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SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK

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The expression of antiviral genes during infection is controlled by inducible transcription factors such as IRF3 (interferon regulatory factor). Activation of IRF3 requires its phosphorylation by TBK1 (TANK-binding kinase) or IKKi (inhibitor of nuclear factor κB kinase, inducible). We have identified a new and essential component of this pathway, the adaptor protein SINTBAD (similar to NAP1 TBK1 adaptor). SINTBAD constitutively binds TBK1 and IKKi but not related kinases. Upon infection with Sendai virus, SINTBAD is essential for the efficient induction of IRF-dependent transcription, as are two further TBK1 adaptors, TANK and NAP1. We identified a conserved TBK1/IKKi-binding domain (TBD) in the three adaptors, predicted to form an α-helix with residues essential for kinase binding clustering on one side. Isolated TBDs compete with adaptor binding to TBK1 and prevent poly(I:C)-induced IRF-dependent transcription. Our results suggest that efficient signal transduction upon viral infection requires SINTBAD, TANK and NAP1 because they link TBK1 and IKKi to virus-activated signalling cascades.
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Introduction

The innate immune system employs two complementary strategies to detect the presence of viruses. One relies on the detection of extracellular virions and initiates signalling without a requirement for the virus-detecting cell to become infected. This pathway is exemplified by Toll-like receptors (TLRs), which survey the contents of endosomes or phagosomes for viral nucleic acids. TLR3, for instance, recognizes double-stranded RNA (dsRNA) (Alexopoulou *et al*, 2001) and initiates signalling via recruitment of the adaptor protein TRIF (TIR domain-containing adaptor inducing interferon β) (also known as TICAM1/Lps2) (Yamamoto *et al*, 2002; Hoebe *et al*, 2003; Oshiumi *et al*, 2003). The other surveillance strategy is to scrutinize the intracellular compartment and to take advantage of the much wider range of viral products created during infection. RNA helicases, for example RIG-I and MDA-5, are thought to recognize replication intermediates of single-stranded RNA viruses (Yoneyama *et al*, 2004; Kato *et al*, 2005; Gitlin *et al*, 2006). Like TLR3 signalling, RIG-I and MDA-5 activation also establishes an antiviral state. However, instead of engaging with TRIF, RIG-I and MDA-5 require participation of the mitochondrial antiviral signalling protein (MAVS) (also known as IPS-1/VISA/CARDIF) for signalling (Kawai *et al*, 2005; Meylan *et al*, 2005; Seth *et al*, 2005; Xu *et al*, 2005). Upon detection of a virus, innate antiviral responses are launched through the coordinated activation of several transcription factors, in particular of the NF-κB (nuclear factor-κB) and IRF (interferon regulatory factor) families. In mammals, the NF-κB family comprises five members, all containing a Rel DNA-binding domain to activate promoters bearing κB elements (Hayden and Ghosh, 2004). Similarly, the nine mammalian members of the IRF family carry helix–loop–helix DNA-binding domains for induction of promoters containing interferon-stimulated response elements (ISRE) (Honda and Taniguchi, 2006).

In resting cells, the transactivating potential of NF-κB and IRFs are kept under tight control. Under such circumstances NF-κB occurs in a complex with I-κB (inhibitor of NF-κB), thereby preventing its accumulation in the nucleus. Exposure to viruses and other agonists induces phosphorylation of I-κB by the I-κB kinase (IKK) complex, followed by degradation of I-κB and release of NF-κB from sequestration (Hacker and Karin, 2006). The IKK complex comprises two related kinases, IKKα and IKKβ (also known as CHUK/IKK1 and IKK2), and an unrelated adaptor NEMO (alternatively called IKKγ/IKKAP/FIP3) (Connelly and Marcu, 1995; DiDonato *et al*, 1997; Mercurio *et al*, 1997, 1999; Regnier *et al*, 1997; Woronicz *et al*, 1997; Zandi *et al*, 1997; Rothwarf *et al*, 1998; Yamaoka *et al*, 1998; Li *et al*, 1999c). Mice deficient for IKKβ fail to activate NF-κB in response to most stimuli, including viral exposure (Tanaka *et al*, 1999; Li *et al*, 1999b, 1999d), whereas deficiency in IKKα prevents NF-κB activation only in response to a limited number of agonists, notably not viral exposure (Hu *et al*, 1999; Takeda *et al*, 1999; Li *et al*, 1999a).

Two further kinases of the IKK family, TBK1 (TANK-binding kinase 1) and IKKi (inhibitor of nuclear factor κB kinase; also referred to as NAK/T2K and IKKε, respectively) mediate TRIF- and MAVS-dependent IRF activation (Pomerantz and Baltimore, 1999; Shimada *et al*, 1999; Bonnard *et al*, 2000; Peters *et al*, 2000; Tojima *et al*, 2000; Fitzgerald *et al*, 2003). These kinases directly phosphorylate IRF3, thereby promoting its dimerization and nuclear accumulation (Sharma *et al*, 2003). TBK1 and IKKi are partly redundant for the activation of IRF3, as single knockout mice display no defect (IKKi−/−) or a significant but still incomplete (TBK1−/−) impairment of IRF3 activation, whereas mice deficient in both genes are more severely affected (Bonnard *et al*, 2000; Hemmi *et al*, 2004; McWhirter *et al*, 2004; Perry *et al*, 2004; Matsui *et al*, 2006).

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The IKKα/IKKβ kinase complex requires the presence of an adaptor subunit, NEMO, to function in NF-κB signalling. Deficiency in NEMO causes incontinentia pigmenti, characterized by early lethality owing to TNF-α-induced liver failure (Makris *et al*, 2000; Rudolph *et al*, 2000; Schmidt-Supprian *et al*, 2000; Smahi *et al*, 2000). NEMO connects the kinases to upstream signals. It employs its C-terminal zinc finger and adjacent coil-zipper domain for signal reception and its N-terminal domain to bind the kinases (Hacker and Karin, 2006). Impairment of either function abrogates NF-κB activation, as demonstrated by mutations in the signal receiving domains in patients with anhidrotic ectodermal dysplasia with immunodeficiency (Zonana *et al*, 2000; Doffinger *et al*, 2001) and by interference with kinase binding using competing peptides (May *et al*, 2000).

In contrast, less is known about the adaptor proteins of TBK1 and IKKi. Two proteins, TANK and NAP1, were described to specifically bind TBK1 and IKKi (Pomerantz and Baltimore, 1999; Fujita *et al*, 2003). NAP1 is required for poly(I:C) and virus-induced IRF activation (Sasai *et al*, 2005, 2006) while no information on the role of TANK in IRF signalling has been published. While this paper was under review, an antiviral role for TANK has been described (Guo and Cheng, 2007).

In this study, we describe a novel component of the antiviral IRF3 signalling pathway, which we named SINTBAD (similar to NAP1 TBK1 adaptor). Using RNA interference, we demonstrate that SINTBAD is required for efficient IRF activation by Sendai virus. SINTBAD binds TBK1 and IKKi via a newly identified TBK1/IKKi-binding domain (TBD), which it shares with TANK and NAP1. Binding to kinases via TBD is essential for poly(I:C)-induced IRF activation. We propose that SINTBAD, NAP1 and TANK connect TBK1/IKKi to virus-activated signalling cascades.

Results

Identification of SINTBAD as a TBK1/IKKi-binding protein

While performing BLAST searches with components of the IRF3 signalling pathway, we discovered homology between NAP1 and KIAA0775, a protein of 596 aa, which we named SINTBAD (Figure 1A). Orthologues of SINTBAD and NAP1 were found in several vertebrates, including fish and human, but were notably absent in lower organisms. Northern blot analysis revealed SINTBAD to be expressed in a wide range of tissues (Figure 1B). SINTBAD, like NAP1, contains three regions predicted to form coiled-coils (CC) (Figure 1C). Sequence similarity between SINTBAD and NAP1, however, is limited to their N-terminal 80 aa. Their region of similarity is therefore distinct from the one shared by NAP1 and TANK, which encompasses CC2 in NAP1 and the singular CC region in TANK. The C-terminal half of SINTBAD contains a proline-rich region and harbours two C2H2 type zinc fingers with similarity to the singular zinc-finger of TANK. Thus, SINTBAD, NAP1 and TANK share distinct regions of homology with each other in a pairwise fashion, that is, each protein contains only two of the three conserved domains.

These sequence similarities prompted us to test whether SINTBAD would also bind kinases of the IKK family. Using LUMIER assays (Barrios-Rodiles *et al*, 2005), we detected specific binding of SINTBAD to TBK1 or IKKi but not to IKKα or IKKβ (Figure 2A). An identical pattern of binding occurred for NAP1 and TANK, while NEMO showed opposite preferences and bound IKKα or IKKβ but not TBK1 or IKKi. These binding patterns are in agreement with previous reports for NAP1, TANK and NEMO and also confirm the binding of TBK1 to KIAA0775, that is SINTBAD, observed in a large-scale proteomics study (Rothwarf *et al*, 1998; Yamaoka *et al*, 1998; Pomerantz and Baltimore, 1999; Fujita *et al*, 2003; Bouwmeester *et al*, 2004).

The ability of SINTBAD to bind selectively only certain members of the IKK family was confirmed using an MBP-SINTBAD (86–330 aa) fusion protein expressed in *Escherichia coli*. The fusion protein specifically precipitated TBK1 but not IKKα from lysates of 293 cells (Figure 2B).

To analyse the interactions between endogenous proteins, we generated a polyclonal antiserum against SINTBAD. In agreement with our LUMIER data, endogenous SINTBAD bound specifically to endogenous TBK1 or IKKi but not to IKKα or IKKβ in 293ET (Figure 2C) and RAW264 cells (data

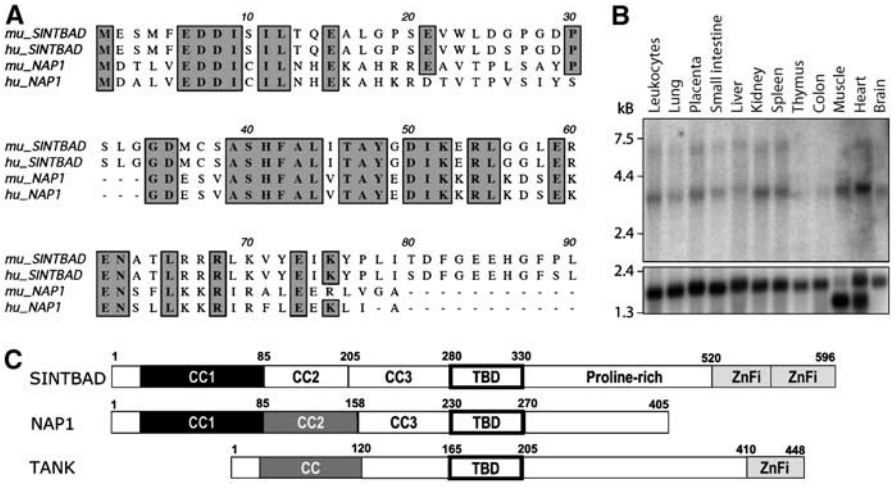

Figure 1 Identification of SINTBAD. (**A**) Alignment of N termini of human and murine SINTBAD and NAP1. (**B**) Northern blot of human tissues probed for SINTBAD (upper panel) and β-actin (lower panel). (**C**) Domain composition of SINTBAD, NAP1 and TANK. Regions with sequence similarity are highlighted. CC—coiled coils forming domain, TBD—TBK1/IKKi-binding domain as identified in this study; ZnFi—C_2H_2 type zinc finger.

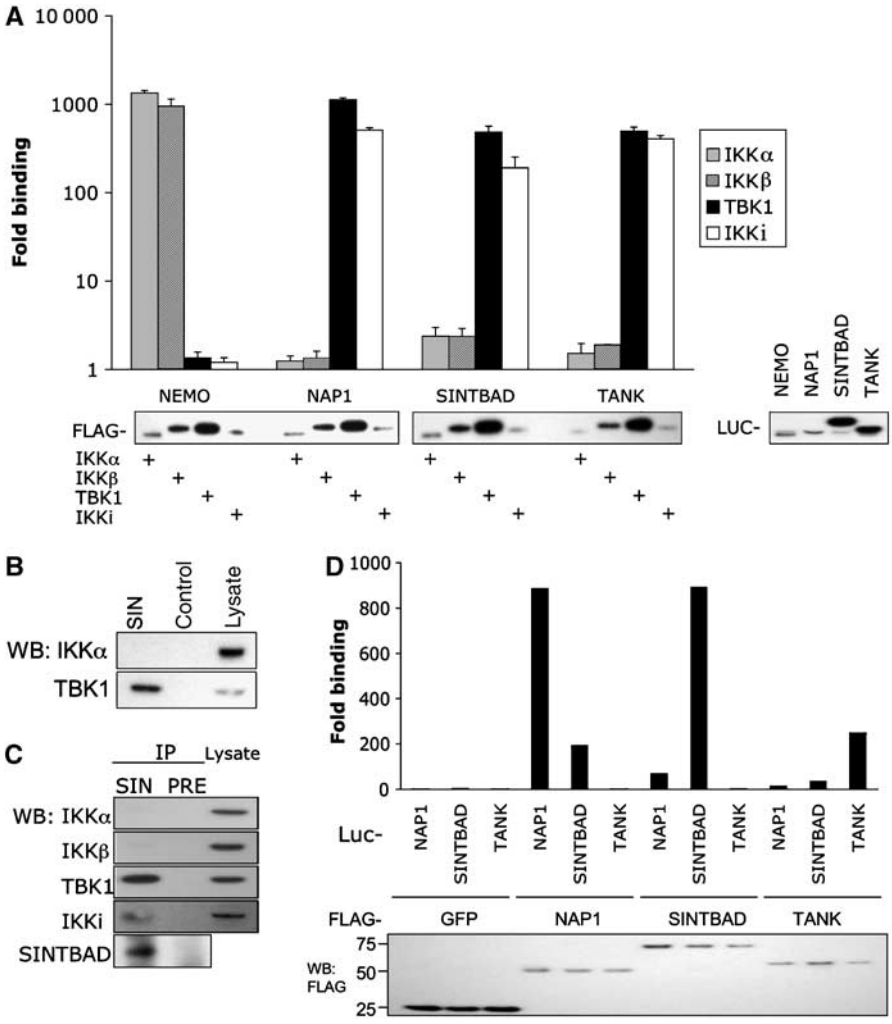

Figure 2 SINTBAD is a TBK1/IKKi adaptor. (**A**) SINTBAD interacts with TBK1 and IKKi. Lysates from 293ET cells transfected with the indicated combination of luciferase-tagged NEMO, NAP1, SINTBAD, TANK and FLAG-tagged IKKα, IKKβ, TBK1, IKKi were precipitated using anti-FLAG agarose. Fold binding indicates the ratio of luciferase activity in precipitates and lysates. The expression of proteins in lysates was detected by Western blotting. (**B**) Recombinant SINTBAD binds TBK1. Lysates of 293ET cells were precipitated using MBP-SINTBAD (86–330 aa) immobilized on amylose resin. Uncharged resin was used as a control. Lysates and eluates were blotted for the presence of IKKα and TBK1. (**C**) SINTBAD binds TBK1 and IKKi at endogenous levels. Lysates of 293ET cells were immunoprecipitated using a polyclonal antiserum against SINTBAD (SIN) or a preimmune serum (PRE). Lysates and precipitates (IP) were blotted for the presence of IKKα, IKKβ, TBK1, IKKi and SINTBAD. (**D**) Oligomerization of NAP1, SINTBAD and TANK. Lysates from 293ET cells transfected with the indicated combination of luciferase-tagged NAP1, SINTBAD, TANK and FLAG-tagged GFP, NAP1, SINTBAD, TANK were precipitated using anti-FLAG agarose. Fold binding indicates the ratio of luciferase activity in precipitates and lysates. The expression of proteins in lysates was detected by Western blotting.

not shown). Taken together, we have demonstrated the occurrence of specific interactions between SINTBAD and TBK1 or IKKi and have proven the existence of such complexes under physiological conditions.

TBK1 and IKKi form high molecular weight complexes *in vivo*. We wondered whether the adaptors contributed to complex formation only by binding the kinases or whether they would also form oligomers with each other. Using LUMIER assays, we detected homo-oligomers of SINTBAD, NAP1 and TANK (Figure 2D). A heterocomplex consisting of SINTBAD and NAP1 was also detected, although its formation was less favoured than either of the homo-oligomers. No significant binding between TANK and SINTBAD or NAP1 was detected. These data suggest that interactions between the adaptors contribute to the assembly of the high molecular weight kinase complexes.

Knockdown of adaptors inhibits virus-induced IRF activation

To test the functional importance of the adaptor proteins for IRF signalling, a panel of newly designed small hairpin RNAs (shRNAs) was used to deplete NAP1, SINTBAD and TANK from cells (Figure 3A). For TBK1, TRIF and MAVS we relied on shRNAs that were previously reported to specifically downregulate their respective targets (Figure 3A) (Fitzgerald *et al*, 2003; Yamamoto *et al*, 2003; Xu *et al*, 2005). Using three different IRF3 inducible reporters, we show that knockdown of TRIF or GFP had no significant effect on Sendai virus-induced IRF activity whereas knockdown of TBK1 or MAVS caused a substantial reduction of IRF activation (Figure 3B–D). Thus, as expected, activation of IRF by TBK1 upon Sendai virus infection required the presence of MAVS but not TRIF in the cells. Knockdown of SINTBAD as well as NAP1 or

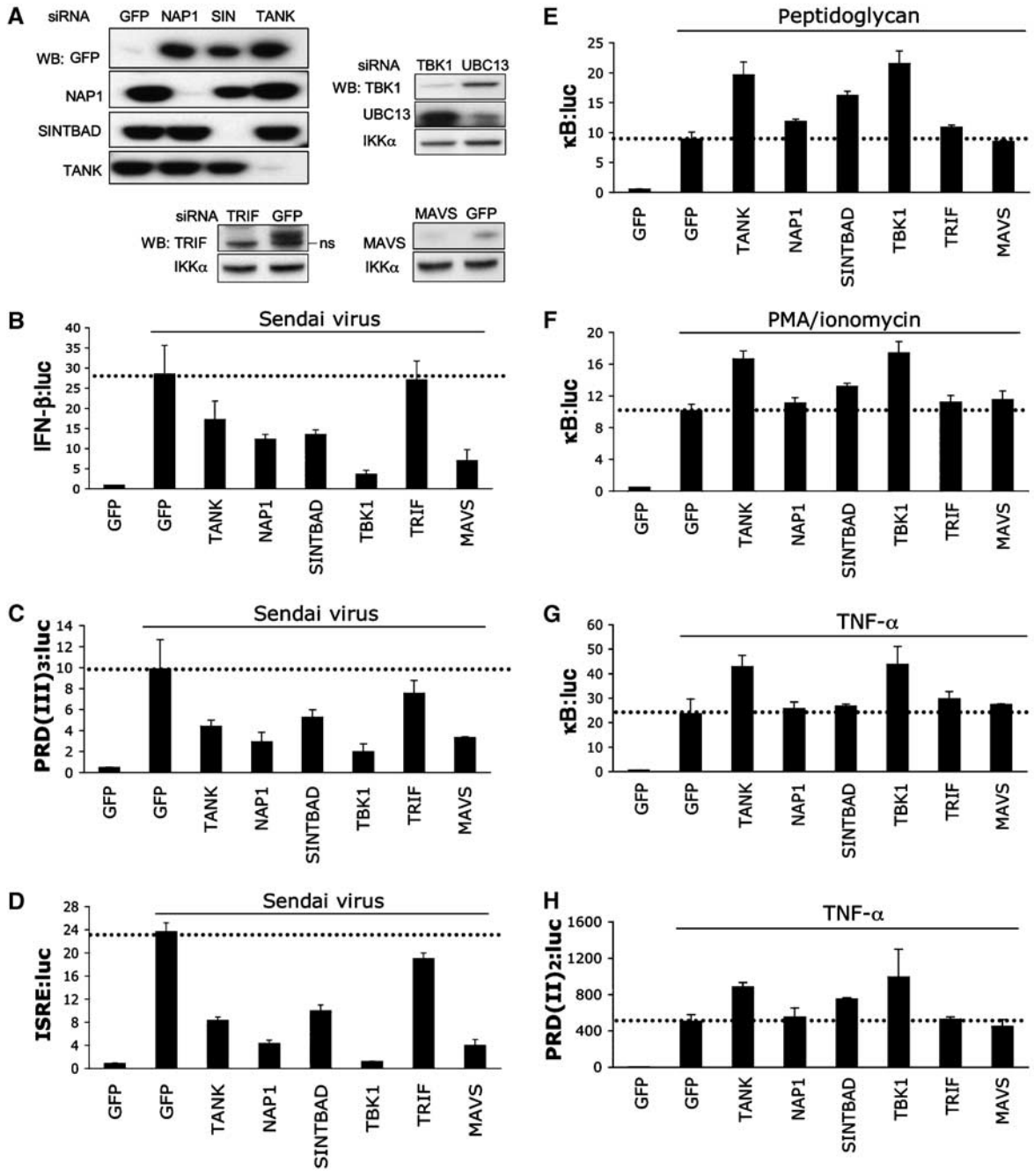

Figure 3 Importance of TBK1 adaptors for Sendai virus-induced IRF but not NF-κB activation. (**A**) Lysates from cells transfected with the indicated combinations of shRNAs and FLAG-tagged GFP, NAP1, SINTBAD, TANK, UBC13, TRIF and MAVS were blotted for the presence of FLAG-tagged proteins. Blots for TBK1 and IKKα detected endogenous protein. (**B–D**) Cells were transfected with the indicated shRNAs and different IRF-dependent luciferase reporters: IFN-β (B), PRD (III-I)$_3$ (C) or ISRE (D). Two days after transfection, cells were stimulated with or without 150 HAU/ml of Sendai virus. Luciferase activity was measured 16 h later. (**E–H**) Cells were transfected with the indicated shRNAs and different NF-κB-dependent luciferase reporters: κB (E–G) and PRD(II)$_2$ (H). Two days after transfection, cells were stimulated with 10 μg/ml of peptidoglycan (E), 50 ng/ml PMA and 1 μM ionomycin (F) or 10 ng/ml of TNF-α (G and H). Luciferase activity was measured 16 h later.

TANK also inhibited Sendai virus-induced activation of the IRF inducible reporter constructs. As a control, we tested the consequences of knockdowns on NF-κB signalling (Figure 3E–H). In agreement with previous studies (Fitzgerald *et al*, 2003; Yamamoto *et al*, 2003; Xu *et al*, 2005), knockdowns of TBK1, MAVS or TRIF did not suppress NF-κB activation by TNF-α, peptidoglycan or PMA. Similarly, knocking down SINTBAD, NAP1 or TANK did not block agonist-induced NF-κB activation either and in some cases even accentuated NF-κB activation. We conclude that upon Sendai virus infection, SINTBAD as well as NAP1, TANK, TBK1 and MAVS are required for IRF activation.

To test the physiological relevance of the reporter-based findings, we investigated whether the regulation of endogenous antiviral genes also required the adaptors (Figure 4). Knockdown of SINTBAD, NAP1 and TANK, as well as knockdown of TBK1 abrogated the expression of IP-10 and ISG15 in response to Sendai virus infection. In contrast, knockdown of

UBC13, which is not required for IRF activation (Yamamoto *et al*, 2006), did not prevent the induction of antiviral genes by Sendai virus.

Identification of a TBK1/IKKi binding domain in SINTBAD, NAP1 and TANK

To gain mechanistic insight into the functioning of the kinase complex, we next sought to localize the regions in SINTBAD, NAP1 and TANK required for homo-oligomerization and for binding to TBK1 and IKKi. Using deletion mutants of SINTBAD and NAP1 in LUMIER assays (Figure 5A and B), we found the CC regions of SINTBAD (1–280 aa) and NAP1 (1–198 aa) were required for homo-oligomerization of the adaptors (Figure 5C and D), but were dispensable for TBK1 binding (Figure 5E and F). Instead, aa 280–330 of SINTBAD and 200–270 of NAP1 were essential for binding to TBK1. An alignment of these regions revealed sequence similarity between NAP1 and SINTBAD, which, interestingly, is present also in TANK (Figure 6A). Further analysis indicated that this region is encoded by a single exon (data not shown).

To directly test whether this region would be sufficient for binding to kinases, we performed LUMIER assays. NAP1 229–270, SINTBAD 280–330, TANK 166–205 bound as potent to TBK1 and IKKi as the respective full-length adaptors (Figure 6B). We conclude that the newly discovered region of homology in NAP1, SINTBAD and TANK is sufficient to mediate binding to TBK1 and IKKi. In the following, we will refer to it as TBD. (TBK1/IKKi binding domain).

A secondary structure prediction indicated TBD to acquire α-helical conformation with conserved residues clustering on one side of the helix (Figure 6C). Guided by this prediction, we created point mutations in NAP1 TBD to study the specificity of binding to TBK1 and IKKi (Figure 6E and F).

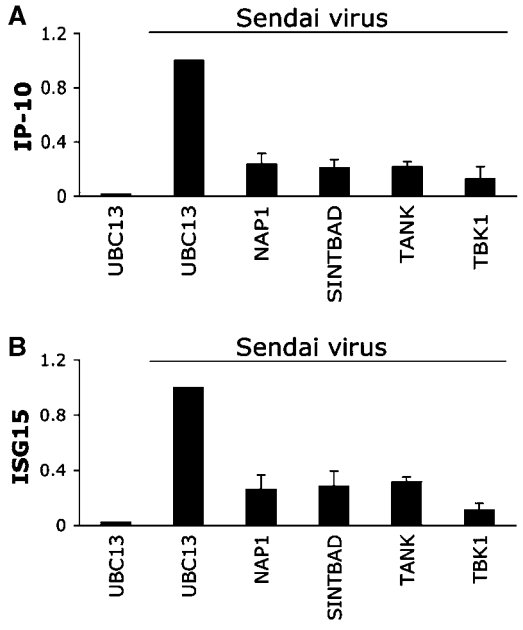

Figure 4 Importance of TBK1 adaptors for the induction of endogenous genes upon infection with Sendai virus. (**A, B**) Cells were co-transfected with GFP and the indicated shRNAs. Two days after transfection, cells were stimulated with or without 150 HAU/ml of Sendai virus for 16 h. mRNA levels of IP-10 (A) and ISG15 (B) were quantified from GFP^+ cells isolated by FACS.

Substitutions Q253A and L257S in NAP1 TBD prevented its interaction with TBK1 and IKKi, while introducing Y236A selectively abrogated binding to TBK1 but not IKKi. In contrast, substitutions W237A and E238A in NAP1 TBD did not interfere with binding to either kinase. These data suggest that TBK1 and IKKi bind the conserved surface of NAP1 TBD in a related but not identical manner.

Isolated TBDs interfere with poly(I:C)-induced IRF activation by blocking TBK1 binding to full-length adaptors

The identification of a conserved binding domain for TBK1 and IKKi in NAP1, SINTBAD and TANK enabled us to test whether interactions between kinases and adaptors via TBD are important for IRF signalling. We speculated that isolated TBDs would compete with full-length adaptors for binding to TBK1 and thereby act as dominant negative inhibitors of agonist-induced IRF activation.

We first measured binding of full-length adaptors to TBK1 in LUMIER assays using fusion constructs between YFP and TBDs as competitors (Figure 7A–C). All TBDs competed potently with binding of full-length adaptors to TBK1. However, while NAP1 TBD and SINTBAD TBD inhibited all adaptors equally well, TANK TBD competed more efficiently with SINTBAD or TANK than with NAP1. NAP1 TBD^{L257S}, chosen for its lack of binding to TBK1, did not to compete with full-length NAP1 for binding to TBK1 (Figure 7E). As a further control, none of the TBDs inhibited homo-oligomerization of TBK1 (Figure 7D).

We next tested the functional consequences of interrupting the binding of TBK1 to its adaptors on signal transduction. Expression of TBDs strongly attenuated poly(I:C)-induced IRF activation but did not reduce NF-κB-dependent reporter activation (Figure 8A and B). NAP1 TBD^{L257S}, chosen for its lack of TBK1 binding and the consequential inability to prevent adaptor recruitment to TBK1, did not attenuate poly(I:C)-induced IRF nor NF-κB activation (Figure 8C and D). Taken together, we conclude that TBDs mediate binding of adaptor proteins to TBK1/IKKi and that this interaction is essential for poly(I:C)-induced IRF signalling.

Discussion

Vertebrates have evolved sophisticated means of detecting and eradicating viruses, while viruses developed a multitude of strategies to interfere with host responses. Much progress has been made recently in identifying the receptors for the detection of viruses and their downstream signalling molecules. However, while we have gained an advanced understanding of the NF-κB pathway, including mechanistic insights into signal flow, our knowledge of IRF signalling is less developed. Here, we describe the discovery of SINTBAD as a novel component of innate antiviral immunity. We show that SINTBAD, like NAP1 and TANK, associates with TBK1 and IKKi and that the presence of all three adaptors is required for efficient IRF induction in response to Sendai virus. Binding of the adaptors to TBK1 and IKKi occurs via TBD, a predicted α-helix conserved between the adaptors, and this interaction is essential for antiviral signalling. We propose that SINTBAD, NAP1 and TANK bind TBK1 and IKKi via their TBDs to provide a functional link between the kinases and other signalling components.

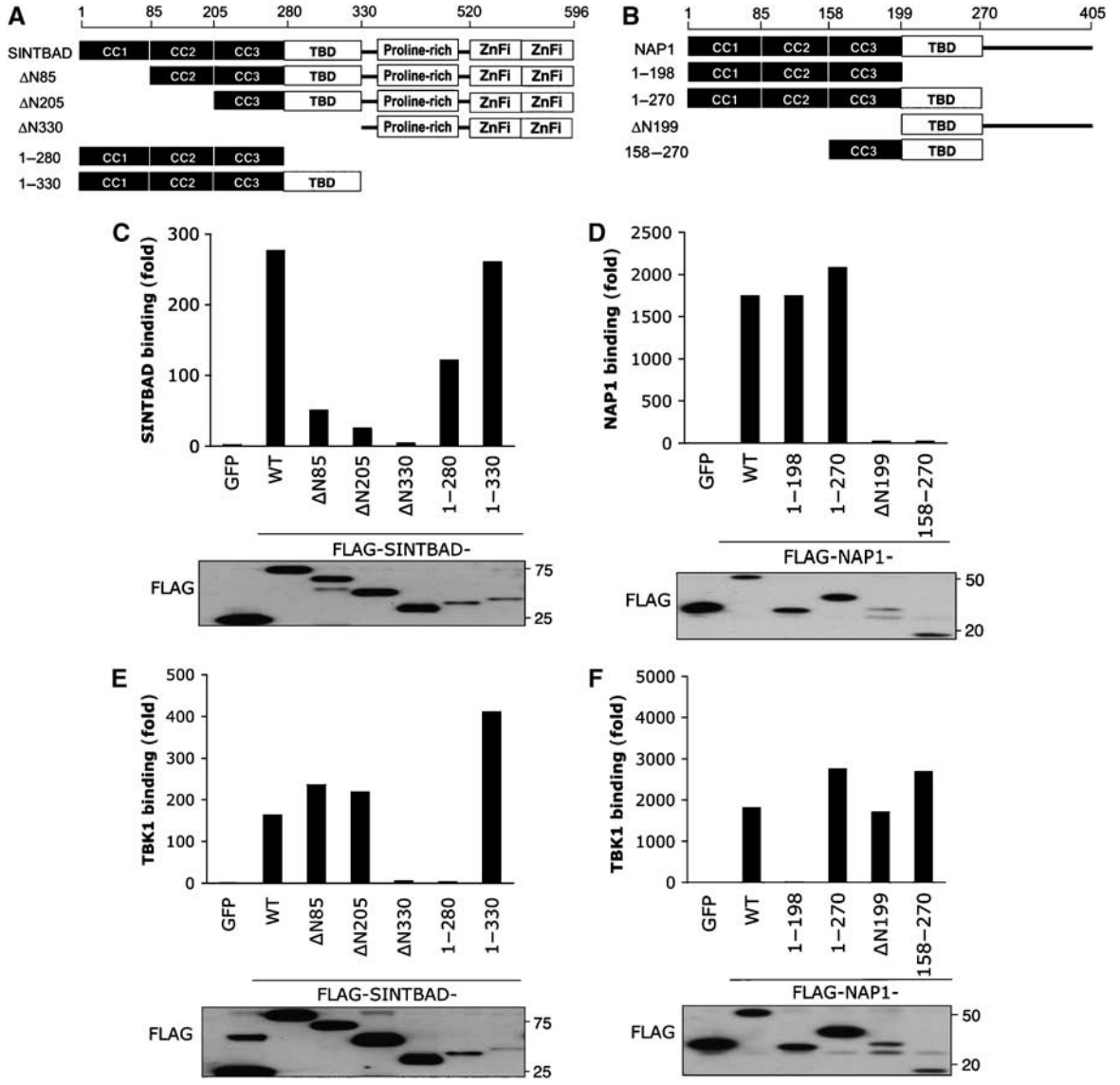

Figure 5 Identification of the TBK1-binding domain in SINTBAD and NAP1. (**A, B**) Schematic representation of deletions in SINTBAD (A) and NAP1 (B). (**C–F**) Cells were transfected with combinations of FLAG-tagged forms of SINTBAD (C and E), NAP1 (D and F) and luciferase-tagged SINTBAD (C), NAP1 (D) or TBK1 (E and F) as indicated. Cell lysates were precipitated using anti-FLAG agarose. Fold binding indicates the ratio of luciferase activity in precipitates and lysates normalized to the binding of GFP. The expression of proteins in lysates was detected by Western blotting.

TBK1 and IKKi activate IRF3 downstream of TLR3 as well as downstream of cytoplasmic RNA helicases, which survey the extracellular and intracellular compartment for the presence of dsRNA, respectively (Bonnard *et al*, 2000; Fitzgerald *et al*, 2003; Hemmi *et al*, 2004; McWhirter *et al*, 2004; Perry *et al*, 2004; Matsui *et al*, 2006). In our experiments, infection with Sendai virus activated IRF3-dependent reporter genes in a TBK1-dependent manner. As expected from previous studies (Yamamoto *et al*, 2003; Xu *et al*, 2005), knockdown of MAVS but not TRIF also abrogated the response, indicating that the recognition of Sendai virus was dependent on the intracellular but not the extracellular surveillance system. SINTBAD as well as TANK and NAP1 were found to be important for signalling during Sendai virus infection. This demonstrates participation of all three adaptors in the cytoplasmic surveillance pathway, confirming a report on NAP1 (Sasai *et al*, 2006). Similar experiments were performed with poly(I:C) to stimulate TLR3 signalling (GR, data not shown). As expected from previous studies (Yamamoto *et al*, 2003; Hemmi *et al*, 2004; McWhirter *et al*, 2004; Perry *et al*, 2004), TBK1 and TRIF were required for poly(I:C)-induced IRF induction. Knockdown of SINTBAD, TANK and NAP1 likewise diminished IRF inducible reporter activity. However, depletion of MAVS also impaired poly(I:C)-induced IRF reporter activity. This result prevents conclusions about specific contribution by the adaptors to the TRIF-dependent extracellular surveillance pathway. The situation appears typical for the use of poly(I:C) in experiments attempting to dissociate the TLR3 signalling pathway from other cascades. Although stimulation with poly(I:C) potently activates signalling, only part of this activation is TLR3-mediated as indicated by the remaining response in TLR3-deficient mice (Alexopoulou *et al*, 2001; Honda *et al*, 2003).

TBK1 and IKKi were initially thought to participate in NF-κB signalling, based on their homology to IKKα and IKKβ and

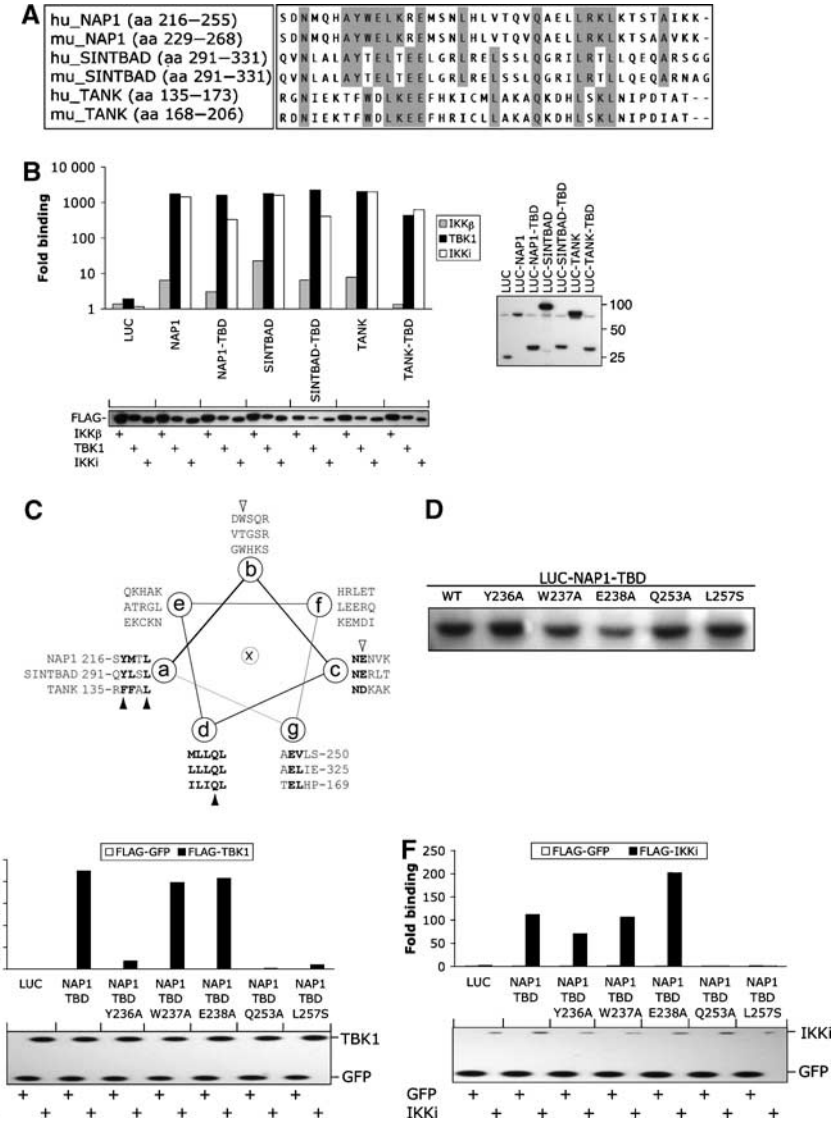

Figure 6 Characterization of the TBD. (**A**) Alignment of TBDs from murine and human TANK, NAP1 and SINTBAD. Borders of the alignment correspond to exon boundaries. (**B**) Cells were transfected with combinations of FLAG-tagged forms of IKKβ, TBK1, IKKi and luciferase-tagged NAP1, SINTBAD, TANK or their respective luciferase-tagged TBDs as indicated. Cell lysates were precipitated using anti-FLAG agarose. Fold binding indicates the ratio of luciferase activity in precipitates and lysates. The expression of proteins in lysates was detected by Western blotting. (**C**) Helical wheel representation of TBD. Human NAP1 (216–250 aa), SINTBAD (291–325 aa) and TANK (135–169 aa) are shown. Bold letters indicate positions conserved in all three adaptors. Positions required and dispensable for TBK1 binding are indicated by black and white arrowheads, respectively. (**D**) Lysates from cells transfected with luciferase-tagged NAP1 TBD carrying the indicated mutations were subjected to Western blotting. (**E, F**) Cells were transfected with different forms of luciferase-tagged NAP1 TBD and FLAG-tagged GFP, TBK1 (E) or IKKi (F) as indicated. Cell lysates were precipitated using anti-FLAG agarose. Fold binding indicates the ratio of luciferase activity in precipitates and lysates normalized to the binding of GFP. The expression of proteins in lysates was detected by Western blotting.

their ability to activate NF-κB upon overexpression (Pomerantz and Baltimore, 1999; Shimada *et al*, 1999; Peters *et al*, 2000; Tojima *et al*, 2000). Given their role as TBK1 adaptors, TANK and NAP1 have also been suggested to mediate NF-κB signalling (Pomerantz and Baltimore, 1999; Fujita *et al*, 2003). Gene targeting initially seemed to confirm a role for TBK1 in NF-κB signalling as TBK1−/− mice die from liver degeneration at E14.5 and are rescued from lethality when TNF-α signalling is prevented (Bonnard *et al*, 2000; Perry *et al*, 2004). However, analysis of cells derived from TBK1-deficient animals did not reveal any defect in NF-κB activation up to the level of DNA binding. Despite this, reduced reporter gene activity was observed initially (Bonnard *et al*, 2000) but could not be confirmed in subsequent studies (Hemmi *et al*, 2004; Perry *et al*, 2004). The lack of effect on NF-κB signalling in our knockdown experiments is therefore expected but provides an additional specificity control for the role of SINTBAD as well as TANK, NAP1, TBK1 and MAVS in Sendai virus-induced IRF signalling.

SINTBAD initially caught our attention because of the sequence similarity between its N terminus and NAP1. The C terminus of SINTBAD also displays sequence homology to TANK and, finally, in a distinct third region, homology exists between NAP1 and TANK. The three proteins hence share an interesting pattern of pairwise sequence homologies. The biological meaning of this pairwise domain sharing is unknown, but seems likely to be of functional significance.

The binding of TBK1 was known to require the N-terminal half of TANK and the region encompassing 158–270 aa of NAP1 (Pomerantz and Baltimore, 1999; Fujita *et al*, 2003),

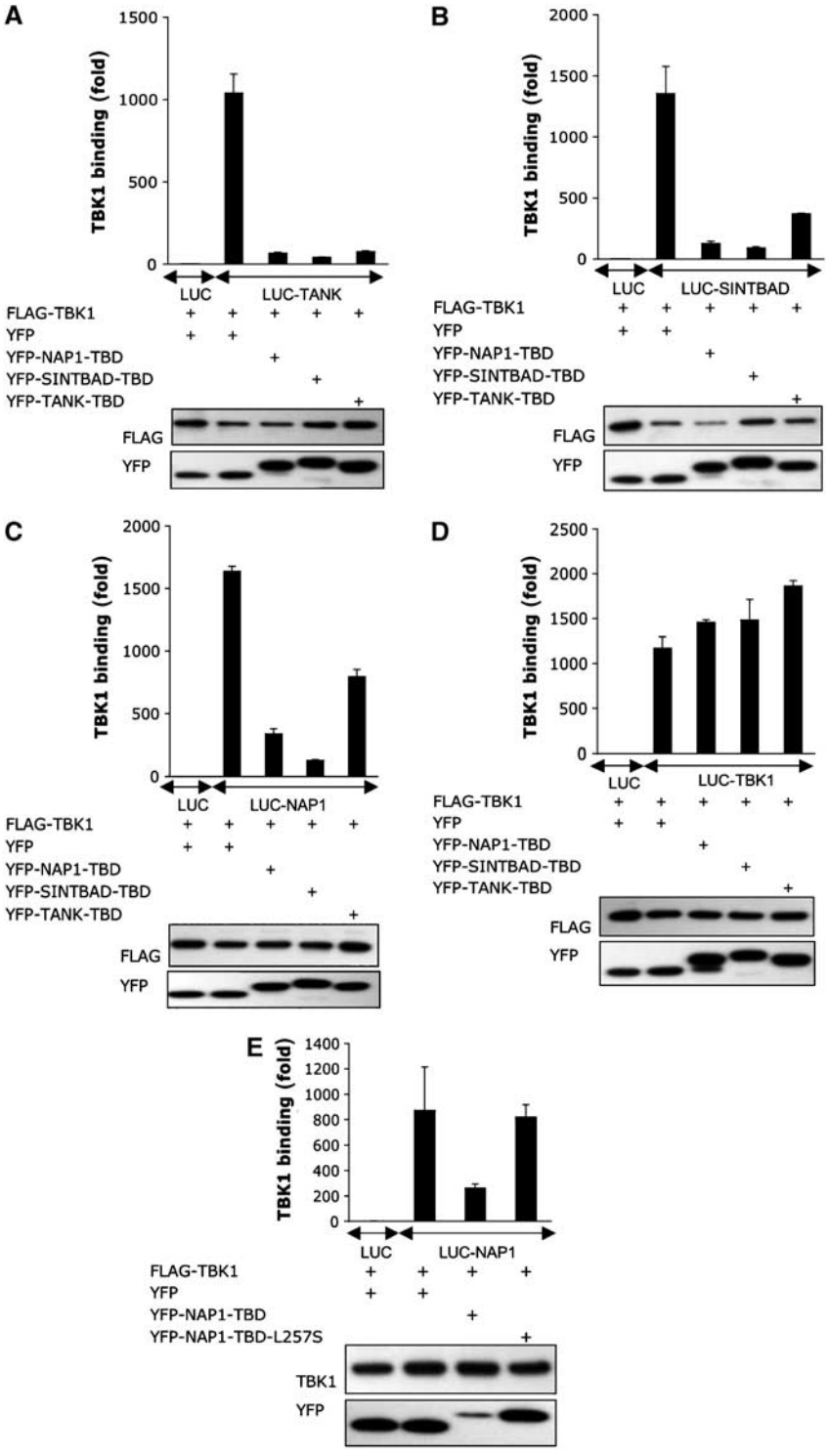

Figure 7 TBDs compete with binding of full-length adaptors to TBK1. (**A**–**D**) Cells were transfected with FLAG-tagged TBK1, YFP-tagged TBDs derived from NAP1, SINTBAD, TANK and luciferase-tagged TANK (A), SINTBAD (B), NAP1 (C), TBK1 (D) as indicated. Cell lysates were precipitated using anti-FLAG agarose. Fold binding indicates the ratio of luciferase activity in precipitates and lysates. The expression of proteins in lysates was detected by Western blotting. (**E**) Cells were transfected with FLAG-tagged TBK1, YFP-tagged NAP1 TBD (wt or L257S) and luciferase-tagged NAP1as indicated. Cell lysates were precipitated using anti-FLAG agarose. Fold binding indicates the ratio of luciferase activity in precipitates and lysates. The expression of proteins in lysates was detected by Western blotting.

thereby excluding the conserved N-terminal CC regions as binding sites. Further delineation of the TBK1-binding site in SINTBAD and NAP1 revealed a region of previously unnoticed sequence similarity present in all the three adaptors. This region, termed TBD, was sufficient for binding of TBK1 or IKKi. TBD is predicted to form an α-helix with conserved residues clustering on one side, suggesting the conserved face of the helix as binding site for the kinases. In support of this model, binding of TBK1 and IKKi to NAP1 TBD was abrogated by mutations targeted to the conserved

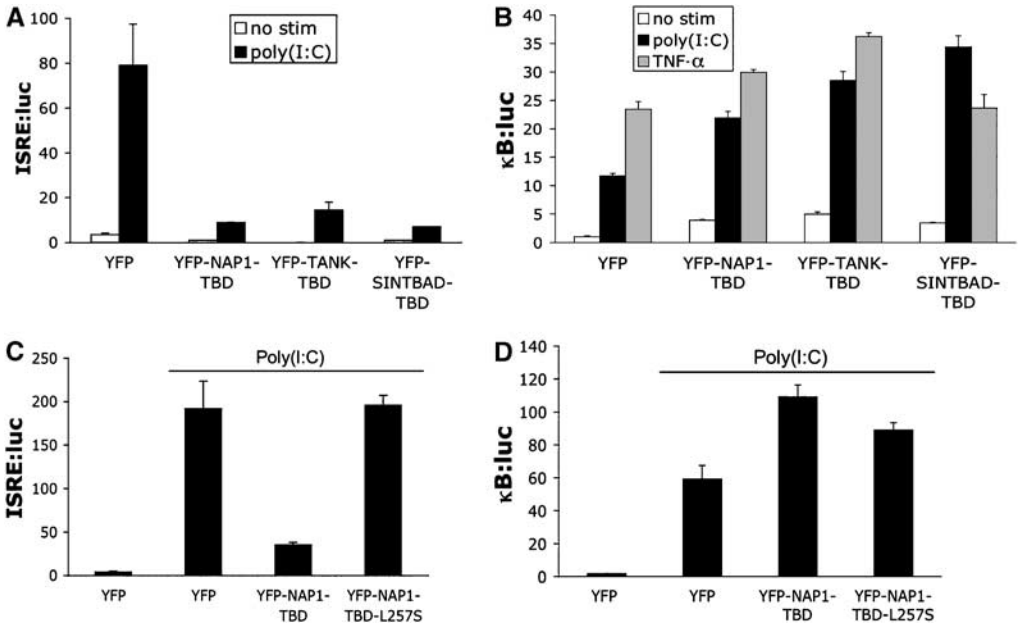

Figure 8 TBDs prevent poly(I:C)-induced IRF but not NF-κB activation. (**A, B**) Cells were transfected with IRF (A) or NF-κB (B)-dependent luciferase reporter genes and YFP-tagged TBDs derived from NAP1, TANK and SINTBAD. Two days after transfection, cells were stimulated with 12.5 μg/ml poly(I:C) or 10 ng/ml of TNF-α. Luciferase activity was measured 5 h later. (**C, D**) Cells were transfected with IRF (C) or NF-κB (D)-dependent luciferase reporter genes and YFP-tagged NAP1 TBD (wt or L257). Two days after transfection, cells were stimulated with 12.5 μg/ml poly(I:C). Luciferase activity was measured 5 h later.

side of NAP1 TBD. Interestingly, NAP1 TBD^{Y236A} selectively disturbed binding to TBK1 but not IKKi. Therefore, despite the common binding site for both kinases, differences in their interactions with the adaptors exist.

Our results are reminiscent of recent work on the IKK complex, where a peptide derived from the NEMO-binding domain (NBD) of IKKβ disrupts binding between NEMO and IKKα/IKKβ (May *et al*, 2000) . Similar to isolated TBDs preventing IRF activation in our study, cell permeable forms of NBD can inhibit agonist-induced NF-κB activation. NBD-derived peptides are currently investigated for their anti-inflammatory potential in models of rheumatoid arthritis (Tas *et al*, 2006). A similar approach using TBDs could be taken to prevent inappropriate activation of IRFs. Our data on NAP1 TBD^{Y236A} suggest that TBDs could be developed into specific inhibitors of individual kinases.

We have shown that TBK1 and IKKi rely on three adaptor proteins, including the newly described SINTBAD. The identification of a conserved TBD in the three adaptors suggests how the kinases might be linked to virus-activated signalling cascades but poses the problem of competition between the adaptors for access to kinases. Characterization of the subunit composition of TBK1 and IKKi complexes and identification of specific functions for the adaptors should therefore be addressed.

Materials and methods

Reagents

Poly(I:C) was purchased from Invivogen, peptidoglycan from Fluka, TNF-α from R&D Systems, monoclonal antibodies against TBK1, IKKi, IKKα and IKKβ from Imgenex, rabbit polyclonal antibodies against GFP and AU1 from Abcam, Sendai virus from Charles River Laboratories, amylose resin from New England Biolabs, PMA, ionomycin, protein G-sepharose, anti-FLAG M2 antibody, FLAG peptide and FLAG agarose from Sigma.

Plasmids

pLuc-IFN-β, pLuc-PRD(III-I)$_3$, pLuc-PRD(II)$_2$ were provided by Tom Maniatis, 4κB-luc by Adrian Ting, 5ISRE(ISG54)-luc by Sasha Radoja. Murine TBK1, NAP1, SINTBAD, TANK and human IKKi, IKKβ, IKKα cDNAs were PCR-amplified from cDNA libraries and cloned into M5P (Randow and Sale, 2006) as AU1, FLAG or *Renilla* luciferase fusions.

Antibody preparation

Murine SINTBAD 1–330 fused to MBP was expressed in *E. coli* and purified on amylose resin. A mixture of native and SDS-denaturated protein was used for immunization (Eurogentech).

Northern blot

A human multiple tissue northern blot (Clontech) was processed according to the instructions of the manufacturer. The most 5′ 700 bases of SINTBAD's coding sequence were used to derive the probe.

Immunoprecipitation and Western blot analysis

Post-nuclear supernatants from cells lysed in IP buffer (150 mM NaCl, 0.1%. Triton-X100, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, proteinase inhibitors)) were incubated for 2 h with preimmune or anti-SINTBAD serum (final dilution 1:100) followed by 2 h incubation with protein G-sepharose. Alternatively, cell lysates were incubated for 2 h with amylose resin, which had been left untreated or had been charged with an MBP-SINTBAD fusion protein (86–330 aa) expressed in *E. coli*. After four washes in IP buffer, precipitates were eluted with SDS loading buffer. Samples were separated on 4–12% denaturing gels (Invitrogen) and visualized by immunoblotting using ECL detection reagents (Amersham Bioscience).

Transfection and reporter gene assays

293ET cells were transfected in 24-well plates using Lipofectamine 2000 (Invitrogen). A total of 50 ng/well reporter plasmid and 50 ng/well pRL-TK *Renilla* luciferase (Promega) were co-transfected to normalize for DNA uptake. After 2 days, some cultures were stimulated with TNF-α (10 ng/ml), peptidoglycan (10 μg/ml), PMA (50 ng/ml) + ionomycin (1 μM) or infected with Sendai virus

(150 HAU/ml) for an additional period of 16 h before luciferase activities were measured using the dual-specific luciferase assay kit (Promega). Data are expressed as mean ± s.d. from triplicate wells of a representative experiment.

RNA interference

shRNAs were expressed from an H1 promoter inserted into M3Psin, a plasmid similar to M5P (Randow and Sale, 2006), but carrying an H1 promoter and a deletion in the 3′ LTR. Oligonucleotides of the general structure 5′-GATCCCC-N19-TTCAAGAGA-N19(reversed/complemented)-TTTTG-3′ were cloned (*Bam*H1 and *Eco*R1 overhangs underlined). Targeting sequences were as follows: GFP 5′-GCCACAACGTCTATATCAT-3′, human NAP1 5′-ATGAGCAGCTACAATCTAA-3′, human SINTBAD 5′-AGCATGAGTTACAGAAGAA-3′, human TANK 5′-GGATAGAGATTCTGCAGTA-3′, human Ubc13 5′-ATCGATCCAGGCCTTGTTA-3′. Targeting sequences for human TBK1 5′-GACAGAAGTTGTGATCACA-3′ (Fitzgerald *et al*, 2003), human TRIF 5′-GACCAGACGCCACTCCAAC-3′ (Yamamoto *et al*, 2003) and human MAVS 5′-GTATATCTGCCGCAATTTC-3′ (Xu *et al*, 2005) have been described.

Quantitative PCR

To evaluate the influence of gene knockdowns on the expression of endogenous genes, 293ET cells were transfected with plasmids encoding shRNAs and GFP. Two days later, cells were infected with Sendai virus for 16 h before isolating GFP-positive cells by fluorescence-activated cell sorting. Cytoplasmic RNA was purified using an RNAeasy mini kit (Qiagen) and cDNA was synthesized using oligo-dT primers and a Superscript III reverse transcription kit (Invitrogen). Quantitative PCRs for ISG15, IP-10 and β-actin were performed in a Taqman machine (Applied Biosystems) using FAM-labelled primers and Taqman Universal PCR master mix (Applied Biosystems) according to the instructions of the manufacturer.

LUMIER assay

For LUMIER assays (Barrios-Rodiles *et al*, 2005), cells were transfected with a pair of putative interactors fused to *Renilla* luciferase or a FLAG antibody tag. Post-nuclear supernatants from cells lysed in IP buffer were incubated with FLAG agarose. After washing, proteins were eluted for 30 min with 150 μg/ml FLAG peptide in *Renilla* lysis buffer (Promega). The ratio between luciferase activity in eluates and lysates is presented as fold binding over a control reaction.

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