Characterization of the RNA Silencing Suppression Activity of the Ebola Virus VP35 Protein in Plants and Mammalian Cells

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Ebola virus (EBOV), a member of the Filoviridae, is a class A priority pathogen. The most pathogenic strains (e.g., Zaire) cause severe hemorrhagic fever in humans, with a fatality rate of up to 80 to 90% (20). Although there have been several promising vaccine approaches and postexposure antiviral therapies in nonhuman primates, no licensed vaccines or specific therapeutics currently exist for the prevention or treatment of human EBOV infections (14, 27, 41, 47, 50). Moreover, EBOV blocks the induction of interferon (IFN) and does not respond to exogenous IFN in vivo or in vitro (1, 17, 18, 22, 24). Most of this antagonism is mediated by EBOV VP35 (1–3), a multifunctional 340-amino-acid (aa) protein that is also an essential viral RNA polymerase cofactor and a structural component of the virion (21, 36, 37). In addition to its ability to bind double-stranded RNA (dsRNA), EBOV VP35 blocks activation of IRF-3 and/or IRF-7. It also enhances the ability of EBOV to replicate. The loss of most of the RSS activity of VP35 to interfere with microRNA-directed silencing could enhance the ability of EBOV to replicate. The loss of most of the RSS activity of VP35 with mutation of arginine 312 to alanine (R312A) suggested to earlier investigators that silencing suppression activity required the ability of VP35 to bind dsRNA (15), since the R312A mutant protein failed to bind dsRNA in vitro (7). However, the mechanism is unknown, VP35’s effect on PKR is apparently RNA independent and prevents the phosphorylation and inactivation of the important translation factor eIF-2α (12, 45).

In addition to its effects on the IFN pathway, VP35 has been demonstrated to be a potent RNA silencing suppressor (RSS) (15). RNA silencing pathways are highly conserved among plants, animals, fungi, and fission yeast (44) and, therefore, likely represent some of the most primordial defense mechanisms. Indeed, it is well established that RNA silencing is an innate antiviral defense in plants, and virtually all plant viruses encode one or more RSS proteins that act as pathogenicity determinants (reviewed in references 10, 31, and 40). Many of these RSSs have been shown to block small interfering RNAs (siRNAs) and/or pathways required for their generation (4, 28). Moreover, many plant virus RSS proteins also interfere with microRNA-directed silencing (25). The role of RSSs in the pathogenicity of mammalian viruses has been the subject of great debate. However, because the microRNA pathway is a major posttranscriptional regulatory mechanism in mammals, the ability of a virus to suppress microRNA-directed silencing globally or specifically could alter the cellular environment to benefit replication. A reporter gene containing specific microRNA target sequences was used to demonstrate that prior expression of wild-type VP35 was able to block establishment of microRNA silencing in mammalian cells. In addition, wild-type VP35 C-terminal domain (CTD) protein fusions were shown to bind small interfering RNA (siRNA). Analysis of mutant proteins demonstrated that reporter activity in RSS assays did not correlate with their ability to antagonize double-stranded RNA (dsRNA)-activated protein kinase R (PKR) or bind siRNA. The results suggest that enhanced reporter activity in the presence of VP35 is a composite of nonspecific translational enhancement and silencing suppression. Moreover, most of the specific RSS activity in mammalian cells is RNA binding independent, consistent with VP35’s proposed role in sequestering one or more silencing complex proteins. To examine RSS activity in a system without interferon, VP35 was tested in well-characterized plant silencing suppression assays. VP35 was shown to possess potent plant RSS activity, and the activities of mutant proteins correlated strongly, but not exclusively, with RNA binding ability. The results suggest the importance of VP35-protein interactions in blocking silencing in a system (mammalian) that cannot amplify dsRNA.
structural studies have revealed that R312 of VP35 is involved not only in interactions with the backbone phosphates of dsRNA, but also in protein–protein interactions at the interface of monomers in the asymmetric dimer of the VP35 CTD that forms in cocryostals with dsRNA (26, 30). Thus, it remains unclear whether RNA silencing inhibition by VP35 depends on dsRNA binding.

To better understand the means by which VP35 suppresses RNA silencing and to investigate its ability to interfere with microRNA silencing, we employed assays that reflect the microRNA-directed silencing pathway that is used by mammals, as well as the siRNA-directed pathway involved in natural antiviral defenses in plants. Plant silencing suppression assays have the additional advantage of avoiding any direct or indirect links with IFN pathways. We also report on the ability of the VP35 CTD to bind directly to the small dsRNAs that are mediators of siRNA- and microRNA-directed responses. The results suggest that microRNA-directed silencing suppression in mammalian cells involves mostly RNA-independent mechanisms. In contrast, in plants that rely heavily on dsRNA amplification, RNA-dependent mechanisms predominate.

MATERIALS AND METHODS

Plasmids. The pCMV-Luc-miR30 reporter and pSuper-miR30 plasmids were obtained from Bryan Cullen and have been described in detail elsewhere (54, 56, 57). pCMV-Luc-miR30 allows high-level constitutive expression of an mRNA containing the firefly luciferase (Fluc) open reading frame (ORF) with 8 tandem repeats of a microRNA 30 (miR30) target sequence in the 3′ untranslated region (UTR). The pSuper-miR21 plasmid was derived by cloning human miR21 sequences into the pSuper promoter III promoter sequences. pRLCMV was obtained from Promega.

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**Transient transfections.** Baby hamster kidney (BHK), human 293T, or 293T derivative cells were seeded into 35- or 60-mm culture dishes in antibiotic-free Dulbecco’s modified minimum essential medium (DMEM) containing 10% fetal bovine serum and incubated overnight at 34°C in a humidified 5% CO2 atmosphere to achieve 50 to 80% confluence. Approximately 1 h prior to transfection, the culture medium was changed to serum- and antibiotic-free DMEM containing 0.1 mM non-essential amino acids. DNA mixtures containing constant amounts of DNA (700 ng/35-mm dish or 2 μg/60-mm dish) and lipofectamine 2000 (Invitrogen; 4.25 μl/dish) were added to cells in Opti-MEM low-serum medium as described previously (51). The DNA mixtures were prepared with the amounts of individual plasmids indicated for each experiment, with the remaining DNA comprised of empty-vector sequences (pUC19 or pCDNA3.1). Four hours after transfection, the medium was replaced with DMEM containing 10% fetal bovine serum, and the cells were incubated as before and were harvested as indicated for each experiment. For some experiments, as indicated, a second transfection was performed 24 h after the first.

**Preparation and cultivation of stable cell lines.** Human 293T cells were transfected with pCEP4-tetR plasmid (700 ng) and incubated for 2 to 4 weeks in medium containing hygromycin (50 μg/ml) to obtain TET repressor (tetR)-expressing cell lines. Culture medium was replenished every 3 to 4 days, and clones resistant to hygromycin were picked and expanded. The clones were functionally screened for the presence of tetR protein by the ability to regulate expression of a test plasmid (pcDNA4/TO-LacZ) containing the Escherichia coli lacZ gene downstream of the TET operator. When transfected with up to 20 ng of pcDNA4/TO-LacZ and in the absence of induction, the tetR-expressing cell line selected (6c3) displayed no detectable β-galactosidase (β-Gal) activity by either X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining or immunoblotting with β-Gal antibody. However, high levels of β-Gal expression were observed when similarly transfected cells were incubated in the presence of DOX (1 μg/ml) for 24 h. Cell line 6c3 was transfected with the zeocin-selectable vector pcDNA4/TO containing either the VP35 (wild type or mutant) or lacZ gene, and clones resistant to zeocin (250 μg/ml) were isolated. Stably transfected cell lines were maintained continuously in medium containing hygromycin (50 μg/ml) and zeocin (250 μg/ml) and were screened by immunoblotting for relevant protein expression in the absence or presence of DOX (1 μg/ml) for 24 h. The C-terminal halves of the VP35 genes from mutant and wild-type cell clones were PCR amplified from purified total cellular DNA, and the sequences were confirmed.

**Antibodies and immunoblotting.** Cells from parallel cultures used for luciferase assays were harvested by scraping the cell monolayers into the medium, followed by low-speed centrifugation. The cell pellets were washed twice in ice-cold phosphate-buffered saline (PBS), flash frozen in liquid nitrogen, and stored at −80°C. The cells were suspended in buffer containing 0.1 M Tris-Cl, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.5% Na deoxycholate and lysed by ultrasonic disruption at 0°C. The suspensions were clarified by centrifugation and the protein content in the supernatant was determined using a Coomassie blue dye-based assay (Bio-Rad) with bovine serum albumin as the standard. Equivalent amounts of protein were separated by SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked in PBS containing 10% nonfat dry milk, washed extensively with PBS containing 0.1% Tween 20, and then incubated with a 1:5,000 dilution of mouse monoclonal antibody 46-0603 to c-myc (Invitrogen), a 1:666 dilution of rabbit polyclonal antibody to β-Gal (5-3′ Inc., West Chester, PA), or a 1:500 dilution of rabbit polyclonal antibody to GFP (Santa Cruz), as indicated. Following extensive washing, the blots were incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Thermo) and developed using the Super Signal West Pico Chemiluminescent kit according to the

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instructions of the manufacturer. The blots were exposed to X-ray film for 1 to 10 s and developed.

**Luciferase assays.** The dual-luciferase reporter assay kit from Promega (Madison, WI) was employed, using an active lysis procedure according to the instructions of the manufacturer. Cells in 35- or 60-mm dishes were seeded and/or pretreated as indicated for each experiment. Twenty-four hours later, the cells were transfected with pCMV-Luc-miR30 (30 ng/35-mm dish or 90 ng/60-mm dish), pCMV-Rluc (2 or 6 ng for 35- or 60-mm dishes, respectively), mixtures of pSuper-miR30 and/or pSuper-miR21 as indicated in each experiment, and vector plasmid filler as described above. In some experiments, test expression plasmids were transfected 24 h after cell seeding, and a second transfection with the luciferase and miR expression plasmids was performed 24 h thereafter. At 48 h after transfection with luciferase plasmids, cells were harvested by scraping and washed twice in ice-cold PBS. Cells from each culture dish were suspended in 1 ml lysis buffer supplied by the manufacturer, flash frozen in liquid nitrogen, and stored at −80°C. The cells were lysed using 3 freeze-thaw cycles, and the lysates were clarified by centrifugation at 13,500 × g for 5 min. The supernatants were immediately according to the manufacturer’s instructions and stored at −20°C prior to assay. Luminescence was read using a microplate luminescence meter (Packard, Meriden, CT). Three readings were taken for each sample, adjusted for background luminescence, and averaged. The average luminescence values from three to six independent replicate cultures were also averaged to obtain each data point reported. A two-way Student’s t test was performed to determine whether observed differences in data were statistically significant (P < 0.05).

**Affinity purification of MBPs and RNA binding assays.** E. coli containing the pMal-5cE plasmid for expression of MBP or plasmid to express an MBP fusion protein with the C-terminal domain (aa 215 to 340) of wild-type or mutated VP35 were grown to mid-log phase (A600 = 0.5) at 37°C. Protein expression was subsequently induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (0.3 mM). Cells from 1 l of culture were harvested 2 h after induction and suspended in 25 ml of column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, and 10 mM 2-mercaptoethanol). The cells were lysed at 0°C by intermittent ultrasonic disruption in 10-s increments for approximately 2 min of sonication time using a Branson model 350 sonicator microprobe at setting 5. After sonication, the cellular membranes were removed by centrifugation at 20,000 × g for 20 min at 4°C. The supernatants (extracts) were collected and used immediately or were stored at −80°C prior to affinity purification of MBPs. Two-milliliter columns of amylose resin (New England BioLabs, Beverly, MA) were prepared and equilibrated with column buffer according to the instructions of the manufacturer. Bacterial extracts (1 ml or equivalent to extract from 40-ml culture) were diluted 1:6 in column buffer and applied to amylose columns. Unbound proteins were removed by washing with 10 column volumes. 32P-labeled dsRNA or oligo(dT)190 prepared as described below, was added to columns containing the bound MBP or MBP fusion protein and incubated at room temperature for 10 min. Unbound nucleic acid was removed by washing with 10 volumes of column buffer. MBP and MBP fusion proteins, together with bound nucleic acid, were eluted by the addition of 10 mM maltose in column buffer. A total of 30 fractions (0.4 ml each) were collected, and 5 μl from each fraction was spotted onto Whatman 3MM filter, dried, and counted by liquid scintillation spectrometry. Nucleic acids were 5’ end labeled with [γ-32P]ATP and T4 bacteriophage polynucleotide kinase and purified by standard procedures. RNA binding assays used 50 μg poly(I)·poly(C) for long dsRNA (specific activity, 1.1 × 106 dpm/μg), 24 ng oligo(dT)190 (4.6 × 104 dpm/μg), or 146 ng siRNA (2.6 × 105 dpm/μg) per column. The lengths of the poly(I)·poly(C) duplexes (Sigma, St. Louis, MO) were variable. Oligo(dT)190 and siRNA were obtained from IDT (Coralville, IA) and Ambion (Austin, TX), respectively. The siRNA contained two annealed 21-nucleotide (nt) RNAs with 19 perfect base pairs and 2-nt 3’ overhangs at each end.

**Plant silencing suppression assays.** Two- and three-component silencing suppressor assays have been described previously (23, 49). Briefly, wild-type Nicotiana benthamiana plants were used in the three-component assay, while the two-component assay utilized transgenic N. benthamiana plants expressing GFP from a Cauliflower mosaic virus 35S promoter (line 16c; provided by David Baulcombe, Cambridge University) (42). Agrobacterium harboring plasmids for expression of the GFP silencing target, a long hairpin of GFP (dsGFP) to induce silencing, and/or a protein of interest or control were mixed at equal volumes (A600 = 1) and coinfiltrated into the underside of 3 leaves per plant using a 1-ml syringe. In all cases, expression was driven by the 35S promoter. GFP fluorescence was analyzed 3, 5, and 7 days postinfiltration with a long-wave UV lamp (Blak-Ray Model B 100YP; UV Products). Photographs of individual leaves were obtained using a Nikon D40 camera with UV and yellow filters at a uniform exposure.

**RNA extraction and Northern blot analysis.** Northern blot procedures have been described previously (49). Leaf discs containing the infiltrated zones were obtained from 3 plants (weighing approximately 0.15 g total), and RNA was extracted using 1 ml TRIzol Reagent (Invitrogen). RNA loaded onto gels for Northern blots contained 5 μg for each sample, with the exception of p19 samples, for which 1 μg of RNA was loaded. Following RNA transfer, the membranes were probed with a GFP antisense riboprobe synthesized with [α-32P]UTP using the Maxiscript T7 kit (Ambion) according to the instructions of the manufacturer.

**RESULTS**

EBOV VP35 can suppress microRNA-directed silencing in mammalian cells. Haasnoot and coworkers (15) were the first to describe EBOV VP35 as an RSS. In their studies, short hairpins were targeted to the open reading frame to silence firefly luciferase reporter activity, and EBOV VP35 was shown to suppress this silencing. Inasmuch as gene silencing in mammalian cells occurs by the targeting of cellular microRNA to predominantly untranslated sequences, and since microRNA silencing could alter the cellular environment to enhance virus replication, we wished to examine the ability and specificity of EBOV VP35 to interfere with microRNA-directed silencing in mammalian cells. A modification of the reporter assay developed by Zeng et al. was employed (57). The reporter construct consisted of the Fluc gene linked to 8 tandem repeats of the miR30 target sequence within the 3’ UTR sequences. When cotransfected with plasmid constructs encoding the target Fluc and the irrelevant Rluc used as a transfection control, the miR30-decoding plasmid severely diminished relative Fluc (Fluc/Rluc) activity compared to the activity observed when no miR-expressing plasmid was added or when cells were cotransfected with an irrelevant miR21 in 2 different cell types (BHk and 293T) (Fig. 1A and B, respectively).

Because our goal was to measure the ability of a viral protein to suppress silencing activity, it was important to determine the dose-response of the silencing activity of the miR30 expression construct. In preliminary experiments, we observed nearly complete silencing of Fluc activity when as little as 0.1 ng of miR30-encoding plasmid was used in some experiments, but the absolute responses were highly variable from experiment to experiment. We observed more consistent dose-responses when cells were transfected with a constant amount of microRNA-expressing plasmid in which the ratio of specific (miR30-expressing) to nonspecific (miR21-expressing) plasmid was varied. Examples of dose-response curves in BHk and 293T cells are shown in Fig. 1C and D, respectively. Regardless of the total amount (10 or 30 ng) of microRNA-expressing plasmid or the type of cell line used, we observed a negative logarithmic relationship between the relative
dose of miR30-expressing plasmid and Fluc activity. In contrast, the level of activity of the irrelevant target gene product, Rluc, displayed no pattern of significant inhibition by miR30 (Fig. 1C and D).

**FIG 1** Specificity and dose-response of microRNA-directed silencing in mammalian cells. Each dish of BHK (A and C) or human 293T (B and D) cells was cotransfected with 30 ng of the Fluc reporter plasmid (pCMV-Luc-miR30) containing miR30 target sequences in the 3' UTR and 2 ng of the Rluc control reporter (pCMV-Rluc) alone or together with plasmid expressing miR30 and/or miR21 as indicated. Fluc and Rluc activities were determined from cells harvested 48 h after transfection using the dual-luciferase reporter kit as described in Materials and Methods. (A and B) Cells were incubated with reporter plasmids only (no miR) or with 10 ng (A) or 30 ng (B) miR-expressing plasmid as indicated. The average Fluc/Rluc activities from 3 replicate samples were determined (± standard deviation) and normalized to that obtained with no microRNA (set at 100%). (C and D) A dose-response for selective reactivity to miRs was determined by cotransfecting cells with reporter plasmids together with a constant amount of miR-expressing plasmids, varying the amount of specific miR30 (indicated on the abscissa) to the amount of the nonspecific miR21-expressing plasmid. (C and D) For BHK cells (C), total miR-expressing plasmid was 10 ng, and for 293T cells (D), total miR-expressing plasmid was 30 ng. The average Fluc (○) and Rluc (□) activities (± standard deviations) of triplicate samples are shown. (E) 293T cells were transfected with protein-expressing plasmids as indicated (2 μg/60-mm dish) and 24 h later supertransfected with luciferase reporter plasmids and miR30 silencing mixture (12 ng miR30- plus 78 ng miR21-expressing plasmids). Luciferase activities from six replicate samples receiving the silencing mixture were averaged and are shown as a percentage of that in samples that received miR21-expressing plasmid only (no silencing). The error bars indicate standard deviations. The statistical significance of differences between samples containing the silencing miR30-expressing plasmid and those containing only the miR21-expressing plasmid (*, P < 0.05) was determined by a two-sided Student's t test. (F) Immunoblots showing expression of test proteins, EGFP (α-EGFP), p19 (α-Flag), or VP35 (α-myc) in 4 μg of extract from parallel cultures of silenced cells (lanes 2, 4, and 6). The same amount of control 293T cell extract was loaded onto the gels (lanes 1, 3, and 5) and probed with the indicated antibodies.

**EBOV VP35 prevents the establishment of miR-directed RNA silencing in mammalian cells.** We first used cotransfection experiments to determine the ability of EBOV VP35 to suppress miR30-directed silencing of Fluc activity. For BHK cells, a mixture
containing 0.15 ng miR30 and 9.85 ng miR21 was selected. This mixture incompletely silenced Fluc activity but left silencing of Fluc in a responsive range. The silencing mixture was cotransfected with expression plasmid encoding either EBOV VP35, an irrelevant protein (EGFP), or the plant virus RSS p19 linked to a FLAG epitope. Luciferase activity was compared to that in nonsilenced cells transfected with 10 ng miR21-expressing plasmid (see Fig. S1 posted at http://go.osu.edu/JVI5741-11Supplementary). Coexpression of EGFP and the silencing miR mixture resulted in an ∼2.5-fold reduction in Fluc activity compared to the nonsilenced control. However, coexpression of EBOV VP35 and the silencing mixture restored Fluc activity. We also observed partial but significant restoration of Fluc activity by the p19 plant virus RSS. Because all plasmids were expressed at the same time, these results could not differentiate between the ability of VP35 to prevent the establishment of or to reverse miR-directed silencing.

A silencing mixture of 4 ng miR30 and 26 ng miR21 for 35-mm plates (or 12 ng miR30 and 78 ng miR21 for 60-mm plates) was selected as the most responsive ratio for silencing suppression experiments in 293T cells or their derivatives (see Fig. ID). Moreover, due to the high transfection efficiency of 293T cells, it was possible to perform sequential transfections to determine whether prior expression of VP35 or other proteins could prevent silencing by miR30. Cells were transfected with test expression plasmid 24 h prior to the addition of the reporter and miR expression plasmids for these experiments (Fig. IF). The miR30-miR21 mixture reduced Fluc expression with prior expression of EGFP compared to that observed when only miR21 was expressed. However, not only did prior expression of VP35 protein prevent the silencing of 293T cells, but VP35 expression led to even greater activity than was observed in nonsilenced cells. In contrast, we observed partial recovery of Fluc expression when cells were preincubated with the p19 plant virus RSS, though Fluc expression remained significantly below that observed in nonsilenced controls. Although this low level of silencing suppression by p19 reached significance in this experiment with 6 replicates, in others using only 3 replicates, we could discern no significant difference between Fluc activity with prior expression of p19 and that with the EGFP control (not shown). Analysis of test protein expression in silenced samples confirmed that all proteins were well expressed (Fig. IF). Thus, the low-level silencing suppression by p19 observed in either 293T or BHK cells (Fig. IF; see Fig. S1 posted at http://go.osu.edu/JVI5741-11Supplementary) is dwarfed by the high RSS activity of VP35 and VP35’s ability to prevent the establishment of miR-directed silencing.

Ability of induced wild-type or mutant VP35 to suppress miR-directed silencing in stable cell lines. (A) Protein expression in the stably transfected 293T-derived cell lines was induced (open bars) by the addition of DOX (final concentration, 1 μg/ml) in culture medium or uninduced (cross-hatched bars) at the same time by the addition of the equivalent amount of vehicle-containing medium (5% dimethyl sulfoxide [DMSO] in DMEM). Twenty-four hours later, the cells were transfected with luciferase reporter plasmid and either 30 ng/dish of control miR21 (not silenced) or a mixture of 4 ng miR30 plus 26 ng miR21 (silenced). Fluc activity was measured in extracts of cells harvested 48 h later. The similarly derived cell line that inductively expresses the irrelevant β-Gal protein (LacZ) was used as a control for the level of silencing that could be established in this system. A representative experiment showing the average amounts of luciferase activity (normalized for the protein amount) from three replicate samples is shown. The fold enhancement of luciferase activity in DOX-induced compared to uninduced samples is indicated for wild-type (WT)- and VP35 mutant-expressing cell lines. The significance of the differences observed was determined by a two-sided Student’s t test (P < 0.05) and is discussed in detail in the text. No significant difference in Fluc expression between induced and uninduced lacZ cells was observed. The error bars indicate standard deviations. (B and C) Protein expression in cells treated with the miR30 silencing complex is shown for uninduced (−) or DOX-induced (+) cells. Equivalent amounts of protein (4 μg) were separated by SDS-PAGE and immunoblotted with antibody to β-Gal (B) or antibody to c-myc (C) to detect protein expression in LacZ or VP35 (wild-type or mutant)-expressing cells, respectively. M, molecular weight marker that contains β-Gal; C, control VP35 expression in 293T cells transfected with VP35 expression plasmid.
The Fluc target reporter plasmid was introduced into the lacZ cell line, together with either 30 ng of miR21-expressing plasmid (not silenced) or a mixture of 4 ng miR30- and 26 ng miR21-expressing plasmid (silenced). The miR30-miR21 mixture was capable of silencing Fluc expression more than 2-fold, regardless of whether lacZ expression was induced by the addition of DOX (Fig. 2A). These results indicate the specificity of silencing by miR30 and the absence of interference by inducible protein expression in the establishment of silencing by miR30. The abilities of wild-type and mutant VP35 genes to suppress the establishment of silencing by miR30 were tested in the relevant uninduced cell lines or those in which gene expression was induced with DOX for 24 h prior to transfection. We observed enhanced Fluc activity in the absence of induction for some of the cell lines (K309A and R312A), suggesting the presence of small but functional amounts of VP35 protein despite its lack of detection by immunoblotting (Fig. 2C). The different absolute levels of Fluc activity without DOX induction therefore likely reflect different basal levels of expression of the proteins. Nevertheless, we observed significantly increased Fluc activity in cells induced to express either the wild-type (3.8-fold) or K309A or R312A mutant (2.2- and 1.7-fold, respectively) VP35 proteins compared to that expressed without induction (Fig. 2A). Moreover, the induced expression of wild-type VP35 or either of the VP35 mutants (K309A and R312A) significantly increased Fluc activity compared to that present in the induced lacZ control cells, demonstrating that much, though not all, of the RSS activity in VP35 (wild-type or mutant)-expressing cell lines was dependent upon induced protein expression. Notably, the R312A and K309A mutations, reported to completely abolish the dsRNA binding ability of VP35 (7, 12), possessed significant RSS activity compared to the lacZ control. However, the RSS activity of the R312A mutant was significantly less than that observed following the induction of the wild-type or K309A mutant gene. Surprisingly, the Fluc activity in K309A-induced cells was as high or higher than that observed in cells induced to express the wild-type VP35 (Fig. 2A). These differences were not due to differences in the levels of induced wild-type or mutant VP35 protein expression, since we observed comparable levels of protein expression in the silenced samples upon DOX induction (Fig. 2C). Taken together, these results indicate that at least some of the RSS activity of VP35 in mammalian cells is RNA binding independent. However, because the RSS activities of the R312A and K309A mutants differed and previous experiments did not investigate the abilities of these mutants to bind to small RNA species, it was important to clarify the abilities of mutant proteins to bind to small RNAs structurally similar to those that mediate microRNA-directed silencing.

Ability of EBOV VP35 to bind siRNA. Although VP35 has been shown to bind to blunt-ended 5’-phosphorylated and nonphosphorylated RNA (7, 19, 26, 30), its ability to bind to mediators of silencing suppression (with 3’ OH overhangs) has not been determined. siRNAs are capable of knocking down gene expression in both plant and mammalian cells (reviewed in references 34, 43, and 48) and, like microRNAs, are incorporated into RNA-induced silencing complexes (RISCs) to mediate posttranscriptional silencing of mRNA. Both siRNAs and microRNAs contain a 19-nt duplex or partially duplex region with 3’ overhangs of 2 nt on each end. Since the VP35 CTD has been shown to be involved in the dsRNA binding activities of the protein (7, 19, 30), we expressed wild-type and mutant forms of this domain (amino acids 215 to 340) as an MBP fusion protein in bacteria. The functionality of purified MBP-VP35 CTD preparations (with a myc tag N terminal to the CTD) was first confirmed. Affinity columns containing either MBP or the MBP-VP35 CTD linked to amylose were prepared as described in Materials and Methods. Different forms of radioactively labeled nucleic acid were incubated with the bound proteins. MBP-containing proteins, together with nucleic acid bound to them, were then eluted by the addition of maltose. In control experiments, we demonstrated that this procedure eluted MBP or MBP-VP35 with a purity of >95% (see Fig. S2 posted at http://go.osu.edu/JVI5741-11Supplementary).

As shown in Fig. 3A and D, neither MBP nor the MBP-VP35 CTD protein was capable of binding a single-stranded DNA, oligo(dT)20. In contrast, MBP-VP35 CTD specifically bound poly(I)·poly(C) (Fig. 3E), whereas MBP failed to bind this long (up to 2-kb) dsRNA (Fig. 3B). MBP-VP35 CTD, but not MBP alone (Fig. 3F and C, respectively), was also capable of binding siRNA. These results demonstrate that MBP-VP35 CTD binds both siRNA and larger dsRNA with high specificity under these conditions. We also tested the abilities of various VP35 mutations within this context to bind siRNA. As expected, we observed no significant binding of the siRNA to the MBP-K309A CTD or the MBP-R312A CTD or to a double mutant containing the R312A and K309A mutations (Fig. 3G to I). These results confirm that wild-type VP35, or at least its CTD, is capable of stably binding siRNA with 2-nt 3’ overhangs and that point mutations at R312 and/or K309 eliminate this binding. Together with the observation that both of these mutant proteins can enhance Fluc expression following miR-directed silencing in mammalian cells, though to different extents, these results also suggest that most of this ability is independent of RNA binding activity.

EBOV VP35 functions as a silencing suppressor in plants. EBOV VP35 has been shown to be a potent IFN antagonist, capable of blocking not only the induction of IFN-β, but also downstream effectors of IFN, such as PKR (1). Indeed, translational inhibition caused by the ability of activated PKR to phosphorylate the essential translation factor eIF-2α is also antagonized by VP35 (12, 45). Inasmuch as miR-directed silencing in mammalian cells can lead to translational inhibition (35, 57), assessment of the RSS activity of VP35 in mammalian cells using a reporter is complicated by the presence of the IFN pathway. In order to evaluate the inherent RSS activity of VP35 (wild type and mutants) independent of any effects it has on the IFN pathway, we used two well-established plant silencing suppressor assays (23), since plants neither encode IFNs nor respond to them. In the three-component system, potent silencing was established by introduction (via Agrobacterium infiltration of N. benthamiana leaves) of plasmids expressing the GFP gene and a long hairpin of GFP (dsGFP), and silencing suppression was determined by the simultaneous addition of a test gene. Expression of the GFP gene is silenced when the dsGFP is converted to short siRNAs, which then associate with RNA-silencing complex proteins (16, 23, 55). The siRNAs may also be amplified by a plant-encoded RNA-dependent RNA polymerase that strengthens and facilitates the spread of the silencing response. In the two-component system, a GFP transgenic line of N. benthamiana (line 16c) was used, and a GFP expression clone (which induces silencing of GFP mRNA originating from the transgene and the expression plasmid) was introduced at the same time as a test gene for silencing suppression. When plant leaves are examined under UV light, they appear red due to the inherent
fluorescence of chlorophyll, and expression of GFP in the areas of infiltration is evident by the bright green color. In both systems, silencing developed over a period of several days, as indicated by the near absence of green color when the leaves were coinfiltrated with the negative-control test plasmid encoding GUS, which failed to suppress silencing (Fig. 4A and B). In contrast, GFP expression persisted even after 7 days when leaves were coinfiltrated with the potent plant virus RSS p19. The wild-type VP35 gene, expressed using the same strong 35S promoter, also suppressed silencing. As an additional control, we tested a G333S VP35 mutant, which was derived by the same PCR-directed mutagenesis and domain switching used to derive all of the mutant expression clones required for these experiments; however, the G333 residue lies outside the central patch of basic residues associated with RNA binding (30). As expected, G333S displayed RSS activity similar to that displayed by wild-type VP35 (Fig. 4A and B). Compared to the negative GUS control, low levels of GFP expression were still observed within the areas of infiltration in the presence of K309A mutants.
and R312A mutant VP35 genes, particularly in the two-component system (Fig. 4B). However, both of these mutations clearly compromised the RSS activity of VP35. The double mutant reduced the RSS activity even further in the two-component system, though low residual GFP expression remained observable even after 7 days (Fig. 4B).

The visual data in these assays were generally confirmed by Northern blots to determine changes in the steady-state levels of...
GFP mRNA within the areas of infiltration. In plants, siRNA generated in response to silencing results in specific cleavage of the target mRNA, leading to reduced steady-state levels. We isolated RNA from infiltrated spots 7 days after inoculation, separated the RNA by denaturing agarose gel electrophoresis, and probed transferred RNA with a GFP riboprobe. Northern blots of the RNA from infiltrated tissues from the three- and two-component assays are shown in Fig. 4C and D, respectively. The RNA load is indicated by the ethidium bromide staining of 28S rRNA in the gel prior to transfer. Because of p19’s potent RSS activity (46), 5-fold less RNA was loaded for p19-containing samples to prevent overexposure of the blots. As expected, an extremely low steady-state level of GFP mRNA (barely detectable in the three-component system) was present in plants that received the negative control (GUS) plasmid compared to the levels in plants that received the positive-control p19 RSS or wild-type VP35 (Fig. 4C and D). Of the VP35 mutants tested, only G333S possessed steady-state levels of GFP mRNA that were significantly greater than those of negative-control samples, confirming that in the presence of wild-type or G333S VP35, GFP mRNA degradation associated with silencing was inhibited. These results are consistent with the visual inspection of GFP expression in the plants (Fig. 4A and B) and demonstrate the ability of EBOV VP35 to act as an RSS in plants independent of the known effects of the protein on IFN response. Moreover, the results show that mutation of the R312 and/or K309 residue, which largely destroys the ability of VP35 to bind to siRNA, also eliminates most of the plant RSS activity. Thus, expression of EBOV VP35 in plants can either prevent the establishment of silencing and/or rapidly reverse it, and this response is highly dependent on the ability of the protein to bind siRNA or other intermediate RNA species in the silencing pathway.

**DISCUSSION**

**Suppression of miR-directed silencing by EBOV VP35 in mammalian cells.** In this report, we analyzed in greater detail the silencing suppressor activity of wild-type and mutant EBOV VP35 first reported by Haasnoot and colleagues (15). Our results demonstrate that wild-type VP35 can specifically suppress microRNA-directed silencing in mammalian cells in which the target sequence is located in the 3′ UTR of the reporter gene, the site where most microRNA targets are found. Our results also demonstrate that some mammalian cell lines differ in their relative susceptibilities to miR-directed silencing. In our hands, BHK cells are exquisitely sensitive to silencing, with <1 ng miR30-expressing plasmid resulting in a 5-fold reduction in target protein expression (Fig. 1C). Although miR-directed silencing of human 293T cells also occurs, similar reductions in reporter silencing required transfection with much larger amounts of miR30-expressing plasmid (compare Fig. 1A and B). By mixing the miR30- and miR21-expressing silencing plasmids, specificity of silencing response and better reproducibility were ensured (Fig. 1C and D). Moreover, dose-response curves suggested ranges of silencing plasmids capable of distinguishing relative RSS abilities of test proteins. By using a small dose of miR30 relative to miR21 plasmid, VP35 consistently suppressed the silencing of target gene expression in both 293T and BHK cells (Fig. 1E; see Fig. S1 posted at http://go.osu.edu/JVI5741-11Supplementary). The potent plant RSS p19, known to bind siRNA (6, 46), possessed low, but significant, RSS activity in BHK and 293T cells, though only partial recovery from silencing occurred in both cell types. The p19 protein was shown previously to possess some RSS activity in miR-directed silencing assays in yet another cell type, HeLa cells (39). Despite some quantitative differences in the amounts of silencing suppression observed in BHK and 293T cells, our results demonstrate conclusively that VP35 can prevent the establishment of miR-directed silencing.

To determine the relative differences in the activities of wild-type versus mutant VP35 genes, it was important to control for cell-line- or species-specific differences, as well as to ensure that essentially all of the cells receiving silencing plasmids also expressed the test RSS. This was achieved by deriving stable inducible cell lines from a single clone of 293T cells that constitutively expresses the TET repressor. These stable cell clones displayed little if any protein expression (detectable by immunoblotting) in the absence of DOX induction (Fig. 2B and C). Introduction of the miR30/miR21-expressing plasmid mixture established effective silencing regardless of the addition of DOX, as shown in the lacZ-expressing clones (Fig. 2A). To reduce the effects of clonal differences, we compared the responses of specific clones with or without induction of test protein expression prior to the establishment of silencing. Based on that comparison, we found that the wild-type VP35 and both mutant VP35 proteins permitted significantly more reporter protein expression following DOX induction than was observed in noninduced cells. Previous analysis of VP35 function demonstrated that the K309A and R312A mutant forms could abrogate PKR activity and eIF-2α phosphorylation as efficiently as the wild-type protein, resulting in enhanced (nonspecific) protein expression (12, 45). Thus, the ability of the R312A mutant protein to increase Fluc expression at significantly lower levels than either the wild-type or K309A mutant in our reporter assays suggests that it is the RSS activity of R312A that is compromised in the miR-directed assay. In other words, the enhancement of reporter activity by R312A is likely due to the nonspecific translational effect of VP35 caused by its PKR antagonism, whereas the cumulative effect of VP35 in mammalian systems (by the wild type and the K309A mutant) reflects both specific RSS activity and nonspecific relief of translational inhibition caused by stress-induced kinases. This interpretation would explain the higher levels of reporter expression observed in silenced cells that express VP35 (wild type or K309A) than in cells that were not silenced (miR21 only) or were silenced but expressed an irrelevant gene (Fig. 1E and 2A).

Our ability to observe wild-type levels of microRNA-directed silencing suppression by the K309A mutant differs from the results of Haasnoot and coworkers, who concluded that neither K309A nor R312A could suppress silencing by short hairpin transcripts (15). Since these investigators introduced the VP35-expressing plasmids at the same time as the reporter and silencing constructs, silencing could have been established prior to significant expression of VP35 protein, or expression of VP35 protein could have been significantly less than we obtained in our studies. In our hands, we observed the most potent silencing suppression when VP35 proteins were present prior to the introduction of silencing plasmids, either by transfecting the VP35 expression plasmid a day prior to transfection with silencing mixtures (Fig. 1E) or by expressing VP35 proteins in stable cell lines by the addition of DOX for 24 h prior to the establishment of silencing (Fig. 2A). Moreover, our use of inducible cell lines resulted in VP35 protein (wild type or mutant) expression in >95% of the cells that were transfected with the silencing mixture. Thus, the inability of
Haasnoot et al. to observe silencing suppression by K309A (15) may be due to the failure of the mutant to reverse established silencing, failure to express the mutant protein at sufficiently high levels in most cells, and/or failure to overcome what could be more potent silencing than was present in our experiments.

It is important to note that the difference between the microRNA-directed silencing suppression activities of K309A and R312A mutants cannot be explained by differences in protein expression levels (Fig. 2C) or by their abilities to bind small siRNAs, since neither mutant CTD could bind siRNAs (Fig. 3G and H). Thus, our results suggest that the microRNA-specific silencing suppression activity of VP35 observed in mammalian cells is not dependent on its ability to bind small RNAs. These results are consistent with a recent report that demonstrated the ability of the RISC-associated proteins PACT and TRBP to bind to EBOV VP35 when overexpressed by transfection (11). These investigators also reported that the VP35 interactions with PACT and TRBP were not dependent upon the presence of siRNA. Thus, it is likely that VP35 can sequester important RISC components, thereby preventing the assembly or activity of silencing complexes.

PACT has also been shown to activate PKR during stress, leading to an inhibition of translation (32). The fact that VP35 can associate with and presumably block the activity of PACT may explain the translational enhancement caused by VP35 that is independent of silencing suppression (for example, with the R312A single mutants but not with the double mutant (Fig. 2A)). These results are consistent with reports that demonstrated an inhibition of host PKR (28) and the translation of viral mRNAs (30) and suggested that VP35 can associate with PACT and TRBP, both of which are important effectors in PKR and silencing complexes, may explain this concordance (11). In animals, could protein signaling molecules, such as IFN, have evolved to amplify the ability of the cells to silence gene expression in the absence of RNA amplification? We have found that a mutated VP35 (R312A) that loses its ability to bind to siRNA (Fig. 3H) but retains PKR antagonism (45) loses most of its ability to counter microRNA-dependent silencing in mammalian cells (Fig. 2) and most of its ability to suppress RNA silencing in plants (Fig. 4). Given the fact that the RNA binding domains of many proteins are also protein binding domains (44), it is possible that the R312A mutation impacts both protein and RNA binding activities of VP35. Indeed, the crystal structure of VP35 with dsRNA shows that the residue is important for interaction between the two monomers arranged in an asymmetric dimer, as well as for backbone RNA binding (26, 30). Since functional silencing complexes in both plant and animal cells require small RNAs as well as proteins that bind to each other and to siRNA or microRNA in the complex, uncovering the precise mechanism(s) of silencing suppression in each system will require the availability of mutants that differentially disrupt RNA binding but not binding to a particular protein target.

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