Carma1, a CARD-containing binding partner of Bcl10, induces Bcl10 phosphorylation and NF-κB activation[1]

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Abstract Bcl10, a caspase recruitment domain (CARD)-containing protein identified from a breakpoint in mucosa-associated lymphoid tissue (MALT) B lymphomas, is essential for antigen-receptor-mediated nuclear factor κB (NF-κB) activation in lymphocytes. We have identified a novel CARD-containing protein and interaction partner of Bcl10, named Carma1. Carma1 is predominantly expressed in lymphocytes and represents a new member of the membrane-associated guanylate kinase family. Carma1 binds Bcl10 via its CARD motif and induces translocation of Bcl10 from the cytoplasm into peri-nuclear structures. Moreover, expression of Carma1 induces phosphorylation of Bcl10 and activation of the transcription factor NF-κB. We propose that Carma1 is a crucial component of a novel Bcl10-dependent signaling pathway in T-cells that leads to the activation of NF-κB.

Key words: Bcl10; Caspase recruitment domain; Carma; Lymphoma; Membrane-associated guanylate kinase; Nuclear factor κB

1. Introduction

Mucosa-associated lymphoid tissue (MALT) lymphomas most frequently involve the gastrointestinal tract and are the most common subset of extranodal non-Hodgkin lymphoma. Chromosomal translocation t(1;14)(p22;q32) is recurrent in MALT lymphomas and is associated with aggressive disease [1]. Molecular cloning of the breakpoint identified a novel gene, *Bcl10*, which is translocated to the immunoglobulin heavy chain locus [2,3]. The human *Bcl10* gene encodes a protein of 233 amino acids (aa) containing an N-terminal caspase recruitment domain (CARD). Several groups have independently characterized Bcl10 as a CARD-containing protein displaying high homology to the equine herpes virus gene product v-E10 [4–6]. Both v-E10 and Bcl10 contain a highly homologous amino-terminal CARD motif that allows for both homo- and heterodimerization of the two proteins [4–6]. v-E10 binds TRAF3 and TRAF6, whereas its cellular homolog Bcl10 interacts with TRAF1, 2 and 5 [4,7]. Despite this structural homology and the capacity of both proteins to associate with TRAFs, they differ in their ability to trigger nuclear factor κB (NF-κB) activation. Overexpression of Bcl10 results in weak NF-κB activation while v-E10 triggers a strong response [4–6]. Bcl10 was also reported to induce apoptosis [5,8,9], however, this function could not be confirmed in cells deficient in the Bcl10 protein [10]. Instead, antigen-driven NF-κB signals and proliferation were found to be defective in Bcl10−/− lymphocytes, corroborating the role of Bcl10 as an important component of the NF-κB signaling pathway.

While events that lead to NF-κB activation downstream of Bcl10 have been studied and include TRAF-binding and subsequent recruitment of the IKK complex [4,7,11], little is known about the upstream activator(s) of Bcl10. Some insight was recently obtained from the study of the molecular function of equine herpes virus protein v-E10, which activates NF-κB via Bcl10 [12]. v-E10 is targeted to the plasma membrane due to a geranylgeranylation consensus site at its C-terminus and is able to recruit Bcl10 from the cytoplasm. This recruitment results in the hyperphosphorylation of Bcl10 and in activation of NF-κB.

To learn more about Bcl10's role in the pathway that connects the antigen receptor to NF-κB activation, we thought to identify the upstream binding partner and activator of Bcl10. Here we present the initial characterization of such a candidate protein, named Carma1 (CARD-MAGUK1). Similar to v-E10, Carma1 interacts with Bcl10 via a CARD–CARD interaction, induces its phosphorylation at several sites, and provokes NF-κB activation.

2. Materials and methods

2.1. Cloning of Carma1 cDNA

EST clones encoding human Carma1 were identified in the dbEST data base at the National Center for Biotechnology Information (EST clones 1283 557 and 2228 571). Both clones were sequenced and EST 2228 571 was used to amplify the C-terminal portion of Carma1 (332–1148) by polymerase chain reaction (PCR) using primers JT2110 (5′-gtatctagaggagaaggaggacctgga-3′) and JT2111 (5′-ggtctagatca cagctggtcctcgtccac-3′) for the N-terminally tagged versions and using JT2110 and JT2112 (5′-ggggatcccagctggtcctcgtccaccca-3′) for the C-terminally tagged constructs. Other Carma1 constructs were cloned using standard procedures.

2.2. Expression vectors

Expression vectors for v-E10 and Bcl10 with an amino-terminal

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[1] The GenBank accession numbers for the human Carma1, Carma2 and Carma3 are AF100338, AF100339 and AF100340, respectively.
[2] These authors contributed equally to this work.
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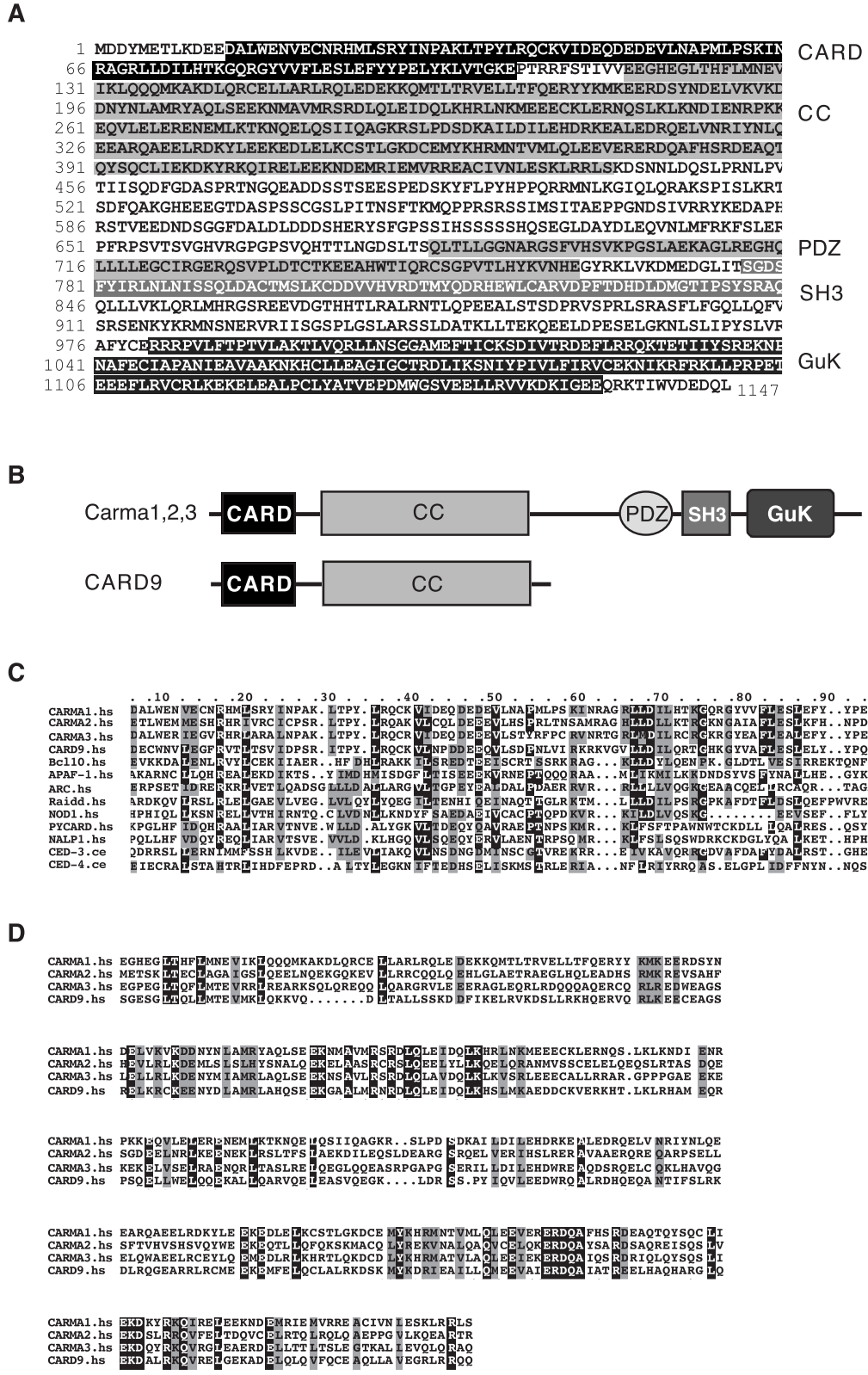

Fig. 1. A: Amino acid sequence of Carma1. The open reading frame for Carma1 reveals the presence of a CARD motif, a CC domain, a PDZ domain, an SH3 domain and a GUK domain (boxed areas). B: Domain organization of the Carma family (Carma 1–3, CARD9). Alignments of (C) CARD from a selected subset of CARD-containing proteins and (D) CC domains from the Carma family members. For each block of aligned sequences, black boxes indicate $>50\%$ (100% for CC domains) aa sequence identity and gray shading indicates $>50\%$ (75% for CC domains) sequence similarity through conservative aa substitutions.

FLAG- or VSV-tag and for dominant negative TRAF1 and IκB have been described previously [4,13]. The point mutation in the CARD motif (L41R) was obtained by amplification on cloned full-length wild-type Bcl10 cDNA using standard PCR conditions and Pwo polymerase (Roche). The following expression plasmids were kindly obtained from the indicated sources: GFP-TRAF2-DN (aa 266–501) from H. Wajant (Stuttgart, Germany); VSV-IKKγ-DN from S. Whiteside and A. Israel, (Paris, France), and NF-κBLuc and β-gal reporter plasmids from V. Jongeneel (Lausanne, Switzerland).

2.3. Northern blot analysis

Northern blot analysis was performed by probing a human multiple tissue Northern blot (Clontech), according to the manufacturer's instructions, with a ^{32}P-labeled antisense RNA probe encompassing the N-terminal 650 nucleotides of Carma1 cDNA.

2.4. Transient cell transfection, immunoprecipitation and NF-κB activation assays

These techniques were performed essentially as described before [4,14]. Antibodies used for Western blotting include anti-FLAG M2 and anti-VSV P5D4 monoclonal antibodies, respectively (Sigma). Cellular (endogenous) Bcl10 was detected using an affinity-purified polyclonal rabbit antibody (AL114) directed against a peptide encompass-

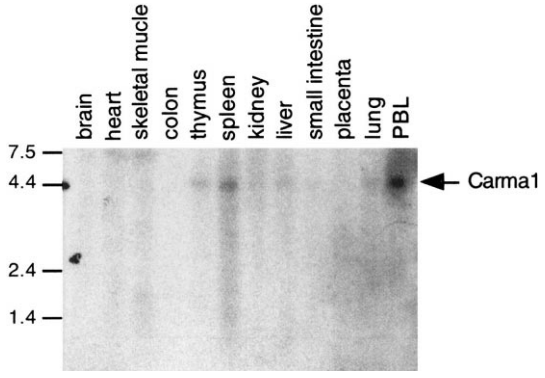

Fig. 2. Tissue distribution of murine Carma1. A Northern blot of various mouse tissues (Clontech) was probed with a ^{32}P-labeled antisense RNA fragment covering the CARD-coding region of Carma1.

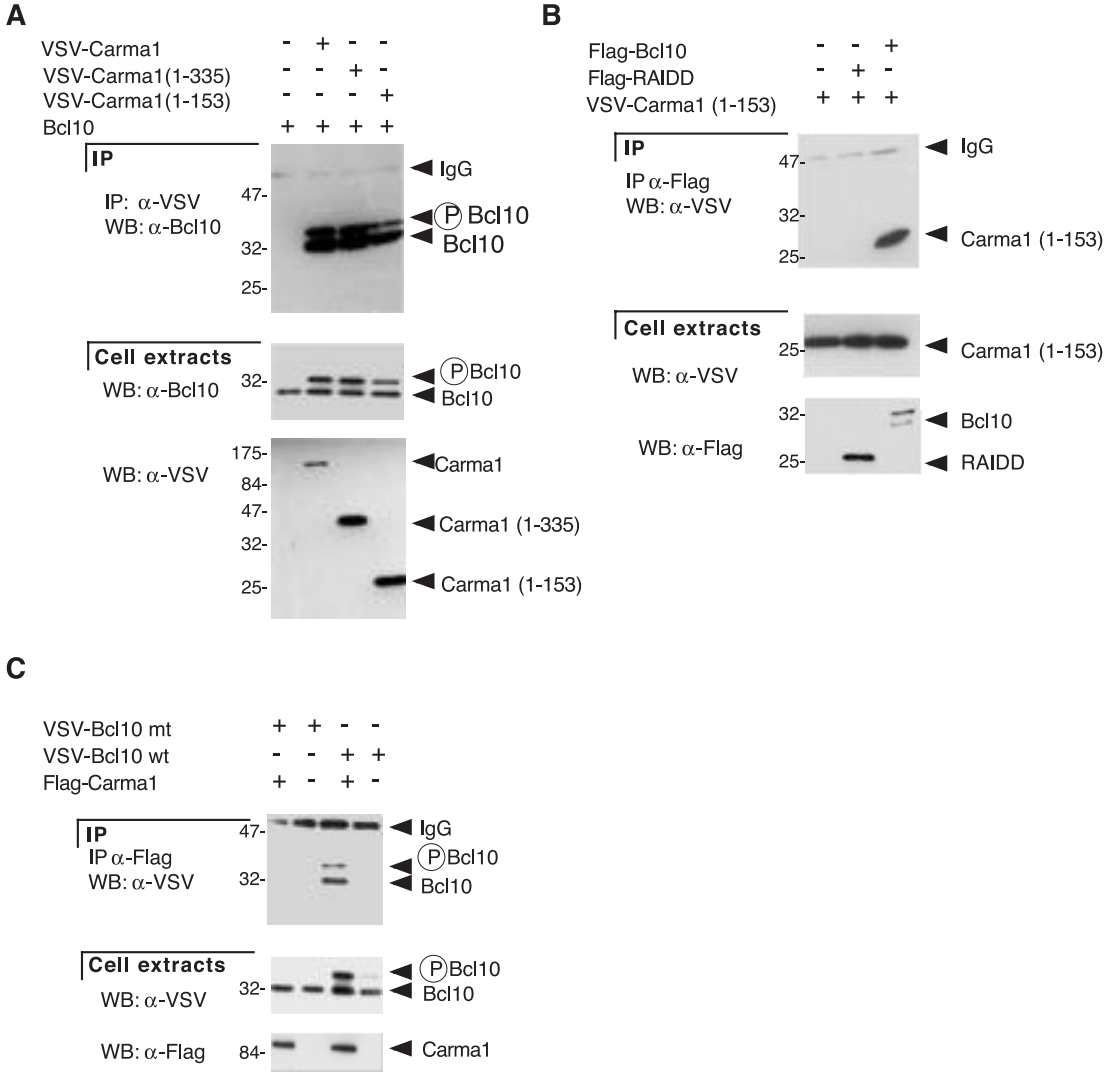

Fig. 3. Interaction of Carma1 with Bcl10. A: 293T cells were co-transfected with expression vectors for various VSV-tagged constructs of Carma1 and for Bcl10, as indicated, and anti-VSV immunoprecipitates were analyzed for the presence of Bcl10 by Western blotting. The expression levels of the Bcl10 and VSV-tagged Carma1s in the cell extracts are shown below. B: 293T cells were co-transfected with an expression vector for VSV-tagged Carma1 (aa 153) and FLAG-Bcl10 and FLAG-RAIDD. Anti-FLAG immunoprecipitates were then analyzed for the presence of Carma1. C: Wild-type and a CARD-mutant Bcl10 were analyzed for their capacity to interact with Carma1.

ing 24 amino-terminal aa (SLTEEDLTEVKKDALENYRVLCEK) of murine Bcl10.

2.5. Protein dephosphorylation

Transfected 293T HEK cells from a 5.5-cm dish were lysed in 1% NP-40 buffer containing 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.25 μg/ml Pefabloc® sc (Serva) on ice. Aliquots (20 μg) of postnuclear lysates were incubated with λ protein phosphatase (New England Biolabs) in a buffer supplied by the manufacturer for 3 h at 30°C.

2.6. Immunostaining and confocal laser scanning microscopy

HeLa cells were transfected with tagged constructs by the calcium phosphate/BES method. Expressed proteins were stained with anti-VSV (Sigma, 1/50 000) or anti-FLAG (Zymed, 0.2 μg/ml) and subsequently with Cy5-labeled (Jackson ImmunoResearch Laboratories) or Alexa-488-labeled (Molecular probes) secondary anti-mouse or anti-rabbit antibody. Confocal microscopy was performed on a Zeiss Axiovert 100 microscope (Zeiss Laser Scanning Microscope 510).

3. Results

3.1. Identification of Carma1 as a novel interaction partner of Bcl10

To identify cellular proteins that possibly interact with the CARD motif of Bcl10, we have used a bioinformatic approach, which is based on the observation that domains of the DD-fold family (DD, DED, CARD motifs) interact best when their respective sequences are highly similar [15]. When we screened genomic databases with a profile (Pfr: CARD) that detects CARD-containing proteins, more than 30 CARD-containing proteins were found. Our attention was attracted by a family of three proteins, designated Carma1, Carma2 and Carma3, which were predicted to contain CARD motifs that are highly homologous to the CARD of Bcl10. The CARDs of Carma1–3 share 23, 27 and 20% identical aa with the Bcl10 CARD, respectively (Fig. 1C), while, for comparison, only 16% identity is found between the CARD motif of Bcl10 and NALP1 [16].

We have focused our interest on Carma1, since several EST clones, covering most of the C-terminus of the predicted protein, were available for Carma1, and the missing cDNA portion encoding the N-terminus could be obtained by nested reverse transcription-PCR. Sequence analysis of the complete cDNA clone validated the genomic sequence, predicting a protein of 1147 aa (132 kDa) (Fig. 1A), comprising five putative functional domains (Fig. 1B): the N-terminal CARD is followed by a region of approximately 300 aa with a predicted coiled-coiled (CC) structure [17], a PDZ domain, an SH3 domain and a GUK (guanylate kinase) domain. The PDZ/SH3/GUK domain arrangement is the signature of members of the MAGUK (membrane-associated GUK) family, which are scaffolding proteins organizing macromolecular complexes at specialized regions of the plasma membrane [18]. A similar structural organization is also predicted for Carma2 and Carma3. The N-terminal part of Carma1 (comprising the CARD and CC domains) displays high sequence similarity with CARD9, a protein recently reported to interact with Bcl10 [18]. Of particular interest is not only the high conservation between the two CARD motifs of Carma1 and CARD9 (52% identical aa) but also the high sequence similarity in the CC region, indicating that CARD 9 is a bona fide member of the Carma family (Fig. 1D). Since most cytoplasmic proteins containing a CC structural motif form dimers [17], it is likely that CARD9 (Carma4) and the other Carma family members form homo- and/or heterodimers.

Northern blot analysis revealed that Carma1 expression was restricted to lymphoid tissues. A single transcript of approximately 4.4 kb was predominantly found in spleen, thymus and, in particular, PBLs (Fig. 2). This tissue distribution suggests a role of Carma1 in cells of the immune system in which Bcl10 was previously shown to play a major role in NF-κB activation [10].

The possible interaction between Carma1 and Bcl10, suggested from the relatively high degree of sequence identity between the CARD of Bcl10 and Carma1, was examined by co-expression of different FLAG-tagged Carma1 constructs with VSV-tagged human Bcl10 in 293T cells, followed by co-immunoprecipitation studies. An interaction was noted

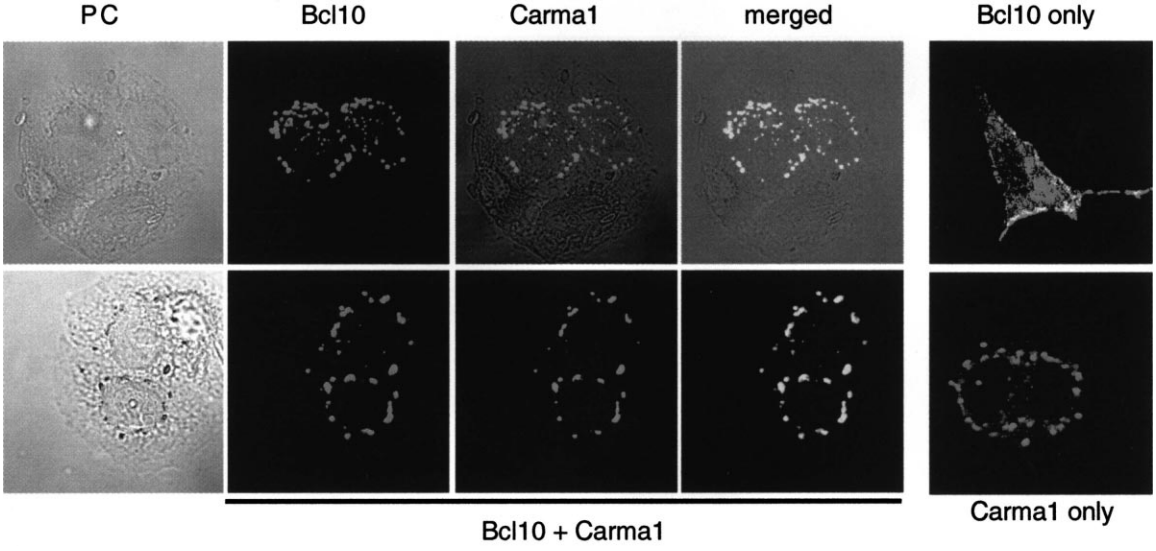

Fig. 4. Carma1 recruits Bcl10 to perinuclear structures. HeLa cells were transfected with expression vectors for both FLAG-Bcl10 and VSV-Carma1 and their subcellular localization determined 30 h after transfection by confocal laser microscopy, using the anti-VSV or anti-FLAG antibodies. The overlay of Bcl10 fluorescence and Carma1 is shown in the column 'merged'. The rightmost panels show HeLa cells that were transfected with either construct alone. PC: Phase contrast.

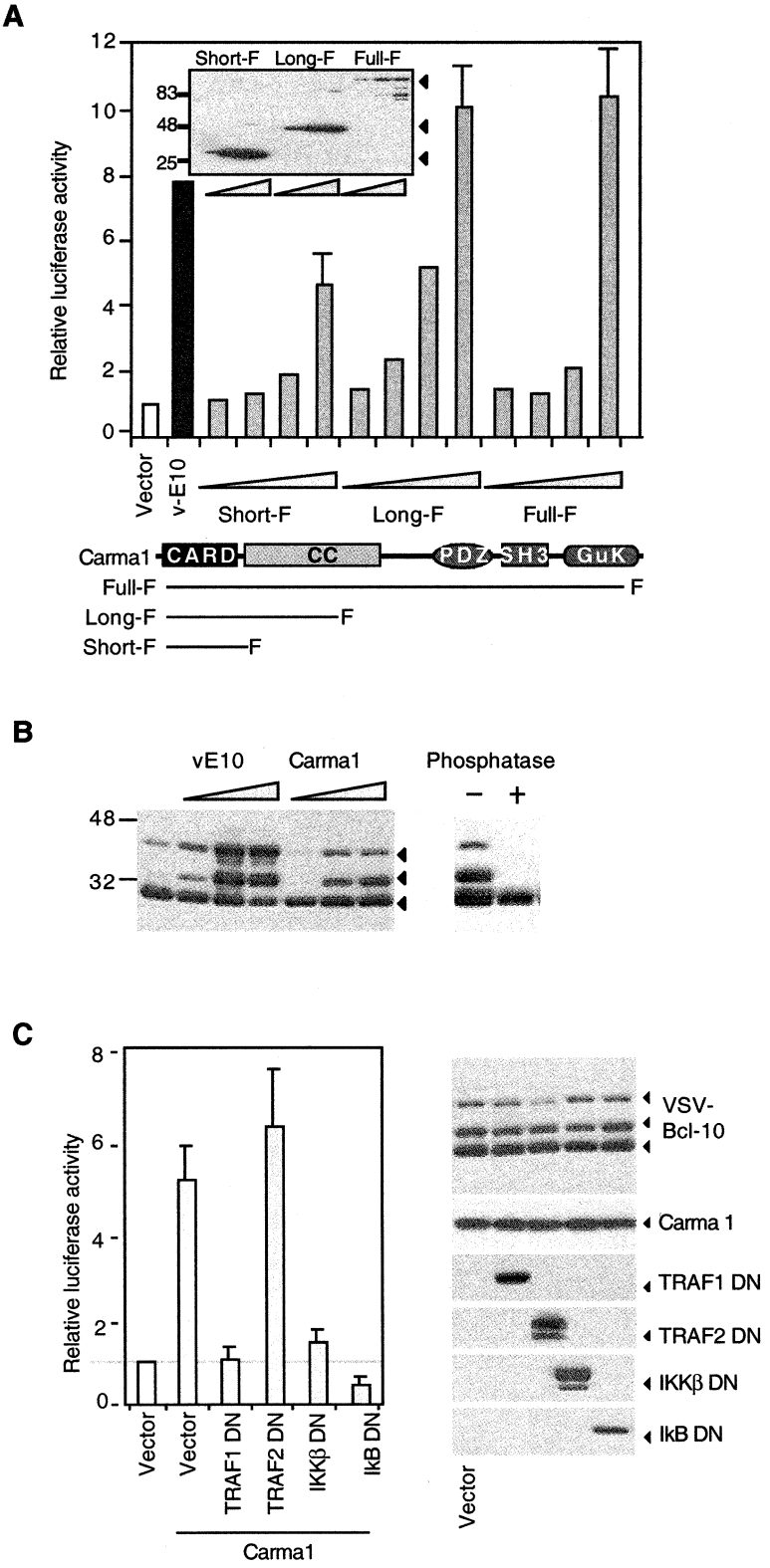

Fig. 5. NF-κB activation and Bcl10 phosphorylation by Carma1. A: 293T cells were co-transfected with an NF-κB luciferase reporter plasmid (pNF-κBluc), β-galactosidase expression vector (pCMV β-gal), and increasing amounts (0.005, 0.05 and 0.5 μg) of the indicated Carma1 constructs with FLAG-tags at their C-terminus (-F). Luciferase activities were determined 24 h after transfection and normalized on the basis of β-galactosidase values as previously described [4]. Values shown are averages for representative experiments in which each transfection was carried out in duplicate. Expression levels of Carma1 constructs are shown in the inset. B: 293T cells were co-transfected with VSV-tagged Bcl10 and increasing amounts (0.005, 0.05 and 0.5 μg) of expression vectors for FLAG-tagged Carma1 or v-E10, respectively. Arrowheads indicate the migration position of the non-phosphorylated and the two hyperphosphorylated forms of Bcl10. Cell extracts of transfected cells were incubated for 3 h in presence or absence of λ-phosphatase, as indicated. C: Inhibition of Carma1-induced NF-κB activation but not Bcl10 phosphorylation by dominant negative (DN) forms of IKKβ TRAF1 and IkB. 293T cells were co-transfected with pNF-κBLuc, pCMV β-gal, 5 μg of Carma1, 5 μg of Bcl10 and/or the indicated DN expression vectors. Samples were analyzed for luciferase activity (left panel) and phosphorylation of Bcl10 (right panel). Expression levels of the transfected proteins are shown in the lower right panel.

only between Carma1 and Bcl10 (Fig. 3A), while RAIDD, caspase-1, -2, -4 and -9, v-E10 or CARDIAK (RIP2) did not interact (Fig. 3B and data not shown). Carma1 did not interact with a mutant Bcl10 whose CARD motif was mutated such that it had lost the capacity to interact with other CARDs [5,6,9,12], indicating that the interaction was CARD-dependent. We were unable to detect an interaction of Carma1 with v-E10 (data not shown).

Association of Carma1 with Bcl10 could also be followed by confocal microscopy. Overexpression of Bcl10 in HeLa cells resulted in a diffuse cytoplasmic staining, in agreement with our previous observation [12] (Fig. 4). Carma1, in contrast, showed a granular perinuclear staining. Upon co-expression with Carma1, most of the cytoplasmic staining of Bcl10 disappeared and Bcl10 now co-localized with Carma1 to perinuclear structures. Taken together, our results demonstrate that Carma1, via its CARD motif, associates with Bcl10.

3.2. Activation of NF-κB by Carma1

Since Bcl10 plays a major role in the NF-κB signaling pathway in lymphocytes [10], we next investigated the capacity of full-length Carma1 and two truncated forms thereof (Carma1-short, containing roughly the CARD motif, and Carma1-long containing the CARD motif and most of the CC domain, see Fig. 5A) to activate the NF-κB luciferase reporter plasmid in 293T cells. Expression of full-length and of Carma1-CARD-CC in 293T cells (which contain endogenous Bcl10, data not shown) resulted in an up to 10-fold increase of the reporter gene (Fig. 5A) and was comparable to the capacity of v-E10 to activate this transcription factor. The CARD motif of Carma1 alone was sufficient for NF-κB activation, albeit at reduced efficiency. DN-IKK2, DN-IκB and DN-TRAF1, but not DN-TRAF2 (which does not bind Bcl10 in our hands [4]), inhibited Carma1-mediated NF-κB activation (Fig. 5B). Together, these results indicate that Carma1, akin to v-E10, initiates the NF-κB signaling cascade upstream of TRAF1 and the IKK signalosome [4,11].

3.3. Carma1 induces phosphorylation of Bcl10

v-E10-induced NF-κB induction leads to the formation of hyperphosphorylated forms of Bcl10 (Fig. 5C) [4]. We therefore investigated whether Carma1 induces phosphorylation of Bcl10. When expressed alone, Bcl10 was mostly in its non-phosphorylated form (Fig. 5C), while in the presence of increased levels of Carma1, a phosphorylation pattern of Bcl10 identical to the one induced by v-E10, i.e. the unphosphorylated (30 kDa) and two slower migrating species (34 and 38 kDa) was observed. These latter two bands correspond to phosphorylated forms as shown by treatment of the lysates with λ-phosphatase (Fig. 5C). DN-IKK2, DN-IκB and DN-TRAF1 did not inhibit Bcl10 phosphorylation (Fig. 5B), suggesting that Bcl10 phosphorylation occurs upstream or in parallel to TRAF1/IKK2-dependent NF-κB induction by Carma1.

4. Discussion

We have identified Carma1 as a CARD-containing member of the MAGUK family and interaction partner of Bcl10. Overexpression of Carma1 results in a CARD-dependent association with Bcl10 and induces phosphorylation of Bcl10 and NF-κB activation. In this respect, Carma1 behaves like v-E10. Phosphorylation of Bcl10 and activation of NF-κB by v-E10 is dependent on Bcl10 translocation from the cytoplasm to the membrane [12]. Carma1, on the other hand, localizes to perinuclear structures in epithelial cells and Bcl10 translocates to these structures when co-expressed with Carma1. This is in complete agreement with a study published during the preparation of our manuscript [19]. It is probable that this translocation triggers Bcl10 to signal NF-κB, but it remains to be seen whether complex formation between Carma1 and Bcl10 in perinuclear structures also occurs in lymphocytes, to which Carma1 expression appears to be restricted. In T-cells, Carma1 and Bcl10 may co-localize in the zone of the immunological synapse where T-cell receptors are being stimulated by antigen-presenting cells. Such a model is suggested from the structural organization of Carma1 identifying it as a member of the MAGUK family. MAGUK members are scaffolding proteins that organize macromolecular complexes at specialized regions of the plasma membrane [18]. MAGUKs possess a characteristic domain structure: one or more PDZ domain(s), an SH3 domain and a GUK domain at the C terminus. PDZ and SH3 domains are well established as protein-binding structures, the former recognizing peptide ligands, usually at the C terminus of target proteins, and the latter recognizing proline-rich peptide motifs. In addition, most MAGUKs contain a motif rich in basic residues which mediates binding to protein 4.1 and/or other ezrin–radixin–moesin family proteins, thus providing anchorage to the cortical actin network. Reorganization of the cortical cytoskeleton is a hallmark of T lymphocyte activation occurring during the formation of the immunological synapse [20]. Thus, it is conceivable that in the immunological synapse, Carma1 plays a role as a modulator of signaling pathways that exist between the T-cell receptor and the cytoskeleton.

Translocation and upregulation of Bcl10 in MALT tumors conceivably mimics antigen receptor signaling by constitutively activating NF-κB, thereby promoting antigen-independent growth and lymphoma progression. Another MALT lymphoma translocation, t(11;18)(q21;q21), fuses the *IAP-2* gene to the *MLT1*/*MALT1* locus [21–23], which encodes the human MLT protein. This fusion activates NF-κB and interestingly, the MLT protein directly interacts with Bcl10 [24], indicating that MLT and Bcl10 are components of the same signaling pathway. Bcl10 regulates lymphocyte proliferation, and its requirement for NF-κB signaling suggests a mechanism for the oncogenic effect. Activation of NF-κB signaling either through mutation of rel genes and their upstream regulators or by expression of viral oncogenes has been implicated in a range of hematopoietic tumor types. The precise knowledge of the Carma1/Bcl10-dependent NF-κB pathway will therefore be of utmost importance for cancer therapy.

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