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Physical and Functional Interaction of CARMA1 and CARMA3 with Iκ Kinase γ-NFκB Essential Modulator*

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CARMA proteins are scaffold molecules that contain a caspase recruitment domain and a membrane-associated guanylate kinase-like domain. CARMA1 plays a critical role in mediating activation of the NFκB transcription factor following antigen receptor stimulation of both B and T lymphocytes. However, the biochemical mechanism by which CARMA1 regulates activation of NFκB remains to be determined. Here we have shown that CARMA1 and CARMA3 physically associate with Iκ kinase γ/NFκB essential modulator (IκKγ-NEMO) in lymphoid and non-lymphoid cells. CARMA1 participates to an inducible large molecular complex that contains IκKγ/NEMO, Bcl10, and IκKα/β kinases. Expression of the NEMO-binding region of CARMA3 exerts a dominant negative effect on Bcl10-mediated activation of NFκB. Thus, our results provide direct evidence for physical and functional interaction between CARMA and the IκK complex and offer a biochemical framework to understand the molecular activities controlled by CARMA-1, -2, and –3 and Bcl10.

The Rel/NFκB signaling pathway and the transcription factors that it activates have emerged as critical regulators of normal immune and inflammatory response, cell proliferation, differentiation, apoptosis, and oncogenesis (1–3). A key event in the canonical NFκB cascade is the activation of the Iκ kinase (IκK)[1] complex, which is composed of three subunits: IκKα, IκKβ, and IκKγ/NFκB essential modulator (NEMO) (4). Whereas IκKα and IκKβ have catalytic kinase activity, NEMO is an important regulatory subunit, and deficiency of this protein results in complete lack of NFκB activation (5). Once activated, the IκK complex is responsible for the phosphorylation and subsequent proteasome-mediated degradation of the inhibitory proteins IκBs (4). Degradation of IκBs frees NFκB and allows its translocation in the nucleus, where it activates transcription of target genes (4).

In T cells, activation of the IκK complex following antigen receptor stimulation requires the activity of PKCθ (6–8) and the function of the caspase recruitment domain (CARD)-containing proteins Bcl10 (9) and CARMA1 (10–16). CARD is a protein-protein interaction motif, originally identified as a conserved sequence present in various molecules involved in regulation of apoptosis, such as RAIDD, several caspases, and the *Caenorhabditis elegans* genes *ced-3* and *ced-4* (17). However, a number of CARD-containing proteins are not implicated in apoptotic signaling but participate in signal transduction pathways that regulate the activation state of NFκB (17). Bcl10 was initially identified in a subset of MALT B cell lymphomas with t (1, 14)(p22;q32) (18, 19). This 233-amino acid protein is ubiquitously expressed and contains an amino-terminal CARD domain. Bcl10$^{-/-}$ lymphocytes show absence of NFκB activation following antigen receptor stimulation or PMA/ionomycin-induced cell activation (9, 29–31). In addition to immunological deficiencies, Bcl10-null murine embryos develop exencephaly that leads to embryonic lethality, indicating that Bcl10 plays pleiotropic roles in the embryonic and adult lives of mammalian organisms (9).

The CARD-containing proteins CARMA1 (also known as CARD11/Bimp2), CARMA2 (CARD14/Bimp3), and CARMA3 (CARD10/Bimp1) share high degrees of sequence, structure, and functional homology (20–23). On the other hand, the three CARMA proteins display tissue-specific distribution, suggesting that they may sub-serve distinct biochemical functions in different cell types (20–23). CARMA proteins belong to the membrane-associated guanylate kinase-like (MAGUK) family of proteins that can function as molecular scaffolds that assist recruitment and assembly of signal transduction molecules and contain an SH3 domain, one or several PDZ domains, and a GUK domain (24).

Similar to Bcl10$^{-/-}$ lymphocytes, CARMA1-deficient lymphocytes exhibit reduced activation of NFκB in response to antigen receptor cross-linking (12). However, the mechanism by which Bcl10 and CARMA proteins activate the IκK complex is still undefined. Here we have shown that CARMA1 and CARMA3 physically associate with NEMO, thereby regulating activation of the NFκB transcription factor.

MATERIALS AND METHODS

Two-hybrid Screening—Two-hybrid screening was performed using the Matchmaker system (Clontech) as previously described (25). Briefly, yeast strain AH109 was transformed with bait plasmids by lithium acetate/PEG 3000 procedure and selected on synthetic dropout plates lacking tryptophane. Transformant colonies were analyzed for expression of the GAL4 bait fusion protein by immunoblot analysis. For library screening, yeast AH109 expressing GAL4·NEMO fusion protein

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[1] The abbreviations used are: IκK, Iκ kinase; NEMO, NFκB essential modulator; CARD, caspase recruitment domain; PMA, phorbol 12-myristate 13-acetate; FCS, fetal calf serum; mAb, monoclonal antibody.

was transformed with a human erythroleukemia cDNA library cloned in pACT2 vector (Clontech). 2×10^6 library clones were screened for interaction with GAL4·NEMO.

Cell Culture and Antibodies—HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and transfected by calcium phosphate precipitation using 10 μg of plasmidic DNA.

Jurkat cells were cultured in RPMI supplemented with 10% FCS. Sources of antisera and monoclonal antibodies were the following: anti-CARMA1, Apotech; anti-FLAG, Sigma; anti-NEMO, anti-c-myc, anti-HA, Santa Cruz Biotechnology, BD Pharmingen; anti-IκB, anti-phospho-IκB, New England Biolabs. Anti-Bcl-10 antibody has been described elsewhere (26). Rabbit antisera to CARMA3 were generated in our animal facility using recombinant CARMA3 as an antigen.

Immunoblot Analysis and Coprecipitation—Cell lysates were made in lysis buffer (150 mM NaCl, 20 mM Hepes, pH 7.4, 1% Triton X-100, 10% glycerol, and a mixture of protease inhibitors). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Blots were developed using the ECL system (Amersham Biosciences). For coimmunoprecipitation experiments, cells were lysed in lysis buffer and immunocomplexes were bound to protein A/G, resolved by SDS-PAGE, and analyzed by immunoblot assay.

Recombinant Protein Production and in Vitro Binding Assays—Histidine-tagged CARMA3 polypeptides were expressed from pET 28 vector (Novagen) in BL21 bacterial strain. Cell lysates from 5×10^6 Jurkat cells were made in lysis buffer, and 300 μl of lysate were mixed with 50 μl of the indicated recombinant protein. Samples were incubated at 4 °C, washed several times by pulse centrifugation in the same buffer, and resuspended in 50 μl of sample buffer. 10 μl of the reaction were loaded for SDS-PAGE and Western blot analysis.

Gel Filtration Analysis—Fifty million Jurkat cells were washed in phosphate-buffered saline and lysed in 500 μl of lysis buffer supplemented with a mixture of proteases and phosphatases inhibitors. Lysates were centrifuged for 60 min at 15,000 rpm, and the supernatant was recovered and loaded on a Superdex 200 gel filtration chromatography column (Amersham Biosciences) precalibrated with tryptophane (0.267 kDa), TTF1 homeodomain (15 kDa), bovine serum albumin (67 kDa), IgG (150 kDa), and thyroglobulin (669 kDa). 300-ml fractions were recovered and analyzed by Western blotting or subjected to immunoprecipitation.

Luciferase Assay—To assess for NFκB activation, HEK293 cells were transfected with the indicated plasmidic DNAs together with pNFκB-luc (Clontech) in 6-well plates. 24 h later, luciferase activity was determined with the luciferase assay system (Promega). A plasmid expressing β-galactosidase was added to the transfection mixture for normalization of the efficiency of transfection.

RESULTS AND DISCUSSION

Two-hybrid Screening—To identify proteins interacting with NEMO, we performed a two-hybrid screening using as bait full-length NEMO fused to the DNA-binding domain of GAL4 (GAL4BD·NEMO). A plasmid library of fusion between the transcription activation domain of GAL4 and cDNAs from a human erythroleukemia cell line was screened for interaction with GAL4BD·NEMO in the yeast reporter strain AH109. Several clones were isolated that activated the β-galactosidase reporter gene. Sequence analysis revealed that one of the isolated plasmids encoded for amino acids 497–1032 of CARMA3.

Further assays were then performed with this library clone in the reporter yeast strain to identify the regions of CARMA3 and NEMO involved in the interaction. As summarized in Fig. 1, the CARMA3$^{497–1032}$ library clone interacted with GAL4BD·NEMO and with truncated forms of NEMO containing the Leu^{51}-Ala^{100} region of the protein. Thus, we concluded that the region Leu^{51}-Ala^{100} of NEMO is essential for binding to CARMA3 in yeast. A reciprocal analysis, using CARMA3 deletion mutants, indicated that the region Ile^{600}-Leu^{800} of CARMA 3, containing a PDZ and an SH3 domain, is required for association with NEMO in yeast (Fig. 1).

Association between CARMA3 and NEMO—To test for a physical association between CARMA3 and NEMO in mammalian cells, a rabbit polyclonal antisera was generated that specifically recognizes CARMA3 (Fig. 2*A*). Because HEK293 cells endogenously express both CARMA3 and NEMO (data not shown), lysates from HEK293 cells were immunoprecipitated with anti-NEMO or an isotype-matched antibody and coprecipitating proteins were analyzed for the presence of CARMA3 by immunoblotting assay. As shown in Fig. 2*B*, NEMO coprecipitates with CARMA3 in lysates from cells cultured in the presence of 10% FCS. We also observed that stimulation of HEK293 cells with PMA and ionomycin enhances association of NEMO with CARMA3 (Fig. 2*C*). However, when HEK293 cells were cultured in the absence of FCS for 12 h, association of CARMA3 with NEMO was detected only upon stimulation with PMA and ionomycin (Fig. 2*D*). In the reciprocal experiment, immunoprecipitation with anti-CARMA3 antisera coprecipitates NEMO from HEK293 lysates (Fig. 2*E*). Taken together, these experiments indicate that CARMA3 associates with NEMO and that the interaction is dependent on cellular stimulation induced by PMA/ionomycin treatment or by stimulating factor(s) present in the FCS.

To define the region of CARMA3 involved in the association with NEMO in mammalian cells, HEK293 cells were cotransfected with plasmids expressing FLAG-tagged CARMA3 polypeptides together with a vector encoding for HA-tagged NEMO. Cell lysates were immunoprecipitated with anti-FLAG-coated beads, and the presence of coprecipitating NEMO was assessed by immunoblot experiments with anti-HA antibody. Consistent with the data obtained from the two-hybrid experiments, results shown in Fig. 2*F* indicate that NEMO specifically coprecipitates with polypeptides containing the region Ile^{600}-Leu^{800} of CARMA3.

Stimulation-dependent Association of CARMA3 with NEMO—To confirm association of CARMA3 and NEMO in a different experimental system, CARMA3 polypeptides were expressed as His-tagged proteins in bacteria and tested for binding to NEMO endogenously expressed in Jurkat cells. As shown in Fig. 3*A*, recombinant polypeptides containing the region Ile^{600}-Leu^{800} of CARMA3 pull down NEMO from Jurkat lysates. Similar to the results obtained in HEK293 cells, stimulation of Jurkat cells with PMA and ionomycin facilitates association of NEMO with CARMA3. Time course experiments indicated that in this experimental system association of CARMA3 and NEMO peaks at 30 min following stimulation (Fig. 3*B*). All tested CARMA3 polypeptides lacking the region Ile^{600}-Leu^{800} failed to pull down NEMO from Jurkat cells lysates (Fig. 3*A* and data not shown).

Association between CARMA1 and CARMA3 with NEMO in Lymphoid Cells—CARMA proteins show different tissue distribution. CARMA1 is expressed in lymphoid tissues, whereas CARMA3 is expressed in kidney, liver, and heart. Given the essential role of CARMA1 in activation of the NFκB transcription factor in lymphocytes, we tested whether CARMA1 also binds to NEMO. To this end, lysates from Jurkat cells left untreated or stimulated with PMA/ionomycin were immunoprecipitated with anti-NEMO or an isotype-matched antibody and coimmunoprecipitated proteins were tested for the presence of CARMA1 by immunoblot assay. As shown in Fig. 3*C*, CARMA1 coprecipitates with NEMO when Jurkat cells were stimulated with PMA and ionomycin.

Gel Filtration Analysis of CARMA1 and NEMO—To confirm that CARMA1 stably associates to NEMO following cellular stimulation, lysates prepared from Jurkat cells left untreated or stimulated with PMA/ionomycin were fractionated on a Superdex 200 gel filtration column and analyzed by immunoblot experiments. As previously reported (5), elution of NEMO, monitored with anti-NEMO antibody, was mostly observed in fractions containing proteins of 600–800 kDa (Fig. 4*A*). When

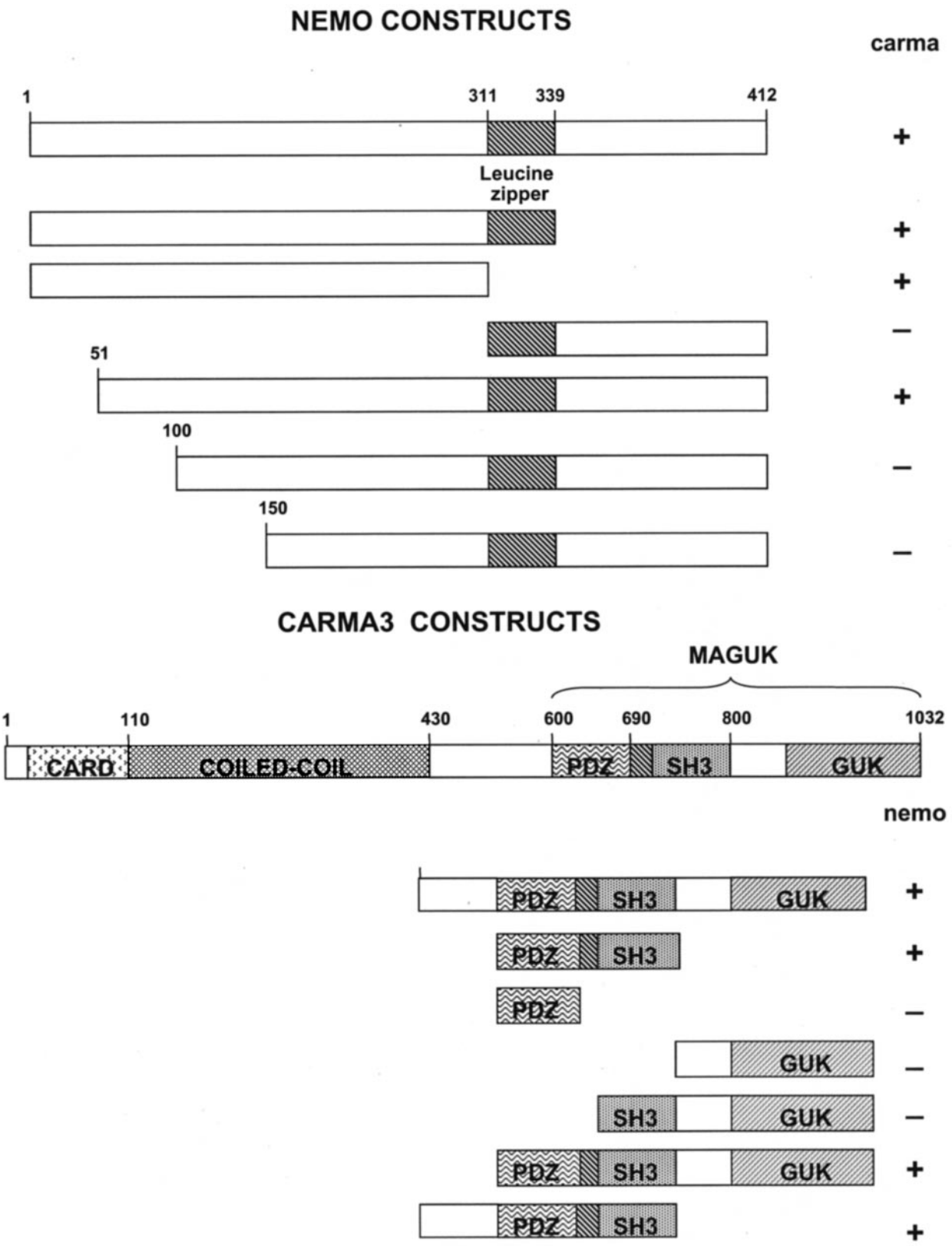

FIG. 1. **Interaction of CARMA3 with NEMO constructs in a yeast two-hybrid assay.** *A* and *B*, schematic representation of the constructs used in the yeast two-hybrid experiments. Yeast strain AH109 was transformed with the indicated plasmids and plated on selective media. Yeast colonies were scored as positive when vigorous growth developed in <5 days. A negative was scored when yeast failed to grow within 12 days. Assays were done for five to ten independent yeast colonies.

we looked for CARMA1 elution, a partially overlapping profile was obtained. We also examined the elution profile of the adapter protein Bcl10, which in lymphoid cells mediates activation of NFκB via interaction with CARMA1. When compared with NEMO and CARMA1, elution of Bcl10 was observed in fractions containing proteins of lower molecular mass (Fig. 4*A*).

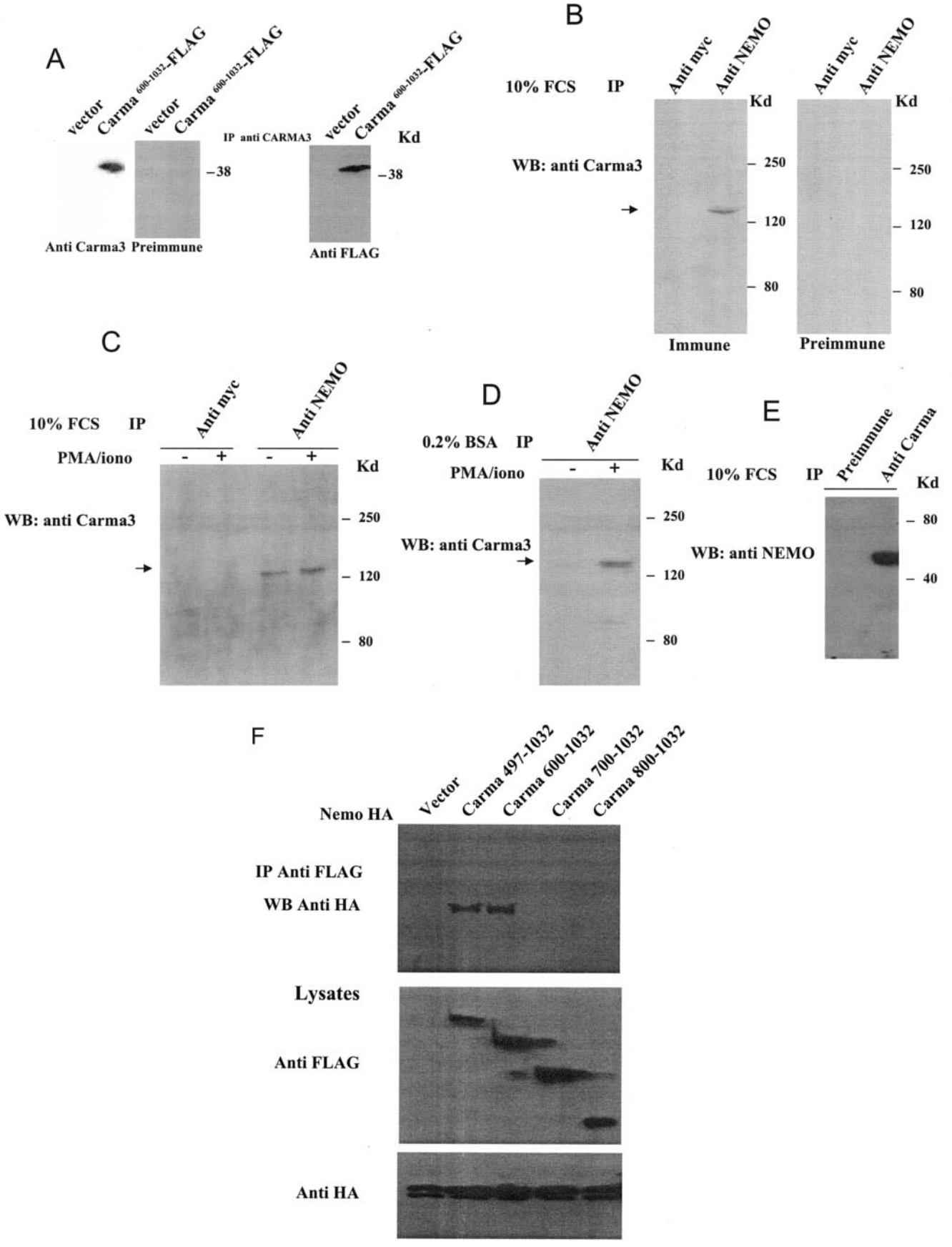

FIG. 2. **Interaction of CARMA3 with NEMO in mammalian cells.** *A*, specificity of the antiserum to CARMA3 used in this study. *Left panel*, HEK293 cells were transfected with a vector that was empty or expressing FLAG-tagged CARMA3$^{600-1032}$. 24 h later, cell lysates were separated by SDS-PAGE and transferred onto membranes subsequently probed with anti-CARMA3 rabbit antiserum or preimmune rabbit serum as indicated. *Right panel*, lysates from HEK293 cells transfected with a plasmid encoding for FLAG-tagged CARMA3$^{600-1032}$ or empty vector were immunoprecipitated with anti-CARMA3 antisera. Immunocomplexes were separated by SDS-PAGE and transferred onto membranes subse-

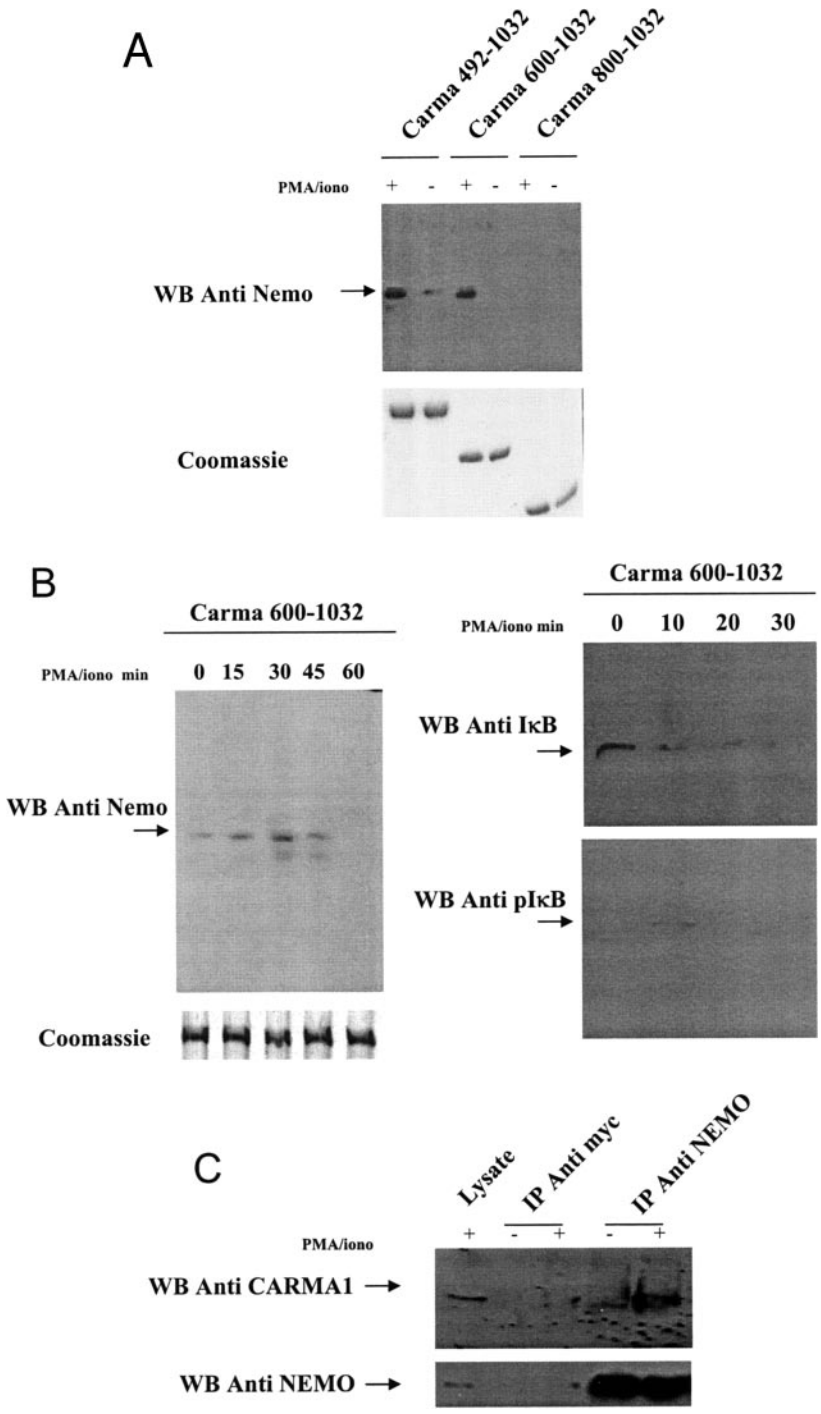

FIG. 3. **Cell stimulation regulates association of CARMAs with NEMO.** *A*, recombinant histidine-tagged CARMA3 polypeptides were purified with nickel-nitrilotriacetic acid-agarose beads and mixed with lysates from Jurkat cells left untreated or treated with PMA (20 ng/ml) and ionomycin (1 μM) for 30 min. After washing, agarose beads were boiled in SDS-sample buffer, separated by SDS-PAGE, and transferred onto nitrocellulose membrane probed with anti-NEMO antibody. A fraction of the reaction mixture was separated by SDS-PAGE and stained with Coomassie Blue to visualize recombinant CARMA3 polypeptides. *B*, *left panel*, lysates from Jurkat cells left untreated or treated with PMA (20 ng/ml) and ionomycin (1 μM) for the indicated time periods were analyzed for binding to recombinant CARMA3 as described in *panel A*. *Right panel*, activation of NFκB following PMA/ionomycin treatment was assessed by examining the phosphorylation state and degradation of IκB. *C*, cell lysates prepared from Jurkat cells left untreated or treated with PMA (20 ng/ml) and ionomycin (1 μM) for 30 min were immunoprecipitated with anti-NEMO mAb or an isotype-matched antibody (*anti-myc*). Immunoprecipitated material was separated by SDS-PAGE and blotted onto membranes hybridized with anti-CARMA1 antibody. Membranes were subsequently probed with anti-NEMO antibody to verify similar amounts of immunoprecipitated NEMO in treated and untreated samples.

Remarkably, when we examined elution of these proteins in lysates of stimulated Jurkat cells, the elution peaks of CARMA1 and Bcl10 both appeared shifted toward the NEMO-containing fractions (Fig. 4*A*).

Immunoprecipitation of the NEMO-containing fractions with anti-NEMO antibody allowed us to coimmunoprecipitate Bcl10, CARMA1, and IκKα/β from lysates of stimulated cells, indicating that both CARMA1 and Bcl10 participate to the

quently probed with anti-FLAG mAb. *B*, lysates prepared from HEK293 cells grown in 10% FCS were immunoprecipitated with anti-NEMO mAb or an isotype-matched antibody (*anti-myc*). Immunoprecipitating material was separated by 7.5% SDS-PAGE and blotted onto membranes subsequently hybridized with anti-CARMA3 rabbit antiserum or preimmune serum as indicated. *C*, lysates prepared from HEK293 cells left untreated or treated with PMA (20 ng/ml) and ionomycin (1 μM) for 30 min were immunoprecipitated with anti-NEMO mAb or ab isotype-matched antibody (*anti-myc*). Coprecipitation of CARMA3 was assessed by immunoblot assay. *D*, lysates prepared from HEK293 cells that were starved for 12 h and left untreated or treated with PMA and ionomycin were immunoprecipitated with anti-NEMO mAb. Immunoprecipitated material was separated by 7.5% SDS-PAGE and blotted onto membranes subsequently hybridized with anti-CARMA3 rabbit antiserum or preimmune serum as indicated. *E*, lysates prepared from HEK293 cells cultured in 10% FCS were immunoprecipitated with anti-CARMA3 rabbit antiserum or preimmune serum. Immunoprecipitated material was separated by 11% SDS-PAGE and blotted onto membranes subsequently hybridized with anti-NEMO mAb. *F*, HEK293 cells were cotransfected with a plasmid encoding for HA-NEMO together with an expression vector encoding for the indicated FLAG-tagged polypeptides of CARMA3. 24 h later, lysates were immunoprecipitated with anti-FLAG-coated beads and analyzed for coprecipitating NEMO by Western blot assay.

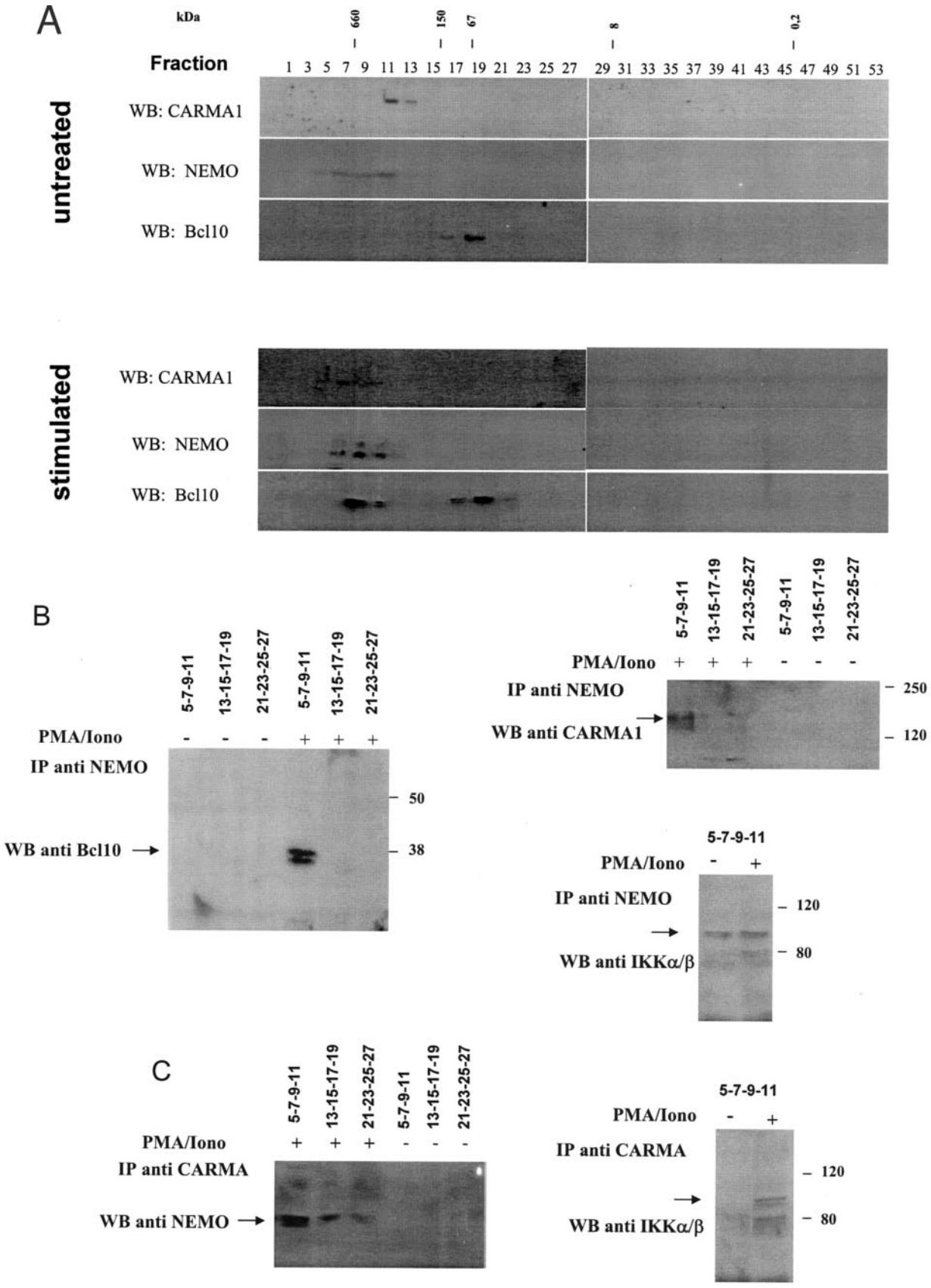

FIG. 4. **CARMA1 participates to the IκK complex.** *A*, gel filtration analysis of NEMO, CARMA1, and Bcl10 in Jurkat cells. Cell extracts were prepared from Jurkat cells left untreated or stimulated with PMA/ionomycin for 30 min and fractionated through a Superdex 200 column. Fractions were analyzed for the presence of CARMA1, NEMO, and Bcl10 by immunoblot assay. *B*, coimmunoprecipitation of CARMA1, Bcl10, and the IκK complex. Fractions were pulled as indicated and immunoprecipitated with anti-NEMO. Immunoprecipitated material was analyzed for the presence of CARMA1, Bcl10, and IκKα/β by immunoblot analysis. *C*, the same fractions as in *panel B* were immunoprecipitated with anti-CARMA and analyzed by immunoblot for the indicated coprecipitating proteins.

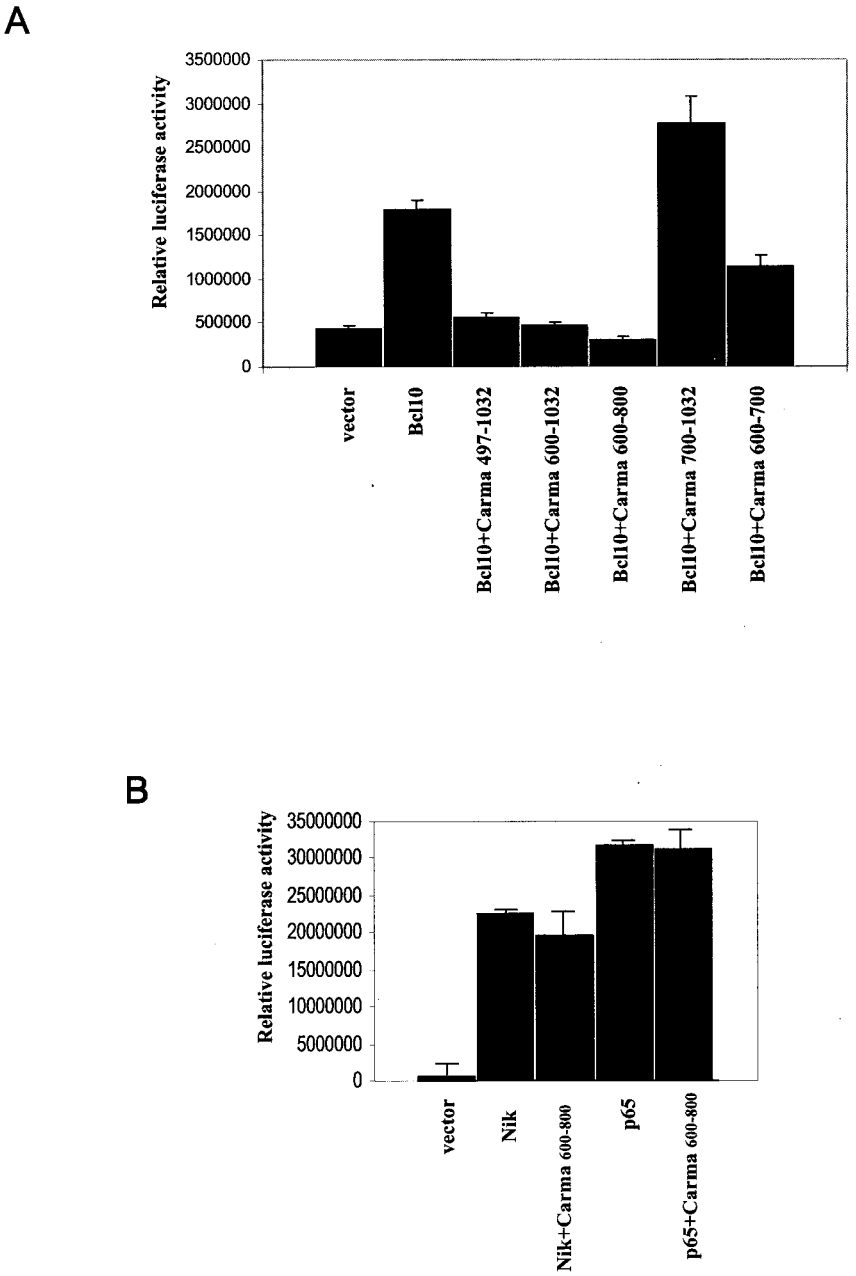

FIG. 5. **The NEMO-binding region of CARMA3 inhibits Bcl10-induced NFκB activation.** *A* and *B*, HEK293 cells were transiently cotransfected with an expression vector encoding for the indicated polypeptides, together with pNFκB-luc and β-galactosidase reporter vectors. The total amount of transfected plasmidic DNA was maintained constant by adding empty vector. 16 h after transfection, cell lysates were prepared and luciferase activity was measured. Data shown represent relative luciferase activity normalized on β-galactosidase activity and is representative of three independent experiments done in triplicate.

600–800-kDa complex containing NEMO, IκKα, and IκKβ (Fig. 4*B*). Similar results were obtained when NEMO-containing fractions were immunoprecipitated with anti-CARMA antisera (Fig. 4*C*).

CARMA3 Modulates Bcl10-induced NFκB Activation—The interaction of CARMA3 and CARMA1 with NEMO prompted us to investigate whether deletion mutants of CARMA3 may influence Bcl10-induced activation of NFκB. Indeed, Fig. 5 shows that expression of polypeptides containing the NEMO-binding region of CARMA3 reduces activation of NFκB mediated by expression of Bcl10 (Fig. 5*A*). This inhibition was specific for Bcl10, because no effect on activation of NFκB induced by other activators was observed (Fig. 5*B*).

The association of NEMO and CARMA reported here provides a biochemical framework to understand the molecular mechanisms by which CARMA-1, -2, and -3 and Bcl10 regulate activation of NFκB. Bcl10 functions as a positive regulator of lymphocyte proliferation and specifically links antigen receptor signaling to NFκB activation (9). In addition, during embryonic development the function of Bcl10 is necessary for the correct neural tube closure, suggesting a general requirement of Bcl10 for proper IκK regulation and NFκB signaling (9) in different cell types. The three CARMA proteins appear to be key players in mediating the tissue-specific, Bcl10-dependent, activation of NFκB. CARMA1 is expressed in a variety of adult tissues, including thymus, spleen, liver, and peripheral blood leukocytes (20, 21). CARMA2 is expressed mostly in placenta, whereas CARMA3 is expressed in fetal lung, liver, and kidney (21). Because Bcl10 is ubiquitously expressed, it may utilize the corresponding tissue-specific CARMA to regulate activation of the IκK complex.

Recent evidence indicates that Bcl10-mediated activation of NFκB requires Lys^{63}-linked ubiquitination of NEMO, which is mediated by the paracaspase MALT1 and the ubiquitin-conjugating complex E2 (27). Together with these observations, our data allow the proposition of the following model (Fig. 6). In resting cells, Bcl10 is found preassociated with the ubiquitin-conjugating complex and MALT1 (27). Following stimulation, Bcl10 binds to CARMA via a CARD-CARD interaction, carry-

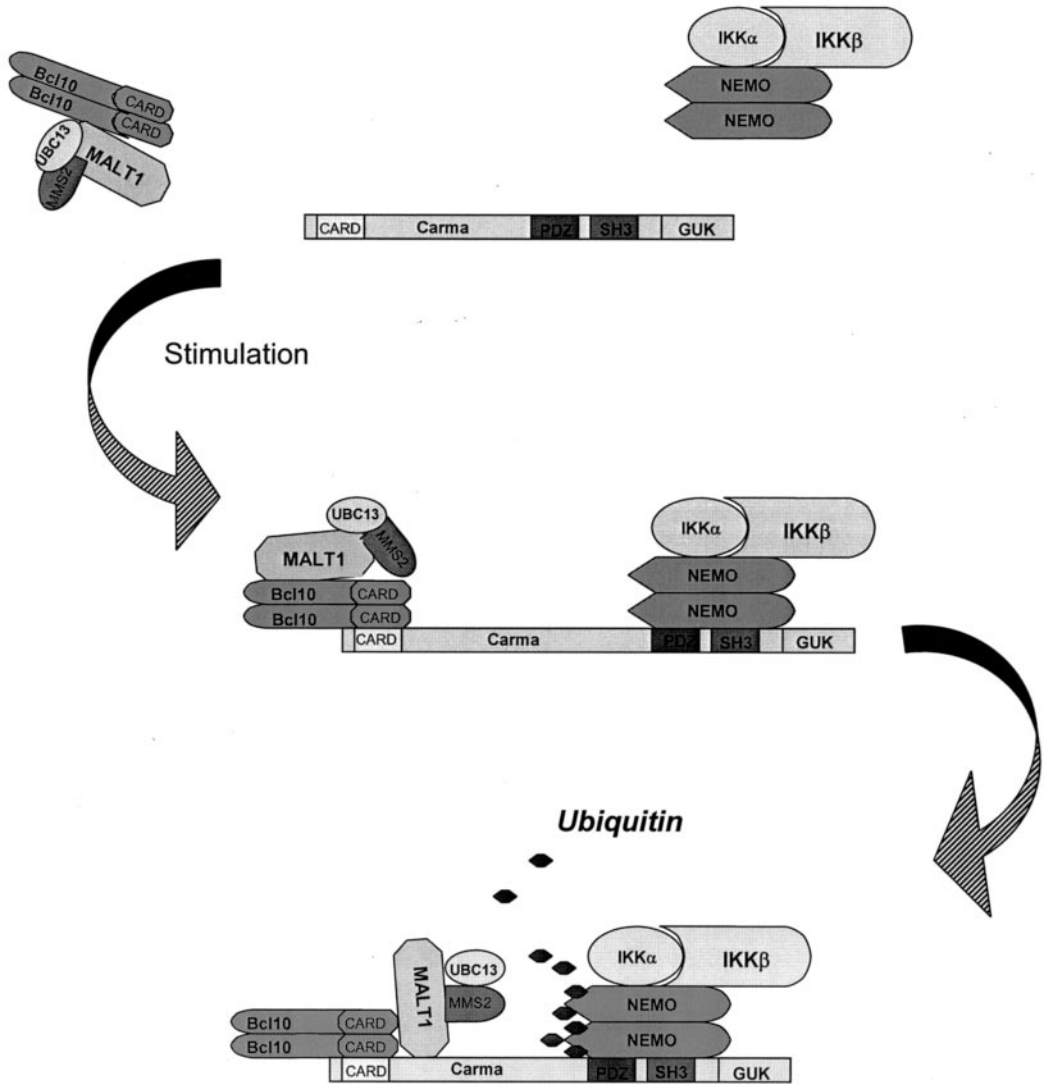

FIG. 6. **Model for NFκB activation via CARMA, Bcl10, and MALT1.** A complex of proteins, comprising Bcl10, MALT1, UBC13, MMS2, and CARMA, associates to the IκK complex following stimulation, facilitating Lys^{63}-linked ubiquitination of NEMO on Lys^{399}. This results in activation of the IκK complex, which triggers NFκB activation.

ing the ubiquitin-conjugating complex in close proximity to NEMO, which associates to the region Ile^{600}-Leu^{800} of CARMA. The close proximity of NEMO to the ubiquitin-conjugating complex results in Lys^{63}-linked ubiquitination of NEMO. This event eventually determines activation of the IκK complex, which, in turn, triggers NFκB activation by phosphorylation of IκB.

Several pieces of evidence support this model. First, lack of either Bcl10, CARMA1, or MALT1 results in the complete absence of NFκB activation following stimulation with PMA and ionomycin (9, 13–16, 28). Second, CARMA mutants unable to recruit Bcl10 act as dominant negative inhibitors of PMA- and ionomycin-induced NFκB activation (12). Third, whereas deletions of the C-terminal GUK domain do not interfere with the ability of CARMA to induce NFκB activity, deletion of the SH3 and PDZ domains reduces the levels of NFκB activation (23).

The importance of CARMA in linking Bcl10/MALT1 to NEMO may extend to cellular processes other than lymphocyte activation and central nervous development. Future studies will be aimed at exploring the potential role of this pathway in a variety of biological systems.

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Physical and Functional Interaction of CARMA1 and CARMA3 with Iκ Kinase γ -NFκB Essential Modulator

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