Activation of the interferon regulatory factors (IRFs) 3 and 7 transcription factors is essential for the induction of type I interferon (IFN) and development of the innate antiviral response. Retinoic acid-inducible gene I has been shown to contribute to virus-induced IFN production independent of the Toll-like receptor pathways in response to a variety of RNA viruses and double-stranded RNA. In the present study, we demonstrate that the NF-κB-inducible, anti-apoptotic protein A20 robustly blocks RIG-I-mediated activation of NF-κB, IRF-3, and IRF-7-dependent promoters but only weakly interferes with TRIF-TLR3-mediated IFN activation. Expression of A20 completely blocked CARD domain containing D-DEDD/ERICH D box RNA helicase domain and two caspase recruitment domains (CARDs); full-length RIG-I can interact with TRIF/TICAM1, leading to TBK1 and IKKβ phosphorylation, which promotes dimerization, cytoplasmic to nuclear translocation, DNA binding, association with CBP/p300 histone acetyltransferases, and transactivation of downstream early genes such as IFNB, IFNA1, and RANTES (regulated on activation normal T cell expressed and secreted). In contrast, IRF-7 protein is synthesized de novo upon IFN stimulation and contributes to the expression of delayed-type genes, including other IFNA subtypes. With IFIR-3, virus infection induces C-terminal phosphorylation and activation of IRF-7 (15, 16). The IKK-related kinases, IKKα (18) and TBK1 (19–21), were shown to be essential signaling components required for IRF-3 and IRF-7 phosphorylation (22–24).

Upon recognition of specific molecular components of viruses or other pathogens, the host cell activates multiple signaling cascades through Toll-like receptor-dependent and -independent pathways, culminating in the production of cytokines and chemokines that disrupt virus replication and initiate innate and adaptive immune responses (1–3). Rapid induction of type I interferon (IFN) expression is a central event in establishing the innate antiviral response and requires pathogen-inducible, activation of transcription factors that function in a synergistic fashion to induce gene expression (reviewed in Refs. 4–9). Among the members of the interferon regulatory factor (IRF) family, IRF-3 and IRF-7 play essential roles in the virus-induced type I IFN gene activation following virus infection (10–17). IRF-3 is activated by C-terminal phosphorylation, which promotes dimerization, cytoplasmic to nuclear translocation, DNA binding, association with CBP/p300 histone acetyltransferases, and transactivation of downstream early genes such as IFNB, IFNA1, and RANTES (regulated on activation normal T cell expressed and secreted). In contrast, IRF-7 protein is synthesized de novo upon IFN stimulation and contributes to the expression of delayed-type genes, including other IFNA subtypes. With IFIR-3, virus infection induces C-terminal phosphorylation and activation of IRF-7 (15, 16). The IKK-related kinases, IKKα (18) and TBK1 (19–21), were shown to be essential signaling components required for IRF-3 and IRF-7 phosphorylation (22–24).

Among the eleven members of the human Toll-like receptor (TLR) family, TLR3, TLR4, TLR7, TLR8, and TLR9 are involved in the initial sensing of viral components. In mice, viral single- and double-stranded RNA, fusion protein of respiratory syncytial virus, single-stranded RNA, and genomic DNA from herpes and cytomegalovirus are recognized by TLR3, TLR4, TLR7, and TLR9, respectively (25–30). Although MyD88 is commonly used by all TLRs, other adapter proteins, including MAL/TIRAP, TRIF/TICAM1, and TRAM/TICAM2, are involved in MyD88-independent pathways (31, 32). TLR3 and TLR4 engage the adapter TRIF/TICAM1, leading to TBK1 and IKKα activation, which in turn activates IRF3 and IFNA/B transcription (33–35).

A separate signaling pathway utilizes the retinoic acid-inducible gene I (RIG-I) to recognize a variety of RNA viruses and trigger the innate antiviral response, independent of the TLR-dependent pathways. RIG-I contains a DEXX(D/H) box RNA helicase domain and two caspase recruitment domains (CARDs); full-length RIG-I can interact with dsDNA through its DEX(D/H) domain with C terminus and augment IFN production in response to viral infection in an ATnPase-dependent manner.

The abbreviations used are: IFN, interferon; IRF, interferon regulatory factor; TLR, Toll-like receptor; RIG-I, retinoic acid-inducible gene I; CARD, caspase recruitment domain; HEK293, human embryonic kidney 293 cells; EMSA, electrophoretic mobility shift assay; ISRE, interferon-stimulated response element; VSV, vesicular stomatitis virus; OTU, ovarian tumor; RLL1, Renilla luciferase activity units; HCV, hepatitis C virus; ZNF, zinc finger domain; aa, amino acid(s); STAT, signal transducers and activators of transcription; IKK, IKB kinase; TBK1; TANK-binding kinase 1; TRIF, TIR domain-containing adaptor-inducing interferon β.

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As a reporter gene activity, the cells were transfected with a luciferase reporter plasmid in the presence of A20 and the reporter gene activities were measured by Dual-Luciferase Reporter Assay, according to manufacturer’s instructions (Promega). Where indicated, cells were treated with Sendai virus (40 hemagglutination units/ml) for the indicated time or 15 h for luciferase assays.

Generation of RIG-I, A20, and NS5/4A-expressing Cell Lines—Plasmids pCDNA3neo, Myc-RIG-I, and Myc-ΔRIG-I were introduced into HEK293 cells by the calcium phosphate method. Cells were selected beginning at 48 h for ~3 weeks in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated calf serum, glutamine, antibiotics, and 100 μg/ml Zeocin (Invitrogen). To generate Myc-ΔRIG-I/Flag A20 and Myc-ΔRIG-I/Flag NS5/4A cell lines, the Myc-ΔRIG-I Puro expression plasmid was co-transfected with Flag A20 pcDNA3.1neo or Flag NS5/4A pcDNA3.1zeo plasmid into HEK293 cells by the calcium phosphate method. Cells were selected beginning at 48 h for ~2 weeks in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated calf serum, glutamine, antibiotics, and 2 μg/ml puromycin (Sigma).

Antibody Preparation—RIG-I (1–228) was expressed in Escherichia coli as a glutathione S-transferase fusion protein and purified by glutathione-Sepharose column chromatography. The recombinant proteins were injected into rabbits to produce antisera against RIG-I (1–228).

Co-immunoprecipitation and Western Blot Analysis—Transient transfection, co-immunoprecipitation, and Western blot analysis were performed as previously described (10).

Analysis of IRF-3 Dimerization by Native PAGE—Whole cell extracts were prepared in Nonidet P-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 30 mM NaF, 5 mM EDTA, 10% glycerol, 1 mM Na3VO4, 40 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of each leupeptin, pepstatin, and aprotinin, and 1% Nonidet P-40), and then were subjected to electrophoresis on 7.5% native acrylamide gels, which were pre-run for 30 min at 4 °C. The electrophoresis buffers were composed of an upper chamber buffer (25 mM Tris, pH 8.4, 192 mM glycine, and 1% sodium deoxycholate) and a lower chamber buffer (25 mM Tris, pH 8.4, 192 mM glycine). Gels were soaked in SDS running buffer (25 mM Tris, pH 8.4, 250 mM glycine, 0.1% SDS) for 30 min at 25 °C and were then electrophoretically transferred on Hybond-C nitrocellulose membranes (Amersham Biosciences) in 25 mM Tris, pH 8.4, 192 mM glycine, and 20% methanol for 1 h at 4 °C. Membranes were blocked in phosphate-buffered saline containing 5% nonfat dry milk and 0.05% Tween 20 for 1 h at 25 °C and then were blotted with an antibody against IRF3 (1 μg/ml) in blocking solution for 1 h at 25 °C. After washing the membranes five times in phosphate-buffered saline/0.05% Tween, they were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) in blocking solution. Immunoreactive bands were visualized by enhanced chemiluminescence (Amer- sham Biosciences).

Electrophoretic Mobility Shift Assay—Briefly, cell pellets were treated with 10 mM HEPES, 50 mM NaCl, 10 mM EDTA, 5 mM MgCl2, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (10 μg/ml), and 1 mM Na3VO4. Suspension was held on ice for 30 min and brought to 0.1% Nonidet P-40 and 10% glycerol concentration. Samples were spun for 5 min at 5,000 rpm at 4 °C. Supernatant was removed, and the pellet was washed in 50 mM NaCl. Nuclear extracts were obtained in a 10 mM HEPES, 400 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), pepstatin (10 μg/ml), aprotinin (10 μg/ml), and 1 mM Na3VO4 solution. Samples were left to rotate at 4 °C for 30 min and spun at 15,000 rpm for 10 min at 4 °C. Whole cell extracts were assayed for IRF-3 binding in gel shift analysis using a 32P-labeled double-stranded oligonucleotide corresponding to
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A20 Disrupts RIG-I Signaling—RIG-I signaling pathway leads to the activation of NF-κB and IRF transcription factors, which are essential for the activation of IFNB promoter (36), and A20 is a potent inhibitor of TLR3- and Sendai virus-induced activation of ISRE promoter (42). To determine the ability of A20 to inhibit RIG-I-mediated activation of IFNB gene transcription, a constitutively active form of RIG-I (ΔRIG-I) and A20-expressing plasmids were co-transfected into HEK293 cells together with an IFNB promoter construct and examined for their ability to activate IFNB reporter gene activity. The IFNB promoter had a low basal activity that was not affected by A20 expression (Fig. 1A). The expression of ΔRIG-I alone resulted in a 275-fold stimulation of IFNB promoter activity, and co-expression of A20 totally inhibited ΔRIG-I-mediated activation of IFNB promoter activity (Fig. 1A). An ISRE reporter construct together with ΔRIG-I and A20-expressing plasmids further demonstrated that RIG-I mediated activation of IRF-3 was blocked by A20. As shown in Fig. 1B, the expression of constitutively active form of RIG-I stimulated the ISRE luciferase reporter gene activity up to 1700-fold, whereas co-expression of A20 with ΔRIG-I almost completely blocked ISRE luciferase reporter gene activity (Fig. 1B). A20 also blocked RIG-I-mediated activation of IRF-7 and the IFNA4 luciferase reporter gene. Expression of ΔRIG-I activated IRF-7 and further enhanced IRF-7-mediated IFNA4 promoter activity 24-fold, whereas co-expression of A20 completely blocked IFNA4 luciferase reporter gene activity (Fig. 1C). Similarly, A20 blocked RIG-I-mediated NF-κB activation (Fig. 1D); the p2 (2)-TK luciferase reporter (two copies of NF-κB binding site from human IFNB promoter linked to minimal TK promoter) was induced 15-fold by ΔRIG-I, an induction that was completely blocked by A20 co-expression. These experiments indicate that A20 is a strong inhibitor of RIG-I signaling to IRF-3 and NF-κB.

A20 Blocks RIG-I-Mediated Transactivation but Only Partially Inhibits TRIF- or IKKe/TBK1-Mediated Transactivation—A20 targets the TLR-3 adapter TRIF and inhibits TLR-3- and Sendai virus-induced activation of ISRE and IFNB promoter (42). In addition, Saitoh and colleagues (41) showed that A20 physically interacted with IKKe/TBK1 and inhibited TLR-3- and Newcastle disease virus-mediated activation of IRF-3. To further investigate the level at which A20 inhibited ISRE activation, a dose-response curve was performed with increasing amounts of A20 and RIG-I, TRIF, IKKe, or TBK1 expression plasmids (Fig. 2). ΔRIG-I (200 ng) resulted in 1500-fold induction of the ISRE promoter, whereas A20 expression plasmid (40 ng) inhibited ΔRIG-I-promoter activity, and co-expression of A20 completely blocked ISRE luciferase reporter gene activity (Fig. 1). An ISRE reporter construct together with ΔRIG-I and A20-expressing plasmids further demonstrated that RIG-I mediated activation of IRF-3 was blocked by A20. As shown in Fig. 1B, the expression of constitutively active form of RIG-I stimulated the ISRE luciferase reporter gene activity up to 1700-fold, whereas co-expression of A20 with ΔRIG-I almost completely blocked ISRE luciferase reporter gene activity (Fig. 1B). A20 also blocked RIG-I-mediated activation of IRF-7 and the IFNA4 luciferase reporter gene. Expression of ΔRIG-I activated IRF-7 and further enhanced IRF-7-mediated IFNA4 promoter activity 24-fold, whereas co-expression of A20 completely blocked IFNA4 luciferase reporter gene activity (Fig. 1C). Similarly, A20 blocked RIG-I-mediated NF-κB activation (Fig. 1D); the p2 (2)-TK luciferase reporter (two copies of NF-κB binding site from human IFNB promoter linked to minimal TK promoter) was induced 15-fold by ΔRIG-I, an induction that was completely blocked by A20 co-expression. These experiments indicate that A20 is a strong inhibitor of RIG-I signaling to IRF-3 and NF-κB.
mediated activation more than 15-fold; increasing the A20 concentration essentially reduced the ΔRIG-I activation to background levels (Fig. 2A). In contrast, when TRIF, IKKε, or TBK1 signaling components were used to activate ISRE promoter activity (2800-, 750-, and 850-fold, respectively), the inhibitory effect of A20 was significantly weaker, with only 2- to 3-fold inhibition observed at the highest concentrations of transfected A20 used (Fig. 2B–D). This result argues that RIG-I signaling is the primary target for A20 inhibition, that TRIF-TLR-3 signaling is not significantly affected, and that the inhibitory effect occurs upstream of the IKKε or TBK1 kinases.

Silencing of A20 Expression Enhances Virus-mediated Activation of ISRE Promoter—Next, to determine whether interference with endogenous A20 expression would modulate ISRE promoter activity, HEK293 cells were transfected with a small interference RNA expression construct directed against A20 (44) and subsequently infected with Sendai virus. Expression of A20 was induced by virus and effectively blocked by A20 specific small interference RNA but not a scrambled small interference RNA (Fig. 3A, lanes 2 and 3). Inhibition of A20 expression correlated with enhanced ISRE-dependent transcription induced by Sendai virus infection (Fig. 3B), thus demonstrating that endogenous A20 is involved in the regulation of ISRE-dependent promoter activity.

A20 Inhibits RIG-I-mediated IRF-3 Activation—Latent IRF-3 is activated in response to virus infection by phosphorylation events that target a cluster of Ser/Thr residues at the C-terminal end of the protein (23). Ser-396 within the C-terminal Ser/Thr cluster is targeted in vivo for phosphorylation following virus infection and plays an essential role in IRF-3 activation (23, 35). Therefore, the phosphorylation state of IRF-3 following ΔRIG-I expression was evaluated by immunoblot using the phosphospecific Ser-396 antibody. ΔRIG-I induced the accumulation of the Ser-396 phosphorylation (Fig. 4A, lane 3), and ΔRIG-I-induced Ser-396 phosphorylation was completely block by A20 (Fig. 4A, lane 4). Expression of TRIF or TBK1 similarly induced Ser-396 phosphorylation (Fig. 4A, lanes 5 and 7); however, A20 only partially reduced the phosphospecific Ser-396 antibody. ΔRIG-I induced the accumulation of the Ser-396 phosphorylation (Fig. 4A, lane 3), and ΔRIG-I-induced Ser-396 phosphorylation was completely block by A20 (Fig. 4A, lane 4). Expression of TRIF or TBK1 similarly induced Ser-396 phosphorylation (Fig. 4A, lanes 5 and 7); however, A20 only partially reduced
TRIF- or TBK1-induced Ser-396 phosphorylation (Fig. 4A, lanes 6 and 8). Complementing the phosphorylation status, the ability of A20 to inhibit ΔRIG-I-induced IRF-3 dimerization was evaluated using native SDS-PAGE and immunoblot with anti-IRF-3 antibody. ΔRIG-I-induced dimerization of endogenous IRF-3 (Fig. 4B, lane 3) was completely abolished with A20 co-expression (Fig. 4B, lane 4). Again, A20 only partially reduced TRIF- or TBK1-induced IRF-3 dimer formation (Fig. 4B, lanes 5–8). Furthermore, EMSA analysis demonstrated that ΔRIG-I, TRIF, or TBK1 expression induced the formation of an IRF-3 protein-DNA complex (Fig. 4C, lanes 3, 5, and 7) that was supershifted with antibody to IRF-3 (Fig. 4C, lane 8). A20 co-expression completely blocked ΔRIG-I-induced IRF-3 protein-DNA complex formation (Fig. 4C, lane 4) but only partially reduced TRIF- or TBK1-mediated IRF-3 protein-DNA complex formation (Fig. 4C, lanes 5, 6, 9, and 10), strongly arguing that A20 specifically targets the RIG-I pathway upstream of TBK1 and does not significantly affect the TRIF-TLR3 pathway.

C-terminal Zinc Finger Domain of A20 Is Both Necessary and Sufficient to Block RIG-I- and Virus-mediated Activation of ISRE Promoter—Wertz et al. (47) demonstrated that A20 down-regulated NF-κB signaling through the cooperative activity of its two ubiquitin-editing domains. To determine which region of A20 was responsible for the inhibition of virus- and RIG-I-mediated signaling, a series of A20 deletions and point mutations were generated to test the inhibitory potential of each mutant on RIG-I induction; all modified proteins were expressed equivalently (Fig. 5). ΔRIG-I activated the ISRE reporter 1200-fold, and expression of both full-length A20 and the C-terminal domain of A20 (aa373–790) inhibited ΔRIG-I transactivation more than 500-fold. Conversely, truncation of the C terminus of A20 (aa1–380) only weakly inhibited transactivation of the ISRE promoter by ΔRIG-I (<3-fold), indicating that the C-terminal ubiquitin ligase domain is important for A20-mediated inhibition.

To assess the role of the seven zinc finger motifs in inhibiting ΔRIG-I-activation, a series of A20 zinc finger deletions and point mutations were examined. Deletion of one, two, or three internal zinc finger domains had essentially no effect on the inhibitory activity of A20; ΔZNF4, ΔZNF4/5, ΔZNF2/3, and ΔZNF2–4 were still able to inhibit RIG-I-mediated transactivation from 100– to 250-fold (Fig. 5B). In contrast, deletion of the one, two, or three C-terminal zinc finger domains (A20(aa1–740), A20(aa1–680), and A20(aa1–517)) reduced the capacity of A20 to inhibit ΔRIG-I-mediated transactivation activity, resulting in inhibition of only 4.2-, 2.9-, and 2.8-fold, respectively.

The deletion analysis indicated that zinc finger domain 7 (ZNF7) was critical to A20-mediated inhibition. To test this idea, full-length A20 with point mutations of conserved cysteines within ZNF4 (C624/627A)
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To further evaluate the antiviral state, the stable HEK293 cell lines were infected with VSV, and viral protein expression was measured at different times after infection. In control or RIG-I-expressing cells, VSV replication was similarly reduced (Fig. 7, lane 3), whereas in virus-infected A20-expressing cells, VSV replication was completely blocked (Fig. 7, lane 4). As a positive control, HEK293 cells were transfected with pRLTK control plasmid (100 ng), ISRE-Luc reporter plasmid (200 ng), ΔRIG-I (Δ), TRIF (B), IKKe (C), or TBK1 (D) expressing plasmid (200 ng) together with an increase amount of A20 expression plasmid (0, 40, 200, and 1000 ng) as indicated. In all of transfection, the pcDNA3 vector was added to bring the total plasmids to 1500 ng. Whole cell extracts were resolved by SDS-PAGE and analyzed by Western blot with anti-FLAG antibody to assess the level of transgene expression.

FIGURE 7. A20 does not significantly reduce the level of RIG-I protein. HEK293 cells were transfected with pRLTK control plasmid (100 ng), ISRE-Luc reporter plasmid (200 ng), ΔRIG-I (ΔI), TRIF (B), IKKe (C), or TBK1 (D) expressing plasmid (200 ng) together with an increase amount of A20 expression plasmid (0, 40, 200, and 1000 ng) as indicated. In all of transfection, the pcDNA3 vector was added to bring the total plasmids to 1500 ng. Whole cell extracts were resolved by SDS-PAGE and analyzed by Western blot with anti-FLAG antibody to assess the level of transgene expression.

A20 Repression of the ΔRIG-I-Induced Antiviral State—To determine whether the A20 could repress the ΔRIG-I-stimulated expression of endogenous IFN and ISG genes, HEK293 cells that stably express RIG-I, ΔRIG-I, ΔRIG-I and A20 and ΔRIG-I and HCV NS3/4A were generated (Fig. 8). The polyclonal FLAG-A20 and control HEK293 cells were infected with Sendai virus or treated with IFNα2 to examine endogenous ISG56 and RIG-I protein expression. As shown in Fig. 8A, ISG56 and RIG-I were highly expressed in virus-infected control HEK293 cells (Fig. 8A, lane 2), whereas in virus-infected A20-expressing cells, ISG56 and RIG-I were inhibited (Fig. 8A, lane 3). These proteins were not detected in uninfected cells (Fig. 8A, lanes 1 and 4), and as a relative measure of specificity, actin expression was not altered by A20. Importantly, IFNα2-mediated induction of ISG56 and RIG-I was not inhibited by A20 expression, indicating the specificity of A20 inhibition for the RIG-I but not the Jak-STAT pathway (Fig. 8A, lanes 2 and 5). The expression of constitutively active form of RIG-I strongly induced ISG56 and RIG-I protein expression in the absence of virus infection or IFN treatment (Fig. 8B, lanes 2 and 3), whereas co-expression of A20 or HCV protease NS3/4A completely blocked the ΔRIG-I-mediated activation of ISG56 and RIG-I gene expression.
were detected at 8 h post-infection, whereas in ΔRIG-I-expressing cells, VSV replication was significantly delayed with viral proteins detected only at a low level beginning at 12 h post-infection (Fig. 9A). Interestingly, in cells that expressed either A20 or NS3/4A, a restoration of the kinetics of VSV expression was observed, with viral proteins again detectable as early as 8 h post-infection (Fig. 9B). Taken together, these results demonstrate that A20 can efficiently block the RIG-I-mediated signaling pathway and down-regulate cellular antiviral response. Although the target of A20 remains unknown, the similarity between A20-mediated inhibition and HCV NS3/4A inhibition suggests that the cellular A20 and the viral NS3/4A may be targeting related components or adapters of the RIG-I pathway.

**A20 Inhibits MAVS/VISA/IPS-1/CARDIF-mediated Activation**—Recently, the adaptor molecule that links RIG-I sensing of incoming viral RNA and downstream activation events was elucidated by four independent groups (48–51). Under the name of Cardif, this protein was demonstrated that the N-terminal de-ubiquitination domain had no effect on MAVS/VISA/IPS-1/CARDIF protein. MAVS/VISA/IPS-1/CARDIF from the insoluble fraction to the soluble fraction and directly targeted MAVS/VISA/IPS-1/CARDIF for proteolytic cleavage (Fig. 10B, lanes 1–4), whereas the expression of increasing amounts of the cellular protein A20 did not alter the subcellular localization or stability of MAVS/VISA/IPS-1/CARDIF (Fig. 10B, lanes 5–7). These results indicated that A20 does not directly target MAVS/VISA/IPS-1/CARDIF protein.

**DISCUSSION**

The results of the present study demonstrate that cellular NF-κB-induced, anti-apoptotic protein A20 efficiently blocks RIG-I-mediated signaling to the IRF and NF-κB pathways. Furthermore, expression of A20 completely blocks ΔRIG-I-induced IRF-3 Ser-396 phosphorylation, dimerization, and DNA binding. Mutational analysis of A20 demonstrated that the N-terminal de-ubiquitination domain had no effect on the inhibitory activity of A20, whereas the deletion of the C-terminal ubiquitin ligase domain almost completely ablated the inhibitory function of A20, an effect that was localized to the distal most zinc finger motifs. Finally, cells stably expressing the active form of RIG-I induced an antiviral state that delayed replication of VSV, an effect that was reversed by stable co-expression of A20 or the HCV-encoded NS3/4A protease. These results demonstrate that virus-mediated activation of A20 functions as a negative regulator of RIG-I-mediated induction of the antiviral state.

**A20 was originally characterized as a TNF-inducible gene in human umbilical vein endothelial cells (52). As an NF-κB target gene (53), A20 is also induced in many other cell types by a wide range of stimuli, including virus infection. Overexpression of A20 has been shown to protect from TNF-α-induced apoptosis and functions via a negative-feedback loop to block NF-κB activation induced by TNF and other stimuli (54). A20-deficient mice (Tnfaip3<sup>−/−</sup>) were generated, and these mice developed severe multiorgan inflammation and were extremely susceptible to TNF due to the enhanced sensitivity to TNF-induced apoptosis (55). A20-deficient fibroblasts displayed prolonged NF-κB activity and were unable to properly terminate NF-κB activation; A20 was also essential for the termination of TLR-induced NF-κB activation macrophages (56). The inhibition of both IRF- and NF-κB-dependent pathways suggests that A20 functions in the physiological context as a negative feedback regulator of immune
response signaling. It will be of interest to determine the response of A20-deficient mice to pathogenic viruses.

It has been reported that A20 inhibits NF-κB signaling through the cooperative activity of its two ubiquitin-editing domains: the N-terminal ovarian tumor (OTU) domain mediates de-ubiquitinating activity, and the C-terminal zinc finger region functions as ubiquitin ligase (47). Our results indicate that the C-terminal ubiquitin ligase domain of A20 is necessary and sufficient to block RIG-I-mediated signaling. Substitution of the indispensable cysteine residue in the catalytic OTU domain with alanine (A103) had essentially no effect on A20-mediated inhibition of RIG-I signaling (Fig. 5). More importantly, expression of the C-terminal ubiquitin ligase domain of A20 reduced activation of ISRE promoter more than 100-fold (Fig. 5). The C-terminal ubiquitin ligase domain of A20 contains the type C ubiquitin ligase domain of A20 consists of seven novel zinc finger motifs of the type Cx2-Cx3-Cx2-Cx3-Cx2-Cx3 (57). Klinkenberg and colleagues (58) reported that the zinc finger motifs of murine A20 are functionally redundant, and A20 mutants containing a minimum of four zinc finger motifs are sufficient to inhibit TNF-induced NF-κB activation to a level comparable to that obtained with the wild-type A20 protein. No strict requirement for a particular zinc finger structure was observed, because A20 mutants containing either the first four or last four zinc finger motifs had full inhibitory activity (58). In contrast, Natoli et al. (59) demonstrated that the last zinc finger of A20 was absolutely required for inhibition of TNF-induced NF-κB activation. Here we show that deletion or mutation of zinc finger motif 7 reduced the inhibitory potential of human A20 more than 70-fold on ΔRIG-I-induced ISRE activation, whereas deletion of internal zinc finger motifs 2 through 4 reduced the inhibitory potential of human A20 only 2- to 4-fold. These results demonstrate that the last zinc finger motifs of human A20 are absolutely required for inhibition of RIG-I-induced ISRE activation.

A20 was shown recently to be involved in negative regulation of TLR-3- and Sendai virus-mediated activation of IRF-3 (42). Wang and colleagues reported that A20 interacted with TRIF and inhibited TRIF-mediated activation of ISRE and IFNβ promoter (42). Saitoh and colleagues (41) demonstrated that A20 physically interacted with TBK1 and IKKe and inhibited TLR-3- and Newcastle disease virus-mediated IRF-3 activation. The present studies confirm that A20-mediated inhibition of ISRE promoter activity correlated with the inhibition of IRF-3 activation; A20 completely blocked the ΔRIG-I-induced IRF-3 phosphorylation, dimerization, and protein-DNA complex formation. However, A20 only minimally reduced TRIF- and TBK1-mediated IRF-3 activation and did not inhibit TBK1- or IKKe-induced gene activation (Fig. 3). The fact that A20 had no effect on the stability of either RIG-I itself or on TBK1/IKKe suggested that the biological target of A20 may be an as yet unidentified adapter molecule that links sensing of virus infection by RIG-I with the downstream kinase activation. In support of this concept, recent studies demonstrated that hepatitis C virus (HCV) gene product NS3/4A protease strongly inhibited virus- and RIG-I-mediated activation of NF-κB and IRF-3 (37, 39), but NS3/4A only weakly inhibited TRIF-mediated induction of NF-κB and IRF-3 (37). Paradoxically, TRIF was identified as a proteolytic substrate of NS3/4A (46) despite the fact that the TRIF pathway does not appear to be a major pathway for IFN response to HCV; in vitro, RIG-I was not a proteolytic substrate for NS3/4A, and expression of NS3/4A did not alter the stability of RIG-I, again in contrast to the strong inhibition of the RIG-I pathway by NS3/4A (37, 39). Recently, Meylan et al. (51) has shown that MAVS/VISA/IPS-1/CARDIF is a direct target by NS3/4A. Although A20 strongly inhibited RIG-I- and MAVS/VISA/IPS-1/CARDIF-mediated transactivation of IRF and NF-κB pathways, no strong association between RIG-I and A20 or MAVS/VISA/IPS-1/CARDIF and A20 was detected by immunoprecipitation; A20 also only weakly inhibited TRIF-mediated induction of NF-κB and IRF-3 (37). Our results indicate that the C-terminal ubiquitin ligase domain of A20 is necessary and sufficient to block RIG-I-mediated signaling. Substitution of the indispensable cysteine residue in the catalytic OTU domain with alanine (A103) had essentially no effect on A20-mediated inhibition of RIG-I signaling (Fig. 5).

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