Control of TANK-binding Kinase 1-mediated Signaling by the γ1 34.5 Protein of Herpes Simplex Virus 1*

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TANK-binding kinase 1 (TBK1) is a key component of Toll-like receptor-dependent and -independent signaling pathways. In response to microbial components, TBK1 activates interferon regulatory factor 3 (IRF3) and cytokine expression. Here we show that TBK1 is a novel target of the γ1 34.5 protein, a virulence factor whose expression is regulated in a temporal fashion. Remarkably, the γ1 34.5 protein is required to inhibit IRF3 phosphorylation, nuclear translocation, and the induction of antiviral genes in infected cells. When expressed in mammalian cells, the γ1 34.5 protein forms complexes with TBK1 and disrupts the interaction of TBK1 and IRF3, which prevents the induction of interferon and interferon-stimulated gene promoters. Down-regulation of TBK1 requires the amino-terminal domain. In addition, unlike wild type virus, a herpes simplex virus mutant lacking γ1 34.5 replicates efficiently in TBK1−/−cells but not in TBK1+/+ cells. Addition of exogenous interferon restores the antiviral activity in both TBK1−/− and TBK1+/+ cells. Hence, control of TBK1-mediated cell signaling by the γ1 34.5 protein contributes to herpes simplex virus infection. These results reveal that TBK1 plays a pivotal role in limiting replication of a DNA virus.

Herpes simplex virus 1 (HSV-1)³ is a large DNA virus that establishes latent or lytic infection, in which the virus triggers innate immune responses. In HSV-infected cells, a number of antiviral mechanisms operate in a cell-type- and time-dependent manner (1). In response to double-stranded RNA (dsRNA), Toll-like receptor 3 (TLR3) recruits an adaptor TIR domain-containing adaptor inducing IFN-β and stimulates cytokine expression (2, 3). In the cytoplasm, RNA helicases, RIG-I (retinoid acid-inducible gene-I), and MDA5 (melanoma differenti-
reveal a previously unrecognized mechanism by which γ1,34.5 facilitates HSV replication.

**EXPERIMENTAL PROCEDURES**

*Cells and Viruses*—Vero, HEL, and 293T cells were from the American Type Culture Collection. TBK1+/+ and TBK1−/− MEF were gifts from Dr. Wen-Chen Yeh. Cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 5% (Vero and 293T) or 10% (MEF and HEL) fetal bovine serum. HSV-1(F) is a prototype HSV-1 strain used in this study (28). In recombinant virus R3616, a 1-kb fragment from the coding region of the γ1,34.5 gene was deleted (28). These viral strains were gifts from Dr. Bernard Roizman (University of Chicago).

*Plasmids*—Plasmids pcDNA3, pTK-Luc, and dN200 have been described elsewhere (29). The FLAG-γ1,34.5 plasmids, WT, Δ30, Δ72, Δ106, Δ146, and N159, were constructed by inserting PCR-amplified fragments into the BamHI and XhoI sites of pcDNA3. To construct GST-IRF3, a DNA fragment encoding amino acids 380–427 from IRF3 was ligated into the BamHI and EcoRI sites of pGEX4-T1. pISG56-Luc was a gift from Ganes Sen (Cleveland Clinical Research Foundation). Plasmids IFNB and FLAG-TBK1 were gifts from R. Lin, J. Herscott (McGill University), and U. Siebenlist (National Institutes of Health). Plasmid GFP-IRF3 was a gift from Nancy Reich (State University of New York, Stony Brook). Plasmid HA-γ1,34.5 was a gift from Youjia Cao (Nankai University). To construct HA-TBK1, the TBK1 insert was PCR-amplified and cloned into the BamHI and XhoI sites of pcDNA3.

*Viral Infections*—Cells were infected with viruses at 0.05, 5, or 10 pfu per cell. At indicated time points, virus yields were determined on Vero cells (26). For interferon assays, cells were untreated or treated with mouse α-interferon (100 units/ml; Sigma) for 20 h. Cells were then infected with viruses. After adsorption for 2 h, the monolayers were overlaid with DMEM and incubated at 37 °C. At indicated time points post-infection, samples were harvested, and viruses were released by three cycles of freezing and thawing and then titrated on Vero cells. For radioisotope labeling, cells were labeled with [35S]methionine (50 μCi/ml; ICN) in DMEM lacking methionine but supplemented with 2% fetal bovine serum 1 h before harvest. At indicated time points, lysates of cell were subjected to electrophoresis and autoradiography (30).

*RT-PCR and Reporter Assays*—Cells were mock-infected or infected with viruses at 5 pfu per cell in serum-free DMEM. At 1 h after infection, cells were grown in DMEM with 1% fetal bovine serum. At the indicated time points, total RNA was harvested from cells using RNaseasy kit (Qiagen). RT-PCR analysis was performed with one-step RT-PCR system according to the manufacturer’s protocols (Invitrogen). Primers used were as follows: mouse ISG54, ATAGATGACAGCAGTGAAG and CTTAGATTCAGACACCTTCTT; mouse ISG56, ATGGGAGAAGTCGTAGGG and TCAGAATGCAGGTCATTT; human ISG54, ATAGATGACAGCAGTGAAG and CTTAGATTCAGACACCTTCTT; human ISG56, ATGAGATGACAGCAGTGAAG and CTTAGATTCAGACACCTTCTT; human or mouse 18S rRNA, CGCAGCTAGGAAATAATGGA and TTATGAGCCGACTTACTGG. Luciferase reporter assays were performed as described previously (29). Briefly, 293T cells grown on 12-well plates were transfected with a control plasmid or plasmid vector expressing TBK1 and γ1,34.5 variants, along with IFN-β or ISG56 reporter plasmid expressing firefly luciferase using Lipofectamine 2000 (Invitrogen). Total levels of transfected DNA were kept constant with empty vector plasmid. As a control for transfection efficiency, a plasmid containing the Renilla luciferase gene driven by the HSV-1 TK promoter was included. At 36 h after transfection, cells were harvested, and luciferase activities were measured using the dual luciferase assay system from Promega.

*Immunoblotting and Immunoprecipitation Analyses*—To analyze protein expression, cells were washed, harvested, and solubilized in disruption buffer containing 50 mm Tris-HCl (pH 7.0), 5% 2-mercaptoethanol, 2% SDS, and 2.75% sucrose. Samples were then sonicated, boiled, subjected to electrophoresis on denaturing 12% polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and reacted with antibodies against eIF2α, phosphorylated eIF2α (Cell Signaling Technology, Inc.), β-actin (Sigma), HSV-1 (Dako Inc.), FLAG (Sigma), HA (Santa Cruz Biotechnology), IRF3 (Santa Cruz Biotechnology), phosphorylated IRF3 (Ser396) (Cell Signaling Technology, Inc.), and γ1,34.5. The membranes were rinsed in phosphate-buffered saline and reacted with donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase. Protein bands were detected by enhanced chemiluminescence (Amersham Biosciences). To examine protein interactions, 293T cells were transfected with the indicated amounts of pcDNA3, FLAG-TBK1, HA-γ1,34.5, FLAG-dN200, and IRF3. At 40 h after transfection, cells were harvested and lysed in 50 mm Tris-HCl (pH 7.4) buffer containing 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mm NaCl, 1 mm EDTA, 1 mm 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 1 μg/ml aprotinin/leupeptin/pepstatin, 1 mm Na3VO4, 1 mm NaF. Lysates were incubated overnight at 4 °C with anti-FLAG M2 affinity gel (Sigma) or anti-HA antibody (Applied Biological Materials Inc.) plus protein A/G-agarose beads (Santa Cruz Biotechnology). Immunocomplexes captured on the affinity gel or protein A/G-agarose beads were subjected to electrophoresis and immunoblotting analysis (29).

*Kinase Assays*—Recombinant GST-IRF3 fusion protein was purified from bacterial lysates by affinity chromatography. 293T cells were transfected with pcDNA3, FLAG-TBK1, and HA-γ1,34.5. At 40 h after transfection, cell lysates were prepared in 20 mm Tris-HCl (pH 7.4) containing 137 mm NaCl, 10% glycerol, 1% Triton X-100, 2 mm EDTA, 50 mm sodium glycophosphate, 20 mm sodium pyrophosphate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mm Na3VO4, and 5 mm benzamidine. TBK1 was immunoprecipitated with anti-FLAG affinity gel (Sigma). Immunocomplexes were incubated with recombinant GST-IRF3(380–427) for 20 min at 30 °C in 25 mm Heps buffer (pH 7.5) containing 10 mm MgCl2, 25 mm sodium β-glycerophosphate, 5 mm benzamidine, 1 mm Na3VO4, 0.5 mm dithiothreitol, and 100 μM ATP. Samples were subjected to electrophoresis and immunoblotting analysis with rabbit anti-phospho-IRF3 (Ser396).

*Fluorescence Microscopy*—After transfection or infection, cells were washed with phosphate-buffered saline and fixed
with ice-cold methanol and acetone for 5 min. Following this step, cells were washed with phosphate-buffered saline and stained with 4',6-diamidino-2-phenylindole (1.5 μg/ml) in the VECTASHIELD mounting medium. Samples were visualized under a fluorescent microscope, and images were captured with Zeiss AxioCam MRm camera.

Cell Fractionation Assays—Infected or transfected cells were lysed in phosphate-buffered saline containing 0.4% Nonidet P-40 and protease inhibitor mixtures (Sigma) and kept on ice with gentle inversion. After centrifugation for 3 min, the nuclei were pelleted, and supernatants were transferred to a tube. The nuclei were resuspended in phosphate-buffered saline with 0.4% Nonidet P-40 and frozen at −80°C for 30 min. The cytoplasmic and nuclear fractions were then solubilized in disruption buffer. Samples were subjected to electrophoresis and Western blot analysis with antibodies against IRF3 (Santa Cruz Biotechnology), GRP78 (glucose-regulated protein 78) (BD Transduction Laboratories), and histone H3 (Cell Signaling), respectively.

RESULTS

γ34.5 Null Mutant Activates Antiviral Immunity Early in HSV Infection—Although expressed as a leaky late gene, γ34.5 is also detectable early in infection (31, 32). To explore the biological function of γ34.5, we measured the induction of ISG54 and ISG56 early in HSV-infected cells. MEF were either mock-infected or infected with viruses, and mRNA levels were determined by RT-PCR. As illustrated in Fig. 1A, the induction of ISG54 as well as ISG56 was seen in cells infected with the γ34.5 null mutant R3616. The mRNA levels of ISG54 and ISG56 increased as virus infection progressed from 3 to 6 h. This stimulation was not observed in cells mock-infected or infected with wild type HSV-1(F), although comparable levels of 18 S RNA were noted in all cells. In correlation, wild type virus, but not the γ34.5 null mutant, expressed the γ34.5 protein at 3 and 6 h after infection (Fig. 1C). Similar results were obtained in human lung fibroblasts (HEL), although there was a delay in the kinetics of ISG54 and ISG56 induction by R3616 (Fig. 1B). Because the onset of viral DNA replication triggers the shutoff of protein synthesis mediated by dsRNA-dependent protein kinase PKR (30), we next asked whether the induction of ISG54 and ISG56 by R3616 was linked to this event. As measured by [35S]methionine labeling, at 3 or 6 h after infection, profiles of protein synthesis were similar in HEL cells infected with HSV-1(F) or R3616 (data not shown). Although eIF-2α was constitutively expressed at comparable levels, there was no detectable eIF-2α phosphorylation regardless of γ34.5 expression (Fig. 1C), suggesting that PKR is not activated early in HSV infection. These phenotypes were also seen in MEF cells. Hence, the expression of γ34.5 abrogated the induction of ISG54 and ISG56 by HSV, which was independent of eIF-2α phosphorylation and the shut-off of protein synthesis.

Previous work has demonstrated that IRF3 activation stimulates ISG56 expression in HSV-infected cells (33). We further evaluated phosphorylation of endogenous IRF3 in infected cells. As revealed by immunoblotting analysis (Fig. 2A), IRF3 was constitutively expressed in HEL cells. Unlike HSV-1(F), R3616 infection resulted in an appearance of the IRF3 phospho-
Together, these results indicate that early expression of γ1,34.5 is required to suppress phosphorylation and nuclear translocation of IRF3 in HSV infection.

γ1,34.5 Null Mutant Replicates More Efficiently in TBK1−/− Cells than in TBK1+/+ Cells—Although HSV induction of antiviral responses involves different components, this process requires TBK1 (6). We hypothesized whether there is a possible link between γ1,34.5 and the TBK1 pathway. To test this, we investigated viral growth properties in TBK1+/+ and TBK1−/− MEF cells. Specifically, cells were infected with either HSV-1(F) or R3616. At 24 h post-infection, virus yields were determined. As shown in Fig. 3A, HSV-1(F) replicated efficiently in both TBK1+/+ and TBK1−/− cells, reaching titers of 4.6 × 10^6 and 1 × 10^6 pfu/ml, respectively. In striking contrast, R3616 replicated poorly in TBK1+/+ cells, with a virus yield less than 10 pfu/ml. There was approximately 10^5-fold decrease in viral growth as compared with HSV-1(F). This reduction was attributable to the lack of γ1,34.5 in R3616. Strikingly, R3616 replicated more efficiently in TBK1−/− cells, with a titer reaching 6.6 × 10^5 pfu/ml. There was approximately 10^5-fold restoration in viral yield. This increase was partial but significant when compared with the replication seen in TBK1+/+ cells. These phenotypes were mirrored by cytopathic effects after viral infection. As illustrated in Fig. 3E, mock-infected cells formed a monolayer, with most cells displaying spindle morphology. HSV-1(F) induced morphological changes in both TBK1+/+ and TBK1−/− cells, where cells formed clumps, indicative of viral replication. In contrast, R3616 induced cytopathic effects only in TBK1−/− cells. Immunoblot analysis revealed that HSV-1(F) produced high levels of viral polypeptides in both TBK1+/+ and TBK1−/− cells, whereas R3616 produced a substantial amount of viral polypeptides only in TBK1−/− cells (Fig. 3B). Collectively, these results show that HSV infection invokes host responses via TBK1 which restricts viral replication.

To examine whether interferon was able to restore the antiviral activity in the absence of TBK1, we assessed viral responses to IFN-α. As indicated in Fig. 3, C and D, HSV-1(F) replicated well in both TBK1+/+ and TBK1−/− cells, with titers ranging from 3.2 × 10^6 to 3.9 × 10^6 pfu/ml at 24 h after infection. Treatment with IFN-α had a marginal effect on viral replication. As expected, R3616 replicated more efficiently in untreated TBK1−/− than in TBK1+/+ cells, with a titer of 2.8 × 10^3 pfu/ml. When cells were treated with IFN-α, R3616 barely replicated, with minimal infectious virus produced. Thus, addition of IFN-α in TBK1−/− cells restored the antiviral activity to mock-infected, infected with either HSV-1(F) or R3616. At indicated time points, distribution of GFP-IRF3 was visualized under a fluorescence microscope. C, quantitation of IRF3 nuclear translocation. A total of 600 GFP-IRF3-positive cells from different fields in 8 were counted. Results are expressed as means ± S.D. from three independent experiments. D, cell fractionation. Cells were treated as in B, and the cytoplasmic and nuclear fractions were prepared as described under “Experimental Procedures.” Samples were subjected to Western blot analysis with antibodies against IRF3 (Santa Cruz Biotechnology), GRP78 (BD Transduction Laboratories), and histone H3 (Cell Signalling), respectively. The protein bands were quantified using NIH Image J software. The ratio represents the relative amount of GFP-IRF3 in the nuclear and cytoplasmic fractions normalized to histone H3 or GRP78, with the mock group arbitrarily set to 1.0. GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole.

**FIGURE 2.** A, γ1,34.5 protein inhibits phosphorylation of endogenous IRF3 in infected cells. Lysates of HEL cells mock-infected or infected with viruses (5 pfu/cell), as described in Fig. 1C, were subjected to immunoblotting analysis with antibodies against IRF3 and phosphorylated IRF3(Ser395), respectively. B, γ1,34.5 protein blocks nuclear translocation of IRF3 in infected cells. 293T cells were transfected with GFP-IRF3. At 24 h after transfection, cells were infected with HSV-1(F) or R3616. At 24 h post-infection, virus yields were determined. C, quantitation of IRF3 nuclear translocation. A total of 600 GFP-IRF3-positive cells from different fields in 8 were counted. Results are expressed as means ± S.D. from three independent experiments. D, cell fractionation. Cells were treated as in B, and the cytoplasmic and nuclear fractions were prepared as described under “Experimental Procedures.” Samples were subjected to Western blot analysis with antibodies against IRF3 (Santa Cruz Biotechnology), GRP78 (BD Transduction Laboratories), and histone H3 (Cell Signalling), respectively. The protein bands were quantified using NIH Image J software. The ratio represents the relative amount of GFP-IRF3 in the nuclear and cytoplasmic fractions normalized to histone H3 or GRP78, with the mock group arbitrarily set to 1.0. GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole.
The growth pattern of R3616 resembled that seen in TBK1/H11001/ cells. Thus, TBK1-induced downstream antiviral molecules likely contribute to the inhibitory effect on viral replication.

γ34.5 Protein Associates with TBK1 and Inhibits Activation of IFN-β and ISG56 Promoters—The functional link between TBK1 and γ34.5 raised a possibility that γ34.5 may interact with TBK1 and suppress its activity. To test this hypothesis, we carried out coimmunoprecipitation experiments in 293T cells transfected with a vector, HA-γ34.5, FLAG-TBK1, and FLAG-dN200, a truncated form of Ebola VP35. As shown in Fig. 4A, the γ34.5 protein was coimmunoprecipitated with TBK1 but not with the control protein dN200. Levels of protein expression were comparable in lysates of transfected cells. These data indicate that the γ34.5 protein specifically associates with TBK1. As TBK1 activates the expression of ISG56 and IFN-β,
we also performed luciferase reporter assays in 293T cells. As indicated in Fig. 4B, the expression of TBK1 activated the IFN-β promoter by ~90-fold. However, coexpression of γ34.5 inhibited this induction in a dose-dependent manner. Likewise, the γ34.5 protein suppressed the induction of the ISG56 promoter by TBK1 (Fig. 4C). We conclude that in the absence of any other HSV proteins, the γ34.5 protein associates with TBK1 and prevents the activation of ISG56 and IFN-β promoters.

**γ34.5 Protein Is Sufficient to Block Phosphorylation and Nuclear Translocation of IRF3**—When bound to IRF3, TBK1 phosphorylates the carboxyl terminus of IRF3, which permits nuclear translocation and activation of IRF3 (7, 34). To gain insight into γ34.5 function, we examined whether the γ34.5 protein directly disrupted this process. Lysates of 293T cells transfected with FLAG-TBK1 and HA-γ34.5 were immunoprecipitated with anti-FLAG antibody. Immunocomplexes were subjected to *in vitro* kinase assays with recombinant GST-IRF3 (Fig. 5A). It is notable that there were some variations in TBK1 expression. Nonetheless, as the expression of γ34.5 was elevated, IRF3 phosphorylation was reduced, indicating that the γ34.5 protein inhibits IRF3 activation. A simple explanation for the inhibitory effect of the γ34.5 protein is that it sequesters TBK1 in an inactive complex and blocks the access of IRF3. To test this idea, we analyzed the TBK1 complex by immunoprecipitation. 293T cells were transfected with FLAG-TBK1, IRF3, and HA-γ34.5. Protein expression was detected in cell lysates (Fig. 5B, upper panels). In parallel, the TBK1 complex was immunoprecipitated with anti-FLAG antibody and subsequently analyzed for the presence of TBK1, γ34.5, and IRF3 (Fig. 5B, lower panels). Although TBK1 remained at similar levels in immunoprecipitates, IRF3 and γ34.5 displayed different patterns. In the absence of γ34.5, IRF3 associated with TBK1 (Fig. 5B, lane 3). As the level of γ34.5 increased, the amount of IRF3 associated with TBK1 diminished (Fig. 5B, lanes 4–7). Thus, expression of the γ34.5 protein displaced IRF3 in the TBK1 complex. To determine whether γ34.5 blocked nuclear translocation of IRF3 stimulated by TBK1, a cellular localization experiment was performed in 293T cells expressing GFP-IRF3 or in combination with TBK1 and γ34.5 (Fig. 5C). When expressed alone, IRF3 remained in the cytoplasm. Addition of TBK1 induced IRF3 redistribution to the nucleus in ~30% of GFP-IRF3-positive cells. This response was suppressed to less than 10% upon expression of the γ34.5 protein as illustrated among cells from different fields (Fig. 5D). Further analysis by cell fractionation revealed similar phenotypes. As illustrated in Fig. 5E, TBK1 strongly stimulated nuclear translocation of GFP-IRF3. However, addition of γ34.5 drastically reduced nuclear accumulation of GFP-IRF3. Control proteins GRP78 and histone H3 remained in the cytoplasmic and
nuclear fractions, respectively. These results suggest that the \(\gamma_{134.5}\) protein blocks IRF3 phosphorylation and nuclear translocation.

**Deletions in the Amino Terminus of \(\gamma_{134.5}\) Disrupt Its Activity on TBK1**—The \(\gamma_{134.5}\) protein consists of 263 amino acids, with a large amino-terminal domain, a linker of triplet repeats, and a carboxyl-terminal domain (35). To map the functional domain, we constructed a series of \(\gamma_{134.5}\) variants with deletions in either the amino terminus or the carboxyl terminus (Fig. 6A). N159 has a deletion in the region spanning amino acids 159–263, whereas \(\Delta 30\), \(\Delta 72\), \(\Delta 106\), and \(\Delta 146\) have deletions in regions encompassing amino acids 1–30, 30–72, 72–106, and 106–146, respectively. We first evaluated these mutants in reporter assays with an IFN-\(\beta\)–106, and 106–146, respectively. We first evaluated these mutations in regions encompassing amino acids 1–30, 30–72, and 159–263, whereas \(\Delta 30\), \(\Delta 72\), \(\Delta 106\), and \(\Delta 146\) have deletions in regions encompassing amino acids 1–30, 30–72, 72–106, and 106–146, respectively. We first evaluated these mutants in reporter assays with an IFN-\(\beta\) promoter construct (Fig. 6B). Like wild type \(\gamma_{134.5}\), N159 suppressed the induction of IFN-\(\beta\) by TBK1 efficiently, indicating that deletion of the carboxyl-terminal domain has no effect. Similarly, \(\Delta 30\), \(\Delta 72\), and \(\Delta 146\) inhibited the IFN-\(\beta\) promoter activity to different degrees. Hence, deletions from amino acids 1 to 72 or from 106 to 146 had little effect on the transcriptional activity of IFN-\(\beta\). Therefore, deletion of amino acids 72–106 in \(\gamma_{134.5}\) substantially relieved its inhibitory effect. We next assessed the ability of \(\gamma_{134.5}\) to bind TBK1 by immunoprecipitation. As illustrated in Fig. 6C, all \(\gamma_{134.5}\) variants, except \(\Delta 106\), coprecipitated with TBK1. These activities paralleled the phenotypes seen in reporter assays. These results indicate that the region spanning amino acids 72–106 in the \(\gamma_{134.5}\) protein is indispensable to inhibit TBK1.

**DISCUSSION**

Here we provide evidence that the \(\gamma_{134.5}\) protein inhibits the induction of antiviral signaling exerted by TBK1. Relevant to this is the finding that \(\gamma_{134.5}\) is essential to promote viral virulence (28). In infected cells, the \(\gamma_{134.5}\) protein prevents translational arrest mediated by the double-stranded PKR (36). In doing so, it forms a high molecular complex with protein phosphatase 1 that dephosphorylates eIF2\(\alpha\) (36), which contributes to HSV replication in vivo (37). This model is generally used to explain the role of \(\gamma_{134.5}\) in HSV infection. Paradoxically, a \(\gamma_{134.5}\) null mutant with secondary-site mutations in the viral genome inhibits PKR activity but remains attenuated (38), suggesting that elements in addition to inhibition of translation shutoff contribute to viral virulence. In support of this notion, we found that TBK1 is a novel target of HSV \(\gamma_{134.5}\), which blocked IRF3 activation and the induction of antiviral genes early in HSV infection. This activity was independent of eIF-2\(\alpha\) phosphorylation and the shutoff of protein synthesis. Indeed, the \(\gamma_{134.5}\) protein associated with TBK1 suppressed the expression of antiviral genes. Notably, unlike wild type virus, the \(\gamma_{134.5}\) null mutant replicated more efficiently in TBK1 \(-/-\) cells than in TBK1 \(+/+\) cells. Therefore, in addition to the PKR pathway, \(\gamma_{134.5}\) interrupts the TBK1 pathway. These results may partly explain why inhibition of PKR alone does not restore HSV replication.

The requirement of \(\gamma_{134.5}\) underscores the vital role of TBK1 against HSV infection, which suggests that TBK1 contributes to the evolutionary maintenance of \(\gamma_{134.5}\) in HSV. TBK1 is at the center of TLR-dependent and -independent pathways (2). It is engaged with multiple sentinel proteins, which include NAP1, TANK, SINTBAD, DAI, and Sec5 (5, 15–18). NAP1, TANK, SINTBAD, and Sec5 activate TBK1 in response to RNA from the cytoplasmic or TLR3 pathways, whereas DAI stimulates TBK1 through double-stranded DNA in the cytoplasm. Nonetheless, TBK1 binds to and phosphorylates IRF3 (7, 34), which stimulates a spectrum of antiviral genes. Because the \(\gamma_{134.5}\) protein associates with and suppresses the TBK1 activity, it is reasonable to propose that this viral protein interferes with cell signaling initiated from TLR3, TLR4, RIG-I/MDA5, or DAI pathways during HSV infection. In this context, it is notable that individuals with TLR3 deficiency are more susceptible to herpes simplex virus 1 encephalitis (3), suggesting a link between HSV and the TLR3 pathway.

The interaction of \(\gamma_{134.5}\) and TBK1 suggests two nonmutually exclusive models. One possibility is that the \(\gamma_{134.5}\) protein may disrupt interactions of TBK1 with one or more of the TBK1

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**FIGURE 6.** A, a schematic diagram of \(\gamma_{134.5}\) variants. B, effect of \(\gamma_{134.5}\) variants on the IFN-\(\beta\) promoter activity. 293T cells were cotransfected with an empty vector, FLAG-TBK1 (50 ng), FLAG-\(\gamma_{134.5}\) variants (900 ng), and an IFN-\(\beta\) luciferase reporter. A plasmid containing the Renilla luciferase gene driven by the HSV-1 TK promoter was included for normalization. At 36 h post-transfection, the cells were harvested, and luciferase activities were assayed. Results are expressed as fold of activation with standard deviations among triplicate samples. C, interaction of \(\gamma_{134.5}\) variants with TBK1. 293T cells were cotransfected with HA-TBK1 and FLAG-\(\gamma_{134.5}\) variants. At 40 h after transfection, lysates were immunoprecipitated (IP) with anti-HA antibody. Proteins in the lysates and precipitates were analyzed by immunoblotting with anti-HA and anti-FLAG antibodies, respectively. **WB,** Western blot.


\section*{\(\gamma_134.5\) Protein and TBK1}

Adaptors and block upstream signaling. Another possibility is that the \(\gamma_134.5\) protein may prevent the access of a downstream target to the TBK1 complex. Consequently, the \(\gamma_134.5\) protein inhibits TBK1-mediated innate immunity. We noted that IRF3 was dislodged from TBK1 as the level of \(\gamma_134.5\) increased in TBK1 immunoprecipitates. Additionally, the \(\gamma_134.5\) protein blocked nuclear translocation of IRF3. These experimental data argue \(\gamma_134.5\) acts as a competitor of IRF3 for TBK1 binding. TBK1 bears a ubiquitin-like domain that regulates the kinase activity (39). When this domain is in close proximity with the kinase domain, TBK1 is in an active state and interacts with IRF3. Hence, binding of the \(\gamma_134.5\) protein likely disrupted this active conformation. Recent studies demonstrate that TBK1 binds to and phosphorylates DEAD box protein 3 (40, 41). Although the underlying mechanism remains unknown, this protein activates the IFN-\(\beta\) promoter upon translocation into the nucleus. These observations suggest that activation of antiviral responses by TBK1 relies on the cooperation of IRF3 and DEAD box protein 3. It is possible that \(\gamma_134.5\) may also interfere with the activity of DEAD box protein 3.

Previous studies indicated that the carboxyl-terminal domain of \(\gamma_134.5\) binds to protein phosphatase 1 and mediates eIF-2\(\alpha\) dephosphorylation (42). This raises the possibility that the \(\gamma_134.5\)-PP1 complex may regulate the TBK1 activity. However, the data present in this study do not support this contention because the amino-terminal domain is sufficient to exert its inhibitory effect on TBK1. We noted that deletion in the region spanning amino acids 70–106 impaired the ability of \(\gamma_134.5\) to bind and inhibit TBK1, which suggests that this region may represent a functional element. The crystal structure of \(\gamma_134.5\) has not been resolved. This region, conserved in \(\gamma_134.5\) from both HSV-1 and HSV-2, is predicted to form an \(\alpha\)-helix followed by a flexible region. It is noteworthy that a cluster of conserved residues centers on this region, with a stretch of leucines and aspartic acids. We speculate that these conserved amino acids are required for protein-protein interactions. Alternatively, they may serve as structural elements. Because deletions in other regions had minimum effects, this latter possibility is less likely. Work is in progress to understand the deletions in other regions had minimum effects, this latter possibility is less likely. Work is in progress to understand the deletions in other regions had minimum effects, this latter possibility is less likely. Work is in progress to understand the

HSV-1 is a large DNA virus that interacts with innate immune systems in a complex way. In addition to \(\gamma_134.5\), Us11 inhibits PKR activation by its RNA binding domain (50). This viral protein further inhibits 2',5'-oligoadenylate synthetase, a cellular protein critical for the antiviral action of interferon (51). Moreover, an immediate early protein ICP0 confers viral resistance to IFN by disseminating promyelocytic leukemia protein (52, 53). Notably, ICP0 also inhibits the induction of IFN-responsive genes, where it sequesters or promotes partial IRF3 degradation (21–23). Recent studies show that another immediate early protein ICP27 is required to suppress cytokine induction mediated by IRF3 in macrophage dendritic cells and embryonic fibroblasts (1, 25). This effect is speculated to result from the regulation of mRNA processing or transport by ICP27 (25). Notably, ICP27 also blocks type I IFN signaling by down-regulating STAT-1 phosphorylation (54). Our observations with \(\gamma_134.5\) indicate that it is essential to suppress the induction of IFN-responsive genes mediated by TBK1. These activities are likely to create a favorable environment for HSV infection. Thus, a question arises as to why HSV employs more than one viral protein to inhibit the induction of antiviral genes. Because HSV replication is regulated in a cascade fashion (55), it is possible that the coordinated action of ICP0, ICP27, and \(\gamma_134.5\) is required to efficiently thwart host defenses. Alternatively, one or more of these viral proteins may function differentially in different cells or tissues \textit{in vivo}. In this regard, it is interesting that HSV induces innate immune responses in a cell type- and time-dependent manner (1). The molecular interplay between the immune systems and ICP0, ICP27, and \(\gamma_134.5\) awaits further investigation. Nonetheless, our work suggests that the interaction of the \(\gamma_134.5\) protein and TBK1 is a critical determinant of viral replication.

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