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The Ebola virus protein VP35, impairs the function of the interferon regulatory factor-activating kinases, IKKε and TBK-1

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Abstract

The Ebola virus (EBOV) VP35 protein antagonizes the early, antiviral interferon (IFN) α/β response. We previously demonstrated that VP35 inhibits virus-induced activation of the IFN β promoter by blocking the phosphorylation of interferon-regulatory factor 3 (IRF-3), a transcription factor crucial for the induction of IFN α/β expression. Further, VP35 blocks IFN β promoter activation induced by any of several components of the retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA-5) activated signaling pathways including: RIG-I, interferon-beta promoter stimulator-1 (IPS-1), TANK-binding kinase-1 (TBK-1) and IκB kinase epsilon (IKKε). These results suggested that VP35 may target the IRF kinases, TBK-1 and IKKε. Co-immunoprecipitation experiments now demonstrate physical interaction of VP35 with IKKε and TBK-1, and use of an IKKε deletion construct further demonstrates that the amino terminal kinase domain of IKKε is sufficient for interaction with either IRF-3 or VP35. *In vitro*, either IKKε or TBK-1 phosphorylate not only IRF-3 but also VP35. Moreover, VP35 over-expression impairs IKKε-IRF-3, IKKε-IRF-7, and IKKε-IPS-1 interactions. Finally, lysates from cells over-expressing IKKε contain kinase activity that can phosphorylate IRF-3 *in vitro*. When VP35 is expressed in the IKKε-expressing cells, this kinase activity is suppressed. These data suggest that VP35 exerts its IFN-antagonist function, at least in part, by blocking necessary interactions between the kinases, IKKε/TBK-1, and their normal interaction partners, including their substrates, IRF-3 and IRF-7.

Introduction

Ebola viruses (EBOVs), members of the family *Filoviridae,* are filamentous, enveloped, negative-sense, single-stranded RNA viruses which cause frequently lethal hemorrhagic fevers in humans and non-human primates (44). EBOV disease is characterized by fever, shock, coagulation defects, and impaired immunity. Fatal infections are also characterized by progressively increasing systemic viral titers and cytokines, consistent with a model in which host innate and adaptive immune responses are unable to control infection, while the inflammatory response becomes over-activated, causing disease (5, 12).

Ebola virus infection blocks cellular interferon (IFN) α/β responses; critical components of the host innate immune response to virus infection (13-15, 23). Two EBOV proteins appear to function in the suppression of IFN α/β responses-VP35 and VP24 (2, 3, 7, 9, 16, 18, 19, 41, 42). The VP35 protein is a multifunctional protein that plays a key role in viral replication and nucleocapsid assembly (22, 35, 36). VP35 possesses a carboxy-terminal domain with a unique fold that allows for dsRNA-binding, a function that may be necessary for inhibition of IFN α/β production (2, 3, 7, 9, 28). The VP24 protein impairs cellular responses to exogenous IFN α/β and IFN γ by blocking the nuclear import of activated STAT1 (41, 42). Recently, mutation of individual basic amino acids within the carboxy-terminus of VP35 rendered recombinant EBOVs less able to inhibit IFN α/β responses in cell culture, resulted in enhanced activation of interferon regulatory factor 3 (IRF-3) and attenuated the virus in cell culture and *in vivo* (16-18). Because VP35 contributes to virus escape from host innate immunity and is required for virulence, understanding the mechanisms by which it acts as an IFN-antagonist is of importance.

IFN α/β activates in cells an antiviral state which can limit spread of infection and also influences adaptive immune responses (11). Upon virus infection, the IFN α/β response can be

triggered by cytoplasmic sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) (24, 53, 54). Signal propagation occurs through the mitochondrial-associated adapter IFNβ-promoter stimulator 1 (IPS-1) (also known as MAVS, VISA, or CARDIF) (25, 33, 45, 52), which subsequently activates Inhibitor of κB kinase epsilon (IKKε) and TANK-binding kinase 1 (TBK-1) (10, 20, 25, 32, 33, 46). These kinases in turn phosphorylate the otherwise inactive transcription factors IRF-3 and/or IRF-7. Phosphorylation results in activation, dimerization and translocation to the nucleus, where IRF-3/-7 contribute to the transcription of IFNα/β genes (26, 30, 55). In most cell types, constitutively expressed IRF-3 is predominately activated during the initial response to virus infection. This triggers expression of IFN β and select IFN α genes. IRF-7 activates a larger number of IFN α genes, and its expression is IFN-inducible. Thus, induction of IRF-7 expression and its subsequent activation provides a means of amplification of the IFN α/β response (29).

VP35 expression prevents the phosphorylation, dimerization, and nuclear translocation of IRF-3 induced by virus infection, thereby inhibiting IFN α/β gene expression (2). Further, VP35 can block activation of the IFNβ-promoter induced by over-expression of any of several components of the RIG-I signaling pathway, including RIG-I, IPS-1, IKKε or TBK-1 (7). However, VP35 does not prevent IFN β promoter activation induced by a constitutively active IRF-3 (IRF-3 5D) (2, 7). These data suggest that VP35 may act proximal to the IRF-3/-7 kinases, IKKε or TBK-1 to suppress IFN α/β gene expression.

In this report, we provide evidence that VP35 physically interacts with IKKε and TBK-1 and is phosphorylated by these kinases. Moreover, we show that VP35 can impair IKKε-IRF-3, IKKε-IRF-7 and IKKε-IPS-1 interactions. Consistent with a model in which VP35 targets the

IRF-3 kinases, the kinase activity of lysates from cells transfected with IKKε is decreased when VP35 is present.

Materials and Methods

Antibodies. Monoclonal antibody 6C5 against the Zaire Ebola virus VP35 (6C5) and Zaire Ebola virus nucleoprotein (NP) were generated in collaboration with the Mount Sinai Hybridoma Center. The monoclonal anti-HA and anti-FLAG (M2) and polyclonal anti-HA and anti-FLAG antibodies were purchased from Sigma (St. Louis).

Cell lines and viruses. 293T cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Sendai virus strain Cantell (SeV) was grown in 10-day-old embryonated chicken eggs for 2 days at 37°C.

Plasmids. The Zaire Ebola virus VP35, FLAG-RIG-I, IPS-1 were cloned into pCAGGS as described elsewhere (3, 7, 37). Plasmids encoding human cDNAs for wild-type TBK-1 and IKKε were kindly provided by John Hiscott (McGill University). FLAG-tagged versions of these cDNAs were amplified by PCR and inserted into the expression plasmid pCAGGS (37). A kinase inactive IKKε (IKKεKN) was generated by introducing the previously described K38A mutation into IKKε (39, 40, 47). A kinase-inactive K38M mutant of TBK-1 (TBK-1KN) was kindly provided by Benjamin tenOever (Mount Sinai School of Medicine). Plasmids encoding human IRF-3 were previously described (2). IRF-3 amino acids 375-427 were amplified by PCR for expression as GST fusions in *Escherichia coli*. The pCAGGS-FLAG-IRF-7 construct was kindly provided by Adolfo García-Sastre (Mount Sinai School of Medicine).

Bacterial expression and purification of GST and GST-IRF-3 C-terminus. GST and GST-IRF-3-C terminus, residues 375-427, (IRF-3-C) were expressed in *E. coli* Origami B BL21(DE3)pLysS host strains (Stratagene). Cultures were grown at 37°C to an $O.D._{600nm}$ of 0.57, and IRF-3 expression was induced by addition of 0.1mM IPTG. Induced cells were grown further at 18°C for 24 hours. Lysates were prepared by sonication for 10 seconds five times in lysis buffer (25mM Tris-HCl (pH 7.5), 200mM NaCl, 0.1% NP-40, 1mM EDTA, 0.1mM DTT and a cocktail of protease inhibitors (Roche)). Bacterially produced protein was purified from cell lysates on a glutathione sepharose (Amersham Biosciences) column. After loading, the column was washed with 25mM Tris-HCL, 1M NaCl, 0.1% NP-40, 1mM EDTA and eluted with 5mM glutathione, 25mM Tris, 200mM NaCl, 1mM EDTA, 1mM TCEP, 5% glycerol, 0.2% CHAPS. Dialysis was then performed overnight in 1L of kinase buffer (20mM HEPES, 1mM beta-glycerophosphate, 50mM NaCl, 1mM EDTA, 1mM dithiotreitol, and 0.1 mM $NaVO_3$).

Transfections. HEK 293T cells were transfected with a 1:1 ratio of Lipofectamine 2000 to plasmid DNA in OptiMEM medium (Gibco) at 37°C for 8 hours. For subsequent infection of cells, the transfection medium was removed and SeV was added, at an MOI of 10, in PBS, 0.3% bovine serum albumin for 1 hr. Infection medium was then replaced with DMEM, 10% fetal bovine serum and cells were incubated at 37°C overnight. Following overnight incubation, cells were lysed in lysis buffer (50mM Tris, pH 8, 1% NP-40, 280mM NaCl, 0.2mM EDTA, 2mM EGTA, and 10% glycerol).

Immunoprecipitations. Lysates were incubated with 1μg of indicated antibody for 4 hours at 4°C, followed by 1 hour incubation with protein A sepharose beads (Roche). Beads were washed

five times with lysis buffer. After washing, beads were resuspended in SDS-PAGE sample loading buffer, separated by 10% SDS-PAGE and analyzed by western blot as indicated.

Purification of FLAG-tagged proteins. HEK 293T cells were transfected with 2μg of expression plasmids for FLAG-tagged IKKε, IKKεKN, TBK-1, TBK-1KN, or VP35. The transfected cell lysates were immunoprecipitated with M2 anti-FLAG affinity gel (Sigma). The FLAG-tagged proteins were eluted from the affinity gel by two sequential incubations with FLAG peptide at 100μg/ml. The eluate was concentrated twenty fold, and buffer was exchanged to kinase buffer with a microcon centrifugal filter device (Millipore). Proteins were stored at -80°C in kinase buffer (without dithiotreitol or $NaVO_3$) supplemented with 4% glycerol.

***In vitro* kinase assays.** Purified FLAG-tagged IKKε, TBK-1, TBK-1KN or IKKεKN was incubated with 5 μCi [γ-^{32}P]ATP (Perkin Elmer), 0.1mM unlabeled ATP and either GST, GST-IRF-3 (375-427) or FLAG- tagged VP35 in 30μl kinase buffer (20mM HEPES, 1mM beta-glycerophosphate, 50mM NaCl, 1mM EDTA, 1mM dithiotreitol, and 0.1 mM $NaVO_3$) (adapted from (27)). Reactions were incubated at 30^{o}C for 30 minutes and terminated by addition of SDS sample loading buffer. Proteins were separated by 12% SDS-PAGE, and phosphorylation was visualized by autoradiography.

To examine IRF-3 phosphorylation by cell lysates, *in vitro* kinase assays were performed as described above using as substrate GST-IRF-3-C and, as a source of kinase, lysates from 12% of 2 x10^6 cells (2.4 x10^5 cell equivalents) co-transfected with FLAG-IKKε expression plasmid and either empty vector, increasing concentrations of VP35 expression plasmid (1μg, 2μg, and 4μg) or EBOV NP plasmid. Kinase reactions were terminated after 1hr by adding glutathione

sepharose (Amersham Biosciences) in lysis buffer for affinity purification of GST-IRF3-C. Following incubation on a nutator mixer for 1hr, beads were washed five times with lysis buffer. SDS-PAGE sample loading buffer was added and proteins were separated by 10% or 12% SDS-PAGE. Phosphorylation was visualized by autoradiography and quantified by ImageJ software. Transfected proteins were visualized by western blot of lysates from 1 $x10^5$ cell equivalents with the indicated antibodies.

Reporter Assay. 293T cells were transfected with the indicated amount of expression plasmid DNA together with 400ng of the IFNalpha4-CAT reporter plasmid (2) and 200ng of the constitutive firefly luciferase reporter plasmid. Twelve hours post-transfection, cells were infected with SeV or mock infected for 1 hour. Twelve hours post-infection, cells were lysed with reporter lysis buffer (Promega) and CAT activities were measured (43). Firefly luciferase activity was determined as recommended by the manufacturer (Promega) and was used to normalize CAT activity. IFNalpha4 reporter gene activation is expressed as fold induction over an empty vector mock infected control.

Results

VP35 interacts with the IRF-3 kinases IKKε and TBK-1. VP35 was previously reported to block phosphorylation of IRF-3 and to inhibit IRF-3-dependent gene expression induced by over-expression of IKKε and TBK-1 (2, 7). To test whether IKKε and TBK-1 are targeted by VP35, co-immunoprecipitation (co-IP) experiments were performed. 293T cells were transfected with expression plasmids for FLAG-tagged kinase inactive forms of IKKε (IKKεKN) (Fig. 1 lanes 2, 5, 7, 8, 10) or TBK-1 (TBK-1KN) (Fig. 1 lanes 1, 4, 6) (39, 40, 47), alone or with VP35 (Fig. 1 lanes 3-7, 9, 10). Inactive kinases were used for co-IP experiments because

interaction of the kinases with their substrate IRF-3 is more readily detected by co-IP when inactive rather than functional kinases are used (data not shown). This presumably reflects the fact that active kinases, upon over-expression, rapidly phosphorylate IRF-3, resulting in its nuclear accumulation. The VP35-kinase interactions, however, can be detected by co-immunoprecipitation with equal efficiency using either the kinase active or inactive forms (data not shown). Twelve hours post-transfection, cells were either mock infected (lanes 1-5) or infected with SeV (lanes 6-10). Twenty four hours post-transfection cells were lysed as described in the methods section. Immunoprecipitations were then performed on lysates by using anti-VP35 monoclonal antibody (lanes 1-7). IKKεKN and TBK-1KN each co-precipitated with VP35 (Fig. 1, lanes 4-7). The reciprocal co-IP was also performed with FLAG-IKKεKN using anti-FLAG monoclonal antibodies (lanes 8-10). When an anti-FLAG immunoprecipitation was performed, VP35 co-precipitated with FLAG-IKKεKN (Fig.1, lane 10). Therefore, VP35 interacts with the IRF kinases.

IKKεKN and TBK-1 can phosphorylate VP35 *in vitro*. Having demonstrated a physical interaction between VP35 and both IKKε and TBK-1, we sought to determine whether IKKε or TBK-1 can phosphorylate VP35 in an *in vitro* kinase assay. FLAG-tagged IKKε, FLAG-tagged TBK-1 and FLAG-tagged VP35 were each purified from separate, transiently-transfected 293T cell cultures. Increasing amounts of FLAG-IKKε (Fig. 2A and B, amounts denoted by wedges) were incubated with constant amounts of GST (Fig. 2A and B, lanes 1-3), GST fused to the C-terminal region of IRF-3 (amino acids 375-427) (IRF-3-C) (Fig. 2A and B, lanes 4-6) or FLAG-VP35 (Fig. 2A and B, lanes 7-9). *In vitro* kinase assays were performed as described in the methods section. The products separated by SDS-PAGE and developed by autoradiography

(Fig. 2A) and by Coomassie blue staining (Fig. 2B). Alternatively, FLAG-TBK-1 was used in *in vitro* kinase assays (Fig. 2C and D, lanes 4-7) with GST (lanes 1, 5), GST-IRF-3-C (lanes 2, 6) or FLAG-VP35 (lanes 3, 7).

As previously reported, both IKKε and TBK-1 undergo apparent auto-phosphorylation (Fig. 2A and C) (47). Neither kinase phosphorylated the negative control protein, GST (Fig. 2A, lanes 1-3 and 2C, lane 5), nor were VP35 or GST-IRF-3-C phosphorylated when the kinase negative forms of the kinases were used (Fig. 2A, lanes 10-11 and Fig. 2C, lane 8). The kinase competent IKKε phosphorylated GST-IRF-3-C as well as VP35, shown by figure 2A lanes 4-6 and 7-9, respectively. TBK-1 also phosphorylated GST-IRF-3-C and VP35 as shown in figure 2C lanes 6 and 7, respectively. Examination of the Coomassie blue stained gels demonstrated that the purified protein preparations did not contain visible amounts of contaminating cellular proteins and that comparable amounts of GST, GST-IRF-3-C and VP35 were present in the kinase reactions (Fig. 2B and D). These data demonstrate that IKKε and TBK-1 can phosphorylate VP35.

VP35 disrupts IKKεKN-IRF-3 and IKKεKN-IRF-7 interaction. The interaction of VP35 with IKKε and TBK-1, coupled with the phosphorylation of VP35 by these kinases suggested that VP35 might act as an alternative substrate which blocks the interaction between IKKε or TBK-1 and their IRF substrates. To address this question co-IP experiments were performed, focusing on IKKε as a representative IRF-3 kinase. Cells were co-transfected with full length FLAG-IKKεKN (Fig. 3A, lanes 1, 4-7) and HA-IRF-3 (lanes 2, 4-7) plasmids in the absence (lane 4) or the presence of increasing amounts of VP35 plasmid (lanes 5-7). IRF-3 was immunoprecipitated using monoclonal anti-HA antibody and co-precipitated FLAG-IKKεKN was analyzed by

western blotting with anti-FLAG polyclonal antibody. HA-IRF-3 pulled-down FLAG-IKKεKN and the amount of IKKεKN that co-precipitated with IRF-3 decreased as the amount of VP35 increased (Fig. 3A, top panel).

VP35 was previously reported to block IRF-3-dependent gene expression induced by SeV infection (2, 7). To determine whether VP35 could block IRF7-dependent gene expression as well, reporter assays were performed utilizing an IRF-7-dependent promoter, IFNalpha4. 293T cells were transfected with an IFNalpha4-CAT reporter plasmid, a constitutive firefly luciferase plasmid and either empty vector (Fig. 3B, samples 1 and 4), FLAG-IRF-7 alone (samples 2 and 5) or FLAG-IRF-7 with VP35 expression plasmids (samples 3 and 6). Cells were subsequently mock infected or infected with SeV, at an MOI of 10. Expression of IRF-7 alone was sufficient to weakly induce IFNalpha4 promoter activation relative to empty vector transfected cells (Fig. 3B, samples 1 and 2). This IRF-7-induced activation of the promoter was decreased upon co-expression with 3ug of VP35 (sample 3). SeV infection of IRF-7 expressing cells resulted in a dramatic induction of the IFNalpha4 reporter (sample 5) compared with that seen for mock infected cells (samples 1 and 2) and SeV infected empty vector expressing cells (sample 4), highlighting the role of IRF-7 in this reporter activation. Co-expression of VP35 with IRF-7 drastically decreased the SeV-induced IFNalpha4 reporter activation (sample 6). Therefore, VP35 is able to block the SeV-mediated activation of an IRF-7-dependent promoter.

Consistent with this functional assay, a biochemical assay similar to that described in figure 3A was performed. 293T cells were co-transfected with full length HA-IKKεKN (Fig. 3C, lanes 1, 3-6) and FLAG-IRF-7 (lanes 2-6) plasmids in the absence (lane 3) or the presence of increasing amounts of VP35 plasmid (lanes 4-6). IKKεKN was immunoprecipitated using monoclonal anti-HA antibody and co-precipitated FLAG-IRF-7 was analyzed by western

blotting with anti-FLAG polyclonal antibody. HA-IKKεKN pulled down FLAG-IRF-7 (lane 3) and the amount of IRF-7 that co-precipitated with IKKεKN decreased when increasing amounts of VP35 were present. Loss if IRF-7-kinase interaction was most dramatic when the highest concentration of VP35 was present (Fig.3C, lane 6). Notably, both in the IRF-3 (Fig. 3A) and IRF-7 (Fig. 3C) experiments, the presence of VP35 results in increased expression of the co-transfected IRF and, to a variable extent, the co-transfected IKKεKN. The molecular basis of this effect is unclear, but this may influence the apparent efficiency with which VP35 appears to affect kinase-IRF interaction in these different experiments. As previously reported in Cárdenas et. al. (7), the amounts of VP35 produced in transfected cells is comparable to what is seen in EBOV infected cells, suggesting that results obtained in transfected cells are likely to be biologically relevant. Cumulatively, these data demonstrate that VP35 can disrupt the physical interaction between full length IKKεKN and either IRF-3 or IRF-7, and this physical disruption contributes to the IFN antagonist function of VP35.

The IKKε amino-terminal kinase domain can interact with IRF-3 and with VP35. The amino-terminal kinase domains of IKKε and TBK-1 are quite homologous, with the two proteins exhibiting approximately 70 percent amino acid identity over their first 350 amino acids (data not shown). To determine if VP35 can interact with the kinase domain of IKKε, a FLAG-tagged IKKεKN deletion mutant consisting of the amino-terminal 315 amino acids (N315) was created (39, 40, 47, 50). This mutant was tested for interaction with either HA-tagged IRF-3 or with untagged VP35 by co-IP assay in figure 4. Cells were transfected with either full length IKKεKN (Fig. 4, lanes 1, 3, 5) or the N315 truncation mutant (lanes 1, 4, 6) and either HA-tagged IRF-3 (lanes 3 and 4) or VP35 (lanes 5 and 6). HA-IRF-3 and VP35 were expressed in the absence of

kinase in lane 2. Anti-HA and anti-VP35 monoclonal antibodies were then added to the transfected cell lysates for immunoprecipitation of IRF-3 and VP35, respectively. As shown in figure 4, VP35 and IRF-3 each physically interacted with the N315 kinase domain (Fig. 4, lanes 4 and 6) as assessed by western blot with anti-FLAG polyclonal antibody; further suggesting that VP35 might physically block the IRF-3-IKKε interaction.

Over-expression of VP35 disrupts IKKεKN N315-IRF-3 interaction. To determine whether VP35 can disrupt binding between the IKKε kinase domain and IRF-3, co-IP experiments were performed using the IKKεKN kinase domain (N315). FLAG-tagged N315 was expressed in 293T cells alone (Fig. 5, lane 1) or with HA-IRF-3 (lanes 3-9) in the absence (lane 4) or presence of increasing amounts of VP35 (lanes 6-9). VP35 was expressed in the absence of kinase domain or HA-IRF-3 in lane 2. IRF-3 was immunoprecipitated with anti-HA antibody and co-precipitation of the N315 kinase domain was assessed by western blot using polyclonal anti-FLAG antibody. IRF-3 interacted with N315, but the amount of N315 co-precipitated decreased as levels of VP35 increased (Fig. 5 top panel). Therefore, the presence of VP35 physically disrupts IKKε kinase domain-IRF-3 interaction in a concentration dependent manner.

VP35 over-expression disrupts the IKKε-IPS-1 interaction. IKKε also interacts with IPS-1 (33). To determine whether VP35 might influence this interaction as well, we performed co-immunoprecipitation experiments similar to those shown in figure 5, using IPS-1 as an IKKε binding partner. Cells were transfected with HA-IPS-1 (Fig. 6, lanes 3-9) and FLAG-IKKε (lanes 2, 4, 6-9) expression plasmids in the absence or presence of increasing amounts of VP35 (lanes 5-9). HA-IPS-1 was immunoprecipitated by adding anti-HA monoclonal antibody to the

cell lysates and co-immunoprecipitation of FLAG-IKKε was assessed by western blot using anti-FLAG polyclonal antibody. The IKKεKN-IPS-1 interaction was impaired, as shown in figure 6 (lane 9, top panel). However, the inhibition did not show a linear dose response to VP35 and was only seen in the samples where the maximum amount of VP35 plasmid was transfected. Cumulatively, these data suggest that the presence of VP35 can disrupt interactions between IKKε and IRF-3, IRF-7 (Fig. 3A and C) and IPS-1 (Fig. 6).

VP35 decreases IRF-3 kinase activity in IKKε-expressing cells. Previous data showed that the presence of VP35 decreased levels of phosphorylated IRF-3 present in cells following SeV infection (2). The data described above suggest that VP35 inhibits the ability of IKKε to interact with IRF-3. To determine whether VP35 expression decreases IKKε ability to phosphorylated IRF-3, an *in vitro* kinase assay was performed using as a source of enzyme lysates from cells transfected with IKKε plasmid in the presence or absence of VP35 plasmid. Cells were transfected with either empty vector (Fig. 7, lane 1), or FLAG-IKKε (lanes 2-6) and increasing amounts of VP35 (1, 2 and 4μg) (lanes 3-5) or Ebola NP (4μg) (lane 6) as an irrelevant protein control. Transfected cell lysates were added to kinase assays where GST-IRF-3-C served as a substrate (lanes 1-6) and subsequently purified on glutathione beads. The precipitated products were then separated by SDS-PAGE, developed by Coomassie blue stain (middle panel) and by autoradiography (upper panel). As shown in Figure 7 equal amounts of IRF-3 phosphorylation (upper panel), as determined by densitometry, were detectable in the IKKε alone or IKKε plus Ebola NP samples. However, phosphorylation of IRF-3 decreased in the presence of VP35 to 89, 79, and 57 percent of the control. Levels of GST-IRF-3 (middle panel), IKKε, NP and VP35 are provided for comparison (western blot using anti-FLAG, anti-NP and anti-VP35 monoclonal

antibodies, bottom three panels). Therefore, the presence of VP35 reduces IRF-3 phosphorylation by IKKε.

Discussion

The Ebola virus VP35 protein antagonizes IFN α/β antiviral response (2, 3). This occurs, at least in part, because VP35 inhibits virus-induced activation of an IFN β promoter by blocking activation of IRF-3 (2). This report demonstrates that VP35 can block activation of an IRF-7-dependent promoter as well. Further studies showed that VP35 can block activation of the IFN β promoter induced by expression of any of several components of the RIG-I/MDA-5 signaling pathway (7). However, VP35 did not detectably inhibit IFN β promoter activation induced by expression of a constitutively active IRF-3 (2, 7). These observations suggested that VP35 acts at the level of the IRF kinases, TBK-1 and IKKε. This report provides the first evidence that VP35 physically interacts with and is phosphorylated by the cellular kinases, IKKε and TBK-1, and suggests that VP35 interaction with IKKε and TBK-1 contributes to suppression of IFN α/β gene expression by EBOV.

We determined that VP35 binds to both IKKε and TBK-1, each of which can activate IRF-3 and IRF-7 in response to RNA virus infection. Several cellular signaling pathways, including the TLR3, TLR4 and the RIG-I and MDA-5-activated pathways signal through either IKKε and/or TBK-1, cellular kinases that phosphorylate IRF-3 and induce IFN α/β production (10, 20, 32, 38, 46, 49). However, the two kinases are not functionally identical. TBK-1 deficient mouse embryonic fibroblasts have impaired IFN α/β responses to virus infection, although residual IKKε may partially compensate for the loss of TBK-1. In contrast, TBK-1 was completely dispensable for IFN α/β-responses to virus infection in mouse bone marrow derived

macrophages (BMM) where IKKε function was predominant (20, 32, 38). Because of this cell type-specific activity, it may benefit EBOV, which productively infects numerous cell types *in vivo*, to encode a mechanism to target both kinases. Our co-IP data suggests that VP35 may succeed in targeting both kinases by interacting with their kinase domains, which are relatively homologous to one another. The presumed consequence of these observations would be the loss of IFN α/β production in many cell types. It should be noted, however, that Toll-like receptor 7 and 9 activate in some cell types, such as plasmacytoid dendritic cells (pDCs), IFN α production through a pathway in which IKK α activates IRF-7 (21). Our data do not determine the impact of VP35 on this alternate source of IFN α. IKKε also phophorylates STAT1, affecting the function of the IFN α/β activated transcription factor complex ISGF3 (48). It will be of interest to determine whether VP35, via its ability to interact with IKKε, might also affect its ability to phosphorylate STAT1.

The EBOV VP35 protein is functionally equivalent to the phosphoproteins (P proteins) of other members of the *Mononegavirales*, a family that includes rhabdoviruses, paramyxoviruses and Borna disease virus. Like other P proteins, VP35 plays an essential role in viral RNA synthesis and interacts with the viral nucleoprotein and the viral RNA-dependent RNA polymerase. It is notable, therefore, that the P proteins of rabies virus (a rhabdovirus) and Borna disease virus also target IKKε and/or TBK-1. Additionally, the V proteins, of several paramyxoviruses (V proteins are encoded by the P gene and share a common amino-terminal domain with P proteins) also target IKKε and/or TBK-1. For example, the Rabies virus P protein blocks IRF-3 phosphorylation by TBK-1, thereby blocking the production of IFN β (6). Similarly, the Borna Disease Virus P protein and paramyxovirus V proteins block IRF-3 phosphorylation by acting as alternative substrates for TBK-1 and IKKε (31, 51). Thus, the

ability to block IKKε and TBK-1 interaction with and phosphorylation of IRF-3/-7 appears to be a function common to this class of viral proteins.

However, VP35-kinase interaction may have broader effects on the activation of IKKε, as higher levels of VP35 also disrupted IKKε-IPS-1 interaction (Fig. 6). IPS-1 is an upstream binding partner of IKKε and TBK-1 that is important for activation of these kinases via the RIG-I and MDA-5 pathways and for production of IFN β during the anti-viral response (10, 20, 25, 32, 33, 45, 46, 52). The capacity of VP35 to target the IKKε-IPS-1 interaction suggests that VP35 may be able to at least partially prevent the activation of IKKε and TBK-1 kinases when high concentrations of VP35 are present.

The observation that VP35 is phosphorylated by IKKε and TBK-1 suggests the possibility that VP35 function may be modulated by these kinases. Consistent with this possibility, metabolic labeling with ^{32}P orthophosphate of 293T cells transfected with VP35 plasmid resulted in VP35 labeling (data not shown). Whether VP35 becomes phosphorylated in EBOV-infected cells is not certain. Previous studies have, however, demonstrated phosphorylation of NP and VP30 (4, 8) and demonstrated functional significance for VP30 phosphorylation (34). The extent to which VP35 may be phosphorylated by IKKε or TBK-1 in EBOV infected cells will obviously be influenced by the extent to which VP35 prevents kinase activation (e.g. by blocking kinase-IPS-1 interaction) versus the extent to which VP35 serves as a decoy substrate for these kinases.

Recent studies employing recombinant EBOVs highlight the importance of VP35 for suppressing host IFN responses. EBOVs with single amino acid substitutions that impair VP35 IFN-antagonist function activated IRF-3 more fully and induced a stronger IFN response, as indicated by global analysis of host gene expression, than did a parental virus with a wild-type

VP35 (17, 18). Mutation of VP35 also impaired virus replication *in vivo* in a non-lethal mouse model of infection (16). The mutations tested in the context of EBOV infection have thus far been demonstrated to impair VP35 dsRNA binding activity (7, 16-18). It will be of interest to determine whether these mutations also impair VP35-IKKε and or VP35-TBK-1 interaction or, whether dsRNA-binding and kinase interaction are independent activities of the VP35 protein.

In humans and non-human primates, fatal EBOV infections are marked by unchecked viral replication and a lack of an effective antiviral response. In order for the virus to overtake the host it must presumably suppress early antiviral innate immune responses. Blocking the phosphorylation of IRF-3/-7 by physically disrupting their interaction with the upstream kinases, IKKε and TBK-1 would presumably accomplish this. We and others therefore hypothesize that VP35 will play a critical role in pathogenesis (1, 5). Direct demonstration of a role for VP35 in the development of Ebola hemorrhagic fever awaits testing of VP35 mutant viruses in appropriate animal models using VP35 mutant viruses. However, the data in this report showing that VP35 targets IKKε and TBK-1 sheds light on at least one of the mechanisms of IFN antagonism by VP35. Further studies on this protein may suggest novel vaccine or antiviral strategies.

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Figure Legends

Fig. 1. IKKε and TBK-1 co-precipitate with VP35. 293T cells were transfected with expression plasmids encoding the indicated proteins (TBK-1KN lanes 1, 4, 6; IKKεKN lanes 2, 5, 7, 8, 10; VP35 lanes 3-7, 9, 10). Twelve hours post-transfection cells were left uninfected (lanes 1-5) or infected with Sendai virus strain Cantell (SeV) for 1 hour (lanes 6-10). Twelve hours later, cells were harvested and lysed. The pre-cleared lysates were immunoprecipitated (IP) using either monoclonal anti-VP35 (IP:VP35) (lanes 1-7) or monoclonal anti-FLAG antibody (IP:FLAG). After SDS-PAGE, western blotting was performed using monoclonal anti-VP35 or anti-FLAG antibody. Expression of VP35 and FLAG-tagged kinase constructs were confirmed by western blot analysis of whole cell lysates (WC), with anti-VP35 and anti-FLAG antibodies, as is shown in the lower panels.

Fig. 2. IKKε and TBK-1 can directly phosphorylate VP35 by *in vitro* kinase assay. In vitro kinase assays were performed with FLAG-tagged kinase, with GST, GST-IRF-3-C (amino acids 375-427) or FLAG-VP35 serving as substrates. Proteins were separated by SDS-PAGE, and phosphorylation was visualized by autoradiography (A, C), and proteins were visualized by Coomassie blue staining (B, D). FLAG-IKKε was used in A and B, lanes 1-9 and TBK-1 was used in B and C, lanes 4-6. The kinase inactive forms were used in A and B, lanes 10 and 11, and in C and D, lane 8.

Fig. 3. VP35 disrupts IKKε interaction with IRF-3 and with IRF-7. (A) FLAG-tagged kinase inactive IKKε (FLAG-IKKεKN), HA-tagged IRF-3 and VP35 expression plasmids were transfected alone (lanes 1-3, respectively) or FLAG-IKKεKN and HA-IRF-3 were co-transfected in the absence (lane 4) or presence of increasing concentrations of VP35 expression plasmid (lanes 5-7). HA-IRF-3 was then precipitated with anti-HA antibody, and the resulting pellets were probed with anti-FLAG and anti-HA antibody to detect IKKεKN or IRF-3, respectively. Levels of FLAG-IKKεKN, HA-IRF-3 and VP35 in whole cell extracts (WC) are also shown. (B) 293T cells were transfected with expression plasmids encoding the IFNalpha4-CAT reporter gene, a constitutive firefly luciferase reporter and either empty vector (samples 1 and 4), FLAG-tagged IRF-7 alone (samples 2 and 5) or FLAG-tagged IRF-7 with VP35 (samples 3 and 6). Twelve hours post-transfection, cells were either mock or infected with SeV, as indicated. Twelve hours post-infection, cells were harvested and CAT and luciferase activities were determined. Values are expressed as fold induction over empty vector mock-infected control. Virus-induced CAT activity was normalized to firefly luciferase activity. Error bars indicate standard deviations. Expression levels of IRF-7 and VP35 were determined by Western blotting (inset). Blots were probed with a monoclonal antibody to VP35 and a monoclonal antibody to FLAG. (C) HA-tagged kinase inactive IKKε (HA-IKKεKN) and FLAG-IRF-7 expression plasmids were transfected alone (lanes 1-2) or HA-IKKεKN and FLAG-IRF-7 were co-transfected in the absence (lane 3) or presence of increasing concentrations of VP35 expression plasmid (lanes 4-6). HA-IKKεKN was then precipitated with anti-HA antibody and the resulting pellets were probed with anti-FLAG, anti-HA and anti-VP35 antibodies. Levels of FLAG-IRF-7, HA-IKKεKN and VP35 in whole cell extracts (WC) are also shown.

Fig. 4. The kinase domain of IKKε is sufficient for interaction with either IRF-3 or VP35. 293T cells were transfected with expression plasmids encoding the indicated FLAG-tagged IKKεKN full length (lanes 1, 3, 5) or the IKKε N315 truncation mutant (lanes 1, 4, 6) in the absence or the presence of either HA-IRF3 (lanes 2-4) or VP35 (lanes 2, 5, 6) expression plasmids. Twelve hours post-transfection cells were infected with SeV. Twelve hours later, cells were harvested and lysed. The pre-cleared lysates were immunoprecipitated (IP) using either monoclonal anti-HA antibody (lanes 1-4) or monoclonal VP35 antibody (lanes 1-2, 5-6). After SDS-PAGE, western blotting was performed using anti-FLAG antibody, anti-HA antibody and anti-VP35 antibody. Expression of VP35, HA-tagged IRF-3 and FLAG-tagged IKKεKN full length and N315 deletion construct was confirmed by western blot analysis of whole cell lysates (WC), with anti-VP35, anti-FLAG and anti-HA antibodies, as is shown in the lower panels.

Fig. 5. VP35 disrupts interactions between the IKKε kinase domain and IRF-3. 293T cells were transfected with expression plasmids encoding the indicated proteins (FLAG-N315 lanes 1, 4, 6-9; VP35 lanes 2, 5-9; HA-IRF-3 lanes 3-9). Twelve hours post-transfection cells were infected with SeV. Twelve hours later, cells were harvested and lysed. The pre-cleared lysates were immunoprecipitated (IP) using monoclonal anti-HA antibody. After SDS-PAGE, western blotting was performed using anti-FLAG antibody (Sigma) and anti-HA antibody (Sigma). Expression of VP35, FLAG-tagged kinase full length, FLAG-tagged kinase N315 mutant and HA-tagged IRF-3 constructs were confirmed by western blot analysis of whole cell lysates (WC), with anti-VP35, anti-FLAG and anti-HA antibodies, as is shown in the lower panels.

Fig. 6. VP35 disrupts IKKε interaction with IPS-1. FLAG-tagged IKKε, HA-IPS-1 and VP35 expression plasmids were transfected alone (lanes 1-3) or HA-IPS-1 was co-transfected with FLAG-IKKε alone (lane 4) or in the presence of increasing amounts of VP35 expression plasmid (lanes 6-9). HA-IPS-1 was then precipitated with anti-HA antibody (IP:HA) and resulting pellets were analyzed by western blot with anti-FLAG and anti-HA antibodies (IB:FLAG, HA). Whole cell lysates (WC) were analyzed by western blot with anti-FLAG, anti-HA and anti-VP35 antibodies (IB:FLAG, HA, VP35)

Fig. 7. VP35 decreases IRF-3 kinase activity in IKKε-expressing cells. *In vitro* kinase assays were performed using as substrate GST-IRF-3-C (lanes 1-6) and as a source of kinase, lysates from cells co-transfected with IKKε expression plasmid (lanes 2-6) and either empty vector (lane 2), increasing concentrations of VP35 expression plasmid (lanes 3-5) or NP expression plasmid (lane 6) (VP35 transfection performed in duplicate). GST-IRF3-C (375-427) was then immunoprecipitated using glutathione sepharose. Top panel. Phosphorylated-GST-IRF-3-C as assessed by SDS-PAGE and autoradiography. Middle panel. Coomassie blue stained GST-IRF-3-C included in the kinase assays. Bottom Panels. Immunoblotting for transfected VP35, IKKε and NP.

Figure 1.

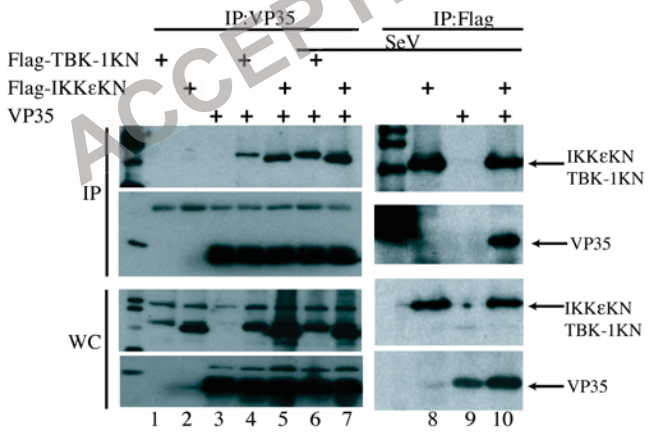


Figure 2.

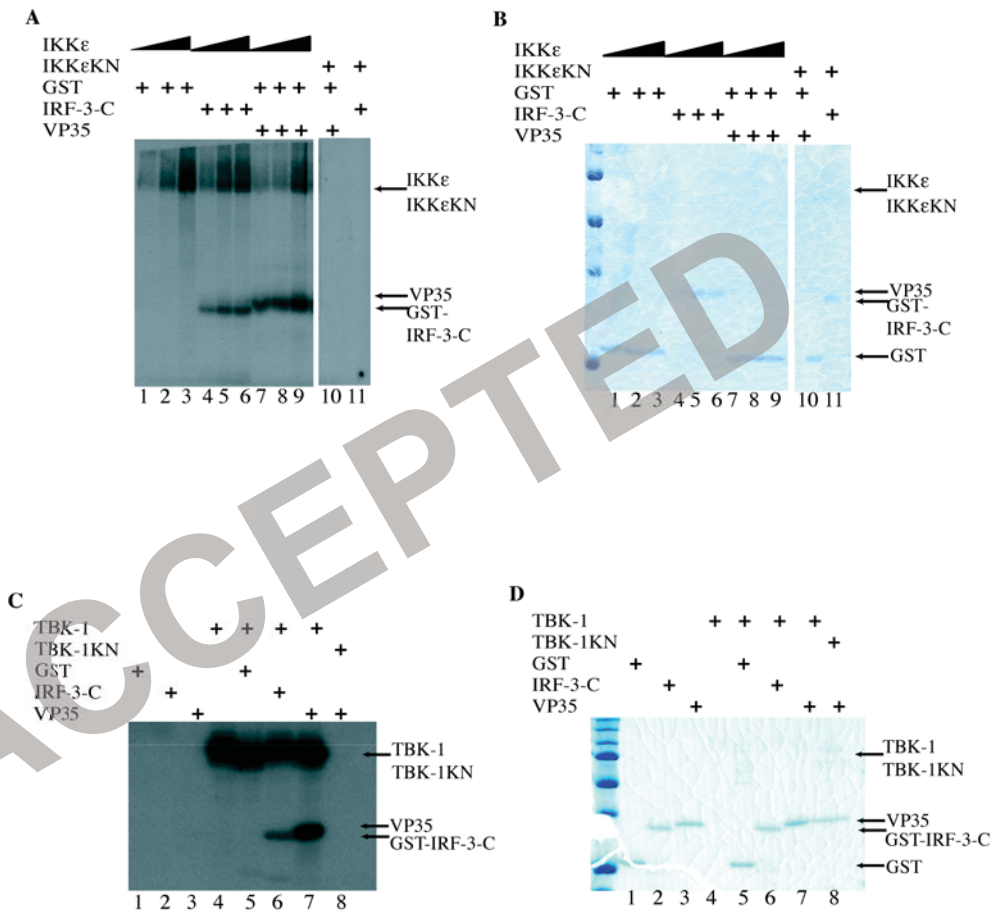


Figure 3

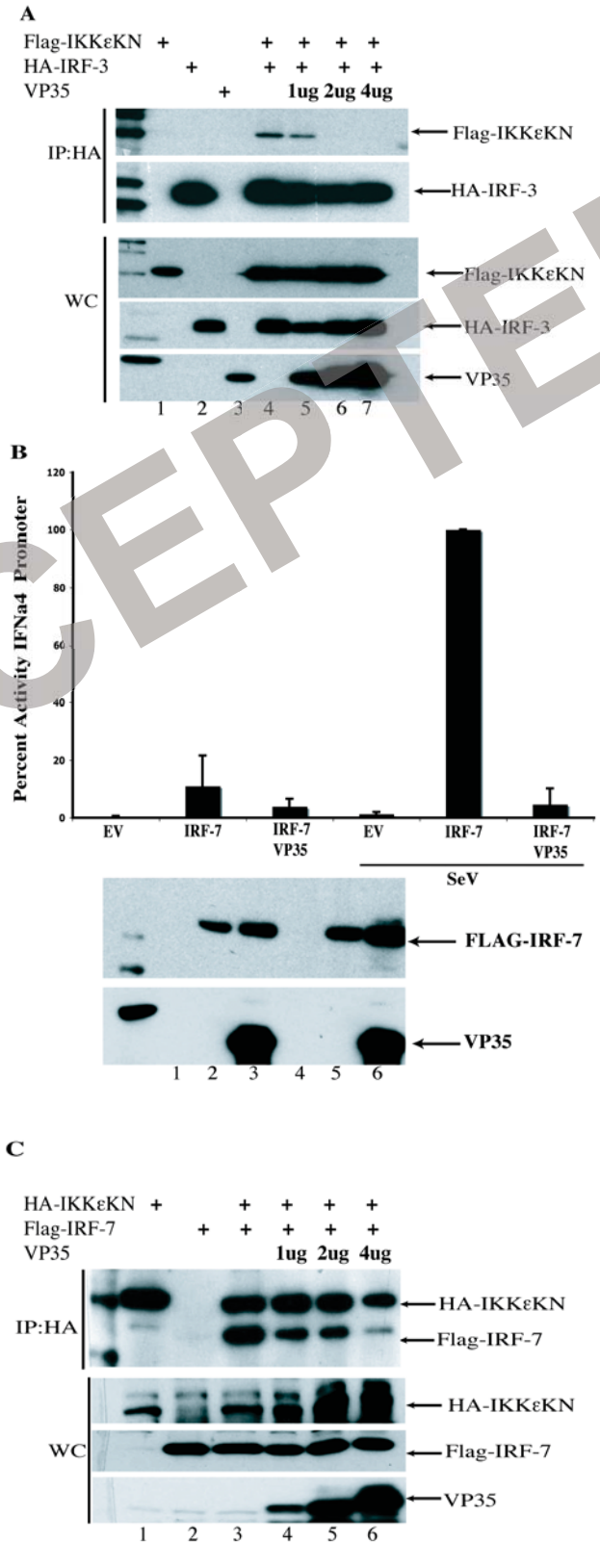


Figure 4

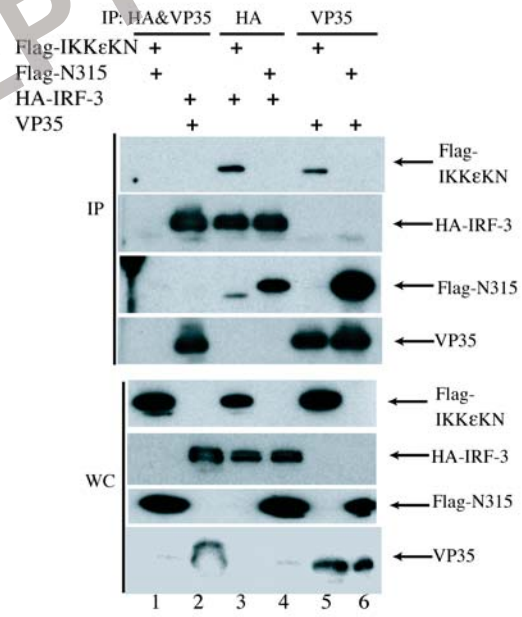
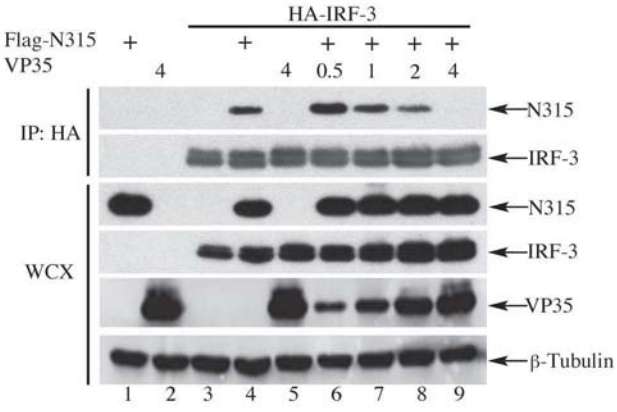


Figure 5.



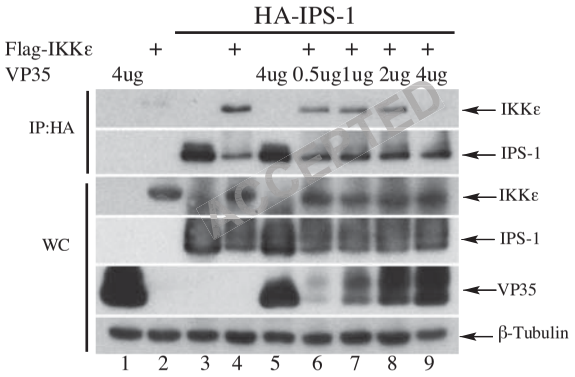

HA-IPS-1
Flag-IKKε + + + + + +
VP35 4ug 4ug 0.5ug 1ug 2ug 4ug
IP:HA
WC
IKKε
IPS-1
IKKε
IPS-1
VP35
β-Tubulin
1 2 3 4 5 6 7 8 9

Fig 7