CARMA3 deficiency abrogates G protein-coupled receptor-induced NF- κ B activation

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G protein-coupled receptors (GPCRs) play pivotal roles in regulating various cellular functions. Although many GPCRs induce NF- κ B activation, the molecular mechanism of GPCR-induced NF- κ B activation remains largely unknown. CARMA3 (CARD and MAGUK domain-containing protein 3) is a scaffold molecule with unknown biological functions. By generating CARMA3 knockout mice using the gene targeting approach, here we show CARMA3 is required for GPCR-induced NF- κ B activation. Mechanistically, we found that CARMA3 deficiency impairs GPCR-induced I κ B kinase (IKK) activation, although it does not affect GPCR-induced IKK α / β phosphorylation, indicating that inducible phosphorylation of IKK α / β alone is not sufficient to induce its kinase activity. We also found that CARMA3 is physically associated with NEMO/IKK γ , and induces polyubiquitination of an unknown protein(s) that associates with NEMO, likely by linking NEMO to TRAF6. Consistently, we found TRAF6 deficiency also abrogates GPCR-induced NF- κ B activation. Together, our results provide the genetic evidence that CARMA3 is required for GPCR-induced NF- κ B activation.

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G protein-coupled receptors (GPCRs) constitute a large family of cell surface receptors, which play pivotal roles in regulating cell migration, differentiation, proliferation, and survival (Marinissen and Gutkind 2001). GPCRs transduce environmental signals by triggering the guanine nucleotide exchange of heterotrimeric G proteins (Gilman 1987). Exchange of GDP for GTP results in activation of the G α subunits and dissociation of the G $\beta\gamma$ subunits. The G α subunits contain several subgroups, including G $_i$, G $_s$, G $_q$, and G $_{12/13}$. These G proteins independently activate their downstream signaling cascades composed of scaffold/adaptor molecules and effector enzymes such as small GTPases, phospholipase, and protein kinases, leading to activation of various transcription factors, including NF- κ B (Marinissen and Gutkind 2001). Previous studies have shown that the stimulation of GPCR ligands, lysophosphatidic acid (LPA), endothelin-1 (ET-1), and angiotensin-II (Ang-II), induces NF- κ B activation (Shahrestanifar et al. 1999; Purcell et

al. 2001). Although some evidence suggests that small GTPases, PI3K, and PKC are involved in GPCR-induced NF- κ B activation, the signaling pathway mediating this activation remains largely undefined (Ye 2001).

The NF- κ B family of transcription factors plays critical roles in controlling expression of survival factors, cytokines, and proinflammatory molecules (Ghosh et al. 1998). Stimulation of various cell surface receptors, including receptors for proinflammatory cytokines such as TNF α and IL-1 β , Toll-like receptors (TLRs), antigen receptors, and GPCRs, activates NF- κ B by initiating distinct signaling pathways that eventually converge on the I κ B kinase (IKK) complex (Hayden and Ghosh 2004). The activated IKK phosphorylates the inhibitory molecule, I κ B, triggering the rapid ubiquitination and proteolysis of I κ Bs. This unmasks the nuclear localization sequence of NF- κ B, resulting in its rapid translocation from the cytoplasm into nucleus, where it regulates the transcription of its target genes (Karin and Ben-Neriah 2000).

The IKK complex contains three subunits: two catalytic subunits, IKK α and IKK β , and an essential regulatory subunit, IKK γ /NEMO (Karin and Ben-Neriah 2000). Activation of the IKK complex is associated with phosphorylation of Ser177 and Ser181 in the activation loop

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of IKK β and Ser176 and Ser180 in IKK α . Genetic studies show that NEMO is required for the activation of NF- κ B by facilitating the formation of the high-molecular-weight IKK complex (Yamaoka et al. 1998). More recent studies have shown that Lys63 (K63)-linked polyubiquitination of NEMO is also essential for activating the IKK complex (Tang et al. 2003; Zhou et al. 2004). However, it is unclear whether IKK α / β phosphorylation and K63-linked polyubiquitination of NEMO are regulated in a linear cascade or two parallel pathways.

CARMA3, Caspase recruitment domain (CARD)-associated and membrane-associated guanylate kinase domain (MAGUK)-containing protein 3 (Gaide et al. 2001; McAllister-Lucas et al. 2001; L. Wang et al. 2001), is a member of the CARMA family that contains three proteins, CARMA1, CARMA2, and CARMA3. These three proteins share similar structural motifs, with an N-terminal CARD domain, followed by a coiled-coil (CC) domain, a PDZ domain, a SH3 domain, and a C-terminal guanylate kinase-like (GUK) domain (Gaide et al. 2001; McAllister-Lucas et al. 2001; L. Wang et al. 2001). However, they have distinct expression patterns with CARMA1 (CARD11) expressed in hematopoietic cells, CARMA2 (CARD14/Bimp2) in the placenta, and CARMA3 (CARD10/Bimp1) in all nonhematopoietic cells (Bertin et al. 2001; Gaide et al. 2001; McAllister-Lucas et al. 2001; L. Wang et al. 2001). Recent studies have revealed a key role for CARMA1 in antigen receptor-induced NF- κ B activation (Gaide et al. 2002; Pomerantz et al. 2002; Wang et al. 2002; Egawa et al. 2003; Hara et al. 2003; Jun et al. 2003; Newton and Dixit 2003). In contrast, although the overexpression of CARMA2 and CARMA3 in HEK293 cells also induces NF- κ B activation (Gaide et al. 2001; McAllister-Lucas et al. 2001; L. Wang et al. 2001), the signaling pathway mediated by these proteins remains completely unknown.

To reveal the signaling pathways mediated by CARMA3, we used a gene targeting approach to generate CARMA3 knockout mice. Using CARMA3-deficient cells, we demonstrate that CARMA3 is required for GPCR-induced NF- κ B activation. This defect is specific because other stimuli such as TNF α , lipopolysaccharide (LPS), and extracellular matrix proteins can activate NF- κ B in CARMA3-deficient cells. Together, our results reveal a new GPCR-induced signaling pathway that leads to NF- κ B activation.

Results

Generation of CARMA3-deficient mice

To investigate the biological function of CARMA3, we constructed a gene targeting vector that replaced the exon 3, which encodes a large part of the CARD domain, of the mouse *Carma3* gene with a PGK-neo cassette (Fig. 1A). The targeting vector was electroporated into mouse embryonic stem (ES) cells. Two ES cell lines with the targeted allele were obtained after homologous recombination (data not shown) and used to generate chimeric mice. The targeted allele successfully underwent germ-

line transmission. We were able to confirm that the *Carma3* gene was disrupted in the targeted mice using genomic PCR, RT-PCR, and Western blot analysis (Fig. 1B–D). Of note, although the PGK-neo cassette (~1 kb) insertion did not abolish *Carma3* mRNA expression (Fig. 1C, lanes 2,3), it disrupted the protein expression of *Carma3* (Fig. 1D), since the PGK-neo cassette was inserted into the exon 3 *Carma3* gene in the reverse orientation (Fig. 1A). To further confirm that the detected *Carma3* mRNA in CARMA3 mutant mice (Fig. 1C, lanes 2,3) could not express a functional CARMA3 protein, we amplified the mutant *Carma3* cDNA by RT-PCR using mRNA from *Carma3*^{-/-} cells. Sequencing analysis indicates that the mutated *Carma3* mRNA contains multiple stop codons in the insertion region of the PGK-neo cassette, thereby completely disrupting the CARMA3 coding sequence (data not shown) and resulting in a lack of expression of CARMA3 proteins (Fig. 1D).

Although *Carma3*-heterozygous (*Carma3*^{+/-}) mice have no developmental defects, ~50% of *Carma3*-homozygous (*Carma3*^{-/-}) mice have the neural tube defect (NTD) phenotype known as anencephaly (Fig. 1E,F; Supplementary Fig. 1). This defect results in perinatal mortality of the mice due to either bleeding out from the skull or infanticide by the mother. We analyzed different stages of *Carma3*^{-/-} embryos and found that this defect occurs before embryonic day 10.5 (E10.5) (data not shown). It has been reported that ~40% of *Bcl10*-null mice also develop the NTD phenotype (Ruland et al. 2001). Consistent with previous observations that CARMA3 associates with *Bcl10*, when overexpressed in HEK293 cells (McAllister-Lucas et al. 2001; L. Wang et al. 2001), the anencephaly phenotype in *Carma3*^{-/-} mice is very similar to that of *Bcl10*^{-/-} mice (Supplementary Fig. 1), suggesting that CARMA3 and *Bcl10* function in the same signal transduction pathway. Also, like the *Bcl10*^{-/-} mice, the remaining 50% of *Carma3*^{-/-} mice do not have the NTD phenotype and grow normally. Unlike CARMA1 deficiency, which resulted in a lymphocyte activation defect (Egawa et al. 2003; Hara et al. 2003; Jun et al. 2003; Newton and Dixit 2003), CARMA3 deficiency does not cause any obvious defects in lymphocyte activation (data not shown), which is consistent with the fact that CARMA3 is not expressed in hematopoietic cells (Gaide et al. 2001; McAllister-Lucas et al. 2001; L. Wang et al. 2001).

CARMA3 is required for LPA-induced NF- κ B activation

We next investigated in which signaling pathway CARMA3 is involved. Our earlier studies showed that CARMA3 could effectively rescue the defect of T-cell receptor (TCR)-induced NF- κ B activation in CARMA1-deficient T cells (Matsumoto et al. 2005), suggesting that CARMA3 and CARMA1 have similar upstream components, but in different signaling pathways. Since PKC functions upstream of CARMA1 in the signaling pathway of antigen receptors in lymphocytes (Matsumoto et al. 2005; Sommer et al. 2005), we postulated that PKC

Figure 1. The generation of CARMA3-deficient mice. (A) The targeting vector containing the PGK-Neo^r gene was used to replace Exon 3 of the *Carma3* gene. Exon 3 of *Carma3* encodes the majority of the CARD domain. Solid arrows indicate the sites of primers used for genotyping the *Carma3* gene, whereas arrows indicate the sites of primers used for RT-PCR of CARMA3 mRNA. (B) Genomic PCR indicates the DNA rearrangement. (C) RT-PCR indicates the insertion of the Neo cassette into the *Carma3* mRNA transcript in CARMA3-deficient mice. (D) Liver cell lysates from *Carma3*^{+/+} or *Carma3*^{-/-} mice were subjected to SDS-PAGE and Western blot analysis using an anti-C-terminal CARMA3 antibody. (E) *Carma3*^{+/+} and *Carma3*^{-/-} E13.5 embryos exhibit the anencephaly phenotype of the neural tube closure defect. (F) Head sections of *Carma3*^{+/+} and *Carma3*^{-/-} E13.5 embryos were stained with H&E.

also functions upstream of CARMA3 in an unknown signaling pathway(s) in nonhematopoietic cells. Earlier studies have suggested that PKC is involved in NF- κ B activation induced by GPCRs (Shahrestanifar et al. 1999; Cummings et al. 2004), integrins (Juliano 2002), and receptor tyrosine kinases (RTKs) (Biswas et al. 2000). Therefore, we hypothesized that CARMA3 might function downstream from PKC in signaling pathways induced by GPCRs, integrins, or RTKs.

To test this hypothesis, we obtained mouse embryonic fibroblasts (MEF) from day 13.5 embryos of *Carma3*^{+/+}, *Carma3*^{+/-}, and *Carma3*^{-/-} mice. These MEF cells were stimulated with or without LPA. LPA is a potent bioactive phospholipid that induces cell survival and proliferation through its GPCRs (Moolenaar et al. 1997). Stimulation of cells with LPA could effectively induce NF- κ B activation in the presence of CARMA3. However, LPA-induced NF- κ B activation was abolished in *Carma3*^{-/-} cells, whereas TNF α could effectively induce NF- κ B activation in the same cells (Fig. 2A, top panel). In addition, consistent with previous observations that PKC is involved in GPCR-induced NF- κ B activation,

treatment of *Carma3*^{-/-} MEF cells with the pharmacological PKC agonists, phorbol-12-myristate-13-acetate (PMA) plus ionomycin (Iono), failed to induce NF- κ B activation (Fig. 2A, top panel). In contrast, LPA or PMA plus Iono-induced AP-1 activation was not significantly affected in the absence of CARMA3 (Fig. 2A, middle panel). Since the NF- κ B complex induced by LPA and PMA plus Iono mainly contained p65 and p50 (Fig. 2B), this result indicates that LPA-induced NF- κ B activation is through the classical NF- κ B pathway. To further confirm the requirement of CARMA3 for GPCR-induced NF- κ B activation, we reconstituted CARMA3-deficient MEF cells with an expression plasmid encoding HA-tagged CARMA3 (Supplementary Fig. 2), and found that NF- κ B activation (Fig. 2C) and I κ B α phosphorylation (Supplementary Fig. 2) induced by LPA or PMA plus Iono were fully restored. Together, these results demonstrate that CARMA3 is required for GPCR-induced NF- κ B activation.

It has been shown that LPA-induced production of cytokines such as MIP-2 (the murine homolog of IL-8), which plays a critical role in cell migration and angio-

Figure 2. CARMA3 is required for LPA-induced NF- κ B activation. (A) MEF cells from *Carma3*^{+/+} and *Carma3*^{-/-} mice were stimulated with or without LPA (10 μ M), PMA (40 ng/mL) plus Iono (100 ng/mL), or TNF α (10 ng/mL) for 30 or 60 min. Nuclear extracts were prepared and subjected to EMSA using ³²P-labeled NF- κ B, AP-1, or OCT-1 probes. (B) Nuclear extracts from unstimulated (UN) or stimulated (LPA or PMA/Iono) *Carma3*^{+/+} MEF cells were incubated with ³²P-labeled NF- κ B probes in the absence or presence of anti-p65 and anti-p50 antibodies for 15 min at room temperature. The resulted mixtures were subjected to electrophoresis and autoradiography. (C) *Carma3*^{-/-} MEF cells were reconstituted with an expression plasmid encoding HA-tagged CARMA3 (C3KO-CARMA3) or empty vector control (C3KO-Vector). The resulting cells were stimulated with or without LPA, PMA plus Iono, or TNF α for 60 min. The nuclear extracts from these cells were subjected to EMSA using ³²P-labeled NF- κ B or OCT-1 probes. (D) Wild-type or CARMA3-deficient MEF cells were starved in media with 0.5% fetal calf serum for 18 h, and then stimulated with or without LPA (30 μ M) or PMA (40 ng/mL) plus Iono (100 ng/mL) for another 20 h. The media from these

cultures were collected and subjected to the MIP-2 ELISA analysis according to the manufacturer's instructions. (E) *Carma3*^{+/+} or *Carma3*^{-/-} MEFs were plated onto dishes precoated with PLL (5 μ g/mL), OPN (2 μ g/mL), or RGDS (45 μ g/mL) for 2 h. For TNF α stimulation, cells were plated onto PLL-coated dishes for 1.5 h, and then stimulated with 10 ng of TNF α for 30 min. Nuclear extracts were prepared and subjected to EMSA using ³²P-labeled NF- κ B probe.

genesis, is dependent on NF- κ B activation (Cummings et al. 2004). Therefore, we next examined the role of CARMA3 in MIP-2 production. The wild-type and CARMA3-deficient MEF cells were stimulated with or without LPA or PMA plus Iono for 20 h. We found that MIP-2 production induced by LPA or PMA plus Iono was significantly defective in CARMA3-deficient cells (Fig. 2D), indicating that CARMA3-mediated NF- κ B activation plays an essential role for LPA-induced cytokine production.

Since earlier studies suggest that PKC is also involved in integrin-induced NF- κ B activation (Juliano 2002), we hypothesized that integrin-induced NF- κ B also depends on CARMA3. However, we found that osteopontin (OPN) and RGD peptides, which can activate the $\alpha_v\beta_3$ integrin molecules, effectively induced NF- κ B activation in wild-type as well as CARMA3-deficient cells (Fig. 2E). In addition, although RTKs have been reported to activate NF- κ B through a PKC-dependent mechanism (Biswas et al. 2000), we were unable to detect NF- κ B activation in both wild-type and CARMA3-deficient MEF cells with FGF, PDGF, and IGF (data not shown). Therefore, we were unable to determine whether RTK-induced NF- κ B is dependent on CARMA3. Furthermore, we found that TLR-induced NF- κ B activation was not defective in CARMA3-deficient cells (data not shown). Together, our results indicate that CARMA3 is selectively involved in GPCR-induced NF- κ B activation.

To investigate the molecular mechanism by which

LPA induces NF- κ B activation, we examined whether I κ B α phosphorylation was defective in CARMA3-deficient cells. Consistent with the nuclear translocation of NF- κ B, stimulation with LPA could induce I κ B α phosphorylation in *Carma3*^{+/+}, but not *Carma3*^{-/-}, cells, whereas TNF α effectively induced I κ B α phosphorylation and degradation in both *Carma3*^{+/+} and *Carma3*^{-/-} cells (Fig. 3A, top panel), indicating that CARMA3 is specifically required for GPCR-induced IKK activation. Of note, LPA-induced I κ B α phosphorylation was significantly weaker than that induced by TNF α (Fig. 3A, top panel), and only a small portion of I κ B α was degraded in wild-type cells (Fig. 3A, middle panel). To further demonstrate that the signal-induced I κ B α degradation is required for LPA-induced NF- κ B, we pretreated cells with the proteasome inhibitor MG132 to block I κ B α degradation. We found that NF- κ B activation induced by LPA, PMA plus Iono, or TNF α was completely inhibited by the treatment of MG132 (Fig. 3B), indicating that the signal-induced I κ B α degradation is required for LPA-induced NF- κ B activation.

Earlier studies suggest that CARMA1, the CARMA3 homolog in lymphoid cells, plays an important role in JNK activation (Gaide et al. 2002; Hara et al. 2003), and our recent studies also reveal that CARMA1 is specifically required for antigen receptor-induced JNK2 activation (Blonska et al. 2007). To investigate whether LPA-induced mitogen-activated protein (MAP) kinase activation is dependent on CARMA3, wild-type and

Figure 3. LPA-induced IκBα phosphorylation is defective in CARMA3-deficient cells. (A) *Carma3*^{+/+} and *Carma3*^{-/-} MEF cells were stimulated with or without LPA (10 μM) or TNFα (10 ng/mL) for indicated time points. Cell lysates were then subjected to SDS-PAGE and Western blot analysis using indicated antibodies. (B) *Carma3*^{+/+} and *Carma3*^{-/-} MEF cells were pretreated with or without MG132 for 15 min, then stimulated with or without LPA (10 μM) or TNFα (10 ng/mL) for 1 h. Nuclear extracts were prepared from these cells, and subjected to EMSA using ³²P-labeled NF-κB or OCT-1 probe. (C) *Carma3*^{+/+} and *Carma3*^{-/-} MEF cells were stimulated with or without LPA (10 μM) for indicated time points. Cell lysates were then subjected to SDS-PAGE and Western blot analysis using indicated antibodies.

CARMA3-deficient cells were stimulated with or without LPA for various time points. We found that LPA-induced JNK and ERK activation was comparable in wild-type and CARMA3-deficient cells (Fig. 3C), indicating that, unlike the role of CARMA1 in antigen receptor signaling pathways, CARMA3 is not required for JNK activation in GPCR signaling pathway. Interestingly, although LPA-induced IκBα phosphorylation was defective in CARMA3-deficient cells (Fig. 3A), we found that LPA stimulation effectively induced IKKβ phosphorylation (Fig. 3D), suggesting that a CARMA3-independent signal can induce IKKβ phosphorylation following LPA stimulation.

CARMA3-regulated IKK complex activation is independent on IKKα/β phosphorylation

To investigate the molecular mechanism that results in the defect of IκBα phosphorylation in CARMA3-deficient cells, we examined IKK activation through an in vitro kinase assay following the stimulation of wild-type and CARMA3-deficient cells with LPA, PMA plus Iono, or TNFα. We found that IKK activation was completely defective in response to the stimulation of LPA and PMA plus Iono in CARMA3-deficient cells, whereas TNFα-induced IKK activation was comparable in both cell types (Fig. 4A). Consistent with this result, IκBα phosphorylation induced by LPA and PMA plus Iono was defective in CARMA3-deficient cells, but not in wild-type cells, whereas TNFα effectively induced IκBα phos-

phorylation in both wild-type and CARMA3-deficient cells (Fig. 4B, top panel). Since IKK-mediated phosphorylation of p65 at Ser 536 has been linked to NF-κB activation (Sakurai et al. 1999), we also examined the phosphorylation of p65 using antibodies against the phospho-Ser536 of p65. We found that the inducible phosphorylation on Ser536 was defective following the stimulation of LPA or PMA plus Iono in CARMA3-deficient cells (Fig. 4B, middle panel). Consistent with the above results, we surprisingly found that IKKβ phosphorylation, induced by the stimulation of LPA or PMA plus Iono, was not defective in CARMA3-deficient cells (Fig. 4B, bottom panel). Together, these results indicate that IKK activation is dependent on CARMA3 in the signaling pathways induced by GPCRs. However, although a CARMA3-independent signaling event can lead to IKKβ phosphorylation in GPCR signaling pathways, the inducible phosphorylation of IKKβ is insufficient to activate the IKK complex. This suggests that the full IKK complex activation is dependent on other CARMA3-associated signaling events.

Earlier studies have suggested that CARMA1 regulates TRAF6 and MALT1, leading to polyubiquitination of NEMO. In addition, it has been shown that CARMA3 physically associates with NEMO/IKKγ (Stilo et al. 2004). To investigate whether CARMA3 also regulates NEMO polyubiquitination, we overexpressed CARMA3 in HEK293T cells. Consistent with earlier studies (Stilo et al. 2004), we found that overexpressed CARMA3 associated with NEMO (Fig. 4C). Importantly, expression

Figure 4. CARMA3 regulates IKK kinase activity by inducing NEMO polyubiquitination but not IKK β phosphorylation. (A) *Carma3*^{+/+} and *Carma3*^{-/-} MEF cells were stimulated with or without LPA (10 μ M), PMA (40 ng/mL) plus Iono (100 ng/mL) (P/I), or TNF α (10 ng/mL) for indicated time points. IKK complex was immunoprecipitated using anti-IKK α /IKK β antibody-conjugated beads. The immunoprecipitated complex was subjected to an in vitro kinase assay using GST-IκB α as substrates. (B) Total cell lysates were subjected to SDS-PAGE and Western blot analysis using indicated antibodies. (C) HEK293 cells (5×10^5 /well) were transfected with the plasmid encoding Flag-tagged CARMA3. Twenty hours later, cell lysates were prepared from the transfected cells and subjected to immunoprecipitation using anti-TAK1- or anti-NEMO-conjugated beads. The immunoprecipitated complexes and cell lysates were subjected to Western blot analysis using indicated antibodies. (D) The lysates from C were also immunoprecipitated using anti-NEMO- or control IgG-conjugated beads. The obtained immunocomplexes were washed with lysis buffer (containing 1% NP-40 and 250 mM NaCl) four times and subjected to Western blot analysis. Parts of the immunocomplexes were treated with 6 M Urea or boiled in 1% SDS for 4 min. Supernatants were diluted 20-fold with lysis buffer and reimmunoprecipitated using IgG or NEMO-conjugated beads and subjected to Western blot analysis using anti-Ubiquitin or anti-NEMO antibodies.

of CARMA3 significantly induced NEMO-associated polyubiquitination. In contrast, expression of CARMA3 did not induce TAK1 (Fig. 4C) and Bcl10 (data not shown) polyubiquitination, suggesting that either NEMO or a NEMO-associated protein was ubiquitinated. To examine whether the observed ubiquitination in NEMO immunoprecipitates was due to NEMO or NEMO-associated proteins, we either treated the immunocomplex with 6 M Urea or boiled the complex in 1% SDS, and then reimmunoprecipitated NEMO. Although we could effectively reimmunoprecipitate NEMO (Fig. 4D, bottom panels), the NEMO-associated polyubiquitination was completely abolished following these treatments (Fig. 4D, top panels). Together, these results suggest that CARMA3 may induce polyubiquitination of one or more proteins that associate with NEMO.

To examine whether stimulation with LPA or PMA plus Iono could induce the NEMO-associated polyubiquitination and whether this polyubiquitination is dependent on CARMA3, we stimulated *Carma3*^{-/-} MEFs (C3KO-Vector) or *Carma3*^{-/-} MEFs reconstituted with HA-tagged CARMA3 (C3KO-CARMA3) (Supplementary Fig. 2) with or without PMA plus Iono and then immu-

noprecipitated NEMO from these cells (Supplementary Fig. 3). However, we were unable to detect signal-induced polyubiquitination in the immunoprecipitated NEMO complex, either in the presence or absence of CARMA3 (Supplementary Fig. 3). These results suggest that the signal-induced, NEMO-associated polyubiquitination was either very transient or only a small amount of proteins were ubiquitinated.

TRAF6 is required for LPA-induced NF- κ B activation

It has been shown that TRAF6 and MALT1 may function as E3 ligases to induce polyubiquitination of NEMO (Sun et al. 2004; Zhou et al. 2004). In addition, TRAF6 deficiency also displays a similar defect of the neural tube closure as CARMA3 deficiency does (Lomaga et al. 2000), suggesting that TRAF6 and CARMA3 may function in the same signaling pathway leading to NF- κ B activation. To determine whether TRAF6 is involved in LPA-induced NF- κ B activation, we stimulated TRAF6-deficient MEF cells with LPA, PMA plus Iono, LPS, or TNF α . Consistent with the role of TRAF6 in TLR but not TNF α signaling pathway, LPS-induced NF- κ B was

defective in TRAF6-deficient cells, whereas TNF α effectively induced NF- κ B activation (Fig. 5A). Although LPA or PMA plus Iono effectively activated NF- κ B in wild-type cells, this activation of NF- κ B was completely defective in TRAF6-deficient cells (Fig. 5A), indicating that TRAF6 is required for GPCR-induced NF- κ B activation. Similar to the role of CARMA3 in IKK activation, LPA or PMA plus Iono could induce IKK β phosphorylation in both wild-type and TRAF6-deficient cells (Fig. 5B). Together, these results indicate that similar to CARMA3, TRAF6 mediates LPA-induced NF- κ B activation through an IKK β phosphorylation-independent mechanism.

Bcl10 is not required for LPA-induced IKK α / β phosphorylation

Earlier studies indicate that CARMA3 is physically associated with Bcl10 upon overexpression, and recent studies by us and others also indicate that Bcl10 is required for GPCR-induced NF- κ B activation (Klemm et al. 2007; McAllister-Lucas et al. 2007; Wang et al. 2007). To determine whether BCL10 is required for GPCR-induced IKK α / β phosphorylation, *Bcl10*^{+/-} or *Bcl10*^{-/-} MEF cells were stimulated with or without LPA or PMA plus Iono. We found that although these stimulations failed

to induce I κ B α phosphorylation in *Bcl10*^{-/-} cells, they effectively induced IKK α / β phosphorylation in both *Bcl10*^{+/-} and *Bcl10*^{-/-} cells (Fig. 5C). Thus, Bcl10 is also required for GPCR-induced NF- κ B activation but is not required for the signal-induced IKK α / β phosphorylation.

CARMA3 functions as a common component in signaling pathways induced by other GPCRs, leading to activation of NF- κ B

To determine whether CARMA3 is also involved in signaling pathways induced by other GPCRs, wild-type or CARMA3-deficient MEF cells were stimulated with or without ET-1. ET-1 is another GPCR ligand that can induce NF- κ B activation (Purcell et al. 2001), and is a potent vasoconstrictor playing a critical role in cardiac hypertrophy (Kedzierski and Yanagisawa 2001). Although ET-1, as well as LPA or PMA plus Iono, effectively activated NF- κ B in *Carma3*^{+/-} and *Carma3*^{-/-} MEF cells, it failed to activate NF- κ B in *Carma3*^{-/-} MEF cells (Fig. 6A). ET-1-induced NF- κ B activation was observed within 30 min and maintained up to 90 min (Fig. 6B), and pretreatment of the cells with cycloheximide did not inhibit ET-1-induced NF- κ B activation (Supplementary Fig. 4), suggesting that the NF- κ B activation in-

Figure 5. TRAF6 and BCL10 play critical roles in LPA-induced NF- κ B activation. (A) Wild-type or TRAF6-deficient MEF cells were stimulated with or without LPA (10 μ M) or PMA (10 ng/mL) plus Iono (100 ng/mL) for 60 min, or LPS (10 ng/mL) and TNF α (1 ng/mL) for 30 min. Nuclear extracts were prepared from these cells and subjected to EMSA using ³²P-labeled NF- κ B or Oct-1 probe. (B) Wild-type or TRAF6-deficient MEF cells were stimulated with or without LPA (10 μ M) or PMA (10 ng/mL) plus Iono (100 ng/mL) for 10 or 20 min. Cell lysates were prepared from these cells and then subjected to Western blot analysis using the indicated antibodies. (C) *Bcl10*^{+/-} or *Bcl10*^{-/-} MEF cells were stimulated with or without LPA (10 μ M) or PMA (10 ng/mL) plus Iono (100 ng/mL) for 10 or 20 min. Cell lysates were prepared from these cells and then subjected to SDS-PAGE and Western blot analysis using the indicated antibodies.

Figure 6. CARMA3 mediates ET-1-induced NF- κ B activation. (A) *Carma3*^{+/+}, *Carma3*^{+/-}, and *Carma3*^{-/-} MEF cells (1×10^6) were stimulated with or without ET-1 (10 nM) and LPA (10 μ M) for 60 min, or PMA (10 ng/mL) plus Iono (100 ng/mL) or TNF α (10 ng/mL) for 30 min. Nuclear extracts were prepared and then subjected to EMSA using the indicated probes. (B) *Carma3*^{+/+}, *Carma3*^{+/-}, and *Carma3*^{-/-} MEF cells were stimulated with or without ET-1 (10 nM) for 15, 30, 60, and 90 min. Nuclear extracts were prepared and subjected to EMSA using the indicated probes. (C) *Carma3*^{+/+} and *Carma3*^{-/-} MEF cells were stimulated with or without ET-1 (10 nM) or TNF α (10 ng/mL) for indicated time points. Cell lysates were then subjected to SDS-PAGE and Western blot analysis using indicated antibodies. (D) Wild-type or CARMA3-deficient MEF cells were stimulated with ET-1 (10 nM) for various time points. Cell lysates were then subjected to SDS-PAGE and Western blot analysis using indicated antibodies.

duced by ET-1 was not due to the secondary stimulation from ET-1-induced cytokines. Similar to LPA stimulation, ET-1-induced phosphorylation of I κ B α was completely defective in CARMA3-deficient cells (Fig. 6C) whereas ET-1-induced IKK phosphorylation was comparable in both wild-type and CARMA3-deficient MEF cells (Supplementary Fig. 5). Together, these results demonstrate that CARMA3 is required for NF- κ B activation induced by different GPCRs. Also similar to LPA-induced MAP kinase activation, ET-1-induced MAP kinase activation was comparable in wild-type and CARMA3-deficient cells (Fig. 6D). Therefore, these results demonstrate that GPCR-induced MAP kinase activation is not dependent on CARMA3.

Since 50% of *Carma3*^{-/-} mice do not display the NTD phenotype, we also prepared MEF cells from embryos with and without the defect. We found that ET-1-induced NF- κ B activation was defective in CARMA3-deficient cells from embryos either with or without the NTD phenotype (Supplementary Fig. 6). This result suggests that either some unknown signaling events can compensate for the defect of CARMA3 during early development of the neural tube, or GPCR-induced NF- κ B is not required for neural tube development.

It has been shown that many GPCRs, such as the receptors for LPA and ET-1, induce NF- κ B activation through G α_q (Ye 2001). Thus, we expressed a constitutively active mutant of G α_q , G α_q (Q209L), which synergistically enhanced CARMA3-induced NF- κ B activation in HEK293 cells (Fig. 7A), supporting the hypothesis that G α_q is functionally linked to CARMA3. To further demonstrate that G α_q -induced NF- κ B activation is depen-

dent on CARMA3, we infected wild-type or CARMA3-deficient MEF cells with lentiviral vector encoded G α_q (Q209L). Although expression of G α_q (Q209L) could induce NF- κ B activation in wild-type cells, it failed to activate NF- κ B in CARMA3-deficient cells (Fig. 7B, top panel). In contrast, expression of G α_q (Q209L) induced comparable levels of AP-1 and MAP kinase activation (Fig. 7B [middle panel], C) in wild-type and CARMA3-deficient cells. Together, these results demonstrate that CARMA3 is selectively involved in G α_q -mediated NF- κ B activation.

Discussion

GPCRs play important roles in cell proliferation, differentiation, migration, and survival (Marinissen and Gutkind 2001). By generating CARMA3 knockout mice and analyzing CARMA3-null cells, we showed that NF- κ B activation induced by LPA and ET-1, two different GPCR ligands, is dependent on CARMA3 (Figs. 2, 6). However, CARMA3 is not required for NF- κ B activation induced by other stimuli, such as TNF α , extracellular matrix proteins, or LPS (Fig. 2; data not shown). Earlier studies have indicated that signal-induced IKK α / β phosphorylation and polyubiquitination of NEMO/IKK γ are required for activation of the IKK complex. Therefore, the current model for IKK activation is that phosphorylation and polyubiquitination of IKK complex proteins are mutually linked signaling events (Chen 2005). However, our studies surprisingly found that CARMA3 deficiency impairs IKK activation without affecting the signal-induced IKK β phosphorylation following the stimu-

Figure 7. $G\alpha_q$ -induced NF- κ B activation is dependent on CARMA3. (A) HEK293 cells were transfected with or without expression vectors encoding CARMA3 or $G\alpha_q(Q209L)$ together with NF- κ B-dependent luciferase (firefly) reporter and EF1 α -dependent luciferase (*Renilla*) vectors. The transfected cells were harvested 20 h post-transfection and lysed in the lysis buffer. Luciferase activities were determined using the dual-luciferase reporter assay kit. NF- κ B-dependent activity was normalized by the constitutively active EF1 α -dependent luciferase activity. Data are the mean of triplicates from a representative experiment. (B) A constitutively active mutant of $G\alpha_q$, $G\alpha_q(Q209L)$, was cloned into a lentiviral expression vector. The lentivirus encoding $G\alpha_q(Q209L)$ was used to infect *Carma3*^{+/+} or *Carma3*^{-/-} MEF cells. Nuclear and cytoplasmic extracts were prepared 72 h following the infection. Nuclear extracts were subjected to EMSA using indicated ³²P-labeled probes. (C) Cytoplasmic extracts were subjected to SDS-PAGE and Western blot analysis using indicated antibodies.

lation of LPA or PMA plus Iono (Fig. 4), indicating that the phosphorylation and polyubiquitination of IKK complex proteins are controlled by different signaling events. Therefore, we propose that full activation of the IKK complex is dependent on signal-induced IKK phosphorylation and CARMA3-dependent polyubiquitination (Fig. 8). Similarly, we also found that signal-induced IKK α / β phosphorylation is not defective in CARMA1-deficient lymphocytes (Shambharkar et al. 2007).

Although several studies show that NEMO is ubiquitinated in response to TNF α , TCR, and Ang-II (Tang et al. 2003; Zhou et al. 2004; McAllister-Lucas et al. 2007), we were unable to detect any meaningful ubiquitination associated with the immunoprecipitated NEMO following either PMA/Iono stimulation (Supplementary Fig. 3) or LPA stimulation (data not shown) in MEF cells. Of note, the signal-induced NEMO ubiquitination is very low in the earlier observations (Tang et al. 2003; Zhou et al. 2004; McAllister-Lucas et al. 2007). Therefore, it is possible that only a very small amount of NEMO or NEMO-associated protein(s) is transiently ubiquitinated following different stimulations. In a parallel study, we also examined whether TCR stimulation could induce NEMO polyubiquitination. We were unable to detect NEMO polyubiquitination following TCR stimulation in Jurkat T cells, but could only detect NEMO ubiquitination in Jurkat T cells ectopically expressing higher levels of CARMA1 and with more potent NF- κ B inducer, PMA plus Iono (Shambharkar et al. 2007).

Although it has been proposed that the IKK complex is ubiquitinated, there is not definite evidence showing NEMO is ubiquitinated. In this study we also observed that expression of CARMA3 induces polyubiquitination of the NEMO-containing complex, but we find that this ubiquitination is abolished following the treatment of the NEMO-containing complex with 6 M Urea or 1% SDS boiling. These results argue that the observed NEMO polyubiquitination is due to the polyubiquitination of an unknown protein(s) that is associated with NEMO. Therefore, it will be important to identify the ubiquitinated component(s) that associates with the IKK complex in the future studies.

It has been shown that TAK1, a MAP3K family member, is involved in NF- κ B activation induced by TNF α and TLR (Sato et al. 2005; Shim et al. 2005), and TAK1 is proposed to phosphorylate IKK β (C. Wang et al. 2001). Consistent with these studies, we found that TAK1 is required for antigen receptor-induced IKK α / β phosphorylation (Shambharkar et al. 2007). However, LPA-induced IKK α / β phosphorylation is not defective in TAK1-deficient MEF cells (data not shown). Therefore, it remains to be determined which kinase is responsible for GPCR-induced IKK α / β phosphorylation.

In the TCR signaling pathway, PKC θ is activated following TCR stimulation, which induces phosphorylation of CARMA1 (Matsumoto et al. 2005; Sommer et al. 2005). The phosphorylated CARMA1 further recruits Bcl10 (Gaide et al. 2002; Hara et al. 2004; Wang et al. 2004), MALT1, and TRAF6 (Sun et al. 2004; Zhou et al. 2004). MALT1 and TRAF6 may then function as E3 ligases to further induce K63-linked polyubiquitination of NEMO, leading to activation of the IKK complex and subsequently NF- κ B (Sun et al. 2004; Zhou et al. 2004). CARMA3 contains the same structural domains as CARMA1, and associates with Bcl10 when overexpressed (McAllister-Lucas et al. 2001; L. Wang et al. 2001). We found that Bcl10, MALT1, and TRAF6 constitutively formed a complex in nonhematopoietic cells (data not shown). Therefore, we hypothesize that the Bcl10-MALT1-TRAF6 complex may function as a common signaling complex downstream from CARMA3 in GPCR signaling pathway (Fig. 8). Consistent with this hypothesis, we demonstrated that TRAF6 (Fig. 5) and Bcl10 (Wang et al. 2007) are required for GPCR-induced NF- κ B activation. Together, our results indicate that GPCR-induced NF- κ B activation involves a signaling cascade containing CARMA3, Bcl10, and TRAF6 in nonhematopoietic cells (Fig. 8). Since CARMA3 is only expressed in nonhematopoietic cells and its homologous protein, CARMA1, is expressed in hematopoietic cells, it will be interesting to investigate whether CARMA1 is required for GPCR-induced NF- κ B activation in hematopoietic cells.

It has been shown that the deficiency of >80 genes results in NTDs (Copp et al. 2003). Interestingly, mice

Working Model

Figure 8. The working model for CARMA3-mediated NF- κ B activation in GPCR signal transduction pathway. GPCR-induced NF- κ B activation involves a signaling cascade of PKC–CARMA3–BCL10–MALT1–TRAF6 leading to polyubiquitination of the IKK complex, whereas a CARMA3-independent and PKC-dependent signal induces IKK β phosphorylation by an unknown kinase in the GPCR signaling pathway. In the absence of CARMA3, GPCR-induced polyubiquitination of the IKK complex is defective, thereby resulting in a defective IKK complex.

deficient in IKK α /IKK β , Bcl10, or TRAF6 display a similar NTD phenotype as CARMA3-deficient mice (Copp et al. 2003), suggesting that the NTD observed in CARMA3-deficient mice is likely associated with impaired NF- κ B activation. Of note, mice deficient in CARMA3, Bcl10, and TRAF6 have a similar penetrance of the NTD phenotype, suggesting that these proteins may mediate the same signaling pathway by an unknown inducer, leading to activation of the IKK complex and NF- κ B in a subset of neural crest cells during neural tube development. Such induction of NF- κ B may be required for the survival of neural crest cells. Therefore, further studies will be needed to determine how CARMA3, Bcl10, and TRAF6 are involved in neural tube development and why only a certain percentage of mice deficient in these genes display the defect during their neural tube development.

Earlier studies suggest that both G α_q and G $\alpha_{12/13}$ mediate GPCR-induced NF- κ B activation. G α_q mediates NF- κ B activation induced by LPA and ET-1 (Ye 2001). Consistent with these observations, we can detect effective induction of NF- κ B upon overexpression of the constitutively active mutant of G α_q , and we found that this induction of NF- κ B is dependent on CARMA3 (Fig. 7). In contrast, overexpression of the similar mutant of G $\alpha_{12/13}$ induced weak NF- κ B activation (data not shown). Therefore, it is difficult to draw a definite conclusion if G $\alpha_{12/13}$ -mediated NF- κ B activation is dependent on CARMA3. Nevertheless, our results indicate that NF- κ B activation induced by those GPCRs that utilize G α_q is dependent on CARMA3.

In summary, our studies revealed a novel signaling cascade induced by GPCRs, in which GPCRs induce NF- κ B through CARMA3, which in turn regulates downstream signaling components such as Bcl10, TRAF6, and NEMO/IKK γ , leading to NF- κ B activation. Therefore, our results not only provide the genetic evidence that CARMA3 is required for GPCR-induced NF- κ B activation, but also provide a key link between the GPCR signaling pathway and the IKK complex. However, it will be important to determine how CARMA3 links to upstream signaling cascades in GPCR-induced signaling pathways and which kinase is responsible for IKK α / β phosphorylation in future studies.

Materials and methods

Antibodies, plasmids, and reagents

Phospho-ERK1/2 (9101S), phospho-IkBa (9246L), phospho-JNK1/2 (9251L), JNK1/2 (9252), phospho-p65 (3036L), and G α_q antibodies were from Cell Signaling. β -Tubulin (D-10), ERK2 (C-14), IkBa (C21), Actin (C-2), IKK α / β (H470), IKK α (H-744), IKK γ (FL-419), Ubiquitin (P4D1), and Bcl10 (H-197) antibodies were from Santa Cruz Biotechnology, Inc. The peptide VRGRILQEQRARLVWVEC, matching to the C terminus of human and mouse CARMA3, was used to immunize rabbits, and the corresponding antibodies were purified using affinity column conjugated with the same peptide. The expression plasmid encoding G α_q (Q209L) was provided by Dr. Richard Ye (University of Illinois at Chicago, Chicago, IL). Electrophoretic mobility shift assay (EMSA) probes were from Promega. ET-1, PMA, and Iono were from Sigma. LPA was from Avanti. Tumor necrosis factor- α was from Endogen.

Targeting vector and gene knockout

Carma3 genomic fragments were amplified from genomic DNA of mouse ES cells, and subcloned into the TK-containing pL2-Neo targeting vector (kindly provided by Dr. Hua Gu, Columbia University, New York) with the PGK-neomycin resistance cassette replacing Exon 3 of the *Carma3* gene. The vector was electroporated into Sv129 ES cells and expanded in culture, and cells were selected under neomycin and gancyclovir. Genomic DNA was isolated from positive cells and screened using Southern blot. Two positive clones were used for injection into C57/Bl6 blastocysts, which were subsequently implanted into pseudo-pregnant C57/Bl6 females. Chimeric mice were intercrossed to generate heterozygous founder mice, which were further intercrossed to generate the *Carma3*^{-/-} animals. *Carma3*^{-/-} mice from both clones exhibited the same neural tube closure defect phenotype. Genomic DNA was isolated from mouse tails through proteinase K digestion and ethanol precipitation, followed by PCR using the following primers: 5'-CATTTCGCTGGGAAACGC-3' (forward, Intron2), 5'-GGGTAGTAGAA TTCCAGGG-3' (reverse 1, Exon 3), and 5'-TGCCTGCTTGC CGAATATC-3' (reverse 2, Neo^r). Total RNA was isolated from MEFs using the RNeasy Protect Mini Kit (Qiagen), according to the manufacturer's protocol. cDNA was prepared from the mRNA using the SuperScript III kit (Invitrogen), according to the manufacturer's protocol. RT-PCR was conducted using the following primers: 5'-TGCTCAGCACCTACCGTTTC-3' (forward, Exon 2) and 5'-CCGAATCTTCTCCTCGCTG-3' (reverse, Exon 6).

Mouse embryos and sections

To isolate embryos, pregnant females were sacrificed at different stages of pregnancy, and embryos were dissected out, separated from surrounding tissues, and photographed. For sections, embryos were fixed in 10% formalin, paraffin embedded, and sectioned at 4–6 μ m diameters. Hematoxylin and eosin (H&E) stains were conducted according to standard protocols.

MEF preparation

MEF cells were prepared by removing day 12.5–13.5 embryos from mothers, separating them from the uterine wall and amniotic sac, and placing them in 0.25% trypsin. The entire embryo was then chopped using a razor blade, digested for 10 min at 37°C, and triturated through a Pasteur pipette. Cells were then split into two 10-cm dishes and grown to confluence in DMEM, and adherent cells were either frozen back as passage 0 or split again for use. TRAF6 KO MEF cells were kindly provided by Dr. Tak Mak (University of Toronto, Canada).

Generation of CARMA3-reconstituted MEF cells

Carma3^{-/-} MEF cells were transfected with a plasmid encoding E1A to immortalize as described previously (Flores et al. 2002). The immortalized *Carma3*^{-/-} MEF cells (C3KO) were further transfected with either HA-tagged mouse CARMA3 in the pCDNA3.1-Hygro vector or empty vector using the calcium phosphate precipitation technique. After 2 d, the cells were selected under hygromycin for 2 wk, and stable clones expressing CARMA3 were selected by Western blot analysis.

Western blot and immunoprecipitation

For detection of CARMA3, livers from adult mice were frozen in liquid nitrogen, followed by grinding with a mortar and pestle

in RIPA buffer (150 mM NaCl, 10 mM Tris at pH 7.2, 0.1% SDS, 1% NP-40, 1% Deoxycholate, 5 mM EDTA, protease inhibitors). One-hundred micrograms of total protein were separated by SDS-PAGE and probed using anti-CARMA3 antibodies. For MAPK and IKK blots, 1×10^6 MEFs were serum starved for 18 h, stimulated, and lysed in 100 μ L of lysis buffer (150 mM NaCl, 50 mM HEPES at pH 7.4, 1 mM EDTA, 1% NP-40, protease inhibitors). Of the resulting lysates, 12–15 μ L were subjected to SDS-PAGE and probed for the specific antibodies.

Electrophoretic mobility shift assay

MEF cells (1×10^6) were starved for 18 h and stimulated for 30–60 min, and nuclear extracts were prepared. Nuclear extracts (5–10 μ g) were then incubated with 1×10^5 cpm of ³²P-labeled probes in 10 mM HEPES (pH 7.9), 40 mM NaCl, 1 mM EDTA, 4% glycerol, 3 μ g Poly-dIc, and 0.5 mM DTT for 15 min at room temperature. The samples were then run on a non-denaturing polyacrylamide gel and exposed to film at -80°C.

MIP-2 ELISA

Wild-type or CARMA3-deficient MEF cells were first starved in the medium with 0.5% fetal calf serum for 18 h, and then stimulated with or without LPA (30 μ M) or PMA (40 ng/mL) plus Iono (100 ng/mL) for another 20 h. The media from these cultures were collected and subjected to MIP-2 ELISA analysis according to the manufacturer's instructions (Quantikine kit from R&D Systems, Inc).

Integrin-induced NF- κ B activation

Recombinant mouse OPN was from R&D Systems, Inc., H-Arg-Gly-Asp-Ser-OH (RGDS) was from Bachem, and poly-L-Lys (PLL) was from Sigma-Aldrich. PLL, OPN, or RGDS was resuspended in PBS and coated onto six-well dishes overnight at 4°C. The dishes were washed with PBS, and 2×10^6 serum-starved MEF cells were plated onto the dishes for 2 h. For TNF α stimulation, 10 ng/mL TNF α were added to PLL-plated cells 30 min before harvest. Following stimulation, the cells were collected and lysed, and nuclear extracts were prepared. Nuclear extracts were then subjected to EMSA for the detection of NF- κ B activation.

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