Engagement of the T-cell receptor (TCR) initiates a signaling cascade that ultimately results in activation of the transcription factor NF-κB, which regulates many T-cell functions including proliferation, differentiation and cytokine production. Herein we demonstrate that Rip2, a caspase recruitment domain (CARD)-containing serine/threonine kinase, plays an important role in this cascade and is required for optimal TCR signaling and NF-κB activation. Following TCR engagement, Rip2 associated with Bcl10, a CARD-containing signaling component of the TCR-induced NF-κB pathway, and induced its phosphorylation. Rip2-deficient mice were defective in TCR-induced NF-κB activation, interleukin-2 production, and proliferation in vitro and exhibited defective T-cell-dependent responses in vivo. The defect in Rip2−/− T-cells correlated with a lack of TCR-induced Bcl10 phosphorylation. Furthermore, deficiency in Bcl10-dependent NF-κB activation could be rescued in Rip2−/− embryonic fibroblasts by exogenous wild-type Rip2 but not a kinase-dead mutant. Together these data define an important role for Rip2 in TCR-induced NF-κB activation and T-cell function and highlight the significance of post-translational modification of Bcl10 by Rip2 in T-cell signaling.

Many diverse stimuli activate NF-κB by inducing the phosphorylation and destruction of inhibitory molecules known as the IκBs that retain NF-κB in the cytoplasm (1). The IκB kinase (IKK) complex, composed of two kinase subunits, IKKα and IKKβ, and a non-catalytic subunit, NEMO, is responsible for the phosphorylation of the IκBs. The association of Bcl10 and CARMA1 (CARD11), two caspase-recruitment domain (CARD)-containing proteins, has been shown to be essential to the transduction of the signal from the T-cell receptor (TCR) to the IKK complex (2). Mice deficient for either Bcl10 or CARMA1 display profound defects in T-cell proliferation and cytokine production due to a lack of NF-κB activation (3–7); however, the mechanism by which the CARMA1/Bcl10 complex activates IKK remains unclear.

**In vitro** experiments have indicated that Bcl10 undergoes phosphorylation when over expressed with its viral homologue, E10 or CARMA1 (8–10). In these studies, Bcl10 phosphorylation correlated with its ability to activate NF-κB, suggesting that this modification was required for NF-κB activation. Indeed, the COOH-terminal domain of Bcl10 is rich in serine and threonine residues and has been proposed as the site of CARMA1-mediated phosphorylation (10). Since CARMA1 itself is not a kinase, the kinase responsible for Bcl10 phosphorylation has remained an open question.

Rip2 is a serine/threonine kinase that contains a CARD domain at its carboxyl terminus and has been shown to induce NF-κB activation in over expression systems (11–13). Rip2 has also been shown to associate in **vitro** with members of the TRAF family, such as TRAF6, that plays an essential role in the innate immune response downstream of Toll-like receptors (TLRs) (14, 15). In addition, Rip2 has been implicated in regulating both the innate and adaptive immune responses (16, 17). Mice deficient in Rip2 mounted only an attenuated immune response against Toll-like receptor agonists such as lipopolysaccharide (LPS) (16, 17). Interestingly, CD4+ T-cells from Rip2-deficient mice were unable to proliferate efficiently in response to antigen-induced T-cell activation, but no mechanism was provided for this striking observation (16, 17). We sought to define the role of Rip2 in antigen-induced NF-κB activation and T-cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Generation of Rip2−/− Mice**—A targeting vector that removed exon I of Rip2 was electroporated into ES cells. Homologous recombinants were used to generate chimeric founder mice by microinjection into C57BL/6J blastocysts. Germ line transmission was confirmed by Southern blot analysis of genomic tail DNA. Two independent ES clone lines resulted in mice with identical phenotypes. All mice used in experiments were backcrossed onto C57BL/6 five to seven generations and were confirmed to be >95% C57BL/6 by PCR analysis of genomic tail DNA.

**Immunoprecipitations**—293 T-cells were transfected with expression vectors for NH2-terminal HA- or Myc/His-tagged Rip2, Rip2 311–541 (ΔKinase) and Rip2 1–454 (ΔCARD) (11), and V5-tagged Bcl10 (18). Tagged proteins were immunoprecipitated using monoclonal antibodies to Rip2 (Genentech, Inc.), V5 (Invitrogen), HA and Myc (Zymed Laboratories Inc.), and actin (ICN). Jurkat cells (4×10^6) were stimulated with PMA (100 ng/ml) (BD Biosciences) with or without irradiated CD4-depleted T-cells for 30 min at 37°C (5% CO2) in 96-well plates. Cells were lysed in RIPA buffer and lysis was performed for 5 min on ice. Cell lysates were clarified by centrifugation at 14,000 rpm for 15 min at 4°C.

**Protein Assays**—Splenic B and T-cells and CD4+ T cells were purified by negative selection using magnetic beads (Miltenyi Biotech) to >95% purity. Purified T-cells were activated with plate bound anti-CD3 (0–10 μg/ml) (BD Biosciences) with or without irradiated CD4-depleted T-cells.
Rip2 Participates in Bcl10 Signaling

APCs or plate-bound anti-CD28 (0–10 μg/ml) (BD Biosciences), phorbol myristate acetate (PMA) (2 μg/ml) plus ionomycin (0.1 μg/ml) in the presence or absence of IL-2 (50 ng/ml) (R & D Systems). B-cells were stimulated with anti-IgM (20 μg/ml) (Jackson Laboratory), LPS (20 μg/ml) (Sigma), or PMA (2 μg/ml) plus ionomycin (0.1 μg/ml). Cells were harvested at 24, 48, 72, and 96 h after an 8-h pulse with [3H]thymidine and are representative of at least three separate experiments.

Neonatal Heart Allograft—Neonatal hearts from BALB/c (H-2d) mice were surgically implanted behind the dorsum of the ear pinna of 12-week-old male Rip2+/− and wild-type mice (Both H-2b). Heart grafts were examined with a stereomicroscope at 10-20-fold magnification every other day until rejection.

Western Blots—Purified T-cells (2 × 10⁶) were stimulated with 10 μg/ml plate-bound anti-CD3 for 0–30 min. Western blots were performed using phospho-specific antibodies for IκBα, ERK1/2, and JNK (Cell Signaling Technology). Blots were stripped and re-probed with antibodies to IκBα and p44 (Cell Signaling Technology).

RESULTS AND DISCUSSION

Rip2 Associates with Bcl10 and Induces Its Phosphorylation—We investigated whether Rip2 could associate with molecules known to play essential roles in the TCR-induced signaling cascade. Initially, we tested whether Rip2 and Bcl10 could associate by overexpressing tagged versions of both proteins in 293 T-cells. V5-tagged Bcl10 could be co-immunoprecipitated with HA-tagged Rip2 (Fig. 1A). Interestingly, two bands representing Bcl10 were observed. The upper band was determined to be a hyperphosphorylated form of Bcl10, since it could be collapsed to the lower band by λ phosphatase treatment (Fig. 1A). Hyperphosphorylation of Bcl10 was also apparent after mobility shift in whole cell lysates from 293T-cells co-transfected with Rip2 and Bcl10, compared with the very low levels of phosphorylation seen with Bcl10 alone (Fig. 1B). To establish the domains of Rip2 responsible for hyperphosphorylation of Bcl10, mutants with deletions of either the kinase domain or the CARD domain were used in co-expression studies. Bcl10 hyperphosphorylation required both a functional kinase domain and CARD domain of Rip2 as neither mutant induced phosphorylation of Bcl10 (Fig. 1B). Moreover, phosphorylation of Bcl10 was specific for Rip2, as overexpression of RIP3 did not induce Bcl10 phosphorylation (Fig. 1C).

To determine whether Rip2 was involved in Bcl10-dependent signaling pathways, we studied the interaction of endogenous proteins in Jurkat cells stimulated with cross-linking antibodies to CD3-TCR. Rip2 and Bcl10 consistently associated in a transient and time-dependent manner after TCR engagement (Fig. 1D, bottom panel). Induction of phosphorylated Zap-70 confirmed TCR activation (Fig. 1E). We next examined the phosphorylation status of Bcl10 using phosphoserine-specific antibodies. Lysates from anti-CD3 treated and untreated Jurkat cells were immunoprecipitated using antibodies to Bcl10 and Western blots were performed using antibodies for phosphoserine and Bcl10. Serine phosphorylated Bcl10 was detected after 15-min treatment with anti-CD3 (Fig. 1F, top panel) and treatment of immunoprecipitates with λ phosphatase (APPase) significantly diminished levels of serine-phosphorylated Bcl10. Phosphorylation of endogenous Bcl10 was also apparent after treatment with anti-CD3, as evidenced by a slower migrating band that could be collapsed by treatment with λ phosphatase (Fig. 1F, bottom panel). Taken together, these results were consistent with Rip2 binding Bcl10 upon TCR engagement and inducing its phosphorylation.

Defective T-cell Proliferation and Function in Rip2+/− Mice—To examine the effects of Rip2 on T cell activation in an in vivo setting, we generated Rip2-deficient mice by homologous recombination. Rip2+/− T-cells were deficient in anti-CD3-induced proliferation.
FIG. 2. Deficient T-cell proliferation and NF-κB activation in Rip2−/− mice. CD4+ T-cells were isolated from Rip2−/− (open squares) and wild-type (closed squares) mice and stimulated ex vivo for 0–72 h with 10 μg/ml CD3 in the presence of CD4-APCs (A) or with 10 μg/ml CD3 alone (B), and cell proliferation was determined by [3H]thymidine incorporation. C, CD4+ T-cells from Rip2−/− (open bars) and wild-type (closed bars) were co-stimulated with 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 or with PMA plus ionomycin for 48 h. D, CD4+ T-cells from Rip2−/− (open bars) and wild-type (closed bars) were stimulated with 10 μg/ml CD3 with or without CD4-APCs in the presence of IL-2 for 48 h, and cell proliferation was determined by [3H]thymidine incorporation. All data are the mean ± S.E. of triplicate samples and are representative of at least three separate experiments. E, neonatal hearts from BALB/c mice (H-2d) were grafted into the ear of 12-week-old male Rip2−/− and wild-type mice (both H-2b), and allograft survival was monitored every other day until rejection. (n = 9 with p value of 0.028 by the Student's t test).
induced proliferation (Fig. 2, A and B). This defect could not be rescued by co-stimulation with anti-CD28 or activation using PMA in combination with calcium ionophore (ion) (Fig. 2C). The levels of IL-2 produced after treatment with anti-CD3 alone, anti-CD3 with anti-CD28, or with PMA and ionomycin were drastically reduced compared with wild-type T-cells (data not shown). Moreover, addition of exogenous IL-2 was not able to rescue the defect in proliferation in Rip2−/− T-cells after stimulation (Fig. 2D). Consistent with previous reports, B-cell proliferation in response to PMA/ionomycin, IgM, and LPS was comparable between Rip2-deficient and wild-type B-cells (data not shown) (16). Taken together, these results suggested that the defect in proliferation in Rip2−/− mice was confined to T-cells and likely due to impairment upstream of IL-2 gene transcription and NF-κB activation.

Previous in vivo experiments on Rip2−/− mice tested T-cell responsiveness using models such as Listeria challenge and T-cell-dependent antibody responses (16, 17), which also involve participation of TLRs and other innate signaling cascades through adjuvant and bacterial components. Therefore, to test T-cell responsiveness in vivo, we designed a high bar functional test, the heart transplant model (18). Hearts from allogeneic neonate BALB/c (H-2d) mice were transplanted into the ear pinna of wild-type and Rip2−/− mice. Hearts from Rip2−/− and wild-type MEFs were transplanted into the ear pinna of wild-type and Rip2−/− mice. Hearts from Rip2−/− and wild-type MEFs were transplanted into the ear pinna of wild-type and Rip2−/− mice. Hearts from Rip2−/− and wild-type MEFs were transplanted into the ear pinna of wild-type and Rip2−/− mice. These results confirmed that the defect was specific for NF-κB signaling downstream of other surface receptors remained intact in Rip2−/− mice.

TCR engagement also elicits activation of the RAS/MAPK (mitogen-activated protein kinase) pathway. Western blotting using phospho-specific anti-ERK1/2 antibodies demonstrated that ERK-1 and ERK-2 were phosphorylated with similar kinetics in wild-type and Rip2−/− T-cells after TCR engagement (Fig. 3C, upper panel). Similarly, activation of the JNK signaling pathway post-TCR engagement was equivalent in both wild-type and Rip2-deficient T-cells (Fig. 3C, lower panel). These results confirmed that the defect was specific for NF-κB signaling downstream of the TCR and that parallel pathways activated by TCR engagement remained intact.

To address the role of Rip2 kinase activity in Bcl10-dependent NF-κB activation, we transfected MEFs from wild-type and Rip2−/− mice with Bcl10 and a luciferase reporter for NF-κB. While Bcl10 could induce NF-κB activation in wild-type MEFs, NF-κB activation by Bcl10 was significantly decreased in Rip2−/− MEFs (Fig. 3D). Transfection of exogenous wild-type Rip2, but not a kinase-dead mutant, K47A, could rescue Bcl10-induced NF-κB reporter activity in Rip2−/− MEFs (Fig. 3D). Therefore, the kinase activity of Rip2 is required for optimal Bcl10-induced NF-κB activation.

Bcl10 Is Phosphorylated after TCR Engagement in Wild-type but Not Rip2−/− Mice—Taken together, our data suggested
that Rip2 functions to regulate T-cell activation by phosphorylating Bcl10. Therefore, we wished to establish whether the lack of NF-κB activation observed in Rip2−/− T-cells correlated with a lack of Bcl10 phosphorylation after TCR engagement. Wild-type and Rip2-deficient T-cells were treated with α-CD3, and cell lysates were fractionated using a phosphoserine/threonine column. Under these lysis conditions, all protein-protein interactions are disrupted, and only phosphorylated proteins bind the column, while unphosphorylated proteins flow through. Western blotting of the phosphorylated protein fractions using antibodies to phospho-ERK and Bcl10 revealed that while phosphorylated ERK1/2 could easily be detected in the purified phosphorylated fractions of both wild-type and Rip2−/− T-cells (Fig. 3E, middle panel), Bcl10 was only present in the purified phosphorylated fractions of α-CD3 treated wild-type T-cells (Fig. 3E, top panel). By contrast, similar levels of Bcl10 were detected in the non-phosphorylated fractions from wild-type and knock-out T-cells (Fig. 3E, bottom panel). To confirm that no unphosphorylated proteins contaminated the phosphorylated protein fraction, lysates from both fractions were Western blotted using antibodies for Hsp60. Hsp60 was abundant in the non-phosphorylated fraction but undetectable in the phosphorylated protein fraction (Fig. 3F). These data demonstrate that Bcl10 is phosphorylated in mouse primary T cells after TCR stimulation, and deficiency of Rip2 precludes phosphorylation of Bcl10.

Herein we provide evidence for the importance of Rip2 in TCR-mediated NF-κB activation and Bcl10-dependent signaling. Phosphorylation of Bcl10 occurs after TCR engagement, and lack of phosphorylation correlates with a defect in NF-κB activation and T-cell proliferation. Earlier studies have shown that Bcl10 is phosphorylated upon over expression of CARMA1; however, the importance of phosphorylation in T-cell signaling was unclear. Our data suggest that phosphorylation of Bcl10 by Rip2 plays a key role in signaling between the TCR and the IKK complex. Recent reports (4–7, 19–21) have demonstrated that CARMA1 is critically involved in TCR-induced NF-κB activation. It remains unclear whether Bcl10 phosphorylation is required for its association with CARMA1. The kinetics of the association between Rip2 and Bcl10 and subsequent Bcl10 phosphorylation in Jurkat T-cells correlates with the kinetics of the published interaction between CARMA1 and Bcl10 in Jurkat T-cells (19). Phosphorylation of Bcl10 may either facilitate its recruitment to lipid rafts or serve to activate other key molecules in downstream signaling events that ultimately activate the IKK complex. For example, MALT1/paracaspase, a death domain-containing caspase-like molecule, has also been shown to associate with Bcl10 and enhance NF-κB activation (22, 23) and is also required for TCR-induced proliferation, cytokine production, and NF-κB activation (24).

Our data are consistent with previous reports that Rip2-deficient mice suffer from defects in the adaptive immune response due to lack of antigen-induced T-cell proliferation and NF-κB activation (16, 17). Similar to published results, we also observed a defect in cytokine production in macrophages stimulated with LPS and other Toll-like receptors, demonstrating an additional defect in innate immunity (data not shown) (16, 17). Since Rip2 associates with key signaling molecules in both the adaptive and innate immune responses, such as Bcl10 and TRAF6 respectively, it is reasonable that the absence of this promiscuous kinase would impinge on multiple signaling pathways and result in broad ranging deficits in immune system function.

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