Casein Kinase 1α Interacts with RIP1 and Regulates NF-κB Activation †

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Casein Kinase 1α Interacts with RIP1 and Regulates NF-κB Activation†

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ABSTRACT: Tumor necrosis factor α (TNFα) triggers a signaling pathway converging on the activation of NF-κB, which forms the basis for many physiological and pathological processes. In a kinase gene screen using a NF-κB reporter, we observed that overexpression of casein kinase 1α (CK1α) enhanced TNFα-induced NF-κB activation, and a CK1α kinase dead mutant, CK1α (K46A), reduced NF-κB activation induced by TNFα. We subsequently demonstrated that CK1α interacted with receptor interacting protein 1 (RIP1) but not with TRADD, TRAF2, MEKK3, IKKα, IKK*β*, or IKK*γ* in mammalian cells. RIP1 is an indispensable molecule in TNFα/NF-κB signaling. We demonstrated that CK1α interacted with and phosphorylated RIP1 at the intermediate domain. Finally, we showed that CK1α enhanced RIP1-mediated NF-κB activation. Taken together, our studies suggest that CK1α is another kinase that regulates RIP1 function in NF-κB activation.

The pro-inflammatory cytokine, tumor necrosis factor α (TNFα), induces a wide variety of biological responses. It has been implicated in the pathogenesis of a number of human diseases, such as sepsis, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases. TNFα exerts its diverse biological properties by binding to and activating two distinct cell surface receptors, TNFR1 and TNFR2. As to TNFα's biological activities, multiple experimental approaches have revealed that TNFR1 initiates the majority of these activities (*1*). Surprisingly, TNFR1 mainly induces two opposing pathways: one is an apoptosis pathway, and the other is a NF-κB activation pathway, which drives the synthesis of a number of pro-inflammatory gene products that play important roles in immune response, inflammation, and anti-apoptosis (*2*).

NF-κB activation by TNFR1 has been well characterized. Upon the stimulation of TNFα, the cytoplasmic segment of TNFR1 recruits and assembles multicomponent signalsome of TNFR1-TRADD-TRAF2-RIP1-IKK. Thus the IKK complex—composed of two catalytic subunits, IKKα, IKK*β*, and a regulatory subunit IKK*γ*—is triggered to activate NF-κB. In fact, it is more complicated concerning several aspects of the pathway, such as signaling specificity and kinetics of activation. A large number of molecules have been reported to influence this pathway. Recently, using a large-scale functional proteomics approach, 221 molecular associations and 80 previously unknown interactors around 32 known and candidate TNF/NF-κB pathway components were mapped (*3*). These studies hint that there may be other undiscovered proteins that may play important roles in TNFα signaling.

In TNFα-induced NF-κB activation, RIP1 is an indispensable factor since RIP1-deficient cells from knockout mice failed to respond to TNFα (*4*). Upon TNFα stimulation, RIP1 undergoes covalent modification, including phosphorylation and ubiquitination, which may be important for the NF-κB activation. Many factors have been reported to influence NF-κB activation by modification of RIP1. For example, RIP3 phosphorylates RIP1 and negatively regulates RIP1-induced NF-κB activation (*5*, *6*). A20, which negatively regulates NF-κB signaling, could disassemble K63-linked ubiquitin chains bound to RIP1 as well as ligate the K48-linked ubiquitin chain to it (*7*, *8*). It is likely that RIP1 with a linked K63 polyubiquitin chain may facilitate the recruitment of TGF-activated-kinase-binding protein 2 (TAB2) (*9*), and the latter is proposed to act as an IKK activator.

Casein kinase 1 (CK1) is one of the early serine/threonine protein kinase families isolated and characterized. Seven CK1 genes encoding α, *β*, *γ*1, *γ*2, *γ*3, *δ*, and *ϵ* isoforms have been characterized in mammals and vertebrates. CK1 has

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been shown to phosphorylate a large number of proteins involved in a wide range of cellular functions. CK1 members associate with components of several signal transduction pathways and bear modulatory function on these pathways. Members of the CK1 family have been reported to phosphorylate and regulate multiple key components of the Wnt signaling (*10*). CK1α phosphorylates β-catenin on serine 45, which is required for subsequent glycogen synthase kinase 3 (GSK-3) phosphorylation and the degradation of β-catenin (*11*). CK1ε phosphorylates Dishecelled (Dvl) to disrupt the complex of Dvl/Axin/GSK-3 and form Dvl/Frat/GSK-3, resulting in the inhibition of GSK-3 activity and stabilization of β-catenin (*12*). Interestingly, CK1ε also functions as a molecular switch that directs Dvl from the JNK pathway to the β-catenin pathway (*13*). CK1 has also been reported to play important roles in NF-AT translocation, Hedgehog, and TGF-β signaling pathways (*14*−*18*). CK1 has also been shown to attenuate the Fas-induced apoptosis and TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (*19*, *20*).

Regulation of many signaling pathways is achieved by the phosphorylation and dephosphorylation of key components of these pathways. Due to the important roles of kinases in the regulation of signaling pathways, we designed a cell-based kinase screening for their potential influence on several signaling pathways in mammalian cells using our kinase library. We found that overexpression of CK1α enhanced the NF-κB activation induced by TNFα. Further studies indicated that CK1α interacted with the intermediate domain of RIP1 and phosphorylated RIP1. Therefore, CK1α may be a new member regulating RIP1 function and influence the TNFα signaling pathway.

MATERIALS AND METHODS

Expression Vectors and Mutagenesis. All mammalian cell expression plasmids were constructed in pEFBOS-Flag (N-terminal Flag tag), pCDEF-Myc (N-terminal Myc tag), or pCDEF-HA (N-terminal HA tag) vectors using standard PCR methods employing custom-designed primers containing appropriate restriction sites. Amino acid mutations were generated using the QuikChange site-directed mutagenesis kit from Stratagene. The presence of the introduced mutation and the fidelity of PCR replication were confirmed by sequence analysis. The pGEX-4T-1 vector (Amersham Biosciences, Inc.) was used for expression of GST-tagged RIP1 truncations in *Escherichia coli*.

Cell Culture, Transfection, and Luciferase Assay. HEK293T and HeLa cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Bio International Limited). Constructs expressing eukaryotic proteins were transiently transfected into mammalian cells using lipofectamine-2000 reagents (Invitrogen). CrmA was included in all transfection at a 1:1 molar ratio to suppress cell death caused by RIP1, RIP2, or RIP3. For NF-κB reporter assays, a dual-luciferase assay system (Promega) was used. Briefly, cells were seeded into 24-well plates, and a mixture of eukaryotic expression plasmids together with pNF-κB-luciferase reporter plasmid were transfected. Cells were cultured for 40 h post transfection, unstimulated or stimulated at 37 °C in the growth medium containing 20 ng/mL TNFα. For IC261 treatment, transfected cells were pretreated for at least 2 h with IC261 prior to the addition of TNFα. After a 12 h TNFα-stimulation period, cells were lysed in 1× reporter lysis buffer (Promega). Luciferase activity was quantitated with Luminometer (Lumat LB 9507, Berthold Technologies) immediately after the addition of luciferin (Promega). Luciferase activity was determined in triplicate for each experimental condition.

Western Blot Analysis and Immunoprecipitation. For immunoprecipitation, HEK293T cells were plated into 10 cm dishes and cells were transiently transfected with indicated plasmids by the calcium phosphate precipitation method. At 24 h post transfection, cells were lysed in lysis buffer containing 1 × Tris-buffered saline, 1% Nonidet P-40, 1 mM PMSF, 1 mM dithiothreitol, 1 × protease inhibitor mixture, and 1 × phosphatase inhibitor cocktail II from Sigma. For western blot analysis, lysates were resolved by SDS-PAGE and transferred to the nitric cellulose membrane. Membranes were blocked with 5% nonfat milk in TBST buffer (10 mM Tris [pH7.8], 150 mM NaCl, with 0.05% Tween detergent). Blots were incubated with the indicated primary antibodies or antiserum followed by a secondary antibody conjugated with horseradish peroxidase and then assayed by enhanced chemiluminescence assay (SuperSignal Western Blotting Kit, PIERCE). For immunoprecipitation, cell lysates were precleared with protein G-Plus agarose (Santa-Cruz), and they were incubated with indicated antibodies and protein G-Plus agarose. After incubation, the immune complexes were washed extensively in lysis buffer, boiled in sample buffer, and subjected to western blot analysis. A goat-anti-CK1α polyclonal antibody was purchased from Santa Cruz Biotechnology. A mouse-anti-RIP1 monoclonal antibody was obtained from BD Biosciences Pharmingen. Mouse anti-phospho-serine and rabbit polyclonal anti-phospho-threonine antibodies were from Sigma and Cell Signaling, respectively.

In Vitro Kinase Assay. The 293T cells transfected with Flag-RIP1 (K45A) or Flag-RIP1 were lysed, and proteins were immunoprecipitated with mouse M2 anti-Flag antibody. After extensive washing with lysis buffer and kinase buffer [20 mM Tris-HCl (pH 7.5), 20 mM β-glycerophosphate, 100 mM NaCl, 10 mM $MgCl_2$, 100 mM Na_3VO_4, 2 mM dithiothreitol, 20 mM ATP, 1 × phosphatase inhibitor cocktails II (Sigma)], the immunoprecipitates were subjected to kinase assay with or without 300 ng of GST-CK1α expressed from insect cells (Invitrogen). The reaction was performed at 30 °C in 20 μL of kinase assay buffer supplemented with 10 μCi [γ-^{32}P]ATP for 30 min. Reaction was terminated by the addition of 6 × SDS-PAGE loading buffer and incubation at 95 °C for 5 min. Samples were resolved on a 10% SDS-PAGE gel, and an autoradiograph was obtained. GST-RIP1 mutant fusion proteins were expressed in the *E. coli.* system, purified by flowing through glutathione-Sepharose 4B column (Amersham Biosciences), and eluting with elution buffer (50 mM Tris, 25 mM glutathione, pH 8.0). A total of 2.0 μg of GST or GST-RIP1 fragments were used as substrates in the presence of 200 ng of insect cell expressed GST-CK1α in in vitro kinase assay.

Statistical Analysis. Statistical significance between groups was determined by a *t*-test of two independent samples.

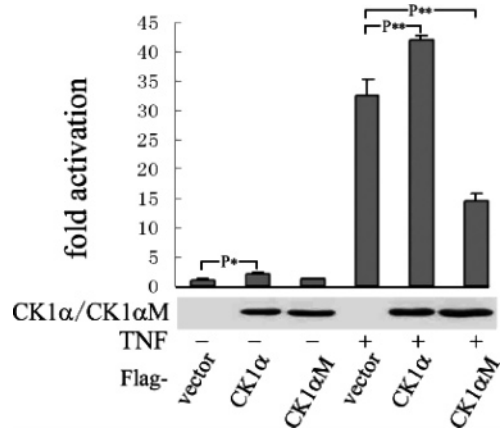

FIGURE 1: Regulation of TNFα/NF-κB signaling by CK1α. Overexpression of CK1α enhanced TNFα-stimulated NF-κB reporter activity, while the catalytic inactive mutant, CK1α (K46A), reduced the activity. HeLa cells were transiently cotransfected with NF-κB luciferase reporter plasmid (0.1 μg) together with control vector (0.4 μg), wild type CK1α (0.4 μg), or CK1α (K46A) (0.4 μg). At 24 h post transfection, cells were exposed to TNFα (20 ng/mL) for 10 h. Cell lysates were prepared, and the luciferase assay was performed using a dual-luciferase assay kit (Promega). Data are the mean of triplicates from one representative experiment. This experiment was repeated three times with similar results. The fold of activation is normalized luciferase activity over the vector control without TNFα treatment. *P** represents $P < 0.05$, and *P*** represents $P < 0.01$.

RESULTS

CK1α Enhancement of TNFα-Induced NF-κB Activation. Phosphorylation and dephosphorylation of key components play important regulatory roles in many signaling pathways. Our lab is focusing on studies of enzymes regulating inflammation-related signaling pathways. We established a full-length protein kinase library by gene cloning into a mammalian expression vector one by one, in which their corresponding kinase dead mutants (dominant negative) were also constructed by mutating the ATP binding site from lysine to alanine. The complexity of TNFα signaling raises the possibility that other undiscovered proteins, including kinases, may play undiscovered roles in modulating the activity of this pathway. We screened the kinase library using a reporter construct containing a key promoter element NF-κB to further elucidate the mechanism of TNFα signaling. In this screen, both wild-type kinases and their corresponding dominant negative mutants were transfected into mammalian cells together with the NF-κB-luciferase reporter, and NF-κB-luciferase activity was measured to determine the effect of these kinases on NF-κB signaling.

During our screen, we found that CK1α had an enhancing effect on the TNFα-induced NF-κB activation. Overexpression of CK1α in HeLa cells resulted in a 28.5% increase of TNFα-induced NF-κB activation ($P < 0.01$). In contrast, overexpression of CK1α (K46A), the kinase inactive mutant, produced a dominant negative effect leading to a 56% reduction in the NF-κB reporter activity ($P < 0.01$) (Figure 1). Similar results were obtained from experiments performed in HEK 293T cells (data not shown). These results suggest that CK1α may be another modulator of the TNFα-induced NF-κB activation pathway.

Interaction of CK1α with the Cytoplasmic Domain of TNFR1 and RIP1. Previous reports have shown that CK1 associated with and phosphorylated TNFR2, and it might also phosphorylate the cytoplasmic domain of TNFR1 in vitro (*21*). However, it remains unclear which isoform of CK1 is responsible for this activity, and the direct interaction of CK1 and TNFR1 has not been verified. In HEK293 and HeLa cells, TNFR1 predominately mediates NF-κB activation by TNFα whereas TNFR2 activates transcription poorly (*22*). Our NF-κB reporter assay results, combined with previous reports that CK1 might be involved in TNFR1 signaling, led us to study the interaction between CK1 and TNFR1. HEK293T cells were cotransfected with HA-CK1α and Flag-TNFR1 (235−455 AA) (AA, amino acid), which encodes the cytoplasmic domain of TNFR1 with a Flag tag. Cell lysates were immunoprecipitated with anti-Flag antibody, and the immunoprecipitates were analyzed by western blotting using anti-HA antibody. As shown in Figure 2A, CK1α specifically associated with the cytoplasmic domain of TNFR1. The result indicates that CK1α is in the receptor-proximal signaling complex.

It is already known that TNFα induced NF-κB activation through the formation of TNFR1/TRADD/TRAF2/RIP1/MEKK3/IKK complex. To test whether CK1α also interacts with other members of the TNFR1 receptor proximal complex, 293T cells were cotransfected with HA-CK1α together with Flag-tagged TRAF2, TRADD, RIP1, IKKα, IKKβ, IKKγ, or MEKK3, respectively, and co-immunoprecipitation assays were performed. In the RIP1 cotransfection experiment, a CrmA expression plasmid was also added to inhibit RIP1-induced apoptosis. Interestingly, CK1α was found to be able to interact with RIP1, but not TRAF2, TRADD, IKKα, IKKβ, IKKγ, or MEKK3 (Figure 2A).

RIP1 is a prototypic kinase of the RIP family of which seven members have been found so far. These members share significant homology in their kinase domains but are different in other regions (*23*). To explore if CK1α specifically binds to RIP1, we tested whether it also interacted with RIP2 or RIP3. The 293T cells were cotransfected with HA-CK1α together with Flag-RIP1, Flag-RIP2, or Flag-RIP3, respectively. Cell lysates were immunoprecipitated with anti-Flag antibody, and the immunoprecipitates were analyzed by western blotting using anti-HA antibody. As shown in Figure 2B, CK1α clearly associated with RIP1 but not with RIP2 or RIP3. Therefore, we concluded that CK1α specifically bound to RIP1.

To examine whether TNFα treatment influences the interaction between CK1α and RIP1, 293T cells were cotransfected with Flag-RIP1 together with control vector or Myc-CK1α. One sample cotransfected with Flag-RIP1 and Myc-CK1α was treated with TNFα (20 ng/mL) for 10 min before harvest. Lysates were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were analyzed by western blotting with anti-Flag antibody. As shown in Figure 2C, TNFα treatment had no noticeable effect on the interaction between CK1α and RIP1, indicating that the interaction between CK1α and RIP1 is constitutive.

Endogenous Interaction between CK1α and RIP1. Since more CK1α was co-immunoprecipitated by RIP1 than TNFR1, we focused our investigation on the relationship between CK1α and RIP1 and the potential influence of this interaction. First, we performed a reciprocal co-immunoprecipitation assay with Flag-RIP1 and Myc-CK1α to confirm the interaction between CK1α and RIP1 (data not shown).

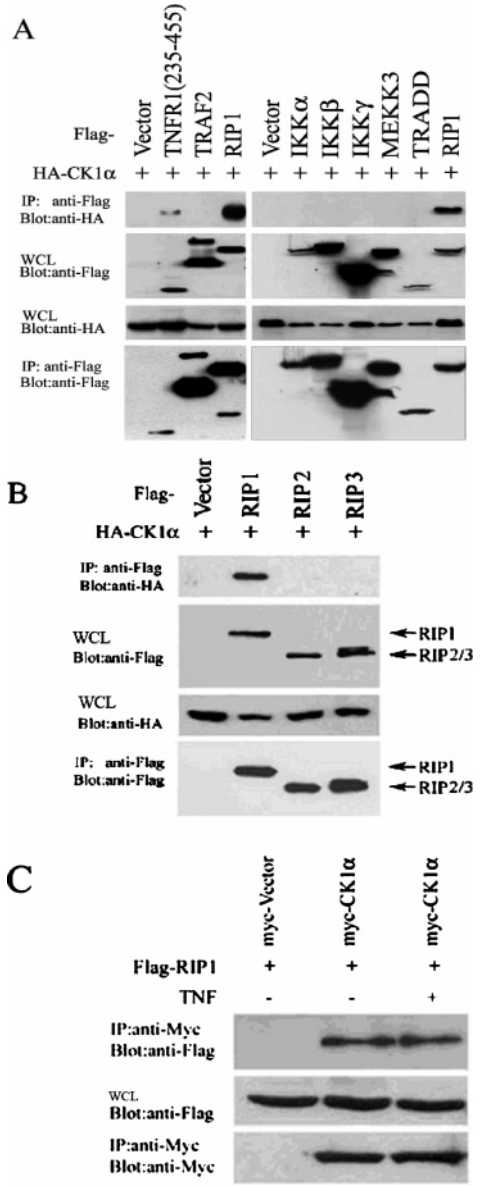

FIGURE 2: Interaction of CK1α with TNFR1 and RIP1 in transfected 293T cells. (A) The 293T cells were cotransfected with HA-CK1α together with control vector, Flag-TNFR1 (235-455AA), Flag-TRAF2, Flag-RIP1, Flag-IKKα, Flag-IKK*β*, Flag-IKK*γ*, Flag-MEKK3, and Flag-TRADD, respectively. Cells were lysed at 24 h post transfection, and lysates were immunoprecipitated with anti-Flag antibody. The presence of HA-CK1α in the immune complex was detected by western blot with anti-HA antibody. The precipitates were also blotted with anti-Flag, and the whole cell lysates were blotted with either anti-Flag or anti-HA. One of three experiments is shown. (B) CK1α associated with RIP1 but not with RIP2 or RIP3. The 293T cells were cotransfected with HA-CK1α together with control vector, Flag-RIP1, Flag-RIP2, or Flag-RIP3, respectively. Cells were lysed at 24 h post transfection, and lysates were immunoprecipitated with anti-Flag antibody. The presence of HA-CK1α in the immune complex was detected by western blot with anti-HA antibody. The precipitates were also blotted with anti-Flag, and the whole cell lysates were blotted with either anti-Flag or anti-HA. One of four experiments is shown. (C) TNFα exhibited no effect on the binding of RIP1 and CK1α. The 293T cells were cotransfected with Flag-tagged RIP1 together with control vector or Myc-CK1α. At 24 h post transfection, one sample cotransfected with Flag-RIP1 and Myc-CK1α was treated with TNFα (20 ng/mL) for 10 min before harvest. Lysates were immunoprecipitated with anti-Myc antibody, and the presence of Flag-RIP1 in the immune complex was detected by western blotting with anti-Flag antibody. The precipitates were also blotted with anti-Myc, and the whole cell lysates were blotted with anti-Flag. One of three experiments is shown.

Later, co-immunoprecipitation at the endogenous level was performed to avoid the overexpression caveat and to further examine their physiological association. HeLa cell lysates were immunoprecipitated with anti-CK1α antibody or a control antibody, and then the immune complex was analyzed by western blotting with a specific anti-RIP1 antibody. As shown in Figure 3A, RIP1 was readily detected in anti-CK1α immune complex, demonstrating the interaction of RIP1 and CK1α at physiological level. Similar to what was observed with overexpressed proteins, TNFα had no effect on the interaction between endogenous RIP1 and CK1α (data not shown).

Further, we determined which domain of RIP1 is responsible for CK1α binding. RIP1 is composed of an N-terminal kinase domain (KD, amino acids 17−289), an intermediate domain (ID, amino acids 290−582), and a C-terminal death domain (DD, amino acids 583−671). Truncation mutants of RIP1 representing disparate domains were constructed. The 293T cells were cotransfected with HA-CK1α together with control vector, Flag-RIP1 (1-292AA), Flag-RIP1 (293-558AA), or Flag-RIP1 (550-671AA), representing the KD, ID, or DD of RIP1. Immunoprecipitation analysis revealed that only ID of RIP1 retained the ability to interact with CK1α, while either the KD or DD exhibited no interaction with CK1α (Figure 3B). These data indicate that the RIP1-CK1α interaction might play an important role in the TNFα pathway.

CK1α Phosphorylation of RIP1. Previous studies showed that CK1α could phosphorylate a large number of proteins associated with it (*12*). We used an in vitro kinase assay to determine whether RIP1 is a new substrate for CK1α. RIP1 itself is a kinase and could autophosphorylate by its own kinase domain in kinase assay. To eliminate this background, a catalytically inert RIP1 (K45A) mutant was constructed. The 293T cells were transfected with Flag-RIP1 (K45A), and cell lysates were immunoprecipitated by anti-Flag antibody to obtain purified RIP1 protein. Immunoprecipitates were subjected to an in vitro kinase assay using insect cell expressed GST-CK1α as kinase. The wild type Flag-RIP1 autophosphorylation band was shown as the molecular weight and phosphorylation control (Figure 4A, lane 1 of upper panel). Radioactivity in the bands representing phosphorylated RIP1 (K45A) was quantitated densitometrically by imageJ software (National Institutes of Health, version 1.38). As shown in Figure 4A, the top diagram shows the relative intensity of RIP1 phosphorylation. In the presence of GST-CK1α, the relative phosphorylation level of RIP1 (K45A) was more than 6-fold higher than that of RIP1 (K45A) alone (lanes 3 and 4 of upper panel), indicating that RIP1 is a substrate of CK1α. Equivalent amounts of RIP1 proteins were present in the kinase assay as shown by western blot of immunoprecipitates (lower panel).

We also examined whether CK1α could phosphorylate RIP1 in mammalian cells. Flag-RIP1 (K45A) was used to avoid RIP1 autophosphorylation. A control vector, HA-CK1α or HA-CK1α (K46A), was cotransfected into 293T cells along with Flag-RIP1 (K45A). After 24 h, Flag-RIP1 (K45A) was immunoprecipitated from the cell lysates by anti-Flag antibody, and the serine/threonine phosphorylation of RIP1 was examined by anti-phospho-serine/threonine immunoblotting. The relative density of the bands representing phosphorylated RIP 1(K45A) at serine/threonine residues

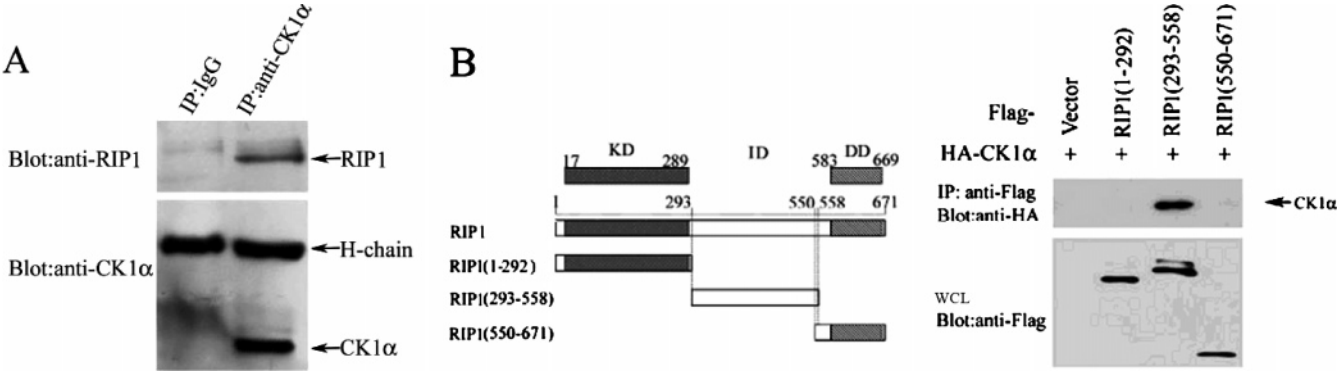

FIGURE 3: The endogenous association of CK1α with RIP1. (A) Endogenous RIP1 associated with CK1α in HeLa cells. CK1α was immunoprecipitated from HeLa cell lysates with anti-CK1α antibody, and an anti-IgG antibody was used as a control. The presence of associated RIP1 in the immune complex was detected with anti-RIP1 monoclonal antibody. The precipitates were also blotted with anti-CK1α. One of three experiments is shown. (B) CK1α interacted with the ID of RIP1. The 293T cells were cotransfected with HA-CK1α together with control vector, Flag-RIP1 (1-292AA), Flag-RIP1 (293-558AA), or Flag-RIP1 (550-671AA), respectively. Immunoprecipitation and western blot were performed as described in Figure 2. One of four experiments is shown. AA = amino acid.

was quantitated by imageJ software and shown in the top diagram. As shown in Figure 4B, there is background phosphorylation of RIP1 (K45A) when it was cotransfected with the control vector. The intensity of background phosphorylation was arbitrarily assigned as 1 (lane 1 of the diagram). Compared with the control vector, CK1α expression increased RIP1 (K45A) phosphorylation on serine/threonine residues in cells (relative intensity of phospho-RIP1 (K45A) was 4.9, lane 2 of the diagram). On the other hand, expression of CK1α (K46A) decreased RIP1 (K45A) phosphorylation on serine/threonine residues in cells (relative intensity of phospho-RIP1 (K45A) was 0.7, lane 3 of the diagram). To further examine whether the CK1α kinase activity was required for the phosphorylation of serine/threonine residues on RIP1, we treated 293T cells with IC261, a specific inhibitor of CK1 (*24*). IC261 treatment lowered the relative intensity of phospho-RIP1 (K45A) cotransfected with HA-CK1α from 4.9 to 1.65 (compare lane 2 to lane 4 of the diagram), indicating that the kinase activity of CK1α is required for serine/threonine phosphorylation of RIP1.

To determine the phosphorylated region of RIP1 by CK1α, we expressed GST-RIP1 (1-301AA), GST-RIP1 (293-558AA), and GST-RIP1 (550-671AA) in *E. coli*, and the purified protein fragments were used as substrates for an in vitro kinase assay. In the presence of insect cell expressed GST-CK1α, a phosphorylated band was observed corresponding to GST-RIP1 (293-558AA) (Figure 4C, lane 6 of upper panel). However, no phosphorylated band was observed when GST-RIP1 (1-301AA) or GST-RIP1 (550-671AA) was used as the substrate, indicating that CK1α phosphorylates RIP1 mainly within the intermediate domain.

CK1α Enhancement of RIP1-Mediated NF-κB Activation. Since CK1α enhanced TNFα-mediated NF-κB activation and associated with RIP1, we asked whether CK1α was a critical member influencing the TNFα signaling pathway. We examined whether CK1α and its catalytic inactive mutant CK1α (K46A) were able to influence RIP1-mediated NF-κB activation. HeLa cells were cotransfected with RIP1 and NF-κB-Luc plasmid, along with a control vector, CK1α, or CK1α (K46A). As shown in Figure 5A, coexpression of CK1α and RIP1 significantly stimulated RIP1-mediated NF-κB activation at 36 h post-transfection ($P < 0.05$) while coexpression of CK1α (K46A) and RIP1 resulted in strong inhibition of NF-κB activation induced by RIP1 overexpression ($P < 0.01$). Transient expression of CK1α or CK1α (K46A) showed similar effects on the NF-κB activation mediated by TNFR1 or TRAF2 (data not shown).

To test whether the interaction of CK1α and RIP1 intermediate domain imposed a functional effect on NF-κB activation, HeLa cells were cotransfected with RIP1-ID and NF-κB-Luc plasmid, along with a control vector, CK1α, or CK1α (K46A). The results showed that RIP1-ID could potently activate NF-κB, although at a lower level compared to full length RIP1 (Figure 5B). Coexpression of CK1α strongly enhanced the NF-κB activation induced by RIP1-ID, suggesting that CK1α might modulate NF-κB activation through binding to the intermediate domain of RIP1 (Figure 5B).

DISCUSSION

TNFα has been implicated in the pathogenesis of a wide spectrum of human diseases. Although this pathway has been extensively studied, many regulatory mechanisms remain unclear, especially concerning the regulation, maintenance, or propagation of the basal and ligand-stimulated activation. A comprehensive functional study of this pathway would shed light on the identification of novel therapeutic strategy. Using NF-κB transcriptional reporter assays in mammalian cells, we systematically screened our kinase library to identify new kinases that might affect the TNFα/NF-κB cascade. CK1α was found to be an important kinase regulating the TNFα pathway by binding to and phosphorylating RIP1.

There are many other reports that connect CK1 with the TNFα signal pathway. The CK1 phosphorylation consensus sequence is found in the cytoplasmic domain of the membrane TNF ligand (mTNF) family, and recombinant CK1 is able to phosphorylate mTNF (*25*). CK1 has been shown to phosphorylate p75-tumor necrosis factor receptor (TNFR2) and negatively regulate apoptosis induced by TNFα signaling (*21*). It is not surprising that CK1α was found to be a new regulator of the TNFα/NF-κB signaling pathway. Although the dominant negative mutant of CK1α has been shown to inhibit TNFα or RIP1's function in NF-κB activation, the inhibition was moderate compared with the inhibition by other dominant negative mutants of key modulators in TNFα signaling pathway, such as TRAF2 and

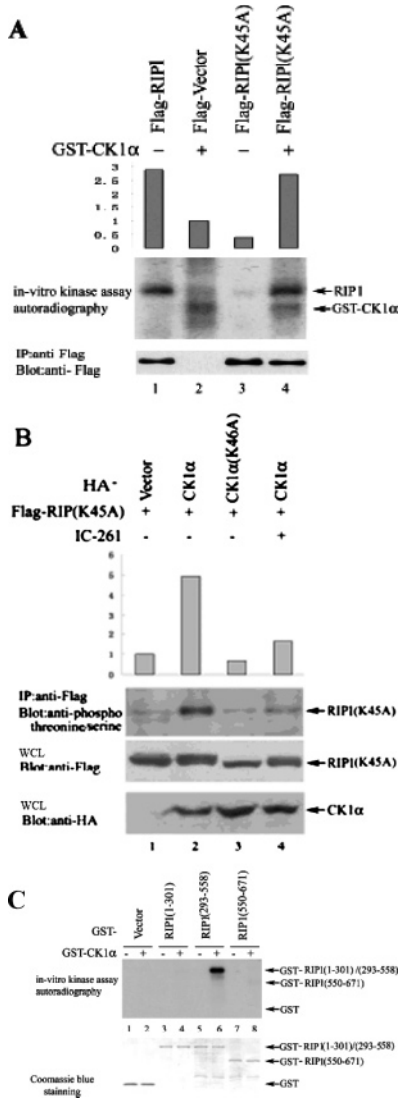

FIGURE 4: CK1α phosphorylates the intermediate domain of RIP1. (A) The 293T cells were transfected with control vector, Flag-RIP1, or Flag-RIP1 (K45A) and were lysed 24 h post transfection, and the lysates were immunoprecipitated with anti-Flag antibody. The immunoprecipitates were subjected to an in vitro kinase assay using [γ^{32}ATP] with or without 300 ng ofGST-CK1α, and they were visualized by autoradiography. Radioactivity in the bands representing phosphorylated RIP1 (K45A) was quantitated densitometrically by imageJ software (National Institutes of Health, version 1.38). The relative intensity of RIP1 phosphorylation is shown in the top diagram. One of four experiments is shown. (B) Phosphorylation of serine/threonine residues within RIP1 following expression of CK1α. 293T cells were cotransfected with Flag-tagged RIP1 (K45A) together with control vector, HA-CK1α, or HA-CK1α (K46A). At 24 h post transfection, cells were treated with or without 40 μM IC261 for 3 h before harvesting. Anti-Flag immunoprecipitates from the respective whole cell lysates (WCL) were analyzed by anti-phospho-serine/threonine (top panel), anti-Flag (middle panel), or anti-HA (bottom panels) immunoblotting. The relative density in the bands representing phosphorylated RIP1 (K45A) on serine/threonine residues was quantitated by imageJ software and is shown in the top diagram. One of three experiments is shown. (C) CK1α phosphorylated RIP1 in the intermediate domain. The GST fusion proteins encompassing the RIP1 KD, ID, or DD were expressed in *E. coli*, and purified fragments were used as substrates. GST protein was used as a control for this reaction. GST-fusion proteins were incubated with insect cell expressed GST-CK1α in the presence of 10 μCi/mL [γ^{32}ATP], resolved by 10% SDS-PAGE, and subjected to autoradiography (upper panel). The loaded GST-fusion proteins were detected by Coomassie blue staining (lower panel). One of three experiments is shown.

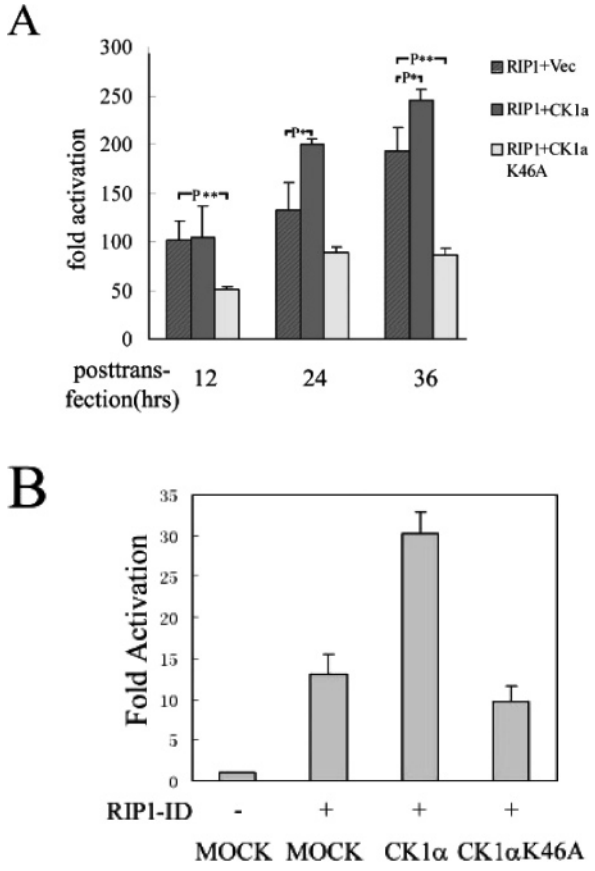

FIGURE 5: CK1α enhances RIP1-mediated NF-κB activation. (A) CK1α increased NF-κB activation mediated by RIP1 overexpression. HeLa cells were cotransfected with 100 ng of NF-κB luciferase plasmid, 50 ng of Flag-RIP1, and 50 ng of CrmA, together with 300 ng of control vector, Flag-CK1α, or Flag-CK1α (K46A). Cells were harvested at different time points post-transfection, and luciferase activity was determined as described in Figure 1. This experiment was repeated four times with similar results. The fold of activation is normalized luciferase activity over control vector without RIP1 overexpression. (B) CK1α enhanced NF-κB activation induced by RIP1-ID overexpression. HeLa cells were cotransfected with NF-κB luciferase plasmid (100 ng) together with the indicated plasmids. After 24 h, luciferase reporter gene assays were performed. This experiment was repeated three times with similar results. The fold of activation is normalized luciferase activity over control vector. *P** represents $P < 0.05$, and *P*** represents $P < 0.01$.

RIP1. Our data suggest that CK1α may not be a signal transducer but rather a regulator in the pathway. It functions by regulating the activity of RIP1, a critical member of the TNFα signal transduction pathway.

Our data show that CK1α binds to TNFR1 and RIP1, suggesting that CK1α is in the receptor proximal complex. The binding is specific because we did not detect its interaction with other key members including TRAF2, TRADD, IKKα, IKKβ, IKKγ, or MEKK3. In another study, the kinase activity of CK1α was measured in a number of tumor cell lines. It was found that Jurkat cells deficient for RIP1 contained the highest CK1α kinase activity (*26*). Though the detailed mechanism is not clear yet, it brings a piece of evidence for the functional correlation between CK1α activity and RIP1.

Further, we found that the intermediate domain of RIP is responsible for CK1α binding, while either the kinase domain or death domain exhibits no interaction with CK1α. Our

phosphorylation studies indicate that CK1α phosphorylates RIP1 mainly within the intermediate domain. Another kinase RIP3, which phosphorylates RIP1, also binds RIP1 in this region via RIP homotypic interaction motif (RHIM). Because the RIP1 intermediate domain is mainly responsible for its NF-κB activation, these data suggest that the RIP1-CK1α interaction might play an important role in the TNFα pathway. We also observed that CK1 increases RIP1-induced NF-κB activation, while RIP3 phosphorylation of RIP1 results in the inhibition of RIP1-induced NF-κB activation (*5*).

The ubiquitination of RIP1 has been identified, which may be critical for limiting inflammation by terminating TNFα-induced NF-κB activation (*27*). Also, it has been reported that Lys377 of RIP1 is the functional ubiquitination site, and mutating this residue to arginine completely abolishes RIP1-mediated NF-κB activation (*28*). Recent reports indicate that c-IAP1 could mediate RIP1 ubiquitination, while A20 was shown to exert two opposing activities: sequential de-ubiquitination and ubiquitination of RIP1, thereby targeting RIP1 to proteasomal degradation (*7*, *8*). It was also reported that HSP90 associates with RIP1, thus regulating the stability of RIP1. Geldanamycin, a specific inhibitor of HSP90, caused RIP1 degradation by the proteasome pathway (*29*). However, previous study showed that the kinase activity of RIP1 is not required for the ubiquitination of itself (*30*). In future experiments, it would be interesting to test whether phosphorylation of RIP1 by CK1α affects its ubiquitination pattern.

Recently, it was reported that CK1α is also a kinase for FADD, a key adaptor in transducing death receptor signals. CK1α phosphorylates FADD at the site of serine 194. The phosphorylated FADD (p-FADD) has an enhanced effect on NF-κB activation, and it also decreases the number of cells in the G2 phase of the cell cycle (*31*). CK1 has a substrate sequence consensus of S/T-PO4-X-X-S/T, where S/T-PO4 indicates a primary phosphorylated serine or threonine. RIP1 contains seven CK1 substrate consensus motifs, which all lie within the intermediate domain, consistent with our kinase assay data. There are up to 15 candidate serine phosphorylation sites and 5 candidate threonine phosphorylation sites in the intermediate domain of RIP1 as predicted by NetPhos 2.0 Server. Our data demonstrate that RIP1 is phosphorylated on serine and threonine residues by CK1α. We have attempted to determine the CK1α phosphorylation sites by mutating some conserved Ser/Thr residues in the intermediate domain but failed to identify any phosphorylation sites. Further studies are needed to identify the CK1α phosphorylation sites on RIP1 to validate the effect of CK1α phosphorylation on NF-κB activation.

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