A20 is a negative regulator of BCL10- and CARMA3-mediated activation of NF-κB

Romania Stilo[1,4], Ettore Varricchio[1], Domenico Liguoro[2], Antonio Leonardi[3] and Pasquale Vito[1,4,*]

[1]Dip. Scienze Biologiche ed Ambientali, Università degli Studi del Sannio di Benevento, Via Port'Arsa 11, 82100 Benevento, Italy
[2]Centro di Endocrinologia ed Oncologia Sperimentale, Napoli, Italy
[3]Dip. di Biologia e Patologia Cellulare e Molecolare, Università degli Studi di Napoli 'Federico II', Via S. Pansini 5, 80131, Napoli, Italy
[4]BioGeM Consortium, Via Camporeale, 83031 Ariano Irpino, Italy
*Author for correspondence (e-mail: vito@unisannio.it)

Accepted 14 January 2008
Journal of Cell Science 121, 1165-1171 Published by The Company of Biologists 2008
doi:10.1242/jcs.021105

Summary

The molecular complex containing CARMA proteins, BCL10 and TRAF6 has been identified recently as a key component in the signal transduction pathways that regulate activation of the nuclear factor κB (NF-κB) transcription factor. Here, we report that the inducible protein A20 negatively regulates these signaling cascades by means of its deubiquitylation activity. We show that A20 perturbs assembly of the complex containing CARMA3, BCL10 and IKKγ/NEMO, thereby suppressing activation of NF-κB. Together, our results further define the molecular mechanisms that control activation of NF-κB and reveal a function for A20 in the regulation of CARMA and BCL10 activity in lymphoid and non-lymphoid cells.

Key words: A20, BCL10, CARMA

Introduction

Nuclear factor κB (NF-κB) is an important transcription factor that plays an essential role in innate and adaptive immunity, inflammation, development, cell death and proliferation (Silverman and Maniatis, 2001; Ghosh and Karin, 2002). Because of its crucial role in cell survival, inappropriate NF-κB activation has been associated with chronic inflammation and tumor formation and progression (Karin, 2006).

The caspase recruitment domain (CARD)-containing proteins CARMA1 (CARD11/Bimp2), CARMA2 (CARD14/Bimp3) and CARMA3 (CARD10/Bimp1) share a high degree of sequence, structure and functional homology (Rawlings et al., 2006). CARMA 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. CARMA proteins contain a Src-homology 3 (SH3) domain, one or several PDZ domains and a GuK domain (Kuhns et al., 2006). Genetic and biochemical studies have identified BCL10 and CARMA1 as crucial components of a complex of proteins that links antigen receptors on B and T lymphocytes to activation of NF-κB (Rawlings et al., 2006; Schulze-Luehrmann and Ghosh, 2006). This complex, which includes CARMA1, BCL10, MALT1 and TRAF6 (Schulze-Luehrmann and Ghosh, 2006), has been shown to activate the IKK complex through a ubiquitylation-dependent pathway (Zhou et al., 2004).

A second member of the CARMA family, CARMA3, is required for activation of NF-κB induced by G-protein-coupled receptors (GPCRs) (Grabiner et al., 2007; McAllister-Lucas, 2007). In fact, it has been demonstrated that a complex of proteins, including CARMA3, BCL10 and MALT1, appears to be responsible for transmitting the signal from the GPCRs to the IKK complex (Grabiner et al., 2007; McAllister-Lucas, 2007; Wang et al., 2007). By contrast, although overexpression of CARMA2 in HEK293 cells also induces NF-κB activation (Bertin et al., 2001; Gaide et al., 2001), the signaling pathway mediated by this protein is still unknown.

A20 is one of the most intensively studied proteins that regulates NF-κB activation by means of ubiquitylation processes (Heyninck and Beyaert, 2005). This inducible and widely expressed cytoplasmic protein contains an N-terminal ovarian tumor (OTU) domain and seven novel zinc-finger structures in its C-terminal domain (Opipari, Jr et al., 1990). A20 inhibits TNFα-mediated NF-κB activation, and the mechanism responsible for the inhibitory function of A20 resides in two opposing enzymatic activities of this protein: a deubiquitylation activity mediated by its OTU domain and a ubiquitin ligase activity mediated by its zinc-finger region (Wertz et al., 2004). Here, we have investigated the involvement of A20 in the activation of NF-κB mediated by BCL10 and CARMA3, and we find that A20 negatively regulates the activity of these proteins.

Results

To analyze the effect of A20 on NF-κB activation induced by BCL10, we transiently transfected HEK293 cells with expression plasmids encoding full-length BCL10 (BCL10FL) and a functionally active form of BCL10 (BCL10 1-127) together with A20 and studied their effects by luciferase reporter tests. The results of these experiments, shown in Fig. 1A, indicated that A20 completely abrogates activation of NF-κB mediated by BCL10, while it has no effect on activation of NF-κB induced by expression of the NF-κB-inducing kinase NIK. The inhibitory effect of A20 on activation of NF-κB promoted by BCL10 was not due to a potential A20-mediated K48-linked polyubiquitylation event and subsequent degradation of BCL10 as transfected BCL10 is expressed at similar levels either in the absence or presence of A20 (Fig. 1A, bottom panel).

The CARD-containing protein CARMA3 is known to associate and cooperate with BCL10 in the induction of NF-κB (Grabiner et al., 2007; McAllister-Lucas, 2007). Thus, we tested whether A20 suppresses activation of NF-κB mediated by CARMA3 in HEK293 cells. As HEK293 cells endogenously express CARMA3, we used

expression plasmids encoding full-length and deleted forms of this protein and observed that the polypeptide encompassing the region Glu11-Val163 of human CARMA3 (CARMA3 11-163) was the strongest activator of NF-κB, as determined by a luciferase reporter test (data not shown). When CARMA3 11-163 was expressed in HEK293 cells, NF-κB activity was induced 80-100 fold compared with empty vector. Nonetheless, when A20 was cotransfected with CARMA3 11-163, NF-κB activity was completely abolished (Fig. 1B). Again, the inhibitory effect exerted by A20 on activation of NF-κB promoted by CARMA3 11-163 was not due to a possible A20-mediated degradation of CARMA3 11-163 as CARMA3 11-163 polypeptide is expressed at similar levels either in the absence or presence of A20 (Fig. 1B, bottom panel). The weak NF-κB activity induced by overexpression of full-length CARMA3 (~4-fold increase compared with the control) was also inhibited by A20 (data not shown). Together, these experiments indicated that A20 suppresses activation of NF-κB induced by overexpression of BCL10 and CARMA3.

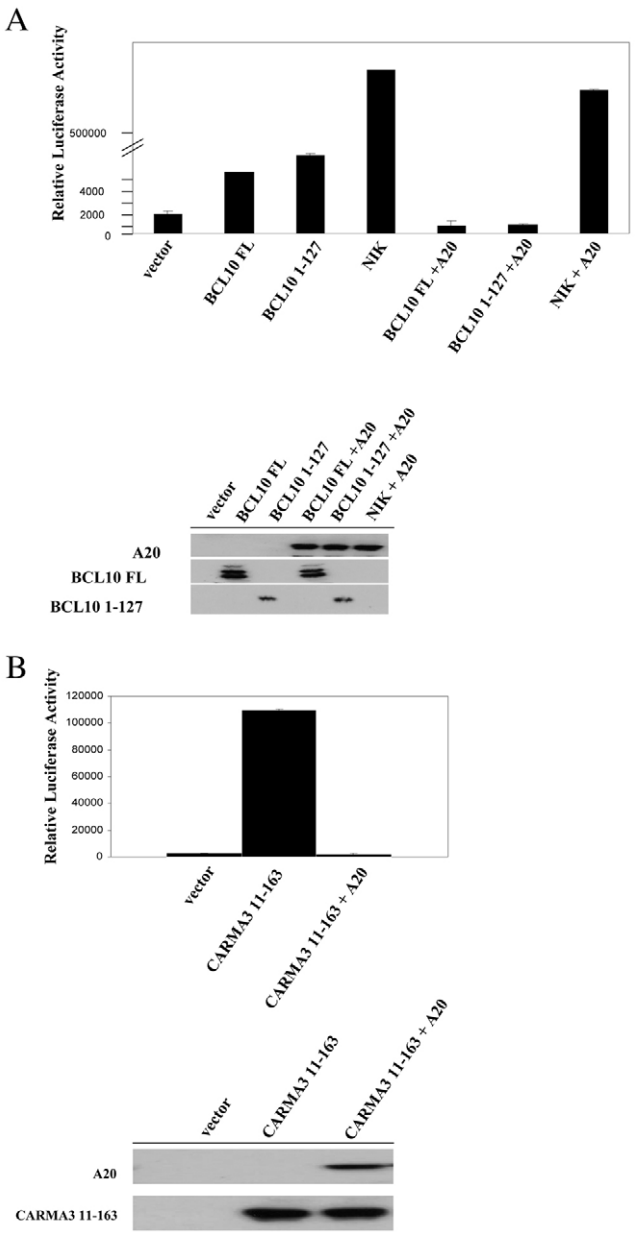

Fig. 1. Selective inhibition of BCL10- and CARMA3-induced NF-κB activation by A20. (A,B) HEK293 cells were transiently cotransfected with an expression vector encoding the indicated polypeptides, together with NF-κB–luciferase and β-galactosidase reporter vectors. The total amount of transfected plasmid DNA was maintained constant by adding empty vector. 16 hours after transfection, cell lysates were prepared and luciferase activity was measured. The data shown represent the relative luciferase activity normalized against β-galactosidase activity and are representative of six independent experiments performed in triplicate. Bottom panels: a fraction of the transfection reaction mixture was separated by SDS-PAGE, blotted onto nitrocellulose membrane and analyzed by immunoblotting to monitor protein expression.

Both CARMA1 and CARMA3 activate NF-κB by recruiting an inducible large molecular complex that includes IKKγ/NEMO, BCL10, TRAF6 and the IKK α/IKKβ kinases (Sun et al., 2004; Bidere et al., 2006; Wang et al., 2004; Hara et al., 2004; Stilo et al., 2004). To analyze whether the NF-κB-inhibitory function of A20 involves the correct formation of this complex, we performed a coimmunoprecipitation analysis. For this, HEK293 cells were transiently transfected with expression plasmids encoding IKKγ/NEMO, BCL10 and A20. 24 hours later, cell lysates were immunoprecipitated with an antibody against IKKγ/NEMO and assayed for coprecipitating proteins by immunoblot experiments. The results of these experiments, shown in Fig. 2A (lanes 1-4), indicate that A20 perturbs the association of BCL10 with IKKγ/NEMO.

It has been shown that A20 negatively regulates NF-κB activation induced by TNFα, Toll-like receptors and TRAF6 by means of the deubiquitylation of signaling molecules, which is catalyzed by the N-terminal OTU domain of A20 (Wertz et al., 2004; Mauro et al., 2006; Boone et al., 2004). Therefore, we tested whether the deubiquitylation activity of A20 is responsible for disruption of the complex containing BCL10 and IKKγ/NEMO, resulting in consequent abrogation of NF-κB activation. For this, we used an expression plasmid encoding a version of A20 (A20mut) harboring the mutations D100A and C103S. These two mutations introduced in the OTU domain of A20 result in the loss of the deubiquitylation activity of A20 on TRAF6 and IKKγ/NEMO (Wertz et al., 2004; Mauro et al., 2006; Boone et al., 2004). Indeed, the results shown in Fig. 2A reveal that wild-type A20 inhibited the BCL10-IKKγ/NEMO association, whereas A20mut did not. The luciferase reporter test confirmed these biochemical data. In fact, A20mut was less efficient in the inhibition of BCL10- and CARMA3-mediated activation of NF-κB (Fig. 2B,C). In these experiments, we also monitored the ubiquitylation state of IKKγ/NEMO. As shown in Fig. 2D,E, transfection of either BCL10 or CARMA3 11-163 in HEK293 cells resulted in ubiquitylation of endogenous IKKγ/NEMO, which was suppressed by A20wt but not by A20mut.

Two recent studies have implicated BCL10 and CARMA3 in lysophosphatidic acid (LPA)-induced NF-κB activation in mouse embryonic fibroblasts (MEFs) (Grabiner et al., 2007; McAllister-Lucas, 2007). As the activity of protein kinase C (PKC) is required for LPA-induced NF-κB activation (Shahrestanifar et al., 1999; Cummings et al., 2004), we first tested whether A20 inhibits activation of NF-κB following activation of PKC. For this, an NF-κB–luciferase reporter plasmid was transfected into HEK293 cells, along with empty vector or expression plasmids encoding A20. Cells were then left untreated or stimulated with ionomycin, a calcium ionophore, plus phorbol 12-myristate 13-acetate (PMA), a surrogate of 1,2-diacylglycerol, the natural activator of conventional and novel

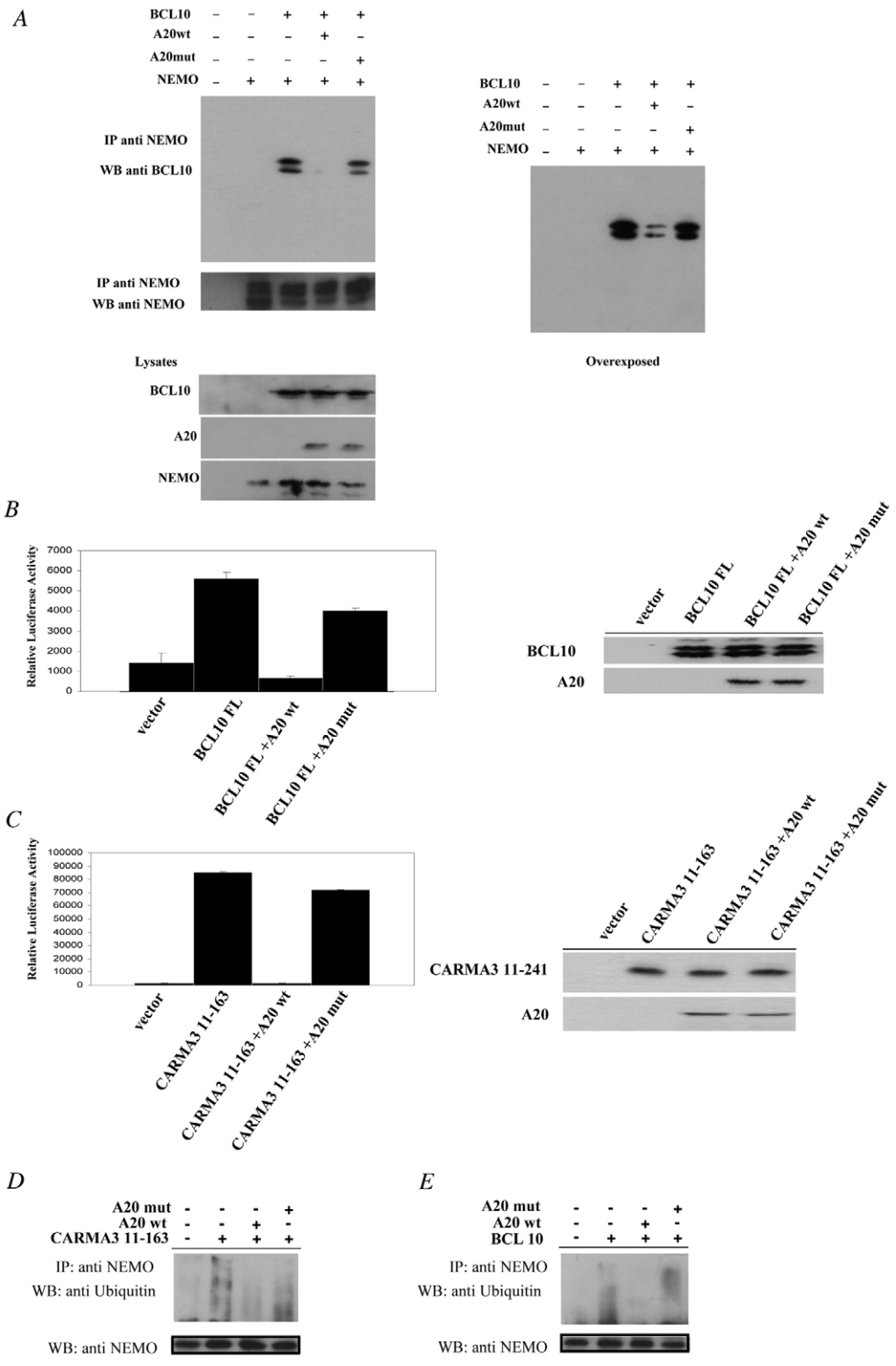

Fig. 2. The deubiquitylation activity of A20 inhibits interaction of BCL10-IKKγ/NEMO in mammalian cells. (A) Lysates from HEK293 cells transfected with the indicated expression vectors were immunoprecipitated with mAb against IKKγ/NEMO. Immunocomplexes were separated by SDS-PAGE, transferred onto membranes and subsequently probed with antisera specific for BCL10 and IKKγ/NEMO. A fraction of the transfection reaction mixture was separated by SDS-PAGE, blotted onto nitrocellulose membranes and analyzed by immunoblotting to visualize protein expression. The results shown are representative of five separate experiments. (B,C) HEK293 cells were transiently cotransfected with an expression vector encoding the indicated polypeptides, together with NF-κB–luciferase and β-galactosidase reporter vectors. The total amount of transfected plasmid DNA was maintained constant by adding empty vector. 16 hours after transfection, cell lysates were prepared and luciferase activity was measured. The data shown represent the relative luciferase activity normalized against β-galactosidase activity and are representative of six independent experiments performed in triplicate. Right panels: a fraction of the transfection reaction mixture was separated by SDS-PAGE, blotted onto nitrocellulose membranes and analyzed by immunoblotting to monitor protein expression. (D,E) Lysates from HEK293 cells transfected with the indicated expression vectors were immunoprecipitated with mAb against IKKγ/NEMO. Immunocomplexes were separated by SDS-PAGE, transferred onto membranes and subsequently probed with antibodies against ubiquitin and IKKγ/NEMO. The results shown are representative of four separate experiments.

PKC enzymes. Consistent with the results obtained from transient-transfection experiments shown in Fig. 2, A20wt, but not A20 mut, repressed activation of NF-κB following the activation of PKC in HEK293 cells (Fig. 3A). In these cells, stimulation with PMA plus ionomycin induced ubiquitylation of endogenous IKKγ/NEMO, which was abrogated by A20wt but not A20mut (Fig. 3B). Activation of NF-κB in HEK293 cells following treatment with PMA and ionomycin was also assessed by monitoring degradation of the inhibitory subunit I-κBα by immunoblot assay. The results of these experiments, shown in Fig. 3C, indicated that A20wt suppressed degradation of the inhibitory subunit I-κBα, whereas A20mut did not.

This latter observation prompted us to investigate further the role of A20 in the signal transduction pathway that links the stimulation of PKC to NF-κB activation. For this, HEK293 cells were transfected either with empty vector or with an expression plasmid encoding A20 and then left untreated or stimulated with PMA and ionomycin. Cell lysates were subsequently immunoprecipitated with an antibody against IKKγ/NEMO and monitored for coprecipitating proteins by western blot analysis. As shown in Fig. 4A, endogenous IKKγ/NEMO associated with endogenous CARMA3 following PMA and ionomycin stimulation, and A20 disrupted formation of this complex. We also found that TRAF6 associates with CARMA3 following PMA and ionomycin treatment, and this association is inhibited by A20 as well (Fig. 4B). Interestingly, coprecipitating TRAF6 displayed a heterogeneous pattern of high-molecular-mass species that is reminiscent of polyubiquitylated forms of TRAF6. In these cells, the association of BCL10 with IKKγ/NEMO induced by treatment with PMA and ionomycin is inhibited by A20 as well (data not shown).

We then tested whether A20 also suppresses the activation of NF-κB induced by LPA stimulation. For this, MEFs were infected with retroviral constructs expressing A20wt or A20mut and subsequently transfected with a luciferase reporter plasmid encoding NF-κB. Infected cells were stimulated with LPA for 5 hours, and luciferase activity was then measured. The results of these experiments, shown in Fig. 5A, indicated that A20wt abrogates activation of NF-κB induced by LPA treatment, whereas A20mut is much less efficient in this inhibitory activity. Biochemical evidence confirmed this observation. In fact, the coimmunoprecipitation experiment shown in Fig. 5B reveals that wild-type A20 inhibits the BCL10-IKKγ/NEMO association following LPA stimulation, whereas A20mut does not.

The negative regulatory activity of A20 upon NF-κB activation mediated by PKC enzymes suggests that A20 might act as a negative-feedback signaling molecule in the PKC-mediated NF-κB pathway. In fact, immunoblot experiments show that the expression of A20 increases following stimulation with PMA plus ionomycin in either HEK293 cells or in a Jurkat lymphoid cell line (Fig. 6A). To further test this hypothesis, we transfected HEK293 cells with a vector encoding a short-hairpin RNA (shRNA) known to target A20 for degradation by the RNAi pathway (Mauro et al., 2006) and monitored NF-κB activity induced by either BCL10, CARMA3 or treatment with PMA plus ionomycin. The results, shown in Fig. 6B-D, indicate that, in cells expressing a shRNA designed to target A20, NF-κB-driven luciferase activity increases by 30-50 fold.

In lymphoid cells, activation of NF-κB following antigen receptor stimulation requires the functions of BCL10 and CARMA1, the member of the CARMA family of proteins expressed in B and T lymphocytes. As CARMA1 shares structural and functional homology with CARMA3, we tested whether A20 inhibits the function of CARMA1 as well. For this, an NF-κB–luciferase reporter was transfected into Jurkat cells, along with empty vector or expression plasmids for A20. Cells were either stimulated with PMA and ionomycin treatment or with antibodies against CD3 and CD28, and luciferase activity was then determined. Consistent with the results obtained in HEK293 cells, A20 repressed activation of NF-κB (Fig. 7A,B). Similarly, stimulation of Jurkat cells with PMA plus ionomycin resulted in ubiquitylation of endogenous IKKγ/NEMO, which was abrogated by A20 (Fig. 7C).

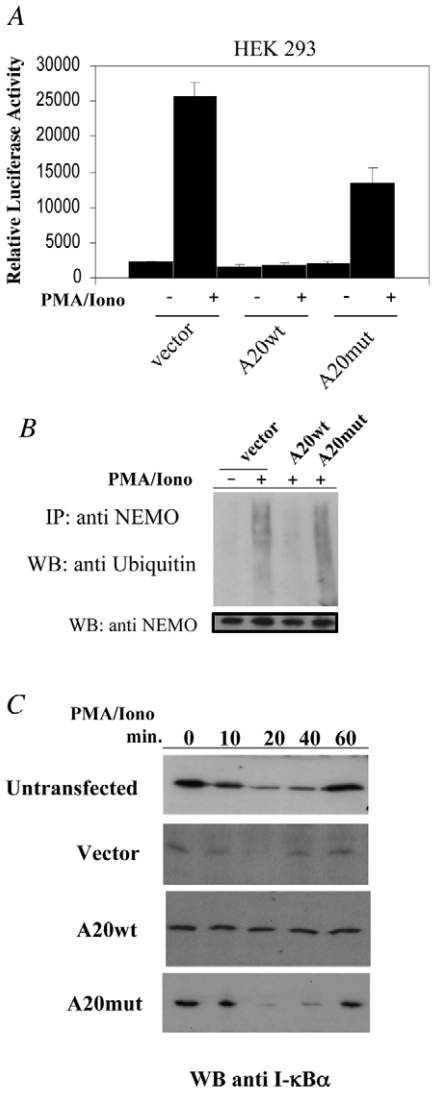

Fig. 3. A20 inhibits PKC-mediated NF-κB activation. (A) HEK293 cells were transfected with empty vector or with the indicated expression vectors, together with an NF-κB–luciferase reporter plasmid. 24 hours later, cells were left untreated or treated with PMA (40 ng/ml) plus ionomycin (1 μM) for 5 hours and luciferase activity was measured. The data shown are representative of six independent experiments. (B) HEK293 cells were transfected with empty vector or with the indicated expression plasmids. 24 hours later, cells were treated with PMA (40 ng/ml) plus ionomycin (1 μM) for 40 minutes. Lysates were then prepared and immunoprecipitated (IP) with mAb against IKKγ/NEMO. Immunocomplexes were separated by SDS-PAGE, transferred onto membranes and subsequently probed with antibodies (WB) against ubiquitin and IKKγ/NEMO. (C) HEK293 cells, transfected with an empty expression vector or vector encoding A20wt or A20 mut, were treated with PMA (40 ng/ml) plus ionomycin (1 μM) for the indicated periods of time. Cell lysates were then prepared and analyzed for degradation of the inhibitory subunit I-κBα by immunoblotting (WP).

Discussion

The results described above allow us to draw several important conclusions regarding the mechanisms that regulate the activation of NF-κB promoted by BCL10 and CARMA proteins. First, we find that BCL10, CARMA1 and CARMA3 signaling is inhibited by A20. A20 was initially identified as a TNFα-inducible gene product that functions as a suppressor of TNFα signaling (Opipari, Jr et al., 1990; Beyaert et al., 2000; Wertz et al., 2004). The physiological function of A20 was elucidated by studies on A20-

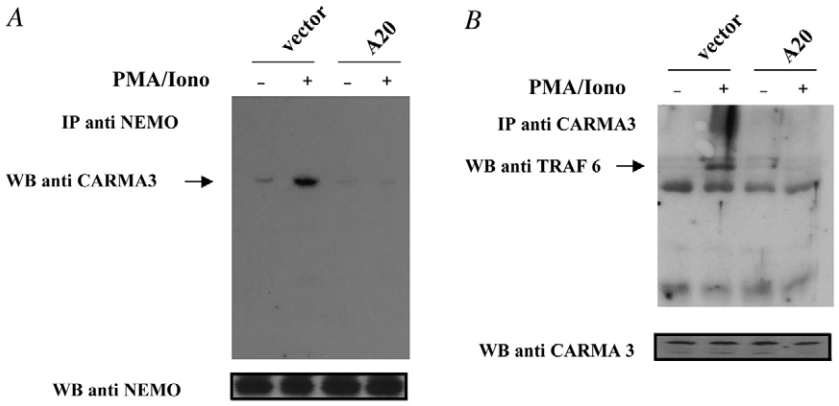

Fig. 4. A20 inhibits formation of molecular complexes following activation of PKC. HEK293 cells were transfected with an empty expression plasmid or plasmid encoding A20. 24 hours later, cells were treated with PMA (40 ng/ml) plus ionomycin (1 μM) for 40 minutes. Cell extracts were immunoprecipitated with the indicated immunoprecipitating antibody (IP) and assayed for coprecipitating proteins by immunoblot experiments probed with the indicated antibody (WB).

deficient mice, which die prematurely because of systemic inflammation and hypersensitivity to LPS and TNFα (Lee et al., 2000). This phenotype correlates with failure of A20-deficient cells to downregulate NF-κB transcriptional activity, owing to a constitutively active IKK complex, indicating that A20 regulates NF-κB signaling upstream or at the level of this complex (Lee et al., 2000). The recent finding that A20 has both ubiquitylation and deubiquitylation activities has shed some light on our understanding of the molecular mechanism by which this protein represses the transcriptional activity of NF-κB. In fact, in the TNF receptor I signaling pathway, A20 functions as a deubiquitylation enzyme for K63-linked polyubiquitylated RIP1, thereby leading to extinction of TNFα-induced NF-κB signaling, and as a ubiquitylation enzyme for K48-linked polyubiquitylation of RIP1, thereby targeting it for degradation (Wertz et al., 2004). In addition to RIP1, also TRAF6 (Boone et al., 2004) and IKKγ/NEMO (Mauro et al., 2006) are both targets for the K63-linked deubiquitylation activity of A20.

Our experiments, showing that the deubiquitylation activity of A20 is required for inhibition of BCL10-mediated activation of NF-κB, are consistent with the observation that BCL10-mediated activation of the NF-κB pathway requires a ubiquitylation event that involves IKKγ/NEMO and TRAF6 (Sun et al., 2004; Zhou et al., 2004). As both IKKγ/NEMO and TRAF6 are targets for the deubiquitylation editing functions of A20 (Mauro et al., 2006; Boone et al., 2004), the inhibitory effect of A20 on BCL10-mediated activation of NF-κB might reside in the direct deubiquitylation of IKKγ/NEMO and/or TRAF6. Thus, deubiquitylation of K63-linked polyubiquitin chains might represent a common strategy that different cell types utilize to downregulate NF-κB-activating pathways. One prediction of this hypothesis would be that cells from A20-deficient mice should display deregulated NF-κB activation. However, although A20-deficient mice have increased numbers of activated lymphocytes (Lee et al., 2000), a fine analysis of NF-κB regulation in cells from A20-deficient mice has not yet been performed, probably because of their deregulated inflammatory pathways (Lee et al., 2000). By contrast, other mechanisms that downmodulate NF-κB activation promoted by BCL10 and CARMA proteins have been proposed (Scharschmidt et al., 2004; Wegener et al., 2006).

The second significant conclusion that can be drawn from these results involves the molecular mechanism by which BCL10 and CARMA proteins activate the IKK complex. An enormous amount of work has been conducted in order to decipher the molecular mechanisms that link the BCL10-CARMA1-MALT1 complex to NF-κB activation (Schulze-Luehrmann and Ghosh, 2006; Kuhns et al., 2006). We have previously shown that both CARMA1 and CARMA3 physically associate with IKKγ/NEMO upon cell stimulation (Stilo et al., 2004). In addition, it has been shown that a BCL10-containing complex recruits TRAF6 and activates its E3-ligase activity, thereby facilitating K63-linked polyubiquitylation of IKKγ/NEMO, which eventually results in NF-κB activation (Sun et al., 2004). In the present work, our experiments clearly show that a ubiquitin-mediated event, sensitive to the deubiquitylating activity of A20, is required for recruitment of IKKγ/NEMO to the BCL10 complex. However, it remains to be determined the molecular target(s) of this ubiquitylation process and whether the ubiquitylation state of IKKγ/NEMO affects its recruitment to the BCL10 complex.

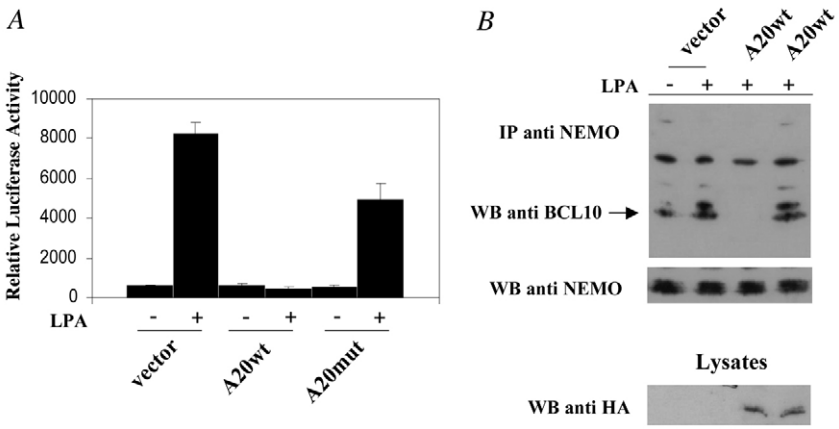

Fig. 5. A20 inhibits LPA-mediated NF-κB activation. (A) MEFs were infected in 100 mm Petri dishes with an empty retroviral construct or construct expressing hemagglutinin (HA)-flagged forms of A20wt and A20mut and subsequently transfected with a luciferase reporter plasmid for NF-κB. Infected cells were left untreated or stimulated with LPA (20 μM) for 8 hours and luciferase activity was measured. The data shown represent the relative luciferase activity and are representative of four independent experiments performed in triplicate. (B) MEFs were infected as in panel A and left untreated or treated with LPA for 60 minutes. Lysates were then prepared and immunoprecipitated (IP) with antibody against IKKγ/NEMO. Immunocomplexes were separated by SDS-PAGE, transferred onto membranes and subsequently probed with antibodies (WB) against BCL10 and IKKγ/NEMO. A20 expression was monitored by immunoblotting with antibody against HA.

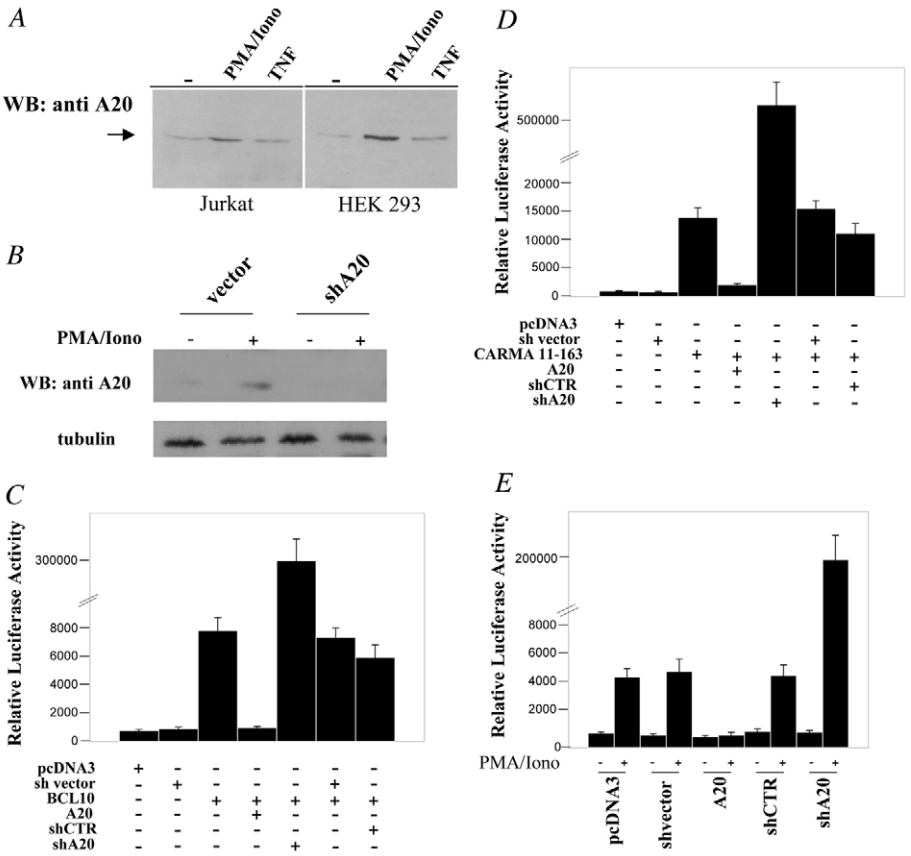

Fig. 6. Negative regulatory activity of A20 on the NF-κB activation mediated by PKC. (A) HEK293 and Jurkat cells were left untreated or treated with PMA (40 ng/ml) plus ionomycin (1 μM) or TNFα (5 ng/ml) for 6 hours. Lysates (80 μg) extracted from treated and untreated cells were separated by SDS-PAGE, blotted onto nitrocellulose membranes and analyzed by immunoblotting (WB) to monitor A20 protein expression. (B) HEK293 cells were transfected with an empty plasmid or plasmid encoding a shRNA against human A20. 24 hours later, the expression level of A20 was monitored by western blot experiments in cells left untreated or stimulated with PMA (40 ng/ml) plus ionomycin (1 μM) for 6 hours. (C,D) HEK293 cells were transiently cotransfected with the indicated plasmid DNA, together with NF-κB–luciferase and β-galactosidase reporter vectors. 16 hours after transfection, cell lysates were prepared and luciferase activity was measured. As a control, a plasmid expressing a shRNA validated to target expression of eGFP was used (shCTR). The data shown represent the relative luciferase activity normalized against β-galactosidase activity and are representative of five independent experiments performed in triplicate. (E) HEK293 cells were transiently cotransfected with the indicated plasmid DNA, together with NF-κB–luciferase and β-galactosidase reporter vectors. 24 hours later, cells were treated with PMA (40 ng/ml) plus ionomycin (1 μM) for 5 hours and luciferase activity was measured. The data shown represent the relative luciferase activity normalized against β-galactosidase activity and are representative of five independent experiments performed in triplicate.

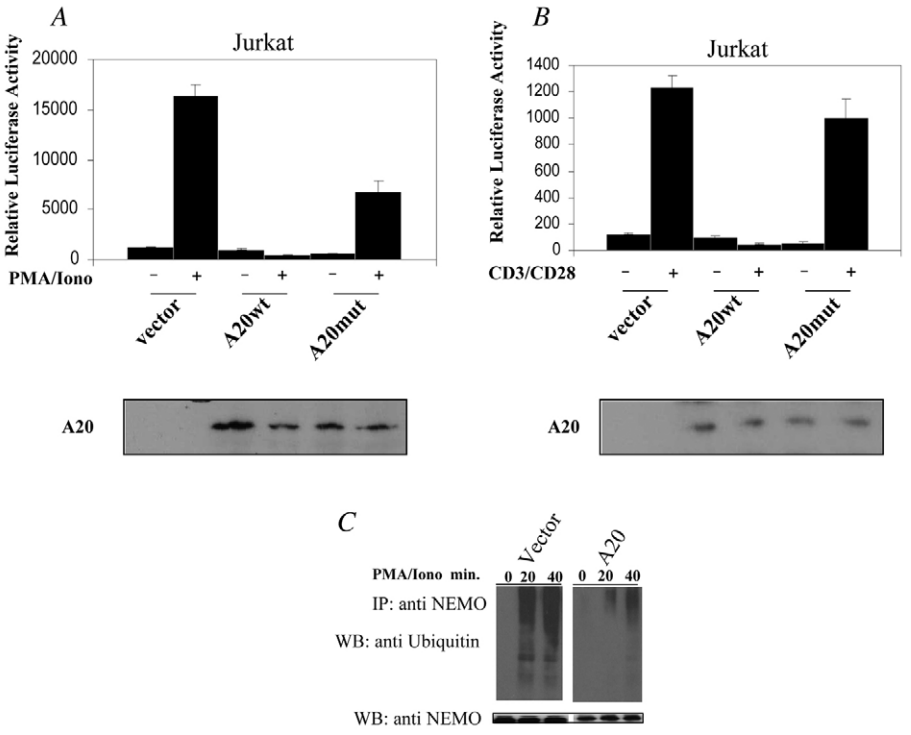

Fig. 7. A20 inhibits CARMA1 and BCL10 function. (A) Jurkat cells were transfected with an empty vector or vector encoding A20wt and A20mut, together with an NF-κB–luciferase reporter plasmid. 24 hours later, cells were treated with PMA (40 ng/ml) plus ionomycin (1 μM) for 5 hours and luciferase activity was measured. The data shown are representative of five separate experiments. The expression level of cotransfected A20wt and A20mut, relative to one experiment, was verified by western blot and is shown in the bottom panel. (B) Jurkat cells were transfected as in panel A and then stimulated for 10 hours with antibodies against CD3 and CD28 (1 μg/ml of anti-CD3 and 2 μg/ml of anti-CD28), after which the luciferase activity was determined. The data shown are representative of four independent experiments. (C) Jurkat cells were transfected with empty vector or with an expression plasmid encoding A20. 24 hours later, cells were treated with PMA (40 ng/ml) plus ionomycin (1 μM) for the indicated periods of time. Lysates were then prepared and immunoprecipitated (IP) with mAb against IKKγ/NEMO. Immunocomplexes were separated by SDS-PAGE, transferred onto membranes and subsequently probed with antibodies (WB) against ubiquitin and IKKγ/NEMO.

Materials and Methods

Reagents and antibodies

Sources of antibodies and reagents were the following: anti-CARMA1, anti-FLAG, Sigma; anti-IKKγ/NEMO, anti-TRAF6, anti-HA, anti-IκBα, anti-IKKγ/NEMO, anti-BCL-10, Santa Cruz Biotechnology; anti-IKKγ/NEMO, anti-CD3, anti-CD28, BD Pharmingen; antibodies against BCL10 and CARMA3 have been generated in our laboratory and have been described previously (Costanzo et al., 1999; Stilo et al., 2004; Guiet and Vito, 2000). PMA, ionomycin and LPA were obtained from Sigma. Horseradish-peroxidase-conjugated secondary antibodies were sourced from Promega.

Plasmids and expression vectors

All plasmids and expression vectors used in this study have been generated by standard procedures and confirmed by sequencing. The vector expressing a shRNA to silence the expression of human A20 has been described previously (Mauro et al., 2006). A shRNA validated to target eGFP (Open Biosystem) has been used as a control for the RNAi experiments.

Cell culture and transfections

HEK293 cells and MEFs were grown in complete DMEM supplemented with 10% fetal calf serum (FCS) and 100 μg/ml penicillin-streptomycin, and maintained at 37°C with 5% CO_2. Jurkat cells were cultured in RPMI supplemented with 10% FCS and 100 μg/ml penicillin-streptomycin. For plasmid transfections, HEK293 cells were transfected in 100 mm Petri dishes by calcium phosphate precipitation. Jurkat cells (30×10^6) were electrotransfected (250 V, 960 μF) with 30 μg of plasmid DNA. Lipofectamine 2000 reagent (Invitrogen) was used to transfect MEFs, following the manufacturer's instructions.

Retroviral stock production and infection

The retroviral system for gene delivery used in our laboratory has been described previously (Guiet et al., 2002). Briefly, LTR-driven retroviral expression constructs were made in the pBMN vector using standard cloning techniques. PBMN expression vectors were then transfected in a packaging cell line by calcium phosphate precipitation. 24 hours after transfection, secreted retrovirus was filtered and used to infect MEFs. Gene expression in infected cells was examined 48 hours after infection by immunoblot assay.

Immunoblot analysis and coprecipitation

For western blot analysis, 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 resolved by 10% SDS-PAGE, and gels were transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour at room temperature in PBS plus 0.05% Tween 20 plus 5% dry nonfat milk. Primary antibody incubations were carried out overnight at 4°C in PBS plus 0.05% Tween 20 plus 2.5% dry nonfat milk. Secondary antibody incubations were performed for 1 hour at room temperature in blocking buffer. Blots were incubated in wash buffer for 10 minutes after blocking, 3×10 minutes after primary antibody, and 5×10 minutes after HRP-conjugated secondary antibody. Reacting bands were detected 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.

Luciferase assay

To determine the activation state of NF-κB, cells were transfected with the indicated plasmid DNAs together with a pNF-κB-luc construct in six-well plates. 24 hours 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.

This work was supported by the EU contract No. 503569 to EMIL (European Molecular Imaging Laboratories Network), MIUR-PRIN 2006 No. 2006051402 and Fondazione Italiana Sclerosi Multipla (2003/R66).

References

Bertin, J., Wang, L., Guo, Y., Jacobson, M. D., Poyet, J. L., Srinivasula, S. M., Merriam, S., DiStefano, P. S. and Alnemri, E. S. (2001). CARD11 and CARD14 are novel caspase recruitment domain (CARD)/membrane-associated guanylate kinase (MAGUK) family members that interact with BCL10 and activate NF-κB. *J. Biol. Chem.* **276**, 11877-11882.

Beyaert, R., Heyninck, K. and Van Huffel, S. (2000). A20 and A20-binding proteins as cellular inhibitors of nuclear factor-κB-dependent gene expression and apoptosis. *Biochem. Pharmacol.* **60**, 1143-1151.

Bidere, N., Snow, A. L., Sakai, K., Zheng, L. and Lenardo, M. J. (2006). Caspase-8 regulation by direct interaction with TRAF6 in T cell receptor-induced NF-κB activation. *Curr. Biol.* **16**, 1666-1671.

Boone, D. L., Turer, E. E., Lee, E. G., Ahmad, R. C., Wheeler, M. T., Tsui, C., Hurley, P., Chien, M., Chai, S., Hitotsumatsu, O. et al. (2004). The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nat. Immunol.* **5**, 1052-1060.

Costanzo, A., Guiet, C. and Vito, P. (1999). c-E10 is a caspase-recruiting domain-containing protein that interacts with components of death receptors signaling pathway and activates nuclear factor-κB. *J. Biol. Chem.* **274**, 20127-20132.

Cummings, R., Zhao, Y., Jacoby, D., Spannhake, E. W., Ohba, M., Garcia, J. G., Watkins, T., He, D., Saatian, B. and Natarajan, V. (2004). Protein kinase C mediates lysophosphatidic acid-induced NF-κB activation and interleukin-8 secretion in human bronchial epithelial cells. *J. Biol. Chem.* **279**, 41085-41094.

Gaide, O., Martinon, F., Micheau, O., Bonnet, D., Thome, M. and Tschopp, J. (2001). Carma1, a CARD-containing binding partner of Bcl10, induces Bcl10 phosphorylation and NF-κB activation *FEBS Lett.* **496**, 121-127.

Ghosh, S. and Karin, M. (2002). Missing pieces in the NF-κB puzzle. *Cell* **109**, S81-S96.

Grabiner, B. C., Blonska, M., Lin, P. C., You, Y., Wang, D., Sun, J., Darnay, B. G., Dong, C. and Lin, X. (2007). CARMA3 deficiency abrogates G protein-coupled receptor-induced NF-κB activation. *Genes Dev.* **21**, 984-996.

Guiet, C. and Vito, P. (2000). Caspase recruitment domain (CARD)-dependent cytoplasmic filaments mediate bcl10-induced NF-κB activation. *J. Cell Biol.* **148**, 1131-1140.

Guiet, C., Silvestri, E., De Smaele, E., Franzoso, G. and Vito, P. (2002). c-FLIP efficiently rescues TRAF-$2^{-/-}$ cells from TNF-induced apoptosis. *Cell Death Differ.* **9**, 138-144.

Hara, H., Bakal, C., Wada, T., Bouchard, D., Rottapel, R., Saito, T. and Penninger, J. M. (2004). The molecular adapter Carma1 controls entry of IκB kinase into the central immune synapse. *J. Exp. Med.* **200**, 1167-1177.

Heyninck, K. and Beyaert, R. (2005). A20 inhibits NF-κB activation by dual ubiquitin-editing functions. *Trends Biochem. Sci.* **30**, 1-4.

Karin, M. (2006). Nuclear factor-κB in cancer development and progression. *Nature* **441**, 431-436.

Kuhns, M. S., Davis, M. M. and Garcia, K. C. (2006). Deconstructing the form and function of the TCR/CD3 complex. *Immunity* **24**, 133-139.

Lee, E. G., Boone, D. L., Chai, S., Libby, S. L., Chien, M., Lodolce, J. P. and Ma, A. (2000). Failure to regulate TNF-induced NF-κB and cell death responses in A20-deficient mice. *Science* **289**, 2350-2354.

Mauro, C., Pacifico, F., Lavorgna, A., Mellone, S., Iannetti, A., Acquaviva, R., Formisano, S., Vito, P. and Leonardi, A. (2006). ABIN-1 binds to NEMO/IKKγ and co-operates with A20 in inhibiting NF-κB. *J. Biol. Chem.* **281**, 18482-18488.

McAllister-Lucas, L. M., Ruland, J., Siu, K., Jin, X., Gu, S., Kim, D. S., Kuffa, P., Kohrt, D., Mak, T. W., Nuñez, G. et al. (2007). CARMA3/Bcl10/MALT1-dependent NF-κB activation mediates angiotensin II-responsive inflammatory signaling in nonimmune cells. *Proc. Natl. Acad. Sci. USA* **104**, 139-144.

Opipari, A. W., Jr, Boguski, M. S. and Dixit, V. M. (1990). The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. *J. Biol. Chem.* **265**, 14705-14708.

Rawlings, D. J., Sommer, K. and Moreno-García, M. E. (2006). The CARMA1 signalosome links the signalling machinery of adaptive and innate immunity in lymphocytes. *Nat. Rev. Immunol.* **11**, 799-812.

Scharschmidt, E., Wegener, E., Heissmeyer, V., Rao, A. and Krappmann, D. (2004). Degradation of Bcl10 induced by T-cell activation negatively regulates NF-κB signaling. *Mol. Cell. Biol.* **24**, 3860-3873.

Schulze-Luehrmann, J. and Ghosh, S. (2006). Antigen-receptor signaling to nuclear factor-κB. *Immunity* **25**, 701-715.

Shahrestanifar, M., Fan, X. and Manning, D. R. (1999). Lysophosphatidic acid activates NF-κB in fibroblasts. A requirement for multiple inputs. *J. Biol. Chem.* **274**, 3828-3833.

Silverman, N. and Maniatis, T. (2001). NF-κB signaling pathways in mammalian and insect innate immunity. *Genes Dev.* **15**, 2321-2342.

Stilo, R., Liguoro, D., Di Jeso, B., Formisano, S., Consiglio, E., Leonardi, A. and Vito, P. (2004). Physical and functional interaction of CARMA1 and CARMA3 with Iκ kinase γ-NF-κB essential modulator. *J. Biol. Chem.* **279**, 34323-34331.

Sun, L., Deng, L., Ea, C. K., Xia, Z. P. and Chen, Z. J. (2004). The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Mol. Cell* **14**, 289-301.

Wang, D., Matsumoto, R., You, Y., Che, T., Lin, X. Y., Gaffen, S. L. and Lin, X. (2004). CD3/CD28 costimulation-induced NF-kappaB activation is mediated by recruitment of protein kinase C-theta, Bcl10, and IκB kinase β to the immunological synapse through CARMA1. *Mol. Cell. Biol.* **1**, 64-71.

Wang, D., You, Y., Lin, P. C., Xue, L., Morris, S. W., Zeng, H., Wen, R. and Lin, X. (2007). Bcl10 plays a critical role in NF-κB activation induced by G protein-coupled receptors. *Proc. Natl. Acad. Sci. USA* **104**, 145-150.

Wegener, E., Oeckinghaus, A., Papadopoulou, N., Lavitas, L., Schmidt-Supprian, M., Ferch, U., Mak, T. W., Ruland, J., Heissmeyer, V. and Krappmann, D. (2006). Essential role for IκB kinase β in remodeling Carma1-Bcl10-Malt1 complexes upon T cell activation. *Mol. Cell* **23**, 13-23.

Wertz, I. E., O'Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., Wu, P., Wiesmann, C., Baker, R., Boone, D. L. et al. (2004). Deubiquitination and ubiquitin ligase domains of A20 downregulate NF-κB signalling. *Nature* **430**, 694-699.

Zhou, H., Wertz, I., O'Rourke, K., Ultsch, M., Seshagiri, S., Eby, M., Xiao, W. and Dixit, V. M. (2004). Bcl10 activates the NF-κB pathway through ubiquitination of NEMO. *Nature* **427**, 167-171.