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Phosphorylation of Carma1, but not Bcl10, by Akt regulates TCR/CD28-mediated NF- κ B induction and cytokine production

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Abstract

Previous studies from our group and others have shown that the Akt kinase can contribute to induction of NF- κ B by antigen receptor signaling. However, the direct targets of Akt in this pathway are not known. Here we show that Akt-mediated NF- κ B activation is mediated at least in part through direct phosphorylation of the adaptor protein Carma1, which we previously demonstrated could interact with Akt in a TCR ligation-dependent manner. The putative Akt phosphorylation sites in Carma1 are distinct from known PKC consensus sites. Mutation of S551, S637 and S645 in Carma1 to non-phosphorylatable residues decreased phosphorylation of GST-Carma1-linker construct by Akt in vitro. In addition, Carma1 S637A/S645A mutants were significantly impaired in their ability to restore TCR-mediated NF- κ B activation and IL-2 expression in Carma1-deficient T cells. Thus, our data reveal Carma1 as a novel target for Akt phosphorylation and suggest that Akt-mediated phosphorylation of Carma1 is an additional regulatory mechanism tuning the NF- κ B response downstream of antigen receptor and co-stimulatory signaling.

Keywords

Signal transduction; NF- κ B; T cells; Phosphorylation; Akt

1. Introduction

Caspase recruitment domain (CARD)-containing membrane-associated guanylate kinase (GUK) (Carma1) proteins are critical adaptors in multiple signaling pathways in many cell types. The Carma family consists of three members: Carma1, Carma2 and Carma3. Carma1 is predominantly expressed in the spleen, thymus, and peripheral blood leukocytes (Gaide et al., 2001); Carma2 is expressed only in the placenta (Gaide et al., 2001); and Carma3 is expressed in a broad range of tissues, at especially high levels in the liver, kidney, heart and brain (McAllister-Lucas et al., 2001). The three members share similar structures: an N-

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terminal CARD, followed by a coiled-coil domain; a linker region; a PDZ domain; a Src homology 3 (SH3) domain and a GUK-like domain (Gaide et al., 2001). The linker region contains crucial phosphorylation sites (Rueda and Thome, 2005). Upon phosphorylation of the linker region, Carma proteins are proposed to adopt a more open conformation, promoting the recruitment of downstream molecules (Matsumoto et al., 2005).

T cell activation is initiated when the T cell receptor for antigen (TCR) recognizes cognate peptide:MHC displayed on the surface of an antigen presenting cell (APC). Following TCR engagement, protein kinase C (PKC) θ , a novel protein kinase C enzyme, is activated, which in turn phosphorylates Carma1 within its linker region, between the coiled-coil and PDZ domains. This phosphorylation initiates a conformational change in Carma1, from an auto-inhibited inactive scaffold to one that is able to interact with downstream proteins, mainly through its CARD (Matsumoto et al., 2005; Sommer et al., 2005). Subsequently, Carma1 interacts with a preexisting complex that includes the CARD protein Bcl10 and the caspase-like protein Malt1 to form the Carma1-Bcl10-Malt1 (CBM) complex. One of the key downstream effects of CBM complex formation is activation of the canonical NF- κ B pathway, and loss of Carma1 causes profound defects in NF- κ B activation by antigen receptors on T and B cells (Thome et al., 2010).

Although PKC θ appears to be the most critical kinase for phosphorylation and activation of Carma1 after T cell activation, other kinases have also been shown to participate in this process. For example, hematopoietic progenitor kinase (HPK1) (Brenner et al., 2009), IKK β (Shinohara et al., 2007) and CaMKII (Ishiguro et al., 2006) have all been shown to contribute to phosphorylation-dependent Carma1 activation. Akt has been shown to regulate TCR-mediated NF- κ B activation and Akt acts upstream of the IKK complex to increase IKK activation, I κ B degradation, and NF- κ B nuclear entry (Cheng et al., 2011; Kane et al., 1999). The molecular details of Akt-mediated IKK activation are still not completely understood. PDK1 and Akt were reported to interact with Carma1 and their regulation of NF- κ B activity was shown to be Carma1-dependent (Park et al., 2009). Also, the interaction between Akt and Carma1 was found to be mediated at least in part by the C-terminal domain of Akt (Narayan et al., 2006). However, inclusion of PDK1 did not augment the association between Akt and Carma1.

We previously showed that Akt activity can modulate formation of the CBM complex (Cheng et al., 2011), thereby implicating Akt, either directly or indirectly, in the phosphorylation of Carma1, and possibly other CBM components. In this study, we show that Akt can directly phosphorylate Carma1 within its linker region. Akt-mediated Carma1 phosphorylation involves mainly the non-PKC consensus residues S637 and S645. Furthermore, mutation of one or both of these serine residues to alanine impairs TCR/CD28-mediated NF- κ B induction and production of the cytokine IL-2, transcription of which is regulated by NF- κ B. However, Akt does not appear to play a major role in CD3/CD28-mediated phosphorylation of Bcl10. Thus, our results indicate that Carma1 serves as a physiological substrate for Akt upon TCR stimulation, and is a direct link between Akt and the canonical NF- κ B signaling pathway.

2. Materials and methods

2.1. Antibodies and reagents

Clonotypic antibody to the Jurkat TCR (C305) was obtained from A. Weiss (University of California, San Francisco). Anti-human CD28 was from Caltag (Burlingame, CA). Anti-Bcl10 and anti-Myc antibody (9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA (12CA5) was from Roche Diagnostics (Indianapolis, IN). Anti-Carma1 was from ProSci Incorporated (San Diego, CA). Phospho-(Ser/Thr)Akt substrate antibody and

GST-GSK3 α/β (Ser21/9) “crosstide” substrate were from Cell Signaling (Beverly, MA). Human IL-2 ELISA kit was from BD Biosciences (San Diego, CA). Staphylococcal enterotoxin (SEE) was from Toxin Technology (Sarasota, FL). Akt1/2, phorbol myristate acetate (PMA) and ionomycin were from EMD Biosciences (La Jolla, CA). Active Akt1/PKB α (N-terminal His-tagged, recombinant, full-length, human Akt, MW=59.9kDa) and active Akt1/PKB α (Δ PH, S473D) (N-terminal His6-tagged, recombinant human Akt1, from residue 118 to the C-terminus containing the mutation S473D, MW=45kDa) were purchased from Millipore (Billerica, MA).

The phospho-Bcl10(S231) antibody was generated by immunization of rabbits with a phosphorylated peptide corresponding to the C-terminal ten residues of Bcl10. An N-terminal cysteine was included for conjugation to KLH. ELISA-positive bulk bleeds were pooled for affinity purification on the phospho-peptide, followed by depletion with the non-phosphorylated peptide. Antibody generation was carried out at Open Biosystems.

2.2. DNA constructs

Expression plasmids for HA-Myr-Akt, Myc-Myr-PKC θ and WT Carma1 were described previously (Narayan et al., 2006). Plasmids encoding the S552A, S637A and S645A mutants of Carma1 were provided by X. Lin. Double mutations of Carma1 were generated using the QuikChange mutagenesis system from Stratagene, according to the manufacturer's instructions. Mutant constructs were verified by sequencing. Plasmids encoding GST fused to wild-type or mutant versions of the human Carma1 linker domain (aa's 432–671) were generated by PCR and sub-cloned into the pGEX-4T1 vector. The resulting expression vectors were transformed into *E. coli* strain BL21. Recombinant GST fusion proteins were purified from bacterial lysates with GST-agarose beads using B-PER bacterial protein extraction reagent (Pierce).

2.3. Cell lines and transfections

Jurkat T cells with stable expression of Carma1 shRNA were generated by lentiviral transduction. Carma1-deficient JPM50.6 cells were described previously (Wang et al., 2002). JPM50.6 cells expressing Carma1 constructs were generated by the reconstitution of JPM50.6 cells with lentiviral vectors encoding WT or mutants of Carma1. HEK293 T cells were transfected by Ca²⁺-phosphate precipitation and Jurkat cells were transfected by electroporation (250 V, 950 μ F). The D10 Th2 cell clone has been described previously (Kane et al., 2004).

2.4. In vitro kinase assays

For detection of Akt-induced phosphorylation of Carma1, GST-tagged WT or mutated Carma1 linker protein was mixed with kinase buffer (20 mM HEPES, pH 7.4, 2 mM MnCl₂, 10 mM MgCl₂, 25 mM beta-glycerophosphate, 0.1 mM sodium orthovanadate, 4 mM sodium fluoride and 1mM DTT), along with 1 μ Ci γ -³²P-ATP and 20 μ M cold ATP, in the presence or absence of recombinant Akt. Samples were incubated for 30 min at 30 degrees. The kinase assay reactions were stopped by adding 20 μ l of 2X SDS loading buffer. Samples were then subjected to SDS-PAGE and autoradiography.

2.5. Luciferase reporter assays

Cells were electroporated with 15 μ g of NF- κ B-luciferase or RE/AP-luciferase reporter plasmid and 5 μ g expression vectors for various Carma1 constructs. The transfected cells were cultured overnight, then stimulated with anti-TCR/CD28 for six hours, after which the cells were lysed and luciferase activity was determined with an Orion luminometer (Zylux, Oak Ridge, TN).

3. Results

3.1. Bcl10 is not a target of Akt phosphorylation in T cells

Our previous study showed that Akt could either directly or indirectly regulate the phosphorylation status and/or stability of Bcl10 (Narayan et al., 2006). Thus, when Myr-Akt was co-expressed with Bcl10, there was an increase in a slower mobility form of the protein, while the co-expression of PTEN led to a nearly complete loss of Bcl10 protein (Narayan et al., 2006). There is one potential site of Akt-mediated phosphorylation within Bcl10 (S231), as predicted by the consensus sequence RxRxxS/T (Fig. 1A). We generated a polyclonal antibody to this site by immunization of rabbits (see *Materials and methods*). We then analyzed whether Bcl10 S231 could be phosphorylated under physiological conditions and whether Bcl10 is a direct substrate of Akt (Fig. 1). Indeed, Bcl10 S231 phosphorylation peaked at fifteen minutes after TCR/CD28 stimulation of Jurkat cells (Fig. 1B). As expected, when Ser231 was mutated to Ala, Bcl10 phosphorylation at S231 was no longer detected (Fig. 1C). However when cells were treated with Akti1/2, a specific allosteric inhibitor of Akt, the phosphorylation of Bcl10 at S231 was retained (Fig. 1D). This suggests that Akt activity is not required for phosphorylation of Bcl10 after TCR/CD28 stimulation in T cells.

In order to search for other possible direct targets of Akt phosphorylation in this pathway, whole cell lysates of CD3/CD28-stimulated T cells were probed with an antibody (pAkt substrate Ab) that is specific for serine and threonine residues preferentially phosphorylated by Akt in the context RxRxxS/T (where x is any amino acid) (Fig. 1E). Consistent with the results above, we did not observe any bands corresponding to the size of Bcl10 that exhibited inducible pAkt substrate immuno-reactivity that was also blocked by Akti1/2. Moreover, when we directly IP'd Bcl10 and probed the resulting western blots with Akt substrate antibody, we failed to see a decrease of Bcl10 phosphorylation with the addition of increasing amount of Akti1/2 (Fig. 1E, lower panel). This finding suggests that there may be no other sites of Akt phosphorylation within Bcl10. Interestingly, we noticed that a band around 150 kD, corresponding to the approximate size of Carma1, showed decreased phosphorylation when Akt was inhibited.

3.2. Direct phosphorylation of the Carma1 linker by Akt

The “linker” region of Carma1 separates the CARD and coiled-coil domains from the MAGUK-domain and must be phosphorylated for NF- κ B activation by the TCR and CD28 (Fig. 2A) (Matsumoto et al., 2005; Sommer et al., 2005). We previously found that Akt can associate with Carma1 (Narayan et al., 2006) and that Akt inhibition impairs the assembly of the CBM complex (Cheng et al., 2011). Given these previous findings, we determined whether Carma1 is a direct substrate of Akt. HEK293T cells were transfected with either constitutively active HA-Myr-Akt or Myc-Myr-PKC θ , and cell lysates were IP'd with either anti-Myc or anti-HA antibody. In vitro kinase assays were then performed using either GST-Carma1-linker (containing aa's 432–671 from human Carma1) as a substrate, or GST-GSK3 α (containing a short stretch of aa's from the N-terminal domain, which is identical in GSK3 α and GSK3 β) as a positive control for Akt activity. Interestingly, Akt phosphorylated the linker region of Carma1 roughly to the same extent as did PKC θ (Fig. 2B). We next proceeded to examine Carma1 phosphorylation by purified recombinant Akt.

Based on analysis of the Carma1 sequence with Scansite (<http://scansite.mit.edu>), we found that the linker region of Carma1 contains several serine residues that are potential, if not ideal, sites of Akt phosphorylation. To further define Akt-dependent phosphorylation sites in Carma1, GST fusion proteins containing S637A and/or S645A mutations within the Carma1 linker region were generated. These proteins were then used as substrates for in vitro kinase

assays with a PH-truncated recombinant Akt. As expected, the GST-tagged Carma1 linker served as a substrate for phosphorylation by recombinant Akt (Fig. 3A). Mutation of the critical PKC θ -specific residue S552 within the Carma1 linker domain still allowed for significant Akt-mediated phosphorylation of the GST-Carma1 linker, suggesting that residues other than S552 serve as Akt-dependent phosphorylation sites. By contrast, phosphorylation of the S637A and S645A mutants by Akt (in this case a full-length activated form) was greatly reduced (Fig. 3A), pointing to these sites as possible Akt phosphorylation sites. Next we made double mutants of S551A/S552A and S637A/S645A within the Carma1 linker domain. S551 was reported to be an HPK1 phosphorylation site (Brenner et al., 2009). Double mutation of S637A/S645A abolished Akt-mediated in vitro phosphorylation of the Carma1 linker. Akt was still able to phosphorylate the S551/S552A mutant (Fig. 3B), although this was significantly weaker than its phosphorylation of the S552A single. This suggests that S551 is another target of Akt phosphorylation. As expected, Akt did not phosphorylate GST alone (data not shown). Importantly, previous in vitro and in vivo studies in human, murine and chicken cells have indicated that both S637 (Huttlin et al., 2010; Moreno-Garcia et al., 2009; Sommer et al., 2005), and S645 (Eitelhuber et al., 2011; Moreno-Garcia et al., 2009; Shinohara et al., 2007; Sommer et al., 2005) can be phosphorylated in intact cells.

3.3. Carma1 linker phosphorylation at S637 and S645 positively regulates NF- κ B activation

To investigate the involvement of S637 and S645 in TCR mediated NF- κ B activation, we mutated both residues in full-length Carma1. Jurkat T cells with stable knock-down of Carma1 were transiently transfected with expression plasmids encoding WT, S637A/S645A or S551A/S552A forms of Carma1, along with an RE/AP- (an NF- κ B-like element in the IL-2 promoter (Shapiro et al., 1997)) or NF- κ B-luciferase reporter plasmid. Both of these elements are responsive to CD28- or Akt-mediated co-stimulation, in conjunction with phorbol esters or TCR/CD3 signals (Kane et al., 2001; Kane et al., 1999). Jurkat T cells with stable knock-down of Carma1 are completely refractory to TCR stimulation for the activation of both of these reporters. Reconstitution of these cells with WT Carma1 rescued TCR-induced activation of both the classical NF- κ B (Fig. 4A, right panel) and RE/AP (Fig. 4A, left panel) reporters. However, the double mutant S637A/S645A could only partially rescue the NF- κ B activation, approximately to the same extent as the S551A/S552A mutant. We also examined the ability of the various Carma1 constructs to re-constitute NF- κ B or RE/AP signaling in the context of stimulation with the more physiological stimuli of antigen presenting cells (the Raji human B cell line) and the superantigen SEE; in addition, we also assessed the effects of single mutation of either S637 or S645 (Fig. 4B). In the case of the classical NF- κ B reporter (right panel) mutation of Carma1 S637 alone had no significant effect on NF- κ B activation, while mutation of S645 alone led to approximately a 40% loss of NF- κ B activity. However, we observed only moderate impairment of RE/AP reporter activity with either S637 or S645 single mutation, suggesting somewhat different signaling requirements for this reporter. Significantly, with both reporters, the double mutants examined above displayed a consistently more profound level of impairment in their ability to rescue reporter activity.

3.4. Role of Carma1 phosphorylation at S637/S645 in T cell activation

Finally, we investigated the physiological role of the putative Akt phosphorylation sites S637 and S645 within Carma1 for TCR-mediated NF- κ B activation. The Carma1-deficient Jurkat T cell line JPM50.6 was stably reconstituted with WT Carma1 or one of the Carma1 mutants. For each mutant, multiple clones were tested for functional analysis. Representative clones are presented. These cells were stimulated with Raji human Burkitt's lymphoma cells loaded with the superantigen staphylococcus enterotoxin E (SEE). Twenty four hours later supernatants were collected and IL-2 concentrations were determined by

ELISA. As expected, Carma1-deficient JPM50.6 cells failed to produce IL-2 after stimulation, while JPM50.6 cells reconstituted with WT Carma1 made IL-2 at levels comparable to parental Jurkat T cells. However, both the S637A/S645A (Akt sites) and S551A/S552A (PKC/HPK1 sites) Carma1 mutants could only partially rescue IL-2 expression (Fig. 5). These defects were observed despite higher levels of expression of the mutant Carma1 constructs, relative to the WT protein. Expression of TCR/CD3 was comparable in Jurkat cell lines expressing these various Carma1 proteins (unpublished data). Although it was previously reported that the S637A mutation had no effect on NF- κ B reporter gene expression in transient transfection assays (Sommer et al., 2005), later studies from the same group showed that mutation of S637 (murine S649) promoted enhanced IKK activation in T cell lines, under conditions of sub-optimal antigen receptor ligation. Our results demonstrate that TCR stimulation in the presence of the Carma1 mutant S637A induced efficient NF- κ B activation. However Carma1 mutant S645A could rescue about 50% of NF- κ B activity, compared with WT Carma1, and the double mutant S637A/S645A could only rescue the NF- κ B activity to the same minimal extent as S551A/S552A. Taken together, our results suggest that S645 of Carma1 is critical for optimal Akt-mediated NF- κ B activity, while S637 plays an auxiliary role, together with S645.

4. Discussion

Phosphorylation of the linker domain in Carma1 is a critical point of regulation in the activation of NF- κ B by antigen receptors. Although PKC β / θ were the first kinases shown to mediate Carma1 phosphorylation and downstream NF- κ B activation, additional kinases are now known to contribute to phosphorylation of Carma1, influencing the strength of signaling through this pathway (Thome et al., 2010). Other kinases that target Carma1 include CaMKII, CK1 α , HPK1, and IKK β . Thus, CK1 α can phosphorylate S608 in human Carma1, IKK β (Shinohara et al., 2007) was reported to phosphorylate S555, and CaMKII was shown to phosphorylate S116 (Ishiguro et al., 2006). The major phosphorylation sites for PKC β / θ were S552 and S645 (Matsumoto et al., 2005), while the HPK1-targeted phosphorylation site was reported to be S551 in Carma1 (Brenner et al., 2009). PKC β was reported to phosphorylate S637 (Moreno-Garcia et al., 2009), although additional kinases might also contribute of this site. Akt is activated downstream of PI3K, and Akt and PKC θ physically and functionally interact to synergistically activate NF- κ B (Bauer et al., 2001; Kane et al., 2001). Thus, identifying direct target(s)/substrate(s) for not only PKC θ , but also Akt in this pathway was a logical next step.

Bcl10 also plays a key role in lymphocytes, as an adaptor protein linking Carma1 and MALT1 in the signaling pathway leading from antigen receptor stimulation to NF- κ B. Structurally, Bcl10 is characterized by an N-terminal CARD and a C-terminal extension of about 130 amino acids that is rich in serine and threonine residues, and which serves as a target for multiple phosphorylation events. Bcl10 is phosphorylated when overexpressed alone or together with various kinases (Thome and Weil, 2007). We previously reported that Bcl10 was phosphorylated in T cells when co-expressed with Myr-Akt, although it was not clear if this was direct (Narayan et al., 2006). In the present study, when we mutated Ser231 to alanine, we did see loss of Bcl10 phosphorylation in T cells. However, Akt activity appears not to be required, either directly or indirectly, for phosphorylation of Bcl10 at this site. Thus, a specific, allosteric Akt1/2 inhibitor, which showed potent inhibition of CBM complex formation and NF- κ B activation (Cheng et al., 2011), had no detectable effect on TCR/CD28 induced Bcl10 S231 phosphorylation, despite the fact that this site appears to be an ideal target for phosphorylation by Akt.

S551 of Carma1 was reported as an HPK1 phosphorylation site, with S552 a major site phosphorylated by PKC θ . The efficient phosphorylation of the S551/S552A mutant by Akt

suggests that Akt targets different sites in the Carma1 linker region to fine-tune Carma1 activity. Two sites (S637 and S645) of apparent Akt-dependent phosphorylation in the linker domain of Carma1 were identified in this study. S637 and S645 were among three sites identified as major sites for linker phosphorylation by PKC β or PKC θ (Moreno-Garcia et al., 2009; Sommer et al., 2005). There are discrepancies regarding the role of PKC in phosphorylation of S637 and S645. Thus, Rawlings and colleagues reported that mutation of S637 (murine S649) or S645 (murine S657) led to about 50 percent reduction in PKC θ -mediated linker phosphorylation (Moreno-Garcia et al., 2009). Mutation of S645 (murine S657) decreased NF- κ B activation to less than 40% of that seen with WT Carma1, while mutation of S637 (murine S649) had no significant effect on NF- κ B activation (Sommer et al., 2005). Lin and colleagues showed that mutation of S645 (murine 657) had little effect on PKC θ -induced phosphorylation in vitro. However, S645A could only rescue 45–50% of the full NF- κ B activity of WT Carma1 (Matsumoto et al., 2005).

S637 and S546 in the Carma1 linker are at least partially redundant with each other, as mutation of both sites was required to abolish Akt phosphorylation of Carma1, as well as to significantly dampen downstream NF- κ B activation and cytokine production. Despite the dramatic effect of the S637/S645 double mutant on Akt-mediated Carma1 linker phosphorylation, we cannot rule out the possibility that Akt might phosphorylate other sites in Carma1. Thus, previous studies identified other functionally important “orphan” sites of phosphorylation (e.g. S565), for which kinases have not yet been identified (Matsumoto et al., 2005). In addition, the role of Akt itself in this pathway is in part redundant with other kinases, as discussed above. These findings are consistent with our previous report that Akt modulates NF- κ B activity in a quantitative fashion, with differential effects on downstream NF- κ B target genes (Cheng et al., 2011). Therefore, we would argue that phosphorylation of the linker domain in Carma1 does not act as a digital switch for NF- κ B activation, but rather provides a sensitive rheostat, with greater phosphorylation increasing the strength and/or duration of NF- κ B activity.

The three members of the Carma family diverge significantly in the linker region. Thus, Carma3, but not Carma2, contains a serine residue (S520) in the homologous position of S552 or S555 in Carma1. Mutation of S520 in Carma3 also abolished its ability to rescue TCR-induced NF κ B activation in Carma1-deficient T cells (Matsumoto et al., 2005). Of note, all three Carma family members contain serine residues in the homologous position of S637 and S645 in Carma1. Since Akt did not target S552, which was mainly regulated by PKC θ , this suggests that Akt might play an important role in regulating other Carma family members (Carma2 or Carma3) in other tissue and organs. Carma3 is an indispensable signaling component in GPCR-induced NF- κ B activation (Grabner et al., 2007). G proteins such as G α i can also activate the PI3K/Akt pathway (Murga et al., 1998). Further research will be necessary to define whether phosphorylation of Carma3 by Akt plays a role in regulating NF- κ B activation in cells and signaling pathways that rely on this member of the Carma family.

In summary, we have found that Akt-mediated phosphorylation of S637/S645 in Carma1 positively regulates NF- κ B activation and IL-2 production in TCR (Raji/SEE) stimulated T cells. These results confirm the physiological relevance of the Akt-specific residue S637 and S645A in Carma1.

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Abbreviations

TCR	T cell receptor
CARD	caspase recruitment domain
CBM	Carma1/Bcl10/MALT1

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Highlights

- S231 in Bcl10 conforms to an Akt consensus site, providing a possible link between Akt and NF- κ B.
- However, phosphorylation of Bcl10 S231 in T cells does not require Akt activity.
- Akt can directly phosphorylate S551, S637 and S645 in human Carma1.
- Mutation of S637 and S645 in Carma1 impairs TCR-dependent NF- κ B induction and IL-2 secretion.
- Phosphorylation of Carma1 down-stream of TCR is regulated in part by Akt.
- Akt-mediated Carma1 phosphorylation may explain the effects of Akt on NF- κ B signaling in T cells.

Fig. 1. Akt-mediated phosphorylation of Bcl10 is not required for NF-κB induction

(A) Sequence alignments of the C-terminal region of human, mouse and rat Bcl10. The consensus sequence for phosphorylation by Akt is also shown; "x" indicates any amino acid, while Φ indicates a hydrophobic residue. (B) Jurkat T cells were stimulated with anti-TCR/CD28 for various times. The lysates were examined by western blots using total Bcl10 and phospho-Bcl10 antibody. (C) Jurkat T cells transiently transfected with WT Bcl10 or S231A-Bcl10 were stimulated with PMA/ionomycin for various times. The lysates were examined as above. (D) Jurkat T cells were pretreated either with or without Akt1/2 for one hour and stimulated with anti-TCR/CD28 for various times. The lysates were examined as in (B). (E) D10 T cells were pretreated with various concentrations of Akt1/2 (0–10 μM) for one hour and stimulated with anti-CD3/CD28 for ten minutes. Whole cell lysates were examined by western blot using phospho-Akt substrate antibody (upper panel). The asterisk

indicates the position of an Akt inhibitor-sensitive substrate corresponding to the approximate MW of Carma1. Blots were then stripped and re-probed with a mAb to beta-actin (lower panel). Results in each part are representative of at least three experiments.

A

Site	Context	Kinase
S551	PP <u>R</u> SRSSIMSI	Akt?
S552	PRSRSS <u>I</u> MSIT	PKC
S637	MFR <u>K</u> FS <u>L</u> ERPF	Akt?
S645	<u>R</u> PFRPS <u>V</u> TSVG	PKC/Akt?

Akt consensus: RxRxx**S/T**Φ

Fig. 2. Efficient phosphorylation of the linker domain of Carma1 by Akt
 (A) Model for Carma1 “activation” by linker domain phosphorylation, and subsequent recruitment of Bcl10 and MALT1. (B) HEK293T cells were transfected with either constitutively active Myc-Myr-PKCθ or HA-Myr-Akt. Cell lysates were IP’d with either anti-Myc or anti-HA antibody. In vitro kinase assay were performed using either GST-Carma1-linker as a substrate or GST-GSK3 as a positive control substrate for Akt activity. Kinase assays were run out on SDS-PAGE gels and transferred to PVDF. After exposure to X-ray film (upper panel), blots were probed with an anti-GST Ab (lower panel). Results are representative of five independent experiments.

A

Site	Context	Kinase
S551	PP <u>R</u> SRSSIMSI	Akt?
S552	PRSRSS <u>I</u> MSIT	PKC
S637	MFRKFS <u>L</u> ERPF	Akt?
S645	<u>R</u> PFRPS <u>V</u> TSVG	PKC/Akt?

B **C** **Fig. 3. Akt phosphorylates S637 and S645 in the linker region of Carma1**

(A) Overview of Carma1 linker domain phosphorylation sites explored in this study. Numbering is for human Carma1 (as in Ref. (Matsumoto et al., 2005)). Phosphorylation sites are in bold, and residues conforming to the Akt consensus site (see Fig. 1) are underlined. (B–C) Recombinant proteins containing GST fused with wild type or mutants of the linker region of Carma1 were used as substrates for recombinant Akt in in vitro kinase assays. Active Akt1/PKB α (δ PH, S473D, MW=45kDa) was used in Panel A, while active Akt1/PKB α (full-length, MW=59.9kDa) was used in panel B. Kinase reactions were subjected to SDS-PAGE and transferred to PVDF membranes and then analyzed by autoradiography. The levels of GST-fusion proteins were determined by western blots with

anti-GST antibody. The asterisk indicates the position of auto-phosphorylated Akt. Results are representative of three independent experiments.

Fig. 4. Residues S637 and S645 of Carma1 regulate TCR-mediated NF-κB activation

Jurkat T cells with stable expression of Carma1-specific shRNA were transfected with the indicated luciferase reporter plasmids, plus expression plasmids encoding wild type Carma1 (WT), or one of various serine mutants. Twenty-four hours later, cells were either left non-stimulated or stimulated with anti-TCR (C305) and CD28 antibodies for six hours (A), or overnight with Raji B cells and superantigen SEE (B), before determining luciferase activity. Results in each panel are the average values of triplicate samples (+/- std. dev.) from a single experiment, representative of three independent experiments in each case.

Fig. 5. Carma1 residues S637 and S645 are important for IL-2 production in T cells

(A–B) Stable cell lines expressing WT or mutated Carma1 constructs were created by transducing Carma1-deficient Jurkat T cells (JPM50.6) with lentiviral vectors containing different Carma1 mutants. Stable cell lines were then stimulated with Raji B cells as antigen presenting cells, plus the superantigen SEE, for 24 hours. IL-2 in the culture supernatant was determined by ELISA (A). Western blots were performed to assess the expression of WT or mutant Carma1 in the various cell lines (B). Two separate clones expressing the S637A/S645A double mutant were analyzed. (C–D) JPM50.6 cells were transiently transfected with the indicated constructs. The next day, cells were stimulated as indicated for 24 hours, and supernatants were collected and analyzed for IL-2 by ELISA. Results shown are the average ($\pm$ std. dev.) of triplicate samples from a single experiment, representative of three that were performed. Statistical significance was determined in comparison to WT Carma1.