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CUTTING EDGE

Cutting Edge: NF-κB-Activating Kinase-Associated Protein 1 Participates in TLR3/Toll-IL-1 Homology Domain-Containing Adapter Molecule-1-Mediated IFN Regulatory Factor 3 Activation[1]

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***TLRs signal the presence of microbial patterns and activate transcription factors. In TLR3 and TLR4, the adapter Toll-IL-1R homology domain-containing adapter molecule (TICAM-1) (also called Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF)) mediates IFN regulatory factor 3 (IRF3) phosphorylation followed by IFN-β production. The regulatory subunit TNFR-associated factor family member-associated NF-κB activator (TANK) couples with the kinase complex IκB kinase-related kinase ε/NF-κB-activating kinase (NAK) (TANK-binding kinase 1 (TBK1)) that involve TICAM-1-dependent IFN-β induction. There are several TANK-homologous proteins. We tested whether TICAM-1 binds and coprecipitates with TANK or its family proteins. The results are: 1) the TANK family protein NAK-associated protein 1 (NAP1), but not TANK, coprecipitates with TICAM-1; 2) NAP1 overexpression markedly enhances TBK1-mediated IFN-β promoter activation; 3) a dominant-negative form, NAP (158–270), suppresses IRF3 activation in response to poly(I:C) or LPS; 4) RNA interference targeting of the NAP1 message results in a failure of poly(I:C)-mediated IRF3 polymerization and IFN-β production. Thus, NAP1 is the kinase subunit responsible for TLR3/4-mediated IFN-β induction in the TICAM-1 pathway. The Journal of Immunology,* 2005, 174: 27–30.

Toll-like receptors consist of extracellular leucine-rich repeats and the cytoplasmic Toll-IL-1R homology domain (TIR).[3] It is generally accepted that the leucine-rich repeats recognize exogenous microbial patterns while the TIR delivers the intracellular signal, which is largely determined by the combination of the TLR and its adapters. In humans, 10 TLRs and four adapters have been identified (1, 2). The early phase of NF-κB activation is known to be MyD88-dependent. TLR3 and TLR4 induce activation of IFN regulatory factor 3 (IRF3) followed by induction of IFN-β independent of MyD88 (1, 2).

Recently, a novel IFN-inducible adapter was identified downstream of TLR3 (2, 3). By yeast two-hybrid and functional analyses, we have identified TLR adapters that participate in TLR-mediated IRF3 activation and IFN-β induction (4, 5). In TLR3, TIR-containing adapter molecule (TICAM)-1 is the adapter directed to IRF3 activation in myeloid dendritic cells and epithelial cells (4, 6), while in TLR4 the TICAM-2-TICAM-1 complex is the adapter (5, 7, 8). Meanwhile, two groups reported that IκB kinase-related kinase ε (IKKε) and TNFR-associated factor (TRAF) family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), kinases which activate IRF3, localize downstream of TICAM-1 (9, 10). The current working concept is that these kinases form a complex with TANK (11, 12). Thus, the pathway converging to IRF3 activation in dendritic cells appeared to have been identified as TLR3/4-TICAM-TANK-IKKε/TBK1-IRF3 and the IFN-β promoter. Recent reports, however, demonstrated that NF-κB-activating kinase (NAK)-associated protein 1 (NAP1) is a subunit for the kinase complex IKKε/TBK1 in the pathway for NF-κB activation (13).

In this study, we tested the virtual molecular association in this pathway, namely the TICAM-1 pathway, particularly focusing on the physical/functional association in the TICAM-1 and NAP1 interaction.

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[3] Abbreviations used in this paper: TIR, Toll-IL-1R homology domain; IRF, IFN regulatory factor; TICAM, TIR-containing adapter molecule; IKKε, IκB kinase-related kinase ε; TRAF, TNFR-associated factor; TANK, TRAF family member-associated NF-κB activator; TBK, TANK-binding kinase; NAK, NF-κB-activating kinase; NAP, NAK-associated protein; HA, hemagglutinin; sih, small interference hairpin-loop; siRNA, small interference RNA.

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Materials and Methods

Cell lines, reagents, plasmids, and immunoprecipitation

HEK293 and HeLa cells were cultured and used as described (4). Poly(I:C) (Amersham Biosciences), LPS (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich), and anti-IRF3 Ab (IBL) were purchased. The p-125 luc reporter was a gift from Dr. T. Taniguchi (Tokyo University, Tokyo, Japan) and contained the human IFN-β promoter region (-125 to $+19$). The Gal4-IRF3, Gal4-DBD, and p55 UASG-Luc for IRF3 activation were gifts form Dr. T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (14). Expression vectors for human TLR3, TLR4, MD-2, and hemagglutinin (HA)-tagged human TICAM-1 in pEF-BOS (5) and those for NAP1 and NAK (TBK1) in pcDNA3.1 (13, 15) were prepared as described previously. Methods for immunoprecipitation and immunoblotting were described previously (4, 5). The monospecific properties of the polyclonal Abs against NAP1 (13) and TICAM-1 (5) were shown in previous reports.

Assay for IRF3 and IFN-β promoter activation

Activation of the IFN-β promoter was measured by reporter assay. HEK293 cells (2×10^5 cells) were transfected in 24-well plates using LipofectAMINE 2000 (Invitrogen Life Technologies) with a p-125 luc (IFN-β) reporter plasmid (0.1 μg) together with the indicated amounts of the TICAM-1, NAK (TBK1), NAP1 full-length and/or NAP1 158–270 plasmids.

Two methods, reporter assay (14) and native gel assay (16) were used to determine the degree of IRF3 activation. In some experiments, cells were stimulated with medium alone, LPS (100 ng/ml), or poly(I:C) (10 μg/ml). Signal intensity was measured by NIH Image Scan. Data were expressed as the means $\pm$ SD. Experiments were performed at least three times in triplicate. The primer sets for RT-PCR were described previously (5, 13, 15).

RNA interference

The gene silencing vector pH1 was constructed from the pH1′ vector (a gift from Drs. M. Okabe and H. Hasuwa, Osaka University, Osaka, Japan) (17). Oligonucleotides were cloned into the pH1 vector to express the small interference hairpin-loop (sih) GFP, sihNAP1-A, and sihNAP1-B hairpins downstream of the human H1 RNA promoter as described (18). The following sequences were targeted: for sihNAP1-A, 5′-AAGCTAATAGCTCGATTTGA AGA-3′; sihNAP1-B, 5′-AAGTGATAATATGCAGCATGCAT-3′. The sequence for sihGFP has been described (18). HeLa cells were transfected with pH1-empty, pH1-GFP, or pH1-NAP1-A/B using PolyFect (Qiagen) and selected bulk populations in 1 μg/ml Puromycin and picked up as single colonies. mRNA was isolated from the cells and silencing efficiency assessed by RT-PCR.

Results and Discussion

We first tested whether TICAM-1 recruits TANK in the HEK293 overexpression system by immunoprecipitation. HA-tagged TICAM-1 failed to coprecipitate Myc-tagged TANK, although sufficient amounts of TICAM-1 and TANK were expressed in HEK293 cells (Fig. 1*A*). The absence of the binding of TICAM-1 to TANK was also confirmed in yeast (data not shown). Under similar conditions, an association between TICAM-1 and another protein with significant similarity to TANK, NAP1, was examined (Fig. 1*B*). HA-tagged TICAM-1 coprecipitated NAP1 when both were expressed in HEK293 cells. Endogenous interaction between NAP1 and TICAM-1 was confirmed in HeLa cells (Fig. 1*C*). NAP1 recruited more to TICAM-1 if the cells were prestimulated with poly(I:C). Thus, TICAM-1 either directly or indirectly associates with NAP1. Physical association studies (Fig. 1) suggest that TANK is not involved in the TICAM-1 pathway, in opposition to the current concept (2). We then undertook investigation of a possible functional effect of NAP1 on IFN-β promoter activation in the TICAM-1 pathway.

HEK293 cells were transfected with vectors with various cDNAs and a reporter gene for the IFN-β promoter. First, TICAM-1 expression induced activation of the IFN-β promoter and this activation level was slightly enhanced if full-length NAP1 was simultaneously expressed (Fig. 2*A*). Previous reports showed that NAP1 interacts with TBK1 and IKKϵ to facilitate their oligomerization and the phosphorylation of the p65/RelA subunit of NF-κB. NAP1 binds TBK1 in the 158–270 region (14). We then tested whether NAP1 (158–270) affects TICAM-1-dependent IFN-β promoter activation (Fig. 2*B*). NAP1 (158–270) blocked TICAM-1-mediated IFN-β promoter activation in a dose-dependent manner although NAP1 (158–270) per se had no effect (Fig. 2*B*). Thus, we designated NAP1 (158–270) to be dominant-negative. Because the NAP1-TBK1 complex activates NF-κB (13), we next checked the synergistic effect of NAP1 and TBK1 on IFN-β promoter activation (Fig. 2*C*). In a dose-dependent manner, NAP1 enhanced IFN-β promoter activation by the functional effect of TBK1. Under similar conditions, TICAM-1-mediated activation of NF-κB, either via TLR3 or TLR4, was suppressed

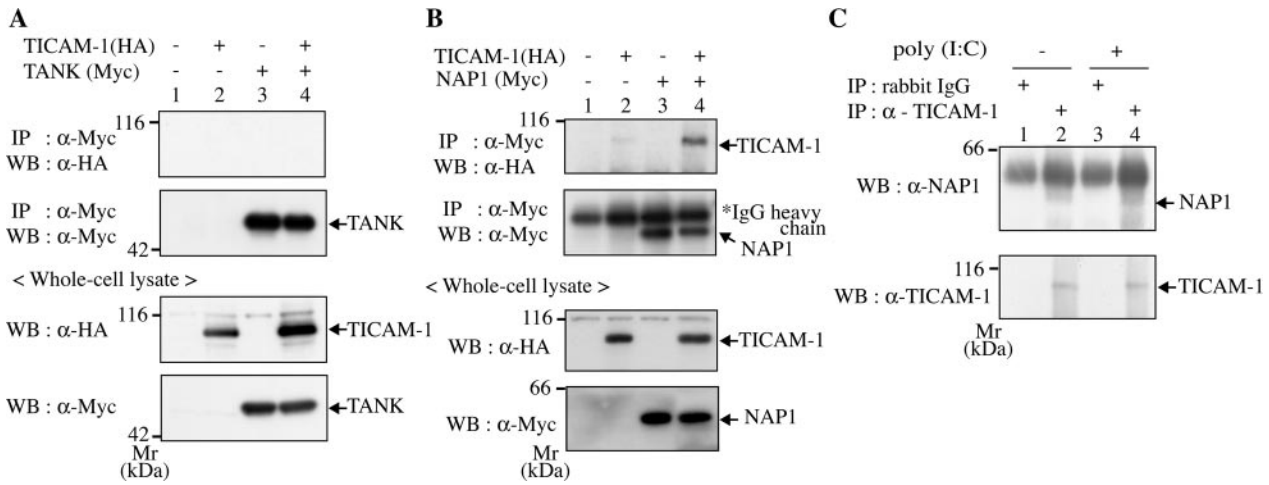

FIGURE 1. NAP1 but not TANK interacts with TICAM-1. HEK293 cells were transiently transfected with expression vectors with TICAM-1 (HA-tagged) and/or TANK (Myc-tagged; *A*), or vectors with TICAM-1 (HA-tagged) and/or NAP1 (Myc-tagged; *B*), and allowed to stand for 24 h. Cells were then lysed and the lysates immunoprecipitated with anti-Myc Ab. The precipitates were resolved on SDS-PAGE and transferred onto membrane. The blot was probed with anti-Myc or anti-HA Ab. Arrows indicate the positions of TICAM-1, NAP1, and TANK. *, The band of IgG H chain. M_r markers are shown to the *left*. Endogenous interaction between NAP1 and TICAM-1 (*C*). HeLa cells were stimulated with poly(I:C) (*lanes 3* and *4*) or saline (*lanes 1* and *2*). After 20 min, lysates were prepared as in *B* and immunoprecipitated with the indicated Ab. The blot was probed with anti-NAP1 or anti-TICAM-1 Ab.

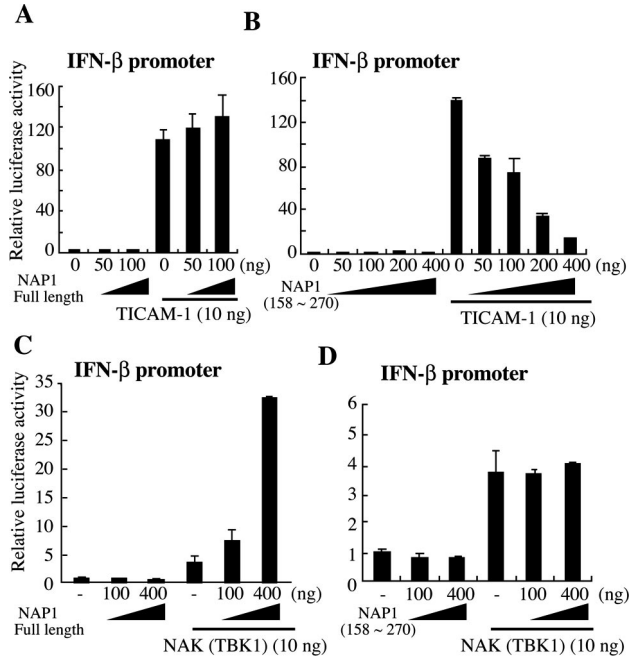

FIGURE 2. NAP1 participates in IFN-β promoter activation via TICAM-1. HEK293 cells were transfected with full-length NAP1 (*A* and *C*) or NAP1 (158-270) (*B* and *D*), together with the IFN-β promoter reporter, and an empty vector and vectors for TICAM-1 (*A* and *B*) or NAK (TBK1; *C* and *D*). After 24 h, luciferase reporter activity was measured and expressed in terms of relative stimulation.

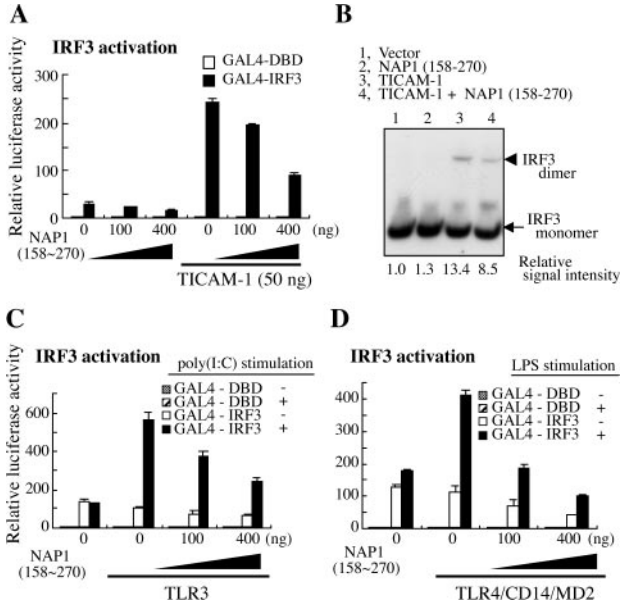

FIGURE 3. NAP1 (158–270) inhibits IRF3 activation. *A*, HEK293 cells were transfected with NAP1 (158–270) and/or TICAM-1, together with the p55 UASG-Luc reporter and Gal4-DBD or Gal4-IRF3, and allowed to stand for 24 h. Cells were lysed, and luciferase activity was assayed and expressed in terms of relative stimulation. *B*, HEK293 cells were transfected with NAP1 (158–270) and/or TICAM-1, and cultured for 24 h. Cells were then lysed and lysates were subjected to native PAGE according to the reported method. Relative signal intensities of the dimer are shown below the lanes. *C* and *D*, HEK293 cells were transfected with vectors for TLR3 (*C*) or TLR4/MD-2/CD14 (*D*) and/or NAP1 (158–270), together with the p55 UASG-Luc reporter gene and Gal4-DBD or Gal4-IRF3. Twenty-four hours after transfection, cells were stimulated with poly(I:C) (10 μg/ml) (*C*) or LPS (100 ng/ml) (*D*) for 6 h and luciferase activities were measured.

by NAP1 (158–270) (data not shown). Hence, the NAP1-TBK1 complex emerges a pathway to IFN-β promoter activation in addition to NF-κB. The dominant-negative form of NAP1 exhibited no suppressive effect on IFN-β promoter potentiation secondary to TBK1 overexpression (Fig. 2*D*), suggesting that NAP1 localizes upstream of TBK1 in the TICAM-1 pathway.

IRF3 is a potent transcription factor for activation of the IFN-β promoter (14, 18), and TLR3 stimulation as well as TICAM-1 transfection results in IRF3 activation (4, 6). Using GAL4-IRF3 (13, 16), we measured the level of IRF3 activation in cells expressing variable levels of NAP1 (158–270) (Fig. 3*A*). Spontaneous IRF3 activation detected in intact cells was inhibited by NAP1 (158–270). The NAP1 (158–270)-dependent IRF3 inhibition was prominent if TICAM-1 cotransfected so as to potentiate IRF3 activation. These results were confirmed by gel-shift assay for the determination of IRF3 activation (16). TICAM-1-mediated IRF3 dimer formation was decreased (13.4 vs 8.5) by coexpression of NAP1 (158–270) (Fig. 3*B*). IRF3 activation followed by poly(I:C) stimulation of TLR3-expressing HEK293 cells was monitored with GAL4-IRF3 (Fig. 3*C*). IRF3 activation was up-regulated in response to poly(I:C) stimulation, and expression of NAP1 (158–270) in this cell system led to the suppression of IRF3 activation (Fig. 3*C*). LPS-mediated IRF3 activation was next tested with cells expressing TLR4/CD14/MD-2 in the GAL4-IRF3 assay system. When HEK293 cells were transfected with an appropriate ratio of TLR4, CD14, and MD-2 (5), IRF3 was sufficiently activatable in response to LPS (Fig. 3*D*). NAP1 (158–270) again inhibited the IRF3 activation induced by LPS and TLR4 (Fig. 3*D*). Thus, IRF3 is evidently activated through the NAP1-TBK1 complex in the TICAM-1 pathway.

Finally, loss-of-function studies were performed with cells depleted of NAP1 using RNA interference. Two small interference (si) RNAs were used to deplete the NAP1 message (Fig. 4*A*), and both introduced NAP1-specific effective gene silencing (Fig. 4*B*). In parallel with NAP1 depletion, IRF3 dimer formation (Fig. 4*C*) and IFN-β mRNA production (Fig. 4*D*) were impaired. These results were confirmed by RT-PCR for IFN-β mRNA (data not shown). Thus, the functional loss of NAP1 abolishes IRF3-mediated IFN-β induction. Taken together, NAP1 physically and functionally associates with TICAM-1 and in conjunction with TBK1, activates IRF3 to stimulate IFN-β promoter activation.

TICAM-1 is the adapter that activates NF-κB and IRF3 (4, 6). Receptor-interacting protein 1 binds the C-terminal domain out of the TIR of TICAM-1 and participates in TICAM-1-mediated NF-κB activation (19). Because IL-1R-associated kinase 1/4 and TRAF6 are responsible for activation of the kinase complex for liberation of NF-κB in the MyD88-dependent pathway (1), TICAM-1 and MyD88 adapters allow the selection of different pathways for NF-κB activation. In this study, our findings suggest that receptor-interacting protein 1 and NAP1 differentially interact with TICAM-1 to induce NF-κB and IRF3 activation, respectively, downstream of TICAM-1. This accounts for the bimodal function of TICAM-1 (1, 3, 20). TANK cannot be replaced for NAP1 in this pathway.

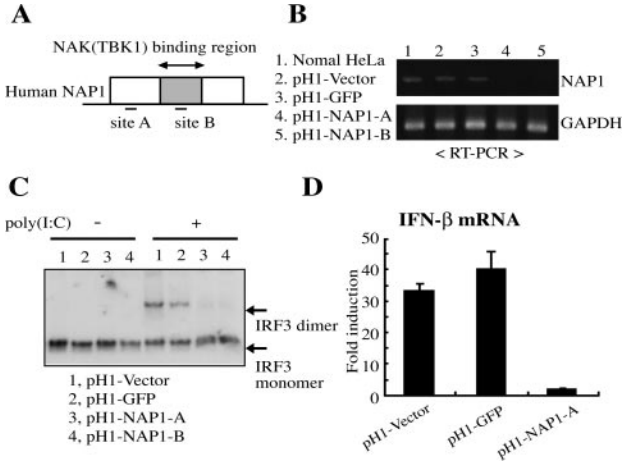

FIGURE 4. NAP1 is required for the activation of IRF3 via TLR3. *A*, The regions of oligonucleotides in human NAP1. siRNAs for NAP1 silencing (sites A and B) were expressed by pH1 vectors (see *Materials and Methods*). *B*, Efficiency of the gene silencing of NAP1. The NAP1 mRNA levels of HeLa cells were assessed by RT-PCR. Notice that the NAP1 message was markedly reduced when cells were treated with NAP1 siRNA site A or B. The GAPDH mRNA levels were used as a control. *C*, IRF3 dimerization assessed by native gel assay. NAP1-silenced HeLa cells were stimulated with poly(I:C) and then lysed. Cell lysates were resolved on native PAGE followed by immunoblotting. Monomeric and dimeric forms of IRF3 are shown by the arrows. *D*, The IFN-β mRNA levels in HeLa cells treated with various siRNAs. NAP1 transfectants were stimulated with poly(I:C) for 6 h. Then, RNAs were recovered to assess the levels of relevant IFN-β mRNA by real-time PCR.

IKKα and IKKβ are the kinases for IκB degradation. These kinases form a complex with IKKγ that is known to be associated with TANK (21). This NF-κB kinase complex cannot activate IRF3, but topologically resembles our current model of the NAP1 kinase complex (13). In fact, the NAP1-TBK1 complex activates NF-κB along with IRF3 (data not shown).

Sharma et al. (10) report that IKKϵ and TBK1 are targets for viral components that are generated by infected viruses. They call these kinases "virus-activated kinases" (22). We favor the interpretation that viral factors target the NAP1 kinase complex. The virus-mediated IRF3-activating pathway may merge with the TLR3-TICAM-1 pathway at the NAP1-TBK1 region. Our present findings would be a key to elucidate the specific relationship between viral infection and TICAM-1-mediated signaling.

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