The C, V and W proteins of Nipah virus inhibit minigenome replication

Katrina Sleeman,1† Bettina Bankamp,1 Kimberly B. Hummel,1 Michael K. Lo,1,2,3 William J. Bellini1 and Paul A. Rota1

1Measles, Mumps, Rubella, and Herpesvirus Laboratory Branch, Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA
2The Research Institute, Nationwide Children’s Hospital, Columbus, OH, USA
3Emory University, Atlanta, GA, USA

Nipah virus (NiV) is a recently emergent, highly pathogenic, zoonotic paramyxovirus of the genus Henipavirus. Like the phosphoprotein (P) gene of other paramyxoviruses, the P gene of NiV is predicted to encode three additional proteins, C, V and W. When the C, V and W proteins of NiV were tested for their ability to inhibit expression of the chloramphenicol acetyltransferase (CAT) reporter gene in plasmid-based, minigenome replication assays, each protein inhibited CAT expression in a dose-dependent manner. The C, V and W proteins of NiV also inhibited expression of CAT from a measles virus (MV) minigenome, but not from a human parainfluenzavirus 3 (hPIV3) minigenome. Interestingly, the C and V proteins of MV, which have previously been shown to inhibit MV minigenome replication, also inhibited NiV minigenome replication; however, they were not able to inhibit hPIV3 minigenome replication. In contrast, the C protein of hPIV3 inhibited minigenome replication of hPIV3, NiV and MV. Although there is very limited amino acid sequence similarity between the C, V and W proteins within the paramyxoviruses, the heterotypic inhibition of replication suggests that these proteins may share functional properties.

INTRODUCTION

Nipah virus (NiV) is a recently emergent, highly pathogenic, zoonotic paramyxovirus. NiV was first identified in Malaysia in 1998 during an outbreak of neurological and respiratory disease in swine, which resulted in severe febrile encephalitis in humans who had contact with infected pigs (Chua et al., 2000). NiV has been associated with subsequent outbreaks of fatal, febrile encephalitis in Bangladesh and India between 2001 and 2005 (Hsu et al., 2004; Luby et al., 2006). Molecular characterization of NiV revealed that it was related closely to Hendra virus, another zoonotic paramyxovirus, which had emerged in Australia in 1994 (Chua et al., 2000; Harcourt et al., 2000, 2001). These two viruses constitute the recently recognized genus Henipavirus (Mayo, 2002).

The genome of NiV is 18 246 nt in length and contains six transcription units encoding six viral structural proteins (3’-N-P-M-F-G-L-5’) and three predicted non-structural proteins, C, V and W (Harcourt et al., 2000). As in other paramyxoviruses, the C protein of NiV is expressed from an alternative open reading frame (ORF) within the phosphoprotein (P) gene, whereas the V and W proteins are expressed by RNA editing (Lamb & Kolakofsky, 2001). At a unique, highly conserved RNA-editing site in the P gene, the polymerase protein (L) inserts a single, non-templated G residue that results in a frame shift and the expression of the V protein. Insertion of two non-templated G residues results in expression of the W protein (Harcourt et al., 2000). Whilst the C proteins of NiV and of other paramyxoviruses are unique and share no sequence similarity with the P protein (Lamb & Kolakofsky, 2001), the V and W proteins share an amino-terminal 407 aa domain with P and each possesses a unique carboxyl-terminal domain consisting of 52 aa for V and 47 aa for W (Harcourt et al., 2000). The sequences of the P proteins of paramyxoviruses are not well conserved (Baron et al., 1993) and, whilst a V protein is not expressed by all paramyxoviruses, the unique carboxyl-terminal domain of V is more conserved than the amino-terminal domain shared with P (Galinski et al., 1992; Matsuoka et al., 1991; Witko et al., 2006). The C, V and W proteins of paramyxoviruses are known to play multiple roles in the viral life cycle, functioning as interferon antagonists (Didcock et al., 1999; Goodbourn et al., 2000; He et al., 2002; Komatsu et al., 2004; Lin et al., 2005; Nanda & Baron, 2006; Ohno et al., 2004; Park et al., 2003; Poole et
al., 2002; Rodriguez et al., 2002; Shaffer et al., 2003), in addition to regulating viral transcription and replication both in vitro and in vivo (Bankamp et al., 2005; Baron & Barrett, 2000; Curran et al., 1991, 1992; Horikami et al., 1996; Kato et al., 1997a, b; Lin et al., 2005; Malur et al., 2004; Parks et al., 2006; Patterson et al., 2000; Reutter et al., 2001; Smallwood & Moyer, 2004; Tober et al., 1998; Witko et al., 2006).

As very little is known about the replication of the henipaviruses, the goal of this research was to investigate the roles that the C, V and W proteins of NiV play in viral transcription and replication. Here, we demonstrate that the C, V and W proteins of NiV inhibit NiV minigenome replication. The NiV C, V and W proteins also inhibited replication of a measles virus (MV) minigenome, and the C and V proteins of MV inhibited NiV minigenome replication. However, the accessory proteins of NiV and MV were not able to inhibit human parainfluenza virus 3 (hPIV3) minigenome replication. Conversely, the C protein of hPIV3 inhibited minigenome replication of both NiV and MV. These findings suggest that these divergent viral proteins share common functional properties, perhaps through interaction with a common host-cell protein(s).

**METHODS**

**Molecular techniques.** Standard DNA manipulations were performed according to methods described by Sambrook & Russell (2001). All plasmids were cloned and prepared by using *Escherichia coli* strain DH5α cells and purified by using the Wizard Plus Maxiprep DNA purification system (Promega); the coding sequences of all inserts were confirmed by sequencing. Sequence reactions were performed by using a cycle-sequencing reaction with fluorescent dye terminators (Perkin-Elmer Applied Biosystems) and the reaction products were analysed by using an ABI 3100 (Perkin-Elmer Applied Biosystems) automatic sequencer. Sequence data were analysed with Sequencher software (Gene Codes Corporation).

**Cells.** Baby hamster kidney cells constitutively expressing T7 polymerase (BHK/rtT7 cells; Buchholz et al., 1999) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 10% tryptose phosphate broth (BD Biosciences), 400 μg G418 ml⁻¹, 4 mM L-glutamine, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ (Mediatech).

**Derivation of plasmids.** Plasmids encoding the N, P and L proteins of NiV and the NiV minigenome have been described previously (Bankamp et al., 2002, 2004; Halpin et al., 2004). hPIV3 plasmids used were a kind gift from Dr Rodriguez (2002). MV (Bankamp et al., 2005) minigenome replication assays.

**Protein expression detected by radioimmunoprecipitation.** BHK/rtT7 cells in 35 mm dishes were transfected with the desired plasmid constructs by using LT-1 transfection reagent (Mirus) according to the manufacturer’s instructions. Cells were labelled approximately 36 h post-transfection with [³⁵S]methionine in methionine-free medium (ICN) for a period of 2 h. Cells were lysed in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl (pH 8.0)] and each lysate supernatant was immunoprecipitated with 2.5 μl mouse antisera specifically recognizing the C, V or W proteins of NiV or rabbit antisera to the HA tag overnight at 4°C, with rotation. Precipitation using GammaBind G–Sepharose (Amersham Pharmacia Biotech) was then performed; protein complexes were separated by SDS-PAGE and bands were visualized by autoradiography.

**Replication assay and CAT ELISA.** BHK/rtT7 cells were transfected with the desired plasmid constructs in Opti-MEM medium (Invitrogen) by using LT-1 transfection reagent (Mirus) according to the manufacturer’s instructions. Total amounts of transfected DNA were kept constant by including additional empty-vector plasmid DNA where applicable. Details of the NiV minigenome replication assay have been described elsewhere (Halpin et al., 2004), in addition to each of these HA/C, HA/V or HA/W expression plasmids, plasmids expressing HA-tagged C, V and W deletion mutants, expressing either the amino terminus or the carboxyl terminus of each protein, were also generated by using the primers described. The template used for PCR amplification of the desired insert was pCDNA/HANiV/C, pCDNA/HANiV/V or pCDNA/HANiV/W. NiV/HANiV/Caa1–83 utilized primers HA/C/F and HA/C/R (5‘-GTACCTGAGCTCTTCTTTCTTCAAATGATCCAATGCT-3’) whilst NiV/HANiV/Caa84–166 utilized the primers HA/C/carboxyl/F (5‘-GATCGAGCTCCACCATGATCAATTACCGTCAATGATG-3’) and HA/C/R to generate the desired insert. NiV/unique/HA/VWaa1–220 was generated by using the primers HA/P/VW/W and HA/P/V/wamin/R (5‘-GTACCTCGAGTTGCTCTTCTTTAACTCCTAGTCG-3’) and HA/C/R to generate the desired insert. NiV/unique/HA/Va432–457 and NiV/unique/HA/Waa122–457 were generated by using a combination of the primers HA/P/VW/wamin/F (5‘-AGTGAGGAGCCCCACCATGATCAATTACCGTCAATGATG-3’) and HA/C/R.

**Micrococcal nuclease treatment of RNA extracts.** Transfected cells in 35 mm dishes were washed with PBS prior to the addition of 200 μl micrococcal nuclease buffer [0.5% sodium deoxycholate, 1% N-ethylmaleimide, 1% Triton X-100, 0.5% sodium citrate, 0.1% SDS and 0.1% sodium deoxycholate]. Cells were treated with micrococcal nuclease (10 U/ml) for 15 min at 37°C, stopped by adding 200 μl of RNase-free water, lysed with RIPA buffer and assayed for CAT activity as described previously (Bankamp et al., 2005).
Micrococcals nuclease-treated RNA that was additionally treated with DNase I (TURBO; Ambion), or poly(A)⁺-selected mRNA, was used in each 25 μl reaction volume. One-step RT-PCR was performed in duplicate reactions using reagents supplied in a TaqMan Gold RT-PCR kit (Applied Biosystems) as described previously (Hummel et al., 2006). Briefly, a reverse-transcription step at 48 °C for 30 min was followed by DNA-polymerase activation at 95 °C for 10 min and 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min. Negative-control reactions included samples run without the addition of template (NTC) or reverse transcriptase.

RNA samples were considered positive only if threshold cycle (Ct) values were <40. CAT RNA was not detected in the cell control, which contained transfection reagent only, or the negative-control reactions (Ct>40). The comparative Ct method (ΔΔCt) was used for the relative quantification of gene expression, using CAT as the target and normalized to the endogenous reference, RNase P (ΔΔCt = CtCAT - CtnRNaseP). Fold change (2⁻ΔΔCt) in CAT RNA was determined by subtracting the ΔΔCt value of the baseline condition, in which the NiV L plasmid was omitted from the replication assay, from each sample (ΔΔCt = ΔCt - ΔCtnRNaseP).

**RESULTS**

The C, V and W proteins of NiV inhibit NiV minigenome replication

To study the abilities of the C, V and W proteins of NiV to regulate viral transcription and/or replication, we used an existing NiV minigenome replication assay (Halpin et al., 2004). Plasmid DNA expressing NiV C, V or W was co-transfected with plasmid DNAs expressing the N, P and L proteins of NiV, in addition to the plasmid containing the NiV CAT minigenome. In the standard replication assay, expression of C from the plasmid DNA expressing P was silenced and the lack of expression of C from plasmids encoding NiV P, V and W was verified by immunoprecipitation (data not shown). In these experiments, the amount of CAT produced from the positive control, consisting of plasmid DNAs expressing the NiV minigenome and the N, P and L proteins, was set at 100 % and the total amount of plasmid DNA in each transfection was kept constant by the addition of empty-vector plasmids encoding NiV P, V and W. RNA samples were considered positive only if threshold cycle (Ct) values were <40. CAT RNA was not detected in the cell control, which contained transfection reagent only, or the negative-control reactions (Ct>40). The comparative Ct method (ΔΔCt) was used for the relative quantification of gene expression, using CAT as the target and normalized to the endogenous reference, RNase P (ΔΔCt = CtCAT - CtnRNaseP). Fold change (2⁻ΔΔCt) in CAT RNA was determined by subtracting the ΔΔCt value of the baseline condition, in which the NiV L plasmid was omitted from the replication assay, from each sample (ΔΔCt = ΔCt - ΔCtnRNaseP).

**Inhibition of NiV minigenome replication by V and W deletion mutants**

To identify which regions of the V and W proteins of NiV were required for the inhibitory effect on NiV minigenome replication, plasmids expressing amino- and carboxyl-terminal deletion mutants of each of the proteins were generated as described in Methods. Each of the deletion mutants was HA-tagged at the amino terminus to enable detection of protein expression. Full-length constructs expressing HA-tagged C, V

---

**Poly(A)⁺-selected mRNA purification.** An OligoDirect mRNA mini kit (Qiagen) was used according to the manufacturer’s recommendations. Final RNA pellets were resuspended in 10 μl RNase-free water and incubated at 55 °C for 10 min. A 30 μl volume of formaldehyde loading buffer (Ambion) was added to each sample and the samples were incubated at 68 °C for 10 min.

**Northern blot analysis.** RNA samples prepared as described above were analysed with RNA Millennium size markers (Ambion) on a 0.5% agarose formaldehyde gel. After electrophoresis at 100 V for 4 h in MOPS–EDTA acetate buffer (pH 7.5), the gel was transferred to a nylon membrane using a wet transfer apparatus in 10 × standard saline citrate (SSC) for 90 min and the membrane was rinsed briefly in 2 × SSC before being crosslinked using the AUTO setting on a UV Stratalinker 1800 (Stratagene). Prehybridization and hybridization to negative-sense probes were described previously (Bankamp et al., 2005). The blots were then equilibrated at room temperature in detection buffer (0.1 M Tris/ HCl (pH 9.5), 0.1 M NaCl) for 5 min prior to chemiluminescent detection with CDP-star (Roche). The membrane was then exposed to X-ray film for various exposure times.

**Real-time RT-PCR assays.** For real-time RT-PCR assays, forward (5’-GCCGCTGCGGCAATTAC-3’) and reverse (5’-TCATTAGCA TACAGGAGCAT-3’) primers were designed to span nt 555–620 of the CAT ORF. A TaqMan CAT gene probe (5’-TTTCATCATGGCCTTGTGATGGCTTCC-3’) was labelled at the 5’ terminus with a fluorescent reporter dye, 6-carboxyfluorescein (FAM), and at the 3’ terminus with a non-fluorescent quencher, black hole 1 (BHQ1). The CAT gene primers and probe were nearly identical to those described by Jeyaseelan et al. (2001) and were used at concentrations of 200 and 100 nM, respectively. A primer and probe set was also used to identify human RNase P (Hummel et al., 2006), a constitutively expressed housekeeping gene, to verify the integrity and quantity of RNA in the samples.
and W proteins were also generated to test whether the inclusion of the HA tag at the amino terminus of each of the proteins affected their function within the minigenome system. Expression of the NiV C, V and W full-length and truncated proteins was confirmed by immunofluorescence assay and immunoprecipitation (data not shown).

Each of the full-length HA-tagged C, V and W proteins inhibited CAT expression to approximately the same extent as the untagged versions of the full-length C, V and W proteins (Fig. 2). Plasmids expressing aa 1–220 of the 407 aa shared amino-terminal domain of V and W, or a protein containing the 52 aa unique carboxyl-terminal domain of V (aa 221–457), inhibited CAT expression from the NiV minigenome to approximately the same extent as the intact V and W proteins (Fig. 2). A plasmid expressing a protein containing the 47 aa unique carboxyl-terminal domain of W (aa 221–451) inhibited CAT expression by approximately 30 %, whilst the full-length W inhibited reporter gene expression by 80–90 % (Fig. 2). Deletion mutants that encoded the first 83 aa or aa 84–166 of the C protein were difficult to detect by immunoprecipitation. The minimal inhibitory effects that these deletion mutants had on CAT expression could have been a result of instability (data not shown).

Heterologous inhibition of paramyxovirus minigenome replication

Due to the lack of sequence similarity and conservation among the non-structural proteins from different genera of the family Paramyxoviridae, it was expected that the NiV C, V and W proteins would not inhibit the replication of other paramyxoviruses. To determine the specificity of inhibition of CAT expression from the NiV minigenome by NiV C, V and W proteins, the C and V proteins of MV were tested for their effects on NiV minigenome expression. Surprisingly, the C proteins from two strains of MV, CAM-70 (CAM) and Edmonston-Zagreb (ZAG), inhibited CAT expression from the NiV minigenome by 50 and 80 %, respectively, compared with 68 % inhibition by the NiV C protein, 78 % by the NiV V protein and 85 % inhibition by the NiV W protein (Fig. 3a). Furthermore, the V protein of CAM-70 also inhibited CAT expression by approximately 70 %. The F protein of the Edmonston strain of MV (MV F) had no inhibitory effect (Fig. 3a) and was included to demonstrate that inhibition was limited to viral accessory proteins. These data were unexpected, due to the high level of sequence divergence between the genera Henipavirus and Morbillivirus.
To investigate the heterologous inhibition of paramyxovirus minigenome replication further, minigenome replication assays for MV (Edmonston strain) and hPIV3 were used. The C, V and W proteins of NiV inhibited CAT expression from the MV minigenome by 30–50%, whilst MV C inhibited reporter-gene expression by 90% (Fig. 3b). It has already been shown that the C protein of hPIV3 inhibits hPIV3 minigenome replication (Malur et al., 2004; Smallwood & Moyer, 2004). The C, V and W proteins of NiV and the C protein of MV had no inhibitory effect on CAT expression from the hPIV3 minigenome (Fig. 3c). However, the C protein of hPIV3 inhibited expression from both the NiV and MV minigenomes by 90 and 70%, respectively, levels comparable to those of the homologous accessory proteins, MV C and NiV W (Fig. 4a, b). This suggests that there may be more than one mechanism by which the C, V and W proteins of paramyxoviruses regulate expression from the minigenome, as hPIV3 C was able to inhibit CAT expression from minigenomes representing all three genera tested with equal effect. In contrast, NiV C, V and W and the MV C and V proteins were more restricted in their inhibitory activities.

**NiV C, V and W inhibit both transcription and replication**

The inhibition observed in the NiV replication assay with the NiV accessory proteins was evaluated further to determine whether it correlated with a reduction in both CAT mRNA and encapsidated minigenome RNA populations. Northern blots showed that the amount of CAT mRNA produced by the NiV minigenome was consistently lower than the amount of RNA produced by the
minigenome of MV (Fig. 5a) and, in the presence of NiV C, V or W proteins, CAT mRNA was undetectable (Fig. 5b). Real-time RT-PCR confirmed that comparable amounts of cellular RNA were loaded in each lane in Fig. 5(b) (data not shown).

Real-time RT-PCR analysis was performed on the mRNA samples, as it is a more sensitive method than Northern blotting for detection of low levels of CAT RNA. The amount of transfected plasmid DNA was kept constant by the addition of plasmid vector. CAT protein concentration in cytoplasmic extracts was measured by ELISA and CAT expression in the absence of inhibiting proteins was set to 100%. Experiments were performed at least three times and results of a single representative experiment are shown. Error bars represent SD between duplicate samples.

DISCUSSION

It is widely acknowledged that the non-structural proteins encoded by many members of the family *Paramyxoviridae* play multiple roles in the viral life cycle, from regulating viral transcription and replication to modulating the host immune response. To determine whether the C, V and W proteins of the henipavirus NiV also possess the ability to regulate replication of the viral genome, a plasmid-based minigenome assay was used. A similar approach has been used to study the regulation of viral genome replication for multiple paramyxoviruses, including MV (Bankamp et al., 2002, 2005; Reutter et al., 2001; Sidhu et al., 1995), hPIV3 (Durbin et al., 1997; Malur et al., 2004; Smallwood & Moyer, 2004), Sendai virus (SeV) (Tapparel et al., 1997), simian virus 5 (SV5) (Lin et al., 2005), respiratory syncytial virus (Atreya et al., 1998), rinderpest virus (Brown et al., 2005), peste-des-petits-ruminants virus (Bailey et al., 2007), J-virus and Beilong virus (Magoffin et al., 2007).

We demonstrated that NiV C, V and W proteins inhibit CAT expression from the NiV minigenome in a dose-dependent manner. The synthesis of both micrococcal nuclease-resistant and poly(A)^+ RNA populations was decreased in the presence of the accessory proteins of NiV. It is important to note that, in the minigenome replication assays used for these studies, any decrease in replication may lead to a decrease in reporter-gene expression. Therefore, these assays cannot be used to distinguish between inhibition of transcription and inhibition of replication, because the inhibition of transcription could...
The observed inhibitory effect of NiV C on NiV minigenome replication is consistent with previously published observations for other paramyxoviruses. MV C has previously been shown to downregulate reporter-gene expression from minigenome constructs (Bankamp et al., 2005; Reutter et al., 2001). Like the C proteins of the respiroviruses hPIV3 (Smallwood & Moyer, 2004; Malur et al., 2004) and SeV (Curran et al., 1992; Grogan & Moyer, 2001; Kato et al., 2002, 2004), MV C inhibits viral RNA transcription and replication specifically (Bankamp et al., 2005). In the case of MV, the C protein binds the large (L) protein, the major subunit of the RNA-dependent RNA polymerase complex (Smallwood & Moyer, 2004), and for SeV, the C–L interaction is required for the inhibitory effect on viral RNA synthesis (Grogan & Moyer, 2001). It is possible that NiV C is acting in a similar manner by interacting with NiV L. Such a potential interaction warrants further investigation.

The V proteins of several paramyxoviruses play a role in regulating viral replication. Recombinant paramyxoviruses lacking V proteins, including rinderpest virus (Baron & Barrett, 2000), SeV (Kato et al., 1997b) and MV (Tober et al., 1998), produced increased levels of genomic RNA, mRNA and viral proteins. In addition, the V protein of SeV inhibited the replication of defective interfering particles (Curran et al., 1991; Horikami et al., 1996), and the V protein of SV5 inhibited viral transcription and replication (Lin et al., 2005). For a number of paramyxoviruses, it has been postulated that the mechanism by which V controls viral replication may be through a direct protein–protein interaction with the nucleocapsid (N) protein (Curran et al., 1991; Horikami et al., 1996; Lin et al., 2005; Svenda et al., 2002; Sweetman et al., 2001). However, recent studies with the V protein of MV discounted the possibility that a V–N interaction is responsible for the inhibitory effect (Witko et al., 2006). The ability of the V protein of MV to bind RNA correlated directly with the ability of MV V to inhibit minigenome replication (Parks et al., 2006), although the mechanism remains to be determined.

A recent publication (Witko et al., 2006) reported results similar to those from our study, as it identified two regions of the MV V protein, one at the amino terminus and one at the carboxyl terminus, that were independently capable of inhibiting CAT expression from the minigenome of MV. Furthermore, we observed that the first 220 aa acids of the shared amino-terminal domain of V/W and a protein containing the unique region of V produced levels of inhibition equivalent to those measured for full-length V and W, whilst a protein containing the unique carboxyl-terminal domain of W was only able to inhibit replication by 30%.

This is the first report of heterotypic inhibition of paramyxovirus minigenome replication by the accessory proteins C, V and W. Despite the lack of amino acid conservation between the C, V and W proteins of NiV, MV and hPIV3, the hPIV3 C protein was able to inhibit NiV and MV minigenome replication, although not to the same extent as the homologous proteins. The C, V and W proteins of NiV and MV inhibited both the homologous and heterologous replication assays, although the levels of heterologous inhibition were not as great as the levels of inhibition achieved with homologous proteins. Taken together, these findings suggest that these divergent viral proteins may share common functional properties. The mechanism(s) of the cross-genus inhibition is currently under investigation and it is likely that conserved host-cell proteins may be involved in a common inhibitory pathway.

ACKNOWLEDGEMENTS

We would like to thank Dr Sue Moyer (University of Florida, Gainesville, FL, USA) for the generous gift of the hPIV3 plasmids and Dr Brian Harcourt (Centers for Disease Control and Prevention, Atlanta, GA, USA) for plasmids expressing NiV C, V and W proteins. K.S. was the recipient of an ASM/NCID post-doctoral fellowship.

REFERENCES


