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SUMO modification of TBK1 at the adaptor-binding C-terminal coiled-coil domain contributes to its antiviral activity

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ABSTRACT

The non-canonical IKK kinase TBK1 serves as an important signal transmitter of the antiviral interferon response, but is also involved in the regulation of further processes such as autophagy. The activity of TBK1 is regulated by posttranslational modifications comprising phosphorylation and ubiquitination. This study identifies SUMOylation as a novel posttranslational TBK1 modification. TBK1 kinase activity is required to allow the attachment of SUMO1 or SUMO2/3 proteins. Since TBK1 does not bind to the E2 enzyme Ubc9, this modification most likely proceeds via trans-SUMOylation. Mass spectrometry allowed identifying K694 as the SUMO acceptor site, a residue located in the C-terminal coiled-coil domain which is exclusively responsible for the association with the adaptor proteins NAP1, Sintbad and TANK. SUMO modification at K694 contributes to the antiviral function of TBK1 and accordingly the viral protein Gam1 antagonizes this posttranslational modification.

1. Introduction

Pathogen-associated patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are sensed by specific receptors which lead to the stimulation of the innate immune system [1]. The activated receptors induce signaling cascades which trigger the activation of specific transcription factors such as nuclear factor κB (NF-κB) and interferon regulatory factors (IRFs). The signaling pathways activating these transcription factors are intertwined at the level of the canonical IκB kinases (IKKs) and the non-canonical IKK-related kinases IKKε and TBK1 (IκB kinase ε/TANK-binding kinase 1) [2,3]. The kinase domains of IKKε and TBK1 share a 64% sequence identity among each other, whereas they exhibit only a 30% identity to the classical IKKs. Similar to the canonical IKKs, IKKε and TBK1 have an N-terminal kinase domain followed by a ubiquitin-like domain (ULD), a leucine zipper (LZ) and a helix-loop-helix (HLH) region [4]. The C-terminal region comprises two coiled-coil (CC) regions and CC2 is responsible for binding to the adaptor proteins TANK (TRAF family member-associated NF-κB activator), Sintbad (similar to NAP1 TBK1 adaptor) and NAP1 (NAK-associated protein 1) [5–8]. The adaptor proteins assemble the non-canonical IKKs together with further interaction partners into distinct, alternative multiprotein complexes [9]. Although lacking intrinsic enzymatic activities, the adaptor proteins are essential for the activation of their client kinases [6,8,10,11]. Activation of TBK1 occurs in proinflammatory signaling cascades triggered either by membrane-bound toll-like receptors (TLRs) or by cytosolic receptors for viral nucleic acids such as RIG-I (retinoic acid-inducible gene I) . These receptor-induced signaling pathways lead to local clustering of TBK1 molecules via adaptor proteins and trans-autophosphorylation of TBK1 dimers at a single phosphoacceptor site in the activation loop. The activation of TBK1 also relies on the inducible attachment of K63-linked ubiquitin chains [12] that allow for protein/protein interactions with further proteins such as NEMO (NF-κB essential modulator) [13].

Major phosphorylation targets of TBK1 are the transcription factors IRF3 and IRF7, which dimerize upon phosphorylation, translocate to the nucleus and lead to expression of type I interferons (IFNs) [14]. These cytokines were named for their ability to interfere with viral proliferation and serve to restrict virus replication and spreading [15]. Accordingly, viruses such as the Rift Valley fever (RVF) virus have developed strategies to disable the production and activities of IFNs [16,17]. We have previously found that TANK and also IKKε can be modified by the attachment of SUMO (small ubiquitin-related modifier), which

Abbreviations: PAMPs, pathogen-associated patterns; DAMPs, damage-associated molecular patterns; NF-κB, nuclear factor κB; IRFs, interferon regulatory factors; IKKs, IκB kinases; IKKε, IκB kinase ε; TBK1, TANK-binding kinase 1; ULD, ubiquitin-like domain; LZ, leucine zipper; HLH, helix-loop-helix; CC, coiled-coil; TANK, TRAF family member-associated NF-κB activator; Sintbad, similar to NAP1 TBK1 adaptor; NAP1, NAK-associated protein 1; TLRs, toll-like receptors; RIG-I, retinoic acid-inducible gene I; NEMO, NF-κB essential modulator; IFNs, interferons; RVF, Rift Valley fever; SUMO, small ubiquitin-related modifier; Ubc9, ubiquitin-conjugating 9; SENPs, sentrin/SUMO-specific proteases; MEFs, mouse embryonic fibroblasts; His, hexahistidine; Ni-NTA, nickel nitrilotriacetic acid

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belongs to the family of ubiquitin-like proteins [18,19]. SUMO modification of a target protein typically does not lead to its degradation, but rather regulates its activity, protein/protein interactions or localization [20]. The three expressed members of the SUMO family comprise SUMO1 and the almost identical isoforms SUMO2 and SUMO3 which are referred to as SUMO2/3 [21]. An isopeptide bond connects the very C-terminal glycine residue of SUMO with a lysine in the target protein. This process critically depends on the activating E1 enzyme (a heterodimer between SAE1 and SAE2) and the SUMO E2 conjugating enzyme Ubc9 (ubiquitin-conjugating 9) [22]. The transfer of Ubc9-bound SUMO to the target protein is typically facilitated by SUMO E3 ligases, but the SUMO conjugating process as such also occurs in the absence of E3 ligases [23,24]. SUMO peptides are removed from their substrate proteins by sentrin/SUMO-specific proteases (SENPs) which also function in the proteolytic processing of the precursor SUMO proteins to the mature form, exhibiting their C-terminal diglycine motifs [25]. SUMO is attached to hundreds of different proteins that are important for large variety of fundamental processes such as signal transduction, chromatin packaging, transcription and DNA repair. Accordingly, aberrant SUMOylation has been implicated in a variety of diseases [20].

Here we show that TBK1 is SUMOylated in a kinase-dependent fashion at K694 within the adaptor-binding CC2 region. TBK1 can be modified by SUMO1 as well as by SUMO2/3, independent from an apparent interaction with Ubc9. TBK1 SUMOylation is important for the antiviral function of the kinase and accordingly the viral protein Gam1 prevents this posttranslational modification.

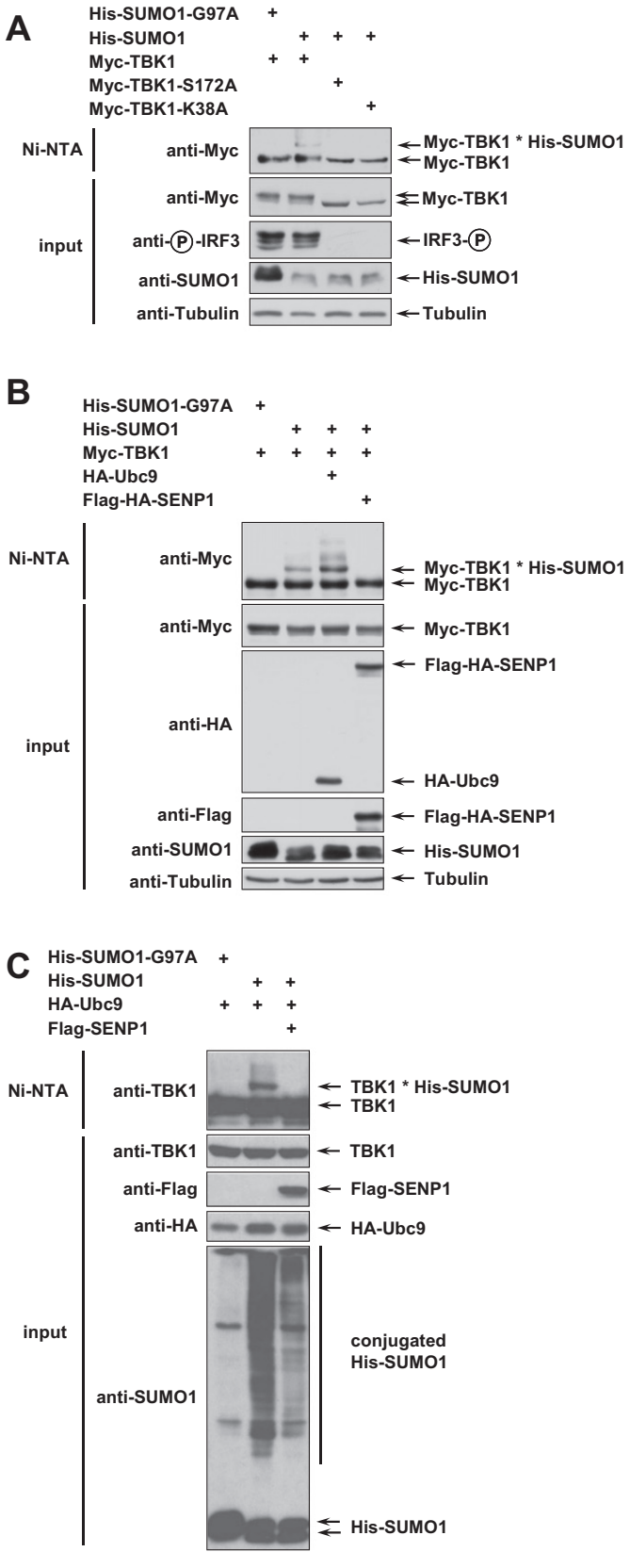


Fig. 1. TBK1 is modified by SUMOylation. (A) Myc–TBK1 and its kinase inactive versions (TBK1–K38A or TBK1–S172A) were coexpressed with His-SUMO1 or His-SUMO1–G97A in 293 T cells as shown. A fraction of cells was lysed in 1 × SDS sample buffer and used for the analysis of protein expression and the phosphorylation of the TBK1 substrate IRF3 with specific antibodies by immunoblotting. Another fraction of cells was lysed in denaturing Gu-HCl lysis buffer to maintain SUMOylation, followed by the enrichment of SUMOylated proteins on Ni-NTA agarose beads. Eluates were analyzed for the occurrence and SUMOylation of TBK1 by immunoblotting. Please note that also unSUMOylated TBK1 alone can already bind to Ni-NTA columns. (B) 293 T cells were transfected to express Myc–TBK1 and His-SUMO1 or His-SUMO1–G97A along with expression vectors encoding Ubc9 and SENP1 as shown. SUMOylated proteins were purified on Ni-NTA beads and subjected to immunoblotting using the indicated antibodies. An aliquot of cells was lysed in SDS sample buffer and analyzed for protein expression by Western blotting. (C) Cells were transfected to express His-tagged SUMO1, SUMO1–G97A, as well as tagged versions of Ubc9 and SENP1 as shown. A fraction of the cell lysate was purified on Ni-NTA beads to enrich SUMOylated proteins, expression and modification of the proteins was analyzed by immunoblotting as shown. The results shown are representative of at least three independent experiments.

2. Materials & methods

2.1. Antibodies, plasmids and reagents

All the information is given in the supplementary Table 1.

2.2. Cell culture and transfections

Human embryonic kidney HEK293T cells and *TBK1*$^{-/-}$ *IKBKE*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were grown in DMEM containing 10% FCS and 1% (w/v) penicillin/streptomycin at 37 °C and 5% CO_2. Cells were seeded in 6 cm dishes and transfected with 2 μl of transfection reagent (Rotifect or PEI, 1 mg/ml) per μg plasmid DNA. After pipetting up and down, complex formation occurred in serum- and antibiotic-free DMEM during 20 min at room temperature. Meanwhile medium was removed from the cells and replaced by antibiotic-free DMEM containing FCS. After adding the transfection mixture, the cells were incubated 3–5 h before the medium was changed and the cells were further grown.

2.3. Cell lysis protocols and Ni-NTA affinity purification

Whole cell lysates were prepared by lysing the cells in a buffer containing the non-ionic detergent NP-40 (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.5 mM sodium orthovanadate, leupeptin (10 μg/ml), aprotinin (10 μg/ml), 1% NP-40

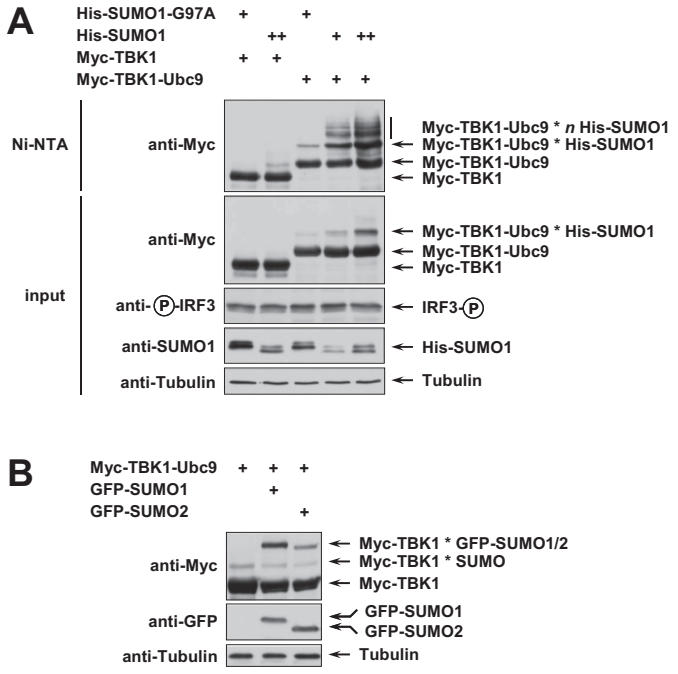


Fig. 2. TBK1 is modified by SUMO1 and SUMO2/3. (A) TBK1 or a TBK1–Ubc9 fusion protein were coexpressed with increasing amounts of His-SUMO1 or the conjugation-defective His-SUMO1–G97A control as shown. Lysates were either analyzed for protein expression (lower) or used for Ni-NTA chromatography to enrich SUMOylated proteins. Immunoblotting was performed to display TBK1 SUMOylation as shown. The positions of mono- and multiSUMOylated TBK1 are indicated. (B) The TBK1–Ubc9 fusion protein was expressed either alone or together with GFP–SUMO1 or GFP–SUMO2. TBK1 SUMOylation was revealed by Western blotting. Western blots were repeated three times, with a representative image shown.

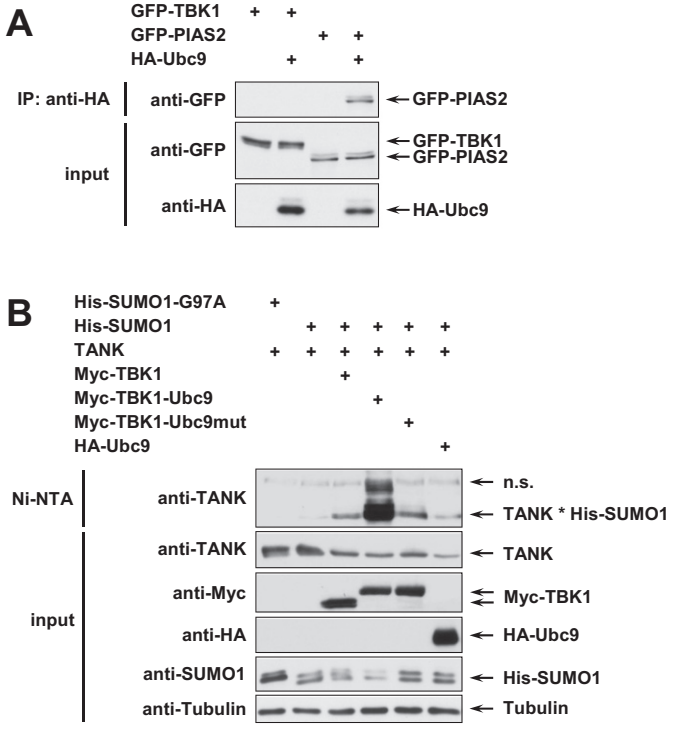


Fig. 3. Trans-SUMOylation between TBK1 and TANK. (A) GFP–TBK1 or GFP–PIAS2 were coexpressed with HA–Ubc9 as shown. The HA-tagged Ubc9 was immunoprecipitated and the bound GFP-tagged proteins were detected by immunoblotting as shown. (B) The indicated constructs were coexpressed in 293 T cells and cells were either lysed in 1× SDS sample buffer to test protein expression or in Gu-HCl buffer followed by enrichment of SUMOylated proteins on Ni-NTA beads. SUMOylation of TANK and protein expression was detected with specific antibodies as shown. A non-specific band (n.s.) is indicated. The results shown are representative of four (A) and two (B) independent experiments.

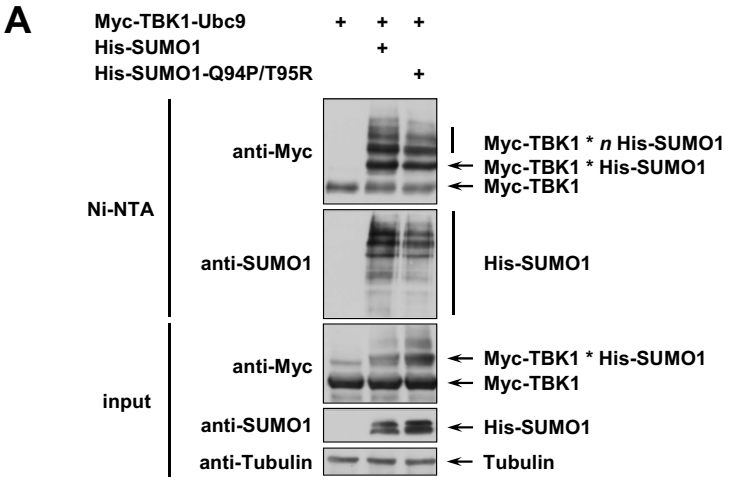
and 10% glycerol). For that, cells were first washed with cold PBS, scraped off and collected in a tube. After centrifugation (1000 ×*g*, 2 min), the cell pellet was resuspended in NP-40 lysis buffer and incubated 20 min on ice. The lysates were cleared by centrifugation and supernatants were transferred into a fresh tube. Lysates were either used for immunoprecipitation or directly mixed with 5× SDS sample buffer and subjected to SDS-PAGE and Western blot analysis. Hexahistidine (His)-tagged SUMO proteins were affinity purified under denaturing conditions by nickel nitrilotriacetic acid (Ni-NTA) pull-down. Cell pellets were lysed in 800 µl Ni-NTA lysis buffer (6 M Gu-HCl; 10 mM Tris; 100 mM NaH_2PO_4 pH 8.0). After shearing the DNA by sonication (2 × 20 s), the samples were cleared by centrifugation (15 000 ×*g*, 10 min, 4 °C). The supernatants were mixed with 50 µl prewashed Ni-NTA agarose (Qiagen) and incubated for 3 h at RT on a rotating wheel. Beads were washed in successive washing steps in 1 ml Ni-NTA washing buffer (8 M Urea; 10 mM Tris/HCl, pH 6.3; 100 mM NaH_2PO_4; 0.1% Triton X-100) and finally eluted by boiling the beads in 50 µl 2.5 × SDS sample buffer containing 200 mM imidazole as described [19].

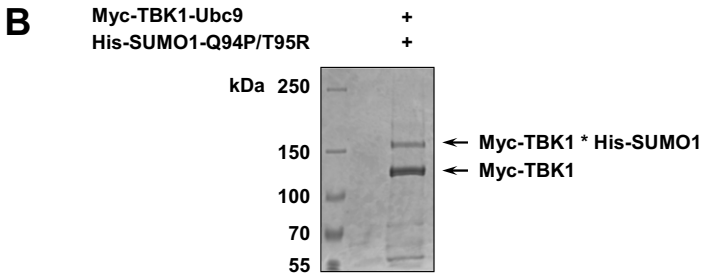
2.4. Immunoprecipitation experiments and Western blotting

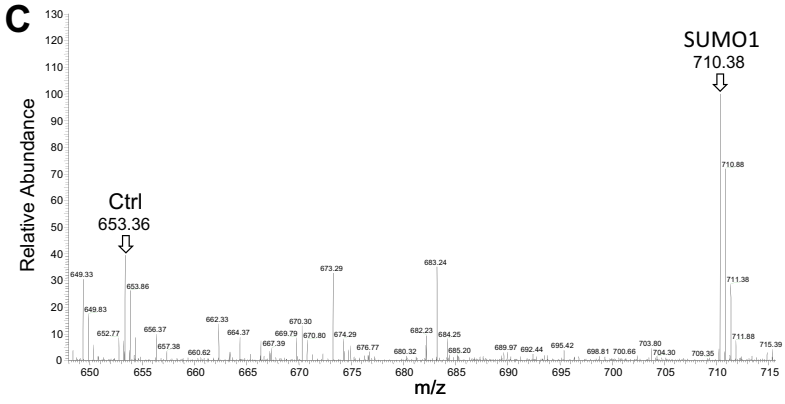
For immunoprecipitation, cleared cell extracts lysed in NP-40 buffer were filled up to a volume of 600 µl with NP-40 buffer and supplemented with 1 µg precipitating antibody or control IgG. After adding 25 µl protein A/G agarose (Santa Cruz), the samples were incubated for 4 h at 4 °C on a rotating wheel. Agarose beads were then washed four times with 1 ml cold NP-40 buffer by inverting the tube five times followed by centrifugation (2000 ×*g*, 1 min). Precipitated proteins were eluted by adding 2× SDS sample buffer. Equal amounts of protein were separated by SDS-PAGE, followed by semidry blotting to a polyvinylidene difluoride membrane (Millipore) as previously described [19].

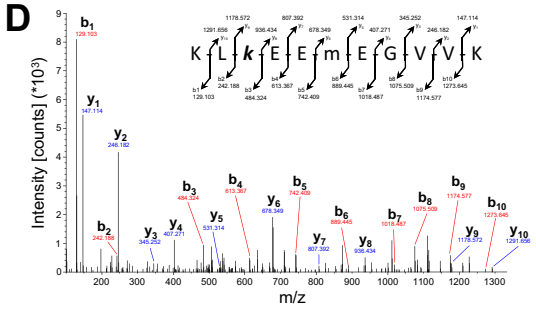
2.5. Purification of SUMOylated TBK1 and mass spectrometry

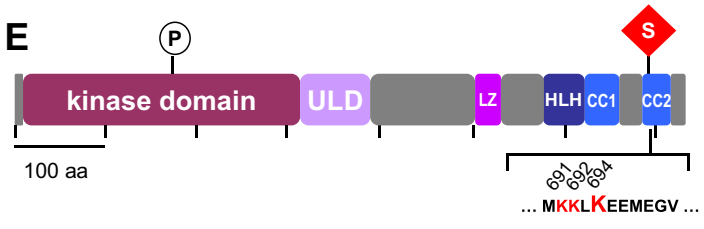
Myc–TBK1–Ubc9 was coexpressed with the trypsin-cleavable His-SUMO1–Q94P/T95R in 293 T cells and purified by immunoprecipitation using anti-Myc antibodies. Prior to electrophoresis, proteins were incubated at 95 °C in 1× SDS sample buffer. Then 1 µl of an acrylamide solution (40%) was added and incubated at room temperature to alkylate cysteine residues for 30 min and directly loaded onto the gel. After electrophoresis, proteins were stained with Coomassie Brilliant Blue for 15 min and the TBK1 bands were digested by trypsin as described [26]. Gel pieces were destained two times with 200 µl 50% ACN, 25 mM ammonium bicarbonate at 37 °C for 30 min and then dehydrated with 100% ACN. Solvent was removed and gel pieces were dried in a vacuum centrifuge and about 20 µl 12 ng/µl sequencing grade Trypsin (Promega) in 10% ACN, 25 mM ammonium bicarbonate were added. Gels were rehydrated in trypsin solution for 1 h on ice and then covered with 10% ACN, 25 mM ammonium bicarbonate. Digestion was performed over night at 37 °C and then stopped, followed by three rounds of peptide extraction by adding 50 µl of 50% ACN, 0.1% TFA and incubation at 37 °C for 1 h. Dried peptide extracts were redissolved in 30 µl 2% ACN, 0.1% TFA for 20 min and agitating at 800 rpm, then centrifuged at 20,000 ×*g* and an aliquot of 20 µl was transferred to a HPLC vial. A sample aliquot containing about 100 fmol of a single protein was injected into a nano-flow ultra-high pressure liquid chromatography system (RSLC, Dionex). The outlet of the LC system was directly connected to the nano-ESI source (Thermo Fisher Scientific) of an LTQ Orbitrap Velos mass spectrometer. The top 10 most intensive ions of charge two or three and a minimum intensity of 2000 were selected for CID fragmentation and subsequent MS/MS analysis. Raw data were processed using Proteome Discoverer software (version 1.3, Thermo Fisher Scientific) and the Mascot search algorithm.


A
Myc-TBK1-Ubc9 + + +
His-SUMO1 +
His-SUMO1-Q94P/T95R +
Ni-NTA
anti-Myc
Myc-TBK1 * n His-SUMO1
Myc-TBK1 * His-SUMO1
Myc-TBK1
anti-SUMO1
His-SUMO1
input
anti-Myc
Myc-TBK1 * His-SUMO1
Myc-TBK1
anti-SUMO1
His-SUMO1
anti-Tubulin
Tubulin


B
Myc-TBK1-Ubc9 +
His-SUMO1-Q94P/T95R +
kDa 250
150
100
70
55
Myc-TBK1 * His-SUMO1
Myc-TBK1


C
Relative Abundance
m/z
Ctrl
653.36
SUMO1
710.38


D
Intensity [counts] (*10³)
m/z
K L k E E m E G V V K


E
P
S
kinase domain
ULD
LZ
HLH
CC1
CC2
100 aa
691
692
694
… MKKLKEEMEGV …

2.6. Reconstitution of MEFs with TBK1

TBK1$^{-/-}$ *IKBKE*$^{-/-}$ MEFs were reconstituted with wildtype and K692/4R TBK1 using a lentiviral vector system (290-pHAGE-hEF1aCAR-PGK puro). To produce lentiviral particles, 293 T cells were transfected with the empty vector or the TBK1 constructs together with the helper plasmids pMDLg/pRRE, pRSV-Rev and pHCMV-G. 48 h after transfection, viruses were harvested, cleared by centrifugation and filtration and MEFs were infected in the presence of 5 μg/ml polybrene (Sigma) for 12 h. One day after infection, the cells were selected with 4 μg/ml puromycin (Invivogen).

2.7. Infection with Rift Valley fever virus

MEFs were grown to 80% density and inoculated with RFV virus lacking the NSs virulence factor [27] at an MOI of 0.01. Virus yields in the supernatants were determined by plaque assay as described [16].

3. Results and discussion

3.1. SUMOylation of TBK1

Since we had previously identified SUMO modification of IKKε [18] and TANK [19], we were interested to determine whether also TBK1 would undergo this posttranslational modification. To address this question we coexpressed active wildtype (wt) TBK1 with His-tagged SUMO1 or a conjugation-defective SUMO1 mutant where the C-terminal glycine was changed to alanine (G97A). After denaturing lysis and purification of His-tagged proteins on Ni-NTA columns, Western blotting allowed detecting the SUMOylated form of TBK1, as revealed by the occurrence of a slower migrating band (Fig. 1A). SUMOylation did not occur in kinase inactive versions of TBK1 that were either mutated in the ATP-binding lysine (TBK1–K38A) or in the activation loop (TBK1–S172A) (Fig. 1A). As expression of TBK1 alone is sufficient to trigger its kinase activity by trans-autophosphorylation [28], this finding suggests that only active TBK1 can be modified by SUMOylation. The kinase dependency could be due to conformational changes within TBK1, as structural data show that TBK1 activation reorganizes the kinase domain into an active configuration while maintaining the compact dimer conformation [12,28,29]. It was then interesting to identify a physiologically relevant stimulus that can regulate the SUMOylation status of TBK1. Neither activation of TLR3 or TLR4 nor overexpression of various proteins, participating in multiple signaling pathways or that have been described to interact with TBK1, had an effect on TBK1 SUMOylation (supplementary Fig. 1A,B). Also a SUMO E3 ligase, responsible for increased TBK1 SUMOylation could not be identified (supplementary Fig. 2A,B). On the other hand, coexpression of the isopeptidase SENP1 abolished the SUMOylation of TBK1, whereas overexpression of the SUMO E2 enzyme Ubc9 triggered its SUMOylation, clearly demonstrating that TBK1 is modified by this posttranslational modification (Fig. 1B). It was also important to investigate the SUMOylation status of the endogenous kinase. As isopeptidases efficiently remove the SUMO moiety in standard lysis buffers [30], we planned to purify SUMO-modified TBK1 under fully denaturing conditions. Towards this goal, cells were transfected to express His-tagged SUMO1 together with Ubc9, followed by enrichment of SUMOylated proteins on Ni-NTA columns. Immunoblotting using a TBK1-specific antibody allowed the detection of SUMO modification for the endogenous TBK1 protein, as indicated by the occurrence of an upshifted band (Fig. 1C). This band did not appear in the presence of overexpressed SENP1 or the conjugation-defective SUMO1 mutant (Fig. 1C), confirming that it corresponds to the SUMOylated form of the kinase.

In order to further increase the SUMOylation level of TBK1, its C-terminus was directly fused to Ubc9, a method that has been described previously [31]. The TBK1–Ubc9 fusion protein showed strongly elevated SUMOylation (Fig. 2A) and coexpression of SUMO1 resulted in the occurrence of additional upshifted bands which may reflect exaggerated TBK1 SUMOylation. The TBK1–Ubc9 fusion protein was not only modified by SUMO1 but also by the paralogue SUMO2 (Fig. 2B). This is in line with the finding that many substrates can be modified by SUMO1 as well as by SUMO2/3 [32]. Consistent with the notion of an overlapping function of the different SUMO isoforms, SUMO1-deficient mice are viable, indicating a compensatory function of SUMO2/3 [33,34]. Since Ubc9 typically binds to its client proteins [35] we were interested to test the interaction between TBK1 and this E2 enzyme by coimmunoprecipitation experiments. While Ubc9 showed a clear interaction with its reported interaction partner PIAS2 [36], no interaction occurred for TBK1 (Fig. 3A). This could be explained either by a very weak interaction that is not stable enough to be maintained during the process of coimmunoprecipitation. Alternatively, it is conceivable that SUMOylation is mediated by trans-SUMOylation, a process where the enzymatic machinery attached to a protein also acts on its interaction partner, thereby allowing the SUMOylation of all partners within a protein complex [37]. To test the potential occurrence of such a process, the TBK1–Ubc9 fusion protein was coexpressed with its interactor TANK and His-tagged SUMO1. Following the enrichment of SUMOylated proteins on Ni-NTA columns the detection of TANK showed a strongly enhanced TANK SUMOylation only in the presence of TBK1–Ubc9, but not by an enzymatically inactive TBK1–Ubc9 mutant (Fig. 3B). These data suggest that the SUMOylation of one protein in a protein complex containing TBK1 and an interaction partner could indeed be due to trans-SUMOylation. In such a scenario the TBK1 interaction partners would provide the indirect contact with Ubc9 and their respective E3 ligases.

3.2. SUMOylation of TBK1 occurs at K694

It was then interesting to identify the SUMO attachment site in TBK1 by a straightforward approach using mass spectrometry. Since tryptic digestion of wildtype SUMO1 is not suitable for mass spectrometric analysis, the C-terminal TGG sequence was mutated to RGG in order to allow trypsin digestion [38]. Besides the SUMO1–T95R mutation, a Q94P point mutation was introduced that was shown to avoid proteolytic cleavage of SUMO1 by SENPs and thus increases the stability of the SUMOylated TBK1 [39]. To confirm the effective attachment of His-SUMO1-Q94P/T95R to TBK1–Ubc9, these constructs were transfected in 293 T cells. Ni-NTA purification followed by Western blotting showed that the conjugation of His-SUMO1-Q94P/T95R to TBK1–Ubc9 was as efficient as the His-SUMO1 wildtype protein (Fig. 4A). Coexpression of TBK1–Ubc9 with the trypsin-cleavable His-SUMO1 mutant allowed the large-scale

Fig. 4. Mapping of the TBK1 SUMOylation site. (A) 293 T cells were transfected to express Myc–TBK1–Ubc9 along with His-SUMO1 or the His-SUMO1-Q94P/T95R mutant which allows the convenient detection of SUMOylated peptides after tryptic digestion. Ni-NTA purification was performed to enrich SUMOylated proteins. Eluates and input samples were analyzed by immunoblotting with the indicated antibodies. The positions of mono- and multiSUMOylated TBK1 are indicated. (B) Myc–TBK1–Ubc9 was coexpressed with His-SUMO1-Q94P/T95R in 293 T cells and purified by immunoprecipitation with anti-Myc antibodies, followed by SDS-PAGE and Coomassie staining. Molecular weights of marker proteins are indicated. Bands were excised and analyzed by mass spectrometry. The results shown are representative of three (A) and two (B) independent experiments. (C) The bands were in-gel digested with trypsin and analyzed by LC-MS as described in Section 2. Part of the extracted ion chromatogram covering the precursor ions of the SUMOylated (SUMO1) peptide and not SUMOylated (Ctrl) peptide is indicated. Other m/z values correspond to different peptides of the analyzed sample. (D) The SUMOylated precursor ion of m/z 710.38 (C) was isolated and subjected to MS/MS analysis in the mass spectrometer. MS and MS/MS data were searched against the human data base entries with the double glycine modification at the ε-amino group of lysine residues. The peptide KLkEEmEGVVK was identified containing the modification at K694. The b- and y-ions are indicated in the spectrum and in the sequence. The SUMOylated lysine residue is shown as a small character in bold and italics. The methionine residue was oxidized and thus also shown as small character. (E) Schematic representation of TBK1. The activation loop phosphorylation (P) and SUMO acceptor sites (S) are indicated, the large K at position 694 marks it as the main SUMOylation site.

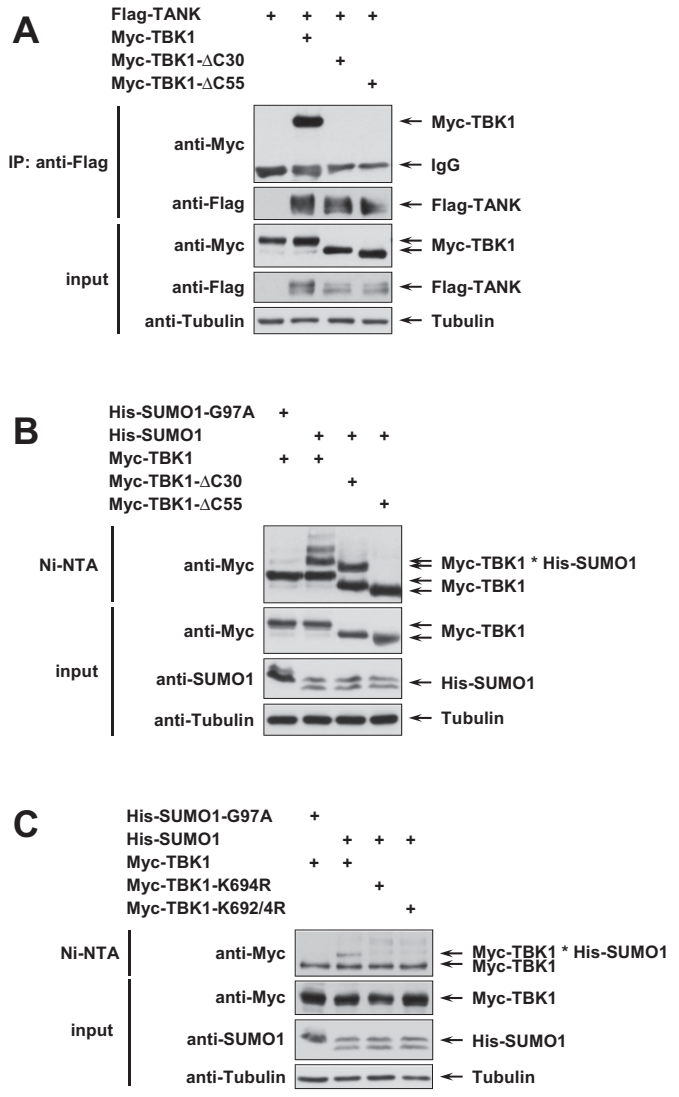


Fig. 5. TBK1 is SUMOylated at K694. (A) The full-length form of TBK1 and two mutants deleted in the C-terminal 30 or 55 amino acids were coexpressed with TANK. After immunoprecipitation of TANK, the bound TBK1 proteins were revealed by immunoblotting with specific antibodies. (B) The indicated TBK1 constructs were coexpressed with His-SUMO1 and TBK1 SUMOylation was revealed by Ni-NTA purification and Western blotting. (C) TBK1 or the indicated point mutants were coexpressed with His-SUMO1. Eluates from Ni-NTA columns were analyzed for TBK1 SUMOylation by immunoblotting. Western blots were repeated three times, with a representative image shown.

purification of SUMOylated TBK1 by immunoprecipitation. SDS-PAGE analysis and Coomassie brilliant blue staining showed highly purified Myc–TBK1–Ubc9 and its SUMO1 conjugated form (Fig. 4B). Bands were subjected to in gel-digestion with trypsin and peptides were analyzed by mass spectrometry (Fig. 4C,D). These experiments identified TBK1–K694 as the major SUMO1 attachment site, while minor SUMOylation was also measured at K691 or K692.

All of the identified sites are located in the second coiled-coil domain of TBK1, as schematically shown in Fig. 4E. Yeast two-hybrid experiments have shown that the C-terminal 43 residues of TBK1 (which contain K694) are sufficient for TANK binding [7]. A further study showed the critical relevance of this CC2 region also for the interaction with the TBK1 adaptor proteins NAP1 and Sintbad [6]. Accordingly, deletion of the C-terminal 30 (TBK1-ΔC30) or 55 (TBK1-ΔC55) amino acids of TBK1 precluded the interaction with TANK (Fig. 5A). It was further tested whether these C-terminal deletion mutants can still be modified by SUMOylation. Coexpression of these mutants with SUMO1 and subsequent analysis of TBK1 SUMOylation showed that deletion of the C-terminal 30 amino acids still allowed for this posttranslational modification (Fig. 5B). In contrast the TBK1-ΔC55 mutant representing the region between positions 1 and 674 and thus lacking K694 was not SUMOylated, in line with the mass spectrometric mapping experiments. We then tested TBK1 point mutants where the major and minor SUMO attachment sites at K694 and K692 where mutated to arginine. Mutation of K694 was sufficient to largely prevent SUMOylation (Fig. 5C), thus confirming its relevance as the main SUMOylation site. TBK1 can also be modified by ubiquitination [12], a posttranslational modification known to be able to compete with SUMO1 for attachment to the same acceptor lysine [40]. We explored this possibility, but the ubiquitination pattern of the SUMOylation-deficient K694/2R mutant resembled that of the wildtype kinase (supplementary Fig. 3), suggesting that the SUMO attachment sites of TBK1 cannot be modified by ubiquitination.

3.3. Functional relevance of TBK1 SUMOylation

The functional role of TBK1–K694 was revealed in a study showing that mutation of this residue still allowed interaction with NAP1 but precluded binding of TANK or Sintbad [6]. The same study also showed in reconstitution experiments that mutation of K694 largely prevented LPS-induced IFN-β induction, suggesting that adaptor binding is important for TBK1 function. Also the mutation of the corresponding region in IKKε showed the relevance of the CC2 region for its ability to trigger the production of type I IFN [41]. Biochemical data suggest that TBK1 does not form a single large multiprotein complex containing all adaptor proteins, but rather forms distinct complexes with each adaptor [6,42,43]. Accordingly, competition experiments show a mutually exclusive binding of the adaptors to TBK1 homodimers [9]. These findings are corroborated by immunofluorescence experiments that revealed distinct intracellular localization of TANK, NAP1 and SINTBAD [6]. Our data suggest that SUMOylation of TBK1 at K694 would sterically prevent

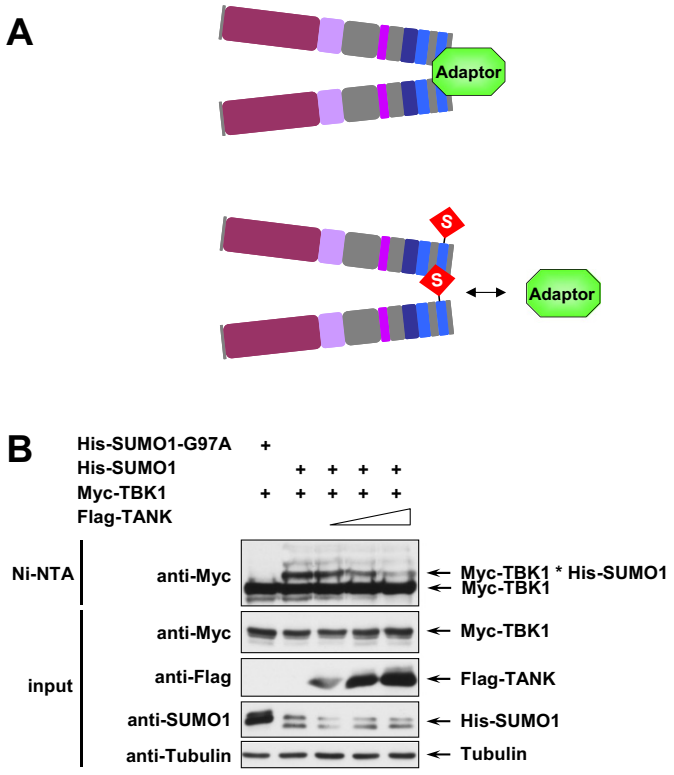


Fig. 6. TBK1 SUMOylation occurs at the adaptor-binding region. (A) Schematic model depicting steric hindrance of adaptor binding by SUMOylation of the TBK1 CC2 domain. (B) TBK1 and His-SUMO1 were coexpressed either alone or together with increasing amounts of TANK. Eluates from Ni-NTA columns were analyzed for TBK1 SUMOylation. The results shown are representative of two independent experiments.

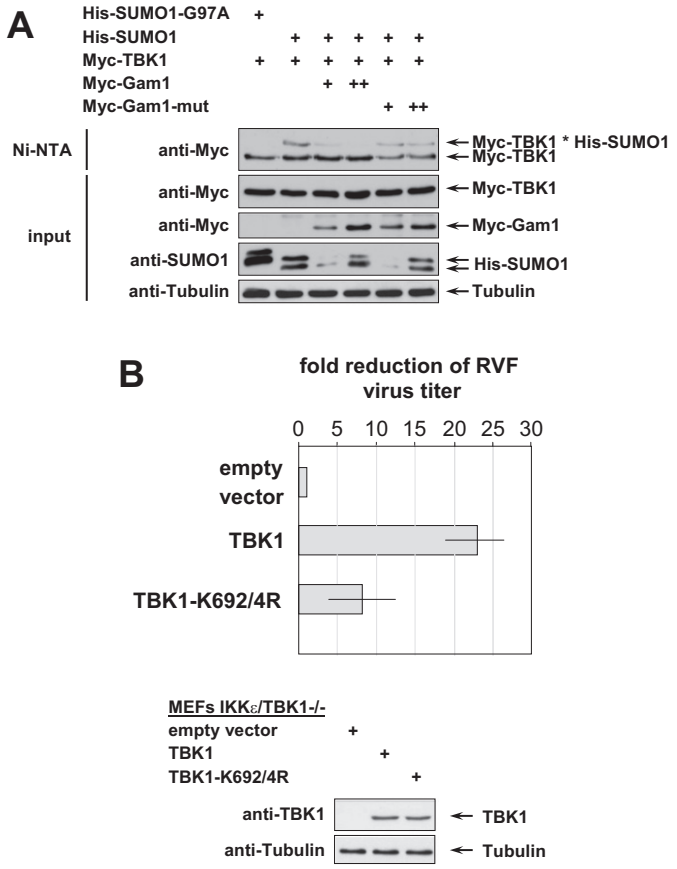


Fig. 7. TBK1 SUMOylation contributes to its antiviral activity. (A) TBK1 and His-SUMO1 were coexpressed with Myc-tagged versions of the adenoviral protein Gam1, followed by the analysis of TBK1 SUMOylation by Western blotting as shown. The results shown are representative of two independent experiments. (B) *TBK1*$^{-/-}$ *IKBKE*$^{-/-}$ MEFs were stably reconstituted to express TBK1 or TBK1–K692/4R (lower). The cells were infected with RVF and virus replication was scored after 48 h by plaque assay of cell culture supernatants. The results display mean values from six different experiments, error bards show standard errors of the mean (SEM).

association of adaptor proteins such as TANK, as schematically displayed in Fig. 6A. In support to this model, the enforced interaction between TBK1 and TANK upon overexpression of this adaptor protein resulted in strongly diminished TBK1 SUMOylation (Fig. 6B). In such a scenario SUMOylation of TANK would serve as a repellent that prevents adaptor protein binding, thus allowing dynamic exchange of TBK1 between different multiprotein complexes. These distinctly assembled complexes can then regulate TBK1 for its specific functions in diverse processes ranging from innate immunity to cell proliferation and autophagy. This is reminiscent to the function of TANK SUMOylation, which weakens the interaction with IKKε and thus allows regulating this protein/protein interaction [19]. Alternatively, SUMOylation often functions as an attractant that functions to enhance protein/protein interactions by SUMO binding to a SUMO interacting motif. However, we failed to detect SUMO-binding of TBK1 and TANK in GST pull-down assays (supplementary Fig. 4), while the control protein Daxx showed SUMO1-binding activity as reported [44]. These results lend further support to the concept that SUMO modification of TBK1 does not serve as a glue but rather as a repellent for protein/protein interactions. TBK1 is an essential component of the host cell machinery leading to the production of antiviral type I interferons [45] and accordingly various TBK1 functions are frequently disabled by proteins encoded by different viruses [46]. We thus screened a number of different viral proteins (listed in supplementary Table 2) for their ability to influence constitutive TBK1 SUMOylation. We found that the adenoviral protein Gam1 inhibited SUMOylation of TBK1, while a mutant Gam1 containing helix-disrupting proline residues had no effect (Fig. 7A). This inhibitory effect is most likely due to the reported inhibitory activity of Gam1 on SAE1/SAE2 activity [47]. It was then interesting to investigate the role of TBK1 SUMOylation for virus replication. MEFs deficient for TBK1 and IKKε were reconstituted to stably express TBK1 or TBK1–K692/4R. These cells were either left untreated or infected with an IFN-sensitive RVF virus mutant at an MOI of 0.01, and virus yields were measured 2 days later. These experiments showed that mutation of the SUMO attachment site strongly impaired the ability of TBK1 to reduce RVF virus titers (Fig. 7B). Given the contribution of TBK1 SUMOylation for its antiviral activity, it will be interesting to study whether also further viral proteins have the ability to interfere with the SUMOylation status of TBK1. In summary, this study shows that the function of TBK1 is not only regulated by phosphorylation and ubiquitination [45,46], but also by SUMO modification. TBK1 serves distinct functions in different signal transduction pathways ranging from autophagy to epithelial–mesenchymal transition and cell survival [46]. It is tempting to speculate that all these different functions are mediated by binding to different adaptor complexes and that the SUMOylation of TBK1 in the adaptor-binding domain may ensure the availability of unbound TBK1 that dynamically shuttles between different complexes. As the signal-dependent relocalization of TBK1 dictates its biological role and substrate specificity [6,28] it will be interesting to study the potential contribution of TBK1 SUMOylation to these processes in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.10.008.

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