Virology 497 (2016) 294–304

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Region of Nipah virus C protein responsible for shuttling between the cytoplasm and nucleus

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ARTICLE INFO

Article history:
Received 15 April 2016
Returned to author for revisions
12 July 2016
Accepted 12 July 2016

Keywords:
Nipah virus
C protein
Subcellular localization
Nuclear localization signal
Nuclear export signal

ABSTRACT

Nipah virus (NiV) causes severe encephalitis in humans, with high mortality. NiV nonstructural C protein (NiV-C) is essential for its pathogenicity, but its functions are unclear. In this study, we focused on NiV-C trafficking in cells and found that it localizes predominantly in the cytoplasm but partly in the nucleus. An analysis of NiV-C mutants showed that amino acids 2, 21–24 and 110–139 of NiV-C are important for its localization in the cytoplasm. Inhibitor treatment indicates that the nuclear export determinant is not a classical CRM1-dependent nuclear export signal. We also determined that amino acids 60–75 and 72–75 were important for nuclear localization of NiV-C. Furthermore, NiV-C mutants that had lost their capacity for nuclear localization inhibited the interferon (IFN) response more strongly than complete NiV-C. These results indicate that the IFN-antagonist activity of NiV-C occurs in the cytoplasm.

1. Introduction

Nipah virus (NiV) is a highly pathogenic zoonotic paramyxovirus in the genus *Henipavirus* within the family *Paramyxoviridae* (Mayo, 2002), which first emerged in Malaysia and Singapore in 1998–1999 (Chua et al., 1999; Paton et al., 1999). During this outbreak, NiV caused severe febrile encephalitis in humans, with about 40% mortality, and it was suggested that the infection was transmitted from pigs with respiratory symptoms (Chua et al., 2000a, 2000b). In Malaysia, the NiV-infected patients were almost all pig handlers, and no person-to-person transmission was evident at the time (Goh et al., 2000). NiV has since emerged in Bangladesh in 2001 and 2003 and in India in 2001 (Chadha et al., 2006; Hsu et al., 2004). The mortality rate during the outbreaks in Bangladesh reached 75%, which was higher than that observed in the initial Malaysian outbreak. In the new outbreaks, no pigs were involved in disease transmission, so it was inferred that the initial victims had direct contact with fruit bats, after which NiV was spread from person to person (Gurley et al., 2007). Recently, some evidence for the existence of Henipavirus in African bats and domesticated pigs has been reported (Drexler et al., 2009, 2012; Hayman et al., 2008, 2011; Iehlé et al., 2007; Peel et al., 2012, 2013; Pernet et al., 2014; Weiss et al., 2012). These findings raise concerns about outbreaks of Henipavirus in Africa.

The NiV genome is a negative-sense nonsegmented RNA, of 18,246 nucleotides (nt; Malaysian isolate) or 18,252 nt (Bangladesh isolate) (Harcourt et al., 2005, 2001). Like other paramyxoviruses, the genome contains six transcriptional units encoding six structural proteins: nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G), and polymerase (L), and three nonstructural proteins, C, V, and W (Harcourt et al., 2000). The NiV C protein (NiV-C) is expressed from an alternative open reading frame at the 5′ end of the P gene and shares no sequence similarity with the P protein, whereas the V and W proteins share the same N-terminal domain with the P protein, because they are translated from the edited P mRNA when one or two nontemplate G residues are inserted into the highly conserved RNA editing site of the P gene. It has been reported that each NiV P gene product, particularly proteins V, W, and C, has interferon (IFN)-antagonist activity (Park et al., 2003; Shaw et al., 2004). The host IFN responses induced by viral infection are involved in the early innate immune response and in the modulation of the subsequent acquired immune response. The common N-terminal domain of the NiV V and W proteins has IFN-antagonist activity (Park et al., 2003). The V protein inhibits the IFN-induced nuclear translocation and phosphorylation of signal transducer and activator of transcription (STAT) (Rodriguez et al., 2002; Shaw et al., 2004). The W protein binds STAT1 after it enters the nucleus and prevents it shuttling back to the cytoplasm (Shaw et al., 2004). In this way, the differential localization of V and W in the cytoplasm and nucleus determines the IFN-antagonist

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http://dx.doi.org/10.1016/j.virol.2016.07.013
0042-6822/

mechanism of each protein. The NiV V and W proteins also inhibit IFN synthesis (Shaw et al., 2005). The V proteins of a number of paramyxoviruses, including NiV, interact with the helicase domain of MDA5, inhibiting its ATPase activity (Childs et al., 2009; Motz et al., 2013; Rodriguez and Horvath, 2013). Unlike the V and W proteins, the mechanism of the IFN-antagonist activity of NiV-C is unknown.

We previously established a reverse genetics system for NiV that retains its severe pathogenicity in a golden hamster model (Yoneda et al., 2006). A study using recombinant NiV lacking the C protein revealed that the C protein plays a key role in the pathogenicity of NiV (Yoneda et al., 2010). Apart from its IFN-antagonist activity, NiV-C regulates the synthesis of viral RNA and inhibits the early host antiviral response (Lo et al., 2012; Mathieu et al., 2012). Although several activities have been reported, the mechanisms of NiV-C action are still unclear.

In other members of the Paramyxovirus, the Rinderpest virus (RPV) C protein localizes in the nucleus (Boxer et al., 2009), the Sendai virus (SeV) C protein localizes in the cytoplasm (Portner et al., 1986), and the Measles virus (MV) C protein shuttles between the nucleus and cytoplasm (Nishie et al., 2007). Therefore, each C protein of the Paramyxovirus displays a different cellular localization pattern, which should relate to their functions. In this study, we focused on the subcellular localization of NiV-C and the residues responsible for its shuttling to clarify the mechanisms of its actions.

2. Materials and methods

2.1. Cell culture

HEK293 cells were propagated in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum (JRH Biosciences), 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco) at 37 °C in 5% CO_2.

2.2. Plasmid construction

To construct pCMV-myc–NiV-C, encoding a c-Myc-tagged NiV-C protein, the C gene open reading frame was amplified with PCR using primers containing the NotI site and Thermo-Start Taq DNA Polymerase (ABgene). The PCR product was digested with NotI and cloned into the pCMV–myc vector (BD Bioscience). To construct pEGFPN1–NiV-C encoding the NiV-C protein upstream from enhanced green fluorescent protein (EGFP), the C gene open reading frame was amplified with PCR using primers NiV-C KpnI F (5′-ATAGGTACCGCCACCATGATGGCCTCAATATTATTGAC-3′) and NiV-C ApaI R (5′-TATGGGCCCCGATCAGTTTCCCGATCTTCT-3′) and Phusion® DNA polymerase (Finzymes). The PCR fragment was digested with KpnI and ApaI, and cloned into the pEGFPN1 vector (Clontech). To construct pEGFPC2 NiV-C, encoding EGFP upstream from the NiV-C protein, the C gene open reading frame was amplified with PCR using NiV-C BglII F (5′-ATAAGATCTCGATGATGGCCTCAATATTAT-3′) and NiV-C EcoRI R (5′-TATGAATTCCTAGATCAGTTTCCCGATCTT-3′) and Phusion® DNA polymerase. The PCR fragment was digested with BglII and EcoRI and cloned into the pEGFPC2 vector (Clontech).

2.3. Site-directed mutagenesis

PCR-based site-directed mutagenesis was used to generate deletions and substitutions in pCMV-myc-NiV-C, pEGFPN1-NiV-C and pEGFPC2-NiV-C. The genes encoded in these plasmids were amplified with PCR and primer pairs containing specific mutations using *Pfu* DNA polymerase (Promega). The PCR fragments were digested with DpnI (Toyobo) and *Escherichia coli* was transformed with the plasmids. The mutated plasmid DNA was extracted from *E. coli* with the Wizard Plus SV Minipreps DNA Purification System (Promega). The introduced mutations were confirmed with vector-specific primers.

2.4. Immunofluorescence analysis

For transfection, cells were plated and grown to 70% confluence on coverslips in 24-well cell culture plates (Corning). HEK293 cells were transfected with the expression plasmids using Lipofectamine LTX (Invitrogen). At 24 h after transfection, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, permeabilized with 0.5% Triton X-100 for 30 min, and blocked with 3% bovine serum albumin in PBS for 30 min at room temperature. C-Myc-tagged NiV-C was detected with a mouse anti-c-Myc monoclonal antibody (Clontech) and a goat anti-mouse immunoglobulin antibody conjugated with Alexa Fluor 488 (Molecular Probes). The coverslips were mounted on glass slides using fluorescent mounting medium (Dako) and immunofluorescence was observed under a confocal fluorescence microscope (Olympus).

2.5. Leptomycin B assay

HEK293 cells were transfected with the EGFP, hPKIα (human cAMP-dependent protein kinase inhibitor α)–EGFP, or NiV-C–EGFP expression plasmid. At 20 h after transfection, the medium was replaced with fresh culture medium containing 10 ng/ml leptomycin B (Sigma) and incubated for another 2 h. Leptomycin B was prepared as a stock solution in 70% ethanol and stored at −20 °C. After incubation, the cells were fixed with 4% paraformaldehyde and the proteins were visualized with confocal microscopy.

2.6. Reporter assay

Assays were performed with HEK293 cells cultured in 24-well plates using the Dual Luciferase Assay System (Promega). The HEK293 cells were transfected with NiV-C- or mutant-expressing plasmid, a plasmid containing the luciferase gene downstream from an IFN-stimulated response element (pISRE–Luc), and a construct encoding the *Renilla* luciferase protein (pRL-CMV). All transfections were performed with Lipofectamine LTX, according to the manufacturer's instructions. The medium was replaced with medium containing 1000 IU of IFNα at 24 h posttransfection. The luciferase assays were performed in triplicate 8 h after stimulation with IFNα.

2.7. Immunoblotting

The whole-cell lysates were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrically transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% skim milk dissolved in Tris-buffered saline and 0.05% Tween-20, and then probed with mouse antibodies directed against c-Myc (1:600) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000). The blots were developed with horseradish-peroxidase-conjugated secondary antibodies and visualized with ECL Western Blotting Detection Reagent (GE Healthcare). Chemiluminescence was scanned with a luminescent image analyzer (LAS-1000 UV Minisystem; Fujifilm).

2.8. Statistical analysis

EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (Kanda, 2013) was used to calculate statistical significance