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**Mechanisms of Signal Transduction:  
Mechanisms of the TRIF-induced  
Interferon-stimulated Response Element  
and NF- $\kappa$ B Activation and Apoptosis  
Pathways**

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*J. Biol. Chem.* 2004, 279:15652-15661.

doi: 10.1074/jbc.M311629200 originally published online January 22, 2004

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Access the most updated version of this article at doi: [10.1074/jbc.M311629200](https://doi.org/10.1074/jbc.M311629200)

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# Mechanisms of the TRIF-induced Interferon-stimulated Response Element and NF- $\kappa$ B Activation and Apoptosis Pathways\*

Received for publication, October 23, 2003, and in revised form, January 13, 2004  
Published, JBC Papers in Press, January 22, 2004, DOI 10.1074/jbc.M311629200

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**Toll-like receptor-3 is critically involved in host defense against viruses through induction of type I interferons (IFNs). Recent studies suggest that a Toll/interleukin-1 receptor domain-containing adapter protein (TRIF) and two protein kinases (TANK-binding kinase-1 (TBK1) and I $\kappa$ B kinase (IKK)- $\epsilon$ ) are critically involved in Toll-like receptor-3-mediated IFN- $\beta$  production through activation of IFN regulatory factor (IRF)-3 and IRF-7. In this study, we demonstrate that TRIF interacts with both IRF-7 and IRF-3. In addition to TBK1 and IKK $\epsilon$ , our results indicate that IKK $\beta$  can also phosphorylate IRF-3 and activate the IFN-stimulated response element. TRIF-induced IRF-3 and IRF-7 activation was mediated by TBK1 and its downstream kinases IKK $\beta$  and IKK $\epsilon$ . TRIF induced NF- $\kappa$ B activation through an IKK $\beta$ - and tumor necrosis factor receptor-associated factor-6-dependent (but not TBK1- and IKK $\epsilon$ -dependent) pathway. In addition, TRIF also induced apoptosis through a RIP/FADD/caspase-8-dependent and mitochondrion-independent pathway. Furthermore, our results suggest that the TRIF-induced IFN-stimulated response element and NF- $\kappa$ B activation and apoptosis pathways are uncoupled and provide a molecular explanation for the divergent effects induced by the adapter protein TRIF.**

The Toll/interleukin-1 receptor (TIR)<sup>1</sup> family members are evolutionarily conserved proteins that are critically involved in host defense from plants to humans (1–3). In mammals, the TIR family members, including interleukin (IL)-1 receptors and Toll-like receptors (TLRs), are important mediators of inflammation and innate and adaptive immune responses (1–3).

\* This work was supported by National Institutes of Health Grant AI49992, the Ellison Medical Foundation, National Natural Science Foundation of China Grants 39925016 and 30100097, Chinese High Technology Program Grant 2001AA221281, and Special Funds for Major State Basic Research of China Grant G19990539. 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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<sup>1</sup> The abbreviations used are: TIR, Toll/interleukin-1 receptor; IL, interleukin; TLR, Toll-like receptor; IRF, interferon regulatory factor; IRAK, interleukin-1 receptor-associated kinase; TRAF6, tumor necrosis factor receptor-associated factor-6; IKK, I $\kappa$ B kinase; LPS, lipopolysaccharide; JNK, c-Jun N-terminal kinase; ISRE, interferon-stimulated response element; IFN, interferon; TBK1, TANK-binding kinase-1; HA, hemagglutinin; DN, dominant-negative; FADD, Fas-associated death domain; RIP, receptor-interacting protein; X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside.

The TIR family members are characterized by a conserved cytoplasmic domain, the TIR domain. Various studies have shown that the TIR domain is required for TIR family member-mediated signaling that leads to activation of transcription factors NF- $\kappa$ B, AP-1, activating transcription factor-2, and interferon regulatory factors (IRFs) and subsequent induction of various chemokines and cytokines that are involved in host defense against the pathogens (1–3).

The TIR family receptors signal through conserved pathways. Upon ligand stimulation, a TIR domain-containing adapter protein such as MyD88 is recruited to the receptor signaling complex (4–6). In turn, MyD88 recruits the serine/threonine kinase IL-1 receptor-associated kinase (IRAK) (6). Once IRAK is recruited to the receptor complex, it is activated and then dissociated from the receptor complex (7). Dissociated IRAK interacts with tumor necrosis factor receptor-associated factor-6 (TRAF6), which then activates NF- $\kappa$ B through a kinase cascade containing transforming growth factor- $\beta$ -associated kinase-1 and I $\kappa$ B kinase (IKK) (7–10).

Gene knockout studies suggest that MyD88 is required for cytokine induction triggered by a variety of ligands that signal through the TIR family members, such as IL-1 $\beta$ , IL-18, lipopolysaccharide (LPS), macrophage-activating lipopeptide-2, CpG DNA, and poly(I-C) (11). However, it has been shown that NF- $\kappa$ B and c-Jun N-terminal kinase (JNK) activation induced by poly(I-C) (ligand for TLR3) and LPS (ligand for TLR4) is delayed (but not abolished) in MyD88<sup>-/-</sup> cells. In addition, poly(I-C) and LPS still trigger dendritic cell maturation (1). These studies suggest that MyD88-independent pathways exist.

Recently, three additional TIR domain-containing adapter molecules (TIRAP/Mal, TRIF/TICAM, and TIRP) were identified (12–16). TIRAP is associated with TLR4. A dominant-negative mutant of TIRAP inhibits NF- $\kappa$ B activation triggered by TLR4, but not by TLR9 or the IL-1 receptor (13, 14). Furthermore, gene knockout studies indicate that TIRAP is essential for LPS-induced cytokine production, suggesting that TIRAP is required for TLR4 signaling. It has also been shown that TIRAP is involved in signaling triggered by ligands for TLR1, TLR2, and TLR6, but not by IL-1, IL-18, and ligands for TLR3, TLR7, and TLR9 (17, 18).

TRIF is the third identified TIR domain-containing adapter molecule. In addition to NF- $\kappa$ B, TRIF also activates the interferon-stimulated response element (ISRE)/interferon- $\beta$  (IFN- $\beta$ ) promoter. A dominant-negative mutant of TRIF inhibits TLR2-, TLR3-, TLR4-, and TLR7-mediated NF- $\kappa$ B activation and TLR3-mediated activation of the IFN- $\beta$  promoter (15, 16). So far, TRIF is the only TIR domain-containing adapter protein that has been implied in TLR3-mediated production of IFN- $\beta$ , suggesting that TRIF is involved in TLR3-induced activation of NF- $\kappa$ B as well as IFN- $\beta$ .

Recently, TRIF-deficient mice were produced by gene knock-

out and chemical mutagenesis approaches (19, 20). These studies indicated that TRIF-deficient mice are defective in both TLR3- and TLR4-mediated expression of IFN- $\beta$ . Inflammatory cytokine production in response to the TLR4 ligand (but not to other TLR ligands) is severely impaired in TRIF-deficient macrophages. The major signaling pathways leading to production of inflammatory cytokines involve the transcription factor NF- $\kappa$ B and various mitogen-activated protein kinases. It was shown that TLR3-mediated NF- $\kappa$ B activation is severely impaired in TRIF<sup>-/-</sup> fibroblasts, suggesting that TRIF plays a major role in TLR3-induced NF- $\kappa$ B activation. In contrast, TLR4-mediated NF- $\kappa$ B and JNK activation is not significantly impaired in either MyD88- or TRIF-deficient fibroblasts. However, double knockout of MyD88 and TRIF completely abolishes TLR4-induced NF- $\kappa$ B and JNK activation (19, 20), suggesting that MyD88 and TRIF are mutually required for TLR4-induced NF- $\kappa$ B and JNK activation.

Interestingly, although poly(I:C)-induced tumor necrosis factor production is abolished in all macrophages, LPS-induced tumor necrosis factor production is abolished only in a fraction of macrophages (19). These data suggest that LPS response in a subset of macrophages is TRIF-independent. Because LPS response in all macrophages is MyD88-dependent, it has been proposed that an unknown adapter protein functioning downstream of MyD88 may be involved in LPS signaling in TRIF-independent cells (19). In this context, the recently identified protein TIRP (12), which is mostly related to TRIF, provides a candidate adapter for the TRIF-independent signaling in the subset of macrophages.

Although TRIF contains a TIR domain, its amino acid sequence is very divergent from other TIR domain-containing adapter protein sequences. The unique sequence and functions of TRIF suggest that it may interact with distinct downstream signaling proteins. It has been shown that TRIF interacts with IRF-3 to activate IFN- $\beta$  (16). Recently, it has been shown that two serine/threonine protein kinases (IKK $\epsilon$  and TANK-binding kinase-1 (TBK1)) can phosphorylate IRF-3 and are involved in TRIF-induced IRF-3 activation (21, 22).

Elucidation of the molecular mechanisms of TRIF-induced downstream signaling events is crucial for understanding TLR3- and TLR4-mediated biological effects. In this work, we show that TRIF induces ISRE and NF- $\kappa$ B activation and apoptosis through distinct intracellular signaling pathways.

#### EXPERIMENTAL PROCEDURES

**Reagents**—Monoclonal antibodies against the FLAG and hemagglutinin (HA) (Sigma) and Myc (Santa Cruz Biotechnology, Santa Cruz, CA) epitopes, the caspase inhibitor ZAD-fm (Enzyme Systems, Livermore, CA), and Sendai virus (American Type Culture Collection, Manassas, VA) were purchased from the indicated manufacturers.

**Yeast Two-hybrid Screening**—To construct a TRIF bait vector, a cDNA encoding full-length TRIF was inserted in-frame into the Gal4 DNA-binding domain vector pGBT (Clontech, Palo Alto, CA). The human B cell cDNA library (American Type Culture Collection) was screened as described (23, 24).

**Constructs**—The ISRE-luciferase reporter construct was purchased from Stratagene. The NF- $\kappa$ B-luciferase reporter construct was provided by Dr. Gary Johnson (University of Colorado Health Sciences Center). The IFN- $\beta$ -luciferase reporter construct was made by PCR amplification of the human IFN- $\beta$  promoter fragment (-300 to +25) and cloning into the pGL3-Basic vector (Promega, Madison, WI). The NF- $\kappa$ B site-mutated IFN- $\beta$  promoter-luciferase reporter construct was made by PCR.

Mammalian expression plasmids for FLAG-TRAF6, FLAG-TRAF6-DN, Myc-IKK $\beta$ , Myc-IKK $\beta$ -KA, FADD-DN, tumor necrosis factor receptor-1, Bcl-2, CrmA, and I $\kappa$ B $\alpha$ (SS/AA) (Dr. David Goeddel, Tularik Inc.); FLAG-IRAK1(K239S), FLAG-IRAK4-KA, and Myc-MyD88 (Dr. Zhao-dan Cao, Tularik Inc.); p53mut (Dr. Bert Vogelstein, John Hopkins University); FLAG-IRF-3 and pCMV-BL-IRF-3/5D (Dr. John Hiscott, Lady Davis Institute for Medical Research); and Myc-IKKE, Myc-IKKE-KA, Myc-TBK1, and Myc-TBK1-KA (Dr. Uli Siebenlist, National Institutes of Health) were provided by the indicated investigators. Mamma-

lian expression plasmids for HA-tagged receptor-interacting protein (RIP), caspase-8(C360S), and FLAG- and HA-tagged TRIF and TIRAP were described previously (12, 24, 25). Mammalian expression plasmids for HA-tagged TRIF deletion mutants, HA-IRF-7, FLAG-IRF-7 $\Delta$ N, FLAG-IRF-3 $\Delta$ N, and FLAG-IRF-3/5D were constructed by PCR amplification of the corresponding cDNA fragments and subsequent cloning into a cytomegalovirus promoter-based vector containing a 5'-HA or 5'-FLAG-tag.

**Cell Transfection and Reporter Gene Assays**—293 cells ( $\sim 2 \times 10^5$ ) were seeded in 6-well dishes and transfected the following day by the standard calcium phosphate precipitation method (26). In the same experiment, each transfection was performed in triplicate; and where necessary, empty control plasmid was added to ensure that each transfection received the same amount of total DNA. To normalize for transfection efficiency, 0.2  $\mu$ g of pRSV- $\beta$ -galactosidase reporter plasmid was added to each transfection. Approximately 16 h after transfection, luciferase reporter assays were performed using a luciferase assay kit (Pharmingen) following the manufacturer's protocol.  $\beta$ -Galactosidase activity was measured using the Galacto-Light chemiluminescent kit (Tropix Inc., Bedford, MA). Luciferase activities were normalized relative to  $\beta$ -galactosidase activities.

All reporter gene assays were repeated at least three times. Data shown are the average from one representative experiment. The S.D. values were <10% of the average values for all samples.

**Co-immunoprecipitation and Western Blot Analysis**—For transient transfection and co-immunoprecipitation experiments, 293 cells ( $\sim 2 \times 10^6$ ) were transfected for 24 h. Transfected cells were lysed in 1 ml of lysis buffer (15 mM Tris, 120 mM NaCl, 1% Triton, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM dithiothreitol, 0.5% Triton X-100, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5). For each immunoprecipitation, a 0.4-ml aliquot of lysate was incubated with 0.5  $\mu$ g of the indicated monoclonal antibody or control mouse IgG and 20  $\mu$ l of a 1:1 slurry of GammaBind G Plus-Sepharose (Amersham Biosciences) for 2 h. The Sepharose beads were washed three times with 1 ml of lysis buffer and 500 mM NaCl. The precipitates were fractionated by SDS-PAGE, and subsequent Western blot analysis was performed as described (12, 24). All immunoprecipitation experiments were repeated at least three times, and similar data were obtained.

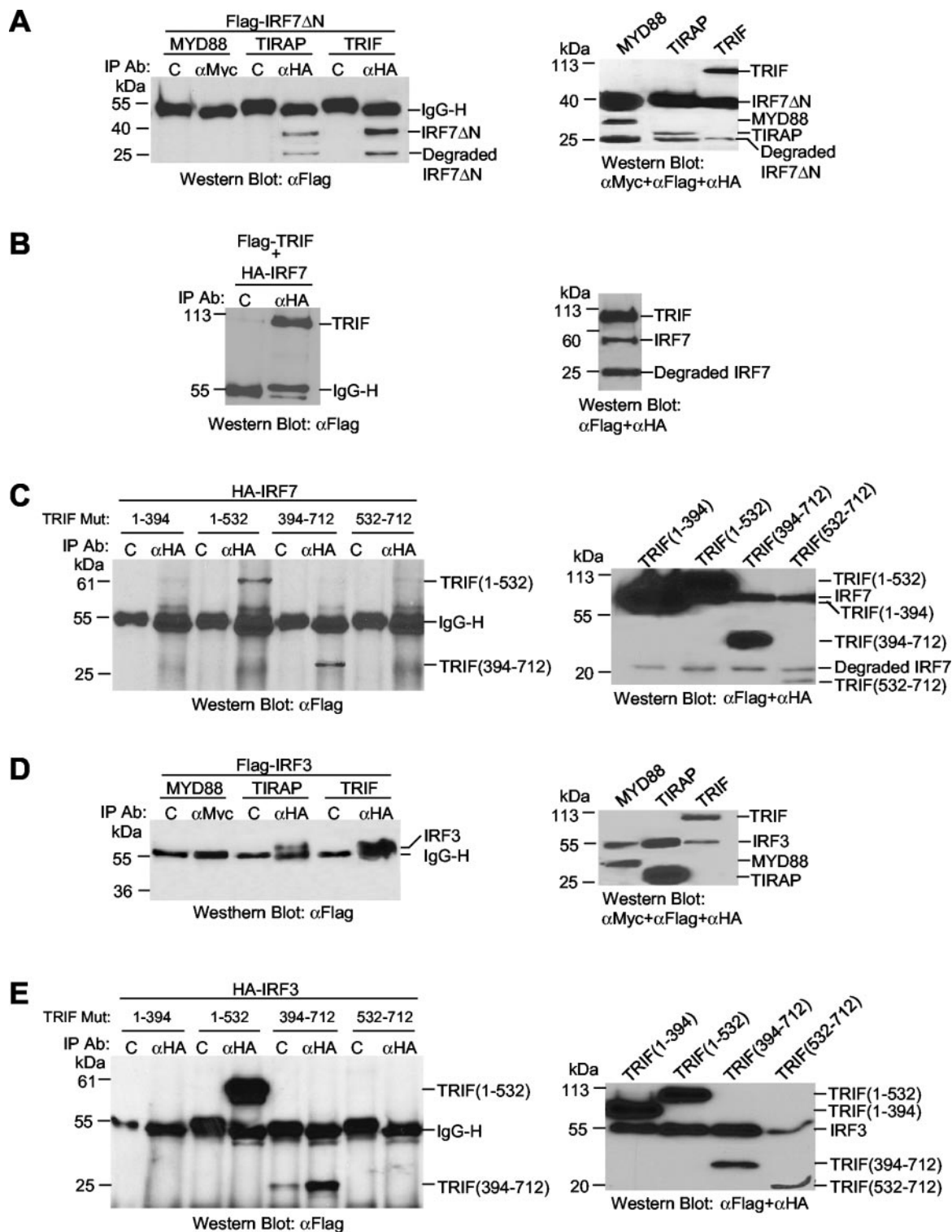
**In Vitro Kinase Assays**—Cell transfection and immunoprecipitation were carried out as described above. The immunoprecipitates were washed twice with kinase buffer (25 mM Tris, 5 mM  $\beta$ -glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 mM MgCl<sub>2</sub>, pH 7.5) and then incubated in 30  $\mu$ l of kinase buffer plus 100  $\mu$ M ATP and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP for 30 min at 30 °C. The beads were spun down and washed once with kinase buffer. The proteins were fractionated by SDS-PAGE. The gel was dried and subjected to autoradiography.

**Apoptosis Assays**— $\beta$ -Galactosidase cotransfection assays for determination of cell death were performed as described (23, 27). Briefly, cells ( $\sim 1 \times 10^5$ /well) were seeded on 12-well dishes and transfected the following day by the standard calcium phosphate precipitation method (26) with 0.1  $\mu$ g of pCMV- $\beta$ -galactosidase and 1  $\mu$ g of the indicated expression plasmids. Where necessary, empty control plasmid was added to ensure that each transfection received the same amount of total DNA. In the same experiment, each transfection was performed in duplicate. Twenty-four hours after transfection, the cells were fixed and stained with X-gal as described previously (23, 27). The numbers of surviving blue cells from five representative viewing fields were counted under a microscope. The dead cells with a shrunk and condensed morphology were not counted. Data from one of at least three representative experiments are shown.

**Sendai Virus Infection**—293 cells ( $\sim 2 \times 10^5$ ) were seeded in 6-well dishes and transfected the next day with 0.5  $\mu$ g of ISRE reporter plasmid and 0.5  $\mu$ g of pRSV- $\beta$ -galactosidase plasmid. Eighteen hours after transfection, cells were washed with medium lacking fetal calf serum (washing medium) and then overlaid with washing medium containing Sendai virus at a multiplicity of infection of 10. After incubation at 37 °C for 60 min, non-adsorbed viruses were removed by repeated washing of the cells. Cells were then cultured in fetal calf serum-containing medium for 18 h before luciferase and  $\beta$ -galactosidase assays were performed.

**DNA Fragmentation Assays**—Transfected cells were washed twice with cold phosphate-buffered saline and lysed in 2% Nonidet P-40 containing 0.2 mg/ml proteinase K. DNA in the lysate was precipitated with 2 volumes of ethanol. The pellets were dissolved in H<sub>2</sub>O and analyzed by electrophoresis on 2% gel.





**FIG. 1. TRIF and TIRAP interact with IRF-7 and IRF-3.** *A*, TRIF and TIRAP (but not MyD88) interact with IRF-7 $\Delta$ N. 293 cells ( $\sim 2 \times 10^6$ ) were transfected with expression plasmids for FLAG-IRF-7 $\Delta$ N and Myc-MyD88, HA-TIRAP, or HA-TRIF. Twenty-four hours after transfection, cell lysates were immunoprecipitated (IP) with anti-HA or anti-Myc antibody (Ab) or control IgG (C). The immunoprecipitates were analyzed by Western blotting with anti-FLAG antibody (left panel). The expression levels of the transfected proteins in the lysates were detected by Western blot analysis (right panel). *B*, TRIF interacts with full-length IRF-7. 293 cells were transfected with expression plasmids for FLAG-TRIF and HA-IRF-7. Co-immunoprecipitation and Western blot analysis were performed as described for *A*. *C*, the TIR domain of TRIF is required for interaction with IRF-7. 293 cells ( $\sim 2 \times 10^6$ ) were transfected with expression plasmids for HA-IRF-7 and the indicated FLAG-tagged TRIF mutants (Mut). Co-immunoprecipitation and Western blot analysis were performed as described for *A*. *D*, TRIF and TIRAP interact with IRF-3. Transfection and co-immunoprecipitation were performed as described for *A*, except that FLAG-IRF-3 was used to replace FLAG-IRF-7 $\Delta$ N. *E*, the TIR domain of TRIF is required for interaction with IRF-3. The experiments were performed as described for *C*, except that IRF-3 was used to replace IRF-7.

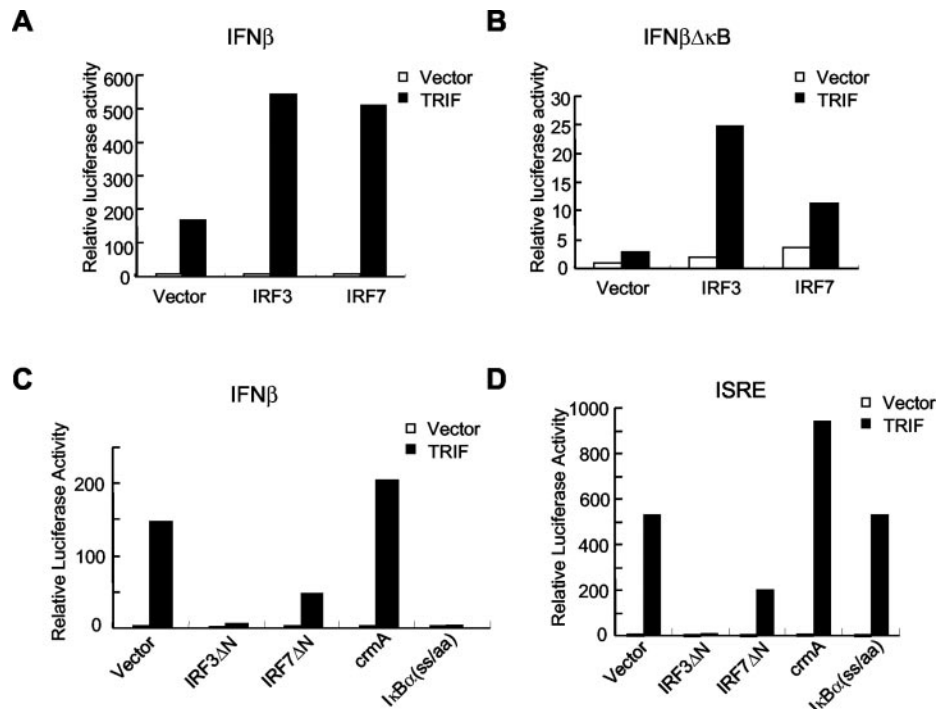
## RESULTS

**TRIF Interacts with IRF-7 and IRF-3**—To unambiguously identify TRIF-interacting proteins, we used the yeast two-hy-

brid system to screen a human B cell cDNA library with full-length TRIF as bait. We screened a total of  $\sim 2 \times 10^6$  independent library clones and obtained 20  $\beta$ -galactosidase-positive

**FIG. 2. IRF-7 and IRF-3 are involved in TRIF-induced IFN- $\beta$  and ISRE activation.**

**A**, TRIF synergizes with IRF-3 and IRF-7 to activate the IFN- $\beta$  promoter. 293 cells ( $\sim 2 \times 10^5$ ) were transfected with the pGL3-IFN- $\beta$ -luciferase reporter construct (0.3  $\mu$ g) and expression plasmids for TRIF and IRF-3 or IRF-7 (1  $\mu$ g each). Luciferase assays were performed 18 h after transfection. **B**, TRIF synergizes with IRF-3 and IRF-7 to activate the NF- $\kappa$ B site-mutated IFN- $\beta$  promoter. The experiments were performed as described for **A**, except that the wild-type IFN- $\beta$  reporter was replaced with the NF- $\kappa$ B site-mutated IFN- $\beta$  reporter (*IFN $\beta$  $\Delta$  $\kappa$ B*). **C**, effects of IRF-3 $\Delta$ N, IRF-7 $\Delta$ N, CrmA, and I $\kappa$ B $\alpha$ (SS/AA) on TRIF-induced IFN- $\beta$  activation. 293 cells ( $\sim 2 \times 10^5$ ) were transfected with the pGL3-IFN- $\beta$ -luciferase reporter construct (0.3  $\mu$ g), expression plasmids for TRIF, and the indicated plasmids (1  $\mu$ g). Luciferase assays were performed 18 h after transfection. **D**, effects of IRF-3 $\Delta$ N, IRF-7 $\Delta$ N, CrmA, and I $\kappa$ B $\alpha$ (SS/AA) on TRIF-induced ISRE activation. The experiments were performed as described for **C**, except that the ISRE-luciferase reporter construct was used.



clones. One of the clones encodes the C-terminal domain (amino acids 297–503) of IRF-7. Previously, it has been shown that IRF-3 interacts with TRIF (15, 16). We next investigated whether IRF-7 is involved in TRIF signaling.

We first determined whether the IRF-7 fragment obtained from the yeast two-hybrid screening, which lacks its N-terminal DNA-binding domain (IRF-7 $\Delta$ N), interacts with TRIF. We transfected expression plasmids for TRIF and IRF-7 $\Delta$ N into 293 cells and performed co-immunoprecipitation experiments. The results indicate that TRIF interacted with IRF-7 $\Delta$ N (Fig. 1A). In the same experiments, TIRAP (but not MyD88) also interacted with IRF-7 $\Delta$ N (Fig. 1A). In similar experiments, TRIF also interacted with full-length IRF-7 (Fig. 1B). These data suggest that TRIF can interact with IRF-7. In these experiments, cleaved fragments of IRF-7 and IRF-7 $\Delta$ N were also detected (Fig. 1, A and B).

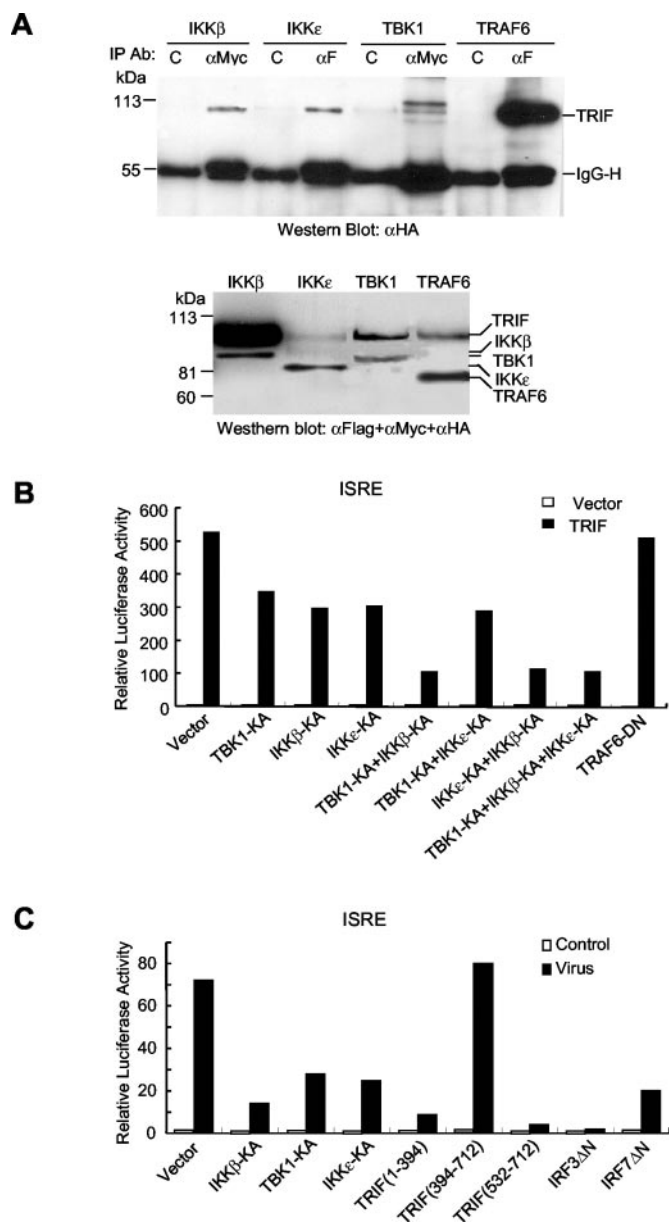
TRIF is a 712-amino acid protein that contains a large N-terminal domain, a middle TIR domain, and a C-terminal domain. To determine which domain of TRIF is responsible for interaction with IRF-7, we made a series of deletion mutants, including TRIF-(1–394) (containing its N-terminal domain), TRIF-(1–532) (containing its N-terminal and middle TIR domains), TRIF-(394–712) (containing its middle TIR and C-terminal domains), and TRIF-(532–712) (containing its C-terminal domain). 293 cells were transfected with HA-tagged IRF-7 and FLAG-tagged TRIF mutants, and co-immunoprecipitation experiments were performed. The results indicate that TRIF-(1–532) and TRIF-(394–712) (but not TRIF-(1–394) and TRIF-(532–712)) interacted with IRF-7 (Fig. 1C). These data suggest that the TIR domain of TRIF is required for its interaction with IRF-7. Similarly, TRIF and TIRAP (but not MyD88) interacted with IRF-3 in co-immunoprecipitation experiments (Fig. 1D), and this interaction required the TIR domain of TRIF (Fig. 1E). Taken together, these data suggest that IRF-7 and IRF-3 are involved in signaling by TRIF and TIRAP, but not by MyD88.

**IRF-7 and IRF-3 Are Involved in TRIF-induced IFN- $\beta$  and ISRE Activation**—It has been shown that TRIF can mediate TLR3- and TLR4-induced IFN- $\beta$  activation (15, 16, 19, 20). To examine the roles of IRF-7 and IRF-3 in TRIF-mediated IFN- $\beta$

activation, we performed reporter gene assays. We found that overexpression of IRF-7 or IRF-3 alone barely activated the IFN- $\beta$  promoter, but both could synergize with TRIF in activating IFN- $\beta$  (Fig. 2A). Because the IFN- $\beta$  promoter contains an NF- $\kappa$ B-binding site (28) in addition to two ISRE-binding sites, we mutated the NF- $\kappa$ B site in the IFN- $\beta$  promoter and determined whether IRF-7 or IRF-3 could synergize with TRIF to activate the mutated IFN- $\beta$  promoter. We found that mutation of the NF- $\kappa$ B-binding site greatly decreased TRIF-induced IFN- $\beta$  activation, from  $\sim 180$ - to  $\sim 3$ -fold (Fig. 2B). However, IRF-3 or IRF-7 could still synergize with TRIF to activate the NF- $\kappa$ B-binding site-mutated IFN- $\beta$  promoter (Fig. 2B).

We then determined the effects of IRF-7 and IRF-3 lacking their DNA-binding domains (mutants IRF-7 $\Delta$ N and IRF-3 $\Delta$ N, respectively) on TRIF-induced IFN- $\beta$  activation. In reporter gene assays, both IRF-7 $\Delta$ N and IRF-3 $\Delta$ N strongly inhibited TRIF-induced IFN- $\beta$  activation (Fig. 2C). Because TRIF activates NF- $\kappa$ B and induces apoptosis (see below), we determined whether CrmA and a non-degradable I $\kappa$ B $\alpha$  mutant, which inhibit TRIF-induced apoptosis and NF- $\kappa$ B activation, respectively (see below), could inhibit TRIF-induced IFN- $\beta$  activation. The results show that CrmA did not inhibit TRIF-induced IFN- $\beta$  activation (Fig. 2C). Surprisingly, the I $\kappa$ B $\alpha$  mutant completely inhibited TRIF-induced IFN- $\beta$  activation (Fig. 2C). Using an independent ISRE reporter that contains five copies of consensus ISRE-binding sites, we confirmed that TRIF-induced ISRE activation was inhibited by IRF-7 $\Delta$ N and IRF-3 $\Delta$ N, but not by CrmA and I $\kappa$ B $\alpha$ (SS/AA). Taken together, our data suggest that IRF-7 and IRF-3 are independently involved in TRIF-induced ISRE activation and that TRIF-induced ISRE activation is uncoupled from TRIF-induced NF- $\kappa$ B activation and apoptosis (also see below).

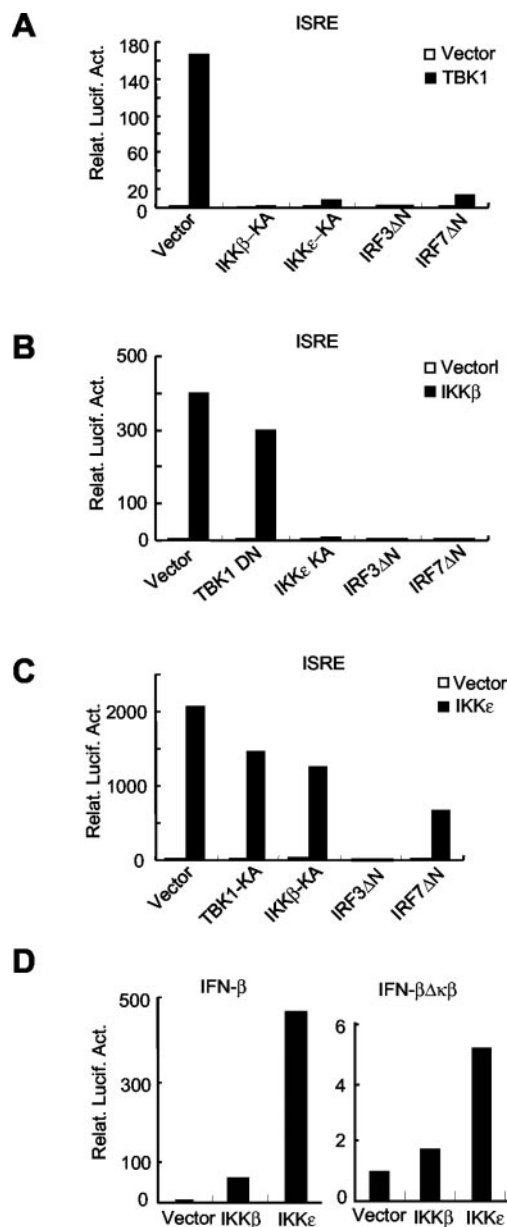
**TBK1, IKK $\beta$ , and IKK $\epsilon$  Are Involved in TRIF- and Sendai Virus-induced ISRE Activation**—Previous studies suggest that TBK1 and IKK $\epsilon$  (but not IKK $\beta$ ) are involved in TRIF-mediated activation of the ISRE sites in the IFN- $\beta$  promoter (20, 21). In transient transfection and co-immunoprecipitation experiments, we found that TRIF was associated with IKK $\epsilon$ , TBK1, and IKK $\beta$  (Fig. 3A). TRAF6 is an adapter protein critically involved in NF- $\kappa$ B activation triggered by TIRs. In the same



**FIG. 3. TBK1, IKK $\beta$ , and IKK $\epsilon$  are involved in TRIF- and Sendai virus-induced ISRE activation.** *A*, TRIF interacts with TBK1, IKK $\beta$ , IKK $\epsilon$ , and TRAF6. 293 cells ( $\sim 2 \times 10^6$ ) were transfected with expression plasmids for HA-tagged TRIF and the indicated FLAG- or Myc-tagged proteins. Cell lysates were immunoprecipitated (IP) with anti-FLAG ( $\alpha$ F) or anti-Myc antibody (Ab) or control IgG (C). The immunoprecipitates were analyzed by Western blotting with anti-HA antibody (upper panel). The expression levels of the transfected proteins were detected by Western blot analysis of the lysates (lower panel). *B*, effects of kinase-inactive mutants of TBK1, IKK $\beta$ , and IKK $\epsilon$  and a dominant-negative mutant of TRAF6 on TRIF-induced ISRE activation. 293 cells ( $\sim 2 \times 10^5$ ) were transfected with the ISRE reporter plasmid (0.3  $\mu$ g), expression plasmids for TRIF (black bars) or empty control plasmid (white bars), and the indicated mutants (1  $\mu$ g each). Luciferase activities were measured 18 h after transfection. *C*, effects of kinase-inactive mutants of TBK1, IKK $\beta$ , and IKK $\epsilon$  and a dominant-negative mutant of TRAF6 on Sendai virus-induced ISRE activation. 293 cells ( $\sim 2 \times 10^5$ ) were transfected with the ISRE reporter plasmid (0.3  $\mu$ g) and expression plasmids for the indicated mutants (1  $\mu$ g each). Eighteen hours after transfection, cells were infected with Sendai virus (black bars) or left untreated (white bars) for an additional 8 h before luciferase activities were measured.

experiments, TRIF strongly interacted with TRAF6 (Fig. 3A).

We then determined the effects of dominant-negative mutants of TBK1, IKK $\beta$ , IKK $\epsilon$ , and TRAF6 on TRIF-induced ISRE activation. As shown in Fig. 3B, the IKK $\beta$ -KA mutant could



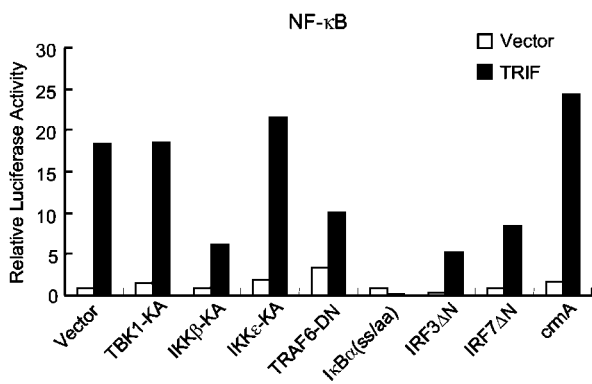
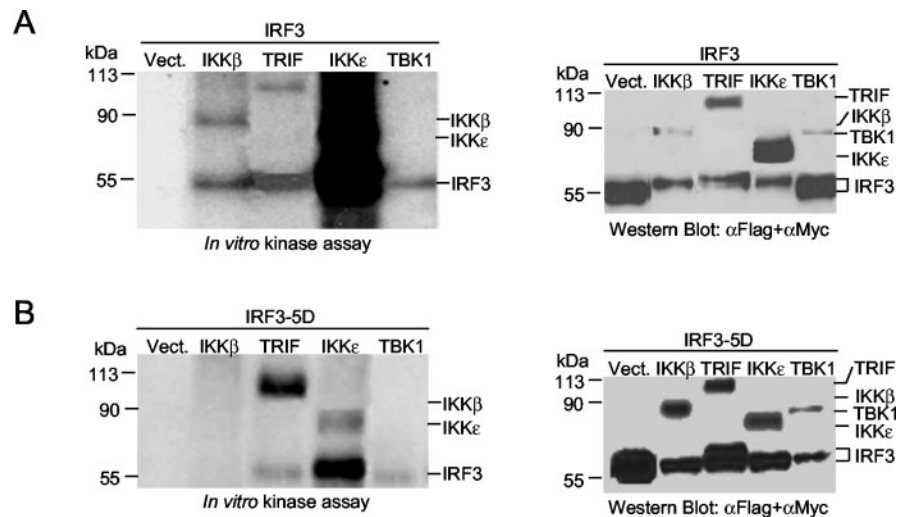
**FIG. 4. Molecular order of ISRE-activating kinases.** *A*, effects of IKK $\beta$ -KA, IKK $\epsilon$ -KA, IRF3 $\Delta$ N, and IRF7 $\Delta$ N on TBK1-induced ISRE activation. 293 cells ( $\sim 2 \times 10^5$ ) were transfected with the ISRE-luciferase reporter construct (0.3  $\mu$ g), expression plasmids for TBK1, and the indicated mutant plasmids (1  $\mu$ g each). Luciferase activities were measured 18 h after transfection. *B*, effects of TBK1-DN, IKK $\epsilon$ -KA, IRF3 $\Delta$ N, and IRF7 $\Delta$ N on IKK $\beta$ -induced ISRE activation. The experiments were performed as described for *A*. *C*, effects of TBK1-KA, IKK $\beta$ -KA, IRF3 $\Delta$ N, and IRF7 $\Delta$ N on IKK $\epsilon$ -induced ISRE activation. The experiments were performed as described for *A*. *D*, effects of IKK $\beta$  and IKK $\epsilon$  on the wild-type and NF- $\kappa$ B site-mutated IFN- $\beta$  promoters. 293 cells ( $\sim 2 \times 10^5$ ) were transfected with a wild-type or NF- $\kappa$ B site-mutated IFN- $\beta$  (IFN $\beta$  $\Delta$  $\kappa$  $\beta$ ) promoter-luciferase reporter construct (0.3  $\mu$ g) and an expression plasmid for IKK $\beta$  or IKK $\epsilon$  (1  $\mu$ g each). Luciferase activities were measured 18 h after transfection. *Relat. Lucif. Act.*, relative luciferase activity.

inhibit TRIF-induced ISRE activation as potently as the TBK1-KA and IKK $\epsilon$ -KA mutants. In the same experiments, the TRAF6-DN mutant, which inhibits TRIF-induced NF- $\kappa$ B activation (see below), did not inhibit TRIF-induced ISRE activation (Fig. 3B).

We then examined the roles of TRIF, TBK1, IKK $\beta$ , IKK $\epsilon$ , IRF-7, and IRF-3 in Sendai virus-induced ISRE activation. We transfected 293 cells with the ISRE reporter construct and expression plasmids for mutants of these proteins, infected the



**FIG. 5. Differential phosphorylation of IRF-3 by IKK $\beta$  and IKK $\epsilon$ .** *A*, both IKK $\beta$  and IKK $\epsilon$  phosphorylate IRF-3. 293 cells ( $\sim 2 \times 10^6$ ) were transfected with expression plasmids for FLAG-IRF-3 and a control vector (*Vect.*), Myc-IKK $\beta$ , HA-TRIF, FLAG-IKK $\epsilon$ , or Myc-TBK1. The transfected cells were lysed, and the lysates were immunoprecipitated with antibodies against their epitope tags. The immunoprecipitates were subjected to *in vitro* kinase assay (left panel). The expression levels of the transfected proteins were detected by Western blot analysis of the lysates (right panel). *B*, phosphorylation of IRF-3/5D by IKK $\epsilon$ , but not by IKK $\beta$ . The experiments were performed as described for *A*, except that IRF-3 was replaced with IRF-3/5D.



**FIG. 6. IKK $\beta$  and TRAF6 (but not TBK1 and IKK $\epsilon$ ) are involved in TRIF-induced NF- $\kappa$ B activation.** 293 cells ( $\sim 2 \times 10^5$ ) were transfected with the NF- $\kappa$ B-luciferase reporter plasmid (0.5  $\mu$ g), an expression plasmid for TRIF (black bars) or an empty control plasmid (white bars), and expression plasmids for the indicated mutant proteins (1  $\mu$ g each). Luciferase reporter assays were performed 18 h after transfection.

transfected cells with Sendai virus, and performed reporter gene assays. As shown in Fig. 3C, Sendai virus-induced ISRE activation was potentially inhibited by both the N- and C-terminal domains (amino acids 1–394 and 532–712, respectively) of TRIF, but not by the TRIF domain-containing mutant of TRIF (TRIF-(394–712)). In the same experiments, IKK $\beta$ -KA as well as TBK1-KA, IKK $\epsilon$ -KA, IRF-3 $\Delta$ N, and IRF-7 $\Delta$ N also inhibited Sendai virus-induced ISRE activation (Fig. 3C). These data suggest that TRIF, IKK $\beta$ , and IRF-7, in addition to the previously identified TBK1, IKK $\epsilon$ , and IRF-3, are also involved in Sendai virus-induced ISRE activation pathways.

**Molecular Order of ISRE-activating Kinases**—Because IKK $\beta$ -KA could inhibit TRIF- and Sendai virus-induced ISRE activation, we investigated whether IKK $\beta$  could activate ISRE. Consistent with previous reports (20, 21), we found that TBK1 and IKK $\epsilon$  could activate ISRE (Fig. 4, A and C). In contrast with the previous reports, we found that IKK $\beta$  could also activate ISRE in the same experiments (Fig. 4B). In fact, IKK $\beta$ -induced ISRE activation was relatively more potent than TBK1-induced ISRE activation in our experiments (Fig. 4, A and B). In these experiments, IKK $\alpha$  did not activate ISRE (data not shown). These data are consistent with our observation that IKK $\beta$ -KA can inhibit TRIF- and Sendai virus-induced ISRE activation.

To determine the reason for the discrepancy in the effects of IKK $\beta$  on ISRE activation observed by others and us, we determined whether IKK $\beta$  could activate reporter constructs driven

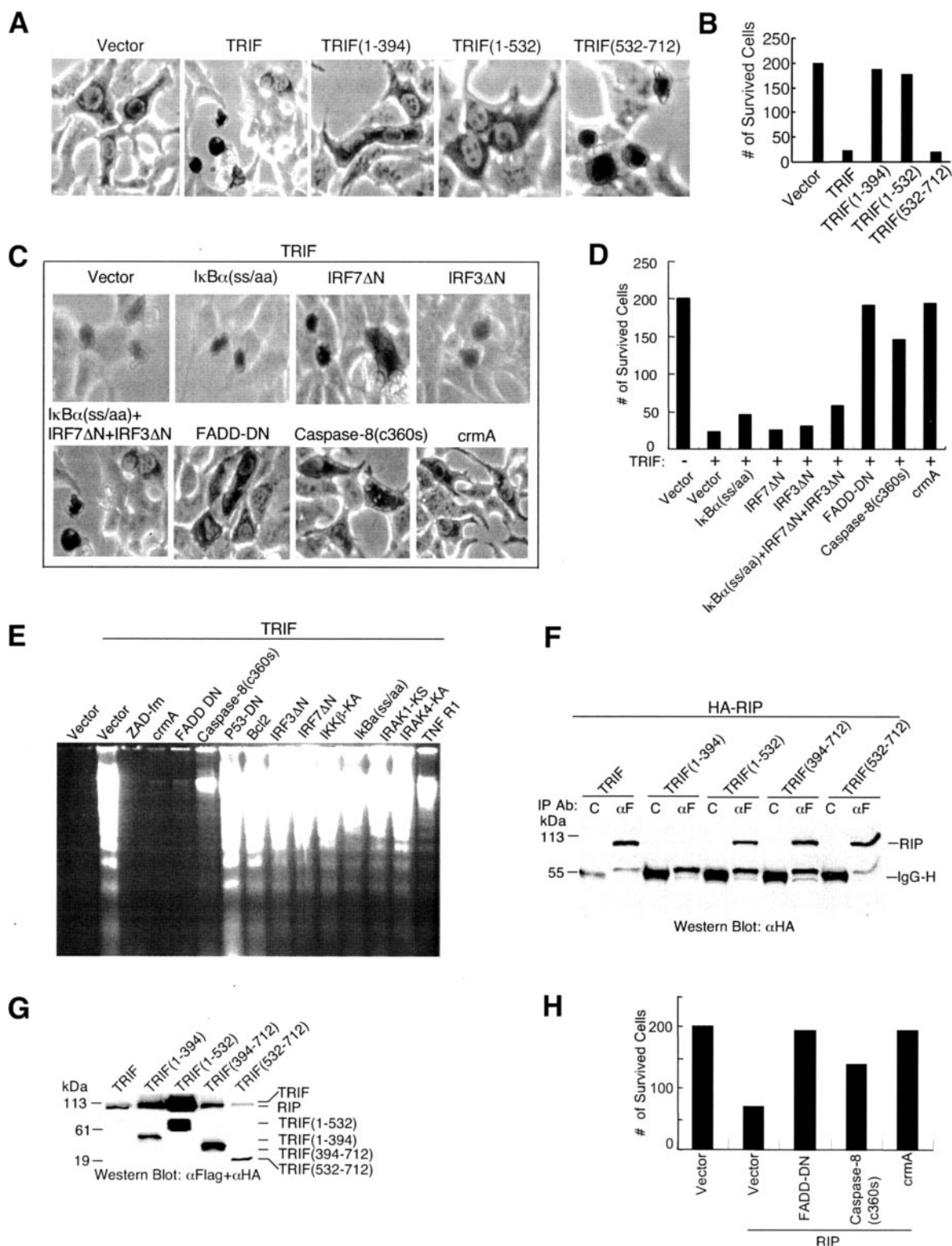
by the IFN- $\beta$  promoter and the NF- $\kappa$ B site-mutated IFN- $\beta$  promoter, which are similar to those used in previous studies (20, 21). Consistent with the previous reports, IKK $\epsilon$  significantly activated these reporters, whereas IKK $\beta$  barely did (Fig. 4D). These studies suggest that IKK $\beta$  may have differential effects on different ISRE reporters.

Because TBK1, IKK $\beta$ , and IKK $\epsilon$  could activate ISRE, we investigated the molecular orders of the three ISRE-activating kinases. In reporter gene assays, TBK1-induced ISRE activation was abolished by kinase-inactive mutants of IKK $\beta$  and IKK $\epsilon$  (Fig. 4A); IKK $\beta$ -induced ISRE activation was completely inhibited by the IKK $\epsilon$  mutant, but not by the kinase-inactive mutant of TBK1 (Fig. 4B); and IKK $\epsilon$ -induced ISRE activation was only slightly inhibited by the TBK1 and IKK $\beta$  mutants (Fig. 4C). These data suggest that TBK1 activates ISRE through IKK $\beta$  and/or IKK $\epsilon$ , whereas IKK $\beta$  can activate ISRE through IKK $\epsilon$ .

In these experiments, we also determined whether dominant-negative mutants of IRF-3 and IRF-7 could block TBK1-, IKK $\beta$ -, and IKK $\epsilon$ -induced ISRE activation. The results indicate that both IRF-3 and IRF-7 mutants inhibited TBK1-, IKK $\beta$ -, and IKK $\epsilon$ -induced ISRE activation (Fig. 4). Interestingly, whereas both mutants could completely abolish IKK $\beta$ -induced ISRE activation, the IRF-7 mutant was significantly less effective than the IRF-3 mutant in inhibiting TBK1- and IKK $\epsilon$ -induced ISRE activation in these assays (Fig. 4).

**Differential Phosphorylation of IRF-3 by IKK $\beta$  and IKK $\epsilon$** —Previously, it has been shown that phosphorylation of IRF-3 and IRF-7 is required for their activation (29, 30). We performed *in vitro* kinase assays to evaluate the abilities of TBK1, IKK $\beta$ , and IKK $\epsilon$  to phosphorylate IRF-3 and IRF-7. Because full-length IRF-7 is highly degradable (31), we used IRF-7 $\Delta$ N for these experiments. Previously, it has been shown that the C-terminal domain (but not the DNA-binding domain) of IRF-7 is phosphorylated following viral stimulation (32). We transfected IRF-7 $\Delta$ N together with TRIF, TBK1, IKK $\beta$ , or IKK $\epsilon$  into 293 cells and performed *in vitro* kinase assays. The results indicate that IRF-7 $\Delta$ N itself was phosphorylated and that overexpression of TRIF, TBK1, IKK $\epsilon$ , and IKK $\beta$  did not increase its phosphorylation (data not shown). In similar experiments, IKK $\epsilon$  potentially phosphorylated IRF-3, whereas TRIF, TBK1, and IKK $\beta$  caused weaker phosphorylation of IRF-3 (Fig. 5A). These data suggest that both IKK $\beta$  and IKK $\epsilon$  can phosphorylate IRF-3.

Previously, it has been shown that five conserved Ser/Thr residues located in amino acids 396–405 at the C terminus of IRF-3 are important for IRF-3 phosphorylation and activation



**FIG. 7. TRIF induces RIP/FADD/caspase-8-dependent, mitochondrion-independent apoptosis.** *A* and *B*, TRIF induces apoptosis through its C-terminal domain. 293 cells ( $\sim 2 \times 10^5$ ) were transfected with 1  $\mu\text{g}$  of the indicated expression plasmids and 0.1  $\mu\text{g}$  of pCMV- $\beta$ -galactosidase plasmid. Twenty-four hours after transfection, the cells were fixed, stained with X-gal, and photographed (*A*). The surviving blue cells were quantitated by counting under a microscope (*B*). *C* and *D*, effects of various mutants and CrmA on TRIF-induced apoptosis. 293 cells ( $\sim 2 \times 10^5$ ) were transfected with 0.1  $\mu\text{g}$  of pCMV- $\beta$ -galactosidase plasmid, 1  $\mu\text{g}$  of an expression plasmid for TRIF, and 1  $\mu\text{g}$  of the indicated expression plasmids. Twenty-four hours after transfection, the cells were fixed, stained with X-gal, and photographed (*C*). The surviving blue cells were quantitated by counting under a microscope (*D*). *E*, TRIF induces DNA fragmentation and effects of various mutants and caspase inhibitors on TRIF-induced DNA fragmentation. 293 cells ( $\sim 2 \times 10^6$ ) were transfected with 10  $\mu\text{g}$  of each of the indicated expression plasmids. DNA was isolated from each sample and analyzed by agarose gel electrophoresis. *F* and *G*, TRIF interacts with RIP through its TIR and C-terminal domains. 293 cells ( $\sim 2 \times 10^6$ ) were transfected with expression plasmids for HA-RIP and FLAG-tagged TRIF or its mutants. Cell lysates were immunoprecipitated (IP) with anti-FLAG ( $\alpha\text{F}$ ) antibody (*Ab*) or control IgG (*C*). The immunoprecipitates were analyzed by Western blotting with anti-HA antibody (*F*). The expression levels of the transfected proteins were detected by Western blot analysis of the lysates (*G*). *H*, RIP-induced apoptosis is blocked by dominant-negative mutants of FADD and caspase-8 and by CrmA. 293 cells ( $\sim 1 \times 10^5$ ) were transfected with 0.1  $\mu\text{g}$  of pCMV- $\beta$ -galactosidase plasmid and 1  $\mu\text{g}$  of each of the indicated expression plasmids. Twenty-four hours after transfection, the surviving blue cells were counted under a microscope.



induced by viral infection and overexpression of IKK $\epsilon$  (22, 33). Surprisingly, we found that an active IRF-3 mutant (IRF-3/5D) in which the five serine residues are mutated to aspartic acids could still be phosphorylated by IKK $\epsilon$ , but not by IKK $\beta$  (Fig. 5B). These data suggest that IKK $\epsilon$  can phosphorylate IRF-3 at additional site(s) other than the five Ser/Thr residues, whereas IKK $\beta$  phosphorylates site(s) belonging to the five conserved Ser/Thr residues.

**Uncoupling and Cross-talk of TRIF-induced NF- $\kappa$ B and ISRE Activation Pathways**—Because dominant-negative mutants of TBK1, IKK $\beta$ , IKK $\epsilon$ , IRF-3, and IRF-7 inhibited TRIF-induced ISRE activation, we examined the effects of these mutants on TRIF-induced NF- $\kappa$ B activation in reporter gene assays. As shown in Fig. 6, the TBK1 and IKK $\epsilon$  mutants slightly increased TRIF-induced NF- $\kappa$ B activation. In contrast, the IKK $\beta$  mutant significantly inhibited TRIF-induced NF- $\kappa$ B activation. A dominant-negative mutant of TRAF6, which did not inhibit TRIF-induced ISRE activation, significantly inhibited TRIF-induced NF- $\kappa$ B activation (Fig. 6). As expected, I $\kappa$ B $\alpha$ (SS/AA) completely inhibited TRIF-induced NF- $\kappa$ B activation, whereas CrmA did not (Fig. 6). Surprisingly, both IRF-3 and IRF-7 mutants significantly inhibited TRIF-induced NF- $\kappa$ B activation (Fig. 6). One explanation for this is that IRF-3 and IRF-7 may bind to IKK $\beta$  and inhibits its ability to activate NF- $\kappa$ B.

Taken together, our data suggest that TBK1 and IKK $\epsilon$  are dedicated to TRIF-induced ISRE activation pathways, whereas IKK $\beta$  is involved in both TRIF-induced ISRE and NF- $\kappa$ B activation pathways. In addition, our data suggest that TRIF-induced NF- $\kappa$ B activation is uncoupled from TRIF-induced apoptosis (also see below).

**TRIF Induces Apoptosis through RIP/FADD/Caspase-8-mediated Pathways**—Infection of cells with Sendai virus induces apoptosis (34). Although TRIF contains a TIR domain, it does not contain a recognizable death domain. However, overexpression of TRIF potently induced apoptosis in 293 cells (Fig. 7, A and B). Domain mapping experiments indicated that the N-terminal and middle TIR domains could not induce apoptosis, whereas the C-terminal 181 amino acids (positions 532–712) were sufficient to induce apoptosis (Fig. 7, A and B).

TRIF-induced apoptosis was not inhibited by I $\kappa$ B $\alpha$ (SS/AA), IRF-3 $\Delta$ N, and IRF-7 $\Delta$ N or by their combination (Fig. 7, C and D). TRIF-induced apoptosis was also not inhibited by kinase-inactive mutants of TBK1, IKK $\beta$ , and IKK $\epsilon$  (data not shown). These data suggest that TRIF-induced apoptosis is independent of TRIF-induced NF- $\kappa$ B and ISRE activation.

Interestingly, TRIF-induced apoptosis was potently inhibited by a dominant-negative mutant of FADD, a caspase-inactive mutant of caspase-8, and the caspase inhibitor CrmA (Fig. 7, C and D). These data suggest that TRIF induces a FADD/caspase-8-dependent apoptosis pathway, which is also utilized by death receptors.

To confirm that TRIF induces FADD/caspase-8-dependent apoptosis, we performed DNA fragmentation assays. As shown in Fig. 7E, TRIF induced DNA fragmentation, a hallmark of caspase-dependent apoptosis. In addition, TRIF-induced DNA fragmentation was inhibited by dominant-negative mutants of FADD and caspase-8 and by the caspase inhibitors ZAD-fm and CrmA (Fig. 7E). TRIF-induced DNA fragmentation was not inhibited by the dominant-negative mutant of p53, Bcl-2, IRF-3 $\Delta$ N, IRF-7 $\Delta$ N, IKK $\beta$ -KA, I $\kappa$ B $\alpha$ (SS/AA), and kinase-inactive mutants of IRAK1 and IRAK4 (Fig. 7E).

We next determined whether TRIF interacts with FADD. In transient transfection and co-immunoprecipitation experiments, we failed to detect an interaction between TRIF and FADD (data not shown). However, we found that TRIF could

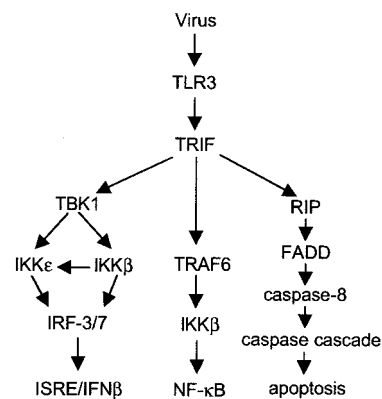


FIG. 8. Model for TRIF-induced intracellular signaling pathways. See “Discussion” for details.

interact with another death domain-containing protein (RIP) in these assays (Fig. 7, F and G). Domain mapping experiments indicated that the TIR and C-terminal domains of TRIF could independently interact with RIP (Fig. 7, F and G). In cell death assays in 293 cells, dominant-negative mutants of FADD and caspase-8 and the caspase inhibitor CrmA inhibited RIP-induced apoptosis (Fig. 7H). Taken together, these data suggest that TRIF induces an RIP/FADD/caspase-8-dependent and mitochondrion-independent apoptosis pathway and that the TRIF-induced apoptosis pathway is uncoupled from TRIF-induced ISRE and NF- $\kappa$ B activation.

#### DISCUSSION

Recognition of specific patterns of microbial components by TLRs in the body leads to activation of innate immunity against pathogens. TLR3 recognizes double-stranded RNA generated during viral infection and is critically involved in host defense against viruses (35). Activation of TLR3 results in induction of type I IFNs, including IFN- $\beta$  and IFN- $\alpha$  family cytokines, which are crucial mediators of the antiviral response.

Recently, it has been shown that a TIR domain-containing adapter protein (TRIF) is critically involved in TLR3-induced IFN- $\beta$  production and NF- $\kappa$ B activation (15, 16). TRIF interacts with IRF-3, a transcription factor that binds to ISRE. It has also been shown that two Ser/Thr kinases (TBK1 and IKK $\epsilon$ ) can phosphorylate IRF-3 and IRF-7 and that these processes are important for TRIF-induced IFN- $\beta$  activation (20, 21). In this study, we further analyzed the mechanisms of TRIF-induced downstream signaling pathways.

In a yeast two-hybrid screening, we found that TRIF could directly interact with IRF-7. This interaction was confirmed by co-immunoprecipitation in mammalian cells (Fig. 1). Domain mapping experiments indicated that the TIR domain of TRIF was required for its interaction with both IRF-7 and IRF-3 (Fig. 1). TRIF could synergize with IRF-7 and IRF-3 to activate the wild-type and NF- $\kappa$ B site-mutated IFN- $\beta$  promoters (Fig. 2). In addition, dominant-negative mutants of IRF-7 and IRF-3 could inhibit TRIF- and Sendai virus-induced IFN- $\beta$  and ISRE activation (Fig. 3). These data suggest that TRIF can signal IFN- $\beta$  activation through either IRF-7- or IRF-3-mediated ISRE activation.

Previous reports suggest that TBK1 and IKK $\epsilon$  (but not IKK $\beta$ ) can activate IRF-3 and IRF-7 (20, 21). Inconsistent with the previous reports, we found that TBK1 and IKK $\epsilon$  (but not IKK $\beta$ ) could activate the IFN- $\beta$  promoter (Fig. 4D). However, using a commercially available ISRE reporter that contains five copies of consensus IRSE-binding sites, we found that IKK $\beta$  could activate ISRE as potently as TBK1 and IKK $\epsilon$  (Fig. 4). That IKK $\beta$  plays a role in virus-induced ISRE activation is

further supported by our observations that a kinase-inactive mutant of IKK $\beta$  could block TRIF- and Sendai virus-induced ISRE activation (Fig. 3). In addition, dominant-negative mutants of IRF-3 and IRF-7 completely inhibited IKK $\beta$ -induced ISRE activation (Fig. 4B).

In *in vitro* kinase assays, both IKK $\beta$  and IKK $\epsilon$  could phosphorylate IRF-3 (Fig. 5). Previously, it has been shown that five conserved Ser/Thr residues located in amino acids 396–405 at the C terminus of IRF-3 are important for IRF-3 phosphorylation and activation induced by viral infection (33) and overexpression of IKK $\epsilon$  (22). Surprisingly, we found that an IRF-3 mutant (IRF-3/5D) in which the five Ser/Thr residues in amino acids 396–405 are mutated to aspartic acids could still be phosphorylated by IKK $\epsilon$ , but not by IKK $\beta$  (Fig. 5B). These data suggest that IKK $\epsilon$  can phosphorylate IRF-3 at additional site(s) other than the five Ser/Thr residues, whereas IKK $\beta$  phosphorylates site(s) belonging to the five conserved Ser/Thr residues within amino acids 396–405. Our data are consistent with a recent study in which it has been shown that IKK $\epsilon$  phosphorylates IRF-3 at Ser<sup>386</sup> (36). Our data point to the possibility that IRF-3 is differentially regulated by IKK $\epsilon$  and IKK $\beta$ .

The IFN- $\beta$  promoter is complicatedly regulated by a combination of various transcription factors (28). Although the IFN- $\beta$  promoter contains an NF- $\kappa$ B site, it is barely activated by overexpression of IKK $\beta$  (Fig. 4D) (20, 21). However, mutation of the NF- $\kappa$ B site in the IFN- $\beta$  promoter dramatically reduced its response to TRIF activation (~60-fold) (Fig. 2, A and B). In addition, I $\kappa$ B $\alpha$ (SS/AA) completely blocked TRIF-induced IFN- $\beta$  promoter activation, but had no effect on TRIF-induced activation of an independent ISRE reporter (Fig. 2, C and D). It is possible that specific IRFs have to collaborate with NF- $\kappa$ B and/or coactivators such as p300/cAMP-responsive element-binding protein-binding protein, TAF<sub>II</sub>250, and proteins with histone acetyltransferase activity to activate the IFN- $\beta$  promoter. In this context, it has been shown that the active form of IRF-3 (but not IRF-7) is associated with these coactivators (29).

Previous studies have demonstrated that both IRF-3 and IRF-7 are required for efficient type I IFN production (29, 30). However, their roles are different in these processes. In the early phase of viral infection, pre-existing IRF-3 is activated and induces expression of IFN- $\beta$  and IFN- $\alpha$ 4. These early produced IFNs transcriptionally induce IRF-7; and upon viral infection, the induced high level IRF-7 is activated and transactivates multiple IFN genes, leading to robust production of IFNs in response to viral infection (29, 30).

In addition to the spatial difference in their functions in virus-induced IFN production, IRF-3 and IRF-7 recognize different DNA-binding sites or have distinct affinities for the same DNA site (29). As mentioned above, the active form of IRF-3 (but not IRF-7) is associated with some coactivators (29). These different properties between IRF-3 and IRF-7 lead to differential gene activation by these two transcription factors. Our studies point to a new possibility that IKK $\beta$  and IKK $\epsilon$  can differentially regulate IRFs and mediate activation of distinct ISREs and type I IFNs.

Previously, it has been shown that TRIF can also activate the transcription factor NF- $\kappa$ B (15, 16). Our studies indicated that the kinase-inactive mutant of IKK $\beta$  (but not those of IKK $\epsilon$  and TBK1) could block TRIF-induced NF- $\kappa$ B activation (Fig. 6). These data suggest that these kinases have differential roles in the TRIF-induced NF- $\kappa$ B and ISRE activation pathways. A dominant-negative mutant of TRAF6 inhibited TRIF-induced NF- $\kappa$ B (but not ISRE) activation (Figs. 3B and 6), suggesting that it plays a role in the TRIF-induced NF- $\kappa$ B (but not ISRE) activation pathway.

In many cases, viral infection leads to apoptosis of host cells.

When overexpressed, TRIF could potentially induce apoptosis (Fig. 7). TRIF-induced apoptosis was mediated by its C-terminal domain. This domain interacted with RIP, a death domain-containing protein kinase. TRIF-induced apoptosis was blocked by the dominant-negative mutants of FADD and caspase-8 and by the caspase inhibitors ZAD-fm and CrmA, but not by a p53 mutant and Bcl-2 (Fig. 7). Our observations suggest that TRIF induces RIP/FADD/caspase-8-dependent, mitochondrion-independent apoptosis.

Previously, it has been suggested that IRF-3 is involved in Sendai virus-induced apoptosis (37). Overexpression of a constitutively active mutant of IRF-3 (IRF-3/5D) induces caspase-dependent apoptosis (37). TRIF-induced apoptosis was not inhibited by IRF-3 $\Delta$ N (Fig. 7), suggesting that TRIF and IRF-3 induce distinct apoptosis pathways. TRIF interacted with RIP and induced apoptosis through death domain-containing FADD (Fig. 7). How IRF-3 induces apoptosis is unknown. In addition, it seems that TRIF is a much more potent inducer of apoptosis than IRF-3. More than 90% of the TRIF-transfected cells died of apoptosis as early as 12 h after transfection (data not shown). In contrast, <40% of the IRF-3/5D-transfected cells died of apoptosis as long as 48 h after IRF-3/5D induction (37).

Based on our and others' results (see above), we have proposed a working model for TRIF-induced intracellular signaling pathways (Fig. 8). Dominant-negative mutants that block one pathway are not able to block the other two pathways, suggesting that the TRIF-induced ISRE and NF- $\kappa$ B activation and apoptosis pathways are uncoupled. The exception is that IKK $\beta$  is probably involved in both TRIF-induced ISRE and NF- $\kappa$ B activation pathways. Our studies may provide a basis for elucidation of the complicated regulation of virus-induced cellular effects.

*Acknowledgments*—We thank Drs. David Goeddel, Zhaodan Cao, John Hiscott, Uli Siebenlist, Bert Vogelstein, and Gary Johnson for reagents.

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