Critical Roles of TRAF2 and TRAF5 in Tumor Necrosis Factor-induced NF-κB Activation and Protection from Cell Death*

Received for publication, May 26, 2001, and in revised form, July 25, 2001
Published, JBC Papers in Press, on July 30, 2001, DOI 10.1074/jbc.M104837200

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Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) were identified as signal transducers for the TNF receptor superfamily. However, the exact roles of TRAF2 and TRAF5 in TNF-induced NF-κB activation still remain controversial. To address this issue, we generated TRAF2 and TRAF5 double knockout (DKO) mice. TNF- but not interleukin-1-induced nuclear translocation of NF-κB was severely impaired in murine embryonic fibroblasts (MEFs) derived from DKO mice. Moreover, DKO MEFs were more susceptible to TNF-induced cytotoxicity than TRAF2 knockout MEFs. Collectively, these results indicate that both TRAF2 and TRAF5 are involved in TNF-induced NF-κB activation and protection from cell death.

Tumor necrosis factor (TNF) exerts a variety of biological effects, including production of inflammatory cytokines, up-regulation of adhesion molecules, proliferation, differentiation, and apoptosis (1). Although such pleiotropic effects are mediated by two cognate TNF receptors, TNF-R1 and TNF-R2, TNF-induced cell death is mediated mainly by TNF-R1. In response to TNF, TNF-R1 is primed and recruits an adapter molecule, TRADD (2). In the apoptotic signaling pathway, the recruited TRADD interacts with FADD (Fas-associated death domain protein) (2), which then recruits and activates caspases, such as caspase-3 and -7, resulting in apoptosis (3). On the other hand, recruited TRADD also interacts with RIP and TNF receptor-associated factor 2 (TRAF2), both of which are implicated in NF-κB and c-Jun N-terminal kinase (JNK) activation (4, 5).

NF-κB is a transcriptional factor that regulates expression of various inflammatory cytokines, chemokines, and adhesion molecules (6). NF-κB is activated by inflammatory cytokines and cellular stresses including TNF, IL-1, lipopolysaccharide, UV, or γ-irradiation. In unstimulated cells, NF-κB is sequestered in the cytoplasm by binding to IκB inhibitory proteins (7). Upon stimulation, N-terminal serine residues of IκBs are phosphorylated, which leads to ubiquitination and subsequent degradation via a 26-S proteasome pathway (8). Then the liberated NF-κB from IκBs translocates to the nucleus and activates transcription of various target genes. Recently, the IκB kinase (IKK) complex, which is responsible for this inducible phosphorylation, was identified and extensively characterized (9). The IKK complex is composed of three subunits, including two structurally related kinases, designated IKKα and IKKβ, and one adapter molecule, designated IKKγ or NEMO (9). Gene-targeting studies showed essential roles of IKKβ and IKKγ/NEMO in cytokine-induced NF-κB activation (10–15). However, it remains to be determined how receptor-mediated signals finally activate IKK. These studies also demonstrated a critical role of NF-κB in the protection of cells from TNF-induced cell death, although the molecular mechanism is not completely understood.

The TRAFs were identified originally as signal-transducing molecules for the TNF-R and the IL-1 receptor superfamilies (16, 17). Ex vivo data demonstrated that TRAF2, TRAF5, and TRAF6 activate NF-κB and are involved in NF-κB activation through these receptors (16, 17). Previous studies suggested that TRAFs interact with NF-κB-inducing kinase (NIK), MAP kinase/ERK kinase kinase 1 (MEKK1), transforming growth factor β-activated kinase, or atypical protein kinase C, and these kinases phosphorylate IKKs resulting in NF-κB activation (18–21). However, the exact contribution of these kinases to cytokine-induced NF-κB activation is not yet clear. So far, RIP and TRAF6 have been shown to play essential roles in TNF- and IL-1/lipopolysaccharide-induced NF-κB activation, respectively (22–24). In contrast, TRAF2- or TRAF5-deficient mice did not show substantial defects in TNF-induced NF-κB activation (25, 26), suggesting that TRAF2 and TRAF5 are not essential or play a redundant role in TNF-induced NF-κB activation.

*This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and by grants from the Human Frontier Science Program, the Tokyo Biochemical Research Foundation, and the Mochida Memorial Foundation for Medical and Pharmaceutical Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; TRADD, TNFR1-associated death domain protein; RIP, receptor-interacting protein; MEF, murine embryonic fibroblast; JNK, c-Jun N-terminal kinase; IKK, IκB kinase; IAP, inhibitor of apoptosis; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; MEKK1, mitogen-activated protein (MAP) kinase/extracellular signal-related kinase (ERK) kinase 1; IL, interleukin; DKO, double knockout; mAb, monoclonal antibody; GST, glutathione S-transferase; HA, hemagglutinin; CHX, cycloheximide.
ivation. To further examine the contribution of TRAF2 and TRAF5 to TNF-induced NF-κB activation, we generated TRAF2 and TRAF5 double knockout (DKO) mice and characterized the response to TNF. We found redundant or nonredundant roles for TRAF2 and TRAF5 in TNF-induced NF-κB activation, JNK activation, and protection from cell death.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Culture—**Recombinant human TNF, murine TNF, and murine IL-1β were purchased from BD Pharmingen. Anti-HA (12CA5) and anti-Flag (M2) monoclonal antibodies (mAbs) were from Roche Molecular Biochemicals and Sigma, respectively. Anti-γC (9E10) was obtained from ATCC. Anti-RIP mAb was purchased from Transduction Laboratories. Anti-IKKα, anti-IKKβ, and anti-IxBα antibodies were purchased from Santa Cruz Biotechnology. HEK293 cells and murine embryonic fibroblasts (MEFs) were cultured in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

**Plasmids—**pcDNA3-HA-TRAF2, pRK5-Flag-RIP, and pRK5-Myc-TRADD were described previously (5, 27). Human TRAF5 was subcloned into pCR-HA vector, designated pCR-HA-TRAF5.

**Generation of traf2−/−traf5−/− (DKO) Mice—**To maximize the yield of double mutant mice, we first generated traf2−/−traf5−/− and traf2−/−traf5+/− mice by crossing traf2−/−traf5−/− and traf2−/−traf5+/− mice. We then crossed traf2−/−traf5−/− and traf2−/−traf5+/− mice to generate traf2−/−traf5−/− mice. Genotypic analysis of these mice was performed as described previously (25, 26).

**Electrophoretic Mobility Shift Assay—**Electrophoretic mobility shift assay was performed essentially as described previously (28). Briefly, MEFs (1 × 10⁶) were stimulated with murine TNF (10 ng/ml) or IL-1β (10 ng/ml) for the indicated time periods. Then, the cells were washed with ice-cold phosphate-buffered saline and harvested. The cells were treated with 0.5% Nonidet P-40, 50 mM HEPES (pH 7.3), 150 mM NaCl, 1 mM EDTA, 1 mM ml/a-prolin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM NaF. Nuclei were removed by centrifugation, and the supernatants were immunoprecipitated with anti-IKKγ/NEMO antibody (30) or anti-JNK1 antibody and protein G-Sepharose. The immunoprecipitates were washed three times with the lysis buffer and twice with the kinase assay buffer. Then, one-half of the lysates was immunoprecipitated with control IgG, anti-γC, or anti-Myc mAb followed by the addition of 30 μl of protein G-Sepharose. The precipitates were washed with lysis buffer, and the eluates were subjected to 10% SDS-polyacrylamide gel electrophoresis and analyzed on a Fuji BAS2500 image analyzer.

**In Vitro Kinase Assay—**In vitro kinase assay was performed essentially as described previously (29). Briefly, MEFs (4 × 10⁶ for IKK assay, 1 × 10⁶ for JNK assay) were plated in 150- or 100-mm dishes. Then the cells were stimulated with murine TNF (10 ng/ml) or IL-1β (10 ng/ml) for the various time periods, and the reaction was stopped with ice-cold phosphate-buffered saline and lysed in 1 ml of a lysis buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.3), 150 mM NaCl, 1 mM ml/a-prolin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM NaF. Nuclei were removed by centrifugation, and the supernatants were immunoprecipitated with anti-IKKγ/NEMO antibody, and the kinase activity (C) was assayed using GST-IκBα(1–100) as a substrate. The levels of RIP and IKKα were verified by Western blotting with anti-RIP and anti-IKKα antibodies. IP, immunoprecipitate; IB, immunoblot. C, degradation of IκBα. After the stimulation as described in B, the levels of IκBα were examined by Western blotting with anti-IκBα antibody.

**RESULTS**

**TNF-induced NF-κB Activation Is Severely Impaired in traf2−/−traf5−/− (DKO) MEFs—**Taken that the cells from traf2−/−(T2KO) or traf5−/− (T5KO) mice did not show a substantial defect in TNF-induced NF-κB activation (25, 26), TRAF2 and TRAF5 might play a redundant role in the NF-κB activation by TNF. To explore this possibility, we generated TRAF2 and TRAF5 DKO MEFs by crossing T2KO and T5KO mice. The percentage of DKO pups at birth was lower than the expected Mendelian ratio, and these pups became runted and died within 2 to 3 weeks. A detailed phenotype of DKO mice will be published elsewhere. To investigate whether NF-κB activation by TNF was impaired in DKO MEFs, we prepared MEFs from E13.5–E14.5 embryos. We first examined nuclear translocation of NF-κB by electrophoretic mobility shift assay. As reported previously (25, 26), TNF-induced NF-κB translocation was not significantly impaired in either T2KO or T5KO MEFs (Fig. 1A). In contrast, DKO MEFs showed severe impairment of nuclear translocation of NF-κB upon TNF stimulation, whereas upon IL-1 stimulation this was intact (Fig. 1A). These results sug-
gusted a redundant role of TRAF2 and TRAF5 in TNF-induced NF-κB activation.

Nuclear translocation of NF-κB requires phosphorylation and subsequent degradation of IkBs. We next assessed the kinase activity of IKK to phosphorylate IkBa. Wild-type and DKO MEFs were stimulated with TNF or IL-1 for 10 or 30 min, and the lysates were immunoprecipitated with anti-IKKγ/NEMO antibody. The precipitates were subjected to in vitro kinase assay using GST-IkBα as a substrate. As shown in Fig. 1B, phosphorylation of GST-IkBα induced by TNF, but not IL-1, at 10 min was dramatically reduced in DKO MEFs as compared with wild type. The late appearance of the kinase activity at 30 min might explain the residual NF-κB binding activity observed in DKO MEFs. We also examined the degradation of IkBa by Western blotting. In wild-type MEFs, IkBa completely disappeared at 10 min and reappeared at 30 min after TNF stimulation (Fig. 1C). In contrast, IkBa levels were only partially reduced at 10 and 30 min after TNF stimulation in DKO MEFs (Fig. 1C). On the other hand, IL-1-induced degradation of IkBa was comparable between wild-type and DKO MEFs. These results suggested that TRAF2 and TRAF5 play a critical role in TNF-induced NF-κB activation by mediating rapid activation of IKK and degradation of IkBa.

**TNF-induced JNK Activation in DKO MEFs**—Previous studies showed a severe impairment of JNK activation by TNF in T2KO MEFs but not T5KO MEFs (25, 26). We next examined TNF-induced JNK activation in DKO MEFs. MEFs were stimulated with TNF or IL-1 for 10 or 30 min, and the lysates were immunoprecipitated with anti-JNK1 antibody. The precipitates were subject to in vitro kinase assay using GST-c-Jun as a substrate. As shown in Fig. 2, phosphorylation of GST-c-Jun induced by TNF, but not IL-1, at 10 min was dramatically reduced in DKO MEFs as compared with wild type. The late appearance of the kinase activity at 30 min might explain the residual NF-κB binding activity observed in DKO MEFs. We also examined the degradation of IkBa by Western blotting. In wild-type MEFs, IkBa completely disappeared at 10 min and reappeared at 30 min after TNF stimulation (Fig. 1C). In contrast, IkBa levels were only partially reduced at 10 and 30 min after TNF stimulation in DKO MEFs (Fig. 1C). On the other hand, IL-1-induced degradation of IkBa was comparable between wild-type and DKO MEFs. These results suggested that TRAF2 and TRAF5 play a critical role in TNF-induced NF-κB activation by mediating rapid activation of IKK and degradation of IkBa.

**Increased Susceptibility of DKO MEFs to TNF-induced Cytotoxicity.** MEFs (5 × 10⁶) were incubated with various amounts of TNF in the absence (A) or presence of CHX (0.25 μg/ml) (B) for 24 h. Cell viability was determined by WST-8 (2-(2-methoxy-4-nitrophenyl)-3(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt). Data are shown as the mean ± S.D. of triplicate samples. Similar results were obtained with three independently established MEFs from DKO mice. WT, wild type.

These results suggested that TRAF5 participates in the TNF-induced NF-κB activation via interaction with RIP.

**Increased Susceptibility of DKO MEFs to TNF-induced Cell Death**—We next examined the susceptibility of wild-type, T2KO, T5KO, and DKO MEFs to TNF-induced cell death in the presence or absence of a protein synthesis inhibitor, CHX. As reported previously (25), T2KO MEFs were only slightly susceptible to TNF-induced cell death in the presence of CHX (Fig. 4A) but was highly susceptible in the presence of CHX (Fig. 4B). In contrast, the susceptibility of T5KO MEFs was almost comparable with that of wild-type MEFs in the absence or presence of CHX. Notably, DKO MEFs were highly susceptible to TNF-induced cell death even in the absence of CHX (Fig. 4A) and were more susceptible than T2KO MEFs in the presence of CHX (Fig. 4B). These results suggested a redundant but critical role of TRAF2 and TRAF5 in protecting MEFs from TNF-induced cell death, which is dependent on protein synthesis, and a predominant role of TRAF2 in the absence of protein synthesis.
DISCUSSION

In the present study, we investigated the relative contributions of TRAF2 and TRAF5 to TNF-induced NF-κB activation, JNK activation, and protection from TNF-induced cell death by utilizing MEFs from T2KO, T5KO, and DKO mice. We found redundant or nonredundant roles for TRAF2 and TRAF5 in these TNF responses.

We and others previously showed that TRAF5, as well as TRAF2, is used by multiple members of the TNF receptor superfamily (16). Thus, we first speculated that TRAF5 is also involved in the TNF-R-mediated signaling pathway and can substitute for TRAF2 in NF-κB activation by TNF. We demonstrated that NF-κB activation by TNF, but not IL-1, was substantially reduced in DKO MEFs (Fig. 1). Moreover, we also showed that TRAF5 physically interacts with RIP (Fig. 3), an essential component of TNF-induced NF-κB activation (22, 32). In contrast to RIP-deficient cells (22, 32), significant levels of TNF-induced NF-κB binding activity (Fig. 1A) and IKK activity (Fig. 1B) were observed in DKO MEFs, especially at a later time point. This suggests that some molecule other than TRAF2 and TRAF5 may mediate the dominant negative effect of truncated TRAF5 on JNK activation were relatively weaker than those of TRAF2 (31). Altogether, TRAF5 seems to play a greater role in TNF-induced NF-κB activation than JNK activation under physiological conditions.

Our present examination of the susceptibility of T2KO, T5KO, and DKO MEFs to TNF-induced cell death has revealed both redundant and nonredundant roles of TRAF2 and TRAF5 in protection from TNF-induced cell death. In the absence of CHX, DKO but not T2KO or T5KO MEFs were highly susceptible to TNF-induced cell death (Fig. 4A). In the presence of CHX, both T2KO and DKO but not T5KO MEFs were highly susceptible to TNF-induced cell death (Fig. 4B). Collectively, these data suggested that TRAF2 and TRAF5 may redundantly mediate NF-κB-dependent anti-apoptotic pathway, which is dependent on protein synthesis. TRAF2 may also mediate an NF-κB-independent anti-apoptotic pathway, which is not dependent on protein synthesis.

Previous studies reported that expression of XIAP, c-IAP1, c-IAP2, and A1/Bfl-1 was induced by TNF in an NF-κB-dependent manner in various types of cells (41–44). Although our RNase protection assay and Northern blot analysis showed that induction of A1/Bfl-1 was severely impaired in DKO MEFs, stable transfection of A1/Bfl-1 did not fully protect DKO MEFs from TNF-induced cell death (data not shown). These results suggest that some molecule(s) other than A1/Bfl-1 induced by NF-κB may be primary responsible for TRAF-mediated NF-κB-dependent protection from TNF-induced cell death. Further studies are now under way to identify such molecules.

Acknowledgments—We thank David Goeddel, Naohiro Inohara, Masahiro Takeuchi, David Wallach, and Ryosuke Takahashi for providing reagents and for helpful discussion.

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