carma1 and BinCARD interact with Bcl10, they do not compete with each other to bind to Bcl10 (data not shown). In addition, phosphorylation of Bcl10 causes changes in its binding partners [14]. Thus, interference of Bcl10 phosphorylation by BinCARD may alter the components of the Bcl10-signaling complex, blocking the activation of NF-κB. The presence of unidentified components in NF-κB signaling triggered by antigen receptor is estimated to fill the remaining gaps in this pathway [17]. Further study aims at molecular architecture of Bcl10-mediated NF-κB-signaling complex and offers an attractive perspective for the understanding of the growth regulation and pathological dysregulation of the NF-κB pathway.

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