Inflammation is a homeostatic mechanism that limits the effects of infectious agents. Tumor necrosis factor (TNF) and interleukin (IL)-1 are two cytokines that induce inflammation through activation of the transcription factor NF-κB. Various studies have suggested that two homologous and structurally related adapter proteins TAB2 and TAB3 play redundant roles in TNF- and IL-1-mediated NF-κB activation pathways. Both TAB2 and TAB3 contain CUE, coiled-coil, and nuclear protein localization 4 zinc finger (NZF) domains. The NZF domains of TAB2/3 are critical for TAB2/3 to bind to Lys63-linked polyubiquitin chains of other adaptor proteins, such as receptor-interacting protein and TRAF6, which are two signaling proteins essential for TNF- and IL-1-induced NF-κB activation, respectively. In a search for proteins containing NZF domains conserved with those of TAB2/3, we identified RBCK1, which has been shown to act as an E3 ubiquitin ligase in iron metabolism. Overexpression of RBCK1 negatively regulates TAB2/3-mediated and TNF- and IL-1-induced NF-κB activation, whereas knockdown of RBCK1 by RNA interference potentiates TNF- and IL-1-induced NF-κB activation. RBCK1 physically interacts with TAB2/3 and facilitates degradation of TAB2/3 through a proteasome-dependent process. Taken together, our findings suggest that RBCK1 is involved in negative regulation of inflammatory signaling triggered by TNF and IL-1 through targeting TAB2/3 for degradation.

Proinflammatory cytokines, such as TNF and IL-1, play critical roles in inflammatory processes and are involved in regulation of immune responses. Stimulation of cells with TNF or IL-1β initiates a cascade of signaling events leading to activation of transcription factors NF-κB and AP1 (activator protein 1) and induction of proinflammatory cytokines (1, 2). NF-κB regulates expression of a large number of genes involved in immune responses, inflammation, cell survival, and cancer (3).

NF-κB is sequestered in the cytoplasm and kept inactive in nonstimulated cells through binding to inhibitory IkB (inhibitor of κB) proteins. Following stimulation with cytokines, infectious agents, or radiation-induced DNA double-stranded breaks, the IkB proteins are phosphorylated, ubiquitinated, and ultimately degraded in a proteasome-dependent manner, which frees NF-κB from IkB proteins to translocate to the nucleus, where it activates transcription of target genes (3).

Considerable progress has been made during the past couple of decades in identifying molecular components involved in TNF- and IL-1-triggered NF-κB activation pathways. Binding of TNF to TNFR-I leads to its trimerization and the recruitment of adaptor proteins, including TRADD, TRAF2, and receptor-interacting protein (RIP) (1). RIP is subsequently polyubiquitinated in a Lys48- or Lys63-linked manner, among which Lys63-linked ubiquitination of RIP is required for the recruitment and activation of TAK1 (TGF-β-activated kinase 1), a kinase associated with adapter proteins TAB2 (TAK1-binding protein 2) and TAB3 (TAK1-binding protein 3) (4, 5). The activated TAK1 subsequently activates downstream kinases IKKα and IKKβ, which phosphorylate IkB proteins, and leads to NF-κB activation (6).

Upon IL-1 stimulation, the IL-1-receptor recruits IL-1-receptor accessory protein to form an activated receptor complex (7–9), which further recruits adaptor proteins, including MyD88, IRAK4, and IRAK1 (10–12). Phosphorylation of IRAK1 by IRAK4 results in recruitment and binding of TRAF6 (TNF receptor-associated factor 6) to the complex, which is followed by the release of IRAK1–TRAF6 from the receptor complex to form another complex with TAK1–TAB1–TAB2 or TAK1–TAB1–TAB3 that are preassociated on the membrane (13–18). In this latter complex, TRAF6 is autopolyubiquitinated with Lys48-linked ubiquitin chains, through which it interacts with adaptor proteins TAB2 and TAB3, leading to the binding and activation of TAK1 and subsequently the activation of IKK and NF-κB (4, 19–21).

As described above, the adaptor proteins TAB2 and TAB3 are critical intermediates for both TNF- and IL-1-induced activation of NF-κB. TAB2 deficiency was embryonic lethal due to liver degeneration and apoptosis. However, TAB2-deficient cells were still capable of activating IKK and JNK in response to TNF, IL-1, and lipopolysaccharide (22), whereas knockdown of
both TAB2 and TAB3 inhibits IKK and JNK activation induced by TNF and IL-1, suggesting that TAB2 and TAB3 play redundant roles in TNF- and IL-1-triggered signaling (23). TAB2 and TAB3 are structurally related, and both contain an N-terminal CUE domain and C-terminal coiled-coil (CC) and nuclear protein localization 4 (Npl4) zinc finger (NZF) domains (24). TAB2 and TAB3 physically link TAK1 to RIP upon TNF stimulation and to TRAF6 upon IL-1β stimulation mainly through their respective NZF domains (4). Collectively, TNF stimulation leads to a RIP-TAB2/3-TAK1 complex, whereas IL-1 stimulation leads to the formation of a TRAF6-TAB2/3-TAK1 complex, both of which are critical for the signaling pathways leading to NF-κB activation.

In this study, we found that the NZF-containing protein RBCK1 (RBCC protein interacting with protein kinase C1) interacts with TAB2 and TAB3 and targets them for degradation in a proteasome-dependent manner. Overexpression of RBCK1 inhibits TAB2/3-mediated and TNF- and IL-1-induced NF-κB activation, whereas knockdown of RBCK1 by RNAi potentiates TNF- and IL-1-induced NF-κB activation. Our findings suggest that RBCK1 plays an inhibitory role in inflammatory signaling pathways.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant TNF, IL-1β, and IFN-γ were purchased from R&D Systems. Mouse monoclonal antibodies against FLAG (M2) and HA epitopes and MGCl32 (Sigma), rabbit polyclonal antibodies against ubiquitin and glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology), were purchased from the indicated manufacturers. Mouse anti-RBCK1, mouse anti-TAB2, and rabbit anti-TAK1 were raised against recombinant human full-length RBCK1, TAB2, and TAK1 proteins, respectively.

Constructs—Mammalian expression plasmids for human HA- or FLAG-tagged RBCK1, TAB1, TAK1, TAB2, and TAB3 and their mutants were constructed by standard molecular biology techniques. Mammalian expression plasmids for HA-RIP, HA-TAK1, HA-TRAF2, and HA-TRAF6 were described previously (25, 26). NF-κB luciferase reporter plasmid was provided by Dr. Gary Johnson.

Transfection and Reporter Gene Assays—293 cells (5 × 10⁴) were seeded on 24-well dishes and transfected the following day by standard calcium phosphate precipitation. Where necessary, empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. To normalize for transfection efficiency, 0.1 μg of pRL-TK Renilla luciferase reporter plasmid was added to each transfection. Approximately 20 h after transfection, luciferase assays were performed using a dual specific luciferase assay kit (Promega, Madison, WI). Firefly luciferase activities were normalized based on Renilla luciferase activities. All reporter gene assays were repeated at least three times. Data shown were average values ± S.D. from one representative experiment.

Co-immunoprecipitation and Western Blot Analysis—Transient transfection and co-immunoprecipitation, as well as endogenous coimmunoprecipitation experiments, were performed as described (27).

RNAi Experiments—Double-stranded oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro RNAi plasmid (Oligoengine Inc.). The following sequences were targeted for human RBCK1 cDNA: oligonucleotide 1, 5’-GACCCCAGATTGCAAGGGA-3’; oligonucleotide 2, 5’-TGAGTTACCTGGCCCTGT-3’; oligonucleotide 3, 5’-GGCCATCCATGAGGACAT-3’; oligonucleotide 4, 5’-CTGCAAGAGATATCAGGAG-3’; oligonucleotide 5, 5’-GGGTATCAGGAGCCTG-3’.

RESULTS

RBCK1 Interacts with TAB2 and TAB3—Previous studies have indicated that the CC domains of TAB2/3 are required for their ubiquitination and protein-protein interaction (23), whereas the NZF domains are critically involved in binding to polyubiquitin chains of RIP and TRAF6, which is key to TNF- and IL-1-induced NF-κB activation (4). To further understand the roles of TAB2/3 in TNF- and IL-1-triggered NF-κB activation pathways, we sought to identify proteins containing NZF domains similar to those of TAB2/3. Data base searches led to identification of RBCK1, also called HOIL-1 (28), XAP3 (29), or UIP28 (30), which contains an NZF domain at its N terminus (Fig. 1, A and B). In addition, it contains an intermediate CC domain and a C-terminal RING-IBR region, also known as RBR (31) or TRIAD domain (32), which contains two zinc finger domains and a cysteine/histidine-rich motif in between (Fig. 1A). It has been shown that RING-IBR region-containing proteins are involved in E3 ubiquitin ligase activity (31).

RBCK1 is an evolutionarily conserved protein and ubiquitously expressed in all examined tissues in rat (33). RBCK1 is localized in both the nucleus and the cytoplasm (34). It has been reported that RBCK1 causes ubiquitin-dependent degradation of heme-oxidized IRP2 (iron regulatory protein-2) as an E3 ligase in iron metabolism (28), facilitates transcriptional coactivation upon HBV infection (29), and interacts with various proteins, including UbcM4 E2 ubiquitin ligase (30), protein kinase C (33), CREB-binding protein, and PML (34).

Since RBCK1 contains an NZF domain that is related to the NZF domains of TAB2 and TAB3, we examined whether it associates with TAB2 and TAB3 through homophilic interactions. We transfected 293 cells with expression plasmids for HA-tagged TAB2 or TAB3 and FLAG-tagged RBCK1. Coimmunoprecipitation experiments indicated that RBCK1 interacted with both TAB2 and TAB3 (Fig. 1C).

To determine whether RBCK1 is associated with TAB2 and TAB3 under physiological conditions, we raised mouse polyclonal antisera against recombinant human full-length RBCK1 and TAB2 (Fig. 1D). Anti-TAB2 also recognized TAB3 because of their structural similarity (data not shown). Endogenous coimmunoprecipitation experiments indicated that RBCK1 was associated with TAB2/3 in untransfected 293 cells (Fig. 1E).

To determine whether other proteins associated with TAB2 and TAB3 interact with RBCK1, we performed transient transfection and coimmunoprecipitation assays. As shown in Fig. 1F, RBCK1 interacted with TAB1, TRAF6, and RIP but not TRAF2 and TAB1 in these experiments.
RBCK1 Inhibits NF-κB Signaling

**A**

![Diagram A](image)

**B**

![Diagram B](image)

**C**

![Diagram C](image)

**D**

![Diagram D](image)

**E**

![Diagram E](image)

**F**

![Diagram F](image)

**FIGURE 1.** **RBCK1 is associated with TAB2, TAB3, TAK1, TRAF6, and RIP.** A, a schematic presentation of human TAB2, TAB3, and RBCK1. C/H, cysteine/histidine-rich. B, an alignment of the amino acid sequences of the NZF domains from human RBCK1, TAB2, and TAB3. C, RBCK1 interacts with TAK1, TAB2, and TAB3. 293 cells (1 × 10⁶) were transfected with the indicated expression plasmids (5 μg each). Cell lysates were immunoprecipitated (IP) with anti-FLAG (αF) or mouse IgG antibody (Ab). The immunoprecipitates were analyzed by Western blot (WB) with mouse polyclonal anti-RBCK1, mouse polyclonal anti-TAB2, or rabbit polyclonal anti-TAK1 antibody. E, RBCK1 interacts with TAB2/3 and TAK1 under physiological conditions. 293 cell lysates were immunoprecipitated with mouse polyclonal anti-RBCK1 or mouse polyclonal anti-TAB2 or anti-TAK1 (bottom) antibody. F, RBCK1 interacts with TRAF6 and RIP but not with TRAF2 or TRAF1. The experiments were performed as in C.

Domain Mapping of the Interactions between RBCK1 and TAB2/3—It has been reported that the interaction between TAB2/3 and TAK1 is dependent on their respective CC domains (19, 23). To determine which domain of RBCK1 facilitates its interaction with TAB2/3, we made a series of RBCK1 deletion mutants (Fig. 2A), including aa 1–220 (carrying NZF), aa 221–500 (carrying CC and RING-IBR), aa 1–270 (carrying NZF and CC), and aa 271–500 (carrying RING-IBR). Coimmunoprecipitation assays indicated that RBCK1 (aa 1–270) interacted with TAB2 as strongly as its full-length did, and RBCK1 (aa 1–220) and RBCK1 (aa 221–500) mutants interacted with TAB2 to a lesser extent compared with the full-length RBCK1 (Fig. 2B). In the same experiment, RBCK1 (aa 271–500) did not interact with TAB2 (Fig. 2B). These observations suggest that the N terminus of RBCK1, which contains the NZF and CC domains, is essential for its interaction with TAB2. Similar results were obtained with TAB3 (data not shown).

Similarly, we made a series of TAB2 deletion mutants and determined which domains of TAB2 are required for its interaction with RBCK1 (Fig. 2A). In transient transfection and coimmunoprecipitation experiments, full-length TAB2, TAB2 (aa 55–693), and TAB2 (aa 361–693) interacted with RBCK1 efficiently (Fig. 2C). In the same experiments, TAB2 (aa 1–665) interacted with RBCK1 weakly, whereas TAB2 (aa 1–360) did not interact with RBCK1 (Fig. 2C). These data suggest that TAB2 interacts with RBCK1 through its C-terminal CC- and NZF-containing fragment. Similar results were obtained with TAB3 and its deletion mutants (data not shown). Taken together, our results suggest that the N terminus of RBCK1 and the C terminus of TAB2/3 are required for their interactions.

**RBCK1 Inhibits TAB2/3-mediated TNF- and IL-1-induced NF-κB Activation**—TAB2 and TAB3 are essential for TNF- and IL-1-induced NF-κB activation in a redundant way, and overexpression of TAB2 and TAB3 potently activates NF-κB (19, 23, 35). Since RBCK1 was found to interact with TAB2 and TAB3, we next investigated the effect of RBCK1 on TAB2/3-mediated and TNF- and IL-1-induced NF-κB activation. Reporter gene assays indicated that RBCK1 inhibited TAB2- and TAB3-mediated NF-κB activation in a dose-dependent manner (Fig. 3A). Moreover, RBCK1 inhibited TNF- and IL-1-induced NF-κB activation (Fig. 3B) but did not affect IFN-γ-mediated IRF-1 activation (Fig. 3C).

We further determined which domains are responsible for the inhibitory effect of RBCK1 on TAB2/3-mediated NF-κB activation. In reporter gene assays, RBCK1 (aa 221–500) inhibited TAB2- or TAB3-mediated NF-κB activation as potently as the full-length RBCK1 did. RBCK1 (aa 221–500) inhibited TAB2- or TAB3-mediated NF-κB activation to a lesser degree, and RBCK1 (aa 1–220) had little inhibitory effect (Fig. 3D).

**Knockdown of RBCK1 Potentiates TNF- and IL-1-induced NF-κB Activation**—Since RBCK1 inhibits TNF- and IL-1-induced NF-κB activation in overexpression experiments, we examined whether RBCK1 regulates TNF- and IL-1-induced
NF-κB activation under physiological condition. To test this, we made five RNAi plasmids targeting different sites of human RBCK1 mRNA. Transient transfection and Western blot analysis indicated that one of these RNAi plasmids (RBCK1-RNAi-4) could markedly inhibit the expression of transfected and endogenous RBCK1 in 293 cells, whereas the other RNAi plasmids had a little or no effect on RBCK1 expression (Fig. 4, A and B). In reporter gene assays, knockdown of RBCK1 by RBCK1-RNAi-4 plasmid activated NF-κB and potentiated TNF- and IL-1-induced NF-κB activation (Fig. 4, C and D). In similar experiments, knockdown of RBCK1 neither activated IRF-1 nor potentiated IFN-γ-induced IRF-1 activation (Fig. 4E). Collectively, these data suggest that RBCK1 is a physiological inhibitor of TNF- and IL-1-induced NF-κB activation pathways.

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**DISCUSSION**

Inflammation is a homeostatic mechanism that limits the effects of infectious agents. TNF and IL-1 are two proinflammatory cytokines, which induce inflammation through activation of the transcription factor NF-κB. In this study, we identified RBCK1 as a negative regulator of TNF- and IL-1-induced NF-κB activation.
Various studies have indicated that two homologous and structurally related adapter proteins TAB2 and TAB3 play redundant roles in TNF- and IL-1-mediated NF-κB activation pathways. TAB2 and TAB3 are associated with TAK1, and these protein complexes are recruited to RIP upon TNF stimulation and to TRAF6 upon IL-1 stimulation (4, 19, 23). Both TAB2 and TAB3 contain CUE, CC, and NZF domains. Among them, NZF domain is highly conserved, and the intact NZF domain is critical for TAB2/3 to bind to Lys63-linked polyubiquitin chains of other adaptor proteins, such as RIP and TRAF6, which are two signaling proteins essential for TNF- and IL-1-induced IKK activation, respectively (4). In a search for proteins containing NZF domains conserved with those of TAB2/3, we identified RBCK1, which has been shown to act as an E3 ubiquitin ligase and is involved in iron metabolism (28). Besides an NZF domain, RBCK1 contains a CC motif and a RING-IBR region, indicating that RBCK1 might be multifunctional through association with other proteins.

Our coimmunoprecipitation results suggest that RBCK1 interacts with TAB2 and TAB3 both in the mammalian overexpression system and in untransfected cells (Fig. 1, C–E). In addition, RBCK1 interacts with TAK1, TRAF6, and RIP, signaling components that are associated with TAB2/3 (Fig. 1, C–F). These findings suggest that RBCK1 is physically associated with TAB2/3-containing protein complexes.

In reporter gene assays, overexpression of RBCK1 inhibits TAB2/3-mediated and TNF- and IL-1-induced NF-κB activation (Fig. 3, A and B), whereas knockdown of RBCK1 by RNAi potentiates TNF- and IL-1-induced NF-κB activation (Fig. 4D). These results suggest that RBCK1 is a physiological suppressor of TNF- and IL-1-induced NF-κB activation pathways.

Previously, it has been demonstrated that the RING-IBR domain exerts E3 ubiquitin ligase activity and is involved in proteasome-dependent protein degradation (31). Our results indicate that the RING-IBR domain is essential for RBCK1 to confer its ubiquitination and proteasome-dependent degradation of TAB2/3 (Fig. 5). Consistently, the RBCK1 mutants that contain the CC and RING-IBR domain also inhibit TAB2/3-mediated NF-κB activation. Furthermore, it has been reported that TAB2/3 are modified by phosphorylation and ubiquitination upon stimulation, which seems to be required for their adaptor functions (23). Taken together, these observations support the following model of the role of RBCK1 in TNF- and IL-1-induced signaling. In this model, RBCK1, which is an E3 ubiquitin ligase, is associated with TAB2/3 through their respective CC and NZF domains. This association causes TAB2/3 ubiquitination and degradation and therefore negatively regulates TNF- and IL-1-induced NF-κB activation.
Rbck1 Inhibits NF-κB Signaling

Host inflammatory responses must be strictly controlled by a variety of negative regulators, because hyperactivation of inflammatory pathways is usually harmful. So far, a number of suppressors of inflammatory signaling have been identified. For example, IraK-M, a homolog of IRAK1 that is induced by lipopolysaccharide stimulation after 24 h, interacts with MyD88 to negatively regulate its signaling to TRAF6 (36). A20 inhibits TNF-, IL-1-, TLR-, and RIG-I-induced NF-κB activation pathways by its ubiquitin-editing function (5, 37). β-Arrestin inhibits NF-κB activation at both upstream and transcriptional levels (37, 38). The inhibitory factors alleviate the inflammatory responses, thereby preventing the host from mounting extraordinary inflammatory responses. The identification of Rbck1 as a novel inhibitor of TNF- and IL-1-induced NF-κB activation pathways provides an additional target and approach for controlling inflammation.

Acknowledgments—We thank members of our laboratory for technical help and stimulating discussion.

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