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Serine 649 Phosphorylation within the Protein Kinase C-Regulated Domain Down-Regulates CARMA1 Activity in Lymphocytes

Miguel E. Moreno-García,* Karen Sommer,* Claudia Haftmann,* Clayton Sontheimer,* Sarah F. Andrews,* and David J. Rawlings1,2†

Phosphorylation of CARMA1 is a crucial event initiating the assembly of IκB kinase and JNK signaling complexes downstream of activated Ag receptors. We previously mapped three protein kinase C (PKC) target sites in murine CARMA1 in vitro, and demonstrated that mutation of two of these serines (S564 and S657) resulted in reduced NF-κB activation, whereas mutation of the third serine (S649) had no clear effect. In this study, we report that when low concentrations of Ag receptor activators are used, loss of S649 (by mutation to alanine) promotes enhanced IκB kinase and JNK activation in both B and T cell lines. Reconstitution of CARMA1−/− DT40 B cells with CARMA1 S649A leads to increased cell death and reduced cell growth in comparison to wild-type CARMA1, likely a result of enhanced JNK activation. To directly determine whether S649 is modified in vivo, we generated phospho-specific Abs recognizing phospho-S649, and phospho-S657 as a positive control. Although phospho-S657 peaked and declined rapidly after Ag receptor stimulation, phospho-S649 occurred later and was maintained for a significantly longer period poststimulation in both B and T cells. Interestingly, phospho-S657 was completely abolished in PKCβ-deficient B cells, whereas delayed phosphorylation at S649 was partially intact and depended, in part, upon novel PKC activity. Thus, distinct PKC-mediated CARMA1 phosphorylation events exert opposing effects on the activation status of CARMA1. We propose that early phosphorylation events at S657 and S654 activate the initial assembly of the CARMA1 signalosome, whereas later phosphorylation at S649 triggers CARMA1 down-regulation.

The transcription factor NF-κB and the MAPK JNK are both activated downstream of Ag receptors, and together regulate the activation, proliferation, apoptosis, survival, and differentiation of lymphocytes (1, 2). In Ag receptor signaling, both NF-κB and JNK share requirements for molecules involved in the proximal tyrosine phosphorylation cascade leading to phospholipase Cγ activation. They also both require protein kinase C (PKC)3-dependent activation of caspase recruitment domain (CARD) membrane-associated guanylate kinase (MAGUK) containing protein 1 (CARMA1), as well as the downstream adaptors Bcl-10 and MALT1 (3). Downstream of MALT1, many of the molecular players required for activation of the classical NF-κB pathway have been described, and are shared with other families of membrane-bound receptors. Activation of JNK is less fully understood, but is known to require phosphorylation mediated by MEK4, MEK7, and MEK6 in lymphocytes (1, 4). Once activated, JNK phosphorylates various downstream substrates, including c-Jun, to promote activation of the AP-1 transcription factor (5).

The adaptor protein CARMA1 is required for activation of both of these pathways. CARMA1-deficient cellular and animal models have demonstrated the importance of this protein in immunity (6–11). CARMA1-deficient lymphocytes exhibit reduced proliferation, differentiation, and survival to Ag receptor engagement or agonists (PMA/Ionomycin (P/I), as chemical activators of PKCs). At the molecular level, BCR or TCR stimulation of CARMA1-deficient lymphocytes induces normal phosphotyrosine signals, Ca2+ flux, and ERK phosphorylation; however, NF-κB and JNK activity remains inactive. Structurally, CARMA1 contains several domains important for the recruitment of signaling proteins as well as for its oligomerization and plasma membrane recruitment (12, 13). The N-terminal portion of CARMA1 includes both a CARD and a coiled-coil domain, which are required for the recruitment of the adaptor protein Bcl-10 (10, 12–15) and CARMA1 oligomerization (16), respectively. These domains are linked to a C-terminal region highly related to the MAGUK family of proteins (17). The MAGUK region contains a PDZ (postsynaptic density 95/disc large/zona occludens 1) domain, a Src homology 3 domain, and a guanylate kinase (GUK)-like domain. The CARD, coiled-coil, Src homology 3, and GUK domains are each required for CARMA1 activity (12, 18) and interact with distinct proteins to coordinate CARMA1 signaling (e.g., CARD with BCL-10, coiled-coil for homodimerization, and so on) (3).

We and others have demonstrated that CARMA1 activation is controlled by PKCβ in B cells and PKCθ in T cells (19, 20). Activated PKCβ or PKCθ phosphorylate a flexible region of CARMA1 linking the coiled-coil and PDZ domains termed the...
PKC-regulated domain (PRD) (3, 21). We demonstrated that specific serine residues within the murine PRD (S564, S649, and S657) were phosphorylated by recombinant PKCβ/δ isoenzymes in vitro, and mutation of these residues to alanine revealed that both S564 and S657 were required for Ag receptor-induced NF-κB activation in Jurkat T cells. In contrast, the S649 residue was apparently not required for the activation of a NF-κB reporter gene (20). Similar results were recently reported for the analogous serine residue (S660) of chicken CARMA1 (22).

These findings suggested two alternative possibilities with regard to the role for S649: that this residue is not a functionally relevant in vivo phosphorylation site in lymphocytes; or that S649 phosphorylation occurs in vivo, but has a separate role from that of activating CARMA1 signaling. In this report, we demonstrate that, although S657 phosphorylation delivers a positive signal for NF-κB and JNK activation, S649 phosphorylation negatively impacts these molecular events in lymphocytes. Functionally, S649 mutation results in a decrease in the survival and growth of reconstituted CARMA1−/− DT40 B cells, compared with wild-type (WT) CARMA1. Using Abs directed to phosphorylated S649 (rho-S649) and phosphorylated S657 (rho-S657), we show that CARMA1 is phosphorylated in vivo at both S657 and S649 in response to Ag receptor engagement, yet with markedly different kinetics. Thus, specific phosphorylation of serine residues in the PRD differentially regulates the downstream signaling activity of CARMA1.

Materials and Methods

Cells lines, reagents, and Abs

Chicken B cell lines with genetic deletions of PKCβ or CARMA1 (PKCβ−/− DT40 and CARMA1−/− DT40) were gifts from T. Kurosaki (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan) (23); human Ramos B cells, Jurkat T cells, and human embryonic kidney (HEK) 293 fibroblasts were obtained from American Type Culture Collection. Cells were cultured as described (20). Rottlerin, Ro31-8220, PMA, and ionomycin were from EMD Biosciences. Abs were used against the following: chicken IgM (Bethyl Labs); myc epitope (9E10; Sigma-Aldrich); human CD28, CD3 (OKT3), a gift from J. Pomerantz (Johns Hopkins University School of Medicine, Baltimore, MD) (12), pRenilla-TK (Promega; transfection control) reporter vector, and several doses of myc-CARMA1 or myc-CARMA1-S649A, myc-CARMA1-S620A, or myc-CARMA1-S620A/649A mutant cDNAs in the pIRE-PURO backbone (Clontech). At 6 h before harvesting, the cells were lysed in ice-cold lysis buffer, and an IKK in vitro kinase assay (Cell Signaling Technology) was conducted as described by the manufacturer.

IKK in vitro kinase assay

DT40 cells (2 × 10^6 cells/condition) were serum-starved for 1 h at 37°C and either left unstimulated or stimulated with P/I for 10 min at 37°C. Cells were lysed in ice-cold lysis buffer, and an IKK in vitro kinase assay (Cell Signaling Technology) was conducted as described by the manufacturer.

NF-κB reporter gene assays

HEK 293 cells (5 × 10^5/sample) or Jurkat T cells (1 × 10^5 cells/sample) were transfected for 24 h with Fugene 6 (Roche) at a DNA to Fugene ratio of 1/5 with 250 ng of NF-κB reporter plasmid IκB-κB-Luciferase, a gift from J. Pomerantz (Johns Hopkins University School of Medicine, Baltimore, MD) (12), pRenilla-TK (Promega; transfection control) reporter vector, and several doses of myc-CARMA1 or myc-CARMA1-S649A, myc-CARMA1-S620A, or myc-CARMA1-S620A/649A mutant cDNAs in the pIRE-PURO backbone (Clontech). At 6 h before harvesting, the cells were left unstimulated or stimulated with P/I (1 μM/ml) for HEK 293 cells or CD3/CD28 (5 μg/ml/1 μg/ml) for Jurkat T cells. NF-κB activation was analyzed measuring the firefly and Renilla luciferase activity according to the Dual-Luciferase Reporter assay from Promega.

Results

IKK and JNK activation in DT40 cells expressing CARMA1 phosphorylation site mutants

Using CARMA1-deficient chicken DT40 cell lines reconstituted with mutant chicken CARMA1 coding sequences, Shimohara et al. (22) were able to validate various potential PKC target sites in CARMA1 downstream of Ag receptor stimulation. We performed an identical set of experiments using P/I stimulation to confirm that the murine CARMA1 PKC target sites that we have previously identified (S564, S649, and S657 (20)), have the same effect on CARMA1 function as the correlating chicken CARMA1 residues. CARMA1−/− DT40 cells were reconstituted with either CARMA1-WT or CARMA1 constructs with S564A, S649A, or S657A mutations. IKK and JNK activation was analyzed after treatment with a high dose of the Ag receptor chemical mimetics P/I (1 μM/1 μg/ml, respectively) (Fig. 1). JNK activity was assessed using phospho-JNK specific Abs, and IKK activity was assessed using an in vitro kinase assay.

Mutation of all three murine serine residues that we have reported to be PKC-specific substrates (CARMA1–3-mut) produced a CARMA1 molecule unable to reconstitute IKK or JNK activation, indicating that some of these residues are required to initiate CARMA1 signaling. To determine the impact of the individual residues, CARMA1−/− DT40 cells expressing CARMA1 single serine mutants were activated and analyzed as described. Consistent with previous reports (19, 20, 22), although S654A and S657A mutations substantially decreased both IKK (Fig. 1A, top) and IKK (Fig. 1B, top) activation, S649A mutation did not show any apparent defect under these activation conditions.

Phosphorylation of CARMA1 at S649 negatively regulates IKK activation

We consistently observed that, following stable transduction of CARMA1−/− DT40 cells using retroviral vectors, protein expression levels of myc-CARMA1-S649A were ~3- to 5-fold lower
than expression levels observed for WT myc-CARMA1, or myc-CARMA1 with other serine mutations (Fig. 1). Shinohara et al. (22) reported a similar phenomenon with the analogous serine residue in the chicken CARMA1 sequence S660. Notably, despite its reduced expression level, myc-CARMA1-S649A reconstituted IKK and JNK activation to levels similar to that induced by the WT CARMA1 protein. This observation strongly suggested that the S649A mutation might enhance the signaling activity of CARMA1. When the levels of phospho-IkBα and phospho-JNK in cells expressing myc-CARMA1-WT and myc-CARMA1-S649A were normalized to the CARMA1 expression levels (Fig. 1, A and B, bottom), we found that both phospho-IkBα and phospho-JNK levels were increased ~3- to 4-fold in cells expressing myc-CARMA1-S649A over those induced by myc-CARMA1-WT.

Next we conducted dose-response assays of these plasmids, using various amounts of myc-tagged CARMA1 WT or S649A expression plasmids transiently transfected, along with constant amounts of NF-κB-luciferase and TK-łuciferase reporter plasmids, into Jurkat T cells followed by CD3/CD28 stimulation. CARMA1-S649A-transfected cells consistently exhibited a nearly 30–50% increase in CD3/CD28-induced NF-κB activation at 24 h posttransfection (Fig. 2A). The activity of the myc-CARMA1 and myc-CARMA1-S649A proteins was also evaluated in transiently transfected and PI-stimulated 293T cells (Fig. 2B, bottom). Comparison of NF-κB reporter gene activation between various doses of myc-CARMA1 or myc-CARMA1-S649A expression vectors showed that myc-CARMA1-S649A-expressing cells consistently exhibited about 30% more NF-κB activity than cells transfected with myc-CARMA1. Anti-myc immunoblotting (Fig. 2B, top) showed equivalent levels of WT and mutant protein expression in transfected cells, indicating that differential protein expression cannot account for these observed differences in NF-κB activation. Of note, transient protein expression in Jurkat cells is difficult to measure due to their relatively low transfection efficiency. However, because we used identical plasmid preparations for reporter assays in both cell lines, our protein measurements in 293T cells are predicted to also reflect relative expression of these constructs in Jurkat T cells in Fig. 2A.

A recent study by Bidère et al. (24) showed that human CARMA1 activity was decreased by phosphorylation of S608, possibly by casein kinase 1α (CK1α). To test whether mutation of this phosphorylation target in murine CARMA1 (S620, the murine analog of human S608) could additively or synergistically enhance down-regulation of CARMA1 activity with S649A, we compared the ability of CARMA1 with single serine mutants vs double mutant myc-CARMA1-S(620, 649)A to enhance NF-κB reporter gene expression at 24 h posttransfection in Jurkat T cells (Fig. 2C). The single mutant myc-CARMA1-S620A promoted enhanced NF-κB activation to levels similar to that of myc-CARMA1-S649A. However, combining these individual serine mutations did not lead to an additive increase in NF-κB reporter gene expression. The double mutant myc-CARMA1-S(620,649)A led to NF-κB activity similar to that observed with the single serine mutants. These data suggest that CARMA1 activation status can be down-modulated by distinct phosphorylation events downstream of alternative signaling pathways (e.g., CK1α vs PKC) and imply that S620 and S649 phosphorylations are likely to target the same mechanism for CARMA1 down-regulation.

**FIGURE 1.** Mutation of SS64 and SS657, but not SS649, interferes with CARMA1-dependent IKK and JNK activation in DT40 B cells. A and B. WT or CARMA1−/− DT40 cells stably expressing myc-CARMA1-WT, myc-CARMA1 (SS64, SS649, SS657A) (3-mut), or the specified single serine-to-alanine mutants (2 X 106 cells/condition) were activated with P/I (1 μM and 1 μg/ml, respectively) for the indicated time (A), or for 0 (−) and 10 (+) min (B). JNK activation was assessed by immunoblotting using anti-phospho-JNK Abs (A), and IKK activity (measured as IkBα phosphorylation) was analyzed (B) as described in Materials and Methods. Phospho-ERK immunoblot was used as an activation control, and anti-myc (CARMA1) and ERK blots were used as loading controls. Phosphorylation was detected using the phosphospecific Abs in whole cell lysates (WCL). Graphs below each blot show the quantification of the phospho-IkBα and phospho-JNK levels normalized to expression of myc-CARMA1-WT or myc-CARMA1-S649A. Relative values at t = 0 were set as 1.

**DT40 B cells expressing myc-CARMA1-S649A exhibit reduced growth and survival**

Several alternative hypotheses could explain why the S649A mutation led to the observed reduction in CARMA1 expression levels in stably transfected cells. The S649A mutation may directly destabilize the CARMA1 protein; enhanced downstream signaling events may directly promote CARMA1 degradation; or cells that express higher levels of the S649A mutant may be counter-selected due to an alteration in cell growth parameters. As protein stability assays showed no difference in protein half-life between CARMA1 WT and S649A (see supplemental Fig. S1),4 we elected to track the selection of ectopic CARMA1 expression in CARMA1−/− DT40 B cells using an IRES-driven cis-linked GFP marker.

Cells were stably transduced using retroviral vectors expressing myc-CARMA1 or myc-CARMA1-S649A and FACS sorted to obtain
FIGURE 2. NF-κB activation is enhanced in Jurkat T cells and HEK 293 fibroblasts transiently expressing the CARMA1-S649A and CARMA1-S620A mutants in Ag receptor and P/I-stimulated cells, respectively. A. Jurkat cells were cotransfected for 24 h with the Igκ2-Luciferase/pRL-TK reporter vectors plus 250 and 62 ng of expression vectors for myc-CARMA1 (WT) and myc-CARMA1-S649A or 250 ng of empty vector DNA. Cells were harvested and left unstimulated (□) or stimulated (■) with plate-coated anti-CD3ε/CD28, and luciferase activity was analyzed as a measurement of NF-κB activation. B, HEK 293 fibroblasts were transfected with different concentrations of myc-CARMA1 (□) or myc-CARMA1-S649A (■) expression vectors, and Igκ2-Luciferase/pRL-TK reporter vectors. Cells were treated with or without P/I, and luciferase activity was analyzed to measure NF-κB activation (bottom). Protein levels of myc-CARMA1 and myc-CARMA1-S649A mutant were analyzed by immunoblotting using anti-myc (top). Anti-actin was used as a loading control. C. Jurkat T cells were cotransfected for 24 h with the Igκ2-Luciferase/pRL-TK reporter vectors plus 250 ng of expression vectors for myc-CARMA1, myc-CARMA1-S649A, myc-CARMA1-S620A, myc-CARMA1-S620A/S649A, or empty vector. Cells were harvested and left unstimulated (□) or stimulated (■) with plate-coated anti-CD3ε/CD28. Luciferase activity was analyzed as a measurement of NF-κB activation. Bar graphs show mean ± SEM of three to four experiments. Student’s t test comparing activated cells expressing myc-CARMA1 vs CARMA1-S649A, CARMA1-S620A, or CARMA1-S620A/S649A was applied. *, p ≤ 0.05 and **, p ≤ 0.01. The relative amount of luciferase to transfection control Renilla is expressed in each experiment.

Increased JNK signaling in B cell lines expressing the CARMA1-S649A mutant

JNK signals have been implicated in the induction of cell death in DT40 B cells, and also in the reduction of cell growth in many other cell types (25, 26). As it is well documented that CARMA1 activation induces both NF-κB and JNK activation in B and T cells (7, 8, 22), we hypothesized that the myc-CARMA1-S649A mutation enhanced JNK activation. To test this idea, we directly evaluated JNK phosphorylation in CARMA1−/− DT40 B cells (Fig. 4). In our initial studies, we did not detect differences in JNK activation in myc-CARMA1−/− vs myc-CARMA1-S649A-expressing cells using 1 μM of PMA (Fig. 1), likely a result of signal saturation. Thus for these studies, we stimulated CARMA1−/− DT40 cells stably expressing either myc-CARMA1, myc-CARMA1-S649A, or empty vector using a range of PMA doses and a fixed ionomycin dose (Fig. 4). As shown in Fig. 4A, whereas JNK phosphorylation was absent in vector-transduced CARMA1−/− DT40 cells, cells expressing myc-CARMA1 or the myc-CARMA1-S649A mutant exhibited reconstituted JNK phosphorylation. The myc-CARMA1-S649A-expressing cells, however, consistently exhibited higher phospho-JNK levels than WT expressing cells (Fig. 4A); increased activity was observed even at the lowest PMA doses tested. In contrast, both cell populations showed similar levels of ERK phosphorylation, implying that this altered activity was specific to JNK signaling.

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To determine whether these events also occurred following direct Ag receptor stimulation, CARMA1−/− DT40 B cells expressing myc-CARMA1-WT vs myc-CARMA1-S649A were activated with anti-chicken IgM (5 μg/ml) for specific time points, and phospho-JNK levels were assessed by immunoblotting (Fig. 4B). Under these conditions, cells expressing the myc-CARMA1-S649A mutant exhibited more rapid and slightly enhanced JNK activation (peaking at 5 min) compared with myc-CARMA1 (peaking at 15 min), although these differences were more subtle than P/I-driven responses. To determine whether these findings were relevant to mammalian B cells, the human B cell line (Ramos) was identically transduced and activated with low P/I doses (Fig. 4C). Consistent with the results using DT40 cells, JNK phosphorylation was significantly increased in Ramos cells expressing myc-CARMA1-S649A compared with that induced by myc-CARMA1-expressing cells; relative ERK phosphorylation remained indistinguishable between the cell lines. When JNK phosphorylation levels were quantified in relation to CARMA1 expression levels, JNK phosphorylation was consistently ~2- to 3-fold higher in myc-CARMA1-S649A than JNK phosphorylation levels in myc-CARMA1-expressing cells (Fig. 4, bottom panels).

Analysis of CARMA1 phosphorylation in vivo using phosphoserine-specific Abs

To understand whether S649 has a physiologically relevant regulatory role in the Ag receptor signaling cascade, we directly assessed whether it was indeed phosphorylated in response to Ag receptor or P/I stimulation in vivo. We generated polyclonal Abs against phospho-S649, as well as phospho-S657 as a positive control (analogous to chicken S668 (22)) to measure in vivo phosphorylation of these residues. To verify their specificity, the resulting Abs were first used to immunoblot bacterially expressed CARMA1 PRD protein fragments with and without in vitro phosphorylation by recombinant PKCB. As shown in Fig. 5A, both Abs reacted only with the phosphorylated WT-PRD, and not with unphosphorylated WT or with phosphorylated PRDs with serine-to-alanine mutations at S649 or S657, respectively.

To analyze the phosphorylation of S649 and S657 downstream of Ag receptor signals in vivo, lymphoid cell lines including B cell (CARMA1−/− DT40 and human Ramos B cells) and T cell (Jurkat) lines were retrovirally transduced to stably express myc-CARMA1 or myc-CARMA1 containing specific mutations of these serine residues. Cells were then stimulated with P/I (Fig. 5, B–D), or Ag receptor-specific Abs (Fig. 5, E and F). CARMA1 phosphorylation was detected by Western blotting using the phosphoserine Abs in either whole cell lysates in CARMA1−/− DT40 cells (Fig. 5, B and F) or in myc-immunoprecipitates (Fig. 5, C, E, and F, myc-IP). P/I stimulation induced phosphorylation of the two serine residues with distinct kinetics in both B cell lines. Although phospho-S649 was rapidly induced, showing a maximum peak at 2–5 min poststimulation and a steady reduction at later time points, phospho-S657 was delayed but more sustained, reaching a maximum at 15–30 min poststimulation. Similarly, we analyzed these phosphorylation events induced by Ag receptor signaling in B and T cell lines (Fig. 5, E and F). Anti-IgM in DT40 CARMA1−/− B cells or anti-CD3/CD28 in Jurkat T cells induced phospho-S657 and phospho-S649 with almost identical kinetics to that induced by P/I Abs. We also determined that endogenous CARMA1 was phosphorylated at residue S649 after P/I stimulation in primary B lymphocytes (Fig. 5G). These findings further support the physiological occurrence of this phosphorylation event.

Due to the substantial disparity between the kinetics of S649 and S657 phosphorylation, we investigated whether there was a hierarchical dependence of phosphorylation between these and other serine
residues. Phosphorylation of CARMA1 with single serine-to-alanine mutations of S649 or S657 was tested in P/I-stimulated cells. Constructs with S564A mutations were also included in the analysis, as S564 is also required for NF-κB activation in lymphocytes (20). As shown in Fig. 5D, phosphorylation of either S649 or S657 was each unaffected by the absence of the other serine residues, indicating that phosphorylation of these individual residues can occur independently from each other.

FIGURE 4. Phosphorylation of S649 negatively regulates CARMA1-dependent JNK activation. A. CARMA1−/− DT40 cells (2 × 10⁶ cells/condition) expressing myc-CARMA1 (WT), myc-CARMA1-S649A, or empty vector were left unstimulated or stimulated with decreasing low doses of PMA (250, 31.2, 7.8, and 3.9 nM) and 1 μg/ml ionomycin for 15 min. Phospho-JNK was analyzed by immunoblotting. B. CARMA1−/− DT40 cells expressing myc-CARMA1 or myc-CARMA1-S649A were left unstimulated or were stimulated with 5 μg/ml IgM for the indicated time points. CARMA1 activity was assessed by phospho-JNK immunoblotting from 2 × 10⁶ cells/condition in whole cell lysate (WCL). C. Ramos human B cells stably expressing myc-CARMA1, myc-CARMA1-S649A, or empty vector (1 × 10⁶ cells/condition) were unstimulated or stimulated with the indicated doses of PMA and 1 μg/ml ionomycin for 15 min at 37°C. Whole cell lysates were immunoblotted as described in A. A–C. Phospho-ERK, ERK, and myc (CARMA1) blots were used as activation and loading controls. Band density of JNK phosphorylation was quantified and expressed relative to those in CARMA1 vs CARMA1-S649A. * p ≤ 0.05.

FIGURE 5. CARMA1 residues S649 and S657 exhibit differential phosphorylation kinetics as detected by phospho-specific polyclonal Abs. A. GST-PRD, GST-PRD-S649A, and GST-PRD-S657A purified from recombinant bacteria were incubated with or without purified PKCα to detect phosphorylation of S649 and S657. B–G. Probes from whole cell lysates (WCL) or lysates immunoprecipitated using anti-myc Abs were transferred to polyvinylidene fluoride membranes, and phospho-S649, phospho-S657, and myc-CARMA1 were detected by immunoblotting.
Requirement for PKC activity in CARMA1 phosphorylation at S649 and S657

Previous studies have shown that CARMA1 phosphorylation, detected in cells lines using total anti-phosphoserine Abs or in vitro using radioactive phospho-labeling of bacterially expressed proteins, required the activity of specific PKC isoforms; PKCβ (found in B cells) and PKCδ (in T cells) (19, 20, 23). Nevertheless, whether phosphorylation of individual serine residues requires the exclusive activity of a particular PKC isoform or kinase in vivo has not been fully determined. It has been reported that chicken S668 (mouse S657) requires PKCζ activity in DT40 B cell line (22); however, whether S649 phosphorylation requires the same kinase is unknown. To assess whether PKCβ is essential for phosphorylation of both S649 and S657 in B cells, PKCβ−/− DT40 cells stably expressing myc-CARMA1 were activated and CARMA1 phosphorylation was analyzed and compared with that observed in myc-CARMA1-reconstituted CARMA1−/− DT40 cells (Fig. 6A). As anticipated, both phospho-S657 and phospho-S649 were markedly reduced in PKCβ−/− DT40 cells. However, although phospho-S657 was abolished in the PKCβ−/− cells, a low level of phospho-S649 was clearly present at later time points. This result suggested that other kinases or alternative PKC isoforms may be involved in CARMA1 phosphorylation of S649. To test this hypothesis, CARMA1−/− DT40 cells expressing myc-CARMA1 were preincubated with either a pan-PKC or a novel PKC inhibitor and subsequently stimulated with P/L (Fig. 6B). Although the pan-PKC inhibitor abrogated both phospho-S657 and phospho-S649, the novel PKC inhibitor (Rottlerin) exhibited no effect on phospho-S657 but partially decreased phospho-S649, indicating that although PKCβ is the major effector for CARMA1 phosphorylation and activation in B cells, other kinases (likely including novel PKC isoforms) also participate in S649 phosphorylation.

Discussion

The current study clearly demonstrates that S649 is an in vivo kinase target downstream of Ag receptor engagement, and that loss of this serine has a positive effect on CARMA1 activity, as measured by both IKK activation and JNK phosphorylation. Thus, in the WT protein, phosphorylation at S649 likely results in CARMA1 down-regulation. These events were conserved in both chicken and human B cell lines, as well as in a human T cell line. Aided by the generation of polyclonal phospho-specific Abs, we found that S649 was phosphorylated in lymphocytes with a distinct kinetic pattern upon cell stimulation. Although the CARMA1-activating phosphorylation at S657 occurs rapidly and is transitory (Fig. 5 and Ref. 22), the inhibitory phosphorylation at S649 is both delayed and more sustained. Interestingly, our data also show that phosphorylation of CARMA1 does not follow a hierarchical process. Phosphorylation of S649 occurred independently of phosphorylation at other residues including S564 and S657 (Fig. 5). As PKCβ and PKCθ are implicated in both rapid, activating phosphorylation and later-occurring deactivating phosphorylation (Fig. 5), the mechanism governing the selective phosphorylation kinetics remains to be determined.

It is well established that PKC isoforms may have both positive and negative regulatory effects on Ag receptor signaling in lymphocytes (27, 28). Additionally, it is well established that signaling proteins can be targeted by multiple kinases that may either positively or negatively affect the target protein activity. For example, Src protein tyrosine kinases and the Tec family kinase Btk are activated by specific tyrosine kinases, and deactivated by other serine/threonine kinases (29, 30). In this study, we demonstrate that CARMA1 activation is both positively and negatively regulated by at least three independent, PKC-mediated phosphorylation events in the PRD induced by Ag receptor cross-linking (and by signals that mimic Ag receptor engagement) in B and T cell lines. In B cells, PKCβ is required for S657 phosphorylation and is a major kinase targeting S649 (Fig. 6). However, we observed that the phospho-S649 signal is partially intact in the absence of PKCβ. Because Rottlerin, an inhibitor of novel PKCs, partially blocked phospho-S649 (but not phospho-S657), we suggest that a novel PKC isoform may also contribute to the phosphorylation of CARMA1 at residue S649 in B cells. Interestingly, previous studies have shown that genetic deletion of the novel PKC isoform PKCθ, which is expressed in B cells, results in B cell hyperactivation and increased proliferation, which under tolerogenic conditions leads to increased NF-κB and JNK activation upon antigenic stimulation (31, 32). Further investigation will be required to determine whether PKCθ, or another kinase, has a role in this phenotype.

It is well established that, although PKCβ/θ phosphorylation is required for CARMA1 activity, other pathways can target CARMA1 phosphorylation, influencing the strength of its signal.
S620 and S649 phosphorylation also do not additively increase CARMA1 activation (Fig. 2). Although in vivo phosphorylation of S608/620 (human/mouse, respectively) by CK1α remains to be directly demonstrated, together these data suggest that at least two alternative phosphorylation events (at S620 or S649) can mediate down-regulation of CARMA1 signaling, and imply these signals promote negative regulation via a similar pathway.

Although significant, phospho-S649 down-regulation of CARMA1 activity was incomplete, and was most evident when low doses of activators were used. We hypothesize that phospho-S649 may set a threshold for Ag receptor-driven IKK and JNK activation. We expect that additional signaling events are likely to be required to fully turn off the CARMA1 signal. Indeed, other negative regulatory processes that have been shown to deactivate the CARMA1 signalingosome as a whole include Bcl-10 degradation (34–37), deubiquitination of substrates including MALTI and TRAFs by A20 (or CYLD) (38–40), and direct CARMA1 ubiquitination and degradation (M.M.-G., K.S., and D.R., manuscript submitted). Furthermore, phosphorylation of some activating residues in CARMA1 is rapid and transitory (Fig. 5), possibly suggesting involvement of a phosphatase in CARMA1 inactivation.

Genetic knockout models in mice have demonstrated that loss of CARMA1 has no effect on early stages of lymphocyte development. However, CARMA1 is required for the development of B1 cells, for maintenance of mature B2 B cells, and for Ag receptor signaling of both B and T cells (6–9). Although it is not known whether overactive or allelic variants of CARMA1 have any role in pathologies of early B cells, there is mounting evidence to suggest that CARMA1 signaling enhances B cell lymphomagenesis in humans (41, 42). A subset of poor prognosis, ABC-DLBCL (activated B cell-like diffuse large B cell lymphomas) requires basal CARMA1 expression and activity to promote NF-κB activation and survival (42). Furthermore, mutations in the coiled-coil domain of CARMA1, which produces a constitutive active CARMA1 molecule, have been described in nearly 10% of these ABC-DLBCL. At first glance, our finding that expression of the overactive CARMA1-S649A mutant is counter-selected (due to increased cell death) in CARMA1−/− DT40 B cells appears to contradict these earlier findings. We hypothesize that because CARMA1 activates both NF-κB (associated with cell survival programs) and JNK (associated with apoptotic programs), overactive CARMA1 may differentially affect specific lymphocyte populations depending on their differentiation stage and on the relative influence these distinct pathways have on their growth and survival. Ag receptor-stimulated immature B cells undergo cell growth arrest and apoptosis, whereas more mature cells (such as the ABC-DLBCL) respond with the opposite biological outcome (43, 44). The DT40 cell line represents an immature B cell stage, and both BCR stimulation and JNK activation are strongly linked with apoptosis in this cell line (45). Additionally, although DT40 cells constitutively activate NF-κB, they down-regulate their BCR over time, indicating a counter-selection for the Ag receptor pathway. Taken together, the results presented here further support the idea that the survival and growth of B lymphoma cells, chicken DT40 cells in this case, are sensitive to the activation status of CARMA1.

In summary, the data presented in this study, combined with previous work, suggest the following working model for CARMA1 activation and subsequent deactivation via specific phosphorylation signals (Fig. 7). In resting cells, most CARMA1 is maintained in an inactive closed-conformation (Fig. 7A). Ag receptor stimulation activates PKCβ or PKCδ in B or T cells, respectively (as well as other kinase pathways), leading to rapid phosphorylation at specific residues including, most notably, S657 (Fig. 7A, left) and S564 in murine CARMA1. These modifications promote interactions with critical proteins (including Bel-10,


Supplemental Figure 1. WT CARMA1 and CARMA1-S649A exhibit similar turnover in the presence of a protein synthesis inhibitor. CARMA1 -/- DT40 cells were stably transduced to express myc-CARMA1-WT, myc-CARMA1-S649A or myc-CARMA1-ΔPRD (e.g. a constitutively activated CARMA1 mutant that serves as positive control for CARMA1 degradation). Cells were incubated in the presence of the protein synthesis inhibitor, cycloheximide (CHX), for the indicated time points. Cell lysates were prepared using RIPA buffer; and western blots were performed to determine the relative levels of CARMA1 and the ERK loading control, respectively.