IkB kinase β–induced phosphorylation of CARMA1 contributes to CARMA1–Bcl10–MALT1 complex formation in B cells

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Protein kinase C (PKC) β has been reported (Shinohara, H., T. Yasuda, Y. Aiba, H. Sanjo, M. Hamadate, H. Watarai, H. Sakurai, and T. Kurosaki. 2005. J. Exp. Med. 202:1423–1431; Sommer, K., B. Guo, J.L. Pomerantz, A.D. Bandaranayake, M.E. Moreno–Garcia, Y.L. Overchchina, and D.J. Rawlings. 2005. Immunity. 23:561–574) to play a crucial role in B cell receptor (BCR)–mediated IkB kinase (IKK) activation through phosphorylation of caspase recruitment domain 11, Bimp3 (CARMA1). However, it remains unclear whether this PKCβ-mediated phosphorylation accounts fully for the activation status of CARMA1, because involvement of other kinases, such as phosphoinositide 3-kinase–dependent kinase 1, has also been suggested. We show that PKCβ mediates phosphorylation of CARMA1 on Ser668, which in turn is essential for BCR-mediated CARMA1–Bcl10–mucosal–associated lymphoid tissue 1 (MALT1) association and subsequent IKK activation. Our analyses also demonstrate that the downstream kinase IKKB contributes to facilitating formation of the complex CARMA1–Bcl10–MALT1 by mediating phosphorylation of CARMA1. Hence, our data suggest that PKCβ is crucial for initial activation of IKK. The activated IKKB does not merely function as an effector enzyme but also modifies the upstream signaling complex through a feedback mechanism, thereby optimizing the strength and duration of the nuclear factor κB signal.
Figure 1. Multiple Ser/Thr residues of CARMA1 are important for BCR-mediated IKK activation. (A) Schematic diagram of various CARMA1 mutants. Arrowheads represent the mutated amino acid indicated as T119A (left). (B) BCR-mediated IKK, ERK, and JNK activation in wild-type and CARMA1-deficient (CARMA1−/−) DT40 B cells. IKK kinase assay was measured by phosphorylation of GST-IkBα as a substrate and detected by anti–phos-pho-IkBα mAb (left).
In this paper, we show that PKCβ mediates phosphorylation of CARMA1 on Ser668, which in turn is essential for the CARMA1–Bcl10–MALT1 association and subsequent IKK activation. We also provide evidence that the activated IKKβ functions as a positive modifier for the upstream signaling complex, at least partly by mediating phosphorylation of CARMA1.

RESULTS

Importance of multiple Ser/Thr residues in BCR-mediated IKK activation

Given the previous evidence that BCR-mediated CARMA1 phosphorylation is decreased in PKCβ-deficient chicken DT40 B cells (6), we searched the chicken CARMA1 sequence with the protein database motif engine NetPhos 2.0 (available at http://www.cbs.dtu.dk/services/NetPhos/) to identify its putative phosphorylation sites mediated by PKCβ. Nine potential residues—chicken Thr119, Ser575, Ser578, Ser631, Ser660, Ser668, Ser715, Ser909, and Ser912—gave a significant score (Fig. 1, A and E). To test whether these potential phosphorylation modifications alter CARMA1 function, each site was mutated, and the resulting mutants harboring C-terminal Flag tag were expressed in CARMA1-deficient DT40 B cells. For each mutant, multiple clones were tested for functional analysis. Representative clones are presented in Fig. 1 C. Expression levels of S575A and S660A mutants were lower than other CARMA1 mutants; clones expressing high amounts of these mutant CARMA1 could not be obtained, despite our tremendous efforts. Expression of BCR, was comparable in DT40 B cell lines expressing these mutant CARMA1 proteins (unpublished data). Then, we examined the effects of these mutations on BCR-mediated IKK activation (Fig. 1 C). Based on the criteria of to what extent BCR-mediated IKK activation is restored, we classified these mutants into three types: (a) similar to wild-type CARMA1, mutants of S660A, S715A, and S912A completely restored IKK activation; (b) restoration was observed, but to a lesser extent compared with wild-type CARMA1 (in the case of S575A and S631A mutants); and (c) mutants of T119A, S578A, S668A, and S909A failed in restoration or barely restored IKK activation after BCR stimulation. Before BCR stimulation, the IKK activation status of the T119A and S909A mutants was reproducibly increased, whereas that of the S578A and S668A mutants was decreased.

Because CARMA1 is also required for BCR-mediated c-Jun N-terminal kinase (JNK) activation (Fig. 1 B) in the second and third classes of mutants, we further examined their effects on JNK activation. Overall, these mutants gave rise to defects in JNK activation similar to those in IKK activation, except that the S631A mutant manifested the same level of JNK activation as wild-type CARMA1 (Fig. 1 D). Collectively, these data indicate that Thr119, Ser578, Ser668, and Ser909 of CARMA1 play a critical role in IKK as well as JNK activation upon BCR engagement. Therefore, we focused our study on the regulation of CARMA1 function through Thr119, Ser578, Ser668, and Ser909.

Thr119, Ser578, and Ser668 on CARMA1 are phosphorylated upon BCR engagement

To examine whether Thr119, Ser578, Ser668, and Ser909 residues on CARMA1 are indeed phosphorylated in BCR signaling, we generated antibodies specific for the phosphorylated peptides centered on Thr119, Ser578, Ser668, and Ser909 (anti-pT119, -pS578, -pS668, and -pS909). The specificity of three antibodies is demonstrated in Fig. 2 A by the lack of detection of the T119A, S578A, and S668A mutants after BCR stimulation. These three sites were also phosphorylated in endogenous CARMA1 upon BCR stimulation (see Fig. 4 C). Thus, we conclude that Thr119, Ser578, and Ser668 undergo phosphorylation upon BCR engagement. However, in the case of Ser909, although anti-pS909 antibody recognized the phosphopeptide more efficiently than the nonphosphopeptide, we could not detect significant differences in the band intensity between wild-type CARMA1 and its S909A mutant (unpublished data). Hence, it is more likely that Ser909 contributes to CARMA1 function by its structural role rather than phosphorylation, although we cannot completely exclude the possibility that the level of Ser909 phosphorylation is low, thereby hindering our detection system.

Deletion of PKCβ almost completely abolished phosphorylation of Ser668. The phosphorylation status of Thr119 and Ser578 was decreased in the absence of PKCβ but still occurred (Fig. 2 B). Because Ser668 was phosphorylated in vitro by the addition of active PKCβ (Fig. 2 C), we conclude that PKCβ is able to phosphorylate Ser668 on CARMA1. In contrast to wild-type CARMA1, the S668A CARMA1 mutant failed to make an inducible association with Bcl10 and MALT1 (Fig. 2 D). Given our previous evidence that wild-type CARMA1 cannot make such inducible association with Bcl10–MALT1 in the absence of PKCβ (6), these above results demonstrate that PKCβ mediates phosphorylation of CARMA1 on Ser668, which in turn is essential for its association with Bcl10–MALT1 and subsequent IKK activation.

Thus, a question arises as to what is a responsible kinase for phosphorylating Thr119 and Ser578, presumably in cooperation with PKCβ. Because the previous report showed that PDK1 participates in IKK activation in the context of TCR

Phospho-ERK and -JNK were analyzed by Western blotting (middle and right). (C) For functional analysis of CARMA1 mutants, IKK kinase assay was performed as in B. Wild-type and mutated Flag-tagged CARMA1 cDNAs were transfected into CARMA1−/− DT40 B cells. Induced IKK activity was quantitated with Multi Gauge software (Fujifilm) and represented as fold activation compared with time zero of the wild type. (Top) Protein expression of wild-type and mutated CARMA1, detected by Western blotting with anti-Flag mAb (1 × 10⁶ cells per lane). (D) For JNK activation, whole-cell lysates (2 × 10⁶ cells per lane) were analyzed by Western blotting with anti–phospho-JNK antibody. (E) Sequence alignments of the important Ser/Thr residues between chicken, mouse, and human CARMA1. wt, wild type.
A FEED-FORWARD REGULATORY LOOP THROUGH IκB KINASE β | Shinohara et al.

This prompted us to consider the idea that IKKβ could participate in the phosphorylation of Thr119 and Ser578. To address this point, we generated IKKβ-deficient DT40 B cells. As expected, BCR-mediated IKK activation was abolished in the mutant cells (Fig. 4, A and B). The phosphorylation status of Ser578 was inhibited in IKKβ-deficient DT40 B cells, although its phosphorylation at 3 and 10 min took place to some extent (Fig. 4, C). To further determine the requirement of its kinase activity, kinase-inactive IKKβ (1) was introduced by using a knock-in method (one allele containing knockout and the second allele containing kinase-inactive IKKβ; Fig. 4, D). As shown in Fig. 4, E, these mutant cells failed to restore phosphorylation of Ser578. Because Ser578 was phosphorylated in vitro by the addition of recombinant, purified IKKβ (Fig. 4, F), we conclude that IKKβ is able to phosphorylate Ser578. Collectively, these data suggest that IKKβ contributes to mediating phosphorylation of CARMA1 on Ser578.

Phosphorylation of Ser578 is mediated by IKKβ

It has been recently reported that IKKβ is required for the initial association of Bcl10 and MALT1 with CARMA1 after T cell activation (13). This prompted us to consider the idea that IKKβ could participate in the phosphorylation of Thr119 and Ser578. To address this point, we generated IKKβ-deficient DT40 B cells, although its phosphorylation at 3 and 10 min took place to some extent (Fig. 4, C). To further determine the requirement of its kinase activity, kinase-inactive IKKβ (1) was introduced by using a knock-in method (one allele containing knockout and the second allele containing kinase-inactive IKKβ; Fig. 4, D). As shown in Fig. 4, E, these mutant cells failed to restore phosphorylation of Ser578. Because Ser578 was phosphorylated in vitro by the addition of recombinant, purified IKKβ (Fig. 4, F), we conclude that IKKβ is able to phosphorylate Ser578. Collectively, these data suggest that IKKβ contributes to mediating phosphorylation of CARMA1 on Ser578.

Figure 2. Thr119, Ser578, and Ser668 on CARMA1 are phosphorylated upon BCR stimulation. (A) Cytosolic extracts (from 2 × 10^7 cells per sample) were immunoprecipitated with anti-Flag mAb and analyzed by Western blotting. The phosphorylated CARMA1 was detected by each phosphospecific antibody (anti-pT119, -pS578, and -pS668). The arrowhead indicates the position of phosphorylated S578 of CARMA1. (B) Phosphorylation status of CARMA1 in wild-type or PKCβ-deficient (PKCβ^−/−) DT40 cells was determined by the same procedures as in A. (C) For in vitro PKCβ kinase assay, purified Flag-tagged CARMA1 protein was used as a substrate. Phosphorylated CARMA1 was analyzed by Western blotting with anti–phospho-S668 antibody. (D) For association of Bcl10 or MALT1 with CARMA1, wild-type and mutated Flag-tagged CARMA1 cDNAs were transfected with pBluescript vectors, as described in Materials and methods, into CARMA1^−/− DT40 B cells. Cell lysates (from 3 × 10^7 cells per sample) were immunoprecipitated by anti-Flag mAb and analyzed by Western blotting using anti-Bcl10 mAb or anti-MALT1 antibody. WCL, whole cell lysate; wt, wild type.
as well as in DT40 B cells harboring kinase-inactive IKK

Because phosphorylation of Thr119 and Ser668 could not be detected by recombinant, purified IKKβ in our experimental conditions, it is likely that IKKβ contributes to the initial phosphorylation of Thr119 and Ser668 in a kinase-independent manner.

Loss of IKKβ inhibited phosphorylation of Ser578 1 min after BCR stimulation, simply suggesting that IKKβ might be activated at 1 min after BCR stimulation, although the level is low. Thus, we examined the time kinetics of IKK activation in more detail. As shown in Fig. 5 A (bottom), IKKβ was indeed activated, albeit to a small extent, 1 min after BCR engagement. Because IKKβ activation is primarily dependent on PKCβ in BCR signaling, these observations imply the idea that the primed IKKβ, in turn, could promote phosphorylation of Ser578 on CARMA1, thereby contributing to maximal IKK activation by enhancing CARMA1–Bcl10–MALT1 association. In support of this idea, the S578A CARMA1 mutant exhibited the reduced level of its association with Bcl10 and MALT1 (Fig. 5 B). Moreover, in IKKβ-deficient DT40 B cells (Fig. 5 C) as well as in DT40 B cells harboring kinase-inactive IKKβ (Fig. 4 E), the initial CARMA1–Bcl10–MALT1 association was significantly suppressed. Like the S578A mutant, the T119A mutant also manifested the reduced association with Bcl10 and MALT1 (Fig. 5 B).

**DISCUSSION**

Previous studies have established the contention that IKKβ functions downstream of PKCβ, CARMA1, Bcl10, and MALT1 on the route of BCR–mediated IKK–NF–κB activation (1, 2). In this study, we present evidence that, in response to BCR activation, IKKβ is not only required for IκB phosphorylation but also modifies assembly of the upstream CARMA1–Bcl10–MALT1 complex. Hence, we propose a model whereby two kinases, PKCβ and IKKβ, sequentially regulate the CARMA1–Bcl10–MALT1 complex. First, PKCβ-mediated phosphorylation of CARMA1 at Ser668 induces the primary CARMA1–Bcl10–MALT1 complex formation and IKKβ activation. Subsequently, IKKβ modifies phosphorylation
Given that the IKK complex is recruited to the CARMA1–Bcl10–MALT1 complex after antigen receptor stimulation, two, although not necessarily mutually exclusive, explanations for the effects of IKK on CARMA1 phosphorylation are possible. First, upon recruitment of IKK to the CARMA1–Bcl10–MALT1 complex, IKKβ would be activated, thereby directly phosphorylating CARMA1. Alternatively, because other kinases such as PKCβ are also thought to be recruited to the CARMA1–Bcl10–MALT1 complex, the recruited IKKβ to the same complex might regulate these kinases.
the mutant DT40 B cells demonstrated normal BCR-mediated NF-κB activation. This difference might reflect a distinct requirement for upstream signaling events in PKC and PKC activation between B and T cells, respectively; BCR-mediated phospholipase C activation might be sufficient for subsequent PKC activation, whereas PKC activation might require PI3K-mediated PDK1 activity in T cells, probably in addition to phospholipase C activation. Supporting this possibility, PDK1 is reported to associate with PKC in CD3/CD28-stimulated T cells (12). Alternatively, as previously proposed (12), recruitment of CARMA1 and PKC into the plasma membrane, presumably raft fractions, requires PDK1 activity in the case of T cell activation. Then, activated PKC catalyzes phosphorylation of CARMA1. In contrast, both functions might be exerted by PKC in B cells.

Ser578, Ser578, Ser578, Ser660, and Ser668 in the CARMA1 linker region are predicted to be potential PKC phosphorylation sites. Among these sites, mutation of Ser578 or Ser668 led to an almost complete defect in BCR-mediated IKK activation, whereas mutation of Ser575 or Ser631 resulted in its partial defect. Although not being formally proven, because of the lack of antibodies toward phospho-Ser575 and phospho-Ser631, the extent of phosphorylation of the S575A or S631A mutant was decreased, as determined by anti–phospho-Ser/-Thr antibody (unpublished data), suggesting that these sites are probably phosphorylated in BCR signaling. In regard to the functional importance of Ser668 (corresponding to Ser657 in mouse and Ser645 in human), two previous papers with Jurkat T cells demonstrated that this site was partially, rather than completely, involved in TCR/CD28-mediated IKK activation (9, 10). The reason why BCR-mediated IKK activation had a more stringent requirement for phosphorylation thereby promoting phosphorylation of CARMA1. In this mechanism, the IKKβ kinase activity might not be necessarily required. In the case of phosphorylation of Ser578, we favor the former idea because of two lines of evidence: (a) the inhibition of phosphorylation on Ser578 in DT40 B cells harboring kinase-inactive IKKβ and (b) the capability of recombinant IKKβ to phosphorylate Ser578. However, the contribution of IKKβ to the initial phosphorylation of Thr119 and Ser668 in a kinase-independent manner suggests that the latter mechanism would also operate. Together, we would like to propose that IKKβ utilizes both mechanisms to promote phosphorylation of the upstream adaptor, CARMA1.

Ser578 does not match the classical IKK consensus motif (DSXXXS) found in all IκB proteins, β-catenin, and FOXO3a (14–16). But, the conservation of this motif seems to reflect the constraints for recognition of SCF-βTRCP E3 ligase and subsequent proteosomal degradation rather than an IKK phosphoacceptor site. The identification of other IKK substrates will allow a better understanding about the molecular parameters for kinase recognition and IKK consensus sequences. Although IKKβ is one of the kinases responsible for phosphorylation of Ser578, the residual phosphorylation of this site in IKKβ-deficient DT40 cells was clearly observed, presumably because of PKCβ, because reduction of phosphorylation status at Ser578 was also reproducibly observed in PKCβ-deficient cells.

PDK1-knockdown Jurkat T cells, generated with the use of short hairpin RNA for PDK1, manifested severe defects in CD3/CD28-dependent NF-κB activation, although these knockdown cells apparently had remaining PDK1 (12). Despite similar levels of remaining PDK1 between knockdown Jurkat cells and DT40 B cells conditionally deficient in PDK1, the mutant DT40 B cells demonstrated normal BCR-mediated NF-κB activation. This difference might reflect a distinct requirement for upstream signaling events in PKCβ and PKCα activation between B and T cells, respectively; BCR-mediated phospholipase Cγ activation might be sufficient for subsequent PKCβ activation, whereas PKCα activation might require PI3K-mediated PDK1 activity in T cells, probably in addition to phospholipase Cγ activation. Supporting this possibility, PDK1 is reported to associate with PKCα in CD3/CD28-stimulated T cells (12). Alternatively, as previously proposed (12), recruitment of CARMA1 and PKCα into the plasma membrane, presumably raft fractions, requires PDK1 activity in the case of T cell activation. Then, activated PKCα catalyzes phosphorylation of CARMA1. In contrast, both functions might be exerted by PKCβ in B cells.

Ser575, Ser578, Ser631, Ser660, and Ser668 in the CARMA1 linker region are predicted to be potential PKCβ phosphorylation sites. Among these sites, mutation of Ser578 or Ser668 led to an almost complete defect in BCR-mediated IKK activation, whereas mutation of Ser575 or Ser631 resulted in its partial defect. Although not being formally proven, because of the lack of antibodies toward phospho-Ser575 and phospho-Ser631, the extent of phosphorylation of the S575A or S631A mutant was decreased, as determined by anti–phospho-sero-Thr antibody (unpublished data), suggesting that these sites are probably phosphorylated in BCR signaling. In regard to the functional importance of Ser668 (corresponding to Ser657 in mouse and Ser645 in human), two previous papers with Jurkat T cells demonstrated that this site was partially, rather than completely, involved in TCR/CD28-mediated IKK activation (9, 10). The reason why BCR-mediated IKK activation had a more stringent requirement for phosphorylation
of Ser668 in DT40 B cells could be explained by the following three possibilities. First, this difference might simply reflect a species difference between chicken and mouse/human. Second, as discussed in the requirement for PDK1 between B and T cells, this difference might reflect a differential requirement for upstream kinases (PKCβ vs. PKCθ) in B and T cells, respectively. Ser668 could be used more dominantly as an in vivo phosphorylation site by PKCθ in B cells, rather than PKCθ in T cells. Finally, because we used the antigen receptor as a stimulant, in contrast to co-stimulation with antigen receptors and coreceptors (CD3/CD28) in the case of Jurkat T cells, phosphorylation of Ser668 might be used more stringently in the antigen receptor signaling context. As CD28 is known to enhance antigen receptor signaling, presumably through PI3K activation (17), such augmented PI3K might lower the threshold for the requirement of Ser668 in IKK activation.

The available evidence indicates that Bc110 is recruited to the CARD of CARMA1 and that MALT1 is recruited to Bc110 through the binding of the MALT1 immunoglobulin domains to the region of Bc110 located just C-terminal of the CARD (for review see reference 5). Indeed, deletion of the CARD of CARMA1 blocked its association with Bc110 and MALT1. More importantly, deletion or mutation of the CARMA1 CARD (for review see reference 5). Indeed, deletion of the CARD of CARMA1 abrogated CARMA1 function to activate NF-κB. The location of phosphorylation site by PKCδ and PI3K respectively. Ser668 could be used more dominantly as an in vivo phosphorylation site by PKCθ in B cells, rather than PKCθ in T cells.

Materials and methods

Cells, antibodies, and reagents. Wild-type and various mutant DT40 cells were cultured as described previously (6). 4-hydroxy tamoxifen (4OH-T) was purchased from Sigma-Aldrich. Anti-phospho–CARMA1 antibodies were obtained by immunizing rabbits with synthetic phosphorylated peptides (pT119, CTRRFST(PO3)IVV-COOH; pS578, IMS(PO3)TTPEPPCOOH; and pS668, CPFRPS(PO3)VTSV-COOH), Anti–chicken IgM mAb, M4 (6), was used for stimulation of BCR. The following antibodies were purchased: anti–extracellular signal-regulated kinase (ERK), anti–JNK, anti–Bc110 mAb (331.3), and anti–Akt (Santa Cruz Biotechnology, Inc.); anti–PKCβII, anti–CARMA1, anti–IKKα/β, anti–IKKβ, and anti–PDK1 (Abcam); anti–IKKα and anti–IKKβ mAbs (BD Biosciences); anti–phospho–ERK, anti–phospho–IKKα/β mAb (5A5), anti–phospho–JNK, anti–phospho–T308-Akt, and anti–phospho–S473-Akt (Cell Signaling Technology); and anti–FLAG mAb (M2, Sigma–Aldrich). Anti–CARMA1 antibody for immunoprecipitation was obtained as described previously (6).

Expression constructs and transfection. Flag–tagged chicken wild-type CARMA1 and its mutant complementary DNAs (cDNAs) were generated by PCR. Each cDNA was cloned into the pAPuro with IRES–GFP expression vector, as previously described (6), or into the knock–in vector with IRES–GFP and the puro–resistant gene (pBHG) for introduction into the endogenous β–actin locus. The knock–in construct places the transgenes under the control of the endogenous β–actin promoter and enables the acquisition of clones expressing the same and proper (approximately twofold higher than endogenous CARMA1) protein expression levels. These constructs were transfected into various mutant DT40 cells by electroporation.

Generation of IKKβ– and PDK1–deficient DT40 cells. Genomic clones of IKKβ and PDK1 were obtained by PCR using oligonucleotides designed by the National Center for Biotechnology Information chicken genome database sequences (available from GenBank/EMBL/DDJB) under accession nos. LOC426792 and LOC416588, respectively. as a primer and DT40 genomic DNA as a template. The targeting constructs for IKKβ were designed for neo and hIgD cassettes to replace exons 1–4 of the chicken IKKβ gene. The targeting vector, pPDK1–hisD, was constructed by replacing the genomic fragment containing exons 3–6 that correspond to the mouse PDK1 ATP–binding domain with hisD cassette. The first allele was disrupted by the pPDK1–hisD, and the second allele was replaced by the conditional construct, which was designed as shown in Fig. 3A. Like the second allele–targeting vector for PDK1, IKKβ knock–in vectors (wild type and its S176/181A [SSAA] mutant) were constructed to make a miniature gene in the endogenous IKKβ locus. The first allele of IKKβ was disrupted by the pIkkβ–hisD, and the second allele was replaced by the knock–in targeting constructs. BCR expression on DT40 B cells deficient in IKKβ and PDK1 was essentially the same as wild–type cells.

Immunoprecipitation and Western blot analysis. Western blot analysis and immunoprecipitation were performed as described previously (6).

Kinase assay. IKK kinase assay was performed as described previously (6). For in vitro kinase assay of PKCβ, PDK1, IKKα, and IKKβ, recombinant active kinases were purchased from Millipore. CARMA1 as a substrate was purified from PKCβ–deficient DT40 cells expressing wild–type Flag–tagged CARMA1 (6). After being lysed by 1% NP–40 lysis buffer without phosphatase

3292
inhibitor, Flag-tagged CARMA1 was immunoprecipitated by M2-beads (Sigma-Aldrich) and eluted by 100 μg/ml of Flag peptide (NDYKDDDD-
KDVKDDDDKDVKDDDDKN). In vitro kinase assay was performed according to the manufacturer’s instructions (Millipore).

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