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IL-17 Receptor Adaptor Protein Act1/CIKS Plays an Evolutionarily Conserved Role in Antiviral Signaling

Grigory Ryzhakov,* Katrina Blazek,* Cheryl Chuk-ke Lai,[†] and Irina A. Udalova*

Double-stranded RNA-induced antiviral gene expression in mammalian cells requires activation of IFN regulatory factor 3 (IRF3). In this study, we show that the IL-17R adaptor protein Act1/CIKS is involved in this process. Small interfering RNA-mediated knockdown of Act1 in primary human skin fibroblasts specifically attenuates expression of IFN- β and IFN-stimulated antiviral genes induced by a synthetic viral mimic, polyinosinic-polycytidylic acid. Ectopic expression of Act1 potentiates the IRF3-driven expression of a synthetic reporter construct as well as the induction of antiviral genes. We demonstrate that this effect is dependent on the ability of Act1 to functionally and physically interact with I κ B kinase ϵ (IKK ϵ), a known IRF3 kinase, and IRF3: 1) Act1 binds IKK ϵ and IRF3; 2) Act1-induced IRF3 activation can be blocked specifically by coexpression of a catalytically inactive mutant of IKK ϵ ; and 3) mutants of IRF3, either lacking the C terminus or mutated at the key phosphorylation sites, important for its activation by IKK ϵ , do not support Act1-dependent IRF3 activation. We also show that a zebrafish Act1 protein is able to trigger antiviral gene expression in human cells, which suggests an evolutionarily conserved function of vertebrate Act1 in the host defense against viruses. On the whole, our study demonstrates that Act1 is a component of antiviral signaling. *The Journal of Immunology*, 2012, 189: 4852–4858.

Antiviral immunity relies on early recognition of viral products by the innate immune system (1, 2). Viruses entering cells via the vesicular/endosomal compartment are sensed by TLRs (1, 3); for example, dsRNA is recognized in mammalian cells by TLR3 (4). Alternatively, viruses invading the cytosol can be detected by RNA helicases (RIG-I-like helicase [RLH]) such as RIG-I and MDA-5 (5).

Virus recognition initiates signaling pathways resulting in activation of IFN regulatory factors (IRFs) (6). IRFs are transcription factors that not only control expression of type I IFNs and a number of IFN-stimulated genes (ISG), but also immune cell differentiation (6). IRF3 is predominantly involved in antiviral responses—for instance, the production of ISG in fibroblasts infected with dsDNA viruses HSV1 or vaccinia (7). IFN- β production is reduced in IRF3 deficient fibroblasts infected with HSV-1, vesicular stomatitis virus, or encephalomyocarditis virus (8). In addition, IRF3 is a component of antibacterial defense; for ex-

ample, it is essential for IFN- β expression by lung epithelial cells infected with *Legionella pneumophila*, an intracellular pathogen (9). IRF3 phosphorylation is essential for activation of this transcriptional factor (10, 11), which results in its dimerization, nuclear translocation, DNA binding, and gene induction in cooperation with p300/CBP (12). Viral infection induces phosphorylation of IRF3 by TBK1 and I κ B kinase ϵ (IKK ϵ) kinases (13, 14), with serines 386 and 396 being the primary phospho-acceptor sites, though some other C-terminal serines can also be phosphorylated (10, 13, 15).

There are a growing number of signaling components known to connect virus-activated receptors and IKKs (1, 5, 16–18). IKK adaptors associate with the TLR3 adaptor Toll/IL-1R domain-containing adapter inducing IFN- β (TRIF) (19), RLH adaptor MAVS (20), and also with a ubiquitin ligase TNFR-associated factor (TRAF) 3 (21).

SEF and IL-17R (SEFIR) domain containing protein Act1/CIKS has been identified by its similarity with TLR adaptors (22), and it is crucially involved in inflammatory processes (23–25). Furthermore, it has been linked to pathology: as a contributing factor in T cell-mediated autoimmunity [e.g., collagen-induced arthritis (26)] or as a protective factor in B cell-mediated diseases such as Sjögren syndrome (27).

The complex physiological role of Act1 is reflected in its versatile molecular functions (28). Act1 transmits signals downstream of IL-17 and IL-25Rs in many cell types (23, 25, 29), mediates angiotensin II-triggered effects (30), and also negatively regulates CD40 and BAFF receptor signaling in B cells (24, 28). Act1 seems to be dispensable for IL-1- and LPS-induced gene expression (29), but its role in other cytokine and TLR signaling pathways, including antiviral signaling, has not yet been addressed.

The C-terminal SEFIR domain of Act1 is important for its homotypic interactions with IL-17R (29), activating downstream signaling (31). In addition to the SEFIR domain, Act1 also contains a TRAF6 binding site at the N terminus, a helix-loop-helix (HLH) region (32), and a Ufd2-box (U-box) domain in the center of the protein (28, 31). This modular domain structure enables Act1 to interact with multiple components of the NF- κ B pathway,

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The online version of this article contains supplemental material.

Abbreviations used in this article: DN, dominant-negative; HLH, helix-loop-helix; HSF, human skin fibroblast; IKK, I κ B kinase; IP, immunoprecipitation; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISRE, IFN-stimulated response element; LMW, low m.w.; NEMO, NF- κ B essential modifier; NFKBIZ, NF- κ B inhibitor ζ ; poly(I:C), polyinosinic-polycytidylic acid; RLH, RIG-I-like helicase; RNAi, RNA interference; SEFIR, SEF and IL-17R; siRNA, small interfering RNA; TRAF, TNFR-associated factor; TRIF, Toll/IL-1R domain-containing adapter inducing IFN- β ; U-box, Ufd2-box.

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TRAF3 and TRAF6 E3 ligases (33, 34), and NF- κ B essential modifier (NEMO), a part of the canonical IKK complex (35, 36). Moreover, IKK ϵ has been shown to bind to and phosphorylate Act1 in IL-17-stimulated cells (37). Importantly, TRAF3, NEMO, and IKK ϵ are all required for antiviral signaling (15, 21, 38).

In this study, we show that Act1 is involved in IRF3-dependent antiviral gene expression induced by a synthetic viral mimic polyinosinic-polycytidylic acid [poly(I:C)]. We demonstrate and map Act1 interactions with the known components of antiviral signaling, IRF3 and IKK ϵ . We also investigate and discuss the mechanisms of how Act1 mediates IRF3 activation in connection with its other functions.

Materials and Methods

Reagents and cells

Primary human skin fibroblasts (HSFs), human lung adenocarcinoma A549, and human embryonic kidney 293 EBNA T cells (or HEK 293 ET, a subclone of 293 cells, which constitutively express SV40 large T Ag and EBV EBNA1 protein to enable enhanced ectopic gene expression) were cultured in DMEM (PAA Laboratories) supplemented with 10% FBS (Life Technologies) and 1% penicillin/streptomycin (PAA Laboratories) at 37°C in 5% CO₂ and 95% humidity. The stimuli were used at the following concentrations: 25 ng/ml human IL-17A (PeproTech), 10 ng/ml human TNF- α (PeproTech), and/or 2.5 μ g/ml high m.w. poly(I:C) (InvivoGen). Myc Ab (9E10) was from Santa Cruz Biotechnology. Rabbit anti-ISG15 and IKK ϵ Abs were from Cell Signaling Technology and Imgenex, respectively. Rabbit polyclonal Ab against phospho-S386 IRF3 was from BD Biosciences. Monoclonal Act1 Ab was purchased from eBioscience and mouse anti- β -actin Ab, FLAG Ab, FLAG peptide, and FLAG agarose from Sigma-Aldrich.

Plasmids

Act1 (*TRAF3IP2*) cDNA was generated from mouse spleen total RNA. pEAK8-myc-Act1 is described in Ryzhakov et al. (34). Act1 deletion and point mutants were generated by PCR mutagenesis and inserted into pEAK8 vector. For evolutionary studies, human and zebrafish Act1 cDNAs were generated from human 293 cell total RNA and zebrafish gill total RNA, respectively, and cloned into pEAK8 vector. Human IRF3 mutants and IKK dominant-negative (DN) mutants were generated by PCR mutagenesis using wild-type IRF3 and IKK cDNAs as templates and inserted into the pENTR vector (Invitrogen) modified to contain the CMV promoter and IRES-linked GFP (pBent). All of the generated constructs were confirmed by sequencing. pNF- κ B-luc and pISRE-luc were purchased from Clontech.

Transfection and reporter assays

Primary HSFs, A549, and human embryonic kidney 293 ET cells were cultured in DMEM (PAA Laboratories) supplemented with 10% FBS (Life Technologies) and 1% penicillin/streptomycin (PAA Laboratories) at 37°C in 5% CO₂ and 95% humidity. For reporter assays, 293ET cells were transfected in 96-well plates using Lipofectamine 2000 (Invitrogen). A mix of 10 ng/well pEAK8-myc-Act1 expression construct and 10 ng/well pNF- κ B-luc plasmid were cotransfected. Alternatively, a mix of 10 ng/well pEAK8-myc-Act1 expression construct, 0.5 ng/well pBent-HA-IRF3, and 10 ng/well pISRE-luc plasmid was used for transfection. To normalize for DNA uptake, 10 ng/well pRL-TK *Renilla* luciferase (Promega) was also cotransfected. One day after transfection, cells were lysed, and luciferase activities were measured in lysates using the Dual-Glo luciferase assay kit (Promega). For RNA extractions, 293 ET cells were seeded in 24-well plates; 200 ng total DNA/well, including 50 ng/well pEAK8-myc-Act1 construct and 2 ng pBent-HA-IRF3, was transfected 1 d after seeding, and 24 h later, the cells were lysed in RLT buffer (Qiagen). For immunoprecipitations (IP), 200 ng myc-Act1 and 500 ng each FLAG-IKK constructs were used per six wells of 293 ET cells.

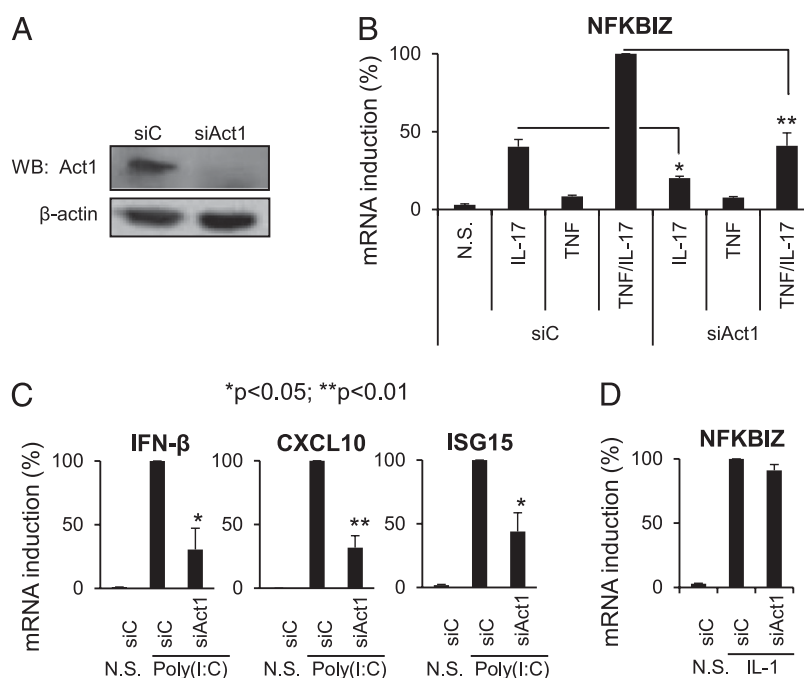
RNA interference

Human Act1 was targeted using On-target Plus siRNA pool (Dharmacon); a nontargeting pool, siC, was used as a control small interfering RNA (siRNA) (Dharmacon). Also, the individual siRNAs from the Act1 pool were screened to validate knockdown specificity and efficiency (Supplemental Fig. 1). The siRNA against human IKK ϵ is described in Fitzgerald et al. (15). HSFs plated 1 d before transfection at 70–90% confluency were transfected with siRNA in a serum-free medium, OptiMEM (Invitrogen), using lipid-based transfection reagent Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The transfection medium was replaced with DMEM containing 10% serum and antibiotics 4 h posttransfection. Twenty-four hours posttransfection, cells were trypsinized and split 1:2 into 12-well plates and incubated for a further 24 h before stimulation with IL-17, TNF- α , IL-1, and/or poly(I:C) and subsequent RNA extraction.

RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA was extracted from cells using Qiagen RNeasy Mini Kit (Qiagen). Cells were lysed in RLT buffer (Qiagen), and cDNA synthesis was performed using extracted RNA, oligo(dT) primer, and Superscript III reverse transcriptase (Invitrogen). The cDNA was PCR amplified using EfficienSee Fast qPCR master mix plus dTTP (Eurogentec). The TaqMan gene expression assays for human RPLP0 (housekeeper control), NF- κ B inhibitor ζ (NFKBIZ), IFN- β , CXCL10, IL-8, and ISG15 were acquired from Applied Biosystems.

FIGURE 1. Act1 is involved in poly(I:C)-induced gene expression in HSFs. (**A–D**) HSFs were transfected with 100 pmol of Act1 siRNA or nontargeting siRNA pool (siC). Cells were incubated with siRNA for 2 d and split 1:2 1 d before 4 h stimulation with IL-17 (25 ng/ml), TNF- α (10 ng/ml), poly(I:C) (2.5 μ g/ml), IL-1 (5 ng/ml), or not stimulated (N.S.). The impact of Act1 knockdown on protein production was determined by Western blotting (WB) using monoclonal Act1 Ab, with β -actin as a protein loading control (A). The effect of Act1 knockdown on mRNA induction of NFKBIZ (B, D) or antiviral genes (C) was determined by quantitative PCR. mRNA induction is expressed as relative fold induction compared with 4 h siC stimulation data point (100%) after normalization to the housekeeper gene RPLP0. Data shown are means \pm SEM of three independent experiments. * p < 0.05, ** p < 0.01 (one-tailed paired t -test).



IP and Western blotting

293 ET cells were lysed in IP buffer (150 mM NaCl, 0.1% Triton X-100, 20 mM Tris-HCl [pH 7.4], and 5 mM EDTA with proteinase inhibitors), and the postnuclear supernatants were incubated for 2 h with myc Ab followed by 2 h incubation with anti-FLAG agarose at 4°C. After four washes in IP buffer, precipitates were eluted with FLAG peptide (150 µg/ml in IP buffer). The eluates were separated by SDS-PAGE using precast 4–12% denaturing gels (Invitrogen), immunoblotted, and the protein bands were visualized using the Amersham ECL detection system (GE Healthcare). Alternatively, siRNA-treated HSFs were lysed in the sample buffer (2×, 0.05% bromophenol blue, 20% glycerol, 4% SDS, 0.25 M TrisHCl [pH 6.8], and 200 mM DTT), and the lysates were subjected to SDS-PAGE and Western blotting.

Statistical analysis

Data are presented as means with error bars of the SEM, or representative data are shown. Statistical significance was calculated on experiments, where $n = 3$, using the paired Student t test.

Results

Act1 is involved in poly(I:C)-induced antiviral signaling

Viruses induce type I IFN production and a subsequent IFN-stimulated antiviral response upon their recognition by TLRs or RLHs (18). Previous studies demonstrated that a ubiquitin ligase, TRAF3, serves as a critical link between TLR adaptors and downstream regulatory kinases important for IRF activation (21). Interestingly, Act1/CIKS can also bind TRAF3 (28). Therefore, we hypothesized that Act1 could participate in antiviral signaling and examined the induction of antiviral genes in primary HSFs in which the levels of Act1 were inhibited by RNA interference (RNAi) using a pool of four individual siRNAs (Fig. 1A). To confirm the efficiency of the RNAi knockdown in HSFs, we first measured the impact of Act1 depletion on IL-17-induced gene expression and observed a reduction of NFKBIZ mRNA in response to IL-17 or combined TNF and IL-17 stimulation, in agreement with previously published data (39) (Fig. 1B). Expression of IFN- β , CXCL10, and ISG15 mRNA in response to poly(I:C) stimulation was significantly lower in Act1-depleted HSFs (Fig. 1C, Supplemental Fig. 1). Of interest, the IL-1-induced expression of NFKBIZ was not altered in the absence of Act1 (Fig. 1D), confirming the signal specificity of Act1 depletion.

Viral products can trigger antiviral gene expression using either endosomal or cytosolic signaling pathways (5, 18). We have previously shown that poly(I:C) signaling occurs via the endosomal, TRIF-dependent pathway in HSFs; however, a low m.w. (LMW) form of poly(I:C) activates cells via the cytosolic pathway

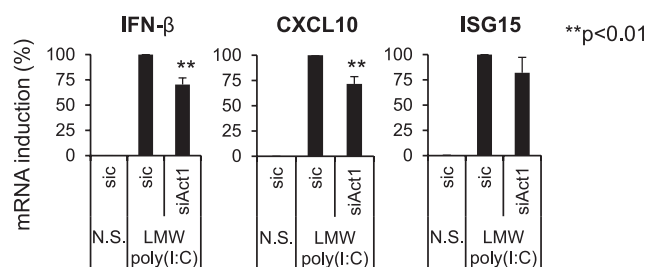


FIGURE 2. Effect of Act1 depletion on antiviral gene expression triggered by a LMW poly(I:C) in HSFs. HSFs were transfected with 100 pmol of Act1 siRNA or nontargeting siRNA pool (siC). Two days after treatment with siRNA, HSFs were transfected with 0.1 µg/ml of LMW poly(I:C) using Lipofectamine 2000 transfection reagent (Invitrogen) and incubated for further 24 h before collection for RNA extraction. mRNA induction is expressed as relative fold induction compared with siC stimulation data point (100%) after normalization to the housekeeping gene RPLP0. Data shown are means \pm SEM of three independent experiments. ** $p < 0.01$ (one-tailed paired t test). N.S., not stimulated.

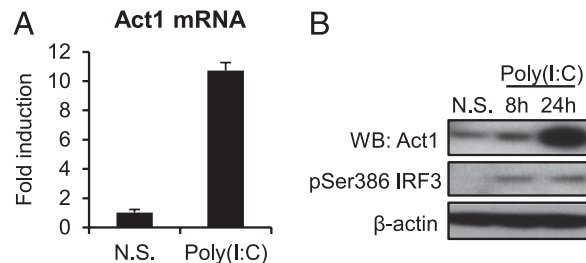


FIGURE 3. Act1/CIKS expression is induced in response to dsRNA. **(A)** HSFs were stimulated overnight with poly(I:C) (2.5 µg/ml) or left not stimulated (N.S.). Total RNA was extracted after that, and Act1 expression was measured by quantitative PCR. mRNA induction is expressed as relative fold induction over nonstimulated control after normalization to the housekeeper gene RPLP0 and is shown as mean \pm SD. **(B)** HSFs were stimulated with poly(I:C) for 8 and 24 h and then immediately lysed in the sample buffer. Proteins in the lysates were separated by SDS-PAGE and analyzed by Western blot (WB) using mAbs against Act1 and β -actin (protein loading control) and a rabbit polyclonal Ab against phosphoserine 386 IRF3.

and only when it is introduced with a lipid carrier (40). We decided to test whether Act1 is also involved in cytosolic antiviral signaling. The transfected LMW poly(I:C) induced antiviral gene expression in HSFs, which was partially diminished in cells with the depleted Act1 (Fig. 2). Thus, both antiviral signaling pathways in HSFs seem to involve Act1, albeit to a different extent.

Act1 is also required for IL-17-induced proinflammatory gene expression (23), but it is unknown if IL-17 could potentially

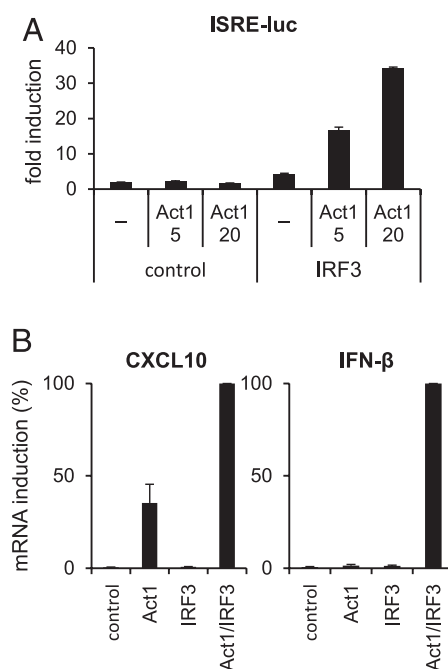


FIGURE 4. Act1 and IRF3 coexpression triggers antiviral signaling. **(A)** 293 ET cells were cotransfected with a luciferase gene reporter driven by IRF binding sites (ISRE-luc) (10 ng/well), increasing amounts of Act1 plasmids (5 or 20 ng/well), and with/without pBent2-IRF3 plasmid (0.5 ng/well). One day after transfection, the cells were lysed, and luciferase activities were measured. The data are shown as the mean \pm SD of a representative out of three independent experiments, each performed in triplicate. **(B)** Total RNA was isolated from 293 ET cells transfected with Act1, IRF3, or combined plasmids. mRNA levels of CXCL10 and IFN- β were measured by real-time PCR and normalized against expression of the housekeeping control (RPLP0). The data are shown as the mean \pm SD of a representative out of three independent experiments.

trigger antiviral gene expression. We stimulated HSFs with either IL-17 or poly(I:C) and measured induction of antiviral genes on the mRNA and protein levels. Unlike poly(I:C), IL-17 did not induce CXCL10 or ISG15 gene expression (Supplemental Fig. 2). Therefore, Act1 mediates antiviral signaling independently of IL-17.

Act1 expression is induced in response to poly(I:C) stimulation

Treatment with dsRNA was previously shown to induce expression of the TLR3 adaptor protein TRIF (41). Because Act1 is also an inducible protein (42), we examined whether its expression was affected by poly(I:C) stimulation. An upregulation of Act1 synthesis was observed in the stimulated cells on mRNA and protein levels (Fig. 3). Therefore, Act1 is both a regulator and a target of antiviral response.

Act1 induces IRF3-dependent gene expression

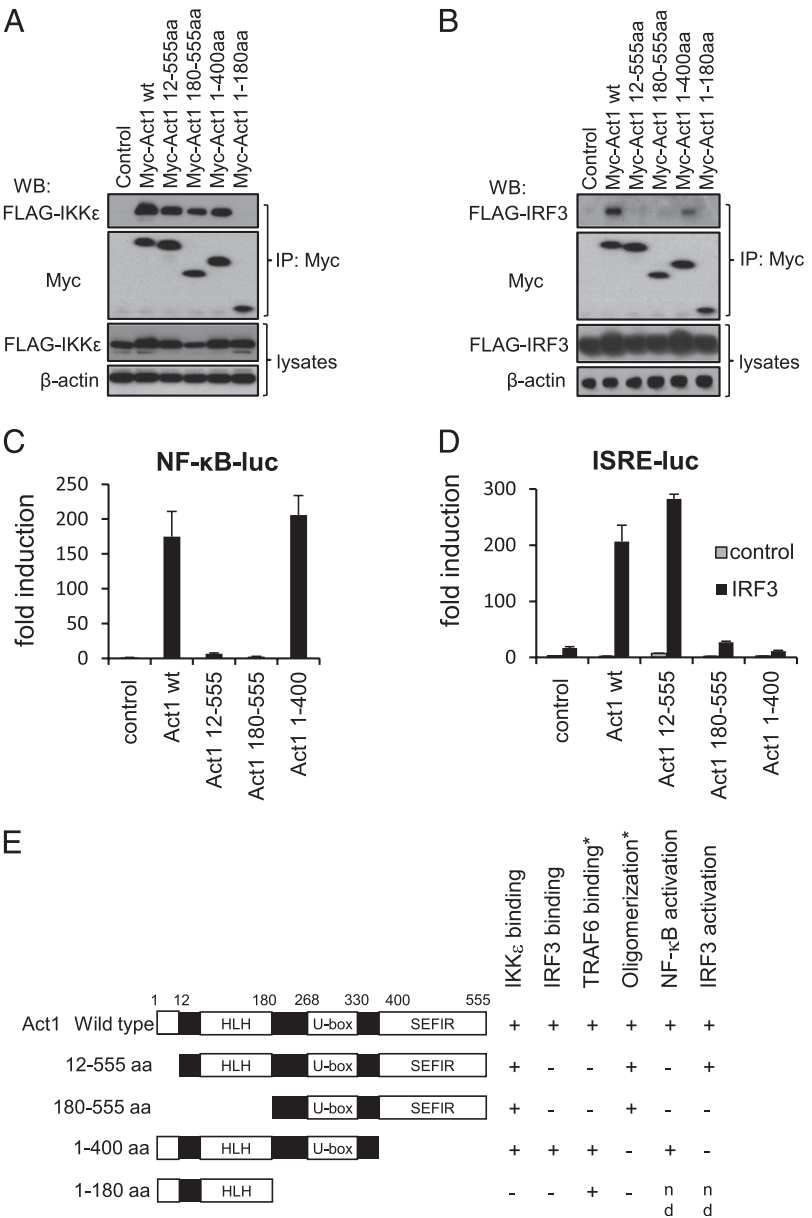
Transcription factors of the IRF family, such as IRF3 and IRF7, control expression of IFN- β and a number of ISGs (6). We next examined whether Act1 can directly activate IRF3 when ectopically expressed in 293 ET cells. Act1 expressed on its own did not

induce the activity of the gene reporter driven by an IFN-stimulated response element (ISRE)-luc. However, when it was coexpressed with IRF3, Act1 triggered ISRE reporter activation in a dose-dependent manner (Fig. 4A). Moreover, Act1 was capable of driving the mRNA expression of endogenous IFN- β and CXCL10 genes (Fig. 4B), suggesting a role in IRF3 pathway activation. Importantly, NF- κ B activation induced by Act1 was not responsible for Act1/IRF3 coinduction of ISRE reporter activity, as the Act1 12–555 aa mutant, unable to bind TRAF6 and activate NF- κ B (34), could still trigger the ISRE reporter activity (Fig. 5C, 5D).

Act1 mediates antiviral signaling via IRF3 phosphorylation

IRF3 is phosphorylated at its C terminus in virus-infected cells (12). This modification leads to IRF3 dimerization and its translocation to the nucleus, where IRF3 can activate transcription (12). To examine the impact of Act1 depletion on IRF3 phosphorylation, HSFs were stimulated with poly(I:C) in the presence or absence of siRNA against Act1. IRF3 phosphorylation at serine 386 was observed 2–4 h after addition of the stimulus, but the knock-down of Act1 expression attenuated it (Fig. 6A).

FIGURE 5. Act1 interacts with IKK ϵ and IRF3 via distinct regions. **(A and B)** 293 ET cells transfected with plasmids encoding myc-tagged Act1 wild-type or mutants or empty vector (control) in combination with FLAG-tagged IKK ϵ (A) or IRF3 (B) were lysed 1 d after transfection in IP buffer. Cell lysates were spun down at $16,000 \times g$, and the supernatants were incubated with a 9E10 anti-myc Ab absorbed on protein G-Sepharose beads. Proteins eluted from the sorbent using the sample buffer were resolved by SDS-PAGE and immunoblotted using Abs against myc-tag, FLAG-tag, and β -actin. **(C and D)** 293 ET cells were cotransfected with an NF- κ B-luc reporter and Act1 deletion mutants **(E)** or a mix containing an ISRE-luc reporter, Act1 deletion constructs, and either GFP (control) or IRF3-containing plasmid. One day after transfection, the cells were lysed, and the luciferase activities were measured. The data are shown as the mean \pm SD of a representative out of three independent experiments, each performed in triplicate. **(E)** A summary of binding and signaling properties of the Act1 deletion mutants used in the study (31). *Previously published data (34, 44). nd, Not determined; WB, Western blot.



We then explored a *trans*-activating potential of IRF3 C-terminal mutants in the ISRE reporter assay. As expected, the IRF3 N380 mutant that lacks the C terminus aa 381–430 did not activate the ISRE reporter in the presence of Act1 (Fig. 6B). Significantly, the mutation of serines 385 and 386, which are known to be targets of TBK1 and IKK ϵ in the antiviral signaling pathway (12, 13), into alanines (IRF3 S1,2A) also blocked Act1-induced IRF3 activation. Of interest, mutations of serines 396 and 398 (IRF3 S3,4A) or serines 402 and 405 and threonine 404 (IRF3 S/T5-7A) had no effect (Fig. 6B). Thus, Act1-induced activation

of IRF3 is dependent on IRF3 phosphorylation at the serine residues 385 to 386 in its C terminus that are targets of TBK1 and IKK kinases.

Act1 mediates antiviral signaling via IRF3 phosphorylation by IKK ϵ

To further dissect the contribution of TBK1 and IKK kinases in Act1-induced IRF3 activation, we have used kinase-dead mutants of IKKs (IKK DN) well characterized in previous studies to act as the DN forms (i.e., to block IRF and NF- κ B signaling pathways) (14). Expression of IKK ϵ DN efficiently blocked Act1/IRF3-induced ISRE reporter activation (Fig. 6C), whereas TBK1 DN, IKK α DN, and IKK β DN failed to suppress reporter activation (Fig. 6C).

IKK ϵ has been previously shown to be important for poly(I:C)-induced antiviral signaling in human embryonic kidney 293 cells (14). However, the IKK ϵ function in stromal cells, which are both responsive to viral agonists and IL-17, has not yet been addressed. In this study, we have used siRNA-mediated knockdown of IKK ϵ expression in human skin fibroblasts (Supplemental Fig. 3A) and observed a significant impairment of antiviral gene expression and IRF3 phosphorylation in these cells (Supplemental Fig. 3B, 3C). Therefore, both the reporter assays using the DN kinase mutants and our RNAi experiments indicate the important role of Act1 and IKK ϵ in dsRNA-induced antiviral signaling in HSFs.

Distinct domains are required for Act1-induced IRF and NF- κ B activation

IKK ϵ mediates IRF3 activation by interacting with several adaptor proteins linking the kinase to upstream signaling pathways (43). IKK ϵ was also recently shown to interact with Act1 in the IL-17 signaling pathway (37). To examine the possibility of IKK ϵ serving as a molecular bridge between Act1 and IRF3, we generated deletion mutants of Act1 and mapped a region within Act1 responsible for binding to IKK ϵ (Fig. 5A, 5E). Recently, we have demonstrated that the N terminus of Act1, aa 1–11, was essential for its interactions with TRAF6 and activation of the NF- κ B pathway (34). However, a TRAF6 binding mutant of Act1 (Myc-Act1 12–555 aa) still interacted with IKK ϵ (Fig. 5A). In addition, mutants lacking HLH and SEFIR domains, 160–555 and 1–400 aa, respectively, could still bind IKK ϵ , whereas a 1–180 aa mutant of Act1 failed to interact with the kinase (Fig. 5A). Therefore, IKK ϵ interacts with the central part of Act1, aa 180–400. Furthermore, we examined the interaction between Act1 and IRF3 and found that the presence of the TRAF6-binding motif, HLH domain, and the central region of Act1 was required (Fig. 5B).

Act1 has been shown to be phosphorylated by IKK ϵ at a serine in the U-box domain in response to IL-17 stimulation (37). We wondered if this modification could be important for Act1-mediated antiviral signaling. We have mutated serines 310 and 311 in Act1 and tested the mutant activity in the reporter assay (Supplemental Fig. 4A). Act1-mediated IRF3 activation was not perturbed by this mutation. Moreover, even the deletion of the whole U-box region of Act1 had no effect on the Act1-induced reporter activation. According to the sequence alignment of several vertebrate Act1 proteins the U-box domain is a later acquisition in the evolution and present only in amniotes (Supplemental Fig. 4B), and the IKK ϵ serine targets are mammal specific. Therefore, the U-box of Act1 had probably emerged in evolutionary terms well after Act1 acquired its antiviral function (discussed below).

To determine which parts of Act1 are responsible for IRF3 activation and how they correlate with the binding data, we set up reporter assays. The TRAF6 binding site, aa 1–11 (34), was required for Act1-dependent NF- κ B but not ISRE reporter induction

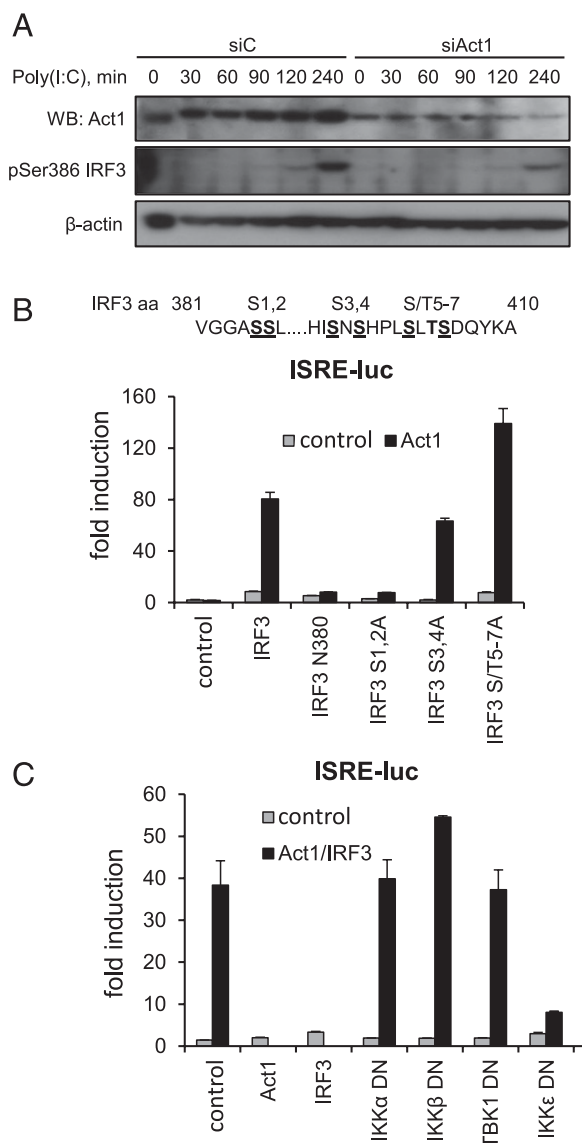


FIGURE 6. Act1 signals via IKK ϵ and the C terminus of IRF3. (A) HSFs were transfected with 100 nmol of the control (siC) or the Act1 siRNA pool (siAct1) (Dharmacon). Three days after transfection, cells were stimulated with poly(I:C) for 30, 60, 90, 120, and 240 min and then immediately lysed in the sample buffer. Proteins in the lysates were separated by SDS-PAGE and analyzed by Western blot (WB) using mAbs against Act1 and β -actin (protein loading control) and a rabbit polyclonal Ab against phospho-serine 386 IRF3. 293 ET cells were transfected with a mix containing ISRE-luc reporter, Act1 construct, and wild-type or mutant IRF3 (B). Alternatively, cells were transfected with plasmid cocktails containing ISRE reporter, Act1, IRF3, and DN forms of I κ B kinases (IKK DN) (C). One day after transfection, the cells were lysed, and luciferase activities were measured. The data are shown as the mean \pm SD of a representative out of three independent experiments, each performed in triplicate.

(Fig. 5C, 5D). The HLH domain, aa 12–180, involved in Act1 binding to IRF3, was required for activation of the ISRE reporter by Act1 (Fig. 5C, 5D). The C-terminal SEFIR domain of Act1, aa 400–555 (23), which was dispensable for Act1 binding to either IKK ϵ or IRF3, impaired the ability of Act1 to activate IRF3 (Fig. 5D), without affecting Act1-induced NF- κ B activation (Fig. 5C). The SEFIR domain of Act1 has been shown to be important for its oligomerization (44). A summary of binding and signaling properties of Act1 (Fig. 5E) shows that Act1 uses distinct sets of domains and binding partners to mediate NF- κ B and IRF3 activation.

The role of Act1 in antiviral signaling is evolutionarily conserved in vertebrates

Act1 and related molecules have been predicted in genomes of all vertebrate taxa and even outside vertebrates (34, 45). We have previously shown that zebrafish Act1 is able to induce NF- κ B activation when expressed in human cells but fails to trigger JNK activation and proinflammatory gene expression (34). Considering that the IFN-based antiviral immunity is found in many vertebrate taxa including fish (46), we examined if zebrafish Act1 could activate IRF3-dependent transcription in human cells. We have coexpressed human or zebrafish Act1 with or without IRF3 in 293 ET cells and looked at reporter activation and endogenous gene expression. Both human and fish Act1 induced ISRE-dependent luciferase expression in the presence of IRF3 (Fig. 7A). In addition, coexpression of both proteins triggered CXCL10 and ISG15 mRNA induction, whereas the expression of IRF3-independent proinflammatory gene IL-8 was only induced by human Act1 (Fig. 7B). These data show that the fish Act1 protein is capable of inducing IRF3-dependent antiviral gene expression in the human system to the same extent as its human counterpart.

Discussion

Act1/CIKS is crucially involved in inflammatory processes (23–25) and contributes to T cell-mediated autoimmunity (26). In this study, we show that Act1 is also involved in poly(I:C)-induced antiviral signaling. It interacts with IKK ϵ and IRF3 and induces IRF3 activation and antiviral gene expression. A recent study showed the formation of a complex between Act1 and IKK ϵ in IL-17-stimulated mouse embryonic fibroblasts, which was crucial for Act1-mediated proinflammatory gene expression (37). Our data therefore support and add to these findings by mapping the molecular interface of the Act1–IKK ϵ interactions and demonstrating their functional involvement in antiviral gene expression.

The broad physiological role of Act1 is reflected in its ability to interact with multiple signaling components (28). Act1 has been shown to activate NF- κ B via its interactions with TRAF6 and the IKK complex (31, 33, 36). In this study, we demonstrate that Act1 activates IRF3 by tethering to IKK ϵ with its central region and recruiting IRF3, which involves the HLH and TRAF6 binding domains of Act1. Therefore, Act1 is likely to use two distinct molecular mechanisms to signal to NF- κ B and IRF3: one depending on the interaction of its N terminus with TRAF6 (34) and another relying on interactions with IKK ϵ via its central region, as well as possible Act1 oligomerization involving the SEFIR domain.

This signaling function of Act1 seems to be evolutionarily conserved in vertebrates. We have previously shown that the TRAF6 binding motif is functional in zebrafish Act1 (34). In this study, we show that the ectopically expressed zebrafish Act1 is able to trigger IRF3-dependent gene expression in human cells. This indicates that little has changed during vertebrate evolution in the molecular surface of Act1 required for antiviral signaling. With emerging knockout/transgenic animal models like zebrafish, it would be interesting to compare the function of Act1 in mammals and lower vertebrates.

This study is a starting point in characterizing Act1 involvement in host defense against viruses. The upstream components of antiviral signaling that may interact with Act1 remain to be elucidated. TRAF6 binding to Act1 was not required for Act1-induced IRF3 activation in our gene reporter assays. However, Act1 can also interact with TRAF3, and both proteins are involved in negative regulation of CD40 and BAFF receptor signaling in B cells (24, 28). TRAF3 was also shown to interfere with the formation of an IL-17R–Act1–TRAF6 signaling complex (47). Considering the role of TRAF3 in virus-induced IRF3 activation (21), it is exciting to speculate that it may connect Act1 to virus-activated receptors.

Participation in both antiviral and proinflammatory signaling pathways is not an unprecedented case. NEMO, an adaptor subunit of the canonical IKK complex, has been previously shown to contribute to the IRF3-dependent antiviral response (38). Interestingly, NEMO has also been shown to interact with Act1 (44) and an IKK ϵ adaptor TANK (48). It would be interesting to explore if all three proteins are able to functionally interact downstream of virus-activated receptors.

In summary, our data map a new function of Act1 in poly(I:C)-induced antiviral response and call for further studies of the physiological role of Act1 in infections that involve different types of viruses and various infected tissues.

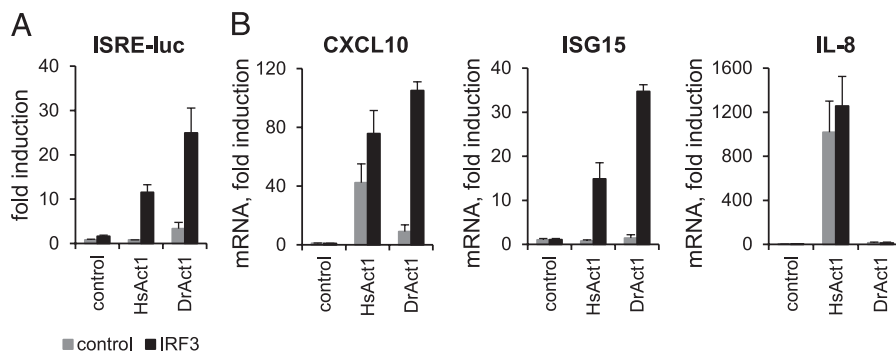


FIGURE 7. Zebrafish Act1 triggers antiviral gene expression in human cells. (A) 293 ET cells were cotransfected with a luciferase gene reporter driven by IRF binding sites (ISRE-luc) (10 ng/well), human (HsAct1), or zebrafish (DrAct1) Act1 plasmids (10 ng/well), and with/without pBent2-IRF3 plasmid (0.5 ng/well). One day after transfection, the cells were lysed, and luciferase activities were measured. (B) Total RNA was isolated from 293 ET cells transfected with Act1, IRF3, or combined plasmids. mRNA levels of CXCL10 and IFN- β were measured by real-time PCR and normalized against expression of the housekeeping control (RPLP0). The data are shown as the mean \pm SD of a representative out of three independent experiments, each performed in triplicate.

Disclosures

The authors have no financial conflicts of interest.

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