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SIKE is an IKKε/TBK1-associated suppressor of TLR3- and virus-triggered IRF-3 activation pathways

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Viral infection or TLR3 engagement causes activation of the transcription factors IRF-3 and NF-κB, which collaborate to induce transcription of type I IFN genes. IKKε and TBK1 are two IKK-related kinases critically involved in virus- and TLR3-triggered activation of IRF-3. We identified a protein termed SIKE (for Suppressor of IKKε) that interacts with IKKε and TBK1. SIKE is associated with TBK1 under physiological condition and dissociated from TBK1 upon viral infection or TLR3 stimulation. Overexpression of SIKE disrupted the interactions of IKKε or TBK1 with TRIF, RIG-I and IRF-3, components in virus- and TLR3-triggered IRF-3 activation pathways, but did not disrupt the interactions of TRIF with TRAF6 and RIP, components in TLR3-triggered NF-κB activation pathway. Consistently, overexpression of SIKE inhibited virus- and TLR3-triggered interferon-stimulated response elements (ISRE) but not NF-κB activation. Knockdown of SIKE potentiated virus- and TLR3-triggered ISRE but not NF-κB activation. Moreover, overexpression of SIKE inhibited IKKε- and TBK1-mediated antiviral response. These findings suggest that SIKE is a physiological suppressor of IKKε and TBK1 and plays an inhibitory role in virus- and TLR3-triggered IRF-3 but not NF-κB activation pathways.

Introduction

Viral infection results in induction of type I interferons (IFNs), including IFN-β and IFN-α family cytokines, which are critical mediators of antiviral responses (Durbin *et al*, 2000; Levy and Garcia-Sastre, 2001; Levy and Marie, 2004; Hengel *et al*, 2005). Transcriptional induction of type I IFN genes requires the coordinate activation of multiple transcription factors and their cooperative assembly into transcrip-

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tional enhancer complexes *in vivo*. For example, the enhancer of the IFN-β gene contains a κB site recognized by NF-κB, a site for ATF-2/c-Jun and two IFN-stimulated response elements (ISREs) recognized by phosphorylated IRF-3 and/or IRF-7 (Maniatis *et al*, 1998; Wathelet *et al*, 1998). It has been shown that transcriptional activation of the IFN-β gene requires coordinate and cooperative assembly of an enhanceosome that contains all of these transcription factors (Maniatis *et al*, 1998; Wathelet *et al*, 1998).

At least two distinct pathways for the activation of the innate antiviral responses by viral infection have been proposed (Levy and Garcia-Sastre, 2001; tenOever *et al*, 2002; Levy and Marie, 2004). First, viruses enter cells by membrane fusion at the plasma membrane or through an endocytic process, leading to the release of viral nucleocapsids (or ribonucleoproteins) into the cytoplasm. The viral dsRNA, accumulated during replication of the viral genome, triggers signaling cascades that lead to the activation of IRF-3 and NF-κB and subsequent production of type I IFNs (Levy and Garcia-Sastre, 2001; tenOever *et al*, 2002). Second, viral dsRNA, released upon lysis of infected cells, binds to TLR3 and triggers signaling pathways leading to IRF-3 and NF-κB activation (Alexopoulou *et al*, 2001; Yamamoto *et al*, 2002, 2003; Hoebe *et al*, 2003; Oshiumi *et al*, 2003; Han *et al*, 2004). It has been proposed that TLR3 is important for systemic responses to viral infection but is not required for the initial, cell-autonomous recognition of viral infection that induces the first wave of type I IFN production (Honda *et al*, 2003; Levy and Marie, 2004; Yoneyama *et al*, 2004).

The molecular components involved in virus-triggered TLR3-independent activation of IRF-3 and NF-κB have begun to emerge. It has been shown that two serine/threonine kinases, TBK1 (also known as NAK or T2K) and IKKε (also known as IKKi), phosphorylate IRF-3 and are involved in virus-triggered induction of type I IFNs in various cell types (Fitzgerald *et al*, 2003; Sharma *et al*, 2003; Hemmi *et al*, 2004; McWhirter *et al*, 2004; Perry *et al*, 2004). More recently, a CARD module-containing RNA helicase protein, RIG-I, has been identified as a sensor of cytoplasmic viral dsRNA (Levy and Marie, 2004; Yoneyama *et al*, 2004). Knockdown or knockout of RIG-I inhibits IFN-β production in response to Newcastle disease virus in different cell types, suggesting that RIG-I is required for virus-triggered signaling (Yoneyama *et al*, 2004; Kata *et al*, 2005). It has been further shown that RIG-I-mediated signaling is independent of TLR3 (Yoneyama *et al*, 2004; Kata *et al*, 2005).

In addition to virus-triggered TLR3-independent signaling pathways, TBK1 and IKKε are also involved in TLR3-mediated IRF-3 activation. It has been shown that TRIF, a TIR domain-containing adapter protein, is associated with TLR3 (Yamamoto *et al*, 2002, 2003; Hoebe *et al*, 2003; Oshiumi *et al*, 2003). TRIF mediates IRF-3 and NF-κB activation through two distinct pathways. TRIF interacts

with TBK1 and/or IKKε, which phosphorylate IRF-3. TRIF interacts with TRAF6 and RIP, which activate NF-κB through TAK1 and IKK (Hoebe *et al*, 2003; Sato *et al*, 2003; Han *et al*, 2004; Jiang *et al*, 2004; Meylan *et al*, 2004).

Since IKKε and TBK1 are critically involved in virus-triggered TLR3-dependent and -independent type I IFN signaling, we investigated whether these kinases are regulated by additional proteins. In this report, we performed yeast two-hybrid screens for potential IKKε-interacting proteins. This effort identified a protein designated as SIKE (for Suppressor of IKKε). Our findings suggest that SIKE is a physiological inhibitor of IKKε and TBK1 and plays an inhibitory role in virus- and TLR3-triggered IRF-3 but not NF-κB activation pathways.

Results

Identification of SIKE as an IKKε- and TBK1-interacting protein

To identify IKKε-interacting proteins, we used the yeast two-hybrid system to screen a human B-cell cDNA library with full-length IKKε as bait. We screened a total of $\sim 8 \times 10^6$ independent clones and obtained 123 β-galactosidase positive clones. Four of these clones encoded an uncharacterized protein of 207 amino acids (aa) that was designed as SIKE. Blast searches of the GenBank databases indicated that SIKE is an evolutionarily conserved protein whose orthologs

could be found from *Xenopus* to human (data not shown). An alignment of human and mouse SIKE aa sequences is shown in Figure 1A. Blast searches of the GenBank databases also indicated that SIKE belongs to an uncharacterized protein family, which includes several uncharacterized proteins, such as FGF receptor 1 oncogene partner 2, hematopoietic stem/progenitor cells 123 (HSPC123), HSCP123-like protein and two invertebrate proteins, Schistosoma mansoni circulating cathodic antigen and *Drosophila* unknown protein CG10158-PA.

Northern blot analysis indicated that human SIKE is ubiquitously expressed as a ~ 5.5 kDa band in all examined tissues, including brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung and leukocytes (Figure 1B). Consistently, blast searches of the GenBank databases identified a human SIKE mRNA sequence of ~ 5.5 kb with an open reading frame of 207 aa.

To determine whether SIKE is expressed in mammalian cells at the protein level, we raised mouse polyclonal antiserum against recombinant human SIKE. Western blot analysis indicated that SIKE was expressed as a ~ 26 kDa protein in all examined cells, including Bjab, HeLa and 293 cells (Figure 1C). The size of endogenous SIKE was slightly smaller than overexpressed HA-tagged SIKE, suggesting that the identified SIKE cDNA encodes a full-length protein. Immunofluorescent staining experiments indicated that SIKE is a cytoplasmic protein (Figure 1D).
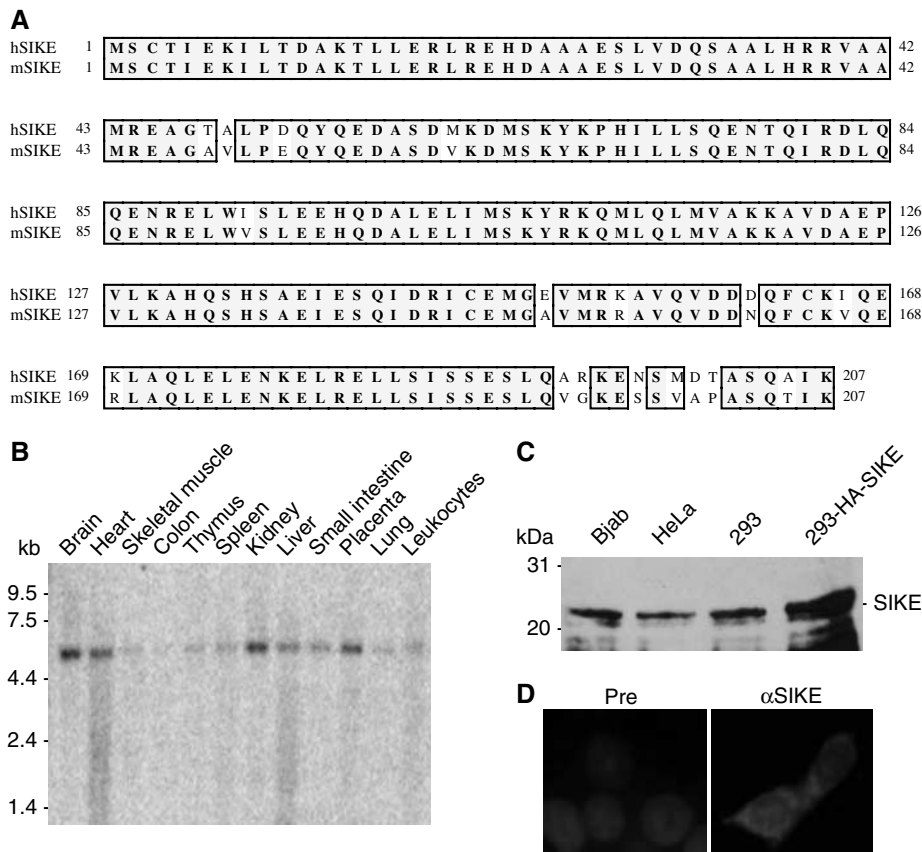

Figure 1 Sequence and expression analysis of SIKE. (**A**) Alignment of amino-acid sequences of human and mouse SIKE. (**B**) Tissue expression of SIKE mRNA. A human multiple tissue mRNA blot was hybridized with a probe corresponding to human SIKE cDNA coding sequence. (**C**) Expression of SIKE protein in mammalian cells. Lysates of the indicated cell types were analyzed by Western blot with a mouse polyclonal anti-SIKE antibody. The 293-HA-SIKE cells were 293 cells transfected with a mammalian expression plasmid for HA-tagged SIKE. (**D**) SIKE is a cytoplasmic protein. The 293 cells were stained with anti-SIKE antibody and DAPI. Pre, mouse preimmune serum; αSIKE, mouse anti-SIKE antiserum.

SIKE is associated with IKKε and TBK1

To determine whether SIKE interacts with IKKε in mammalian cells, we transfected 293 cells with expression plasmids for HA-tagged SIKE and Flag-tagged IKKε. Co-immunoprecipitation experiments indicated that SIKE interacted with IKKε (Figure 2A). In these experiments, SIKE also interacted with TBK1 but not RIP (Figure 2A), a kinase that is involved in TNF receptor and TLR3-induced NF-κB activation (Hsu *et al*, 1996; Meylan *et al*, 2004).
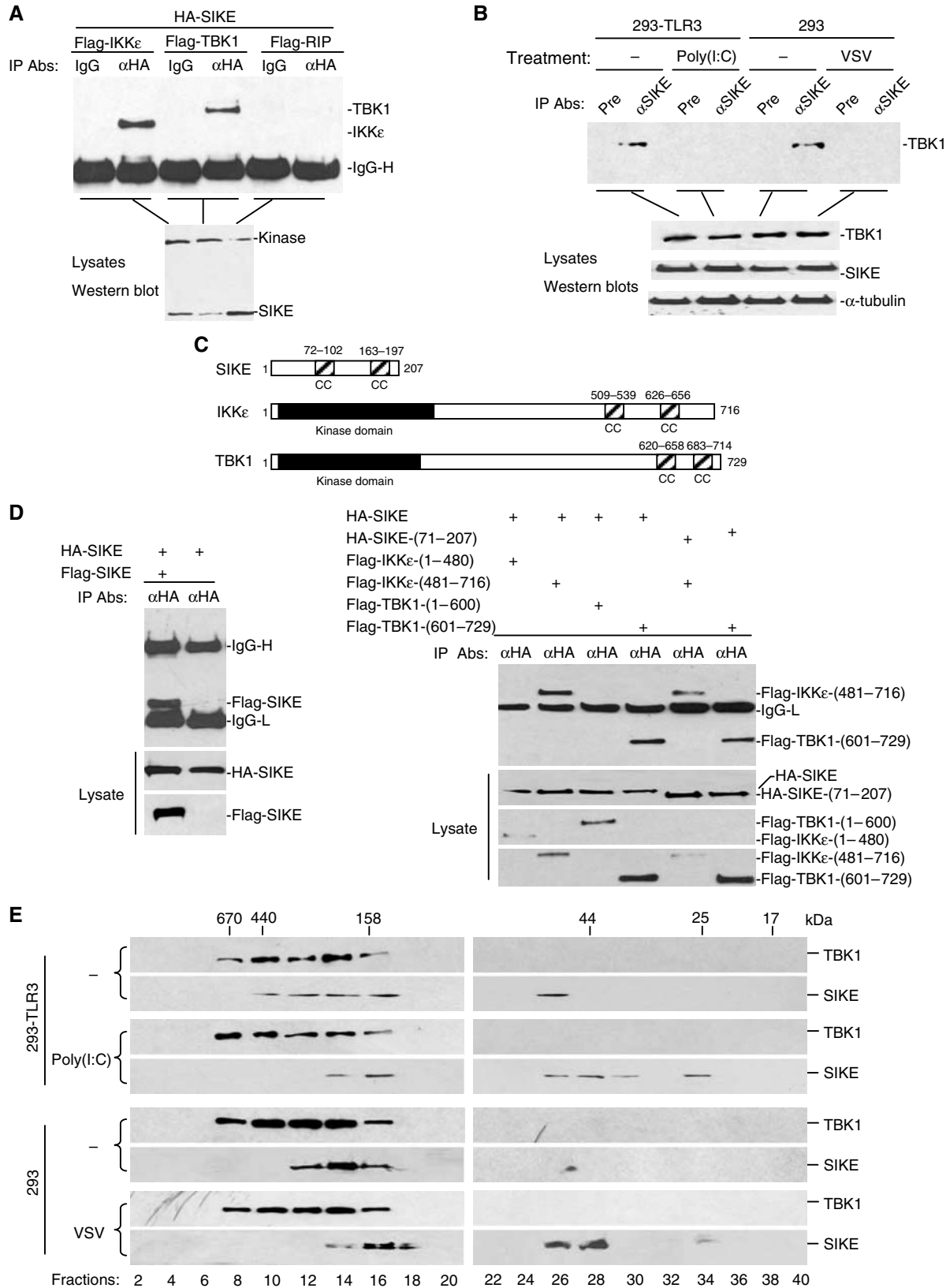
Previously, it has been demonstrated that TBK1 is constitutively expressed in most cell types, while IKKε is undetectable under physiological conditions but inducible in lymphoid and other cell types (Pomerantz and Baltimore, 1999; Shimada *et al*, 1999; Peters *et al*, 2000). Therefore, we examined whether SIKE is associated with TBK1 in untransfected cells. In co-immunoprecipitation experiments, endogenous SIKE interacted with endogenous TBK1 under physiological condition. This interaction was significantly decreased following virus infection or poly(I:C) treatment of TLR3-expressing 293 cells (Figure 2B). These data suggest that SIKE is constitutively associated with TBK1 under physiological condition, but dissociated from TBK1 upon virus infection or TLR3 activation.

Since SIKE interacts with IKKε and TBK1, we performed structural analysis to determine whether they are structurally related to each other. Using the paircoil program, we found that SIKE contains two coiled-coil motifs at aa72–102 and aa163–197, respectively (Figure 2C). Interestingly, IKKε and TBK1 also contain two potential coiled-coil motifs at their C-terminuses (Figure 2C). Since coiled-coil motifs mediate homophilic multimerization, we determined whether SIKE could dimerize or oligomerize. In co-immunoprecipitation experiments, Flag-tagged SIKE could interact with HA-tagged SIKE (Figure 2D), suggesting that SIKE could form homo-dimmers or -oligomers.

We also made series of mutants of SIKE, IKKε and TBK1 and performed domain mapping experiments. We found that SIKE interacted with IKKε and TBK1 through their respective coiled-coil domains (Figure 2D).

To further characterize the association of SIKE with TBK1, we examined the biochemical nature of SIKE and TBK1 in untransfected cells. In these experiments, 293-TLR3 cells were treated with poly(I:C) for 10 min or left untreated, or 293 cells were infected with vesicular stomatitis virus (VSV) for 4 h or left uninfected before lysis. Cell lysates were separated by size-exclusion chromatography and individual fractions were analyzed by Western blots with antibodies against SIKE and TBK1, respectively. As shown in Figure 2E, SIKE and TBK1 exist in overlapping fractions that eluted in the 158–670 kDa range in uninfected cells. However, following poly(I:C) treatment or VSV infection, a significant part of SIKE was shifted to fractions peaking at about 25 and 44 kDa. Based on these data, we propose that SIKE is associated with TBK1, as well as unidentified proteins, as complexes of 158–670 kDa under physiological conditions. Upon poly(I:C) stimulation or viral infection, SIKE is dissociated from the TBK1-containing complexes to become free monomer (in 25 kDa fraction) or dimer (in 44 kDa fraction) (Figure 2E).

In the size-exclusion chromatography experiments, the molecular weights of TBK1-containing complexes were not decreased following virus infection or poly(I:C) treatment. It is possible that the molecular weight of dissociated SIKE is too small to have apparent effect on the molecular weights of TBK1-containing complexes. Alternatively, virus infection or poly(I:C) treatment may cause both dissociation of SIKE from the TBK1-containing complexes and recruitment of other proteins to the TBK1-containing complexes.

SIKE inhibits IKKε- and TBK1-mediated activation of ISRE and the IFN-β promoter

Overexpression of IKKε and TBK1 activates both ISRE and NF-κB (Pomerantz and Baltimore, 1999; Peters *et al*, 2000; Fitzgerald *et al*, 2003; Han *et al*, 2004). Since SIKE interacts with IKKε and TBK1, we determined the effects of SIKE on IKKε- and TBK1-mediated signaling. In reporter gene assays, SIKE inhibited both IKKε- and TBK1-mediated activation of ISRE and the IFN-β promoter (Figure 3A). In similar experiments, SIKE did not inhibit IKKε- and TBK1-mediated NF-κB activation (Figure 3A). These results suggest that SIKE specifically inhibits IKKε- and TBK1-mediated IRF-3 pathways.

SIKE inhibits TLR3-mediated activation of ISRE and the IFN-β promoter

Since IKKε and TBK1 are critically involved in TLR3-triggered ISRE but not NF-κB activation pathways (Fitzgerald *et al*, 2003; Sharma *et al*, 2003; Hemmi *et al*, 2004; McWhirter *et al*, 2004; Perry *et al*, 2004), we determined the effects of SIKE on these pathways. TRIF is a signaling component upstream of IKKε and TBK1 in TLR3-mediated signaling. In reporter gene assays, SIKE inhibited TRIF-mediated activation of ISRE and the IFN-β promoter (Figure 3B), but had no effect on TRIF-mediated NF-κB activation (Figure 3B). In a poly(I:C)-responsive 293 cell line that stably expresses TLR3, SIKE inhibited poly(I:C)-induced activation of ISRE and the IFN-β promoter but not NF-κB (Figure 3C). In similar experiments, SIKE did not inhibit IRF3-mediated activation of ISRE and the IFN-β promoter (data not shown). These data suggest that SIKE specifically inhibits TLR3-mediated ISRE but not NF-κB activation pathways at TBK1 and/or IKKε level.

SIKE disrupts the interactions of IKKε or TBK1 with TRIF and IRF-3

One of the simplest explanations for the inhibitory role of SIKE on TLR3-mediated ISRE activation is that SIKE sequesters IKKε and/or TBK1 in an inactive complex and blocks their interactions with TRIF and/or IRF-3, two signaling components that are associated with IKKε and TBK1 in the

Figure 2 SIKE is associated with IKKε and TBK1. (**A**) SIKE interacts with IKKε and TBK1 but not RIP in mammalian overexpression system. The 293 cells (1×10^6) were transfected with the indicated plasmids (5 μg each). Co-immunoprecipitations were performed with anti-HA antibody (αHA) or control mouse IgG (IgG). Western blot was performed with anti-Flag antibody (upper panel). Expression of the transfected plasmids was confirmed by Western blot analysis of the lysates with anti-HA and anti-Flag antibodies (lower panel). (**B**) Association of SIKE with TBK1 and effects of stimulation on the association in untransfected cells. The 293-TLR3 or 293 cells (5×10^7) were left untreated or treated with poly(I:C) (50 μg/ml) for 10 min or infected with VSV for 4 h. The cells were lysed and the lysates were immunoprecipitated with anti-SIKE antiserum or preimmune control serum. The immunoprecipitates were analyzed by Western blots with anti-TBK1 antibody (upper panel). The expression levels of the endogenous proteins were detected by Western blot analysis with anti-TBK1, anti-SIKE and anti-α-tubulin antibodies (lower panels). (**C**) A schematic presentation of the coiled-coil motifs identified in SIKE, IKKε and TBK1. CC, coiled coil. (**D**) SIKE interacts with itself, IKKε and TBK1 through their respective coiled-coil motifs. The 293 cells (1×10^6) were transfected with the indicated plasmids (5 μg each) and co-immunoprecipitations were performed as in (A). (**E**) Analysis of protein complexes containing SIKE and TBK1 by size-exclusion chromatography. The 293-TLR3 (2×10^8) cells were treated with poly(I:C) (50 μg/ml) for 10 min or left untreated. The 293 cells were infected with VSV for 4 h or left uninfected before lysis. Cell lysates were analyzed by size-exclusion chromatography on Superdex 200 column. The individual fractions were analyzed by Western blots with anti-TBK1 and anti-SIKE antibodies, respectively.
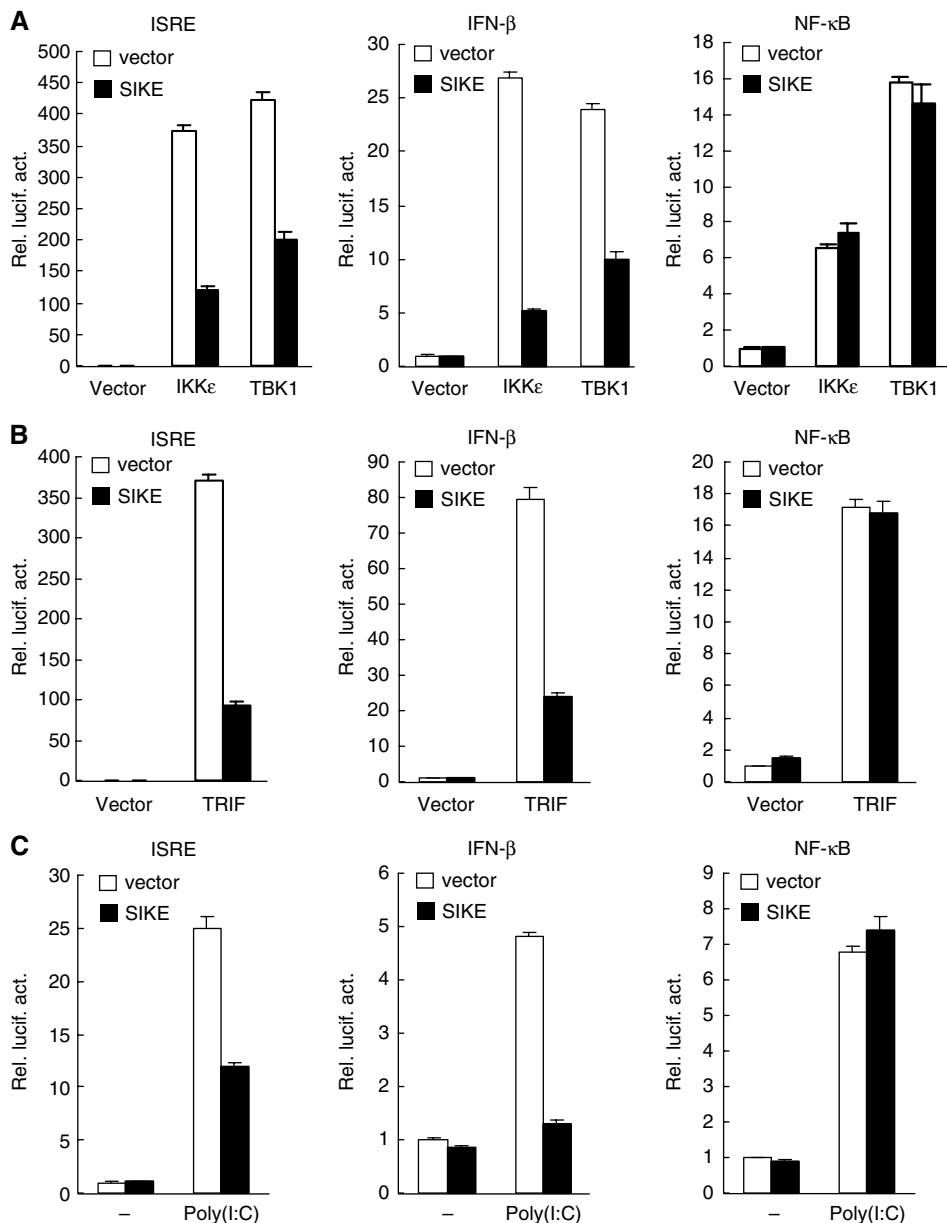
Figure 3 SIKE inhibits IKKε-, TBK1-, TRIF- and TLR3-mediated activation of ISRE and the IFN-β promoter but not NF-κB. (**A**) SIKE inhibits IKKε- and TBK1-mediated activation of ISRE and the IFN-β promoter but not NF-κB. The 293 cells (1×10^5) were transfected with the indicated luciferase reporter plasmid (0.1 μg), an expression plasmid for IKKε or TBK1 (0.1 μg) and an empty control plasmid (open bars) or an expression plasmid for SIKE (filled bars) (1 μg). Luciferase assays were performed 16 h after transfection. (**B**) SIKE inhibits TRIF-mediated activation of ISRE and the IFN-β promoter but not NF-κB. The experiments were similarly performed as in (A), except that a TRIF expression plasmid (0.1 μg) was used to replace the IKKε or TBK1 plasmid. (**C**) SIKE inhibits poly(I:C)-induced activation of ISRE and the IFN-β promoter but not NF-κB. The 293-TLR3 cells (1×10^5) were transfected with the indicated luciferase reporter plasmid (0.1 μg) and an empty control vector (empty bars) or a SIKE expression plasmid (filled bars) (1 μg). At 14 h after transfection, cells were treated with poly(I:C) (50 μg/ml) or left untreated for 6 h before luciferase assays were performed.

TLR3-mediated ISRE activation pathway. To test this, we performed transient transfection and co-immunoprecipitation experiments. In these experiments, either IKKε or TBK1 interacted with TRIF and IRF-3 as expected. The interactions were completely disrupted with the addition of SIKE (Figure 4A and B). In similar experiments, SIKE did not disrupt the interactions of TRIF with TRAF6 and RIP, two components that are involved in TLR3-mediated NF-κB activation pathway (Figure 4C). Since SIKE did not interact with TRIF and IRF-3 in co-immunoprecipitation experiments (data not shown), these results suggest that SIKE specifically blocks

the interactions of IKKε or TBK1 with TRIF or IRF-3 by its association with IKKε or TBK1.

SIKE inhibits virus-triggered TLR3-independent activation of ISRE and the IFN-β promoter

In addition to TLR3-mediated ISRE activation, IKKε and TBK1 are also involved in virus-triggered TLR3-independent activation of ISRE and the IFN-β promoter. Recent studies have indicated that RIG-I is a sensor of viral dsRNA in the cytoplasm and is critically involved in virus-triggered TLR3-indepenent signaling (Levy and Marie, 2004; Yoneyama *et al*,
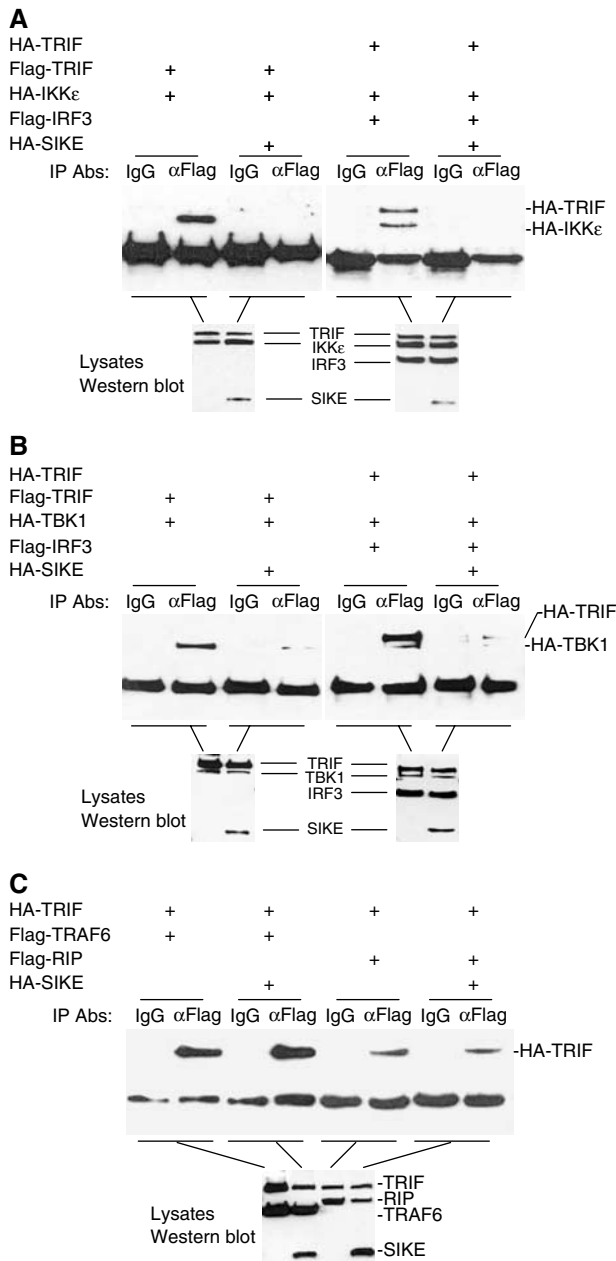
Figure 4 Effects of SIKE on assembly of the TRIF-IKKε-IRF-3, TRIF-TBK1-IRF3 and TRIF-TRAF6-RIP complexes. (**A**) SIKE disrupts the TRIF-IKKε-IRF-3 complex. The 293 cells (1×10^6) were transfected with 5 µg of the indicated plasmids. Cell lysates were immunoprecipitated with control mouse IgG (IgG) or anti-Flag (αFlag) antibody. The immunoprecipitates were analyzed by Western blot with anti-HA antibody (upper panel). The expression levels of the transfected proteins were detected by Western blot analysis with anti-HA and anti-Flag antibodies (low panel). (**B**) SIKE disrupts the TRIF-TBK1-IRF-3 complex. The experiments were carried out similarly as in (A), except that TBK1 plasmid was used to replace IKKε plasmid. (**C**) SIKE does not disrupt the interactions of TRIF with TRAF6 or RIP. The 293 cells were transfected with the indicated plasmids and the co-immunoprecipitation and Western blot analysis were carried out as in (A).

2004). Overexpression of the CARD modules of RIG-I activates ISRE, NF-κB and the IFN-β promoter (Yoneyama *et al*, 2004). Therefore, we examined the effects of SIKE on virus-induced and RIG-I-mediated TLR3-independent signaling. The parent 293 cells do not express TLR3 and are not responsive to
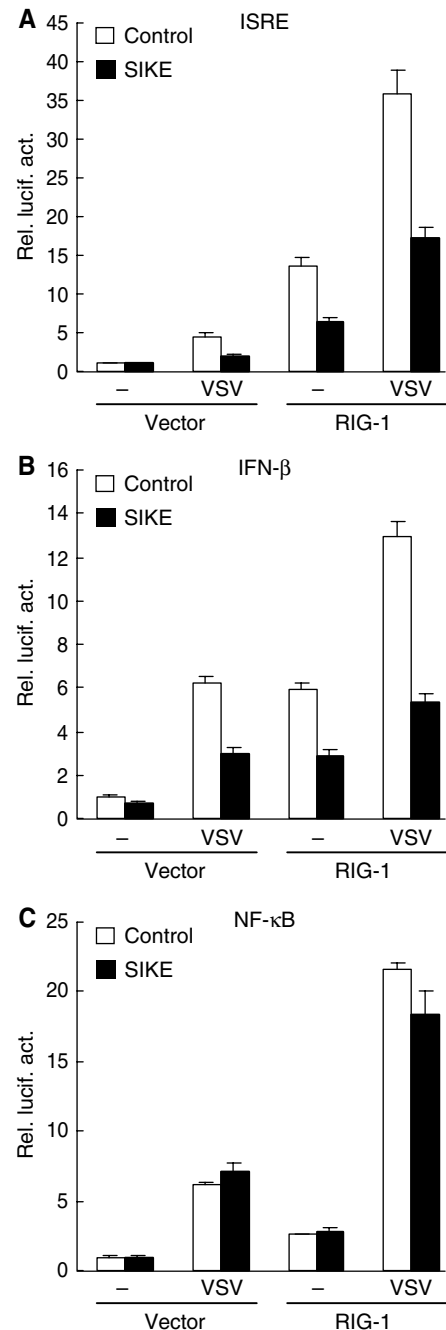

Figure 5 SIKE inhibits VSV-induced and RIG-I-mediated activation of ISRE (**A**) and the IFN-β promoter (**B**) but not NF-κB (**C**). The 293 cells (1×10^5) were transfected with the indicated luciferase reporter plasmid (0.1 µg), an empty control vector or an expression plasmid for RIG-I (1 µg) as indicated, and an empty control vector (empty bars) or an expression plasmid for SIKE (filled bars). At 14 h after transfection, cells were infected with VSV or left infected for 18 h before luciferase assays were performed.

poly(I:C) (Yoneyama *et al*, 2004 and data not shown). In these cells, SIKE inhibited VSV-induced and RIG-I-mediated activation of ISRE and the IFN-β promoter (Figure 5A and B). In similar experiments, SIKE did not inhibit VSV-induced and RIG-I-mediated activation of NF-κB (Figure 5C).

SIKE blocks the interaction of IKKε and TBK1 with RIG-I

It has been demonstrated that both RIG-I and IKKε or TBK1 are involved in virus-triggered TLR3-independent signaling

(Hemmi *et al*, 2004; Levy and Marie, 2004; Perry *et al*, 2004; tenOever *et al*, 2004). However, the relationship between RIG-I and IKKε or TBK1 has not been revealed. We found

A

Flag-RIG-I   +   +
HA-IKKε   +   +   +   +
Flag-RIG-I-CARD   +
Flag-RIG-I-helicase   +
HA-SIKE   +
IP Abs: IgG αFlag IgG αFlag IgG αFlag IgG αFlag

-HA-IKKε

Lysates
Western blot   -RIG-I
                -IKKε   -RIG-I-helicase
                -RIG-I-CARD
                -SIKE

B

Flag-RIG-I   +   +
HA-TBK1   +   +   +   +
Flag-RIG-I-CARD   +
Flag-RIG-I-helicase   +
HA-SIKE   +
IP Abs: IgG αFlag IgG αFlag IgG αFlag IgG αFlag

-HA-TBK1

Lysates
Western blot   -RIG-I
                -TBK1
                -RIG-I-helicase
                -RIG-I-CARD
                -SIKE

Figure 6 SIKE disrupts the interactions of RIG-I with IKKε or TBK1. (**A**) SIKE disrupts the interaction of IKKε with RIG-I. The 293 cells (1×10^6) were transfected with 5 μg of the indicated plasmids. Cell lysates were immunoprecipitated with control mouse IgG (IgG) or anti-Flag (αFlag) antibody. The immunoprecipitates were analyzed by Western blot with anti-HA antibody (upper panel). The expression levels of the transfected proteins were detected by Western blot analysis with anti-HA and anti-Flag antibodies (low panels). (**B**) SIKE disrupts the interaction of TBK1 with RIG-I. The experiments were carried out as in (A), except that the TBK1 plasmid was used.

that RIG-I could interact with IKKε and TBK1 in co-immuno-precipitation experiments (Figure 6A and B). The CARD modules but not the helicase domain of RIG-I is responsible for its interaction with IKKε and TBK1 (Figure 6A and B). In these experiments, the interactions of IKKε and TBK1 with RIG-I and its CARD modules were completely disrupted by the addition of SIKE (Figure 6A and B).
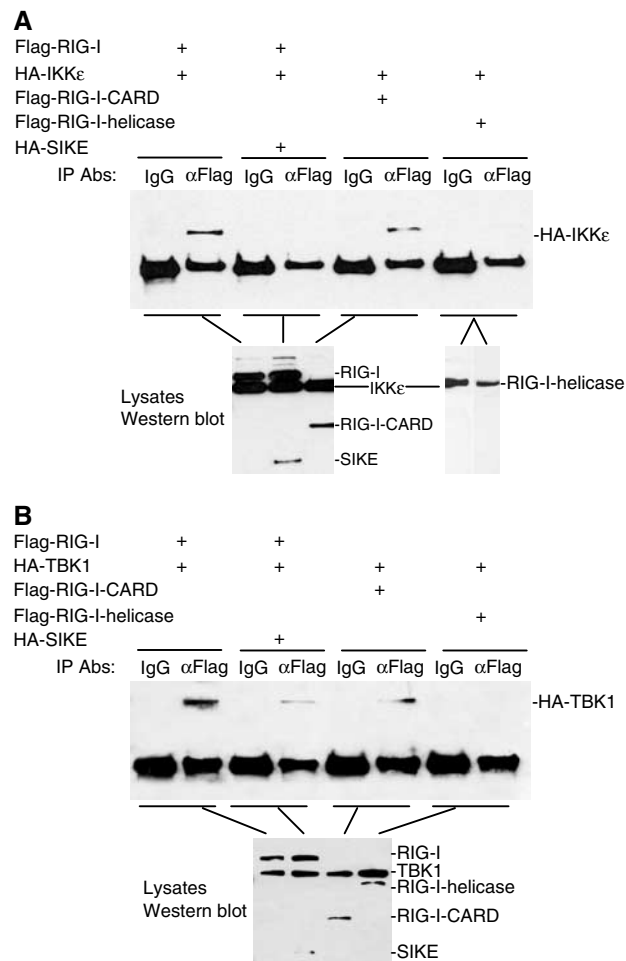
Knockdown of SIKE expression potentiates poly(I:C)- and VSV-induced activation of ISRE and the IFN-β promoter

To further determine whether SIKE is involved in the regulation of the ISRE activation pathways under physiological conditions, we constructed five human SIKE RNAi plasmids. One of these RNAi plasmids (#4) could significantly inhibit the expression of transfected and endogenous SIKE in 293 cells as suggested by Western blot analysis (Figure 7A). In reporter gene assays, this SIKE RNAi plasmid potentiated poly(I:C)- and VSV-triggered activation of ISRE and the IFN-β promoter but not NF-κB (Figure 7B–G). These data suggest that SIKE is a physiological inhibitor of TLR3- and virus-triggered ISRE but not NF-κB activation pathways.

Since SIKE inhibits TLR3- and virus-triggered ISRE activation, we further examined whether SIKE inhibits TLR3-mediated and virus-triggered expression of endogenous IFN-β and Rantes. In reverse-transcription polymerase chain reaction (RT–PCR) experiments, we found that SIKE RNAi potentiated basal as well as poly(I:C)- and virus-triggered expression of endogenous IFN-β and Rantes mRNA (Figure 7H and I). These data suggest that SIKE is a physiological inhibitor of TLR3-mediated and virus-triggered gene expression.
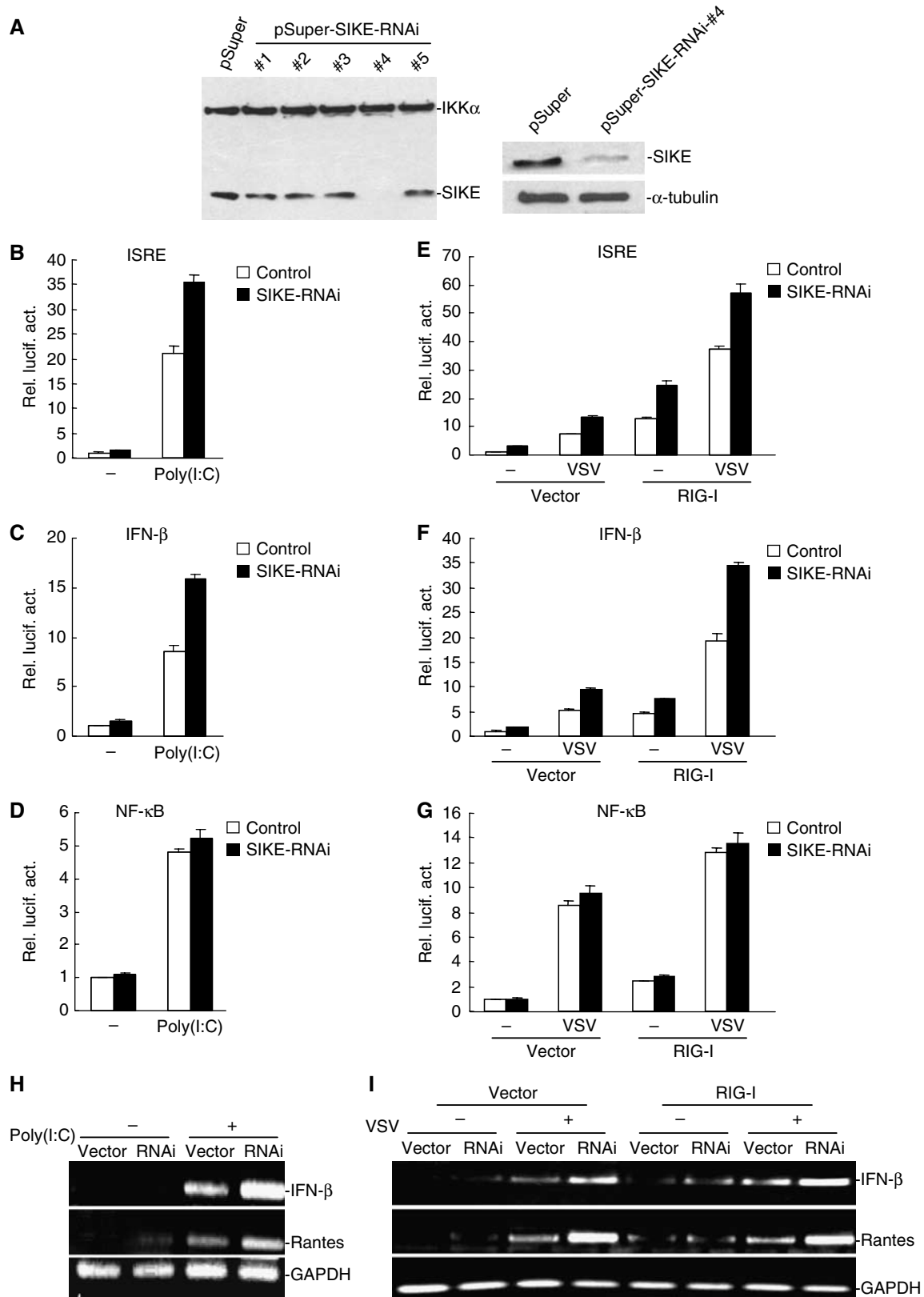
SIKE inhibits IKKε- and TBK1-mediated antiviral response

IKKε and TBK1 have been demonstrated to be required for establishing the antiviral state and this activity is IRF3-dependent. Since SIKE could inhibit IKKε- and TBK1-mediated activation of ISRE and the IFN-β promoter, we determined whether SIKE has effects on IKKε- and TBK-mediated antiviral response. We transfected 293 cells with IKKε/TBK1, together with a control plasmid or a SIKE expression or RNAi plasmid. The transfected cells were infected with VSV and replicated viruses released into the medium were quantitated by standard plaque assays. The results indicated that VSV production in the presence of IKKε/

Figure 7 Effects of SIKE RNAi on poly(I:C)- and VSV-induced activation of ISRE, NF-κB and the IFN-β promoter. (**A**) Effects of SIKE RNAi plasmids on the expression of transfected and endogenous SIKE. In the left panel, 293 cells (1×10^5) were transfected with expression plasmids for Flag-SIKE and Flag-IKKα (0.5 μg each), and the indicated RNAi plasmids (1 μg). At 24 h after transfection, cell lysates were analyzed by Western blot with anti-Flag antibody. In the right panels, 293 cells (1×10^5) were transfected with 1 μg of pSuper.Retro or the #4 SIKE RNAi plasmid for 24 h. Cell lysates were then analyzed by Western blots with a mouse anti-SIKE (upper panel) or a monoclonal anti-α-tubulin (lower panel) antibody. (**B–D**) SIKE RNAi potentiates poly(I:C)-induced activation of ISRE and the IFN-β promoter but not NF-κB in 293-TLR3 cells. The 293-TLR3 cells (1×10^5) were transfected with 0.1 μg of the indicated reporter plasmid, 0.1 μg of pRL-TK *Renilla* luciferase plasmid, 1 μg of the #4 SIKE RNAi or pSuper.Retro control plasmid as indicated. At 36 h after transfection, cells were treated with poly(I:C) (50 μg/ml) (filled bars) or left untreated (open bars) for 6 h before luciferase assays were performed. (**E–G**) SIKE RNAi potentiates VSV-induced RIG-I-mediated activation of ISRE and the IFN-β promoter but not NF-κB in 293 cells. The 293 cells (1×10^5) were transfected with 0.1 μg of the indicated reporter plasmid, 0.1 μg of pRL-TK *Renilla* luciferase plasmid, 1 μg of an expression plasmid for RIG-I, 1 μg of the #4 SIKE RNAi (filled bars) or pSuper.Retro control plasmid (open bars) as indicated. At 36 h after transfection, cells were infected with VSV or left uninfected for 18 h before luciferase assays were performed. (**H**) SIKE RNAi potentiated basal as well as poly(I:C)-induced expression of endogenous IFN-β and Rantes mRNA. The 293-TLR3 cells were transfected with control or SIKE RNAi plasmid. At 36 h after transfection, cells were treated with poly(I:C) (50 μg/ml) for 6 h before RT–PCR was performed. (**I**) SIKE RNAi potentiated virus-triggered RIG-I-mediated expression of endogenous IFN-β and Rantes mRNA. The 293 cells were transfected with control or SIKE RNAi plasmid, together with empty control or RIG-I expression plasmid as indicated. At 36 h after transfection, cells were infected with VSV or left uninfected for 18 h before RT–PCR was performed.

TBK1 decreased by ∼4 logs to 10^4 plaque-forming units (PFU)/ml at 12 h postinfection (Figure 8), which is consistent with the data reported previously (Sharma *et al*, 2003). Overexpression of SIKE restored the VSV production to more than 10^6 PFU/ml (Figure 8A), while SIKE RNAi potentiated IKKε- and TBK1-mediated inhibition of VSV production (Figure 8B). These data indicate that SIKE is a physiological inhibitor of IKKε- and TBK1-mediated antiviral response.
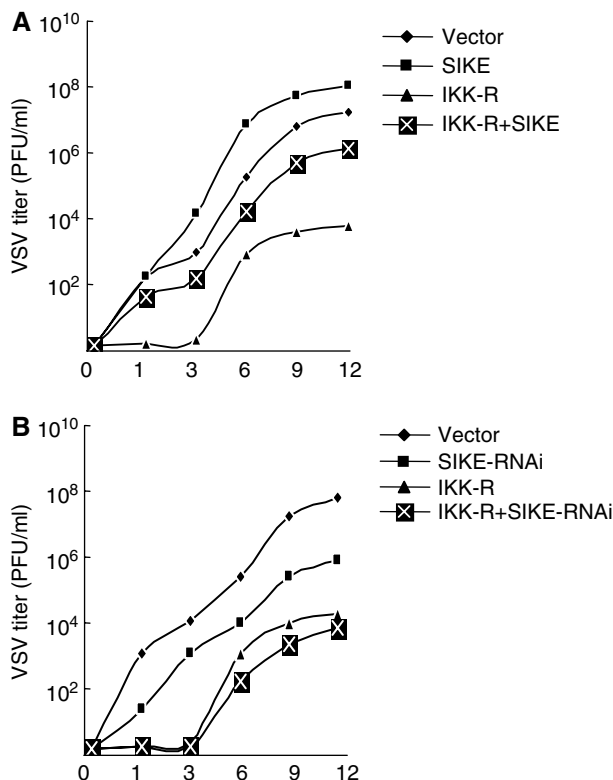
Figure 8 Effects of SIKE on IKKε- and TBK1-mediated antiviral response. (**A**) SIKE inhibits IKKε- and TBK1-mediated antiviral response. The 293 cells (1×10^5) were transfected with IRF-3 expression plasmid (2 μg) together with an empty control plasmid (2 μg), SIKE (2 μg), IKK-R (IKKε + TBK1, each 0.5 μg) or IKK-R + SIKE (0.5 μg of IKKε, 0.5 μg of TBK1 and 2 μg of SIKE). At 24 h after transfection, cells were infected with VSV (MOI 10) and supernatants were harvested at 0, 1, 3, 6, 9 and 12 h postinfection. Supernatants were analyzed for VSV production using standard plaque assays. Plaques were counted and titers calculated as PFU/ml. (**B**) Knockdown of SIKE potentiates IKKε- and TBK1-mediated antiviral response. The experiments were carried out similarly as in (A), except that SIKE-RNAi plasmid was used to replace SIKE expression plasmid and the 293 cells were infected with VSV at 36 h after transfection.

Discussion

The immune system has evolved distinct virus recognition strategies. Viruses can enter cells by membrane fusion or through endocytosis. The viral dsRNA accumulated during replication of the viral genome is detected by the RNA helicase protein RIG-I or MDA5 in the cytoplasm (Andrejeva *et al*, 2004; Yoneyama *et al*, 2004). During late stage of infection, viral dsRNA is released upon lysis of infected cells and recognized by TLR3 (Alexopoulou *et al*, 2001; Levy and Marie, 2004). These distinct virus recognition strategies trigger signaling pathways that converge on activation of the transcription factors NF-κB and IRF-3, leading to transcription of the antiviral type I IFNs. In these converged pathways, two noncanonical IKK family members, IKKε and TBK1, phosphorylate IRF-3 and are critically involved in virus-triggered TLR3-independent and TLR3-mediated induction of type I IFNs (Fitzgerald *et al*, 2003; Hoebe *et al*, 2003; Oshiumi *et al*, 2003; Sharma *et al*, 2003; Yamamoto *et al*, 2003; Han *et al*, 2004; Hemmi *et al*, 2004; McWhirter *et al*, 2004; Perry *et al*, 2004).

Although it is clear that IKKε and TBK1 play essential roles in antiviral responses in various cell types, how their activities are regulated is unknown. To get insight into this, we searched for additional IKKε-interacting proteins by the yeast two-hybrid system. This effort identified SIKE, an evolutionarily conserved protein.

In mammalian overexpression systems, SIKE interacts with both IKKε and TBK1 (Figure 2A). These interactions are mediated through their respective coiled-coil motifs (Figure 2D). We further demonstrated that SIKE is associated with TBK1 in untransfected cells. Upon viral infection or TLR3 engagement, SIKE is dissociated from the TBK1 complex as suggested by co-immunoprecipitation and gel filtration experiments (Figure 2B and E). Since IKKε is undetectable under physiological conditions in most cell types (Shimada *et al*, 1999; Peters *et al*, 2000), an endogenous interaction between SIKE and IKKε is not confirmed. It is possible that SIKE is associated with IKKε when IKKε is induced by certain stimuli.

In mammalian overexpression system, SIKE blocked the interactions of IKKε or TBK1 with TRIF, RIG-I and IRF-3 (Figures 4 and 6), signaling components required for TLR3-mediatd or virus-triggered IRF-3 activation (Yamamoto *et al*, 2002; Honda *et al*, 2003; Levy and Marie, 2004; Yoneyama *et al*, 2004). In reporter gene assays, SIKE inhibited activation of ISRE and the IFN-β promoter mediated by IKKε, TBK1, TRIF and RIG-I, as well as triggered by poly(I:C) and VSV (Figures 3 and 5), while knockdown of SIKE potentiated activation of ISRE and the IFN-β promoter triggered by poly(I:C) and VSV infection (Figure 7). In VSV replication experiments, SIKE inhibited IKKε- and TBK1-mediated antiviral response, while knockdown of SIKE potentiated IKKε- and TBK1-mediated antiviral state (Figure 8).

SIKE does not disrupt the interactions of TRIF with TRAF6 and RIP (Figure 4C), components required for TLR3-mediated NF-κB activation (Hoebe *et al*, 2003; Sato *et al*, 2003; Han *et al*, 2004; Jiang *et al*, 2004; Meylan *et al*, 2004). Consistently, SIKE does not inhibit NF-κB activation triggered by TNF, poly(I:C) and VSV. These results suggest that SIKE specifically inhibits IKKε- and TBK1-mediated ISRE activation pathways.

Since SIKE is associated with TBK1 under physiological conditions and dissociated with TBK1 upon TLR3 engagement or viral infection (Figure 2), the simplest explanation for our observations is that SIKE functions as a physiological suppressor of IKKε and TBK1 by sequestering IKKε and TBK1 in inactive complexes. SIKE-mediated regulation of signaling is not caused by change of its protein level, because SIKE protein level was not significantly changed following TLR3 engagement or VSV infection (Figure 2B and data not shown). Although the experiments carried out in this study are not enough to offer a precise explanation on the regulatory mechanisms of SIKE in TLR3-mediated or virus-triggered signaling, some plausible scenarios can be envisioned. For example, upon TLR3 engagement or viral infection, the recruitment of TRIF to TLR3, or binding of RIG-I to dsRNA, may cause their conformational changes and thus provide a high-affinity binding site for IKKε/TBK1, causing dissociation of SIKE with IKKε/TBK1 and subsequent activation of IKKε/TBK1. Alternatively, poly(I:C) engagement or viral infection may directly signal certain post-translational modifications or conformational changes of IKKε/TBK1 or SIKE, causing their dissociation and enabling the association of IKKε/TBK1 with TRIF or RIG-I.

Since type I IFNs play a vital role in innate antiviral response and provide potent regulatory interactions with the adaptive immune system for efficient virus elimination, they are tightly regulated at transcription level. Expression of type I IFNs is nearly undetectable in uninfected individuals, but rapidly induced to high concentrations following infection. The identification of a physiological suppressor of IKKε and TBK1 provides an explanation on the inability of these kinases to activate the IFN pathways under physiological conditions.

Materials and methods

Reagents
Mouse monoclonal and rabbit polyclonal antibodies against Flag and HA epitopes (Sigma, St Louis, MO), and rabbit polyclonal antibodies against NAK (TBK1) (eBioscience 14-6351) were purchased from the indicated manufacturers. VSV was provided by Dr Hong-Kui Deng. Mouse anti-SIKE and anti-IKKε antisera were raised against recombinant human SIKE(aa26–207) and IKKε proteins.

Yeast two-hybrid screening
To construct an IKKε bait vector, a cDNA fragment encoding full-length human IKKε was inserted in-frame into the Gal4 DNA-binding domain vector pGBT (Clontech, Palo Alto, CA). A human B-cell cDNA library (ATCC, Manassas, VA) was screened following protocols recommended by Clontech.

Immunofluorescent staining
The 293 cells cultured on glass coverslips were plunged sequentially into methanol and acetone at -20°C, each for 10 min. The cells were rehydrated in PBS, blocked with 1% bovine serum albumin in PBS for 15 min and stained with primary antibody in blocking buffer for 1 h at room temperature. The cells were rinsed with PBS and stained with Texas Red-conjugated Affinipure goat anti-mouse IgG (1:200 dilution) for 45 min at room temperature. The cells were then rinsed with PBS containing DAPI and mounted in Prolong Antifade (Molecular Probes). The cells were observed with a Leica DMR/XA immunofluorescent microscope using a ×100 plan objective.

Northern blot analysis
Human multiple tissue mRNA blot was purchased from Clontech. The blot was hybridized with ^{32}P-dCTP-labeled cDNA probe corresponding to human SIKE coding sequence. Hybridization was performed in the Rapid Hybridization Buffer (Clontech) under high stringency conditions.

Constructs
Mammalian expression plasmid pcDNA3-Flag-IKKε was provided by Dr Tom Maniatis. Mammalian expression plasmids for SIKE and its deletion mutants were constructed by PCR amplification of SIKE cDNA from human B-cell library and subsequently cloning into CMV promoter-based vectors containing an HA or Flag tag. HA-IKKε and its deletion mutants were constructed by PCR amplification of the IKKε cDNA from the pcDNA3-Flag-IKKε plasmid and subsequently cloning into a CMV promoter-based vector containing a tag. TBK1 deletion mutants were constructed by PCR amplification of the TBK1 cDNA from the PRK-Flag-TBK1 plasmid and subsequently cloning into a CMV promoter-based vector containing a tag. Mammalian expression plasmids for Flag-IKKα, Flag-TBK1, HA-TBK1, Flag-RIP, Flag- and HA-TRIF, Flag-TRAF6, Flag-IRF-3, Flag-RIG-1 and its deletion mutants were described previously (Hu *et al*, 1999; Bin *et al*, 2003; Han *et al*, 2004). ISRE-luciferase reporter plasmid was purchased from Stratagene (West Cedar Creek, TX). NF-κB luciferase reporter plasmid was provided by Dr Gary Johnson. Human IFN-β promoter luciferase reporter plasmid was described previously (Han *et al*, 2004).

Transfection and reporter gene assays
The 293 cells ($\sim 1 \times 10^5$) were seeded on 12-well dishes and transfected the following day by standard calcium phosphate precipitation. In the same experiment, where necessary, 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 pRL-TK *Renilla* luciferase reporter plasmid was added to each transfection. Approximately 16 h after transfection, luciferase assays were performed using a dual-specific luciferase assay kit (Promega, Madison, WI). Firefly luciferase activities were normalized based on *Renilla* luciferase activities. All reporter gene assays were repeated for at least three times. Data shown were average values $\pm$ s.d. from one representative experiment.

Co-immunoprecipitation and Western blot analysis
For transient transfection and co-immunoprecipitation experiments, 293 cells ($\sim 1 \times 10^6$) were transfected for 24 h. The transfected cells 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, 1 mM phenylmethylsulfonyl fluoride). For each immunoprecipitation, 0.4 ml aliquot of lysate was incubated with 0.5 μg of the indicated antibody or control IgG and 25 μl of a 1:1 slurry of GammaBind G Plus-Sepharose (Amersham Biosciences) for 2 h. The Sepharose beads were washed three times with 1 ml of lysis buffer containing 500 mM NaCl. The precipitates were analyzed by Western blots as described (Huang *et al*, 2004).

For endogenous co-immunoprecipitation experiments, cells (5×10^7) were treated with poly(I:C) (50 μg/ml) for 10 min or infected with VSV virus for 4 h or left untreated. Cells were then lysed in 5 ml lysis buffer and the lysate was incubated with 1 μl of the indicated antiserum or preimmune control serum. The subsequent procedures were carried out as described above.

Size-exclusion chromatography
Cells (2×10^8) were lysed in 1.5 ml lysis buffer. The lysate was centrifuged for 1 h at 15 000 r.p.m. The supernatant was recovered and loaded onto a Superdex 200 gel filtration chromatography column pre-equilibrated with lysis buffer. The samples were eluted from the column in lysis buffer at a flow rate of 0.5 ml/min and collected in fractions of 1 ml. The fractions were precipitated with 10% trichloroacetic acid and analyzed by Western blots with antibodies against IKKε, TBK1 and SIKE, respectively.

RNAi experiments
Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro RNAi plasmid (Oligoengine Inc.). The following sequences were targeted for human SIKE cDNA: (1) GAGGATGCATCCGATATGA; (2) AGCTCACCAGTCTCACTCT; (3) GGAACTTCGAGAATTATTG; (4) GGACATGTCCAAATACAAA; and (5) ATACAAACCTCACATTCTG.

RT–PCR
Total RNA was isolated from 293 cells using Trizol reagent (Tianwei, China) and subjected to RT–PCR analysis to measure expression of IFN-β, Rantes and GAPDH according to the manufacturer's instructions. Gene-specific primer sequences were as follows: IFN-β, 5′-CAGCAATTTTCAGTGTCAGAAGCT-3′ and 5′-TCATCCTGTCCTTGAGGCAGTAT-3′; Rantes, 5′-ATGAAGGTCTCCGCGGCACGCCT-3′ and 5′-CTAGCTCATCTCCAAAGAGTTG-3′; and GAPDH, 5′-AAAATCAAGTGGGGCGATGCT-3′ and 5′-GGGCAGAGATGATGACCCTTT-3′.

VSV plaque assay
The 293 cells (1×10^5) were transfected with the indicated plasmids for 24 or 36 h prior to VSV infection (MOI of 10.0). At 1 h after infection, cells were washed with PBS for three times and then medium was added. The supernatants were harvested at 0, 1, 3, 6, 9 and 12 h after washing. The supernatants were diluted $1:10^6$ and then used to infect confluent BHK21 cells cultured on 24-well plates. At 1 h postinfection, supernatant was removed and 3% methylcellulose was overplayed. At 3 days postinfection, overlay was removed, cells were fixed with 4% formaldehyde for 1 h and stained with 0.2% Crystal violet in 20% methanol. Plaques were counted, averaged and multiplied by the dilution factor to determine viral titer as PFU/ml.

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