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CARMA1 Is Required for Akt-Mediated NF-κB Activation in T Cells

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Many details of the generic pathway for induction of NF-κB have been delineated, but it is still not clear how multiple, diverse receptor systems are able to converge on this evolutionarily conserved family of transcription factors. Recent studies have shown that the CARMA1, Bcl10, and MALT1 proteins are critical for coupling the common elements of the NF-κB pathway to the T-cell receptor (TCR) and CD28. We previously demonstrated a role for the serine/threonine kinase Akt in CD28-mediated NF-κB induction. Using a CARMA1-deficient T-cell line, we have now found that the CARMA complex is required for induction of NF-κB by Akt, in cooperation with protein kinase C activation. Furthermore, using a novel selective inhibitor of Akt, we confirm that Akt plays a modulatory role in NF-κB induction by the TCR and CD28. Finally, we provide evidence for a physical and functional interaction between Akt and CARMA and for Akt-dependent phosphorylation of Bcl10. Therefore, in T cells, Akt impinges upon NF-κB signaling through at least two separate mechanisms.

Inducible activation of transcription through the NF-κB family of factors is essential for the regulation of many immune response genes (19, 27). Some of the best characterized of these include the cytokine interleukin 2 (IL-2), the alpha chain of its high-affinity receptor, and the prosurvival molecule Bcl-x_L. NF-κB can be induced by signals from the T-cell receptor (TCR)/CD3 complex and the costimulatory receptor CD28. Study of the signaling pathways used by receptors for proinflammatory cytokines, such as tumor necrosis factor (TNF), has revealed a common pathway used by most, if not all, receptors to induce NF-κB (24). Access of NF-κB transcription factor dimers to the nucleus is controlled by inducible degradation of an inhibitory protein known as IκB, which normally sequesters NF-κB in the cytoplasm. IκB degradation occurs after its inducible phosphorylation, which results in ubiquitination and degradation by the proteasome. Phosphorylation is catalyzed by a trimeric complex that includes two catalytic subunits, IκB kinase α (IKKα) and IKKβ, and an adaptor subunit, IKKγ. Another level of NF-κB regulation involves phosphorylation or acetylation of the NF-κB proteins themselves (6).

The IKK complex, and downstream NF-κB function, can be activated by the ligation of a diverse array of receptors, including antigen receptors like the TCR, costimulatory receptors like CD28, TNF family receptors, and Toll-like receptors. At this point, it is not clear how all of these receptors couple to the IKK complex, but since these receptors all use different types of proximal signaling strategies, it seems likely that they employ different components to couple to the IKK complex. Recent studies have identified a complex of three proteins that links antigen receptors on B and T cells to the IKK complex, proteins known as CARMA1, Bcl10 (B-cell lymphoma 10), and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1) (reviewed in reference 41). Deletion of any one of these proteins impairs the ability of antigen receptors to activate NF-κB but appears not to affect other pathways. More specifically, in T cells, this complex lies downstream of protein kinase C (PKC) θ, deletion of which also inhibits NF-κB induction.

We and others previously reported that Akt plays a role in NF-κB induction in T cells (15–19, 22). Others also reported a role for Akt in NF-κB induction in other cell types. Some studies, including our own, demonstrated that Akt acts upstream of the IKK complex to increase IKK activation, IκB degradation, and NF-κB nuclear entry (22, 31, 32). However, others suggested that the main effect of the phosphatidylinositol (PI) 3-kinase/Akt pathway is to increase NF-κB phosphorylation and coactivator binding, possibly downstream of Ras (28, 36). Recent data suggest that these discrepancies may be due in part to cell type-specific differences in IKK expression (9).

At least in T cells, it appears that Akt cannot induce NF-κB on its own, but rather it cooperates with signals from PKC θ in order to do so (4, 18). This costimulatory activity of Akt appeared indistinguishable from that provided by CD28, and indeed, we observed that expression of activated Akt could replace the CD28 costimulatory signal for upregulation of IL-2 and gamma interferon, although it could not replace the CD28 signal for upregulation of IL-4 and IL-5 (18).

We have reexamined the issue of where in the NF-κB pathway Akt acts, taking advantage of the recently described CARMA1-deficient Jurkat T-cell line JPM 50.6 (48). These cells have a specific defect in TCR/CD28-induced NF-κB, but not in TCR-induced NFAT or induction of NF-κB by other pathways, including TNF. The NF-κB defect in these cells lies upstream of IKK activation, resulting in a failure of TCR/CD28 to induce IKK kinase activity, IκB phosphorylation, and NF-κB nuclear entry. JPM 50.6 cells therefore provide a useful genetic model with which to test the role of CARMA1 (and its associated proteins) in Akt-dependent NF-κB induction.

MATERIALS AND METHODS

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-murine anti-CD3 and anti-CD28 and anti-GFP were from BD-Pharmingen (San Jose, CA). Anti-IκB and phos-

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pho-specific antibodies against glycogen synthase kinase 3 (GSK-3) and PKC θ were obtained from Cell Signaling, Inc. (Beverly, MA). Phospho-specific antibody to Akt (S473) was from Biosource (Camarillo, CA). Anti-β-actin, Cy3-conjugated antibody (9E10), chicken conalbumin, and ionomycin were from Sigma (St. Louis, MO). Akti 1/2 (Akt1 and Akt2 inhibitor) and phorbol myristate acetate (PMA) were from EMD Biosciences (La Jolla, CA). Anti-Bcl10, anti-PKC θ, and anti-Myc antibody 9E10 were from Santa Cruz Biotechnology (Santa Cruz, CA). Staphylococcal enterotoxin E (SEE) was from Toxin Technology (Sarasota, FL). Fluorophore-conjugated secondary antibodies and Alexa 488-conjugated antihemagglutinin (anti-HA) antibody were from Molecular Probes (Eugene, OR). Purified 12CA5 antibody was obtained from Roche (Indianapolis, IN). Lambda phosphatase was obtained from New England Biolabs (Beverly, MA).

DNA constructs. Luciferase reporters were as described previously (22). HA-tagged myristylated Akt (HA-Myr-Akt) was obtained from P. Tsichlis (Tufts Medical School, Boston, MA). CARMA1 and Bcl10 cDNAs were obtained from X. Lin (M. D. Anderson, Houston, TX). The Flag-IKKβ construct was provided by Tularik (South San Francisco, CA). EE-tagged phosphoinositide-dependent kinase 1 (PDK-1) and phosphatase and tensin homolog (PTEN) were obtained from D. Stokoe (University of California, San Francisco). GFP-Akt constructs have been described previously (20).

Cell lines and transfections. Parental and CARMA1-deficient (JPM 50.6; originally obtained from Xin Lin) Jurkat T cells were transfected by electroporation as described previously (22). A fast-growing derivative of the D10 murine T-cell clone has been described previously (20). Human embryonic kidney fibroblasts (293T) were transfected as described previously (21).

Luciferase reporter assays. The day after transfection, cells were washed and stimulated for 6 hours in U-bottom 96-well plates; luciferase assays were then performed as described previously (22). Luciferase activity was determined with an Orion luminometer (Zylux, Oak Ridge, TN).

Conjugate formation, staining, and confocal microscopy. The day after transfection, live D10 cells were purified over Lympholyte-M (Accurate Chemicals, Westbury, NY) and then recultured for 3 to 5 h without IL-2. Conjugates were formed and stained as described previously (20), with CH27 B cells that had been incubated overnight with chicken conalbumin. Images were collected on an Olympus FluoView confocal microscope (University of Pittsburgh Center for Biologic Imaging), using a 60× 1.4-numerical-aperture objective. TIFF images (24 bit) were assembled in Canvas 8.

Cell lysis, IP, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting. Cell lysates were prepared in lysis buffer containing 1% NP-40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 1 mM EDTA, in addition to protease [4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, aprotinin, and leupeptin; all used according to the manufacturer's recommendations (EMD Biosciences)] and phosphatase (sodium orthovanadate [EMD Biosciences]; beta-glycerol phosphate [Sigma]) inhibitors. Immunoprecipitation (IP) and Western blotting were carried out as described previously (21, 43). Enhanced-chemiluminescence Western blots were developed with SuperSignal West Pico substrate (Pierce, Rockford, IL) and imaged on a Kodak Image Station 2000R.

IKK kinase assays. IKK IP kinase assays were performed essentially as described previously (21), except that only cold ATP was used in kinase reactions, and the proteins from the kinase reactions were separated by SDS-PAGE and Western blotted. Blots were probed with a polyclonal antibody specific for phosphorylated IκB.

RESULTS

We were interested in characterizing signaling downstream of the Akt-PKC θ module in cells lacking CARMA1, a key regulator of TCR/CD28-induced NF-κB activation in T cells. Jurkat T cells lacking CARMA1 are reported to be defective in NF-κB induction through the TCR and CD28, while NFAT induction still proceeds normally (48). We assessed this using superantigen and Daudi B cells to stimulate parental Jurkat cells or the CARMA1-deficient JPM 50.6 cells in transcriptional reporter assays. As shown in Fig. 1A, when superantigen and antigen-presenting cells (APCs) are used to stimulate these cells, the JPM 50.6 cells can induce NFAT-dependent transcription as efficiently as parental Jurkat cells can. However, JPM 50.6 cells are profoundly deficient in the activation

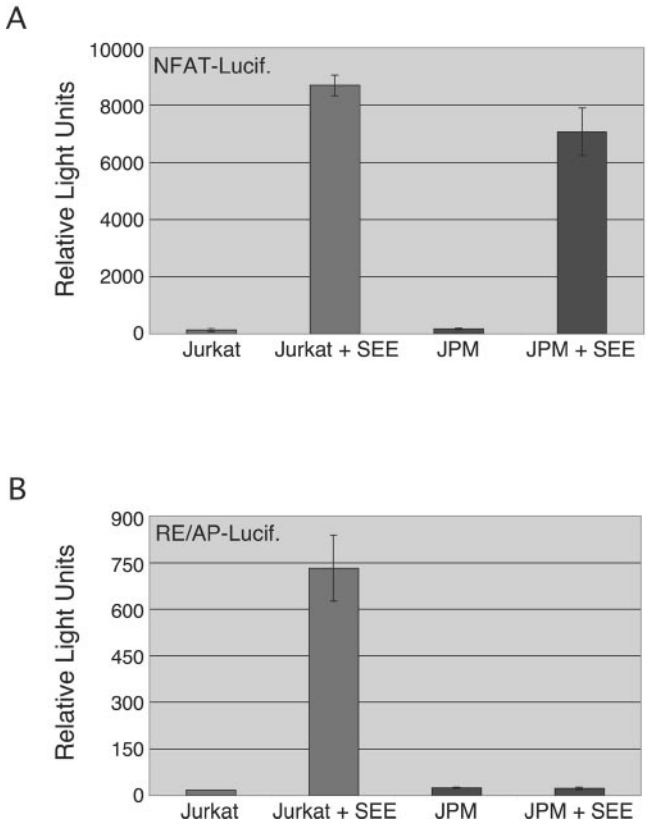

FIG. 1. CARMA1-deficient JPM 50.6 Jurkat cells fail to activate NF-κB in response to superantigen. JPM 50.6 (JPM) and parental Jurkat T cells were transfected with an NFAT/AP-1 or NF-κB luciferase (Lucif.) reporter. The next day, cells were stimulated with Daudi B cells plus SEE, and luciferase activity was determined. Results displayed are the average relative light units from three values from a single experiment, which was representative of three experiments that were performed.

of an NF-κB-dependent RE/AP reporter (35) in comparison to parental Jurkat cells (Fig. 1B). This confirms previous reports, using antibody and pharmacological stimulation, which showed a specific NF-κB defect in T cells lacking CARMA1.

Since our previous studies indicated that Akt lies on the pathway from CD28 to NF-κB induction, one would predict that Akt could no longer activate this pathway in the absence of CARMA1, which impairs CD28 signaling to NF-κB. As shown in Fig. 2A, this is indeed the case. Thus, an NF-κB-luciferase reporter was transfected into parental or CARMA1-deficient Jurkat cells, along with empty vector or expression plasmids for Akt and/or CARMA1. Cells were then stimulated with PMA alone or PMA plus anti-CD28. As we previously reported (18, 22), a low concentration of PMA that is incapable of activating the NF-κB reporter on its own can do so either in the presence of an anti-CD28 antibody or when Akt is overexpressed (Fig. 2A, lanes 1 and 2). Neither of these regimens led to NF-κB activation in the JPM 50.6 cells, while responses to both could be rescued when CARMA1 was also transfected into the JPM 50.6 cells (Fig. 2A, lanes 3 to 6).

Induction of NF-κB-dependent transcription depends upon activation of the IKK complex (19, 25). We previously showed that Akt-mediated NF-κB induction also proceeds through

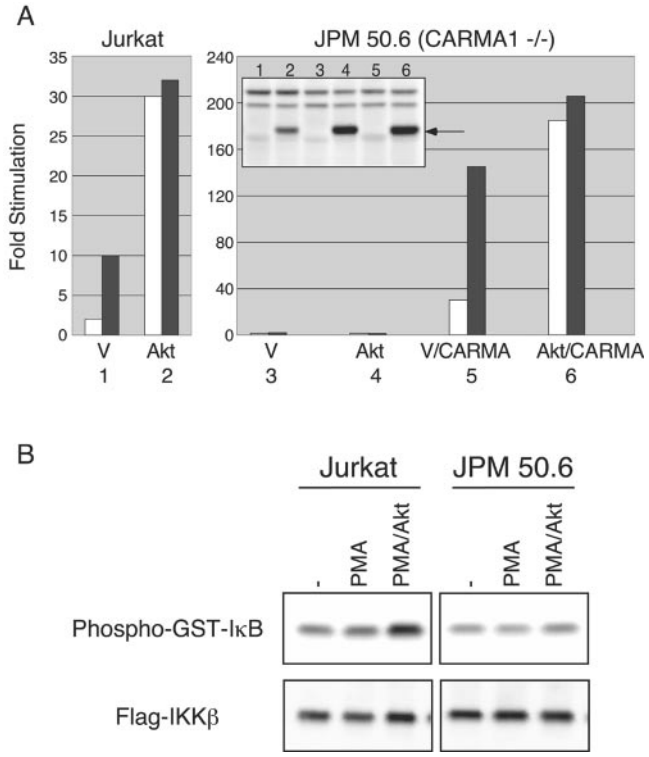

FIG. 2. NF-κB activation by Akt is impaired in the absence of CARMA1. (A) Jurkat and JPM 50.6 cells were transfected with an NF-κB luciferase reporter and the indicated plasmids. Cells were stimulated the next day for 6 hours, and luciferase activity was determined. Open bars indicate samples stimulated with PMA alone, and filled bars indicate samples stimulated with PMA plus anti-CD28. Results are displayed as the average fold stimulation over unstimulated samples, from duplicate samples of a single experiment, representative of 10 experiments that were performed. The Western blot insert shows expression of HA-Akt (indicated by the arrow) in the transfections assayed for luciferase activity. V, empty vector. (B) Jurkat and JPM 50.6 cells were transfected with a Flag-tagged IKKβ construct, along with empty vector or Akt. The next day, cells were left unstimulated (−) or treated with 10 ng/ml PMA for 30 min. IKK-β was immunoprecipitated from cell lysates and incubated with recombinant glutathione *S*-transferase (GST)–IκB substrate. Phosphorylated substrate was revealed by blotting with a phospho-IκB antibody. This experiment is representative of three experiments that were performed.

activation of the IKK complex (22). We therefore wanted to determine whether Akt could still induce IKK kinase activity in the JPM 50.6 cell line. Jurkat or JPM 50.6 cells were transfected with empty vector or active Akt, along with a Flag-tagged IKKβ construct. Cells were then left unstimulated or stimulated with a low concentration of PMA, followed by IP of the tagged IKK and in vitro kinase assays. Consistent with our previous findings, this low concentration of PMA alone is insufficient to activate IKK kinase activity in T cells but can synergize with an activated allele of Akt to do so (Fig. 2B, top left panel). However, no significant IKK activation is seen with PMA treatment, either with or without active Akt transfection, in the CARMA1-deficient cells (Fig. 2B, top right panel). These results demonstrate that Akt-mediated induction of NF-κB in T cells proceeds through the CARMA complex.

We next wanted to confirm that Akt itself is capable of being activated in JPM 50.6 cells. Consistent with previous results

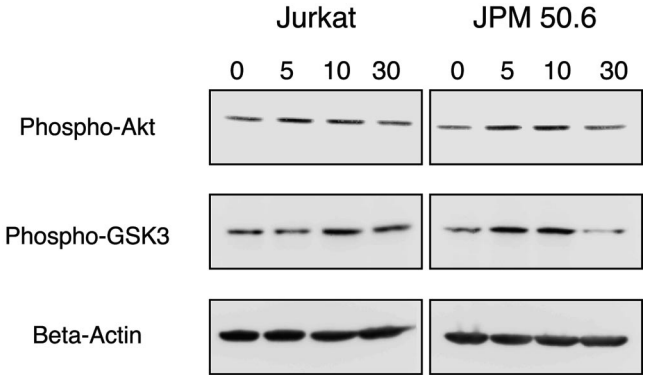

FIG. 3. Normal Akt and GSK-3 phosphorylation in JPM 50.6 cells. Jurkat and JPM 50.6 cells were stimulated with anti-TCR and anti-CD28 antibodies for the indicated times (in minutes). Lysates from one million cells per lane were subjected to SDS-PAGE and Western blotting. Blots were probed with phospho-specific antibodies to Akt or GSK-3, stripped, and reprobed with a monoclonal antibody specific for beta-actin. All blots are from a single experiment, representative of three experiments that were performed.

(48), we found that Akt is phosphorylated at serine 473 comparably in parental and CARMA1-deficient Jurkat T cells (Fig. 3, top panels). Phosphorylation of Akt is an indirect measure of its function, as it indicates only that Akt has been phosphorylated by one of two upstream kinases. We therefore examined a target that is itself known to be phosphorylated by Akt, i.e., GSK-3. As shown in Fig. 3 (middle panels), GSK-3 phosphorylation proceeded with similar kinetics and to a similar magnitude in parental and CARMA1-deficient Jurkat cells. Thus, activation of Akt is intact in CARMA1-deficient cells, yet the downstream function of this kinase that leads to the NF-κB pathway is compromised.

One of the limitations in identifying targets and pathways regulated by Akt has been the lack of a specific, cell-permeable Akt kinase inhibitor. PI 3-kinase inhibitors are widely used to inhibit Akt activation, but these inhibitors (such as wortmannin and LY294002) have the drawback that they also inhibit the modulation of other PI 3-kinase targets. In addition, the development of specific Akt inhibitors has been hampered by the fact that Akt belongs to a family of kinases, including PDK-1, PKA, PKC, and SGK (serum- and glucocorticoid-induced kinase), which contain kinase domains with significant homology to each other. Recently, a new compound (Akti 1/2) has been reported as a potent and specific inhibitor of Akt1 and Akt2 (50% inhibitory concentrations [IC_{50}s] of 58 and 210 nM), but it does not inhibit the related kinases described above or 14 other serine/threonine and tyrosine kinases (in vitro IC_{50} values all greater than 50 μM) (2, 3, 49). Also, remarkably, the IC_{50} of Akti 1/2 for Akt3 is 2.1 mM. This compound appears to act as an allosteric, PH (plekstrin homology) domain-dependent inhibitor of Akt, possibly through promotion of an inactive conformation (2, 3, 7). As shown in Fig. 4A, Akti 1/2 completely inhibited Akt activity in T cells stimulated through the TCR/CD3 complex and CD28, while not affecting Akt expression in this short-term experiment (data not shown). This effect occurs at a concentration (20 μM) well below that at which inhibition of Akt3 or other related kinases would occur and is consistent with our earlier finding that Akt1 and

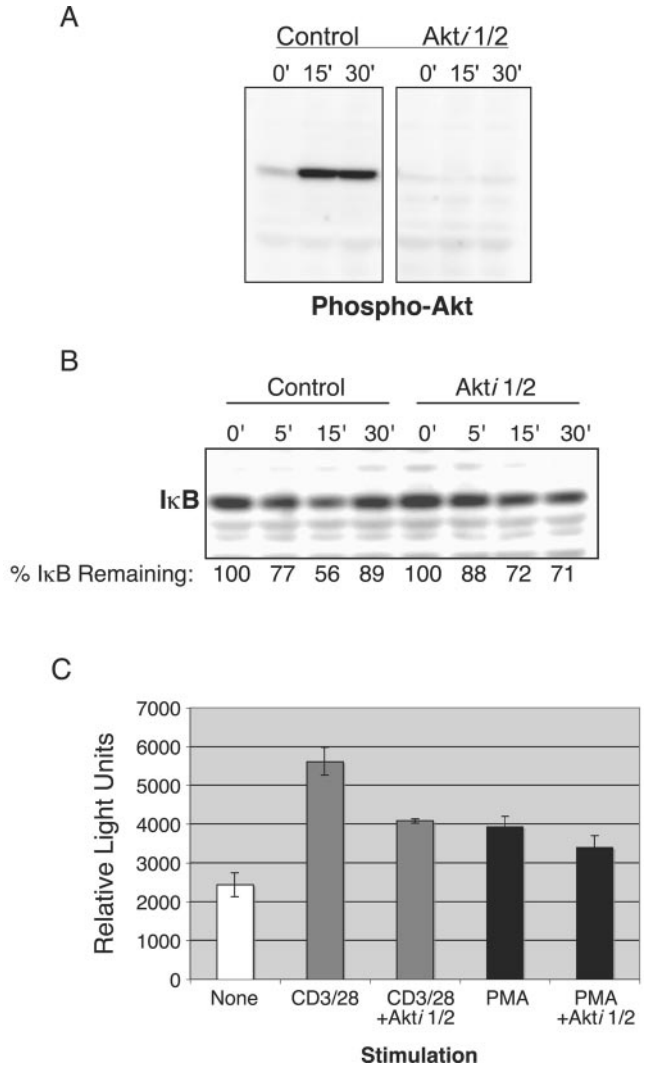

FIG. 4. A selective Akt inhibitor partially impairs CD3/CD28-mediated NF-κB activation. (A) D10 T cells were preincubated for 30 min at room temperature without or with 20 μM Akti 1/2. Cells were then stimulated at 37°C for the indicated times (0, 15, and 30 min) with biotinylated anti-CD3/CD28 antibodies (1 μg/ml each plus 5 μg/ml streptavidin). Western blots of cell lysates were probed with a polyclonal antibody specific for phospho-serine 473 in Akt. (B) D10 T cells were pretreated with Akti 1/2 and stimulated as described above for panel A. Western blots of cell lysates were probed with a polyclonal antibody specific for IκB-α. (C) D10 T cells were transfected with an NF-κB-luciferase reporter and stimulated under the conditions indicated. PMA was used at 10 ng/ml, and Akti 1/2 was used at 20 μM. Results in all cases are representative of three experiments. Values are the average number of light units ± standard deviations (error bars) from three values of a representative experiment. The inhibition of CD3/CD28-mediated NF-κB was determined to be significant by a Student's *t* test analysis ($P < 0.05$).

Akt2 are the predominant isoforms of this kinase expressed in T cells (L. P. Kane, unpublished data).

Having confirmed its ability to inhibit Akt activation, we performed functional experiments with Akti 1/2. Thus, treatment of D10 T cells with 20 μM Akti 1/2 results in partial inhibition of IκB degradation after stimulation with anti-CD3/CD28 antibodies. Note the significant degradation of IκB at the 5- and 15-minute time point in control cells compared with the cells treated with Akti 1/2 (Fig. 4B). Also, in the control cells, IκB levels begin to increase again at 30 min (since IκB is itself regulated by NF-κB), while the level of IκB in cells treated with Akti 1/2 is still decreasing (note the values at the bottom of the blot). We next examined the ability of this compound to inhibit NF-κB-dependent transcription. As shown in Fig. 4C, Akti 1/2 partially inhibited CD3/CD28-mediated activation of an NF-κB luciferase reporter in D10 T cells (about 30% inhibition). This compound did not significantly inhibit PMA-induced NF-κB activation, providing further evidence of its specificity and general lack of toxicity. Similar results were obtained with an RE/AP-luciferase reporter (data not shown), another NF-κB-dependent element that is found in the promoter of the IL-2 gene (35). These data are consistent with a modulatory role for Akt in the NF-κB pathway.

Given that Akt-mediated NF-κB induction in T cells requires the CARMA complex, we wanted to determine whether an interaction between Akt and CARMA1 could be detected. A Myc-tagged CARMA1 construct was cotransfected into 293T cells, along with either empty vector or an HA-tagged myristylated Akt construct. Thus, as shown in Fig. 5, when transfected CARMA1 was immunoprecipitated with an anti-Myc antibody, it was able to coprecipitate HA-Akt (second lane). We also performed these transfections with the addition of EE-tagged PDK-1, which was recently reported to play a role in CARMA1 membrane recruitment (26). However, inclusion of PDK-1 did not augment the ability of Akt to be coprecipitated with CARMA1 (Fig. 5A, rightmost lane). Recent studies from our lab and others have shown a role for a conserved proline-rich motif in the C-terminal domain of Akt in its activation and localization (14, 20). Since CARMA1 is predicted to contain an SH3 (Src homology region 3) domain, we considered the possibility that it might interact directly with the proline-rich sequence in Akt. We transfected a Myc-tagged CARMA1 construct into 293T cells, along with either a green fluorescent protein (GFP) fusion protein containing the C terminus of Akt or a control GFP construct. We noted that the GFP fusion containing only the C-terminal domain of Akt could coprecipitate CARMA1 (Fig. 5B, GFP-Akt lanes). However, mutation of the prolines at residues 421 and 424 did not abolish the interaction between GFP-Akt and CARMA1 but rather had a partial effect [Fig. 5B, GFP-Akt (Pro Mut) lanes]. Thus, the interaction between CARMA1 and Akt appears to be mediated at least in part by the C-terminal domain of Akt, although not exclusively through the proline-rich sequence.

In order to examine the putative association between Akt and CARMA1 by an independent assay, we transfected Myc-CARMA1, alone or together with HA-Myr-Akt, into D10 T cells, and examined CARMA1 localization by confocal microscopy. If Akt and CARMA1 can associate, we reasoned that coexpression of myristylated Akt, which resides in lipid rafts might recruit more CARMA1 into that compartment. Thus, when CARMA1 was transfected alone into D10 cells, some overlap with lipid raft staining was observed, although substantial nonraft, apparently cytoplasmic, staining was also seen (data not shown). However, when Myr-Akt was cotransfected with CARMA1, the latter displayed a tighter ring of staining around the periphery of cells and almost complete overlap with lipid raft staining (data not shown).

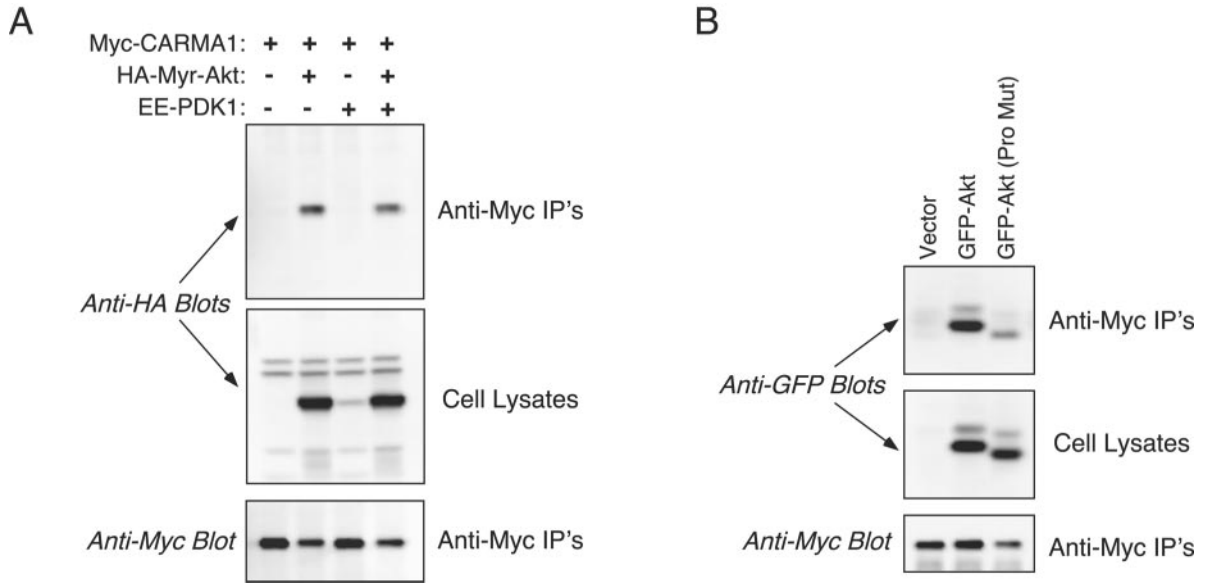

FIG. 5. Association between Akt and CARMA1. HEK 293T cells were transfected with Myc-tagged CARMA1 and the indicated constructs. The next day, IPs were performed with anti-Myc antibody and immunoprecipitated proteins were separated by SDS-PAGE. Western blots were probed with anti-HA (A) or anti-GFP (B) antibody to detect Akt in the immunoprecipitates (IP's) or cell lysates. Blots were stripped and reprobed with anti-Myc antibody to control for levels of Myc-CARMA in the immunoprecipitates (Anti-Myc IP's). Results are representative of three (A) or five (B) experiments that were performed. Pro Mut, proline mutation.

Having observed that Akt and CARMA1 can interact, we wished to determine under somewhat more physiological circumstances whether the PI 3-kinase/Akt pathway might modulate CARMA1 localization. We have attempted to visualize endogenous CARMA1 by direct staining but have not yet found an appropriate antibody for this application (Kane, unpublished). We therefore transfected Myc-CARMA1 into D10 T cells, along with an Akt PH-GFP fusion protein, as a marker for PI 3-kinase activation. We then formed conjugates between these cells and antigen-loaded APCs and followed the subcel-

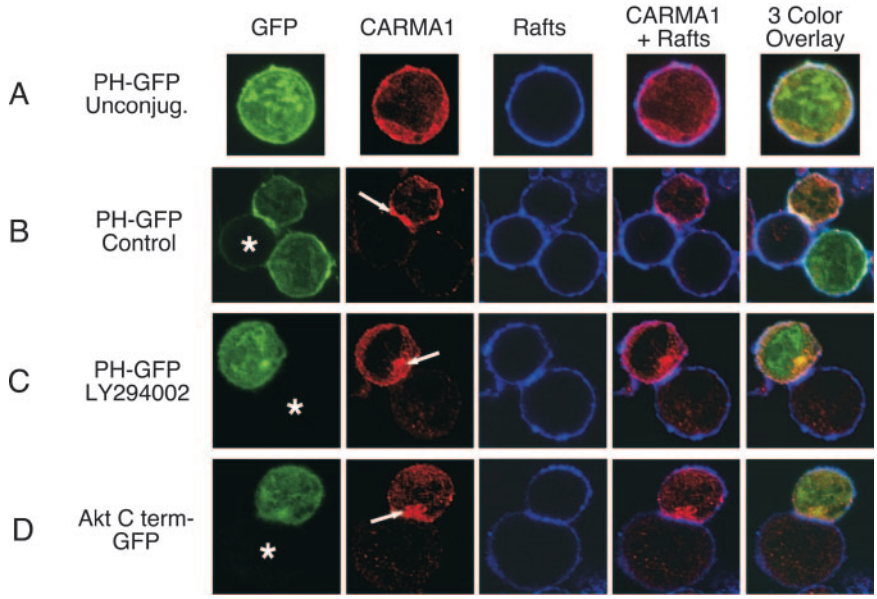

FIG. 6. CARMA1 localization during T-cell activation by APCs. D10 T cells were transfected with the indicated GFP constructs and Myc-tagged CARMA1. The next day T cells—alone (A) or in conjugates (B to D) with antigen-loaded APCs—were settled onto slides. Slides were stained with Cy3-conjugated anti-Myc antibody and Alexa 647-conjugated cholera toxin B subunit to reveal lipid rafts and analyzed by confocal microscopy. (C) T cells were pretreated for 1 hour with 20 μM LY294002 before formation of conjugates. The fields in row B include a cell expressing PH-GFP but not Myc-CARMA1 (lower right). Images are representative of 20 to 30 cells analyzed in each case. The asterisks indicate the location of the APC in each series of conjugates. Unconjug., unconjugated; Akt C term-GFP, GFP fusion containing the C-terminal domain of Akt (Akt C term-GFP).

lular localization of CARMA1 during this early phase of T-cell activation. As shown in Fig. 6, a large proportion of CARMA1 relocalizes to the contact site between the T cell and APC, similar to the PH-GFP fusion protein (compare CARMA1 staining in Fig. 6A and B). This localization also coincides with clustering of lipid raft components, as revealed by cholera toxin B subunit staining (note the white area in three-color overlay of Fig. 6B). Coexpression of the PH-GFP construct does not appear to affect CARMA1 localization, since similar results were noted when CARMA1 was transfected alone (data not shown). We next investigated whether inhibition of the PI 3-kinase pathway could impact this localization of CARMA1 during conjugate formation. As shown in Fig. 6C, pretreatment of transfected D10 T cells prevented membrane localization of PH-GFP. This treatment also had a noticeable effect on CARMA1 relocalization, which is most obvious in the CARMA1 and raft overlay panel. Thus, there was less CARMA1 in tight association with the plasma membrane; in addition, many cells displayed a "bunching up" of CARMA1 in the vicinity of the APC contact site (indicated by the white arrow), although this was never as closely positioned to the APC as in control cells (compare with CARMA1 staining in Fig. 6B). As shown in Fig. 6D, very similar results were obtained when CARMA1 was cotransfected with a GFP fusion containing the C-terminal domain of Akt (Akt C term-GFP). This GFP fusion protein can interact with CARMA1 (Fig. 5B) but cannot localize to the plasma membrane or T-cell–APC contact site (20). As with LY294002 treatment, cotransfection with Akt C term-GFP resulted in less CARMA1 recruitment to the plasma membrane and APC contact site, although once again accumulation of CARMA1 in the vicinity of the contact site was observed in many conjugates (white arrow).

Recent studies have indicated that signals that impinge on NF-κB, including TCR ligation, result in phosphorylation of Bcl10 (5, 33, 42), although the functional consequences of this phosphorylation are still not clear. Given the role of the CARMA1-Bcl10-MALT1 (CBM) complex in Akt-mediated NF-κB induction, we sought to determine whether the Akt pathway might modulate Bcl10 phosphorylation. We expressed Bcl10 by itself, with activated Akt or with the PTEN phosphatase, which negatively regulates the PI 3-kinase/Akt pathway. Lysates were made and analyzed by Western blotting with a Bcl10 antibody. As shown in Fig. 7A, two prominent bands (and a weaker, third, top band) are detected when Bcl10 is transfected into 293T cells; the top band appeared to be a phosphorylated form, since cotransfection with activated Akt resulted in an increase in its intensity. Furthermore, cotransfection with the phosphatidylinositol 3,4,5-trisphosphate (PIP_3) phosphatase PTEN led to a decrease in the intensity of the top band. Neither condition detectably affected the intensity of the lower band. This finding is consistent with previous reports that Bcl10 can be phosphorylated in HeLa and T cells, although no kinase was identified (33, 42). To directly address whether the top band results from phosphorylation, Bcl10 was transfected with or without Myr-Akt, followed by IP of the transfected Bcl10. One half of each immunoprecipitate was treated with lambda phosphatase (Fig. 7B, Ppase), a broad-spectrum phosphatase. As shown in Fig. 7B, treatment of Bcl10 immunoprecipitates with the phosphatase led to almost complete elimination of the prominent top band (and the weaker topmost band), while the lower band increased in intensity (compare Myr-Akt lanes in the two gels). Thus, the top anti-Bcl10-reactive bands result from phosphorylation that can be increased by activated Akt.

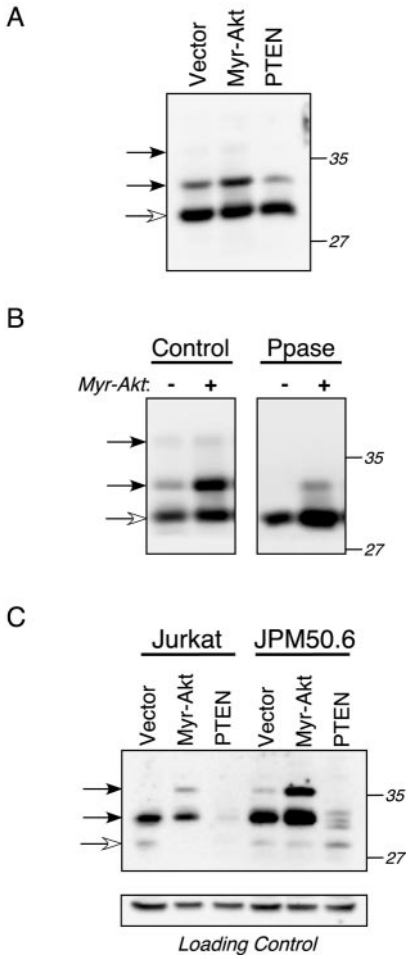

FIG. 7. Akt-dependent, but CARMA1-independent, phosphorylation of Bcl10. (A) Human embryonic kidney (HEK) 293T cells were transfected with Bcl10 and the plasmids indicated above the lanes. Cytoplasmic lysates were separated by SDS-PAGE and Western blotted with an antibody to Bcl10. (B) HEK 293T cells were transfected with Bcl10, with (+) or without (−) Myr-Akt. Lysates were made, followed by IP of Bcl10. Immunoprecipitates were divided into two, and half were treated with lambda phosphatase (Ppase). SDS-PAGE and Bcl10 Western blotting were then performed. (C) Parental Jurkat T cells or CARMA1-deficient JPM 50.6 cells were transfected with Bcl10 and the indicated plasmids. Cell lysates were subjected to SDS-PAGE and Western blotting for Bcl10. The lower, open arrow indicates the location of nonphosphorylated Bcl10, while the top, closed arrows point to the phosphorylated forms of the protein. The positions of molecular mass markers (in kilodaltons) are indicated on the right-hand side of each blot. Results are representative of five (A), two (B), and three (C) experiments that were performed.

Given our finding that CARMA1 expression is required for Akt-mediated NF-κB activation, we examined whether the phosphorylation of Bcl10 also requires CARMA1. Therefore, Bcl10 was transfected into parental or CARMA1-deficient Jurkat cells with either Myr-Akt or PTEN as described above. As shown in Fig. 7C, expression of Bcl10 in Jurkat T cells also led to the appearance of multiple Bcl10-reactive bands, al-

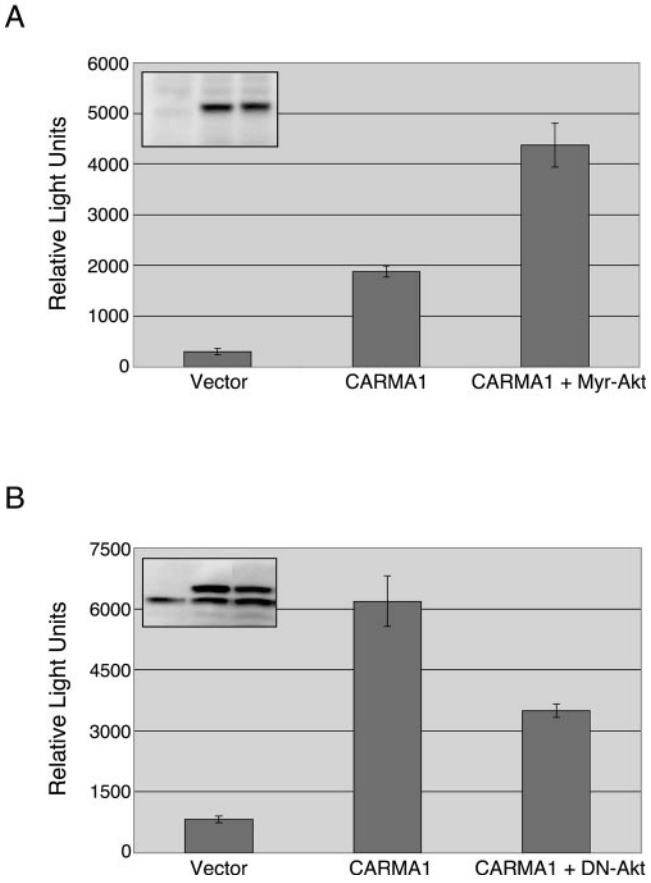

FIG. 8. Modulation of CARMA1-induced NF-κB by Akt. Jurkat T cells were transfected with an NF-κB-luciferase reporter and 7 μg of empty vector or CARMA1, plus either 5 μg of myristylated Akt (Myr-Akt) (A) or 3 μg of dominant-negative (DN) Akt (B). Luciferase activity was determined the next day, and is presented as the average number of light units from triplicate samples ± standard deviations (error bars) of a single experiment, representative of five experiments that were performed in each case. The inserts show Western blot analysis of Myc-CARMA1 expression. The blot in panel B includes an additional nonspecific band (lower band), because the anti-Myc (9E10) antibody used in this experiment was from a different source.

though a greater fraction of the protein appeared to be phosphorylated than in the experiments conducted in 293T cells shown in Fig. 7A (note closed arrows). Consistent with those experiments, coexpression of Myr-Akt led to a further decrease in Bcl10 mobility (top closed arrow). Curiously, in the Jurkat cells, coexpression of PTEN led to a nearly complete elimination of Bcl10 protein, which did not appear to be the result of nonspecific cell death or inhibition of transfection (data not shown). The same pattern was observed in lysates of JPM 50.6 cells after Bcl10 transfection; these cells often transfected with greater efficiency than the parental Jurkat cells did (data not shown), which more than likely accounts for the overall higher levels of Bcl10 protein seen in this experiment. These results demonstrate that the Akt pathway can regulate the phosphorylation status of Bcl10, which may explain in part the effect of Akt on NF-κB activation.

Finally, we sought to determine more directly whether modulation of the Akt pathway could affect NF-κB activation driven by CARMA1. We overexpressed CARMA1 in Jurkat T cells to induce NF-κB and cotransfected either activated (myristylated) Akt or a dominant-negative form of Akt that had been mutated at both sites of regulatory phosphorylation (S473 and T308) as well as the ATP-binding site in the kinase domain (K179). As shown in Fig. 8A, cotransfection of Myr-Akt along with CARMA1 resulted in a two- to threefold increase in the degree of NF-κB activation induced by CARMA1 alone. Conversely, cotransfection of the dominant-negative Akt (Fig. 8B) resulted in an approximately twofold decrease in CARMA1-mediated NF-κB activation. These results are consistent with our model that CARMA1 lies downstream of Akt, which can modulate NF-κB activation by the TCR and CD28.

DISCUSSION

Recent years have witnessed a significant amount of progress in understanding how T- and B-cell antigen receptors are coupled to activation of the IKK complex and induction of NF-κB. Currently, much attention is focused on three proteins—CARMA1, Bcl10, and MALT1—hypothesized to function as part of a complex (the CBM complex (39) upstream of the IKKs and downstream of PKC θ. Previously we reported that Akt can induce NF-κB in T-cell lines and substitute for the CD28 costimulatory signal. We have shown here that NF-κB induction in T cells by the Akt kinase requires the expression of CARMA1, although Akt activation appears to proceed unimpaired in the absence of this protein. Finally, we have provided evidence that Akt acts proximally to the putative CBM complex, possibly through a direct interaction with CARMA1 as well as by modulating phosphorylation of another member of the complex, Bcl10. This is the first report to demonstrate connections between Akt and the CBM complex. The role of Akt upstream of the IKK complex, on the basis of our work and that of others, is summarized in the model contained in Fig. 9.

We had previously demonstrated that Akt induction of NF-κB in T cells, unlike certain other cell types, requires activation of PKC, which can provided by phorbol ester, overexpression of PKC θ, or ligation of the TCR (18, 22). A previous report had suggested that PKC θ localization to the immunologic synapse and/or lipid rafts is impaired in CARMA1-deficient Jurkat T cells (47), although this was not observed in murine T cells lacking CARMA1 (11). Since we wanted to assess the ability of Akt (and PKC) to activate NF-κB in the CARMA1-deficient Jurkat T cells, it was important to determine whether there is indeed any impairment in PKC θ activation and/or localization in these cells. Thus, although we observed somewhat lower levels of total PKC θ protein in CARMA1-deficient JPM 50.6 Jurkat cells, we noted that this protein could still be inducibly phosphorylated and recruited to the immunological synapse (data not shown).

A recent report has suggested that CARMA1-deficient murine T cells do not have a total block in activation but can proliferate in response to high concentrations of cognate peptide on antigen-presenting cells (11). This is in contrast to previous reports that noted a nearly complete lack of proliferation by CARMA1 knockout cells in response to stimulation with anti-CD3/CD28 antibodies (8, 12, 29). We found that longer incubation of JPM 50.6 cells after transfection of activated PKC θ actually resulted in substantial NF-κB activation

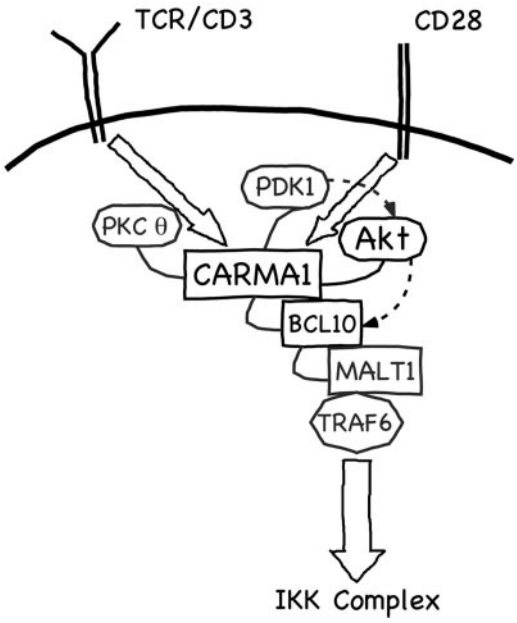

FIG. 9. Model for Akt participation in NF-κB induction by the TCR and CD28. Thick open arrows denote major pathways from which details have been omitted for simplicity. Curved lines indicate protein-protein interactions, which may or may not be direct, that have been demonstrated in this and other reports. Curved, dashed arrows indicate likely (not necessarily direct) phosphorylation events.

(after at least 24 h, compared with the usual 6-hour assay), while treatment for the same amount of time with PMA/ionomycin or anti-CD3/CD28 did not. These results suggest that stimulation through another receptor (or receptors) for which there is a ligand on APC, provides a more sustained signal that can overcome the block to proliferation in CARMA-deficient cells. This interaction is presumably missing from the Daudi/SEE/Jurkat system employed in Fig. 1, since we did not observe detectable NF-κB activity when JPM 50.6 cells were stimulated under these conditions.

Early in the course of T-cell activation, Akt and PKC θ appear to act through the CBM complex, which may directly interface with the IKK complex (38, 40, 50). We have provided evidence for both physical and functional interactions between Akt and the CBM complex. First, we have observed an interaction between CARMA1 and either full-length Akt or its C-terminal domain (Fig. 5). The nature of this interaction is still not clear, but the interaction may rely at least in part on the proline-rich sequence in the C-terminal domain of Akt. Our results also demonstrate that inhibition of Akt membrane recruitment results in less efficient relocalization of CARMA1 to the plasma membrane and APC contact site during the initial phase of T-cell activation by antigen/APC (Fig. 6). Intriguingly, these findings correlate with those of Wang et al. (47), who showed apparently constitutive membrane association of wild-type CARMA1 in Jurkat T cells, which display high basal levels of PIP_3 and Akt activity, due to lack of PTEN expression (34).

We have investigated CARMA1 relocalization in the presence of the novel Akt inhibitor employed in Fig. 4 (Akti 1/2) but have not observed any effects (data not shown). This is not entirely surprising, since published data demonstrate that while Akti 1/2 may require the PH domain of Akt to effect inhibition, it does not bind directly to that domain (7). Thus, the inhibitor appears to stabilize the closed, inactive confirmation of Akt, rather than competing with PIP_3 for binding to the PH domain. Under these circumstances, the inhibitor would be expected to have little if any impact on Akt (or CARMA1) localization to the plasma membrane.

The results presented in Fig. 7 indicate that the PI 3-kinase/Akt pathway can regulate the phosphorylation status of Bcl10. Previous groups have reported on the phosphorylation of Bcl10 (5, 33, 42), with one group providing evidence that this phosphorylation occurs downstream of the receptor-interacting protein 2 (RIP2) protein (33). This latter observation is particularly intriguing in light of another recent report that placed the related protein RIP1 upstream of Akt in the TLR4 (Toll-like receptor 4) signaling pathway (46). Although this has not been shown in T cells, it may be that one or more RIP proteins (of which at least four are expressed in mammals) links the TCR and CD28 to Akt, leading to Bcl10 phosphorylation. In addition, it is still unclear which kinase(s) is directly responsible for the phosphorylation of Bcl10, an issue that is often difficult to resolve. Nonetheless, there is one potential site of Akt-mediated phosphorylation within Bcl10, as predicted by the consensus sequence RXRXXS/T (1).

Finally, the functional consequences of Bcl10 phosphorylation are still unclear, e.g., does it affect Bcl10 localization or assembly of the CBM complex? Clearly, Akt-dependent phosphorylation of Bcl10 is not sufficient to increase NF-κB activation, since this still occurs in CARMA1-deficient T cells (Fig. 7C). Intriguingly, our results in T cells also suggest that the Akt pathway may regulate Bcl10 protein stability or translation, since ectopic expression of PTEN results in a loss of cotransfected Bcl10 protein (Fig. 7C). This would be consistent with recent reports demonstrating selective regulation of protein stability by PTEN (13) and Akt (33). We are currently assessing this possibility. We are also currently attempting to map the sites of phosphorylation within Bcl10, which will aid in the identification of the kinase(s) responsible for its phosphorylation, as well as the consequences of such phosphorylation.

Given our finding that a specific Akt inhibitor partially blocks activation of an NF-κB reporter (Fig. 4), it appears that Akt is not an obligate component of CD3/CD28-induced NF-κB signaling, at least in T cells. However, our data are consistent with Akt playing a role as a kind of "rheostat," which may modulate the strength and/or duration of NF-κB activation in these cells. In this regard, it should be instructive to obtain a global view of gene transcription in the presence or absence of Akt inhibitors, as such compounds may have relatively specific effects on new gene transcription that accompanies T-cell activation. This type of analysis has recently revealed such a role for the GSK-3 kinase in modulating NF-κB function, such that only a subset of NF-κB-regulated genes is affected by loss of GSK-3 function (37). In addition to this physiological pathway downstream of CD3 and CD28, NF-κB-dependent transcription may also play a pathological role in the transformation of cells that occurs as a result of increased Akt expression and/or kinase activity (10, 23, 30, 45).

Reports that appeared within the past year have significantly complicated the picture of signaling from the TCR and CD28 to

the IKKs, which now includes the PDK-1 kinase, TRAF6 (TNF receptor-associated factor 6)-mediated ubiquitination of IKKγ, and activation of the protease caspase 8 (44). Thus, recent data suggest that the CBM complex, along with TRAF6, leads to IKK activation in part by mediating ubiquitination of the IKKγ subunit (40, 50), although it is still not clear how ubiquitination regulates IKK function. Another protein that is now thought to lie between antigen receptors and the IKK complex is caspase 8 (39), although again it is still not clear precisely how this enzyme contributes to IKK activation. It may be of interest to determine whether Akt can modulate the function of any of these novel components of the NF-κB pathway.

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We have no conflicts of interest to disclose.

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