Determination of the henipavirus phosphoprotein gene mRNA editing frequencies and detection of the C, V and W proteins of Nipah virus in virus-infected cells

Michael K. Lo,1,2,4,5 Brian H. Harcourt,1 Bruce A. Mungall,3 Azaibi Tamin,1 Mark E. Peeples,4,5 William J. Bellini1 and Paul A. Rota1

Correspondence
Paul A. Rota
prota@cdc.gov

1Measles, Mumps, Rubella and Herpesviruses Laboratory Branch, 1600 Clifton Road, MS-C-22, Atlanta, GA 30333, USA
2Emory University School of Medicine, Department of Microbiology and Immunology, 1510 Clifton Road, Atlanta, GA 30322, USA
3Commonwealth Scientific Industrial Research Organization, Australian Animal Health Laboratory, 5 Portarlington Road, East Geelong, Victoria, Australia
4The Research Institute at Nationwide Children’s Hospital, Center for Vaccines and Immunity, 700 Children’s Drive, Columbus, OH 43205, USA
5The Ohio State University, College of Medicine, Department of Pediatrics, Columbus, OH 43205, USA

Received 12 September 2008
Accepted 13 October 2008

The henipaviruses, Nipah virus (NiV) and Hendra virus (HeV), are highly pathogenic zoonotic paramyxoviruses. Like many other paramyxoviruses, henipaviruses employ a process of co-transcriptional mRNA editing during transcription of the phosphoprotein (P) gene to generate additional mRNAs encoding the V and W proteins. The C protein is translated from the P mRNA, but in an alternate reading frame. Sequence analysis of multiple, cloned mRNAs showed that the mRNA editing frequencies of the P genes of the henipaviruses are higher than those reported for other paramyxoviruses. Antisera to synthetic peptides from the P, V, W and C proteins of NiV were generated to study their expression in infected cells. All proteins were detected in both infected cells and purified virions. In infected cells, the W protein was detected in the nucleus while P, V and C were found in the cytoplasm.

Nipah virus (NiV) and Hendra virus (HeV) are paramyxoviruses in the genus Henipavirus, the subfamily Paramyxovirinae, within the family Paramyxoviridae. HeV causes febrile respiratory illness in humans and animals; of all the febrile illnesses caused by HeV in Australia from 1994 to 2007, there were 17 equine deaths and two human fatalities out of three cases (Hanna et al., 2006; Hooper & Williamson, 2000; Murray et al., 1995a, b). The first human NiV infections were detected during an outbreak of severe febrile encephalitis in peninsular Malaysia and Singapore from autumn 1998 to spring 1999 (Chua et al., 2000). NiV has subsequently been established as the cause of fatal human encephalitis in Bangladesh in 2001, 2003–2005, 2007 and 2008 (Banerjee, 2007; Hsu et al., 2004) as well as in India in 2001 and 2007 (Chadha et al., 2006).

Fruit-eating bats of the genus Pteropus are a natural reservoir for NiV and HeV (Chua et al., 2002; Halpin et al., 2000; Yob et al., 2001). Humans are infected by exposure to infected fruit bats or material contaminated by infected bats (Hsu et al., 2004), but are also infected via intermediate hosts such as pigs (Amal et al., 2000; Chew et al., 2000; Paton et al., 1999) or by direct human-to-human contact (Gurley et al., 2007). No vaccines or specific antiviral drugs are currently available for NiV. Although patients from the Malaysian outbreak who received ribavirin showed a lower mortality rate (Chong et al., 2001), ribavirin was unable to protect NiV-infected hamsters from fatal disease (Georges-Courbot et al., 2006).

The genomes of NiV and HeV are 18,246 and 18,234 nt, respectively, making them significantly larger than most other paramyxoviruses, with the exceptions of rodent-borne Beilong and J viruses (Jack et al., 2005; Li et al., 2006). The N, P and L proteins are required and sufficient for mini-genome replication, similar to other members of the subfamily Paramyxovirinae (Halpin et al., 2004). The P genes of the subfamily Paramyxovirinae, which are 2,704 nt
for NiV and 2.698 nt for HeV (Supplementary Fig. S1, available in JGV Online), contain several open reading frames (ORFs) in addition to the P ORF. Like most other paramyxovirinae, henipaviruses have a UC-rich region that acts as an editing site and facilitates the non-templated addition of G residues into P gene mRNA transcripts. The additional G residues allow access to the reading frames of V and W proteins which share amino termini with P, but have unique carboxy termini (Fig. 1a) (Kolakofsky et al., 2005). The C ORF, which is embedded in the P gene and is translated via an alternative translational start site, is also in the P gene of both HeV and NiV. In this study, we present the initial characterization of the editing frequencies of the P genes of the henipaviruses and the first demonstration of the expression of these additional P gene products during NiV infection.

To determine the mRNA editing frequency of the P genes of the henipaviruses, RT-PCR was performed on poly-A-plus mRNA purified from infected cell lysates at 24 h to amplify the P gene mRNAs of HeV, and the Maylasian (NiV-M) and Bangladesh (NiV-B) strains of NiV (the full methods used to obtain all the data described here are available in JGV Online). The PCR products were separately cloned into the plasmid cloning vector, pCR2.1 TA; approximately 100 plasmid clones were isolated and the editing site for each PCR product was sequenced. When similar techniques were used to analyse the P gene transcripts of other paramyxoviruses such as measles virus (MeV), two-thirds of the mRNAs transcribed from the P gene were not edited and approximately 90% of edited mRNAs had a single non-templated G residue added (Bankamp et al., 2008; Vanchiere et al., 1995). In contrast, the majority of henipavirus transcripts were edited; 67% in NiV-M, 66% in NiV-B and 71% in HeV (Fig. 1b). Therefore, the henipaviruses produced edited transcripts at twice the frequency of other viruses (Bankamp et al., 2008; Hausmann et al., 1999; Kato et al., 1997; Mebatsion et al., 2003; Vanchiere et al., 1995), with the exception of human parainfluenza virus 3, which edits approximately half of its P gene mRNA transcripts (Galinski et al., 1992). Though it is not known how the addition of a GGG triplet encoding a glycine residue or multiple glycine residues in any of these proteins might alter their function, we assumed that transcripts with an additional three, six or nine G residues would encode a functional P protein. Similarly, transcripts with four, seven or ten G residues would encode a functional V protein, and those with 5, 8, 11 or 14 G residues would encode a functional W protein. Given these assumptions, 50% of all henipavirus P gene mRNA transcripts would encode the P protein, 25% would encode the V protein and 25% would encode the W protein (Fig. 1c).

We generated polyclonal mouse anti-peptide antisera against the unique regions of P, C, V and W to individually detect each protein (Fig. 1a). Radioimmunoprecipitations (RIPs) of transfected 293 cells individually expressing the P, C, V or W proteins confirmed that the antisera made against each of these proteins recognized the correct protein (Fig. 2a). The C protein, predicted to be 19.7 kDa, migrated at an apparent molecular mass of 20 kDa. The V and W proteins migrated at an apparent molecular mass of 55 kDa, slightly larger than the predicted 50.6 kDa. This was also observed for the P protein, which is predicted to be 78.3 kDa, but migrated at an apparent molecular mass of 80–85 kDa (Shiell et al., 2003). RIPs of lysates from Vero cells transfected with the P-, V- or W-expressing plasmids precipitated with PVW antisera that recognizes the shared N-terminal region of the P protein (Fig. 2a, right-hand three lanes) confirmed that the migration of each protein was similar to that shown in 293 cells (Fig. 2a).

To confirm the presence of NiV P, C, V and W in NiV-infected cells, Western blots were performed on NiV-infected and HeV-infected Vero cells (Fig. 2b). The antisera against NiV V and W also detected the corresponding V and W proteins of HeV, though the bands for HeV V and W were less intense than the bands for NiV V and W. The antisera to NiV C detected a protein with an apparent molecular mass of 16 kDa, smaller than the expected size of C, that did not react with the HeV-infected lysate. Upon further investigation using Western blot and RIPs, we found that denatured samples of plasmid-expressed NiV C migrate as a doublet of approximately 19 and 16 kDa, and that the protein detected by NiV C antisera in NiV-infected lysates co-migrated with the 16 kDa band (Supplementary Fig. S2, available in JGV Online). The antisera to NiV P did not detect a protein migrating at the expected size of HeV P, although the band detected in the HeV-infected lysates could be a degradation product of HeV P (Fig. 2b). None of the antisera recognized proteins in the mock-infected cell lysates.

Sucrose gradient-purified NiV virions were also examined by Western blot to determine which of the P, C, V and W proteins were present. All of these proteins were detected and each protein migrated with the same apparent molecular mass as the proteins detected in lysates of infected cells (Fig. 2c). Compared with the Western blots of infected cell lysates (Fig. 2b), the P protein band detected in the purified NiV was markedly more abundant than the C, V and W bands, and putative degradation products running below each specific band were not present (Fig. 2c). The antisera to NiV C detected a small amount of an additional protein which had an electrophoretic mobility similar to that of NiV P. This may indicate that C associates with P in virions. Incorporation of V or C proteins into virions is not universal among viruses in the subfamily Paramyxovirinae. While the presence of V protein has been demonstrated in the virions of simian virus 5, mumps virus and HeV, it has not been detected in MeV or Sendai virus (SeV) (Curran et al., 1991; Kato et al., 1997; Paterson et al., 1995; Shiell et al., 2003; Takeuchi et al., 1990). However, the C protein has been detected in MeV and SeV virions (Devaux & Cattaneo, 2004; Yamada et al., 1990).
To determine the subcellular location of NiV P, V, W and C in NiV-infected cells, immunofluorescence assays were performed using the respective anti-peptide antisera (Fig. 3). The P protein was detected throughout the cell and did not co-localize with the nucleus, but appeared to be concentrated at the plasma membrane. The speckled distribution of the P protein within the cytoplasm of NiV-infected cells and its accumulation near the plasma membrane is in contrast with the homogeneous cytoplasmic distribution shown when the P ORF was expressed individually in mammalian cells (Shaw et al., 2004). As expected, the distribution of the NiV N protein was similar to that of NiV P, as the interacting domains of the NiV N and P proteins have been mapped (Chan et al., 2004). The C protein was detected throughout the cytoplasm in a punctate pattern and was visibly excluded from the nucleus. The V protein was also found exclusively in the cytoplasm, but the distribution was more uniform than...
that of C, resulting in a pronounced boundary between the nucleus and the cytoplasm. The W protein was detected exclusively in the nucleus and was distributed evenly throughout, with the exception of the nucleoli. The cellular distributions of V and W proteins were consistent with previous studies in which the V and W ORFs were individually expressed via plasmids (Rodriguez & Horvath, 2004; Rodriguez et al., 2002; Shaw et al., 2004, 2005).

The C, V and W proteins of NiV inhibit both transcription and replication in a NiV minigenome assay (Sleeman et al., 2008). The distinct cellular distributions of each protein shown in this study imply that the inhibitory effects of these proteins utilize different mechanisms. In cells infected with MeV, a specific interaction between C and L proteins is required in order to inhibit viral RNA transcription and replication, while the inhibition mediated by the V protein correlates specifically with its ability to bind RNA (Bankamp et al., 2005; Grogan & Moyer, 2001; Parks et al., 2006; Smallwood & Moyer, 2004; Witko et al., 2006). Since the W protein localizes to the nucleus, the mechanism behind its inhibition of replication is likely to be different from that of C and V, as it is unlikely that it interacts with the cytoplasmic viral polymerase.

Fig. 2. Detection of NiV P, C, V and W proteins with anti-peptide sera. (a) 293 cells transfected with NiV P, C, V, W or empty vector plasmid (M) were labelled with [35S]methionine for 2 h; these were immunoprecipitated with antiserum against each respective NiV protein and electrophoresed on a 12% SDS-PAGE gel. Letters below the gel indicate the gene expressed in the cell lysate for each well and letters above the gel indicate the specific antiserum used for the immunoprecipitation. In the three far right lanes, radiolabelled Vero cell lysates expressing P, V and W were immunoprecipitated with the PVW peptide antiserum to confirm their electrophoretic mobilities. (b) NiV- or HeV-infected (m.o.i. of 1) Vero cells were examined by Western blot. Cell lysates (from 24 h infection) were run on a 4–12% gradient SDS-PAGE gel. The antiserum used to probe each protein is indicated above the blots. Odd-numbered lanes indicate samples from NiV-infected lysates and even-numbered lanes are from HeV-infected lysates. M, Mock-infected lysates. Well loading control blots against cellular β-actin are shown below the main blot. (c) Western blots were performed on sucrose-gradient purified NiV as described in (b).
A number of plasmid expression studies have demonstrated various interactions between NiV P, V and W proteins with certain host cell proteins including STAT-1 (P, V and W), mda-5 (V) and karyopherin a (KPNA) 3 and 4 (W). These interactions were all linked to the ability of the P, V and W proteins to block interferon (IFN) signalling and/or IFN induction (Childs et al., 2007; Rodriguez et al., 2002, 2003; Shaw et al., 2004, 2005). PLK-1, another host protein, has been shown recently to interact with the V and W proteins independently of the STAT-1 protein interaction (Ludlow et al., 2008). While it was demonstrated that PLK-1 binding did not affect NiV minigenome replication, the implications of this interaction have yet to be determined in NiV infection.

This is the first study in which the expression and cellular distribution of the NiV C protein has been characterized in NiV-infected cells. Plasmid-expressed NiV C rescued an IFN-sensitive Newcastle disease virus, but the mechanism behind this effect remains undefined (Park et al., 2003). Studies to identify host proteins that interact with C merit further investigation. The C proteins of paramyxoviruses inhibit antiviral responses and serve as virulence factors (Devaux & Cattaneo, 2004; Devaux et al., 2008; Escoffier et al., 1999; Garcin et al., 2001; Gotoh et al., 2001; Nakatsu et al., 2006, 2008; Takeuchi et al., 2005). This study is also the first demonstration of NiV W expression and localization in infected cells, and we have thus far confirmed the interaction between W and KPNA3 in NiV-infected cells by co-immunoprecipitation (Supplementary Fig. S3, available in JGV Online). With the availability of a full-length NiV reverse genetics system, further investigation of the roles of C, V and W in viral replication and pathogenesis is now possible (Yoneda et al., 2006). A report indicating that NiV can cause immunosuppression in pigs suggests that dissecting the impact of the C, V and W proteins during NiV infection in vivo will be key to understanding the molecular mechanisms of pathogenesis (Berhane et al., 2008).

Acknowledgements

We would like to thank Dr Glenn Marsh, Dr Tony Sanchez, Dr Brian Shiell, Dr Lin-fa Wang, Mohammed Aljofan, Hayley Snelling and Elena Virtue for helpful discussions and technical assistance. We thank Dr Thomas Ksiazek and Dr Pierre Rollin for critical reading of the manuscript. M. K. L. was supported by an NIH grant (AI069014) to M. E. P.

References


