Virus Research 177 (2013) 108–112

Contents lists available at ScienceDirect

Virus Research

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

Short communication

Human respiratory syncytial virus N, P and M protein interactions in HEK-293T cells

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ARTICLE INFO

Article history:
Received 19 March 2013
Received in revised form 6 July 2013
Accepted 11 July 2013
Available online 23 July 2013

Keywords:
Human respiratory syncytial virus
Nucleoprotein
Phosphoprotein
Matrix
Protein interaction
Optimized genes

ABSTRACT

Characterization of Human Respiratory Syncytial Virus (HRSV) protein interactions with host cell components is crucial to devise antiviral strategies. Viral nucleoprotein, phosphoprotein and matrix protein genes were optimized for human codon usage and cloned into expression vectors. HEK-293T cells were transfected with these vectors, viral proteins were immunoprecipitated, and co-immunoprecipitated cellular proteins were identified through mass spectrometry. Cell proteins identified with higher confidence scores were probed in the immunoprecipitation using specific antibodies. The results indicate that nucleoprotein interacts with arginine methyl-transferase, methylosome protein and Hsp70. Phosphoprotein interacts with Hsp70 and tropomysin, and matrix with tropomysin and nucleophosmin. Additionally, we performed immunoprecipitation of these cellular proteins in cells infected with HRSV, followed by detection of co-immunoprecipitated viral proteins. The results indicate that these interactions also occur in the context of viral infection, and their potential contribution for a HRSV replication model is discussed.

Human Respiratory Syncytial Virus (HRSV) is a negative-sense single-stranded RNA virus that belongs to the Paramyxoviridae family and the Pneumovirinae subfamily (Collins et al., 2001). HRSV is considered the most important pathogen causing respiratory disease in infants and neonates worldwide, which may present clinical complications like pneumonia and bronchiolitis (Holberg et al., 1991). This virus is responsible for a significant amount of the lower respiratory tract infections in infants up to one year of age and it is estimated that half of these infants have re-infections after one year (Schmidt et al., 2004). HRSV is also a significant cause of respiratory disease in the elderly (Han et al., 1999), immune-compromised patients, such as bone marrow transplant patients (Hall et al., 1986), and is related to the development of asthma in childhood (Lemanske, 2004). Since its discovery in the 1960s, efforts have been made toward the development of a vaccine, but to date this has proved unsuccessful (Kim et al., 1969; Collins and Melero, 2011).

HRSV is enveloped and carries a negative-sense RNA genome of about 15 Kb that encodes 11 proteins. The nucleoprotein (N) associates with the viral RNA, and phosphoprotein (P) interacts with N and with the RNA-dependent RNA polymerase (L) to form the nucleocapsid. The genome also encodes three envelope proteins (F, G, SH), a matrix protein (M), a nucleocapsid-associated transcription factor (M2-1), another protein involved in genome replication (M2-2, the second product of the M2 gene) and two nonstructural proteins (NS1, NS2) (Collins et al., 2001).

In this work we searched cellular partners for the viral proteins N, P and M. N protein is highly conserved among pneumoviruses and interacts with viral RNA, generating a helicoidal nucleocapsid (Maclellan et al., 2007) which is responsible for genome and anti-genome resistance to RNAse (Collins et al., 2001). P protein interacts with N protein, giving specificity for viral RNA encapsidation (Spehner et al., 1997) and interacts with L protein (the major unit of the virus replication complex), conferring stability and the correct placement in the ribonucleo-complex for RNA synthesis

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http://dx.doi.org/10.1016/j.virusres.2013.07.010

(Bowman et al., 1999). M protein is involved in viral assembly and budding. During viral infection M is present in cytoplasmic inclusion bodies and is associated with ribonucleo-particles containing N, P, L and M2-1 proteins (Ghildyal et al., 2002, Carromeu et al., 2007). The association of M protein with the viral nucleocapsid is mediated by M2-1 protein (Li et al., 2008). There is also evidence that like the M protein of other negative-stranded RNA viruses, HRSV M protein may function as a virus transcription inhibition factor (Ghildyal et al., 2002, 2003).

These viral proteins most likely interact with host cell components and their identification is essential for understanding virus replication and devising antiviral strategies. We have cloned N, P (Simabuco et al., 2009) and M (reported in this work) genes, with optimized codons for expression in human cells. Here we report the tagging of M, N and P proteins with FLAG peptide; allowing co-immunoprecipitation experiments for partners' detection and identification by mass spectrometry (see the experimental design in Fig. 1A). The cellular partners were confirmed by immunoblotting in non-infected cells expressing viral Flag-tagged proteins or in HRSV infected cells expressing the wild type viral proteins. We observed that Heat-shock protein 70 (Hsp70), Arginine methyltransferase (PRMT5), Methylosome protein (WDR77), Tropomysin (Tm) and Nucleophosmin (Npm) proteins interact with N, P and M, indicating their potential cooperation with the HRSV replication complex, and this will be discussed in more detail later.

The HRSV (strain A2) M, N and P genes were codon optimized for human cell expression and synthesized by GeneArt (see nucleotide sequences in Supplementary Figure 1). The genes were delivered in the pCR4-zero plasmid which allows subcloning in pFlag vector. The plasmids were digested with specific restriction endonucleases, BamHI-PmeI for M, and BamHI-EcoRI for N and P, and cloned into pFlag vector previously digested with the same enzymes. The pFlag vector is based on the pcDNA3 (Invitrogen) and contains the FLAG peptide coding sequence upstream from the multiple cloning site (Zerbini L.F., unpublished data).

The plasmids obtained, named pFlagMopt, pFlagNopt and pFlagPopt, were transfected in HEK293T cells and in all experiments pFlag (empty vector) was used as a control. Briefly, DNA was mixed with Lipofectamine PLUS Reagent (Invitrogen) in a serum free media and incubated for 15 min. Lipofectamine (Invitrogen) was added and the mixture was incubated for another 15 min. The transfection solution was added to the cells and after 48 h expression was analyzed by immunoblotting. Briefly, proteins were subjected to electrophoresis on SDS-PAGE and after being transferred to nitrocellulose membrane followed by incubation with anti-Flag antibody. Detection was performed using secondary antibodies conjugated with peroxidase, SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposition to Hyperfilm (Amersham Biosciences). As can be seen in Fig. 1B, the expression was strong for the three genes and similar to that obtained for optimized N and P genes cloned into the pShuttle vector (Simabuco et al., 2009).

For immunoprecipitation, 48 h after transfection, proteins were extracted using Cell Lysis Buffer (Cell Signaling) containing protease and phosphatase inhibitor cocktails (Sigma). Cell debris was removed by centrifugation and the supernatant subjected to immunoprecipitation with anti-Flag antibody coupled to agarose beads (Sigma). Briefly, after16 h, agarose beads were washed 5 times with TBS and proteins were eluted with Flag peptide (150 ng/μl). Eluted proteins of the immunoprecipitation were separated on SDS-PAGE, stained with Coomassie blue (Supplementary Figure 2) and the differentially expressed proteins were excised, trypsinized and analyzed in a MALDI TOF/TOF Analyzer (Applied Biosystems). Mass spectrometry was performed at the BIDMC Genomics Center/DFHCC Proteomics Core, Boston, USA. The peptides were identified using Protein Pilot software (Applied Biosystems) and the SwissProt database. Table 1 summarizes the data for the selected human proteins with higher confidence scores. All identified proteins presented ProtScores above 2.0, which represent, by definition, a confidence above 99%.

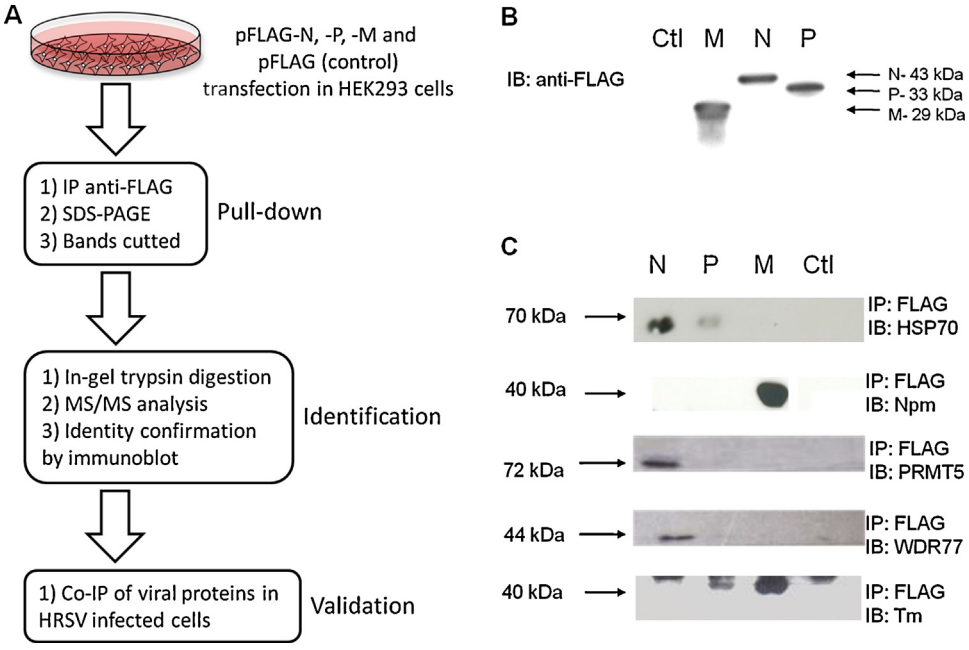

Fig. 1. Immunoprecipitation of HRSV Flag-tagged N, P or M co-imunoprecipitates cellular proteins. (A) Representation of experimental rationale. (B) Expression of Flag tagged proteins. HEK293T cells were transfected with plasmids pFlag (Ctl), pFlagMopt (M), pFlagNopt (N) or pFlagPopt (P), collected after 48 h, cell lysates subjected to SDS-PAGE, and the Western Blot result is presented for detection (IB) with antibodies against the FLAG tag. (C) HEK293T cells were transfected with plasmids pFlag (Ctl), pFlagMopt (M), pFlagNopt (N) or pFlagPopt (P), and collected after 48 h. Cell lysates were immunoprecipitated with anti-FLAG antibody (IP) and subjected to SDS-PAGE (indicated on top). The Western Blot results are presented for detection with specific antibodies (see text) against Hsp70, Npm, PRMT5, WDR77 or Tm, as indicated (IB). The expected molecular weight of proteins is indicated by arrows.