The Ebola Virus VP35 Protein Inhibits Activation of Interferon Regulatory Factor 3

Christopher F. Basler,1* Andrea Mikulasova,1 Luis Martinez-Sobrido,1 Jason Paragas,2 Elke Mühlerberger,3 Mike Bray,2† Hans-Dieter Klenk,3 Peter Palese,1 and Adolfo García-Sastre1

Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029; Virology Division, US Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Frederick, Maryland 21702-5011; and Institut für Virologie, Philipps-Universität Marburg, D-35037 Marburg, Germany

Received 21 March 2003/Accepted 1 May 2003

The Ebola virus VP35 protein was previously found to act as an interferon (IFN) antagonist which could complement growth of influenza delNS1 virus, a mutant influenza virus lacking the influenza virus IFN antagonist protein, NS1. The Ebola virus VP35 could also prevent the virus- or double-stranded RNA-mediated transcriptional activation of both the beta IFN (IFN-β) promoter and the IFN-stimulated ISG54 promoter (C. Basler et al., Proc. Natl. Acad. Sci. USA 97:12289-12294, 2000). We now show that VP35 inhibits virus infection-induced transcriptional activation of IFN regulatory factor 3 (IRF-3)-responsive mammalian promoters and that VP35 does not block signaling from the IFN-α/β receptor. The ability of VP35 to inhibit this virus-induced transcription correlates with its ability to block activation of IRF-3, a cellular transcription factor of central importance in initiating the host cell IFN response. We demonstrate that VP35 blocks the Sendai virus-induced activation of two promoters which can be directly activated by IRF-3, namely, the ISG54 promoter and the ISG56 promoter. Further, expression of VP35 prevents the IFN-α-dependent activation of the IFN-α4 promoter in response to viral infection. The inhibition of IRF-3 appears to occur through an inhibition of IRF-3 phosphorylation. VP35 blocks virus-induced IRF-3 phosphorylation and subsequent IRF-3 dimerization and nuclear translocation. Consistent with these observations, Ebola virus infection of Vero cells activated neither transcription from the ISG54 promoter nor nuclear accumulation of IRF-3. These data suggest that in Ebola virus-infected cells, VP35 inhibits the induction of antiviral genes, including the IFN-β gene, by blocking IRF-3 activation.

* Corresponding author. Mailing address: Dept. of Microbiology, Box 1124, Mount Sinai School of Medicine, 1 Gustave L. Levy Pl., New York, NY 10029. Phone: (212) 241-5923. Fax: (212) 534-1684. E-mail: chris.basler@mssm.edu.
† Present address: National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD 20892.

Ebola viruses cause sporadic outbreaks of viral hemorrhagic fever in humans, such as recent outbreaks in Gabon and the Republic of Congo (89–91). Fatality rates for Ebola hemorrhagic fever have ranged from 50 to 90% in different outbreaks (88). Because it is highly lethal and causes dramatic symptoms, and because effective vaccines and therapies are lacking (14), Ebola virus is classified by the Centers for Disease Control and Prevention and the National Institutes of Health as a category A bioterrorism agent (17, 41). The molecular mechanisms contributing to the high virulence of Ebola virus are only beginning to be unraveled (21, 78). It is likely that virus-encoded proteins that inhibit the host cell interferon (IFN) response play a significant role in Ebola virus pathogenesis (6, 12). Data from several other viruses have demonstrated an essential role for virus-encoded IFN antagonists in virulence. For example, mutants of influenza A viruses (23, 80), Bunyamwera virus (15, 87), paramyxoviruses (22), vaccinia virus (8, 11), and herpes simplex virus type 1 (19, 42, 49), which lack or which possess altered viral IFN antagonists, are attenuated in mice. Evidence also suggests that full virulence of Rift Valley Fever virus requires the anti-IFN activity of the viral NSs protein (10).

Ebola virus infection has been shown to block host cell responses to IFNs, to inhibit double-stranded RNA (dsRNA)-mediated induction of antiviral gene expression, and to block alpha/beta IFN (IFN-α/β) production in infected cells (7, 13, 27–29), although Ebola virus infection has been reported to induce IFN production under some circumstances (31). We previously demonstrated that the Ebola virus VP35 protein, an essential component of the Ebola virus RNA replication machinery (52, 53), functions as an IFN antagonist (7). VP35 expression rescued growth of a mutant influenza virus, influenza delNS1 virus, which lacks the influenza A virus NS1 protein, a previously described inhibitor of the IFN response (7), demonstrating the ability of VP35 to counteract the antiviral effects of the host IFN system. Furthermore, expression of VP35 was able to inhibit the dsRNA or virus-induced activation of the beta IFN (IFN-β) promoter or the IFN-inducible ISG54 promoter (7). However, these experiments did not identify specific component(s) of the IFN response affected by VP35.

IFN regulatory factor 3 (IRF-3) is a constitutively expressed transcription factor. Inactive IRF-3 shuttles between the nucleus and cytoplasm and is predominantly cytoplasmic. However, following its activation by phosphorylation, IRF-3 accumulates in the nucleus, where it acts as a transcription factor.
(40, 45, 94). Activation of IRF-3 is associated with serine/threonine phosphorylation near its carboxy terminus, on serines 385 and 386 (46) and on a cluster of threonine phosphorylation near its carboxy terminus, on (40, 45, 94). Activation of IRF-3 is associated with serine/threonine phosphorylation near its carboxy terminus, on (40, 45, 94, 95). Activation of IRF-3, Vero cells were transfected using LF2000 with 0.2 μg of the indicated expression plasmid. Transfected cells were divided into two parts and lysed in reporter lysis buffer (Promega), and CAT or luciferase activities were determined.

To measure the ability of Ebola viruses to activate the ISG-54 promoter, 1 × 10⁵ 293T cells were transfected in suspension with 0.3 μg of the plasmid pHISG-54-CAT by using the calcium phosphate method. Transfected cells were seeded into 10-cm-diameter dishes and incubated for 24 h at 37°C. Thereafter, cells were washed twice with DMEM and infected with either SeV strain Cantell, Ebola virus Zaire, or Ebola virus Reston, at an MOI of 3, or mock infected. After an incubation period of 24 h in DMEM with 2.5% fetal calf serum, the cells were harvested and lysed in reporter lysis buffer (Promega), and CAT or luciferase activities were determined.

To measure activation of the mouse IFN-α promoter, transient transfection of 293T cells was performed by using the calcium phosphate method (68). Each transfection included 0.5 μg of the indicated expression plasmid and 54-CAT by using the calcium phosphate method. Transfected cells were seeded into 10-cm-diameter dishes and incubated for 24 h at 37°C. Thereafter, cells were washed twice with DMEM and infected with either SeV strain Cantell, Ebola virus Zaire, or Ebola virus Reston, at an MOI of 3, or mock infected. After an incubation period of 24 h in DMEM with 2.5% fetal calf serum, the cells were harvested and lysed in reporter lysis buffer (Promega), and CAT or luciferase activities were determined.

To measure activation of the mouse IFN-α promoter, transient transfection of 293T cells was performed by using the calcium phosphate method (68). Each transfection included 0.5 μg of the indicated expression plasmid and 4 promoter, transient transfection of 293T cells was performed by using the calcium phosphate method. Transfected cells were seeded into 10-cm-diameter dishes and incubated for 24 h at 37°C. Thereafter, cells were washed twice with DMEM and infected with either SeV strain Cantell, Ebola virus Zaire, or Ebola virus Reston, at an MOI of 3, or mock infected. After an incubation period of 24 h in DMEM with 2.5% fetal calf serum, the cells were harvested and lysed in reporter lysis buffer (Promega), and CAT or luciferase activities were determined.

To measure activation of the mouse IFN-α promoter, transient transfection of 293T cells was performed by using the calcium phosphate method (68). Each transfection included 0.5 μg of the indicated expression plasmid and 4 promoter, transient transfection of 293T cells was performed by using the calcium phosphate method. Transfected cells were seeded into 10-cm-diameter dishes and incubated for 24 h at 37°C. Thereafter, cells were washed twice with DMEM and infected with either SeV strain Cantell, Ebola virus Zaire, or Ebola virus Reston, at an MOI of 3, or mock infected. After an incubation period of 24 h in DMEM with 2.5% fetal calf serum, the cells were harvested and lysed in reporter lysis buffer (Promega), and CAT or luciferase activities were determined.

To measure activation of the mouse IFN-α promoter, transient transfection of 293T cells was performed by using the calcium phosphate method (68). Each transfection included 0.5 μg of the indicated expression plasmid and 4 promoter, transient transfection of 293T cells was performed by using the calcium phosphate method. Transfected cells were seeded into 10-cm-diameter dishes and incubated for 24 h at 37°C. Thereafter, cells were washed twice with DMEM and infected with either SeV strain Cantell, Ebola virus Zaire, or Ebola virus Reston, at an MOI of 3, or mock infected. After an incubation period of 24 h in DMEM with 2.5% fetal calf serum, the cells were harvested and lysed in reporter lysis buffer (Promega), and CAT or luciferase activities were determined.

To measure activation of the mouse IFN-α promoter, transient transfection of 293T cells was performed by using the calcium phosphate method (68). Each transfection included 0.5 μg of the indicated expression plasmid and 4 promoter, transient transfection of 293T cells was performed by using the calcium phosphate method. Transfected cells were seeded into 10-cm-diameter dishes and incubated for 24 h at 37°C. Thereafter, cells were washed twice with DMEM and infected with either SeV strain Cantell, Ebola virus Zaire, or Ebola virus Reston, at an MOI of 3, or mock infected. After an incubation period of 24 h in DMEM with 2.5% fetal calf serum, the cells were harvested and lysed in reporter lysis buffer (Promega), and CAT or luciferase activities were determined.

To measure activation of the mouse IFN-α promoter, transient transfection of 293T cells was performed by using the calcium phosphate method (68). Each transfection included 0.5 μg of the indicated expression plasmid and 4 promoter, transient transfection of 293T cells was performed by using the calcium phosphate method. Transfected cells were seeded into 10-cm-diameter dishes and incubated for 24 h at 37°C. Thereafter, cells were washed twice with DMEM and infected with either SeV strain Cantell, Ebola virus Zaire, or Ebola virus Reston, at an MOI of 3, or mock infected. After an incubation period of 24 h in DMEM with 2.5% fetal calf serum, the cells were harvested and lysed in reporter lysis buffer (Promega), and CAT or luciferase activities were determined.
mid. Translocation of IRF-3 was induced by SeV infection at an MOI of 10. The percentage of cells with nuclear GFP-IRF-3 localization was then determined by examining 200 to 300 green fluorescent cells per sample. To analyze the colocalization of IRF-3 and VP35, cells were transfected with 0.8 μg of pEGFP-C1-hIRF3 and 0.4 μg of pCAGGS-VP35(R) HA, which encodes an HA-tagged VP35 derived from Ebola virus Reston. Cells were transfected in suspension and seeded in individual wells of 96-well chamber slides (Lab-Tek) at 1 x 10^4 cells per well. Twenty-four hours posttransfection, translocation of IRF-3 was induced by SeV infection at an MOI of 10. Cell monolayers were then fixed for 5 min with methanol and permeabilized for 30 s with acetone. Fixed cells were washed with PBS, and where indicated, the HA-tagged Ebola virus VP35(R) protein was detected with a mouse anti-HA monoclonal antibody 12CAS (Hybridoma Core Facility, Mount Sinai School of Medicine) at a dilution of 1:100. As a secondary antibody, Texas red-conjugated anti-mouse immunoglobulin G (IgG) (Rockland) was used at a dilution of 1:100. All antibodies were diluted in PBS with 3% bovine serum albumin. Cells were affixed to slides using Pro-Long antifade agent (Molecular Probes). Samples were examined under an Olympus IX-70 fluorescence microscope.

Localization of IRF-3 in Ebola virus-infected cells. VeroE6 cells were transfected, in suspension, with 5 μg of pEGFP-C1-IRF-3 by using LF2000. Transfected cells were plated on 8-well chamber slides (Lab-Tek) and incubated for 24 h. Chamber slides were transferred into the BSL-4 laboratory at USAMRIID and infected with Ebola virus Zaire at an MOI of 1. Infected cells were washed with PBS, and then fresh viral growth medium was added. Twenty-four and forty-eight hours postinfection, infected cells were fixed for 24 h with 10% formalin before the slides were developed for the Ebola virus GP.

Slides were washed with water for 10 min. Fixed cells were further washed extensively with 1× PBS, treated with protease K for 10 min at room temperature, and washed again with 1× PBS. Cells were incubated with a mouse monoclonal antibody directed against Ebola virus Zaire GP (dilution, 1:2,000). Washed cells were then incubated with an anti-mouse IgG (dilution, 1:400) secondary antibody (Alexa Fluor 594, Molecular Probes). All antibodies were diluted with an antibody diluent containing background-reducing components (DAKO). Coverslips were affixed to glass slides using mounting media containing 4',6'-diamidino-2-phenylindole (DAPI) (Vector). Slides were imaged using a Nikon TE2000 inverted fluorescent microscope. Ebola virus infections were conducted within the Biological Safety Level 4 (BSL-4) laboratory at USAMRIID. Personnel wore positive-pressure protective suits (ILC, Dover, Frederica, Del.) equipped with high-efficiency particulate air filters and supplied with umbilical-fed air.

Western blot analyses. 293T cells were transfected using LF2000. Each transfection of 1 x 10^6 cells contained 1 μg of the hIRF-3 expression vector pcAGGS-hIRF3 and 2 μg of the indicated expression plasmid. Twenty-four hours posttransfection, cell monolayers were infected with SeV at a MOI of 10 or mock infected. Four hours postinfection, cell lysates were then made in NP-40 lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, 0.1 μg of leupeptin/ml, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), as described previously (34). These lysates were then subjected either to native gel electrophoresis as described previously (34) or to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane in a buffer containing 40 mM glycine, 50 mM Tris base, 0.037% SDS and 20% methanol for 2 h. Human IRF-3 was detected by using a mouse anti-IRF-3 monoclonal antibody SL-12 (kindly provided by Peter M. Howley, Harvard Medical School) at a dilution of 1:1,000 in a solution of 5% nonfat dry milk in PBS. The secondary antibody was anti-mouse IgG conjugated to horseradish peroxidase (Boehringer Mannhein Corp.) (1:10,000 dilution in a solution of 5% nonfat dry milk in PBS). The secondary antibody was anti-mouse IgG conjugated to horse-radish peroxidase (Boehringer Mannhein Corp.) (1:10,000 dilution in a solution of 5% nonfat dry milk in PBS). The Western blots were developed with Lightning chemiluminescence reagent (PerkinElmer). Direct visualization of Western blot signals was detected with a mouse anti-HA monoclonal antibody (Promega) (1:1,000 dilution) or Texas red-conjugated anti-mouse immunoglobulin G (IgG) (Rockland) (1:1,000 dilution).

RESULTS

VP35 blocks IFN-independent virus-induced activation of the ISG54 promoter. It was previously demonstrated that VP35 could inhibit in 293 cells the activation of the IFN-responsive ISG54 promoter in response to dsRNA treatment or to SeV infection (7). In these previous experiments, activation of the ISG45 reporter could have been mediated by either of two mechanisms. First, IFN produced in response to dsRNA treatment or to viral infection might have activated the ISG54 promoter. Alternatively, because it has been reported that IRF-3 can directly activate the promoters of several IFN-stimulated genes (ISGs), including the ISG54 and ISG56 promoters (26, 63, 70), virus- or dsRNA-mediated activation of the transcription factor IRF-3 could have directly stimulated the ISG54 promoter. We therefore asked whether VP35 can block the virus-induced activation of the ISG54 or ISG56 promoters independently of IFN production. Vero cells, which do not produce IFN-α/β, were transfected with an ISG54-CAT reporter plasmid or a human ISG56-luciferase reporter plasmid plus a constitutively expressed Renilla luciferase plasmid and a mammalian expression plasmid. The expression plasmids were empty vector, a positive control plasmid encoding the influenza A virus NS1 protein (which inhibits IRF-3 activation [80]), an Ebola virus Zaire VP35 expression plasmid, or a plasmid encoding an HA-tagged version of the Ebola virus Reston VP35 protein. Cells were subsequently mock infected or infected with SeV, a potent viral activator of IRF-3 (45), at an MOI of 4. Infection of cells transfected with empty vector resulted in a dramatic up-regulation of expression from either the ISG54 or the ISG56 promoters compared with that seen for uninfected, empty-vector transfected cells (Fig. 1A and B). Expression of the influenza virus NS1 protein or the VP35 protein of Ebola virus Zaire dramatically decreased the activation of either reporter gene (Fig. 1A and B). Interestingly, the HA-tagged VP35 protein of Ebola virus Reston also blocked ISG54 activation in Vero cells (Fig. 1A). Similar results were obtained with an untagged Ebola virus Reston VP35 clone (data not shown). Transfection of a different Ebola virus protein, the nucleoprotein (NP), did not significantly affect virus-induced reporter gene expression (Fig. 1B). NP is expressed from this plasmid has been previously demonstrated (7). To determine whether VP35 also affects IFN-induced gene activation, Vero cells were again transfected with either the ISG54 or the ISG56 promoter reporter genes in the presence of empty vector, an Ebola virus Zaire VP35 plasmid, an HA-tagged Ebola virus Reston VP35 plasmid, or an Ebola virus NP plasmid. One day posttransfection, the cells were treated with IFN-β and subsequently assayed for reporter gene expression. Neither the Zaire or Reston VP35 proteins were able to inhibit IFN-β-induced activation of the ISG54 or the ISG56 promoters (Fig. 1C and D). Transfection, in parallel, of a plasmid encoding the Nipah virus V or W proteins, previously shown to inhibit IFN-α/β signaling, was able to block IFN-β-mediated ISG54 promoter activation (data not shown) (62). We therefore conclude that the Ebola virus VP35 protein is able to block the virus-mediated, but not the IFN-mediated, activation of the ISG54 and ISG56 promoters.

VP35 blocks the IRF-3 dependent, virus-induced activation of the mouse IFN-α promoter. The ISG54 and ISG56 pro-
motors can both be transcriptionally activated directly by IRF-3, without production of IFN, in response to viral infection (26, 54, 63), suggesting that VP35 blocks IRF-3-dependent transcription. We therefore investigated the ability of VP35 to prevent the induction of an additional IRF-3-stimulated promoter, the mouse IFN-β/H9254 promoter. Overexpression of IRF-3 has previously been reported to cooperate with viral infection to activate the IFN-β/H9254 promoter (35). We were therefore able to establish a transfection system where the virus-induced activation of the IFN-β/H9254 promoter was dependent on overexpression of IRF-3, because in the absence of IRF-3 overexpression, SeV infection did not lead to reporter gene induction (data not shown). Using this system, the Ebola virus Zaire VP35 was tested for its ability to inhibit the virus-induced activation of mouse IFN-β/H9254 promoter in the presence of overexpressed IRF-3. Cells were transfected with an IRF-3–CAT plasmid, an IRF-3 expression plasmid, and either empty vector, NS1 expression plasmid, or VP35 expression plasmid. In the absence of viral infection, little CAT activity was detected, and in the presence of VP35, the basal CAT activity was lower in empty vector-transfected cells (Fig. 2A). When the cells were infected with SeV, activation of the IFN-α4 promoter increased fivefold. The presence of VP35 completely blocked this virus-induced activation (Fig. 2A). As expected, a previously described IRF-3 inhibitor, the influenza A virus NS1 protein, also blocked promoter activation (Fig. 2A). Similar results were found when influenza delNS1 virus, a mutant influenza virus lacking the IFN antagonist NS1 protein (23), was used instead of SeV to induce IFN-α4 promoter activity (Fig. 2B). As in the SeV experiment, almost no CAT activity could be detected in cells, whether mock infected or delNS1 virus-infected, which were not overexpressing IRF-3 (data not shown). Combined, these data suggest that the Ebola virus VP35 protein can block virus-induced, IRF-3-dependent gene expression.

**VP35 blocks IRF-3 nuclear translocation.** Given the ability of VP35 to inhibit IRF-3-mediated gene expression, it was important to define how IRF-3 function was blocked. Virus infection can induce serine/threonine phosphorylation near the...
carboxy-terminus of IRF-3 (45, 46, 74, 94). This phosphorylation leads to the dimerization and nuclear accumulation of IRF-3 (40, 46, 74, 94). In order to begin to assess the mechanism by which VP35 inhibits IRF-3-dependent gene activation, we assessed the ability of VP35 to inhibit the nuclear accumulation of IRF-3 by using GFP–IRF-3 fusions, which have previously been ascribed to phosphorylation of serine and threonine residues near its carboxy terminus (72). The expression of IRF-3 was drastically reduced (Fig. 3B). Similarly, expression of the influenza virus NS1 protein, previously reported to inhibit nuclear accumulation of IRF-3 in response to viral infection, also reduced GFP–IRF-3 nuclear accumulation (Fig. 3B). In contrast, a different Ebola virus protein, VP24, did not block virus-induced nuclear accumulation of GFP–IRF-3 (Fig. 3B). Our ability to differentiate nuclear from cytoplasmic IRF-3 is illustrated in Fig. 3A.

**VP35 blocks virus-induced IRF-3 dimerization and phosphorylation.** Experiments were then performed to determine whether the VP35-mediated inhibition of IRF-3 nuclear localization might be due to an inhibition of IRF-3 phosphorylation and/or dimerization. 293T cells were transfected with a human IRF-3 expression plasmid and either empty vector or plasmids expressing Ebola virus Zaire VP35, HA-tagged Ebola virus Reston VP35, or Ebola virus Zaire NP proteins and subsequently mock infected or infected with SeV. We employed a previously described native PAGE method which separates IRF-3 monomers and dimers and detects IRF-3 by immunoblotting (34). This is a sensitive method to distinguish inactive (monomeric) from active (dimeric) IRF-3 (34). By this method, SeV infection was found to induce formation of IRF-3 dimers by 4 h postinfection. Expression of VP35 blocked IRF-3 dimerization, whereas that of Ebola virus NP did not (Fig. 4A, upper panel). The same cell extracts used for native gel electrophoresis were also analyzed by standard SDS-PAGE and Western blotting. Infection of empty vector-transfected cells with SeV induced a slower migrating form of IRF-3 (Fig. 4A, middle panel). Similar virus infection-induced shifts in IRF-3 have previously been ascribed to phosphorylation of serine and threonine residues near its carboxy terminus (72). The expression of VP35 blocked the formation of the slower migrating form of IRF-3, whereas that of Ebola virus NP proteins did not (Fig. 4A, middle panel), suggesting that VP35 inhibits IRF-3 phosphorylation. Further confirmation that VP35 blocks IRF-3 phosphorylation was shown by immunoprecipitating IRF-3 from SeV-infected cells radiolabeled with [32P]orthophosphate. Whereas IRF-3 phosphorylation was greatly enhanced by SeV infection in empty vector-transfected cells or NP-expressing cells, IRF-3 phosphorylation was completely blocked in VP35-expressing cells (Fig. 4B). In order to determine whether the ability of VP35 to inhibit IRF-3 transactivation is dependent upon its ability to...
block IRF-3 phosphorylation, we asked whether VP35 could block transcriptional activation mediated by a constitutively activated IRF-3 mutant, IRF-3 5D. In this mutant, phosphomimetic aspartic acid residues are substituted for five serine and threonine residues (Ser 396, 398, 402, and 405 and Thr 404) which become phosphorylated in response to virus infection (45). IRF-3 5D activates transcription in 293 cells independent of virus infection (but reportedly did not constitutively

FIG. 3. The Ebola virus VP35 protein prevents the nuclear translocation of hIRF-3 after SeV infection. (A) Fluorescence images showing expression of HA-tagged VP35(R) protein (red) and the corresponding distribution of GFP–hIRF-3 (green). Cells expressing both HA-tagged VP35 and GFP–IRF-3 are indicated by the large white arrows. Examples of cells with nuclear GFP–IRF-3 are indicated by the small yellow arrows. Vero cells were transfected with 0.4 μg of VP35(R) expression plasmid plus 0.8 μg of pEGFP-C1-hIRF3 and infected 24 h later with SeV. Eight hours postinfection, cells were fixed and stained with anti-HA monoclonal antibody (red). (B) The percentage of GFP–IRF-3-expressing cells with nuclear GFP–IRF-3 in cells transfected with the indicated plasmids and either mock infected or infected with SeV is shown. Vero cells were transfected with 0.4 μg of empty vector or expression plasmids for Ebola virus Zaire VP35 [VP35(Z)], HA-tagged Ebola virus Reston VP35 [HAVP35(R)], influenza virus NS1 protein (NS1), or Ebola virus Zaire virus VP24 protein (VP24), plus 0.2 μg of pEGFP-C1-hIRF3. At 24 h posttransfection, the cells were mock infected or infected with SeV at an MOI of 10. Eight hours postinfection, the cells were examined for GFP localization. The results are the average of two independent experiments where 200 to 300 cells were counted per transfection.
VP35 blocks IRF-3 activation by preventing IRF-3 phosphorylation.

**FIG. 4.** VP35 blocks dimerization and phosphorylation of IRF-3 in response to SeV infection. (A) Immunoblot analysis of IRF-3 in mock-infected (mock) and SeV-infected (SeV) 293 cells transfected with empty vector, VP35 expression plasmid, or NP expression plasmid. IRF-3 monomers and dimers were visualized following native gel electrophoresis, and different IRF-3 forms, including virus-induced, phosphorylated forms (indicated by asterisks), were visualized following SDS-PAGE of the same extracts used on the native gel. An antiguyceraldehyde-3-phosphate dehydrogenase (GAPDH) blot is shown as a loading control. (B) Phosphorylated IRF-3 detected by [32P]orthophosphate labeling and immunoprecipitation of IRF-3 from mock- or SeV-infected 293 cells previously transfected with 1 µg of IRF-3 expression plasmid and 2 µg of empty vector, VP35 expression plasmid, or NP expression plasmid. (C) The relative ability of IRF-3 3D to activate an ISG56 promoter-driven reporter gene in Vero cells cotransfected with empty vector, VP35 expression plasmid, or NP expression plasmid. The values are reported as fold induction of the reporter gene relative to induction in cells receiving empty vector and no IRF-3 3D plasmid. All values were normalized to expression from a cotransfected, constitutively expressed Renilla luciferase reporter gene.

**DISCUSSION**

This study demonstrates that the Ebola virus VP35 protein inhibits the activation of IRF-3 in response to viral infection. IRF-3 is a cellular transcription factor that plays a central role in initiating the host cell IFN response to viral infection (for recent reviews, see references 3, 5, 43, 66, 72, 81, and 93). Activation of IRF-3 contributes to the immediate activation of IFN-β gene expression and of selective IFN-α genes as well as the immediate expression of several other genes with potential antiviral activity (26). Previously, we had found that expression of VP35 could complement the growth of a mutant influenza virus, influenza delNS1 virus, which lacks the influenza virus IFN antagonist NS1 protein (7). VP35 had also been found to block activation of the IFN-β promoter by dsRNA or by virus infection, to inhibit SeV- or influenza delNS1 virus-induced transcription of the endogenous IFN-β promoter, and to inhibit the virus-induced activation of the ISG54 promoter (7). In the present study, we have demonstrated that VP35 expression inhibits the virus-induced activation of both the ISG54 promoter and the ISG56 promoter in Vero cells. In contrast, VP35 was not able to block the activation of either promoter following treatment of cells with IFN-β. Because Vero cells do not produce IFN-α/β, which might otherwise activate the ISG54 and ISG56 promoters (51), and because both the ISG54 and ISG56 promoters can be activated directly by IRF-3 (26, 54), these results suggested that VP35 could block IRF-3-mediated activation of these genes. Furthermore, VP35 expression inhibited virus-induced, IRF-3-dependent activation of
FIG. 5. Ebola virus infection does not induce the activation of the ISG54 promoter or IRF-3 nuclear accumulation. (A) Ebola virus infection does not activate the ISG54 promoter. 293 cells were transfected with the IFN and IRF-3-responsive reporter plasmid pHIG54-CAT and, 24 h posttransfection, infected with the indicated viruses at an MOI of 3. Twenty-four hours posttransfection, the cells were either fixed and stained for the viral GP surface antigen (left panels) or lysed for CAT assays. The mock-infected immunofluorescence shows a background of red staining, but this is clearly distinguishable from the viral antigen staining in the infected cells. (B) Ebola virus infection did not induce GFP–IRF-3 nuclear accumulation. Cells were mock infected or infected with Ebola virus Zaire. Vero cells were transfected with the GFP–IRF-3 expression plasmid. Twenty-four hours posttransfection, the cells were mock infected or infected with Ebola virus Zaire at an MOI of 1. At 48 h postinfection, the cells were fixed and stained with an anti-GP antibody (red) and with DAPI stain (blue) to identify the nucleus. The top panels show GFP–IRF-3 and viral antigen staining; the bottom panels show the same fields as the top panels but with DAPI staining included to identify the nuclei. One field of uninfected cells is shown on the left. Two fields of virus-infected cells are shown, one in the center and one on the right.
the mouse IFN-α4 promoter (Fig. 2). These results are consistent with earlier studies demonstrating that Ebola virus infection blocks host cell responses to both dsRNA, which is often used as an experimental inducer of IFN responses, and IFN (28, 29). They are also consistent with studies showing that Ebola virus infection does not induce production of IFN in some cell types and can block the dsRNA-induced production of IFN-α/β in primary human peripheral blood mononuclear cells and monocytes (27).

Our results also address the manner in which IRF-3 is inhibited by VP35. Our data using a GFP-IRF-3 fusion protein as a marker for cellular IRF-3 localization indicate that the presence of VP35 prevents the virus-induced nuclear accumulation of IRF-3 (Fig. 3). In order to define how VP35 affects the IRF-3 activation process, we examined the dimerization and phosphorylation of IRF-3. Expression of VP35 was able to prevent IRF-3 dimerization and virus-induced phosphorylation of IRF-3 (Fig. 4). That expression of VP35 was unable to inhibit the function of IRF-3 5D, which does not need to be phosphorylated to stimulate gene expression, argues strongly for a model whereby VP35 functions specifically by blocking IRF-3 phosphorylation.

Several viruses, including human cytomegalovirus, are known to activate IRF-3 through interactions with the host cell prior to viral entry (55, 65). Several other viruses encode proteins which are known to inhibit the function of IRF-3. The influenza A virus NS1 protein prevents IFN-3 activation and blocks production of IFN-α/β in influenza virus-infected cells (23, 79). This activity appears to require the dsRNA-binding activity of NS1, suggesting a model where NS1 binds to dsRNA produced during viral infection and prevents the activation of dsRNA-downstream pathways leading to IRF-3 phosphorylation (79). Similarly, the vaccinia virus E3L protein, also a dsRNA-binding protein, inhibits IRF-3 activation (76); whereas a vaccinia virus lacking E3L activates IRF-3 upon infection, wild-type vaccinia virus does not (92). The human papillomavirus 16 E6 protein has been shown to bind IRF-3 and to inhibit IRF-3 transcriptional activity (44, 67). HHV-8 vIRF-1 and vIRF-3 have also been shown to bind to IRF-3 (3, 44). The reported ability of vIRF-1 to interact directly with IRF-3, to associate with CBP/p300, to inhibit CBP/p300 transcriptional activation, and to compete with IRF-3 for binding to CBP/p300, may play a role in the ability of vIRF-1 to affect IRF-3-related transcriptional activity (16, 44, 71). The ability of vIRF-3 to influence virus-induced IFN-α gene expression may be related to its ability to interact with IRF-3 and/or CBP/p300 (3). Recently, the rotavirus NSP1 protein was shown to interact with IRF-3, although it has not yet been reported to inhibit IRF-3 function (25). Also, noncytopathogenic bovine viral diarrhea virus blocks IFN-α/β production by preventing formation of IRF-3-DNA complexes, although the mechanism by which this occurs is, as yet, undefined (2). Finally, the V proteins of paramyxoviruses, which were previously shown to block signaling from the IFN-α/β receptor by promoting degradation of STAT proteins (1, 20, 39, 60, 61), have been reported to inhibit IFN-β gene expression and to block dsRNA-induced nuclear translocation of an IRF-3-GFP fusion protein (30, 64).

Our data suggest that VP35 inhibits IRF-3 activation primarily by inhibiting phosphorylation. The identification of host cell factors that interact with VP35 would shed light on its mechanism of action, and experiments to identify such proteins are under way. Experiments to determine whether VP35 interacts directly with IRF-3 have thus far yielded only negative results. The ability of VP35 to prevent IRF-3 phosphorylation suggests the possibility that VP35 may interact with some component of the virus-activated signaling pathway which leads to IRF-3 activation. It will also be of interest to examine the effect of VP35 on other IRF family members. The influenza virus NS1 and vaccinia virus E3L proteins reportedly also prevent IRF-7 activation (48, 76), and other viral IRF-7 inhibitors have been described (96). IRF-7 and IRF-5 are of particular interest because they are involved in direct virus-induced activation of IFN-α genes (4, 48, 50, 70).

We have found that the Ebola virus Reston VP35 behaves similarly to the Ebola Zaire virus VP35 with regard to IFN antagonist functions, at least in the cell types tested. The Reston VP35 inhibits activation of the ISG54 promoter. Like the Zaire VP35, Reston VP35 blocks GFP-IRF-3 nuclear accumulation and appears to inhibit IRF-3 phosphorylation in response to SeV infection. In addition, infection of human-derived 293 cells with Ebola virus Reston did not activate the ISG54 promoter. Ebola virus Reston causes lethal disease in nonhuman primates but did not cause disease in the few documented cases of human exposure. Animal handlers caring for Ebola virus-infected cynomolgus macaques did not become ill but did seroconvert, suggesting that they experienced subclinical infections (12, 18). In contrast, the Zaire subtype of Ebola virus appears to be the most lethal filovirus in humans, and outbreaks of Ebola virus Zaire have had reported fatality rates approaching 90 percent (69). Based on these observations, some have suggested that the Reston strain is avirulent in humans; however, the possibility exists that Ebola virus Reston may cause human disease under some circumstances (12). Given the possible differences between Ebola Zaire and Ebola Reston viruses in terms of human virulence, comparisons of these viruses are therefore of great interest. At this point our data do not suggest differences in IFN antagonist function for Zaire versus Reston virus VP35s. However, we cannot presently rule out the possibility that there exist species-specific or cell type-specific differences between the VP35s of different filoviruses.

The IFN antagonist activity of the VP35 protein is likely to play a significant role in Ebola virus pathogenesis. Data from several viruses have demonstrated that functional IFN antagonists are required for virulence in animal models. For example, an influenza virus lacking the IFN-antagonist NS1 protein, influenza delNS1 virus, is avirulent in BALB/c mice, whereas the isogenic wild-type influenza A/PR/8/34 (H1N1) virus (PR8 virus) readily kills mice (23). However, the influenza delNS1 virus kills STAT1-null mice, which lack a critical component of the IFN-α/β system (23). The ability of influenza delNS1 virus to kill mice with defective IFN-signaling pathways demonstrates that its attenuation in wild-type mice is related to the IFN system. Similarly, we would predict that an Ebola virus lacking an effective IFN antagonist activity would be attenuated, and the inability of wild-type Ebola viruses to cause disease in mice may reflect an inability of Ebola viruses to counteract the mouse IFN system (13). Our present results would predict that in the absence of VP35 IFN antagonist...
function, large amounts of IFN-α/β would be produced, inducing in cells an antiviral state and leading to inhibition of viral spread. In addition, VP35 may assist Ebola virus in evading other aspects of the host antiviral response, as IFN-α/β appear to influence adaptive immune responses (6). In particular, IFN-α/β production has been shown to influence the production of other immunoregulatory cytokines, including interleukin-15 and IFN-γ, and to influence NK cell and dendritic cell function (reviewed in references 9 and 59). Interestingly, Ebola virus infection has recently been reported to impair human dendritic cell function (47). It is possible that the ability of VP35 to inhibit IFN production may contribute to the ability of Ebola virus to inhibit dendritic cell functions.

While our data provide insights into the mechanism by which VP35 blocks IFN production, the overall impact of the IFN antagonist function of VP35 on Ebola virus replication remains to be determined. Several recently developed technologies are likely to prove useful in elucidating the functional significance of the VP35 IFN antagonist activity. Reverse genetics methods have been established for Ebola virus, permitting the introduction of specific alterations into the viral genome (56, 83). These techniques hold the promise of creating viruses devoid of VP35 anti-IFN activity. However, the prospect of creating such a mutant VP35 virus is complicated by the other functions of VP35, including its essential role in viral RNA synthesis and its role in viral assembly (33, 52, 53). We are seeking to identify mutations that eliminate VP35 anti-IFN function without significantly impairing its other functions. The developing genomics and proteomics technologies also hold great promise in helping to define the impact of viral infection and viral gene expression on the host cell (37). Recently, microarray technology has been employed to study the impact of both wild-type and mutant NS1 IFN-antagonist proteins of influenza A viruses (24). Such techniques could be employed to analyze the IFN response to Ebola virus infection in different cell types and to compare the IFN responses in cells infected with different subtypes of Ebola virus. These techniques could also be used to examine the specific influence of VP35s of different filovirus strains in different cell types. Ultimately, it is hoped that our current and future studies on VP35 will enhance our understanding of the pathogenesis of filoviruses and identify new targets for antiviral therapies.

ACKNOWLEDGMENTS

C.F.B. and A.M. contributed equally to this work.

This work was supported by NIH grants to C.F.B., A.G.-S., and P.P. C.F.B. is an Ellison Medical Foundation New Scholar in Global Infectious Diseases. J.P. is a recipient of a National Research Council Fellowship.

We thank Neva Morales and Estanislao Nistal-Villan for expert technical assistance. We thank John Hiscott (McGill University) for helpful discussions and providing the ISG56 promoter reporter plasmid. We thank Peter Howley (Harvard Medical School) for providing the SL-12 antibody.

REFERENCES


9756  BASLER ET AL.  J. VIROL.


