NF-κB activation following engagement of the antigen-specific T cell receptor involves protein kinase C-δ-dependent assembly of the CARMA1-BCL10-MALT1 (CBM) signalosome, which coordinates downstream activation of IκB kinase (IKK). We previously identified a novel role for the adhesion- and degranulation-promoting adapter protein (ADAP) in regulating the assembly of the CBM complex via an interaction of ADAP with CARMA1. In this study, we identify a novel site in ADAP that is critical for association with the TAK1 kinase. ADAP is critical for recruitment of TAK1 and the CBM complex, but not IKK, to protein kinase C-δ. ADAP is not required for TAK1 activation. Although both the TAK1 and the CBM complex binding sites in ADAP are essential for IκBα phosphorylation and degradation and NF-κB nuclear translocation, only the TAK1 binding site in ADAP is necessary for IKK phosphorylation. In contrast, only the CARMA1 binding site in ADAP is required for ubiquitination of IKKγ. Thus, distinct sites within ADAP control two key activation responses that are required for NF-κB activation in T cells.

In the immune system, the NF-κB transcription factor pathway plays a central role in T cell activation and survival (1, 2). The canonical NF-κB pathway involves activation of the IκB-kinase (IKK) complex, which consists of the catalytic IKKα and IKKβ subunits and the regulatory IKKγ (NF-κB essential modulator (NEMO)) subunit. Activated IKK mediates phosphorylation of IκBα, resulting in IκBα degradation and translocation of NF-κB to the nucleus. In T cells, stimulation of the T cell receptor and the CD28 co-stimulatory receptor results in activation of the PKCθ isoform and association of the IKK complex with PKCθ(3). PKCθ phosphorylates the membrane-associated guanylate kinase (MAGUK) family member adapter CARMA1, which coordinates downstream activation of IKK, resulting in IκB phosphorylation and IKKγ-dependent NF-κB nuclear translocation, only the TAK1 binding site in ADAP is necessary for IKK phosphorylation. In contrast, only the CARMA1 binding site in ADAP is required for ubiquitination of IKKγ. Thus, distinct sites within ADAP control two key activation responses that are required for NF-κB activation in T cells.

**NF-κB Activation in T Cells Requires Discrete Control of IκB Kinase α/β (IKKα/β) Phosphorylation and IKKγ Ubiquitination by the ADAP Adapter Protein**

Rupa Srivastava, Brandon J. Burbach, and Yoji Shimizu

From the Department of Laboratory Medicine and Pathology, Center for Immunology, Masonic Cancer Center, University of Minnesota Medical School, Minneapolis, Minnesota 55455

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Independent Sites in ADAP Control NF-κB Activation

**RESULTS**

**ADAP Regulates IKKα/β Phosphorylation and Recruitment of TAK1 to the PKCθ Signalosome**—We analyzed TAK1-mediated regulation of NF-κB by first examining ADAP-dependent IKKα/β phosphorylation following stimulation of naïve mouse T cells with anti-CD3 and anti-CD28 antibodies that engage the T cell receptor and the CD28 co-stimulatory receptor. Although CD3/CD28 stimulation of control T cells resulted in IKKα/β phosphorylation observed within 10 min of stimulation, IKKα/β phosphorylation was only detectable at late time points (30–40 min) after stimulation of ADAP−/− T cells (Fig. 1A). This was not due to impaired IKK recruitment of the PKCθ signalosome in the absence of ADAP as CD3/CD28 stimulation resulted in increased IKK in PKCθ IPs from control and ADAP−/− T cells (Fig. 1B). CD3/CD28 stimulation has also been reported to induce the recruitment of TAK1 and the CBM complex to PKCθ (11). Thus, we also analyzed PKCθ IPs and found that recruitment of TAK1, as well as CARMA1 and BCL-10, was dramatically impaired following CD3/CD28 stimulation of ADAP−/− T cells (Fig. 1C). Similar results were observed when we specifically immunoprecipitated TAK1 from control and ADAP−/− T cells (Fig. 1D). Expression of wild-type ADAP in ADAP−/− T cells restored CD3/CD28-mediated recruitment of TAK1, CARMA1, and BCL-10 to the PKCθ signalosome (Fig. 1E). Together, these results show that ADAP is critical for CD3/CD28-mediated IKKα/β phosphorylation and recruitment of TAK1 and the CBM complex to the PKCθ signalosome.

**A Novel Site in the C-terminal End of ADAP Is Critical for TAK1 Association**—To define the region of ADAP critical for CD3/CD28-mediated association with TAK1, we expressed a series of HA-tagged ADAP truncation mutants in Jurkat T cells and tested their ability to co-IP TAK1 following CD3/CD28 stimulation. Both wild-type ADAP and mutant ADAP containing amino acids 1–719 were able to co-IP TAK1 in CD3/CD28-stimulated T cells (Fig. 2B). In contrast, an ADAP mutant containing amino acids 1–604 was not able to co-IP TAK1. This suggests that the region between amino acids 604 and 719 in ADAP is critical for TAK1 association.
ADAP is critical for TAK1 association. The region of ADAP critical for TAK1 association was further mapped to amino acids 691–708 within ADAP (amino acid sequence DASDFP-PAPAEMSQQGSMV) as a mutant lacking this region (ADAPΔCAR) was unable to co-IP TAK1 following CD3/CD28 stimulation of Jurkat T cells (Fig. 2B).

We previously showed that a distinct ADAP mutant (ADAPΔCAR lacking amino acids 426–541) is critical for ADAP association with CARMA1 and CD3/CD28-mediated assembly of the CBM complex in primary naive mouse T cells (20). In contrast to ADAPΔTAK, immunoprecipitation of ADAPΔCAR was able to co-IP TAK1 in both Jurkat T cells (Fig. 2B) and ADAP−/− T cells expressing the ADAPΔCAR mutant (Fig. 2C). Conversely, only the ADAPΔTAK mutant was able to co-IP BCL-10. Similar results were observed when we examined anti-ADAP IPs of ADAP−/− T cells expressing these mutants (Fig. 2C). Furthermore, expression of the ADAPΔTAK mutant restored the recruitment of CARMA1 and BCL-10, but not TAK1, to PKCθ, whereas the ADAPΔCAR mutant restored only the recruitment of TAK1 to PKCθ (Fig. 2D).

ADAP Mediates NF-κB Activation via IKKα/β Phosphorylation and IKKγ Ubiquitination—Activation of the IKK complex involves CARMA1-dependent ubiquitination of IKKγ and TAK1-dependent phosphorylation of IKKα/β (8–10). We found that CD3/CD28-mediated ubiquitination of IKKγ was dramatically impaired in ADAP−/− T cells (Fig. 3A). Expression of wild-type ADAP or ADAPΔTAK in ADAP−/− T cells restored CD3/CD28-mediated ubiquitination of IKKγ to levels observed with control T cells, whereas ADAPΔCAR was unable to restore IKKγ ubiquitination (Fig. 3A). Expression of wild-type ADAP in ADAP−/− T cells also restored CD3/CD28-mediated IKKα/β phosphorylation (Fig. 3B). Although ADAPΔTAK was able to restore IKKγ ubiquitination, this mutant was unable to restore IKKα/β phosphorylation (Fig. 3B). In contrast, expression of ADAPΔCAR in ADAP−/− T cells restored CD3/CD28-mediated phosphorylation of IKKα/β (Fig. 3B). Thus, distinct sites within ADAP independently control CARMA1-dependent IKKγ ubiquitination and TAK1-dependent IKKα/β phosphorylation.

We also assessed the role of ADAP in CD3/CD28-mediated increases in TAK1 enzymatic activity. CD3/CD28 stimulation of both wild-type and ADAP−/− T cells resulted in similar increases in TAK1 activity in TAK1 IPs, as assessed by in vitro phosphorylation of a GST-IKK fusion protein (Fig. 3C). Expression of wild-type ADAP, the ADAPΔTAK mutant, or the ADAPΔCAR mutant did not alter CD3/CD28-mediated activation of TAK1. These results suggest that CD3/CD28-mediated activation of TAK1 in T cells does not require ADAP expression. Fig. 3D demonstrates that all cell samples analyzed were infected with recombiant adenovirus, as assessed by flow cytometric analysis of expression of the Thy1.1 expression marker encoded by our recombiant adenovirus. In addition, Western blotting analysis demonstrates expression of wild-type ADAP and ADAP mutants at levels comparable with ADAP expression in control wild-type T cells.

Both the TAK1 Binding Site and the CARMA1 Binding Site in ADAP Are Required for IkBα Phosphorylation and Degradation and NF-κB Nuclear Translocation—To determine whether both the TAK1 binding site and the CARMA1 binding site in ADAP are important for NF-κB activation, we analyzed IkBα phosphorylation and degradation, as well as nuclear translocation of NF-κB. Each of these sites is independently critical for full activation of NF-κB as expression of either the ADAPΔTAK or the ADAPΔCAR mutant in ADAP−/− T cells did not restore CD3/CD28-mediated IkBα phosphorylation and degradation (Fig. 4A). Impaired nuclear translocation of p65 in ADAP−/− T cells was also not restored by expression of either the ADAPΔTAK or the ADAPΔCAR mutant (Fig. 4B). CD3/CD28-mediated phosphorylation of Erk was comparable in all samples analyzed, demonstrating that CD3/CD28-mediated signaling was not globally impaired (Fig. 4A).

DISCUSSION

In this study, we have defined the mechanism by which the adapter protein ADAP regulates NF-κB activation in T cells. Because our previous work showed that ADAP associates with CARMA1 and regulates CD3/CD28-mediated assembly of the CBM complex (20), we analyzed signaling responses that are required for IKK complex activation. Ubiquitination of the IKKγ regulatory subunit has been shown to be dependent on CARMA1 expression (11). Consistent with this earlier work, we demonstrate here that ADAP−/− T cells exhibit impaired IKKγ...
ubiquitination following CD3/CD28 stimulation. In addition, expression of the ADAP ΔCAR mutant, which does not interact with CARMA1 (20), in ADAP−/− T cells cannot restore CD3/CD28-mediated IKKγ ubiquitination. These results highlight the critical role that ADAP plays in CARMA1-dependent activation of IKK and are consistent with other evidence linking CARMA1 to IKKγ ubiquitination (11).

We also revealed that CD3/CD28-mediated recruitment of TAK1 to the PKCθ signalosome is dependent on ADAP, and TAK1 can also be co-immunoprecipitated with ADAP. We traced this activity to a small region in the C-terminal end of murine ADAP between amino acids 691 and 708 (amino acid sequence DASDFPPPPAEMSQGMSV) that is highly conserved in the human, monkey, and chicken orthologs of ADAP. TAK1 has been proposed to play a key role in activating IKK by phosphorylating IKKα/β (9, 10). However, a requirement for TAK1 in T cell receptor-mediated activation of NF-κB appears to be dependent at least in part on the differentiation state of the T cell as NF-κB activation is impaired in TAK1-deficient thymocytes and mature single-positive thymocytes but not in TAK1-deficient effector T cells (14, 15). The expression system in this study utilized recombinant adenovirus and transgenic mice expressing the hCAR receptor (20, 21). Because this system allows for efficient transduction of primary, non-cycling naive T cells (22), our findings are consistent with a requirement for TAK1 in NF-κB activation in naive T cells.

Regulated recruitment of the IKK complex and proteins that regulate IKK complex activation to PKCθ is a critical early step in CD3/CD28-mediated activation of NF-κB (1, 3, 25). 3-Phosphoinositide-dependent kinase 1 (PDK1) has been proposed to play a dual role in this recruitment by regulating PKCθ-mediated recruitment of the IKK complex and the recruitment of CARMA1 to the PKCθ signalosome (26). ADAP also appears to play a dual role in regulating recruitment of NF-κB signaling proteins to the PKCθ signalosome as distinct sites in ADAP are critical for the recruitment of TAK1 and CARMA1 to PKCθ. Although TAK1 and CARMA1 have been proposed to interact with each other, TAK1 recruitment to PKCθ and IKKα/β phosphorylation is not altered in CARMA1-deficient T cells (11, 16, 17). We suggest that ADAP plays a central role in this CARMA1-independent recruitment of TAK1 to PKCθ. PDK1 is also critical for the phosphorylation and activation of PKCθ (26). In contrast, our earlier studies demonstrated that CD3/CD28-mediated phosphorylation of PKCθ is not dependent on ADAP (20). This is consistent with our findings in this study of the normal recruitment of IKK to PKCθ in ADAP−/− T cells. Similarly, loss of ADAP does not alter CD3/CD28-mediated activation of TAK1. Thus, these results suggest that ADAP functions to recruit active TAK1 to the PKCθ signalosome, where it can then promote the activation of the IKK complex by phosphorylating IKKα/β.

ADAP IPs were probed for TAK1, CARMA1, BCL-10, and ADAP. D, control hCAR+ T cells (Ctrl) and hCAR− ADAP−/− T cells were transduced and stimulated as in C. PKCθ IPs were probed for ADAP, TAK1, BCL10, CARMA1, and PKCθ.
Our identification of sites within ADAP that independently control association with CARMA1 and TAK1 provide a biochemical basis for the independent control of CARMA1-mediated IKK\(\gamma\) ubiquitination and TAK1-dependent IKK\(\gamma\)/\(\beta\) phosphorylation in T cells (1, 11, 25). Both of these sites are critical for NF-\(\kappa\)B signaling as expression of either the ADAP\(\Delta\)CAR or the ADAP\(\Delta\)TAK mutant was not able to restore CD3/CD28-mediated phosphorylation and degradation of I\(\kappa\)B\(\alpha\), as well as subsequent nuclear translocation of p65, in ADAP\(\Delta\)CAR/\(\Delta\)TAK T cells.

Thus, our results support a central role for ADAP in regulating both CARMA1-dependent IKK\(\gamma\) ubiquitination and TAK1-mediated IKK\(\alpha/\beta\) phosphorylation, two key events that lead to IKK complex activation and NF-\(\kappa\)B activation in T cells.

**FIGURE 3.** Independent control of IKK\(\gamma\) ubiquitination and IKK\(\alpha/\beta\) phosphorylation by ADAP. Control hCAR\(^{+}\) T cells (Ctrl) and hCAR\(^{-}\) ADAP\(^{-}\) T cells were transduced with adenovirus encoding Thy1.1 alone (Thy) or wild-type ADAP (WT), the ADAP\(\Delta\)CAR mutant, or the ADAP\(\Delta\)TAK mutant prior to CD3/CD28 stimulation for 15 min (A) or 45 min (B). A, lysates were immunoblotted with antibodies specific for ubiquitin and IKK\(\gamma\). B, IKK\(\alpha/\beta\) IPs were probed for phosphorylated IKK and IKK. C, in vitro kinase assays were performed with TAK1 IPs. Phosphorylation of GST-IKK was assessed by Western blotting with an anti-phospho-IKK antibody (p-GST IKK). Samples were also probed with an anti-IKK antibody (GST IKK). D, flow cytometry analysis of T cells infected with adenovirus with an anti-Thy1.1 antibody, which detects the Thy1.1 cell surface protein expressed by all recombinant adenoviruses used in this study. Cell lysates were also immunoblotted with an anti-ADAP antibody to confirm ADAP expression (bottom).

Phosphorylation of GST-IKK was assessed by Western blotting with an anti-phospho-IKK antibody (p-GST IKK). Samples were also probed with an anti-IKK antibody (GST IKK). D, flow cytometry analysis of T cells infected with adenovirus with an anti-Thy1.1 antibody, which detects the Thy1.1 cell surface protein expressed by all recombinant adenoviruses used in this study. Cell lysates were also immunoblotted with an anti-ADAP antibody to confirm ADAP expression (bottom).
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