FEBS Letters 576 (2004) 86–90 FEBS 28819

A20 is a potent inhibitor of TLR3- and Sendai virus-induced activation of NF-κB and ISRE and IFN-β promoter

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Received 20 June 2004; revised 18 August 2004; accepted 20 August 2004

Available online 15 September 2004

Edited by Frances Shannon

Abstract Toll-like receptor 3 (TLR3) recognizes dsRNA generated during viral infection and activation of TLR3 results in induction of type I interferons (IFNs) and cellular anti-viral response. TLR3 is associated with a TIR domain-containing adapter protein TRIF, which activates distinct downstream pathways leading to activation of NF-κB and ISRE sites in the promoters of type I IFNs. We show here that A20, a NF-κB-inducible zinc finger protein that has been demonstrated to be an inhibitor of TNF-induced NF-κB activation and a physiological suppressor of inflammatory response, potently inhibited TLR3- and Sendai virus-mediated activation of ISRE and NF-κB and IFN-β promoter in reporter gene assays. A20 also inhibited TRIF-, but not its downstream signaling components TBK1-, IKKβ-, and IKKε-mediated activation of ISRE and NF-κB and IFN-β promoter. Moreover, A20 interacted with TRIF in co-immunoprecipitation experiments. Finally, expression of A20 could be induced at protein level by Sendai virus infection. These data suggest that A20 targets TRIF to inhibit TLR3-mediated induction of IFN-β transcription and functions as a feedback negative regulator for TLR3 signaling and cellular anti-viral response.

Keywords: Toll-like receptor 3; IFN-β; Interferon-stimulated response element; NF-κB; A20

1. Introduction

Recognition of specific patterns of microbial components by Toll-like receptors (TLRs) in the body leads to activation of innate immunity against pathogens [1–3]. TLR3 recognizes dsRNA generated during viral infection and activation of TLR3 results in induction of type I interferons (IFNs), including IFN-β and IFN-α family cytokines, which are crucial mediators of the cellular anti-viral response.

Transcriptional activation of the promoters of type I IFNs requires coordinated activation of multiple transcriptional factors and their cooperative assembly into a transcriptional enhancer complex in vivo. For example, the enhancer of human IFN-β gene contains a κB site recognized by NF-κB, a site for ATF-2/c-Jun, and two ISRE (interferon-stimulated response element) sites recognized by IRF-3 and/or IRF-7 [4,5]. It has been shown that transcriptional activation of IFN-β gene requires coordinated and cooperative assembly of an enhancersome that contains all of these transcriptional factors [4,5].

Recently, major progress has been achieved on the mechanisms of TLR3-mediated IFN-β transcriptional induction. It has been shown that TRIF, a TIR domain-containing adapter protein, is responsible for transducing TLR3 signals to downstream effectors [6–9]. TRIF activates NF-κB through a RIP-TRAF6-IKKβ dependent pathway [6–8]. TRIF activates ISRE through TBK1-, IKKβ- and IKKε-mediated phosphorylation of IRF-3 and/or IRF-7 [6,8]. In addition, it has also been shown that TRIF can induce apoptosis through a RIP-FADD-caspase-8 dependent pathway [6].

Negative regulation of TLR3-mediated production of type I IFNs is less understood. In this report, we identified A20 as a potent inhibitor of TLR3- and Sendai virus-induced ISRE activation and IFN-β transcription. A20 was originally identified as a TNF-inducible protein in human endothelial cells [10]. Subsequently, it has been shown that A20 is induced by a variety of proinflammatory reagents through activation of NF-κB [11]. A20 contains seven zinc finger motifs having characteristics of $CX_{2-4}CX_{11}CX_2C$ at its C-terminal domain [10,11]. It has been shown that A20 interacts with TRAF2, RIP, IKKγ and TRAF6, which are signaling components involved in TNF, IL-1 and TLR-mediated NF-κB activation pathways [12–17]. Overexpression of A20 inhibits NF-κB activation triggered by various stimuli, including TNF, IL-1, and LPS [10–19]. A20-deficient cells fail to terminate TNF-induced NF-κB activation [20]. Mice deficient for A20 develop severe inflammation and cachexia, are hypersensitive to both LPS and TNF, and die prematurely [20]. These studies suggest that A20 is a physiological inhibitor of NF-κB activation and inflammatory response.

In this report, we found that A20 interacted with TRIF and potently inhibited TLR3- and Sendai virus-induced activation of ISRE and IFN-β promoter. Our findings suggest that A20 is an inducible protein that blocks both inflammatory and cellular anti-viral responses.

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doi:10.1016/j.febslet.2004.08.071

2. Materials and methods

2.1. Reagents

Recombinant human TNF and IFN-γ (R&D Systems), human embryonic kidney 293 cells and Sendai viruses (ATCC), monoclonal antibodies against Flag and HA epitopes (Sigma), A20 (Imgenex) and β-tubulin (Sigma) were purchased from the indicated companies. The 293 cells expressing TLR3 (293-T3Y) were provided by Drs. Kate Fitzgerald and Tom Maniatis.

2.2. Constructs

ISRE-luciferase reporter construct was purchased from Stratagene (La Jolla, CA). NF-κB-luciferase reporter construct (Dr. Gary Johnson) and mammalian expression plasmid for Flag-IKKβ (Dr. David Goeddel) were provided by the indicated investigators. Human IFN-β promoter luciferase reporter construct, and mammalian expression plasmids for Flag- or HA-tagged TRIF, TBK1 and IKKε were previously described [6]. Mammalian expression plasmid for Flag-tagged A20 and its deletion mutants were constructed by insertion of PCR amplified human A20 or its mutant cDNAs into a pCMV based expression vector containing a N-terminal Flag tag.

2.3. Cell transfection and reporter gene assays

293 cells (1×10^5) were seeded on 12-well dishes and transfected the following day by standard calcium phosphate precipitation. For each transfection, 0.1 μg of the tested reporter plasmid and the indicated amounts of expression plasmids were added. Within the same experiment, each transfection was performed in duplicate. Empty control plasmid was added to ensure that each transfection receives the same amount of total DNA. To normalize for transfection efficiency, 0.1 μg of pTK-RL (*Rellina* Luciferase) or RSV-β-galactosidase plasmid was added to each transfection. Firefly luciferase activities were normalized on the basis of β-galactosidase or *Rellina* luciferase activities.

2.4. Co-immunoprecipitation and Western blot analysis

Transfected 293 cells from each 100-mm dish were lysed in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). For each immunoprecipitation, 0.4 ml aliquot of lysate was incubated with 0.5 μg of the indicated monoclonal antibody and 25 μl of a 1:1 slurry of GammaBind G Plus Sepharose (Amersham Pharmacia, Piscataway, NJ) for 1 h. The Sepharose beads were washed three times with 1 ml lysis buffer containing 500 mM NaCl. The precipitates were fractionated on SDS–PAGE and subsequent Western blot analysis was performed with the indicated antibodies.

2.5. Sendai virus infection

293 cells (~1×10^5) were seeded in 12-well dishes and transfected the next day with 0.1 μg of the indicated reporter plasmid. Fourteen hours after transfection, cells were washed with medium lacking fetal calf serum (washing medium) and 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 washing of the cells. Cells were then cultured in fetal calf serum-containing medium for 6 h before luciferase assays were performed.

3. Results and discussion

3.1. A20 inhibits TLR3- and Sendai virus-induced activation of ISRE and NF-κB and IFN-β promoter

Previously, it has been shown that A20 can inhibit NF-κB activation induced by various stimuli, including TNF, IL-1 and TLR4 [10–19]. We examined whether A20 could also inhibit signaling by TLR3. To do this, we performed reporter gene assays utilizing a TLR3-expressing stable cell line. As shown in Fig. 1A, ectopic expression of A20 potently inhibited ISRE activation triggered by stimulation with the TLR3 ligand Poly(I:C). In similar experiments, A20 also inhibited Poly-(I:C)-induced activation of NF-κB and IFN-β promoter (Fig. 1B and C).

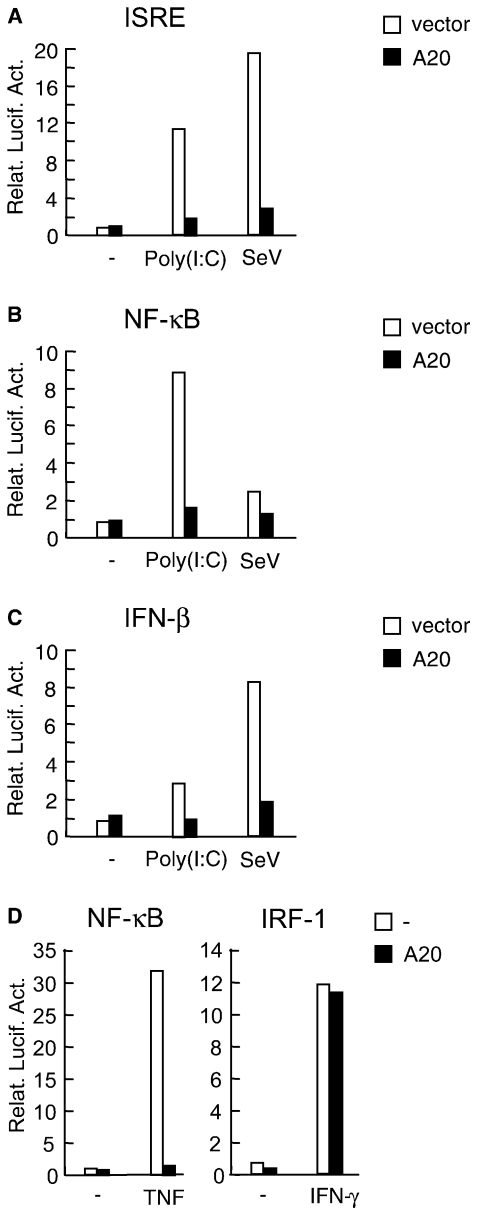

Fig. 1. A20 inhibits Poly(I:C)- and Sendai virus-induced activation of ISRE, NF-κB and IFN-β promoter. (A) A20 inhibits Poly(I:C)- and Sendai virus-induced activation of ISRE. The TLR3-expressing 293-T3Y cells (1×10^5) were transfected with 0.1 μg of ISRE reporter plasmid and 1 μg of an expression plasmid for A20 (filled bars) or an empty control plasmid (open bars). Fourteen hours after transfection, cells were treated with Poly(I:C) (25 μg/ml) or infected with Sendai virus or left untreated for 6 h before reporter assays were performed. (B) A20 inhibits Poly(I:C)- and Sendai virus-induced activation of NF-κB. The experiments were done similarly as in A except that NF-κB-luciferase reporter plasmid was used. (C) A20 inhibits Poly(I:C)- and Sendai virus-induced activation of IFN-β promoter. The experiments were done similarly as in A except that human IFN-β promoter luciferase reporter plasmid was used. (D) A20 inhibits TNF-induced NF-κB activation but not IFN-γ-induced IRF-1 promoter activation. 293 cells (1×10^5) were transfected with 0.1 g of NF-κB or IRF-1 promoter reporter plasmid as indicated and 1 μg of an expression plasmid for A20 (filled bars) or an empty control plasmid (open bars). Fourteen hours after transfection, cells were treated with TNF (20 ng/ml) or IFN-γ (100 ng/ml) or left untreated for 6 h before reporter assays were performed. SeV, Sendai virus.

Since TLR3 is a receptor for dsRNA produced in viral infection, we determined whether A20 could inhibit Sendai virus-induced ISRE and NF-κB activation and IFN-β transcription. In reporter gene assays, A20 potently inhibited Sendai virus-induced activation of ISRE, NF-κB and IFN-β promoter (Fig. 1). In similar experiments, A20 inhibited TNF-induced NF-κB activation, but not IFN-γ-induced activation of the IRF-1 promoter (Fig. 1D).

Taken together, these data suggest that A20 is a potent and specific inhibitor of TLR3-mediated activation of ISRE and NF-κB and IFN-β promoter.

3.2. A20 inhibits TRIF-, but not TBK1-, IKKβ- and IKKε-mediated activation of NF-κB, ISRE and IFN-β promoter

It has been shown that TLR3 is associated with the TIR-domain containing adapter protein TRIF, which in turn activates ISRE and NF-κB through distinct downstream pathways [6,8,21–23]. To determine which component in TLR3 signaling is targeted by A20, we determined whether A20 could inhibit signaling mediated by various components in TLR3 pathways. In reporter gene assays, A20 could potently inhibit TRIF-, but not TBK1-, IKKε- and IKKβ-induced activation of ISRE, NF-κB and IFN-β promoter (Fig. 2). These data suggest that A20 inhibits TLR3 signaling by targeting TRIF or its upstream component(s).

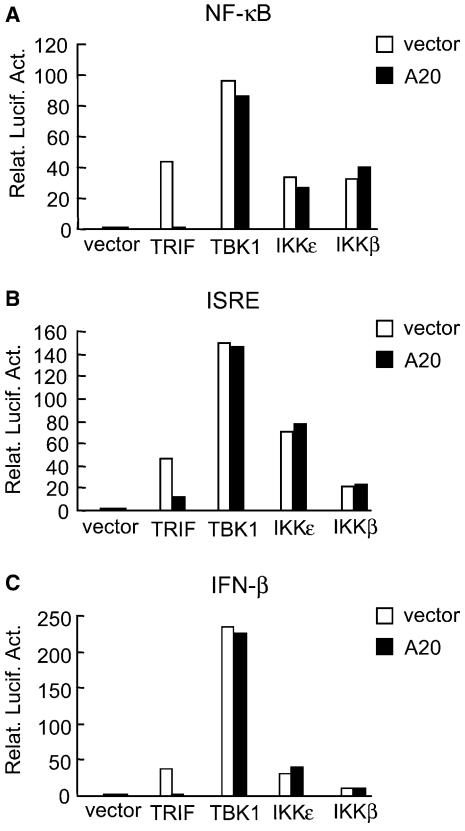

Fig. 2. A20 inhibits TRIF-, but not TBK1-, IKKβ- and IKKε-mediated activation of ISRE, NF-κB and IFN-β promoter. (A) A20 inhibits TRIF-, but not TBK1-, IKKβ- and IKKε-mediated NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg of NF-κB reporter plasmid and 0.5 μg of each of the indicated plasmid. Reporter assays were performed 14 h after transfection. (B) A20 inhibits TRIF-, but not TBK1-, IKKβ- and IKKε-mediated ISRE activation. The experiments were done similarly as in A except that ISRE-luciferase reporter plasmid was used. (C) A20 inhibits TRIF-, but not TBK1-, IKKβ- and IKKε-mediated activation of IFN-β promoter. The experiments were done similarly as in A except that human IFN-β promoter luciferase reporter plasmid was used.

3.3. A20 interacts with TRIF

Since A20 inhibits TLR3 signaling by targeting TRIF or its upstream components, we determined whether A20 could interact with TRIF. In transient transfection and co-immunoprecipitation experiments, A20 could interact with TRIF (Fig. 3A). One possible mechanism responsible for A20-mediated inhibition of TLR3 signaling is that A20 competes with downstream components for interaction with TRIF. To test this, we determined whether A20 could compete with TRAF6 for interaction with TRIF. In co-immunoprecipitation

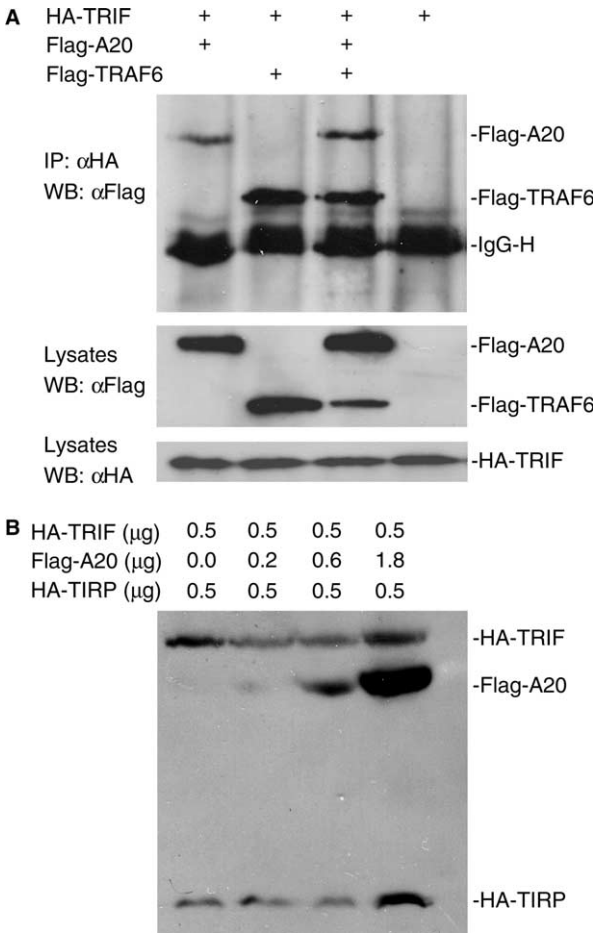

Fig. 3. A20 interacts with TRIF but does not cause downregulation of TRIF protein level. (A) A20 interacts with TRIF. 293 cells ($\sim 2 \times 10^6$) were transfected with 5 μg of each of the indicated expression plasmids. Cell lysates were immunoprecipitated with a monoclonal anti-HA antibody. The immunoprecipitates were analyzed by Western blot with anti-Flag antibody (upper panel). The expression levels of the transfected proteins were detected by Western blot analysis with anti-Flag (middle panel) or anti-HA (low panel) antibody. (B) A20 does not cause downregulation of TRIF protein level. 293 cells ($\sim 2 \times 10^5$) were transfected with 0.5 μg of HA-TRIF plasmid, 0.5 μg of HA-TIRP plasmid, and increased amounts of Flag-A20 plasmid as indicated. Sixteen hours after transfection, cell lysates were analyzed by Western blot with a mixture of anti-Flag and anti-HA antibodies.

experiments, A20 did not significantly compete with TRAF6 for binding to TRIF (Fig. 3A).

3.4. A20 does not downregulate TRIF protein level

We further examined whether A20 caused downregulation of TRIF protein level. We co-transfected 293 cells with constant amounts of TRIF expression plasmid, increased amounts of A20 expression plasmid, and constant amounts of an expression plasmid for a control protein TIRP. Western blot analysis indicated that A20 did not significantly affect the level of TRIF expression (Fig. 3B). These data suggest that A20-mediated inhibition of TRIF signaling is not due to down-regulation of TRIF expression.

3.5. A20's de-ubiquitination activity is not required for its inhibitory effects on Sendai virus-induced ISRE activation

Recently, it has been shown that A20 is a de-ubiquitinating enzyme [23]. However, its de-ubiquitination activity is not required for its inhibition of TNF-induced NF-κB activation [23]. Consistent with the previous observation [23], we found that the C-terminal zinc finger domain of A20, aa370–775, which lacks the N-terminal de-ubiquitinating catalytic domain

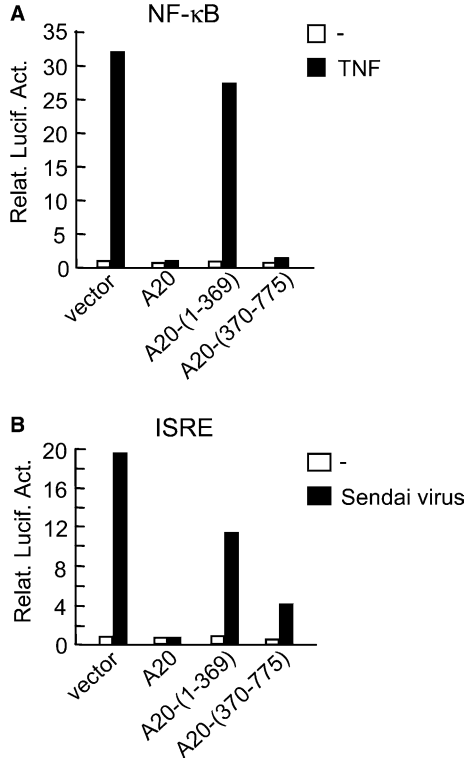

Fig. 4. The de-ubiquitinating activity of A20 is not required for its inhibitory effects on NF-κB and ISRE activation. (A) The de-ubiquitinating activity of A20 is not required for its inhibitory effect on TNF-induced NF-κB activation. 293 cells (1×10^5) were transfected with 0.1 μg of NF-κB reporter plasmid and 1 μg of the indicated expression plasmids. Fourteen hours after transfection, cells were treated with TNF (10 ng/ml) (filled bars) or left untreated (empty bars) for 6 h before reporter assays were performed. (B) The de-ubiquitinating activity of A20 is not required for its inhibitory effect on Sendai virus-induced ISRE activation. 293 cells (1×10^5) were transfected with 0.1 g of ISRE-luciferase reporter plasmid and 1 μg of the indicated expression plasmids. Fourteen hours after transfection, cells were infected with Sendai virus (filled bars) or left uninfected (empty bars) for 6 h before reporter assays were performed.

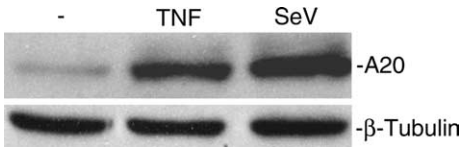

Fig. 5. Induction of A20 expression by Sendai virus infection and TNF stimulation. U937 cells (3×10^5) were treated with TNF (20 ng/ml) or infected with Sendai virus (SeV) or left untreated for 18 h. Cells were then lysed and the lysates were analyzed by Western blot with a monoclonal anti-A20 antibody (upper panel). The same blot was re-probed with a monoclonal anti-β-tubulin antibody (lower panel).

[23], was as effective as the full-length A20 in inhibiting TNF-induced NF-κB activation in reporter gene assays (Fig. 4A). In these assays, the N-terminal domain of A20, aa1-369, had little inhibitory effect on TNF-induced NF-κB activation (Fig. 4A). Similarly, A20-(aa370–775) also significantly inhibited Sendai virus-induced ISRE activation (Fig. 4B). These data suggest that the de-ubiquitinating activity of A20 is not required for its inhibitory effects on NF-κB and ISRE activation by various stimuli.

3.6. Sendai virus infection induces expression of A20 at protein level

Previous studies have shown that A20 is a feedback negative regulator of TNF-induced NF-κB activation and inflammation [10–20]. Since A20 is a potent inhibitor of TLR3- and Sendai virus-induced transcription of IFN-β, we wondered whether A20 could act as a negative feedback regulator of viral infection. Therefore, we examined whether Sendai virus infection could induce expression of A20. As shown in Fig. 5, infection of the monocytic U937 cells with Sendai virus significantly induced A20 at protein level. As a positive control, TNF also upregulated A20 in the same experiment (Fig. 5). Interestingly, both Sendai virus infection and TNF treatment did not upregulate A20 in 293 cells (data not shown). These data suggest that both Sendai virus and TNF can upregulate A20 in a cell-type specific manner.

The exact biochemical mechanisms responsible for A20-mediated inhibition of TLR3 signaling need to be further investigated. Nevertheless, our discovery of A20 as a potent inhibitor of TLR3-induced ISRE and NF-κB activation and IFN-β transcription provides an interesting link between inflammatory response and innate immunity against viruses. Our data point to the following model: viral infection leads to upregulation of A20, which in turn inhibits virus-induced IFN transcription and cellular anti-viral response.

Acknowledgements: This work was supported by grants from the National Natural Science Foundation of China (#39925016 and 90208016), the Chinese High-Technology Program (#2003AA221030), and the Special Funds for Major State Basic Research of China (G19990539).

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