Virology 518 (2018) 64–76

Contents lists available at ScienceDirect

Virology

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

An alanine residue in human parainfluenza virus type 3 phosphoprotein is critical for restricting excessive N^0-P interaction and maintaining N solubility

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ARTICLE INFO

Keywords:
Human parainfluenza virus
Phosphoprotein
Inclusion boides

ABSTRACT

The phosphoprotein (P) of human parainfluenza virus type 3 (HPIV3) plays a pivotal role in viral RNA synthesis, which interacts with the nucleoprotein (N) to form a soluble N^0-P complex (N^0, free of RNAs) to prevent the nonspecific RNA binding and illegitimate aggregation of N. Functional regions within P have been studied intensively. However, the precise site (s) within P directly involved in N^0-P interaction still remains unclear. In this study, using a series of deleted and truncated mutants of P of HPIV3, we demonstrate that amino-terminal 40 amino acids (aa) of P restrict and regulate N^0-P interaction. Furthermore, using in vivo HPIV3 minigenome replicon assay, we identify a critical P mutant (P_{A28P}) located in amino-terminal 40 aa, which fails to support RNA synthesis of HPIV3 minigenome replicon. Although P_{A28P} maintains an enhanced N-P interaction, it is unable to form N^0-P complex and keep N soluble, thus, resulting in aggregation and functional abolishment of N-P complex. Moreover, we found that recombinant HPIV3 with mutation of A28P in P failed to be rescued. Taken together, we identified a residue within the extreme amino-terminus of P, which plays a critical role in restricting the excessively N-P interaction and keeping a functional N^0-P complex formation.

1. Introduction

Human parainfluenza virus type 3 (HPIV3), an airborne pathogen, belongs to the *Respirovirus* genus of the *Paramyxoviridae* family, which is a significant cause of lower respiratory illness in newborns and infants (Moscona, 2005). No effective antiviral therapy or vaccine for HPIV3 is currently available. Therefore, investigation of its replication mechanism could facilitate the development of novel therapeutic approaches for HPIV3 infection. The genome of HPIV3 is a nonsegmented, negative-strand RNA with 15,462 nucleotides encapsidated by the nucleoprotein (N; 68 kDa) to form the N-RNA template (Banerjee et al., 1990). The N-RNA template associates with virus-encoded phosphoprotein (P; 90 kDa) and a large polymerase protein (L; 255 kDa) to form an active ribonucleoprotein (RNP) complex, which is the central unit for viral transcription and replication (Banerjee, 1987; Banerjee et al., 1990). The RNP generates six capped and polyadenylated mRNAs encoding the viral structural proteins and an antigenome intermediate (Banerjee et al., 1990). Besides encoding P, the mRNA of the P gene also encodes several accessory proteins, such as the C protein, the D protein, and possibly the V protein, by using an alternative open reading frame (Luk et al., 1986). All three proteins encoded by the P gene may play a role in interferon escape (Durbin et al., 1999; Fontana et al., 2008).

Like the Ns of many other nonsegmented negative-strand RNA viruses (NNSVs), the N of HPIV3 is composed of two main parts: a highly conserved N-terminal moiety (about 80% of the sequence) designated N_{core} and a hypervariable disordered C-terminal moiety (about 20% of the sequence) designated N_{tail} (Longhi et al., 2003). N_{core} comprises a multimerization domain for N self-assembly and an RNA-binding domain to form the N-RNA template (Buchholz et al., 1994; Myers et al., 1997). The disordered N_{tail} is required for the association of P and the N-RNA template (Liston et al., 1997; Longhi et al., 2003; Zhang et al., 2002). When N is expressed alone, it forms two types of N: N^0 (free of RNA) and N-RNA. Both types of N interact with P. Encapsidation of RNA is an intrinsic property of N, but N cannot distinguish viral RNAs from cellular RNAs. The N^0-P interaction forms a soluble N^0-P complex that prevents the aggregation of N and the nonspecific encapsidation of cellular RNAs (Curran et al., 1995b), resulting in specific association of N with viral genome RNA, which serves as a template for transcription and replication (Mellon and Emerson, 1978; Whelan et al., 2004). P is an indispensable cofactor of the viral

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https://doi.org/10.1016/j.virol.2018.02.008
Received 10 October 2017; Received in revised form 9 January 2018; Accepted 6 February 2018

RNA polymerase L and plays a central role in viral replication. The L cannot bind to the N-RNA template to initiate the synthesis of RNA by itself. P acts as a bridge to bring the L protein to the N-RNA template and initiate viral RNA synthesis (Horikami and Moyer, 1995). Moreover, P of HPIV3 interacts with the matrix protein (Zhang et al., 2015) and can be packed into the virus-like particles of HPIV3by the matrix protein (Zhang et al., 2014). Ps of NNSVs are also organized into two moieties: a highly variable amino-terminal domain and a conserved carboxyl-terminal domain (Leyrat et al., 2010). Several previous studies have provided insight into the functional domains in P. The roles of the amino- and carboxyl-termini of P have been carefully studied and the variable disordered amino-terminus of P also contains conserved motifs, such as the soyuz1 motif and soyuz2 motif (De et al., 2000; Karlin and Belshaw, 2012). The soyuz1 motif within the first 40 aa of P is predicted to be the site for N^0-P binding, and the soyuz2 motif may play an important role in blocking the interferon pathway (De et al., 2000; Karlin and Belshaw, 2012). However, the precise site (s) required for the N^0-P interaction in HPIV3 should be further investigated. The carboxyl-terminus of P comprises an L-P binding domain and an N-RNA-P binding domain, as well as a domain responsible for the oligomerization of P (Choudhary et al., 2002; Gerard et al., 2009; Ribeiro et al., 2008). And the extreme carboxyl-terminal 20 aa of P are required for the interaction of P with the N-RNA template (De et al., 2000). A previous study by Choudhary et al. indicated that the oligomerization of P occurred via a coiled-coil motif (Choudhary et al., 2002), and the oligomerization of P was likely involved in the interaction of P with the RNA polymerase complex to synthesize RNA during transcription and replication. The oligomerization domain was mapped between aa 423 and 457 of HPIV3 P (Choudhary et al., 2002).

Our previous studies showed that the interaction of HPIV3 N and P provides the minimal requirement for the formation of inclusion bodies (IBs), which are viral replication factories (Zhang et al., 2013), and acetylated α-tubulin enhances viral replication by regulating IB fusion (Zhang et al., 2016). Disruption of N-P interaction by deletion of the carboxyl-terminal of P definitely abolished the HPIV3 IB formation (Zhang et al., 2013). However, (i) whether the N^0-P interaction is also critical for HPIV3 IB formation and (ii) the precise site(s) within the amino-terminus of P that contributes to the N^0-P interaction remain unclear.

Here, we identified a critical residue within the amino-terminus of P that is indispensable for N^0-P interaction, IB formation, and virial RNA synthesis.

2. Materials and methods

2.1. Cells and virus

HeLa and LLC-MK2 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 8% fetal bovine serum (FBS) (PAN-Biotech GmbH). 293T cells were maintained in DMEM supplemented with 20% FBS. 293T-T7 cells were cultured in DMEM containing 20% FBS and 1 μg/ml puromycin. HPIV3 (NIH 47885) and recombinant HPIV3 were propagated in LLC-MK2 cells via inoculation at a multiplicity of infection (MOI) of 0.1. Recombinant vaccinia virus (vTF7-3) expressing bacteriophage T7 RNA polymerase was propagated in HeLa cells at an MOI of 0.1.

2.2. Plasmid constructs

The plasmids pGEM4-N, pGEM4-P, pGEM4-L, pOCUS-HPIV3-MG, and pOCUS-HPIV3, which encode untagged N, P, L, HPIV3 minigenome, and HPIV3 genome, respectively, have been described previously (Hoffman and Banerjee, 2000), as were the plasmids pcDNA3.0-N and pCAGGS-N_{L478A} (Zhang et al., 2013). cDNA encoding the wild-type of P and its mutants were amplified via polymerase chain reaction (PCR)-based cloning techniques with pOCUS-HPIV3 as the template. PCR products were cloned into pCAGGS and pGADT7, GST-fused PN40 was amplified with pGEX-6P3 and pOCUS-HPIV3 as templates. The resultant PCR products were mixed as templates to amplify the cDNA encoding the GST-fused PN40. The re-amplified GST-fused PN40 PCR product was cloned into pCAGGS and pGADT7. The PCR-based approach was also applied to construct the point mutation in the HPIV3 genome. PCR products of the mutational HPIV3 genome (HPIV3-P_{A28P}, HPIV3-P_{A28K}, HPIV3-P_{A28E}, HPIV3-P_{A28F}, HPIV3-P_{A28N}, HPIV3-P_{A28G}, and HPIV3-P_{A28S}) were cloned into pOCUS-HPIV3 and digested with *Sal*I and Eco91I via ligase-independent cloning. All constructs were verified by DNA sequencing.

2.3. In vivo HPIV3 minigenome replicon assay

The in vivo HPIV3 minigenome assay was performed as described previously (Hoffman and Banerjee, 2000) with minor modifications. HeLa cells in 12-well plates grown to 90% confluence were infected with vTF7-3 at an MOI of 1. One hour post-infection, pGADT7-P (62.5 ng) or P mutants were transfected in the presence of pcDNA3.0-N (125 ng), pGEM4-L (100 ng), and a plasmid encoding the HPIV3 minigenome (50 ng) by Lipofectamine 2000 (Invitrogen). To determine whether P_{A28P} has a dominant negative effect on the RNA synthesis function of wild-type P, we transfected increasing amounts of P_{A28P} or wild-type P (15.625 ng, 31.25 ng, 62.5 ng, and 125 ng) with pcDNA3.0-N (125 ng), pGADT7-P (31.25 ng), pGEM4-L (100 ng), and HPIV3 minigenome (50 ng). The transfection medium was replaced with DMEM containing 4% FBS 5 h later. At 24 h post-transfection, the cells were harvested and lysed in 150 μl lysis buffer. We used 20 μl aliquots to assess luciferase activity according to the manufacturer's instructions. All assays were repeated at least three times for accuracy.

2.4. Coimmunoprecipitation assay

Appropriate plasmids were transfected into 293T cells via calcium phosphate transfection. To detect the association of P mutants and N^0, Myc-tagged N_{L478A} and HA-tagged P mutants or Myc-tagged N_{L478A} and wild-type P were transfected into 293T cells. At 48 h post-transfection, cells were harvested and lysed with 800 μl lysis buffer (50 mM Tris-HCl [pH, 7.4], 150 mM NaCl, 1% [wt/vol] Triton X-100, 1 mM EDTA [pH, 8.0], 0.1% [vol/vol] sodium dodecyl sulfate [SDS], and protease inhibitor cocktail) for 30 min. The soluble cytoplasmic extracts were separated via centrifugation at 13,000 rpm for 30 min at 4 °C. We then boiled 40 μl aliquots of the lysates in SDS protein-loading buffer for input analysis, and the remaining lysates were precleared via incubation with 25 μl protein G-Sepharose 4 Fast Flow medium for 1 h at 4 °C with rotation. The precleared lysates were collected via centrifugation at 5000 rpm for 2 min at 4 °C and incubated with monoclonal anti-HA antibody (Sigma) for another 2 h at 4 °C with rotation. After brief centrifugation, the lysates were mixed with 40 μl protein G-Sepharose 4 Fast Flow medium and incubated overnight at 4 °C with rotation. Beads were collected via centrifugation at 5000 rpm for 2 min and then washed with washing buffer (5% [wt/vol] sucrose, 5 mM Tris-HCl [pH, 7.4], 5 mM EDTA [pH, 8.0], and 500 mM NaCl, and 1% [vol/vol] Triton X-100). The beads were then boiled in SDS protein-loading buffer for 10 min, and the bound proteins were analyzed via Western blotting. Quantity one software was used to quantify the band intensities of the co-immunoprecipitated N.

2.5. Distribution assay of N after centrifugation

HeLa cells in 6-well plates were transfected with 0.25 μg Myc-tagged N and 1 μg wild-type P or P mutants with Lipofectamine 2000. At 24 h post-transfection, cells were harvested and lysed in 200 μl lysis buffer for 30 min. The supernatants and pellets were separated via centrifugation at 13,000 rpm for 30 min at 4 °C. The supernatants were then collected and boiled for 10 min in SDS protein-loading buffer for