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A20 Is a Negative Regulator of IFN Regulatory Factor 3 Signaling

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IFN regulatory factor 3 (IRF-3) is a critical transcription factor that regulates an establishment of innate immune status following detection of viral pathogens. Recent studies have revealed that two IkB kinase (IKK)-like kinases, NF-κB-activating kinase/Traf family member-associated NF-κB activator-binding kinase 1 (TBK1) and IKK-i/iIKKe, are responsible for activation of IRF-3, but the regulatory mechanism of the IRF-3 signaling pathway has not been fully understood. In this study, we report that IRF-3 activation is suppressed by A20, which was initially identified as an inhibitor of apoptosis and inducibly expressed by dsRNA. A20 physically interacts with NF-κB-activating kinase/Traf family member-associated NF-κB activator-binding kinase 1 and IKK-i/iIKKe, and inhibits dimerization of IRF-3 following engagement of TLR3 by dsRNA or Newcastle disease virus infection, leading to suppression of the IFN stimulation response element- and IFN-β promoter-dependent transcription. Importantly, knocking down of A20 expression by RNA interference results in enhanced IRF-3-dependent transcription triggered by the stimulation of TLR3 or virus infection. Our study thus demonstrates that A20 is a candidate negative regulator of the signaling cascade to IRF-3 activation in the innate antiviral response. The Journal of Immunology, 2005, 174: 1507–1512.

The innate immune system is an important, evolutionarily conserved mechanism that confers host defense against viral and microbial infection (1–3). IFN regulatory factor 3 (IRF-3) is a ubiquitously expressed transcription factor that regulates primary induction of type I IFN, IFN-αβ, and plays a critical role for establishing innate immune status in response to invasion of pathogens (1–2, 4). Although IRF-3 is retained in the cytoplasm of unstimulated cells, it is phosphorylated and forms a dimer upon viral infection, which then translocates to the nucleus, binds to IFN stimulation response element (ISRE) and enhances the transcription of a set of genes including IFN-β (2, 4, 5). Recent studies revealed that two noncanonical IkB kinase (IKK)-like kinases, NF-κB-activating kinase (NAK)/Traf family member-associated NF-κB activator-binding kinase 1 (TBK1) and IKK-i/iIKKe, could induce the dimerization of IRF-3 by enhancing phosphorylation of IRF-3 and play essential roles for IRF-3-dependent transcriptional activation (6, 7).

Viral and microbial pathogens can be detected by TLR3 and TLR4, which recognize viral dsRNA and bacterial LPS, respectively (3, 8). Engagement of these TLRs triggers the IRF-3 and NF-κB signaling pathways and confers the rapid induction of IFN-β. Although MyD88 is a common adaptor protein for TLRs and plays important roles for NF-κB activation, TLR3 and TLR4 still mediate both IRF-3 and NF-κB activation in MyD88-deficient mice, indicating that the MyD88-independent signaling pathways for production of IFN-β are triggered by engagement of these TLRs (3, 8). We and others identified adaptor molecules, TOLL/IL-1R domain-containing adaptor inducing IFN-β (TRIF) (also called TICAM-1) and TRIF-related adaptor molecule (TRAM; also called TICAM-2 or TIRP) as mediators in the MyD88-independent signaling pathways (8–11). TRAM interacts with TLR4 and is specifically involved in TLR4-induced IRF-3 activation. TRIF, which interacts with TLR3 and TRAM, is involved in both TLR3- and TLR4-mediated signaling pathways and associates with NAK/TBK1 to initiate IRF-3 activation (12).

Although recent discoveries identified critical mediators of IRF-3 signaling, regulatory mechanisms of these molecules are not fully understood. Because NAK/TBK1 and iIKKe are IKK-like molecules, we hypothesized that the regulatory mechanism of IRF-3 kinases might be similar to that of the canonical IKKa/IKKe/NF-κB essential modulator (NEMO) complex. We therefore examined regulators of the IKK complex activation for participation in IRF-3 signaling pathways and have found that A20 potently inhibits IRF-3 activation. A20 is a 90-kDa protein whose expression is induced by a variety of stimuli including poly(I)poly(C) (polyIC), LPS, and TNF-α (13, 14). A recent study indicated that viral infection also induced A20 expression through activation of protein kinase R (15). A20 has a deubiquitinase domain at the N terminus and seven repeats of zinc finger domain at the C terminus, although the role of these domains for A20 function is not fully understood (13, 16). A recent study using mice deficient in A20...
provided genetic evidence that A20 worked as a negative regulator in NF-κB signaling pathways. Injection of TNF-α in A20-deficient mice induced severe inflammation due to persistent activation of NF-κB (17). These mice exhibited elevated sensitivity to endotoxin shock, suggesting that A20 might negatively regulate TLR-initiated signaling pathways (17, 18). However, involvement of A20 in IRF-3 signaling pathways triggered by engagement of TLR or virus infection has not been reported.

In this study, we demonstrate that A20 interacts with IRF-3 kinases, NAK/TBK1 and IKK-α/IKKε, and inhibits TLR3- or virus-induced IRF-3 dimerization and ISRE-dependent transcriptional activation.

Materials and Methods

Reagents

Monoclonal anti-HA Ab (12CA5) was a kind gift from Dr. A. Israel (Institut Pasteur, Paris, France). Monoclonal anti-FLAG Ab (M2) and anti-HA Ab (HA-7) were purchased from Sigma-Aldrich. Anti-actin Ab (C-2) and anti-IRF-3 Ab (FL-425) were purchased from Santa Cruz Biotechnology. Polyclonal anti-human IRF-3 (phospho-Ser386) Ab was described previously (20). Sequences inserted immediately downstream of U6 promoter were as follows (only sense sequence is shown): specific to A20, 5′-GGAAAACAGACACACGCAAC-3′; the unrelated control, 5′-GTAGCGGGTTGATTATAC-3′. The resultant plasmids were referred as pU6-A20i or pU6-Ctli, respectively.

Cell culture, transfection, and reporter assay

293/TLR3 cells were described previously (22). The 293, 293/TLR3, and HeLa cells used in this study were all maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. THP-1 cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin. 293 and 293/TLR3 cells were transfected by the calcium-phosphate method as described previously (23). HeLa cells were transfected by FuGene6 transfection reagent (Roche) according to the manufacturer’s instructions. THP-1 cells were transfected by DMRIE-C transfection reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. 293, 293/TLR3, and HeLa cells were transfected with 250 ng of pEF1-lacZ, and 250 ng of pISRE-luc or p125-luc along with the indicated effector plasmid. THP-1 cells were transfected with 500 ng of EF1-lacZ and 500 ng of pISRE-luc along with the indicated effector plasmid. After the transfection, cells were treated as indicated and lysed in lysis buffer (25 mM Tris-HCl, 8 mM MgCl₂, 1 mM DTT, 1% Triton X-100, 15% glycerol). Luciferase activity was normalized based on β-galactosidase activity. All of the experiments were conducted at least six times. The results were essentially reproducible.

Preparation of whole-cell extracts, immunoblotting, and immunoprecipitation

Cells were suspended in lysis buffer (20 mM HEPES (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, and 0.1% Nonidet P-40) supplemented with 1 mM DTT, 1% Triton X-100, 15% glycerol). Luciferase activity was normalized based on the β-galactosidase activity. The values shown are means ± SD from three separate transfections. R.L.U., Relative luciferase unit.

A20 suppresses IRF-3-dependent transcription. A and B, 293/TLR3 cells were transfected with 1 μg of the indicated expression plasmid (A) or the indicated amounts of pcDNA3-HA-A20 (B) along with pISRE-luc and pEF1-lacZ. Total amount of effector plasmid (1 μg) was kept constant by addition of pcDNA3. Twenty-four hours after transfection, the cells were subjected to luciferase reporter assays. The luciferase activity was normalized based on the β-galactosidase activity. The values shown are means ± SD from three separate transfections. R.L.U., Relative luciferase unit.
The engagement of TLR3 with polyIC and infection with NDV, which are well-characterized stimuli to trigger IRF-3 signaling pathways, were conducted at least twice. The results were essentially reproducible. 

ECL detection system (Amersham Biosciences). All of the experiments were conducted at least twice. The results were essentially reproducible.

Detection of IRF-3 dimer by native PAGE

Cells were suspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40) supplemented with 1 mM PMSF and 1 mM Na~2~VO~4~. Extracts were cleared by centrifugation. Whole-cell extracts (10 μg) were fractionated by 8% SDS-PAGE, and transferred onto Immobilon membranes (Millipore), and blots were revealed with an ECL detection system (Amersham Biosciences). All of the experiments were conducted at least twice. The results were essentially reproducible.

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Results

A20 interacts with NAK/TBK1 and IKK-ι/IKKe

Because NAK/TBK1 is a common regulator of TLR- or virus-induced IRF-3 activation, we next examined interaction of A20 with NAK/TBK1 and found that A20 was coimmunoprecipitated with NAK/TBK1 (Fig. 3A). In addition, A20 was associated with another IRF-3 kinase, IKK-ι/IKKe (Fig. 3B). Interestingly, when A20 was cotransfected with NAK/TBK1 or IKK-ι/IKKe, A20 was found to migrate more slowly in immunoblot studies (Fig. 3A and B), as had been shown for TRIF, which associates with NAK/TBK1 (12). A20 has a deubiquitinase domain at the N terminus and repeated zinc finger domains at the C terminus (Fig. 3C). Previous studies demonstrated that TNFR-associated factor (TRAF)2 and TRAF6 interact with the N terminus of A20, whereas A20-binding inhibitor of NF-κB interacts with the C terminus of A20 (13). NEMO was reported to interact with both the N and C termini of A20 (24). To determine which functional domain(s) of A20 is required for the interaction with these kinases, we generated deletion constructs capable of expressing the N terminus (aa 1–378) or C terminus (aa 379–790) of A20 (Fig. 3C). Immunoblot analyses detected these mutants at expected positions, although the level of expression of the N terminus of A20 was higher than the others (Fig. 3D). Immunoblotting studies coupled with immunoprecipitation revealed that NAK/TBK1 and IKK-ι/IKKe interacted with the C-terminal zinc finger and N-terminal deubiquitinase domains of A20 (Fig. 3, E and F).

Because A20 interacts with NAK/TBK1, which phosphorylates IRF-3, we examined whether A20 inhibits NAK/TBK1-induced IRF-3 phosphorylation. Immunoblotting with phosphospecific anti-IRF-3 Ab following native PAGE demonstrated that NAK/TBK1-induced phosphorylation of IRF-3 at Ser~386~, one of the important residues for the dimerization of IRF-3 (5), was impaired in cells expressing the full length or C terminus of A20 (Fig. 3G, upper panels). In contrast, NAK/TBK1-induced phosphorylation of IRF-3 was not inhibited in cells expressing the N terminus of A20. As reported previously (7), slowly migrating, multiply phosphorylated species of IRF-3 were detected by SDS-PAGE followed by immunoblotting with phosphospecific or conventional anti-IRF-3 Abs when IRF-3 was coexpressed with NAK/TBK1, and these modifications were weakened by coexpression of the full length or C terminus of A20 (Fig. 3G, lower panels). We verified in transfection studies that the biochemical activity of each A20 domain correlated with the functional requirement for inhibition of TLR3- or NDV-induced ISRE-dependent transcription (Fig. 3, H and I). The C-terminal zinc finger domain of A20 suppressed ISRE-dependent transcription induced by TLR3 stimulation or indeed activated ISRE-dependent transcription in a manner that depends on NAK/TBK1 and IRF-3 (Fig. 1, A and C). As a result, we found that A20 inhibited IRF3 stimulation- or NDV infection-induced ISRE-dependent transcription in a dose-dependent manner (Fig. 1, B and D). In contrast, expression of A20 did not affect IFN-α-induced activation (Fig. 1B), indicating that A20 specifically regulates IRF-3 signaling. We next asked whether A20 modulates the activity of the IFN-β promoter, which harbors ISRE recognized by IRF-3. Transfection studies revealed that A20 potently inhibited TLR3- or NDV-induced IFN-β promoter activation (Fig. 1, E and F). A20 also inhibited NAK/TBK1-induced, but not the constitutively active IRF-3 5D mutant-induced ISRE-dependent transcription (Fig. 2, A and B). In addition, A20 inhibited the polyIC- or NDV-induced dimerization of endogenous IRF-3 (Fig. 2, C and D). These results strongly suggest that A20 acts on a common mediator(s) that regulates IRF-3 activation induced by TLR3 engagement or NDV infection.
NDV infection as efficiently as the full-length A20, whereas the N terminus of A20 failed to do so.

**siRNA to A20 enhances the signal-induced IRF-3-dependent transcription**

To verify the role of endogenous A20, we generated a siRNA expression construct capable of knocking down the A20 expression. Expression of siRNA specific to A20 effectively reduced the level of transfected A20, whereas it did not affect that of IRF-3 (Fig. 4A). Expression of this siRNA specifically enhanced ISRE-dependent transcription induced by TLR3 stimulation or NDV infection, whereas it barely caused additional ISRE-dependent transcription in unstimulated 293 and 293/TLR3 cells or in 293/TLR3 cells stimulated with IFN-α (Fig. 4, B and E). Similar results were obtained with HeLa cells (Fig. 4C) and immunologically relevant THP-1 cells (D). Consistent with the ability of A20 to suppress NAK/IKK-ε-induced ISRE-dependent transcription (Fig. 2A), expression of siRNA to A20 enhanced this transcriptional activation (Fig. 4F). In contrast, it did not alter the constitutively active IRF-3 5D mutant-induced ISRE-dependent transcription (Fig. 4G). These results indicate that endogenous A20 negatively regulates IRF-3 signaling following initial stimulation of the system.
stimulated with polyIC or IFN-\(\beta\)/H9251 cells (\(\text{lacZ}\) and pISRE-luc. Thirty-six hours after transfection, the cells were transfected with 500 ng of pU6-Ctli or pU6-A20i along with pEF1-\(\beta\)-lacZ for 36 h. The harvested cells were subjected to luciferase reporter assay.

**FIGURE 4.** Suppression of A20 expression by RNA interference enhances the signal-induced IRF-3-dependent transcription. A, 293 cells were transfected with 500 ng of pU6-Ctli or pU6-A20i along with 100 ng of pCDNA3-HA-A20 and 100 ng of pEF-HA-IRF3. Cells extracts (10 \(\mu\)g) were subjected to Western blotting analysis with anti-HA and anti-actin Abs. Citi, Control siRNA; A20i, siRNA specific to A20. B and C, 293/TLR3 cells (B) or HeLa cells (C) were transfected with 500 ng of pU6-Ctli or pU6-A20i along with pEF1-lacZ and pISRE-luc. Thirty-six hours after transfection, the cells were transfected with 1 \(\mu\)g of pU6-Ctli or pU6-A20i along with pEF1-lacZ and pISRE-luc. Thirty-six hours after transfection, the cells were stimulated with polyIC or IFN-\(\alpha\) for 8 h. D, 293 cells were transfected with 500 ng of pU6-Ctli or pU6-A20i along with pEF1-lacZ and pISRE-luc. Thirty-six hours after transfection, the cells were infected with NDV for 12 h. E and F, 293 cells were transfected with 50 ng of pCDNA3-FLAG-NAK (F) or 15 ng of pEF-p50-IRF-3 5D (G) along with indicated siRNA expression plasmid, pEF1-lacZ and pISRE-luc for 36 h. The harvested cells were subjected to luciferase reporter assay.

**Discussion**

Previous studies identified critical mediators in TLR signaling pathways that play important roles in innate immune responses. In addition, recent reports showing that mice deficient in suppressor of cytokine signaling-1, IL-1R-associated kinase M, or ST2, a negative regulator of NF-\(\kappa\)B signaling, are hypersensitive to endotoxin shock, strongly suggest that negative feedback regulation of TLR signaling is important to protect the host from excessive immune response (25–28). Because IRF-3 is known to mediate endotoxin shock or virus-induced cell death, the IRF-3 activity should also be strictly controlled (29, 30). Several negative regulators of TLR-triggered NF-\(\kappa\)B signaling were identified, but those of IRF-3 signaling have not been described except for IRF-2, which competes with IRF-3 for the recruitment of CREB binding protein (31). In this study, we have demonstrated for the first time a negative regulation of IRF-3 signaling triggered by TLR3 engagement or virus infection at the level of IRF-3 kinases. Because A20 was identified as a polyIC- or LPS-inducible protein (13–14), A20 might participate in the negative feedback regulation of IRF-3 signaling. Thus, our present study provides a molecular basis for a role of A20 in evasion of fatal excessive immune response in hosts suffering from virus infection. Pitha and colleagues (32) reported that pretreatment of cells with LPS impaired virus-induced phosphorylation and subsequent nuclear translocation of IRF-3. Because A20 is induced by LPS stimulation, our results that A20 interferes with the NAK/TBK1 and IKK-\(\alpha\)/IKKe-mediated IRF-3 signaling could partly explain the impaired IRF-3 activation after LPS treatment. Studies using A20-deficient mice will clarify the roles of A20 for the negative regulation of antiviral innate immune responses in vivo.

We showed potent suppression of the IFN-\(\beta\) promoter by A20. This may partly be due to A20 inhibition of NF-\(\kappa\)B activation (data not shown). Because A20 was reported to inhibit NF-\(\kappa\)B activation through TRAF6 or receptor interacting protein, which transduce signals from TLR3-TRIF and interact with NEMO, it is reasonable to assume that A20 interferes with the TRAF6, receptor interacting protein, and NEMO signaling axis triggered by TLR3 stimulation or virus infection (12, 13, 24, 33, 34).

We have demonstrated the important role of the C-terminal zinc finger domain of A20 for inhibition of IRF-3 activation induced by TLR3 stimulation or NDV infection (Fig. 3, H and I). The C terminus of A20 interacts with NAK/TBK1 and inhibits NAK/TBK1-induced phosphorylation and subsequent dimerization of IRF-3 (Fig. 3G). In contrast, the N terminus of A20 does not inhibit NAK/TBK1-induced IRF-3 activation, although it also interacts with NAK/TBK1. These results indicate that A20 inhibits NAK/TBK1 from phosphorylating IRF-3, and that this inhibition cannot simply be explained by the binding of A20 or A20 mutant to NAK/TBK1. Beyaert and colleagues (33, 35) reported that the C terminus of A20 was also required for the inhibition of NF-\(\kappa\)B activation, and that the zinc finger motifs of A20 compensate the function each other. However, the functional consequences of the A20 C terminus remain to be clarified, and further study will be required to define the molecular mechanism responsible for the inhibition of IRF-3 activation by A20.

Recently, several proteins that contain A20-like functional domain were reported. Cezanne and Tribad share deubiquitinase and zinc finger domains with A20, and Cezanne inhibits TNF-\(\alpha\)-induced NF-\(\kappa\)B activation (36). ZNF216 is another A20-like protein that has zinc finger domain and inhibits TNF-\(\alpha\)-, IL-1\(\beta\)-, and TLR4-induced NF-\(\kappa\)B activation via interaction with NEMO (37). It would be interesting to examine whether these A20-like molecules also regulate IRF-3 activation.
A2O NEGATIVELY REGULATES IRF-3 SIGNALING

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