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Neuroepithelial Transforming Gene 1 (*Net1*) Binds to Caspase Activation and Recruitment Domain (CARD)- and Membrane-associated Guanylate Kinase-like Domain-containing (CARMA) Proteins and Regulates Nuclear Factor κB Activation*[S]

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Background: The CARMA-BCL10-MALT1 complex recruits signaling components leading to activation of NF-κB transcription factor.
Results: The Rho guanine nucleotide exchange factor Net1 binds to BCL10, CARMA1, and CARMA3.
Conclusion: Net1 modulates activation of NF-κB transcription factor.
Significance: The results shown are important to understand events that control NF-κB activation and the mechanisms by which Net1 promotes cellular transformation.

The molecular complexes containing CARMA proteins have been recently identified as a key components in the signal transduction pathways that regulate activation of nuclear factor κB (NF-κB) transcription factor. Here, we used immunoprecipitation coupled with mass spectrometry to identify cellular binding partners of CARMA proteins. Our data indicate that the Rho guanine nucleotide exchange factor Net1 binds to CARMA1 and CARMA3 in resting and activated cells. Net1 expression induces NF-κB activation and cooperates with BCL10 and CARMA proteins in inducing NF-κB activity. Conversely, shRNA-mediated abrogation of Net1 results in impaired NF-κB activation following stimuli that require correct CARMA-BCL10-MALT1 complex formation and functioning. Microarray expression data are consistent with a positive role for Net1 on NF-κB activation. Thus, this study identifies Net1 as a CARMA-interacting molecule and brings important information on the molecular mechanisms that control NF-κB transcriptional activity.

The NF-κB signaling pathway is a major regulator of normal immune and inflammatory response, cell proliferation, differentiation, apoptosis, and oncogenesis (1). A key event in the canonical NF-κB cascade is the activation of the IκB kinase complex, which, upon activation, is responsible for phosphorylation and subsequent proteasome-mediated degradation of the inhibitory protein IκBα. Degradation of IκBα frees NF-κB, allowing its translocation in the nucleus where it activates transcription of target genes (1).

Previous studies have demonstrated that signal-dependent formation of the CARMA[2]-BCL10-MALT1 complex (commonly known as the CBM complex) recruits downstream signaling components, leading to the activation of NF-κB (2, 3). Genetic and biochemical studies have identified CARMA1 as a crucial component of the CBM complex that links antigen receptors on B and T lymphocytes to activation of NF-κB (3, 4). In addition to antigen receptor signaling, the CBM complex mediates NF-κB activation induced by multiple immunoreceptors (5–7). In natural killer cells, activation of immunoreceptor tyrosine-based activation motif-coupled receptors leads to CBM-dependent induction of NF-κB and production of proinflammatory cytokines (6, 7). Furthermore, activation of the Fcϵ receptor I on mast cells also engages BCL10 and MALT1 to activate NF-κB (8, 9). Similarly, a CBM complex that comprises CARMA3, BCL10, and MALT1 plays an important role in activation of NF-κB in cells outside of the immune system. Thus, independent studies indicate that CARMA3 and BCL10 are implicated in the signal transduction pathways elicited by G protein-coupled receptors, a large family of cell surface receptors that regulate cell migration, differentiation, proliferation, and survival (10–12). Recent data also show that the less characterized members of the CARMA family of proteins, CARMA2 and its splice variants, regulate activation of NF-κB through formation of a similar CBM complex (13). Hence, it appears that the CBM complex serves as a molecular platform to recruit signaling components responsible for activation of

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[S] This article contains supplemental data S1–S3.
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[2] The abbreviations used are: CARMA, caspase activation and recruitment domain- and membrane-associated guanylate kinase-like domain-containing; CBM, CARMA-BCL10-MALT1; LPA, lysophosphatidic acid; MAGUK, membrane-associated guanylate kinase; Net1, neuroepithelial transforming gene 1; PMA, phorbol 12-myristate 13-acetate.

NF-κB transcription factors in response to dissimilar stimuli (2, 25).

Structurally, CARMA1–3 proteins belong to the membrane-associated guanylate kinase (MAGUK) family of proteins, which can function as molecular scaffolds that assist recruitment and assembly of signal transduction molecules (2, 14). All three CARMA proteins contain a CARD domain, a Src homology 3 domain, one or several PDZ domains, and a GuK domain (2, 14).

So far, a number of proteins have been implicated in regulating the assembly and proper functioning of the CBM complex. These include the Ca^{2+}-dependent phosphatase calcineurin (15), the serine-threonine protein phosphatase PP2A regulatory subunit Aα (16), the COP9 signalosome subunit 5 (17), the casein kinase 1α (18) and the deubiquitinating protein A20 (19). Altogether, this evidence indicates that there are a number of cellular modulators that operate on the CBM complex, thereby modulating the consequent activation of NF-κB.

The neuroepithelial transforming gene 1 (Net1) is a Rho guanine nucleotide exchange factor that was identified as a transforming gene in a screen for novel oncogenes in NIH3T3 cells (20). Net1 possesses a C-terminal PDZ domain binding site, which is required for Net1-dependent cell transformation (21). In fact, Net1 has been shown to interact through its PDZ binding motif with the PDZ domain-containing proteins of the MAGUK family, including Dlg1/SAP97, SAP102, and PSD95 (22, 23).

Here, we demonstrate that Net1 associates with CARMA1 and CARMA3 in resting and activated cells. The functional significance of this interaction is highlighted by the evidence here showing that shRNA-mediated Net1 depletion results in a marked reduction of NF-κB activation following stimuli that require correct assembly and functioning of the CBM complex.

MATERIALS AND METHODS

Reagents—Sources of antibodies and reagents were the following: anti-FLAG, anti-HA, and anti-actin, Sigma; anti-CARMA1, anti-ubiquitin (P4D1), anti-Net1, anti-MALT1, anti-JNK 1/3, anti-phospho-JNK (p54 p46), Santa Cruz Biotechnology; anti-CD28 (clone ANC 28.1) Ancell Corporation; anti-CD3 (clone TR66), Alexis Biomedicals; anti-IκBα, anti-phospho-IκBα, anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, Cell Signaling. Anti-BCL10 and anti-CARMA3 were generated in our laboratory and have been described elsewhere (24, 25). Recombinant tumor necrosis factor α (TNFα) was from Miltenyl. Interleukin-1β (IL-1β), lipopolysaccharide (LPS), lysophosphatidic acid (LPA), phorbol 12-myristate 13-acetate (PMA), and ionomycin were from Sigma.

Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% FBS. DNA plasmids were transfected into cultured cells by calcium-phosphate methods or using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Lentiviral vectors expressing shNet1 RNAs were obtained from Sigma and used according to the manufacturer's instructions. Retroviral infections were performed as described previously (26). Expression plasmids were generated using standard methodologies and confirmed by sequencing.

Purification of Peripheral Blood Mononuclear Cells—Peripheral blood mononuclear cells were purified from human heparinized venous blood from healthy donors by Ficoll-Hypaque (density 1.077 g/liter; Sigma-Aldrich) density gradient centrifugation at 2000 rpm for 30 min at 20 °C with no break according to the manufacturer's protocol. Mononuclear cells collected on the top of Ficoll-Hypaque layer were washed twice in 1× PBS and centrifuged for 10 min at 1300 rpm to separate them from platelets. Mononuclear cells were resuspended in complete RPMI 1640 medium supplemented with 20% FBS, and peripheral blood lymphocytes were separated from monocytes and macrophages by the adherence method. After 1 h in 37 °C, 5% CO_2 humidified incubator nonadherent lymphocytes were collected, washed for 10 min at 1400 rpm, and resuspended in RPMI 1640 medium and 20% FBS. For the co-precipitation assay, cell lysates were prepared from peripheral blood mononuclear cells left untreated or stimulated with 40 ng/ml PMA and 1 μM ionomycin for 15 min.

Immunoblot Analysis and Co-precipitation—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 co-immunoprecipitation experiments, cells were lysed in lysis buffer, and immunocomplexes were bound to protein A/G (Roche Applied Science), resolved by SDS-PAGE, and analyzed by immunoblotting assay.

In Vitro Binding Assay—The *in vitro* binding assay was performed as described previously (43). Briefly, recombinant histidine-tagged proteins were made in *Escherichia coli* BL21 strain using the pET expression system (Novagen) and purified with nickel-nitrilotriacetic acid-agarose beads (Qiagen) as described. Cells were lysed in lysis buffer (25 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and a protease inhibitor mixture), and 300 μl of lysates was mixed with 50 μl of histidine-tagged 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 was loaded for SDS-PAGE and Western blot analysis.

For MALT1 ubiquitination assay cells were lysed in denaturing condition with lysis buffer supplemented with 1% SDS to separate interacting proteins. After 10-fold dilution (to 0.1% SDS final concentration) MALT1 was immunoprecipitated from lysates with protein G-bound antibody overnight, and the ubiquitination state was checked by SDS-PAGE.

Luciferase Assay—To assess for NF-κB activation, HEK293 and Jurkat cells were transfected with the indicated plasmidic DNAs together with pNF-κB-Luc (Clontech) in 6-well plates. After transfection and treatments, luciferase activity was determined with the Luciferase Assay System (Promega). Plasmids expressing RSV-β-galactosidase or TK-*Renilla* were used in transfection mixtures to normalize efficiency of transfection.

T Cell Activation and ELISA—96-wells plates were coated with 1 μg/ml anti-CD3 and anti-CD28 (1 μg/ml) antibodies in sterile PBS and kept at 4 °C overnight. The quantitative detection of secreted IL-2 was performed using Biotrak Easy ELISA kit from GE Healthcare according to the manufacturer's instructions.

Real-time PCR—Real-time PCRs were performed in triplicate by using the SYBR Green PCR Master Mix (Qiagen) in a 7900HT sequence-detection system (Applied Biosystems). The relative transcription level was calculated by using the ΔΔCt method.

RNA Microarray—Single stranded biotinylated cDNA was generated as follows: 100 ng of total RNA was subjected to two cycles of cDNA synthesis with the Ambion WT expression kit (Applera). The first cycle was performed using an engineered set of random primers that excludes rRNA-matching sequences and includes the T7 promoter sequence. After second strand synthesis, the resulting cDNA was transcribed *in vitro* with the T7 RNA polymerase to generate a cRNA. This cRNA was subjected to a second cycle of first strand synthesis in the presence of dUTP in a fixed ratio relative to dTTP. Single strand cDNA was then purified and fragmented with a mixture of uracil DNA glycosylase and apurinic/apirimidinic endonuclease 1 (Affymetrix) that cleaves in correspondence of incorporated dUTPs. DNA fragments were then terminally labeled by terminal deoxynucleotidyl transferase (Affymetrix) with biotin. The biotinylated DNA was hybridized to the Human Genechip Gene 1ST Arrays (Affymetrix), containing almost 29,000 genes selected from *Homo sapiens* genome databases RefSeq, ENSEMBL, and GenBank. Chips were washed and scanned on the Affymetrix Complete GeneChip Instrument System, generating digitized image data (DAT) files.

Microarray Data Analysis—DAT files were analyzed by Expression Console (Affymetrix). The full data set was normalized by using the Robust Multialignment Algorithm (RMA). The expression values obtained were analyzed by using GeneSpring 10.3 (Agilent Technologies). Further normalization steps included a per chip normalization to 50th percentile and a per gene normalization to median. Results were filtered for -fold change >1.5. Statistical analysis was performed with ANOVA using as p value cutoff 0.05.

RESULTS

To search for CARMA1-interacting proteins, we performed a preparative immunoprecipitation of CARMA1 from cultured Jurkat cells. Immunoprecipitated proteins were separated by SDS-PAGE and subjected to MALDI-MS analysis. When blasted against the NCBI data base, two peptides from an excised band exactly matched the human p65 Net1 (data not shown).

To confirm association of Net1 with CARMA1 in mammalian cells, lysates from Jurkat cells were immunoprecipitated with anti-Net1 or control antibody (anti-HA), and co-precipitating proteins were analyzed for the presence of CARMA1 by immunoblotting assay. As shown in Fig. 1*A*, immunoprecipitation of endogenous Net1 revealed association with CARMA1 in Jurkat cell lysates. Cell stimulation with PMA plus ionomycin did not influence the association, indicating that interaction between the two proteins is not induced by stimulation. Based on pulldown assays, His-fused recombinant CARMA1 bound to endogenous Net1 in HEK293 cell extracts (Fig. 1*B*). No pulldown was detectable using beads alone, indicating that Net1 specifically binds CARMA1 *in vitro* as well as in intact mammalian cells. Also in this experimental system, cellular stimulation with PMA/ionomycin did not influence association of recombinant CARMA1 with endogenous Net1 (Fig. 1*B*). In transfection experiments, Net1 also associates with CARMA3 and with WT BCL10, but not with a mutated inactive version of BCL10 (L41Q) (Fig. 1*C*). Finally, an interaction between Net1 and CARMA1 was also detected in lysates extracted from human peripheral blood leukocytes (Fig. 1*D*).

Because CARMA proteins are implicated in NF-κB signaling pathways, we first determined whether Net1 expression can induce NF-κB activity using a luciferase reporter assay. When Net1 was expressed in HEK293 cells, NF-κB activity was induced at least 4–6-fold compared with empty vector (Fig. 2). In addition, Net1 expression potentiates the NF-κB-inducing activity of BCL10 and CARMA proteins (Fig. 2). The ability of Net1 to induce activation of NF-κB is abolished by the presence of the inactive mutant BCL10 (L41Q) (17), indicating that induction of NF-κB by Net1 requires a functional BCL10 molecule (Fig. 2).

We next investigated the effect of short hairpin RNAs (shRNA)-mediated knockdown of Net1 on the activation of NF-κB elicited by stimuli that require the CBM complex. For this, we used a lentiviral expression system encoding shRNAs designed to target human Net1 for degradation by the RNAi pathway. As shown in Fig. 3*A*, three of six shRNAs targeting Net1 induce a reduction of about 70–80% of the expression of Net1, both at mRNA and protein levels. In Jurkat cells, Net1 silencing reduced activation of NF-κB induced by treatment with LPA, PMA/ionomycin, and IL-1β (Fig. 3*B*). In the same cells, NF-κB activity induced by TNFα stimulation was not affected by Net1 depletion. Similar results were observed when Net1 knockdown was obtained in HEK293 cells (Fig. 3*B*). In addition, Net1 deficiency in Jurkat cells results in decreased production of IL-2 following stimulation (Fig. 3*C*). Finally, Net1 depletion represses NF-κB activation promoted by expression of active forms of BCL10, CARMA3, and CARMA1 (Fig. 3*D*).

To determine which step in the activation of NF-κB was altered by the lack of Net1, we monitored degradation of the inhibitory subunit IκBα following stimulation. As shown in Fig. 4*A*, Net1 depletion affects phosphorylation and degradation of the inhibitory subunit IκBα following stimulation with PMA in Jurkat cells. In contrast, degradation of IκBα proceeds normally following treatment with TNFα.

Because it has been shown that MALT1 ubiquitination is critical for the engagement of CBM and IK kinase complexes (27), we monitored the ubiquitination state of MALT1 in the absence of Net1. Immunoblot assays indicate that Net1 deficiency impairs MALT1 ubiquitination following stimulation (Fig. 4*B*).

Given this result, we next examined whether the IK kinase complex was recruited to the CBM complex following cellular stimulation. The experiment shown in Fig. 4*C* indicates that in

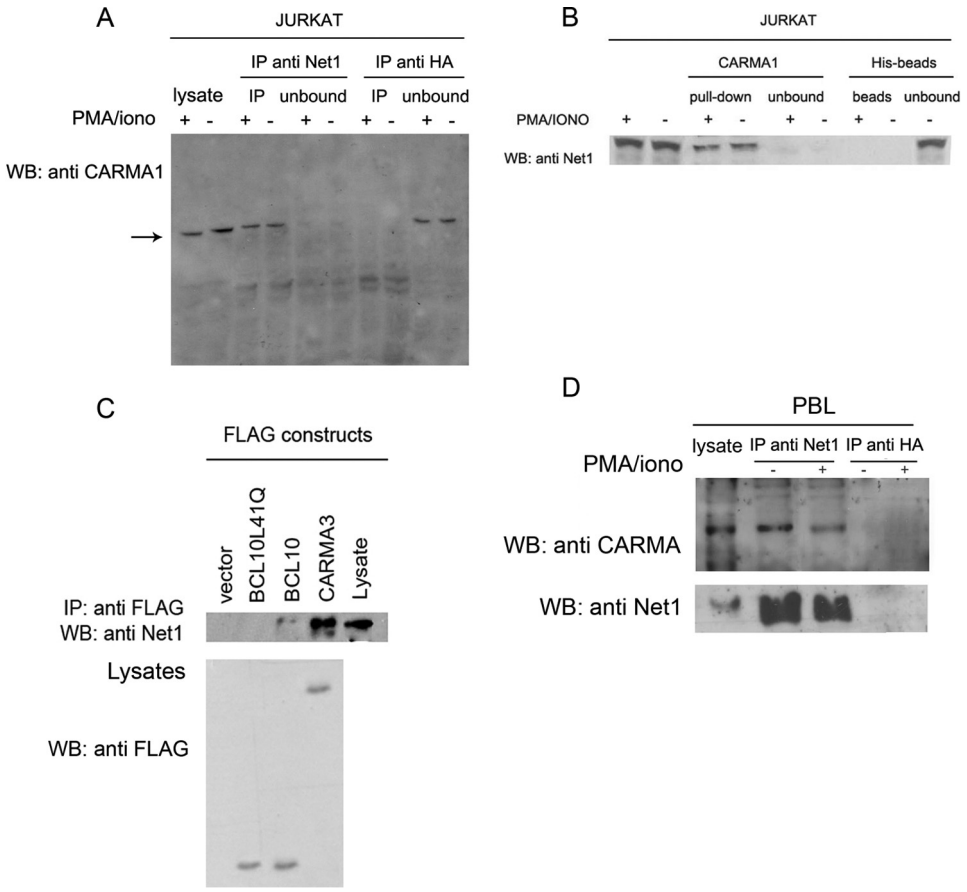

FIGURE 1. **CARMA proteins bind to Net1.** *A*, Jurkat cells were left untreated or stimulated for 15 min with 40 ng/ml PMA plus 2 μM ionomycin. Cell lysates were immunoprecipitated (*IP*) with anti-Net1 antibody or control antibody (anti-HA) and analyzed by immunoblotting (*WB*) probed with anti-CARMA1. *B*, cell lysates were prepared from Jurkat cells left untreated or stimulated for 15 min (40 ng/ml PMA plus 2 μM ionomycin) and incubated with His_6-CARMA or beads alone. Pulled-down proteins were probed with anti-Net1 antibody. Autoradiographs shown are representative of four independent experiments. *C*, HEK293 cells were transfected with a vector expressing the indicated FLAG-tagged polypeptides. 24 h later, cell lysates were immunoprecipitated with anti-FLAG antibody and analyzed by immunoblotting probed with anti-Net1. *D*, peripheral blood mononuclear cells were left untreated or stimulated for 15 min with 40 ng/ml PMA plus 2 μM ionomycin. Cell lysates were immunoprecipitated with anti-Net1 antibody or control antibody (anti-HA) and analyzed by immunoblotting probed with anti-CARMA1.

the absence of Net1, the NEMO subunit of the IK kinase complex is normally recruited to the CBM complex. Similarly, Net11 deficiency does not impair p38, JNK, and ERK activation following stimulation (Fig. 4*D*).

Because Net1 is a guanine nucleotide exchange factor, we next determined whether this enzymatic activity is required for facilitating NF-κB induction. For this, we generated the inactive mutant Net1L321E, which does not possess guanine exchange activity (33). The experiments shown in Fig. 5 indicate that the mutant Net1L321E still cooperates with CARMA3 and BCL10 in inducing NF-κB activation. Thus, the guanine nucleotide exchange activity of Net1 is dispensable for its positive regulation of NF-κB.

Finally, to assess the role of Net1 in global transcriptome, we compared the transcriptional profiles of Net1-silenced *versus* nonspecific siRNA (scramble)-infected cells following PMA stimulation on Affymetrix microarrays. Analysis was conducted in triplicate, and significant gene expression variation was considered for a -fold change $\geq$1.5, corresponding to the average reduction of Net1 gene expression in silenced cells. Comparing the two PMA-treated samples (shNet1 cells *versus* scramble cells) the analysis indentified 247 varying genes, of which 137 (55%) were up-regulated and 110 (45%) were down-regulated. Data analysis shows that following stimulation, the absence of Net1 results in a down-regulation of genes clustered in immunological and inflammatory pathways (supplemental data S1 and S2). Importantly, genes down-regulated in Net-silenced cells included the NF-κB regulated gene I-κBα, which is considered a canonical marker for NF-κB activation (43), and the cell cycle regulators cyclin D2 and $p27^{kip1}$, both direct targets of NF-κB (44–46) (supplemental data S2). Down-regulation of these NF-κB target genes was confirmed by real-time PCR (Fig. 6).

DISCUSSION

There are at least two aspects that make the finding shown in this paper worthy of particular attention. The first one is that our data identify Net1 as a critical player for proper activation of NF-κB following signals that require correct assembly and functioning of the CBM complex. This is *per se* a major novelty that adds a new element to our knowledge about the molecular mechanism through which the CBM complex controls activation of NF-κB transcription factor. Given the key role played by NF-κB in a wide range of biological contexts and considering the impor-

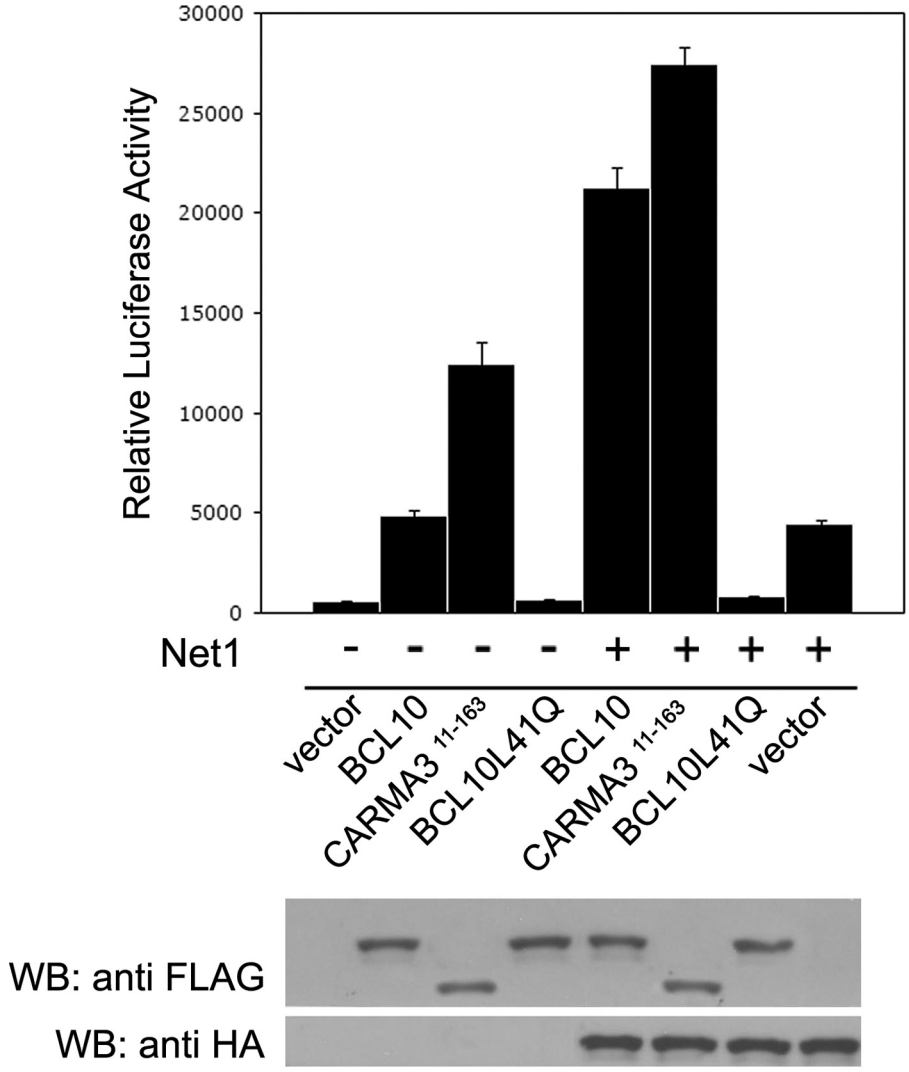

FIGURE 2. **Net1 potentiates NF-κB activation.** *Upper*, HEK293 cells were co-transfected with pNF-κB-luciferase plasmid, a β-galactosidase reporter vector, and expression vectors encoding for the indicated polypeptides. 24 h after transfection, cell lysates were prepared, and luciferase activity was measured. Data shown represent relative luciferase activity normalized against β-galactosidase activity and are representative of at least 10 independent experiments performed in triplicate. *Lower*, a fraction of cell lysates was analyzed by immunoblotting (*WB*) to verify constructs expression.

tance that the CBM complex has in both immune and nonimmune cells (1–4), this finding brings a valuable advancement in the field.

Second, Net1 is a Rho guanine nucleotide exchange factor that was cloned as a transforming gene in a screen for oncogenes in NIH3T3 cells (16). Subsequently, Net1 was involved in different types of tumors and metastases, including gastric cancer (28, 29), hepatocellular carcinoma (30, 31), and glioma (32). The mechanism by which Net1 stimulates cell proliferation and transformation is complex and in many ways still obscure. It is known that to cause cell transformation Net1 must be enzymatically active in the cytoplasm and requires the presence of a C-terminal PDZ domain binding site (16, 33). Importantly, the PDZ domain binding site of Net1 is not required for catalytic activity toward RhoA, indicating that interaction with one or more PDZ domain-containing proteins is required only for cell transformation (21). In addition, through its PDZ binding motif Net1 binds to PDZ-containing proteins of the MAGUK family (22, 23). In this general context, our finding may shed some light in understanding the mechanisms by which Net1 causes cell transformation. In fact, our data show that Net1 binds to the MAGUK domain-containing proteins CARMA1 and CARMA3, and previous studies indicate that CBM proteins are deeply involved in tumoral pathogenesis (34–37). Chromosomal translocations, which lead to the overexpression of BCL10 and MALT1 or generation of API2-MALT1 fusion protein, were found in MALT lymphoma, and the activation of NF-κB by these oncogenic proteins is believed to be one of the hallmarks of MALT lymphoma (34–36). In addition, pathogenic CARMA1 expression was found in adult T cell leukemia (38), primary gastric B cell lymphoma (39), and diffuse large B cell lymphoma (37, 40, 41). It is certainly remarkable that deregulated activation of NF-κB was invoked for tumoral transformation involving proteins participating in the CBM complex because it is firmly established that deregulated NF-κB activation is associated with tumor development and progression (42). Thus, the evidence here showing that Net1 binds to components of the CBM complex and has a positive

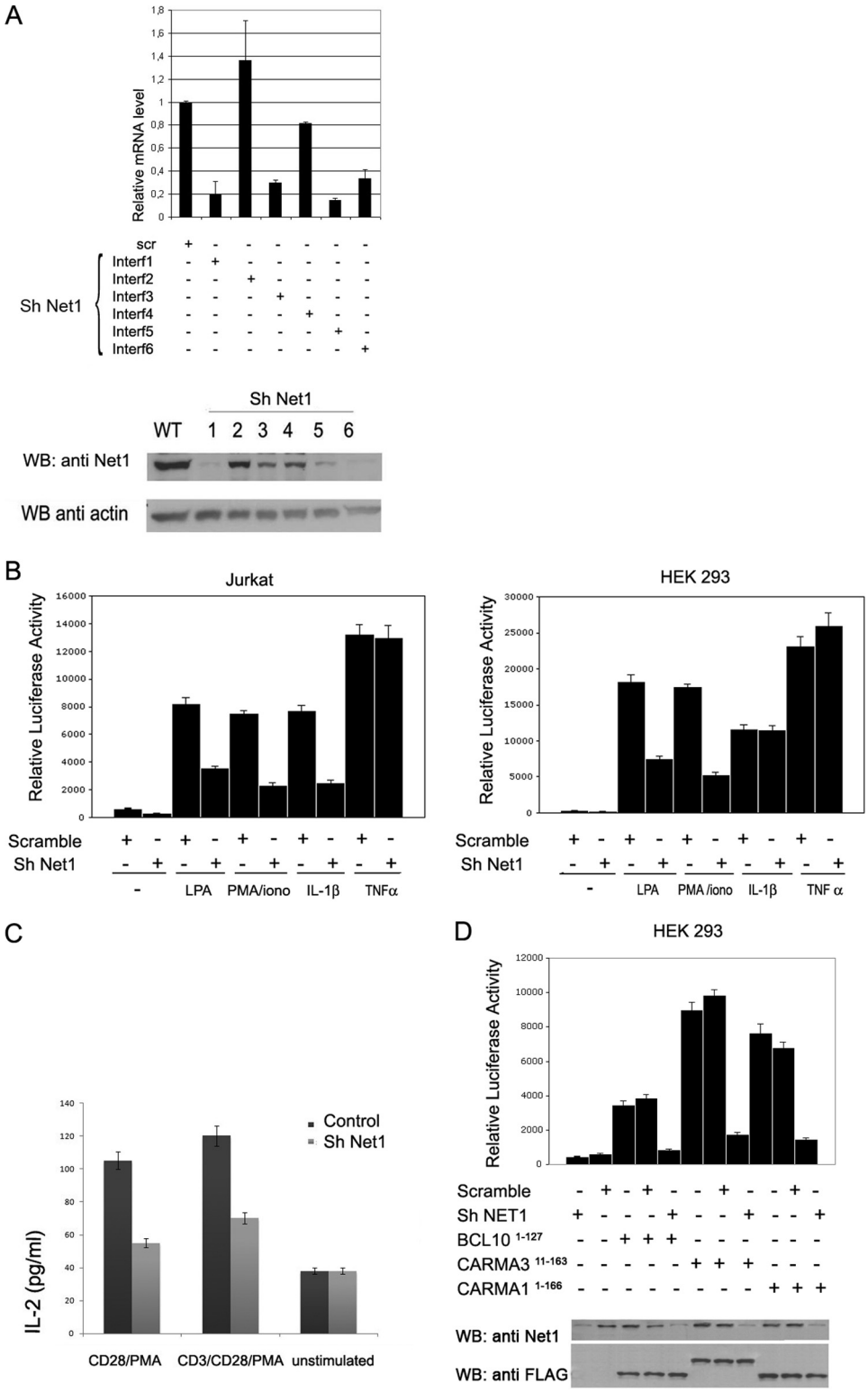

FIGURE 3. **Net1 depletion reduces NF-κB activation.** *A*, *upper*, Jurkat cells were infected with lentiviral vectors encoding for six different shRNAs targeting human Net1 or a control shRNA (scramble). After selection, Net1 mRNA levels normalized to GAPDH were quantified by real-time PCR. *A*, *lower*, Net1 expression level in silenced Jurkat cells was monitored by immunoblotting assay. *B*, Jurkat cells (*left*) and HEK293 cells (*right*) depleted of Net1 were transfected with an NF-κB-luciferase reporter plasmid. 24 h later, cells were treated with the indicated stimuli (10 μM LPA, 40 ng/ml PMA plus 2 μM ionomycin, 20 ng/ml IL-1β) for 4 h, and luciferase activity was determined. Data shown represent the relative luciferase activity normalized against β-galactosidase activity and are representative of at least 10 independent experiments performed in triplicate. *C*, Jurkat cells depleted of Net1 were stimulated for 24 h with anti-CD3/CD28 and PMA, as indicated. Secreted IL-2 was then measured by ELISA. *D*, *upper*, HEK293 cells were infected with lentiviral vectors encoding for shRNAs targeting Net1 or a control sequence (scramble). After selection, cells were transiently co-transfected with an expression vector encoding the indicated polypeptides, together with NF-κB-luciferase and β-galactosidase reporter vectors. 24 h after transfection, cell lysates were prepared, and luciferase activity was measured. Data shown represent relative luciferase activity normalized against β-galactosidase activity and are representative of at least 10 independent experiments performed in triplicate. *D*, *lower*, a fraction of cell lysates was analyzed by immunoblotting (*WB*) assay to verify constructs expression.

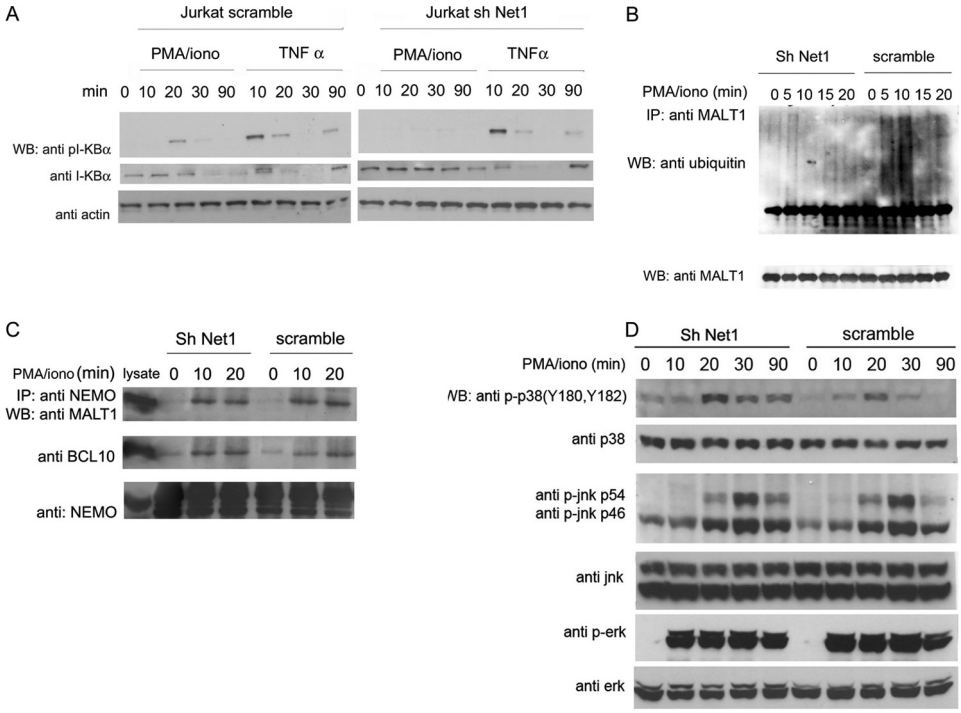

FIGURE 4. **Net1 deficiency impairs IκBα phosphorylation and degradation.** *A*, Jurkat cells expressing shRNAs targeting human Net1 or a control shRNA (*scramble*) were treated with 10 μM PMA plus 2 μM ionomycin and 20 ng/ml TNFα for the indicated time periods, and phosphorylation and degradation of IκBα were monitored by immunoblotting assay. *B*, Net1-depleted or control Jurkat cells were treated with 10 μM PMA plus 2 μM ionomycin for the indicated time periods. Cell lysates were then prepared, immunoprecipitated (*IP*) with anti-MALT1 antibody, and analyzed by immunoblotting (*WB*) probed with anti-ubiquitin (P4D1). *C*, Net1-depleted or control Jurkat cells were stimulated for the indicated time periods. Cell lysates were then immunoprecipitated with anti-NEMO antibody and analyzed for co-precipitating MALT1 and BCL10 by immunoblotting assay. *D*, Net1-depleted or control Jurkat cells were stimulated as indicated. Cell lysates were then prepared and analyzed by immunoblotting assay.

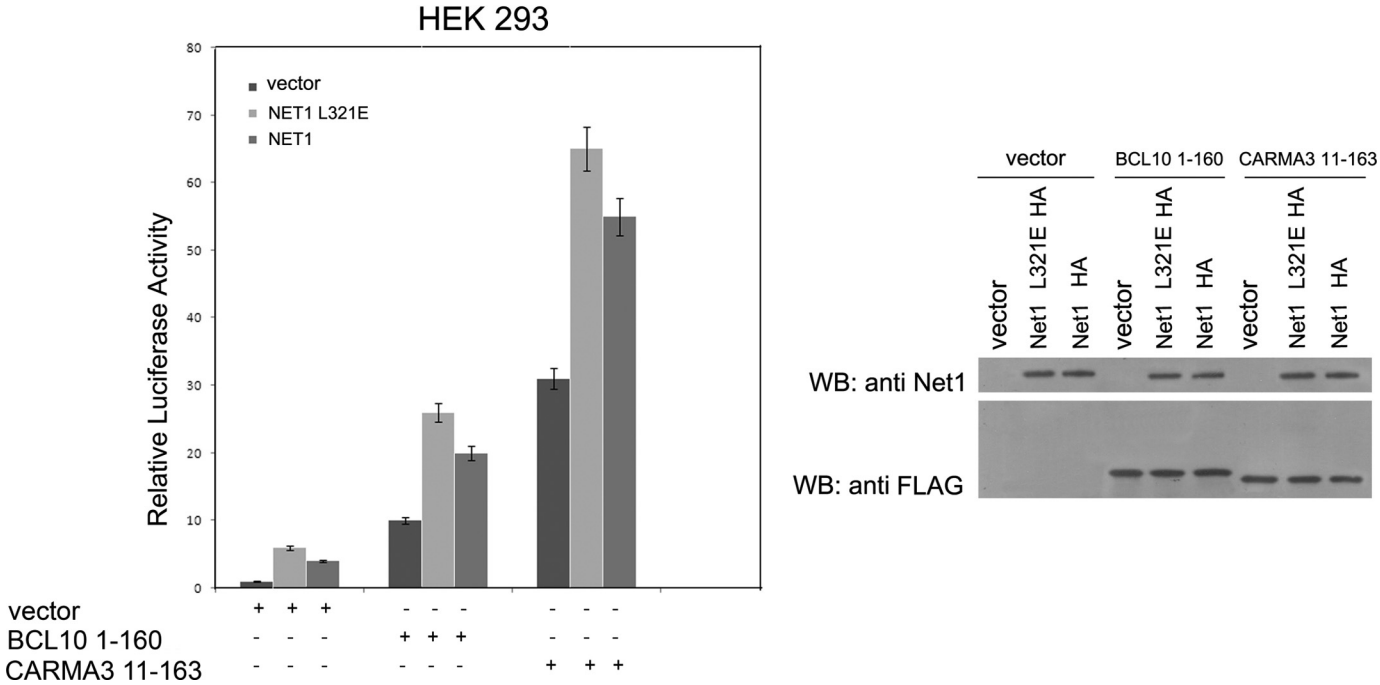

FIGURE 5. **Net1L321E mutant still cooperates in activating NF-κB.** HEK293 cells were co-transfected with pNF-κB-luciferase plasmid, a β-galactosidase reporter vector and expression vectors encoding for the indicated polypeptides. 24 h after transfection, cell lysates were prepared, and luciferase activity was measured. *Left*, data shown represent relative luciferase activity normalized against β-galactosidase activity and are representative of at least six independent experiments performed in triplicate. *Right*, a fraction of cell lysates was analyzed by immunoblotting assay to verify constructs expression.

effect on NF-κB activation may surely represent a track to understand the mechanisms by which Net1 promotes cellular transformation.

Clearly, our work opens many interesting questions concerning Net1 function which require further investigation. In this context, the generation of animal models genetically modified

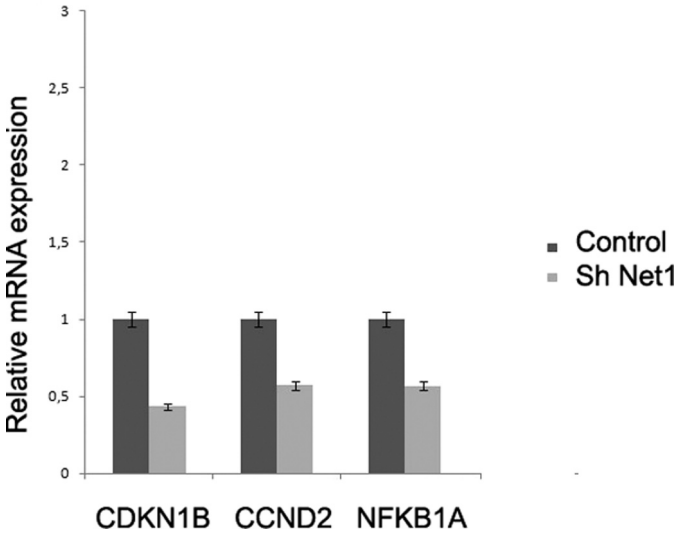

FIGURE 6. **Real-time PCR quantitation of mRNA levels of selected genes normalized to GAPDH in Jurkat cells silenced with shRNA targeting Net1.** Oligonucleotides used for real-time PCR quantitation are detailed in Supplemental data S3.

in the locus encoding for Net1 will be certainly of enormous value finally to define the physiological role of this protein.

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