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Essential Role for IκB Kinase β in Remodeling Carma1-Bcl10-Malt1 Complexes upon T Cell Activation

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Summary

T cell receptor (TCR) signaling to IκB kinase (IKK)/NF-κB is controlled by PKCθ-dependent activation of the Carma1, Bcl10, and Malt1 (CBM) complex. Antigen-induced phosphorylation of Bcl10 has been reported, but its physiological function is unknown. Here we show that the putative downstream kinase IKKβ is required for initial CBM complex formation. Further, upon engagement of IKKβ/Malt1/Bcl10 with Carma1, IKKβ phosphorylates Bcl10 in the C terminus and thereby interferes with Bcl10/Malt1 association and Bcl10-mediated IKKγ ubiquitination. Mutation of the IKKβ phosphorylation sites on Bcl10 enhances expression of NF-κB target genes IL-2 and TNFα after activation of primary T cells. Thus, our data provide evidence that IKKβ serves a dual role upstream of its classical substrates, the IκB proteins. While being essential for triggering initial CBM complex formation, IKKβ-dependent phosphorylation of Bcl10 exhibits a negative regulatory role in T cell activation.

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Introduction

T cell activation is initiated by the simultaneous engagement of the T cell receptor (TCR) and the CD28 costimulatory receptor. TCR/CD28 coligation causes the assembly of receptor molecules, signaling mediators, and lipid rafts to form the immunological synapse at the interface between the T cell and an antigen-presenting cell (APC) (Acuto and Michel, 2003). The formation of the immunological synapse triggers several downstream signaling pathways that ultimately induce the activation of transcription factors, including NF-AT, AP-1, and NF-κB (Okamura and Rao, 2001).

Activation of the IκB kinase (IKK)/NF-κB signaling pathway is essential for activation, clonal expansion, and effector function of T cells (Kane et al., 2002). The mammalian NF-κB family consists of five different DNA binding subunits: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), c-Rel, RelB, and p65 (RelA). TCR/CD28 engagement induces the canonical NF-κB signaling pathway, which involves phosphorylation-dependent degradation of small cytosolic IκBs and subsequent nuclear translocation of predominately p65- and c-Rel-containing NF-κB dimers (Hayden and Ghosh, 2004). IκB phosphorylation is mediated by the IKK complex that consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO). Therefore, the upstream pathways that control IKK activation are critical for an understanding of the mechanisms that govern T cell activation.

Through genetic ablation in mice, PKCθ, Carma1, Bcl10, and Malt1 were shown to link TCR proximal signaling events to IKK and NF-κB activation (Egawa et al., 2003; Hara et al., 2003; Jun et al., 2003; Pfeifhofer et al., 2003; Ruefli-Brasse et al., 2003; Ruland et al., 2001, 2003; Sun et al., 2000). However, the underlying molecular mechanisms by which these proteins promote IKK/NF-κB activation are not fully understood. Physical interactions between overexpressed Carma1/Bcl10 and Bcl10/Malt1 and Carma1/Malt1 proteins as well as their recruitment into lipid raft microdomains upon TCR ligation have suggested that inducible complex formation is required for signal propagation (Che et al., 2004; Gaide et al., 2002; Lucas et al., 2001; Wang et al., 2004). The MAGUK (membrane-associated guanylate kinase) protein Carma1 apparently acts as a molecular scaffold that also controls the entry of IKKs into the lipid rafts and the immunological synapse (Hara et al., 2004; Wang et al., 2004). Recently, it was shown that the central linker domain of Carma1 is phosphorylated by PKCθ or PKCβ after lymphocyte activation (Matsumoto et al., 2005; Sommer et al., 2005). Phosphorylation seems

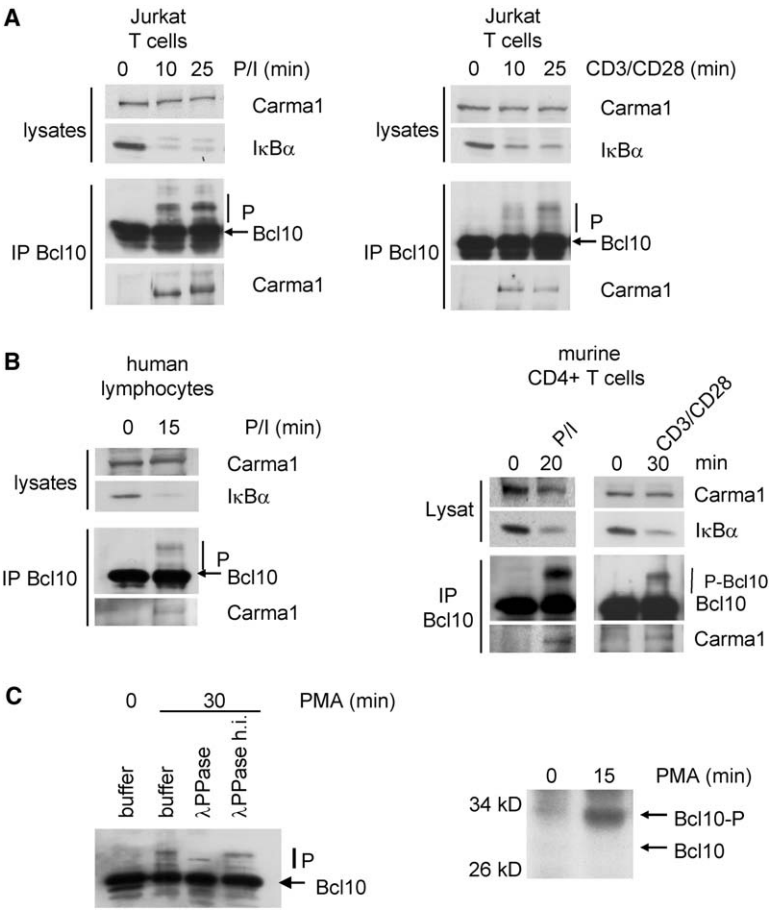

Figure 1. Bcl10 Phosphorylation and Bcl10/Carma1 Interaction upon T Cell Activation

(A) Carma1 and Bcl10 association in activated Jurkat T cells. Bcl10 immunoprecipitations from either P/I- (left panel) or CD3/CD28- (right panel) stimulated Jurkat T cells are shown. After activation, Carma1 precipitates with Bcl10, and in parallel Bcl10 is phosphorylated.

(B) Carma1 and Bcl10 association in activated primary cells. Analysis of Bcl10 immunoprecipitates shows inducible Bcl10/Carma1 interaction and Bcl10 phosphorylation in P/I-stimulated human lymphocytes (left panel) and in P/I- or CD3/CD28-stimulated murine CD4+ T cells (right panels).

(C) Bcl10 phosphorylation upon T cell activation. Slower migrating PMA-induced Bcl10 species are sensitive to treatment with λ-phosphatase (left panel), but not heat-inactivated phosphatase. Analysis of Bcl10 immunoprecipitates from metabolically labeled Jurkat cells reveals a radioactive Bcl10 phosphorylation signal after PMA stimulation.

to control the assembly of Carma1 with Bcl10/Malt1 (Sommer et al., 2005). Further, the potential IKKβ kinase TAK1 was shown to associate with phosphorylated Carma1 in lymphocytes (Shinohara et al., 2005).

It has been observed that, besides Carma1, Bcl10 is highly phosphorylated upon lymphocyte activation (Cannons et al., 2004). Bcl10 phosphorylation is dependent on the presence of Carma1 (Jun et al., 2003) and can be induced by overexpression of PKCθ or Carma1 (Gaide et al., 2002; Wang et al., 2002). Even though the phosphoacceptor sites on Bcl10 have not been mapped and the protein kinases remain unknown, the temporal correlation of inducible Bcl10 phosphorylation suggests a function for T cell activation.

The current literature presents strong evidence that the inducible clustering of signaling mediators and the formation of higher-order multiprotein complexes is important for T cell activation. The Carma1, Bcl10, and Malt1 (CBM) complex is apparently essential for triggering IKK/NF-κB activation upon TCR/CD28 coligation, but the signals that elicit CBM complex formation and downstream effector activation remain elusive. Using biochemical and genetic approaches, we demonstrate that IKKβ is a critical protein kinase for the regulation of the CBM complex. IKKβ is required for initial formation of the CBM complex but also triggers disengagement of Bcl10 and Malt1 by phosphorylation of the C terminus of Bcl10 and thereby negatively influences TCR signaling. Our analysis shows that the CBM complex is a highly dynamic structure and that the formation of multiprotein aggregates supports nonlinear signaling networks and feedback regulations.

Results

IKKβ Triggers CBM Complex Formation and Phosphorylation of Bcl10

To investigate the mechanistic requirements for activation of Carma1, Bcl10, and Malt1 upon T cell activation, we determined the phosphorylation status of Bcl10 and the interactions between Carma1 and Bcl10 by immunoprecipitations (Figure 1). Bcl10 is phosphorylated upon T cell activation by PMA/ionomycin (P/I) or CD3/CD28 crosslinking in Jurkat T cells, primary human lymphocytes, or murine CD4+ T cells, as evident from the appearance of slower migrating Bcl10 species (Figures 1A and 1B). The slower migrating Bcl10 signals are largely due to phosphorylation, as evident from their sensitivity to λ phosphatase treatment and a strong Bcl10 phosphoband after PMA stimulation of 33-phosphorus-labeled Jurkat cells (Figure 1C). Coimmunoprecipitation studies reveal that, upon T cell activation, Bcl10 is rapidly recruited to Carma1 (Figures 1A and 1B). Even though phosphorylation of Bcl10 coincides with its inducible Carma1 association, we reproducibly noted that the efficient binding of Bcl10 to Carma1 slightly preceded the phosphorylation of Bcl10 (see Figure 1A, right, and Figure 2A), suggesting that

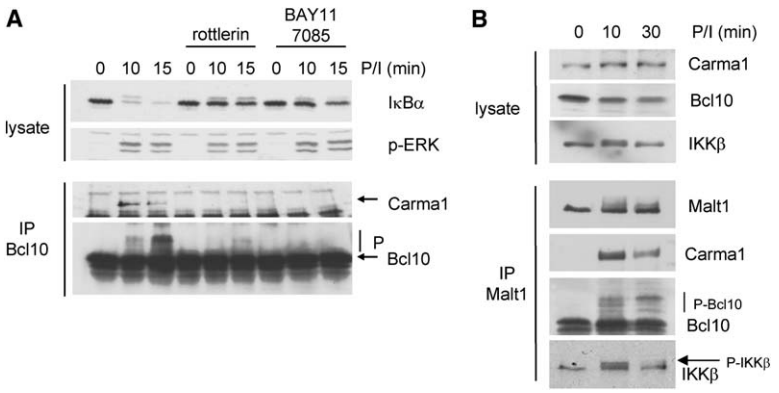

Figure 2. Requirement of IKKβ Kinase Activity for Bcl10 Phosphorylation and CBM Complex Formation

(A) Pharmacological inhibition of PKCθ and IKKβ interferes with CBM complex formation. Jurkat T cells were pretreated with either PKCθ inhibitor rottlerin or IKKβ inhibitors BAY11-7085 for 30 min. Analysis of Bcl10 immunoprecipitates reveals suppression of P/I-induced Bcl10/Carma1 interaction, Bcl10 phosphorylation, and IκBα degradation, while Erk phosphorylation is unaffected. (B) Association of IKKβ with the CBM complex. Analysis of Malt1 immunoprecipitates shows constitutive Malt1 association with IKKβ and Bcl10 in Jurkat T cells. After P/I stimulation, activated (phosphorylated) IKKβ is recruited to the CBM complex.

Bcl10 phosphorylation might occur after complex formation rather than directly promote the interaction to Carma1.

To identify the kinase(s) involved in Bcl10 phosphorylation, we administered pharmacological inhibitors prior to stimulation. Rottlerin, a potent inhibitor of PKCθ-mediated T cell activation (Sasahara et al., 2002), prevents inducible phosphorylation of Bcl10, association of Bcl10 and Carma1, and degradation of IκBα (Figure 2A), underscoring the critical role of PKCθ for upstream IKK/NF-κB signaling upon T cell activation. In contrast, activation of Erk1/2 was unaffected by rottlerin. Unexpectedly, inhibition of the potential downstream kinase IKKβ by BAY11-7085 (Figure 2A) does not only inhibit downstream signaling to induce degradation of IκBα but also strongly impedes Bcl10 phosphorylation and Carma1 association. The results indicate that IKKβ is involved in CBM complex formation and Bcl10 phosphorylation. Further, we performed immunoprecipitations using an anti-Malt1 antibody. Whereas Malt1 constitutively associates with Bcl10 in Jurkat T cells (Figure 2B) and primary lymphocytes (data not shown), the entire Malt1/Bcl10 complex is inducibly recruited to Carma1 upon P/I stimulation. Thus, the cellular CBM complex is a dynamic structure that is rapidly formed after T cell activation. Moreover, IKKβ is bound to Malt1/Bcl10 in untreated Jurkat T cells, and upon activation phospho-IKKβ is transported to the CBM complex. As suggested previously, the inducible recruitment of IKKβ to the Carma1-containing complex may be involved in regulating downstream phosphorylation of IκBs (Su et al., 2005). Additionally, the association of IKKβ with the CBM complex may also promote upstream functions, e.g., Bcl10 phosphorylation, in response to TCR signaling.

To dissect the molecular components that control Bcl10 phosphorylation and CBM complex formation, we inactivated potential mediators of this process by RNA interference (RNAi) (Figure 3; and see Figure S1 in the Supplemental Data available with this article online). As expected from the knockout mice, downregulation of the CBM complex components Carma1 or Malt1, as well as the IKK complex member IKKβ by short hairpin RNAs (shRNAs), impaired degradation of IκBα in response to T cell activation (Figure S1A). Inactivation of Carma1 by shRNAs or Carma1 deficiency in Jurkat T cells inhibited Bcl10 phosphorylation (Figure S1B). Consistent with the results obtained by pharmacological inhibition of IKKβ activity, downregulation of IKKβ by using two different shRNAs reduced Bcl10 phosphorylation in response to T cell-activating stimuli (P/I, Figure 3A; CD3/CD28, Figure S1C). Moreover, signal-induced Bcl10 recruitment to Carma1 was also strongly impaired by IKKβ knockdown, indicating a function of IKKβ in initial CBM complex formation. The hypothesis is underscored by the defective association of Malt1 to Carma1 upon downregulation of IKKβ (Figure 3A). In all cases, shRNA transfection did not affect activation of Erk1/2, indicating that receptor proximal signaling is functional.

To substantiate the role of IKKβ on CBM complex formation, we purified naive CD4+ T cells from CD4-Cre/IKKβ$^{FL/FL}$ (T lineage-specific deletion of IKKβ) and CD4-Cre control mice (Schmidt-Supprian et al., 2003; Figure 3B). Genetic inactivation of IKKβ strongly inhibited recruitment of Bcl10 to Carma1 and prevented phosphorylation of Bcl10 in response to T cell activation. Together, these data provide evidence that upon T cell activation IKKβ is not only required for the phosphorylation and degradation of cytosolic IκBs but is also essentially involved in the recruitment of Bcl10/Malt1 heteromers to Carma1 and the phosphorylation of Bcl10. Also, Malt1 deficiency in primary T cells impairs P/I-induced Bcl10 phosphorylation and Bcl10/Carma1 association (Figure 3C), indicating that for Bcl10 phosphorylation and for Bcl10 recruitment to Carma1 the integrity of the Bcl10/Malt1 complex is required.

Next we asked whether this proposed upstream function of IKKβ also depends on IKKγ, the regulatory subunit of the IKK complex. A strong shRNA-mediated decrease in IKKγ protein levels did not affect Bcl10 phosphorylation and CBM complex formation (Figure 3D). Further, in IKKγ-deficient Jurkat T cells (Harhaj et al., 2000), no impairment of stimulus-induced association between Bcl10 and Carma1 (Figure 3D) was observed. Thus, our studies establish that IKKβ function in the formation of the CBM complex does not depend on a mechanism that requires the regulatory IKKγ subunit. Notably, despite efficient CBM complex formation in IKKγ− and IKKγ+ (IKKγ rescued) Jurkat cells, we observed very weak phosphorylation of Bcl10 upon P/I treatment in both Jurkat clones (data not shown), which again suggests that Bcl10 phosphorylation is not directly required for CBM complex formation.

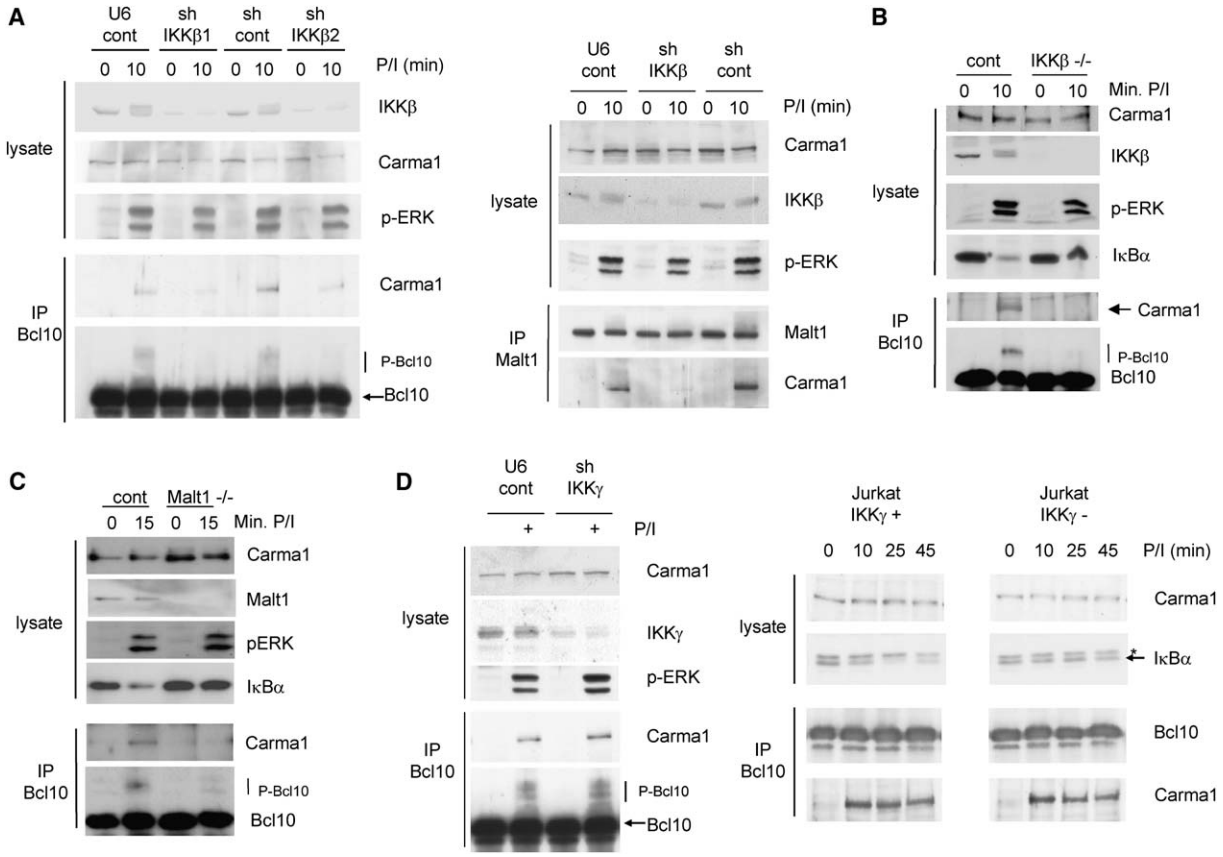

Figure 3. IKKβ Is Required for CBM Complex Formation and Bcl10 Phosphorylation

(A) Inactivation of IKKβ blocks P/I-induced interaction of Bcl10/Malt1 with Carma1 and Bcl10 phosphorylation. In Jurkat T cells transfected with two different shRNAs against IKKβ, Bcl10 phosphorylation and Bcl10 interaction with Carma1, as well as Malt1 association to Carma1, are impaired (left and right panels).

(B) T lineage-specific deletion of IKKβ in CD4-Cre IKK2$^{FL/FL}$ mice interferes with CBM complex formation and Bcl10 phosphorylation. Compared to CD4-Cre control mice, analysis of Bcl10 precipitates from purified IKKβ-deficient CD4+ T cells reveals impaired Bcl10/Carma1 interaction and Bcl10 phosphorylation after stimulation with P/I.

(C) Malt1 deficiency impairs Bcl10 phosphorylation and Carma1 association. CD4+ T cells from Malt1$^{-/-}$ mice and control mice were stimulated with P/I. Bcl10 precipitates were analyzed as indicated.

(D) Inactivation of IKKγ has no effect on CBM complex formation. P/I-stimulated Jurkat T cells transfected with IKKγ shRNA do not exhibit impaired Bcl10/Carma1 interaction or impaired Bcl10 phosphorylation (left panel). Analysis of Bcl10 immunoprecipitates from IKKγ-deficient (IKKγ−) and IKKγ rescued (IKKγ+) Jurkat T cells also shows intact induction of Bcl10/Carma1 association, confirming that IKKβ function in CBM complex formation is independent of IKKγ (right panel). IκBα degradation is blocked in IKKγ-deficient cells. The presence and absence of IKKγ in the respective cells was verified by Western blotting (data not shown). * indicates nonspecific bands.

IKKβ Phosphorylates the C Terminus of Bcl10

To identify potential IKKβ-dependent phosphoacceptor sites on Bcl10, we analyzed the Bcl10 phosphorylation status in cotransfection studies. Coexpression of IKKβ and Bcl10 in HEK293 cells induced the appearance of retarded Bcl10 phosphobands (Figure 4A). A pattern of Bcl10 phosphorylation comparable to that found in 293 cells was observed in primary CD4+ T cells after activation with P/I. Further, coexpression analysis reveals that Bcl10 phosphorylation requires an active IKKβ kinase, while IKKα and PKCθ are unable to trigger Bcl10 phosphorylation in 293 cells (Figure 4B). In addition, recombinant Bcl10 was also efficiently phosphorylated in in vitro immunoprecipitation kinase assays (IP-KAs) after pulling down the endogenous IKK complex from activated Jurkat T cells with either anti-IKKγ or anti-IKKβ antibodies (Figures 4A and 4E). However, compared to IκBα, Bcl10 is only poorly phosphorylated by recombinant, purified IKKβ in in vitro kinase reactions (Figure S2A). Yet, pharmacological inhibition demonstrates that IKKβ activity is essential for Bcl10 phosphorylation in this kinase reaction (Figure S2B). Thus, IKKβ is a Bcl10 kinase that most likely requires a cellular cofactor for full activity.

Using deletion mutants and site-directed mutagenesis, we mapped the IKKβ phosphorylation sites on Bcl10 (Figure 4C). Whereas destruction of the amino-terminal CARD of Bcl10 did not affect IKKβ-driven phosphorylation (data not shown), successive truncation of the C terminus of Bcl10 diminished the phosphorylation (Figure 4D). Deletion of the C-terminal 93 amino acids (Bcl10 1–140) completely abrogated IKKβ-induced Bcl10 phosphorylation. Similar, purified GstBcl10 1–140 was not phosphorylated in IP-KAs using either IKKγ or IKKβ antibodies (Figure 4E). Point mutations of the five serines in the region between amino acids 134 and 144 show that multiple serine residues are phosphorylated and contribute to the considerable mobility shift of

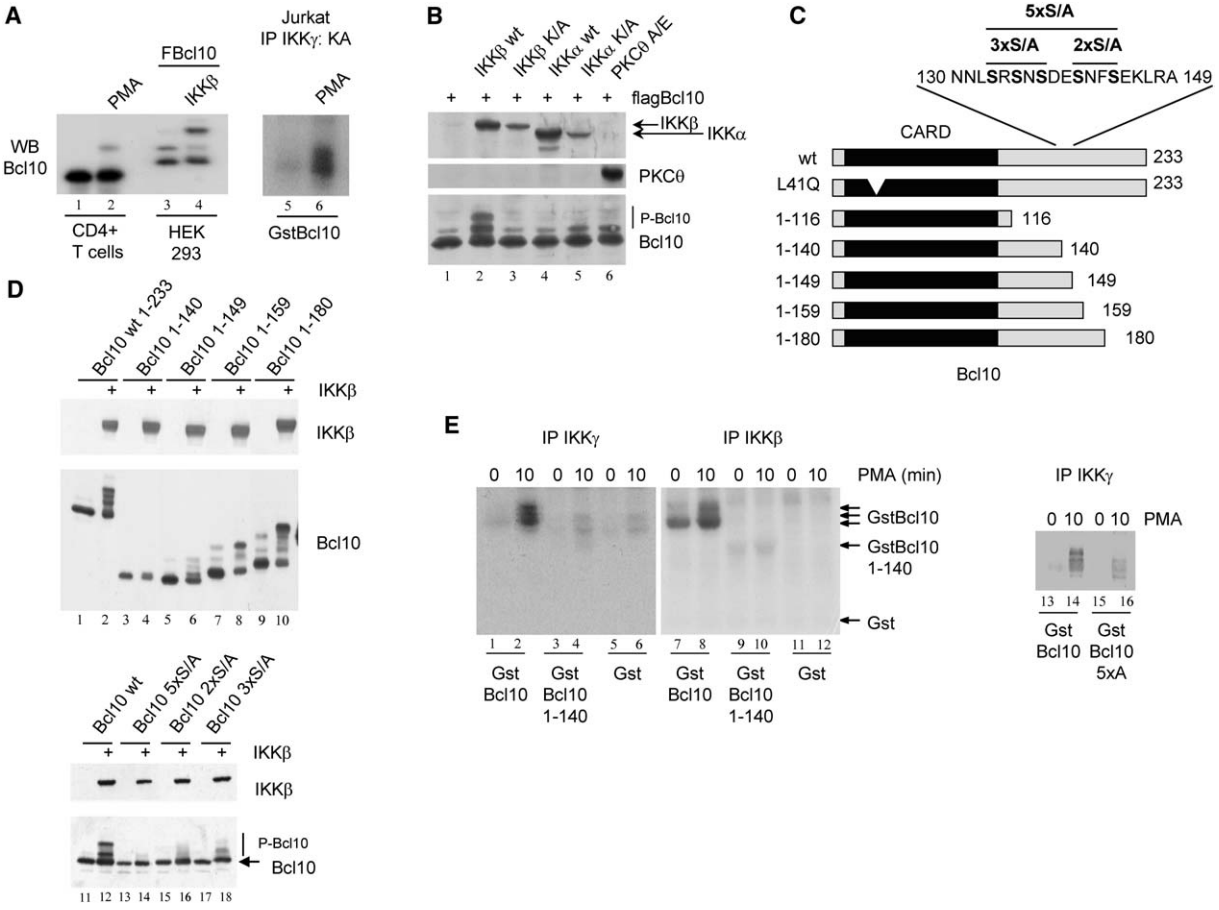

Figure 4. IKKβ Phosphorylates Bcl10 in the C Terminus

(A) Bcl10 phosphorylation in vitro and in vivo. Comparison of Bcl10 phosphorylation patterns of endogenous Bcl10 after P/I activation of primary murine T cells (lanes 1 and 2), phosphorylated FlagBcl10 in MycIKKβ-expressing 293 cells (lanes 3 and 4), and bacterially expressed GstBcl10 in an in vitro kinase reaction using IKKγ precipitates from P/I-stimulated Jurkat T cells (lanes 5 and 6).

(B) Bcl10 is an IKKβ substrate. Coexpression analysis of FlagBcl10 and either epitope-tagged IKKβ, IKKα (wt or kinase dead, K/A), or PKCθ A/E (constitutive active) indicates that Bcl10 is specifically phosphorylated by IKKβ (lanes 2 and 3).

(C) Schematic representation of Bcl10 wt and Bcl10 point and deletion mutants used for phosphoacceptor site mapping and functional assays. Serine to alanine substitutions around amino acid 140 in Bcl10 mutants (Bcl10 5×S/A, Bcl10 3×S/A, Bcl10 2×S/A) are indicated. Destruction of CARD interaction is observed in Bcl10 L41Q.

(D) C-terminal Bcl10 phosphorylation by IKKβ in 293 cells. Analysis of Bcl10 after IKKβ coexpression in 293 cells reveals IKKβ-mediated phosphorylation in the C-terminal part of Bcl10 (upper panel). For mapping the relevant phosphoacceptor site, three point mutants were generated to remove serines 134, 136, and 138 (Bcl10 3×S/A); or 141 and 144 (Bcl10 2×S/A); or all serine residues in the region (Bcl10 5×S/A) (lower panel). Only removal of all five serine residues in Bcl10 5×S/A completely prevents phosphorylation (lanes 13 and 14).

(E) C-terminal Bcl10 phosphorylation by IKK in in vitro kinase assays. Mutant GstBcl10 phosphorylation was analyzed using IKKγ or IKKβ precipitates from activated Jurkat T cells. Phosphorylation of GstBcl10 1–140 (left panel) and GstBcl10 5×S/A (right panel) was significantly decreased when compared to GSTBcl10 wt.

modified Bcl10 in SDS gel electrophoresis (Figure 4D). Only by replacing all five serines in the relevant region (Bcl10 5×S/A) was the appearance of phosphorylated Bcl10 completely prevented. Further, the extent of Bcl10 5×S/A mutant phosphorylation was strongly reduced in IP-KAS using IKK from activated Jurkat T cells (Figure 4E) and in stimulated Jurkat T cells that express the mutant protein (Figure 5A). An antibody against Bcl10 phosphorylated on S134 (anti-Bcl10 P-S134) specifically recognizes phospho-Bcl10 wt in an in vitro kinase reaction using recombinant IKKβ (Figure S2C), showing that IKKβ is able to phosphorylate Bcl10 directly at the C terminus. Since only mutation of all serines 134, 136, 138, 141, and 144 completely prevented signal-induced Bcl10 phosphorylation, the Bcl10 5×S/A mutant was used to elucidate the effects of C-terminal Bcl10 phosphorylation on downstream signaling.

Phosphorylation of Bcl10 Disrupts Bcl10/Malt1 Association and Bcl10-Mediated IKKγ Ubiquitination

To analyze the function of Bcl10 phosphorylation on primary CBM complex formation, we tested its effect on Bcl10/Carma1 association. Inducible interaction of wt and mutant Bcl10 proteins with Carma1 was investigated in Jurkat T cells after transfection (Figure 5A). FlagBcl10 shows a constitutive phosphorylation that is slightly increased after P/I stimulation. As expected, in Jurkat T cells the CARD of Bcl10 mediates inducible association with Carma1 (see Bcl10 L41Q and Bcl10 1–116). In the C-terminal deletion mutants (Bcl10 1–140

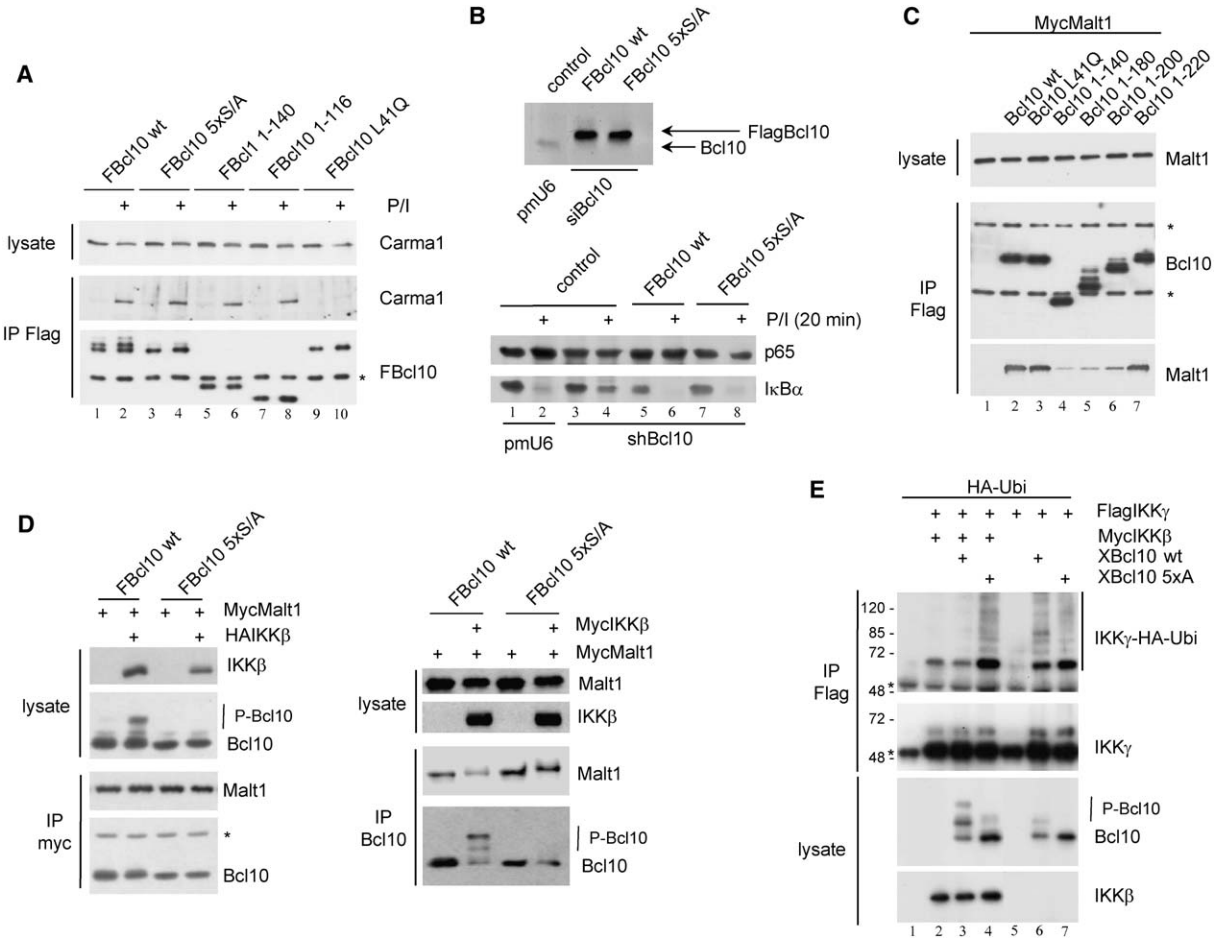

Figure 5. C-Terminal Bcl10 Phosphorylation Is Not Involved in Primary CBM Complex Formation but Prevents Bcl10/Malt1 Interaction and IKKγ Ubiquitination

(A) The CARD of Bcl10 is necessary and sufficient to mediate inducible interaction with Carma1. Jurkat T cells were transfected with FlagBcl10 wt and mutant constructs. Analysis of Flag immunoprecipitates revealed that a functional Bcl10 CARD (compare Bcl10 wt and Bcl10 L41Q) is necessary for P/I-induced Carma1 interaction. C-terminal mutations of Bcl10, including Bcl10 5×S/A, have no impact on CBM recruitment. * indicates migration of immunoglobulin chains.

(B) C-terminal Bcl10 phosphorylation is not required for IKK signaling in Jurkat T cells. Jurkat T cells were transfected with shRNA against Bcl10 to reduce endogenous protein levels, and rescue was performed by coexpression of Bcl10 wt and Bcl10 5×S/A (upper panel). Constructs for rescue contain multiple silent mutations within the shRNA target sequence. Inactivation of Bcl10 results in impaired IKK activation as indicated by reduced IκBα degradation (lower panel). Bcl10 wt or Bcl10 5×S/A cotransfection restores inducible IκBα degradation.

(C) The C terminus of Bcl10 mediates Malt1 interaction. COS7 cells were cotransfected with MycMalt1 and FlagBcl10 constructs as indicated. Bcl10 wt and mutants were analyzed for their ability to interact with Malt1. While a functional Bcl10 CARD is dispensable for Bcl10/Malt1 interaction, successive deletion from the C terminus of Bcl10 prevents efficient association.

(D) IKKβ-triggered phosphorylation prevents interaction of Bcl10 and Malt1. COS7 cells were cotransfected with epitope-tagged Malt1, Bcl10, and IKKβ as indicated. Malt1 immunoprecipitations demonstrate that unphosphorylated, but not phosphorylated, Bcl10 interacts with Malt1 (left panel). Vice versa, phosphorylation of Bcl10 wt by IKKβ reduces the ability of Bcl10 to interact with Malt1 (right panel). In contrast, the presence of IKKβ does not alter the affinity between Bcl10 5×S/A and Malt1.

(E) IKKβ interferes with Bcl10-induced IKKγ ubiquitination in COS7 cells. COS7 cells were transfected with epitope-tagged constructs as indicated. IKKγ ubiquitination was determined by detection of HA-ubiquitin after immunoprecipitation of FlagIKKγ.

or Bcl10 1–116) or in Bcl10 5×S/A mutant, Bcl10 phosphorylation is strongly decreased, but recruitment to Carma1 is not diminished, which demonstrates that Bcl10 phosphorylation is not required for primary CBM complex formation. However, the finding that Bcl10 phosphorylation is absent in the Bcl10 L41Q mutants indicates that Bcl10 phosphorylation itself depends on the recruitment of Bcl10 to Carma1.

To investigate whether Bcl10 phosphorylation might be required for signal propagation to NF-κB in a process other than CBM complex formation, we tested the ability of Bcl10 phosphomutant to activate IKK/NF-κB signaling (Figure 5B). shRNA-mediated downregulation of endogenous Bcl10 in Jurkat T cells delayed degradation of IκBα in response to P/I. This defect was rescued by the expression of either Bcl10 wt or Bcl10 5×S/A, which demonstrates that the C-terminal phosphorylation of Bcl10 is not required for primary signal transduction from the TCR to IKK/NF-κB.

Bcl10 is prone to proteolytic degradation following antigenic stimulation of lymphocytes (Hu et al., 2006; Scharschmidt et al., 2004). To test whether phosphorylation might influence Bcl10 stability, we determined Bcl10 protein levels after pharmacological inhibition

(Figure S3A). Whereas PKCθ inhibitor rottlerin inhibited IκBα and Bcl10 degradation, the IKKβ inhibitor Bay 11-7085 only interfered with IκBα degradation, without affecting Bcl10 stability. In addition, neither mutation of the C-terminal IKKβ phosphoacceptor sites (Figure S3B) nor deletion of the C terminus of Bcl10 (Scharschmidt et al., 2004) abrogated Bcl10 degradation, which strongly suggests that IKKβ-mediated phosphorylation of the C terminus is not triggering Bcl10 degradation.

Deletion of a short region directly adjacent to the CARD of Bcl10 has been shown to abrogate Bcl10-Malt1 association (Lucas et al., 2001). To characterize the interaction, we carried out a more extensive mapping of the Bcl10-Malt1 interface in 293 cells (Figure 5C). Destruction of the CARD (Bcl10 L41Q) does not impair the binding of Bcl10 to Malt1, but stepwise deletion from the C terminus of Bcl10 leads to a weakening of the Bcl10-Malt1 interaction. This demonstrates that a long stretch in the C terminus of Bcl10, which includes the five serine residues between amino acids 134 and 144, mediates the association with Malt1. We therefore asked whether IKKβ-triggered Bcl10 phosphorylation modulates the binding of Bcl10 to Malt1 (Figure 5D). IKKβ-induced phosphorylation of Bcl10 completely prevents its interaction with Malt1, as revealed by the lack of phospho-Bcl10 in Malt1 coimmunoprecipitations (Figure 5D). Vice versa, a strong decrease of Malt1 binding to Bcl10 can be observed in Bcl10 immunoprecipitates when a considerable amount of Bcl10 is phosphorylated by IKKβ (Figure 5D). The reduced affinity of Bcl10 to Malt1 in the presence of IKKβ was only evident with Bcl10 wt but not with Bcl10 5×S/A phosphodefective mutant.

Bcl10 activates IKK/NF-κB through ubiquitination of IKKγ in a Malt1-dependent process (Zhou et al., 2004). Therefore, we tested whether IKKβ phosphorylation of Bcl10 and the concomitant interference with Bcl10/Malt1 association might negatively influence Bcl10-mediated IKKγ ubiquitination (Figure 5E). In the absence of IKKβ, Bcl10 wt and Bcl10 5×S/A induce IKKγ polyubiquitination to a similar extent. Importantly, IKKβ cotransfection strongly inhibits IKKγ ubiquitination by Bcl10 wt, but not by Bcl10 5×S/A. Taken together, these data indicate that IKKβ mediates inhibition of Bcl10/Malt1 interaction, interferes with Bcl10-induced IKKγ ubiquitination, and as a result negatively regulates Bcl10-dependent IKK/NF-κB signaling.

Bcl10 Phosphorylation Interferes with IL-2 and TNFα Production

To investigate the hypothesis that C-terminal Bcl10 phosphorylation negatively regulates TCR signaling, we tested the effects of Bcl10 phosphorylation on cytokine production in T cells. To avoid potential interference with endogenous Bcl10 and to directly investigate the physiological consequences of Bcl10 phosphorylation in primary cells, we purified naive CD4+ T cells from Bcl10$^{-/-}$ mice (Ruland et al., 2001). Subsequently, these primary Bcl10-deficient cells were retrovirally transduced with IRES sequence-containing vectors for simultaneous expression of FlagBcl10 constructs and the cell surface protein Thy1.1 as a marker for infection. The analysis of Thy1.1+ cells shows equivalent levels of Bcl10 wt and Bcl10 5×S/A protein expression in reconstituted cells (Figure 6A; Figure S4A). Similar FlagBcl10 wt and FlagBcl10 5×S/A expression levels are also observed after Bcl10 immunoprecipitation and Western blotting from retrovirally infected DO11.10 T cells (Figure S4B).

To analyze the function of Bcl10 phosphorylation on Bcl10-dependent signaling, we determined the induction of IκBα as a prototype NF-κB target gene after 2 hr P/I stimulation using RT-PCR (Figure 6B). In Bcl10 5×S/A reconstituted cells, IκBα mRNA expression was enhanced when compared to Bcl10 wt cells (3.2-fold versus 2.3-fold), indicating that Bcl10 phosphorylation negatively influences IKK/NF-κB signaling. We next examined in single cells the induction of IL-2 and TNFα, which require IKK/NF-κB-dependent activity after antigenic stimulation (Klemm et al., 2006; Ruland et al., 2001). Cytokine production was measured in CD4+ T cells activated by either P/I stimulation (Figure 6C) or CD3/CD28 coligation (Figure 6D). Intracellular IL-2 and TNFα production was determined in viable retrovirally infected Thy1.1+ cells, while nontransduced Thy1.1− cells served as an internal control. Whereas in nontransduced cells (Thy1.1−) or in cells infected with Thy1.1 vector control (Thy1.1+ control) no significant IL-2 and TNFα synthesis was observed, reintroduction of Bcl10 wt enables a significant proportion of cells to produce both cytokines. Moreover, expression of the C-terminal phosphorylation site mutant (Bcl10 5×S/A) in Bcl10$^{-/-}$ T cells resulted in a higher number of IL-2- and TNFα-producing cells when compared to Bcl10 wt transduced Bcl10$^{-/-}$ cells (Figures 6C and 6D; quantification Figure 6E). The difference in TNFα and IL-2 production was not due to changes in the Th1/Th2 subpopulation between Bcl10 wt and Bcl10 5×S/A rescued cells, as evident from the efficient production of IFNγ in the absence of IL-4 in both samples (Figure 6D). The slight increase in IFNγ production in Bcl10 5×S/A-Thy1.1− cells compared to Bcl10 wt-Thy1.1− cells is most likely a bystander effect in which the rescued cells influence the noninfected cells. Importantly, the strong induction of IFNγ in Thy1.1− cells compared to no induction of IL-2 or TNFα in those cells demonstrates a clear difference in the requirement of Bcl10 for the production of Th1 effector cytokines. The higher efficiency with which phosphorylation-defective Bcl10, compared to wt Bcl10, reconstitutes T cell activation in Bcl10$^{-/-}$ cells indicates that Bcl10 and downstream IKK/NF-κB signaling are negatively regulated through Bcl10 C-terminal phosphorylation.

Discussion

Recruitment of Bcl10/Malt1 to Carma1 is a critical step in the process of T cell activation, but the molecular mechanisms underlying this process have not been fully elucidated. In our study, we present strong evidence for the unexpected finding that, in response to T cell activation, IKKβ is not solely required for IκB phosphorylation but also controls remodeling of CBM complexes. We propose a model whereby IKKβ has a dual function in the regulation of the CBM complex (Figure 7): (1) IKKβ is required for the recruitment of Bcl10/Malt1 to Carma1 after TCR engagement and thus initial CBM complex formation. (2) Subsequently, IKKβ catalyzes C-terminal

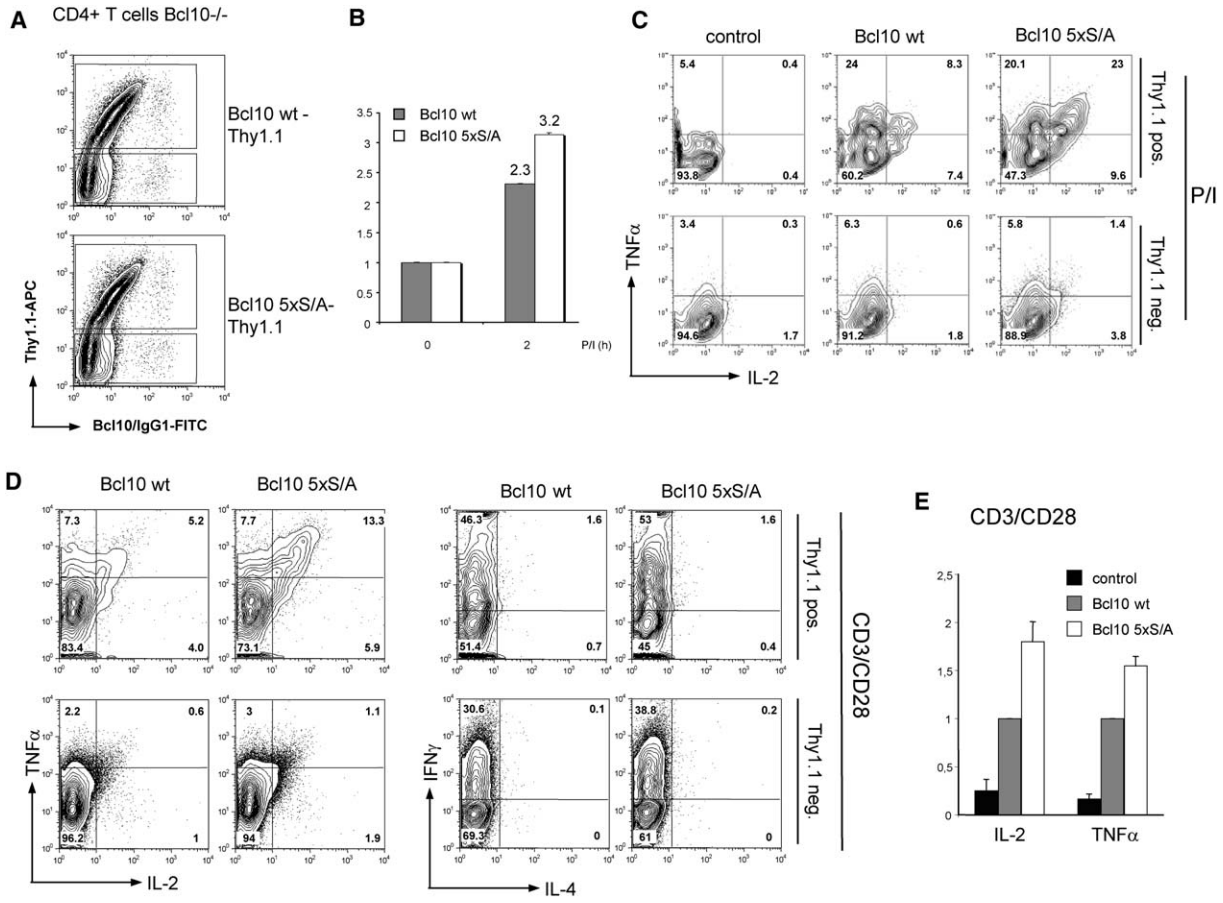

Figure 6. C-Terminal Bcl10 Phosphorylation Negatively Regulates T Cell Activation

(A) Expression levels of Bcl10 wt and Bcl10 5×S/A in retrovirally reconstituted Bcl10$^{-/-}$ T cells. FACS analysis of surface Thy1.1 and intracellular Bcl10 shows equivalent expression of FlagBcl10 proteins. Antibodies did not show crossreactivity as determined in DO11.10 cells infected with Thy1.1 retroviruses (data not shown).

(B) Mutation of the IKKβ-dependent Bcl10 phosphorylation site enhances IκBα gene expression after T cell activation. Real-time RT-PCR analysis using RNA from reconstituted Bcl10-deficient T cells reveals that expression of FlagBcl10 5×S/A increases the induction of the IκBα gene compared to FlagBcl10 wt reconstituted P/I-stimulated cells. Bars represent mean induction and standard deviation.

(C and D) Mutation of the IKKβ-dependent Bcl10 phosphorylation sites enhances IL-2 and TNFα production after T cell activation. T cells from Bcl10-deficient mice were reconstituted with either Bcl10 wt or Bcl10 5×S/A. After stimulation with P/I (C) or CD3/CD28 (D), intracellular cytokine expression was measured by FACS analysis. Cytokine production was rescued by either expression of Bcl10 wt or Bcl10 5×S/A, but expression of the phosphorylation-defective mutant leads to a higher number of IL-2- and TNFα-producing cells as well as a higher expression of both cytokines. The presence of IFNγ and the lack of IL-4 indicate a Th1 phenotype of analyzed cells.

(E) Quantification of IL-2 and TNFα production in reconstituted primary T cells. Numbers of IL-2- and TNFα-producing Bcl10 wt Thy1.1+ cells after CD3/CD28 activation were set to 1 (gray) and directly compared to either Thy1.1 vector control cells (black) or to Bcl10 5×S/A Thy1.1+ cells (white). Bars represent mean induction and standard deviation of three independent experiments.

Bcl10 phosphorylation and disengagement of Bcl10 from Malt1, resulting in a decreased ability of Bcl10 to induce IKKγ ubiquitination. Thereby, IKKβ induces a remodeling of the CBM complex that interferes with antigen signaling and cytokine production. Thus, IKKβ balances T cell activation by controlling positive and negative regulatory mechanisms upstream of its classical substrates, the IκB proteins.

We have shown that IKKβ is constitutively associated with Bcl10/Malt1 in Jurkat T cells. In a recent study caspase-8 was shown to bridge Bcl10/Malt1 to IKKβ (Su et al., 2005). Since we cannot detect direct association of IKKβ and Malt1 or Bcl10 after overexpression (data not shown), caspase-8 might be the critical link. Alternatively, PDK1 was shown to simultaneously recruit PKCθ/IKK and the CBM complex into lipid raft microdomains upon TCR/CD28 coligation (Lee et al., 2005), suggesting that a PDK1/PKCθ-dependent pathway could induce Bcl10 phosphorylation by IKKβ. Regarding initial CBM complex formation, IKKβ could have a structural role, but substrate phosphorylation might also be involved in this process. Further studies will lead to the identification of other upstream IKKβ functions, besides Bcl10 phosphorylation, to decipher IKKβ function for CBM complex formation. Clearly, Bcl10 phosphorylation by IKKβ is not obligatory for CBM complex formation and T cell activation but rather seems to dampen T cell activation.

At a first glance, the upstream role of IKKβ in CBM complex formation seems to contradict previous reports that established IKKβ function downstream of Carma1, Bcl10, and Malt1 on the route to IKK/NF-κB

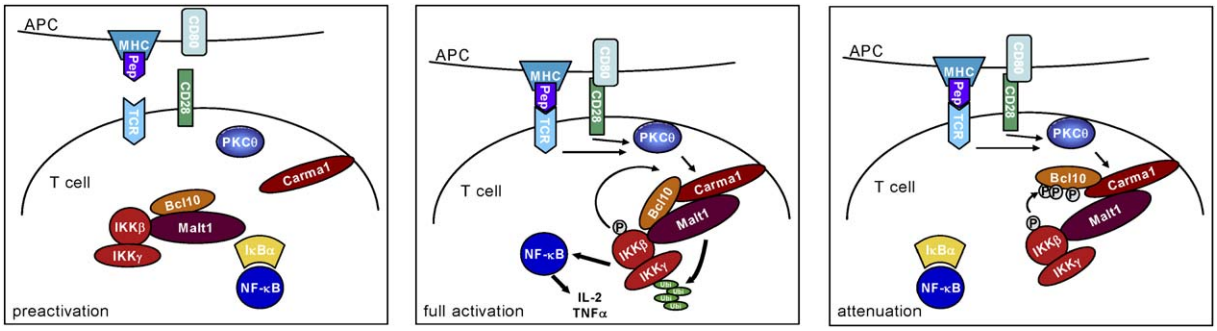

Figure 7. Schematic Model for Functions of IKKβ in CBM Complex Remodeling

In resting T cells, IKKβ is associated with Bcl10/Malt1 (left). Upon T cell activation, phosphorylation of Carma1 by PKCθ induces association of Bcl10/Malt1 to Carma1. IKKβ is required for stable CBM complex formation by an unknown mechanism. Stable association between Carma1, Bcl10, and Malt1 induces maximal IKK activation through ubiquitination of IKKγ (middle). Besides its positive regulatory function on CBM complex formation and IκB phosphorylation/degradation, IKKβ phosphorylates Bcl10 in the Malt1 interaction domain, which causes a disengagement of Bcl10 from Malt1 and interferes with IKKγ ubiquitination. As a result, IKK/NF-κB signaling and cytokine production is attenuated (right), establishing a negative regulatory function for IKKβ.

(Thome, 2004). It is conceivable that TCR-dependent phosphorylation of Carma1 by PKCθ induces a primary weak CBM complex formation and IKKβ activation (Matsumoto et al., 2005; Sommer et al., 2005), but that a stable CBM complex requires additional modifications, which are induced by IKKβ. A similar dual function has been shown for PKCθ, which regulates downstream Bcl10/Carma1 interaction and functions upstream in immunological synapse formation (Sasahara et al., 2002).

Intriguingly, the function of IKKβ for CBM complex formation is independent of IKKγ. At the moment it is not clear whether a complex only containing IKKβ exists under physiological conditions or whether a single IKKβ/γ complex is able to fulfill such different tasks. Similarly, there is not yet definitive evidence that IKKα homodimers, which are not bound to IKKγ, exist in vivo and mediate signaling via the alternative pathway (Senftleben et al., 2001).

Here we present experimental evidence that Bcl10 is a substrate of IKKβ. Bcl10 phosphorylation upon T cell activation is blocked by pharmacological inhibition of IKKβ, overexpression of IKKβ induces Bcl10 phosphorylation, and phosphorylation of Bcl10 in in vitro kinase reactions after immunoprecipitation of IKK complex components is blocked by IKKβ inhibitors. In all assays, IκBα is a better substrate of IKKβ than Bcl10, which might be the reason for the slower kinetic of Bcl10 phosphorylation after T cell activation. Maximal Bcl10 phosphorylation is detected at much later time points than phosphorylation-induced IκBα degradation. Multiple phosphorylations target the Bcl10 protein, as revealed by the massive mobility shift of Bcl10 in SDS gels and by the analysis of point mutants. We cannot rule out that IKKβ-driven Bcl10 phosphorylation is augmented by another so-far-unknown protein kinase. Bcl10 does not contain the classical IKK consensus motif (DSΨXXS) as found in all IκB proteins, β-catenin, and the recently identified IKKβ target FOXO3a (Hu et al., 2004; Maniatis, 1999). However, the conservation of this motif seems to reflect the constraints for the recognition by the SCF-βTRCP E3 ligase and the subsequent proteasomal degradation rather than an IKK phosphoacceptor site (Maniatis, 1999). Since destruction of the CARD prevents phosphorylation, not only the potential IKKβ phosphoacceptor site, but also the recruitment of Bcl10 to Carma1 is essential for phosphorylation. The identification of other IKK substrates will allow a better understanding about the molecular parameters for kinase recognition and IKKβ consensus sequences.

We and others have shown that Bcl10 is degraded after T cell activation (Hu et al., 2006; Scharschmidt et al., 2004). Still, phosphorylated Bcl10 did not show an altered half-life (data not shown), and the phosphorylation-defective Bcl10 mutant 5×S/A is inducibly degraded with equivalent efficiency compared to Bcl10 wt. In addition, inhibition of IKKβ kinase activity has no effect on signal-induced Bcl10 degradation, while inhibition of the upstream kinase PKCθ stabilizes Bcl10. This indicates that there is no direct link between C-terminal Bcl10 phosphorylation and Bcl10 instability. Thus, IKKβ-dependent Bcl10 phosphorylation attenuates TCR signals shortly after activation, whereas at later time points degradation of Bcl10 promotes the termination of downstream signaling.

Functionally, IKKβ-driven phosphorylation in the C-terminal region of Bcl10 interferes with the association of Bcl10 and Malt1, suggesting that phosphorylation might trigger a disengagement of the complex. Since phosphorylated Bcl10 is still immunoprecipitated by Malt1 from activated T cells (Figure 2B), phosphorylation per se does not disrupt the CBM complex but rather induces a remodeling of the components (Figure 7). Previously, it was shown that the caspase-like domain of Malt1 can directly interact with the coiled-coil region of Carma1 in a Bcl10-independent manner (Che et al., 2004). Thus, Carma1 could function as a molecular scaffold and keep endogenous Malt1 in one complex with phosphorylated Bcl10. Nevertheless, the enhanced cytokine production in Bcl10 5×S/A-expressing Bcl10$^{-/-}$ T cells indicates that phosphorylation of Bcl10 is part of a negative feedback mechanism that counteracts cytokine production and T cell activation (Figure 7). Thus, loosening of Bcl10/Malt1 interactions within the CBM complex apparently impairs activation of downstream signaling pathways. This is also supported by our finding that IKKβ-mediated Bcl10 phosphorylation reduces the ability of Bcl10 to induce IKKγ ubiquitination. Clearly, Bcl10-mediated IKKγ ubiquitination is a critical

step for TCR-triggered IKK activation and requires the presence of Malt1 (Zhou et al., 2004). Consequently, interference with Bcl10/Malt1 association will directly inhibit IKK activation. Certainly, signaling to other transcription factors, e.g., NF-AT and AP-1, is required for full IL-2 and TNFα induction in response to T cell activation (Altman et al., 2000). Since Malt1-deficient T cells display defects in IKK/NF-κB, JNK, and p38 activation (Ruland et al., 2003), other signaling pathways downstream of the CBM complex could also be influenced by Bcl10 phosphorylation.

Our analysis indicates that, upon T cell activation, the clustering of proteins around the CBM complex promotes nonlinear and nonhierarchical signal propagation by facilitating complex interactions between protein networks. IKKβ acts on multiple layers of the signaling pathway and thereby functions as a positive and negative regulator for T cell activation.

Experimental Procedures

Cell Culture

Standard cell culture and transfections have been described previously (Scharschmidt et al., 2004). Primary lymphocytes were isolated from spleen and lymph nodes. Positive selection of naive CD4+ T cells was performed using Dynabeads (Invitrogen). Primary cells were cultured in RPMI supplemented with 10% FCS, 1% Pen/1% Strep, 1% MEM, 1% sodium pyruvate, and 0,1% β-mercaptoethanol. For RNAi, Jurkat T cells were electroporated and cultured for 72 hr before lysis.

Retroviral Infection of CD4+ T cells

Retroviral infections were carried out as follows: purified CD4+ T cells from $Bcl10^{-/-}$ mice (Ruland et al., 2001) were simulated with plate-bound CD3/CD28 antibodies for 48 hr. Cells were resuspended in retroviral supernatant supplemented with Polybrene. After 6 hr incubation, cells were resuspended in media supplemented with IL-2 and cultured for another 72 hr. Cells were then activated for 6 hr with plate bound CD3/CD28 antibodies or P/I. After 4 hr, Brefeldin A was added. Activated cells were fixed, stained, and analyzed by FACS. Statistics for cytokine production, including standard deviation, was done from three independent experiments.

Retroviral supernatant was produced from Phoenix packaging cells. The cells were transfected using standard $CaPO_4$ protocols, and supernatant was collected after 48 hr.

Reagents

The following reagents or enzymes were purchased and used at final concentrations as indicated: PMA (200 ng/ml, Calbiochem), Ionomycin (300 ng/ml, Calbiochem), IL-2 (20 U/ml, Roche), Polybrene (8 μg/ml, Sigma), Brefeldin A (10 μg/ml, Sigma), Bay 11-7085 (5 μg/ml, Calbiochem), sc-514 (22,5 μg/ml, Calbiochem), λ-phosphatase (20 U/ml, NEB), and Lipofectamine 2000 (Invitrogen). The following antibodies were used: Bcl10, Malt1, IκBα, IKKγ, p65, Myc-9E10, and HA Y-11 (all Santa Cruz); Carma1 (Alexis Biochemicals); IKKβ and pERK (Cell Signaling); Flag M2 (Sigma); Thy1.1, IL-2, and TNFα (Pharmingen); and IFNγ XMG1.2 and IL-4 11B11 (eBioscience). Monoclonal Malt1 antibody was a gift from V. Dixit (Genentech).

Plasmids

Bcl10 constructs were cloned into Flag or Xpress pEF vector (Invitrogen) or MycpRK5 vector. Malt1 cDNA was obtained from RZPD clone BM016367. For vector-based expression of shRNAs, double-stranded oligonucleotides were cloned into mU6pro vector (see Supplemental Data). Retroviral constructs were generated using the Gateway system (Invitrogen), and Bcl10 cDNAs were cloned via pENTR11 into pMSCV Thy1.1.

Immunoprecipitation, Kinase Assays, and Ubiquitination

Cells were lysed in coimmunoprecipitation buffer (see Supplemental Data). Precipitations were carried out overnight. For kinase assays, lysates from Jurkat T cells were subjected to immunoprecipitation for 4–5 hr. Precipitates were washed and used in in vitro kinase assays, and GST fusion proteins purified from *E. coli* served as substrates. IKKγ ubiquitination was determined as previously described (Scharschmidt et al., 2004).

RT-PCR

Five hundred nanograms of total RNA was used to perform first strand synthesis using Superscript II (Invitrogen) and random hexamer oligonucleotide primers. For quantitative real-time PCR, the LightCycler 2 system (Roche) was used (for primers see Supplemental Data). Reactions were normalized to endogenous 18S RNA. Each reaction was carried out in triplicate.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four figures, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/23/1/13/DC1/.

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