Virology 525 (2018) 40–47

Contents lists available at ScienceDirect

Virology

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

CDC25B promotes influenza A virus replication by regulating the phosphorylation of nucleoprotein

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ARTICLE INFO

Keywords:
Influenza A virus
CDC25B
Nucleoprotein
Self-oligomerization
Nuclear export
Phosphorylation

ABSTRACT

Cell division cycle 25 B (CDC25B) is a member of the CDC25 phosphatase family. It can dephosphorylate cyclin-dependent kinases and regulate the cell division cycle. Moreover, siRNA knockdown of CDC25B impairs influenza A virus (IAV) replication. Here, to further understand the regulatory mechanism of CDC25B for IAV replication, a CDC25B-knockout (KO) 293T cell line was constructed using CRISPR/Cas9. The present data indicated that the replication of IAV was decreased in CDC25B-KO cells. Additionally, CDC25B deficiency damaged viral polymerase activity, nucleoprotein (NP) self-oligomerization, and NP nuclear export. Most importantly, we found that the NP phosphorylation levels were significantly increased in CDC25B-KO cells. These findings indicate that CDC25B facilitates the dephosphorylation of NP, which is vital for regulating NP functions and the life cycle of IAV.

1. Introduction

Influenza A virus (IAV) belongs to the Orthomyxoviridae family and has a genome consisting of eight negative-sense, single-stranded RNA segments encoding 17 viral proteins (Hu et al., 2018; Samji, 2009). IAV can infect a wide range of birds and mammals, including humans. Seasonal influenza epidemics caused by IAV result in high morbidity and mortality, seriously threatening human health (Punpanich and Chotpitayasunondh, 2012). Nucleoprotein (NP) is an abundant IAV protein and involved in multiple functions (Boulo et al., 2007; Zheng and Tao, 2013). It envelopes viral RNA and combines with the viral polymerase (PA, PB1, and PB2) to form the viral ribonucleoprotein (vRNP) complex (Baudin et al., 1994). NP contains a head and a body domain that are separated by a RNA binding domain (Ng et al., 2008; Ye et al., 2006). NP itself oligomerizes through the insertion of the tail-loop from one NP monomer into the body domain of a neighboring NP (Chan et al., 2010). Hence, NP self-oligomerization is essential for maintaining vRNP complex structure and function (Elton et al., 1999; Kingsbury and Webster, 1969). In addition, NP also plays a pivotal role in mediating nuclear-cytoplasmic shuttling of the vRNP complex (Whittaker et al., 1996a, 1996b). Two nuclear localization signals on NP control the nuclear import of vRNP (Cros et al., 2005; Weber et al., 1998), and three NP nuclear export signals participate in the export of vRNP from the nucleus to the cytoplasm (Yu et al., 2012).

NP phosphorylation plays an important role during viral infection by regulating nuclear-cytoplasmic shuttling, oligomerization of NP, and polymerase activity. For instance, nuclear import of NP is modulated by phosphorylation of residue S3 in influenza virus A/Puerto Rico/8/1934(H1N1) (Bullido et al., 2000). The phosphorylation and dephosphorylation of residues S9, Y10, Y296, and T188 affect the nuclear-cytoplasmic shuttling of NP and regulate influenza virus replication (Li et al., 2018; Zheng et al., 2015). The phosphorylation of NP S407 and S486 decreases viral polymerase activity and NP oligomerization, which are important for viral replication (Mondal et al., 2015).

Many host factors target NP to regulate the replication of IAV. For instance, Moloney leukemia virus 10 is an inhibitor of IAV replication that acts by inhibiting the polymerase activity and nuclear transport of NP (Zhang et al., 2016). Cellular nuclear transport factor 2 like export protein 1 binds the C-terminal region of NP and thus stimulates NP-mediated nuclear export by the CRM1-dependent pathway (Chutiwitoonchai and Aida, 2016). The interaction of host nucleolus proteins with NP results in increased expression of M1 and HA in infected cells (Kumar et al., 2016). In addition, cell division cycle 25B (CDC25B) is thought as a pro-IAV host factor. It is likely that CDC25B

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https://doi.org/10.1016/j.virol.2018.09.005
Received 28 June 2018; Received in revised form 6 September 2018; Accepted 6 September 2018

supports NS1 nuclear function to hijack host transcription machinery in favor of viral RNA synthesis by RNA interference (RNAi) (Perwitasari et al., 2013), but the exact mechanism remains unknown.

The members of CDC25 phosphatase family play important roles in transitions between cell cycle phases by dephosphorylating and activating cyclin-dependent kinases (CDKs) (Igarashi et al., 1991; Norbury et al., 1991). In mammalian cells, CDC25 is a family comprised of CDC25A, CDC25B and CDC25C (Galaktionov and Beach, 1991; Nagata et al., 1991; Sadhu et al., 1990). CDC25A acts at the G1/S transition. CDC25B and CDC25C play a vital role at the G2/M transition (Aressy and Ducommun, 2008; Boutros et al., 2007; Lammer et al., 1998). CDC25B shows a high substrate specificity for cdc2/cyclinA during S-phase, but during G2-phase, its activity increases toward cdc2/cyclinB as a substrate (Lammer et al., 1998). Additionally, CDC25B has oncogenic properties and is overexpressed in many cancers (Gasparotto et al., 1997; Kristjánsdóttir and Rudolph, 2004; Kudo et al., 1997; Nishioka et al., 2001; Sasaki et al., 2001; Takemasa et al., 2000). Therefore, the CDC25B phosphatase is an attractive candidate for cancer therapeutics.

In the present study, CDC25B-knocked out (KO) 293T cells were generated using the CRISPR/Cas9 system. The results suggested that CDC25B is critical for influenza virus replication and transcription, NP self-oligomerization, and NP nuclear export. In addition, CDC25B inhibited the phosphorylation of NP. Therefore, CDC25B promotes IAV replication by regulating the functions of NP.

2. Materials and methods

2.1. Viruses and cells

The influenza virus A/WSN/ 1933(H1N1) strain was propagated in 10-day-old specific pathogen-free embryonic chicken eggs. CDC25B-KO 293T cells were generated with CRISPR/Cas9 gene editing technology. Madin-Darby canine kidney (MDCK) cells, human embryonic kidney (293T) cells, and CDC25B-KO 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (both from Invitrogen) at 37 °C and 5% CO_2.

2.2. Antibodies and reagents

Anti-CDC25B antibody was purchased from ABGENT. Mouse anti-β-actin and anti-GAPDH monoclonal antibody were purchased from Amrebio (Beijing, China). Anti-HSP70 (3A3) antibody, anti-Lamin B1 (A-11) antibody, anti-p-Tyr (sc-508) antibody, anti-p-Thr (sc-5267) antibody, anti-p-Ser (sc-81514) antibody, and anti-c-Myc (9E10) antibody were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody against NP and mouse monoclonal antibody against M1 were generated as previously described (Liu et al., 2009). Mouse anti-FLAG antibody and anti-FLAG (M2) Affinity Gel were purchased from Sigma-Aldrich. Pierce™ Protein G Agarose (20397) was purchased from Thermo Scientific. Complete protease inhibitor cocktail tablets and the phosphatase inhibitor phosSTOP were purchased from Roche.

2.3. Plasmid construction

For co-immunoprecipitation assays (co-IPs), luciferase assays, and immunofluorescence assays (IFAs), the NP sequence obtained from the A/WSN/33 virus was cloned into the pcDNA3.0-Flag, pCMV-Myc, and pCDNA4/TO vectors, respectively.

For the construction of sgRNAs and Cas9 Plasmids, and to improve KO efficiency, three pairs of guide RNAs (gRNAs) were designed against CDC25B in this experiment (Table 1). These guide sequence oligonucleotides were annealed to generate double-stranded gRNAs and cloned into the *Bsa*I site of the Precut-PCS vector including the U6 promoter. The positive monoclone was first detected using PCR and the following PCR primers: KO-CDC25B forward (5′- AGCCAGCTGTGCCGGCGTTT GTT-3′) and reverse (5′- GGCACAATGAGGGAGGAGGGAGT-3′). Then, the selected positive clone was sequenced with the anthropogenic U6 promoter universal primer: 5′-ATGGACTATCATATGCTTACCGTA-3′.

Table 1
Sequences of guide RNAs against CDC25B.

Guide RNAs	Sequences
KO-CDC25B-gRNA1-up	CACCGGCTCGGCTCTCAGTCCAGC
KO-CDC25B-gRNA1-down	AAACGCTGGACTGAGAGCCGAGCC
KO-CDC25B-gRNA2-up	CACCGGCCTCCTGCTGGGATCTCA
KO-CDC25B-gRNA2-down	AAACTGAGATCCCAGCAGGAGGCC
KO-CDC25B-gRNA3-up	CACCGTGCGGTGGCGCCCAGCGTC
KO-CDC25B-gRNA3-down	AAACGACGCTGGGCGCCACCGCAC

2.4. Generation of the CDC25B-KO 293T cell line

The 293T cells were grown on 10-cm culture dishes and co-transfected with three cas9 plasmids (4 μg per plasmid in a dish) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, the cells were screened with 3 μg/mL puromycin for 7 days, and then many monoclonal cells were obtained by flow cytometry in 96 well plates. After amplification, a monoclonal cell line was obtained, and genomic DNA was extracted. We preliminarily detected the PCR products of target DNA using nucleic acid electrophoresis. Then, these genomic DNAs were sequenced. Finally, the expression of CDC25B protein was assessed by western blotting.

2.5. Multi-cycle growth curves

WT-293T and KO-293T cell monolayers in 10-cm cell culture dishes were washed with PBS and infected with virus at a multiplicity of infection (MOI) of 0.001 for 1 h at 37 °C and 5% CO_2. The virus inoculums were removed by washing with PBS. Cell monolayers were incubated in DMEM with 1% FBS and 0.5 μg/mL TPCK-treated trypsin at 37 °C. After infection, 500 μL of supernatant was collected at 24, 36, 48, 60, and 72 h and stored at − 80 °C. At the same time, 500 μL of fresh medium was added back to each dish. Finally, virus titers were determined by plaque assays.

2.6. Immunoprecipitation and western blotting analyses

WT-293T and KO-293T cells were lysed in lysis buffer (150 mM NaCl, 20 mM HEPES (pH = 7.4), 1 mM EDTA, 1% Triton X-100%, and 10% glycerol) including protease inhibitor. After centrifugation, the supernatants were subjected to immunoprecipitation with different antibodies. Following four washes with wash buffer (300 mM NaCl, 20 mM HEPES (pH = 7.4), 1% Triton X-100, 1 mM EDTA, and 10% glycerol), the precipitated proteins were separated by SDS-PAGE and then transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Billerica, MA). The membranes were blocked for 2 h at room temperature in blocking solution (5% skim milk powder and 0.5% Tween 20 in PBS), and target proteins were detected using corresponding antibodies followed by the addition of anti-rabbit or anti-mouse secondary antibody coupled to horseradish peroxidase. Then, proteins were observed by chemiluminescence detection reagents.

2.7. RNA extraction, cDNA synthesis, and real-time quantitative PCR

Total RNA was extracted from WT-293T and KO-293T cells using TRIzol (Invitrogen) according to the manufacturer's instructions. The samples were digested with DNase I and subjected to RT-PCR. RNA was reverse-transcribed using the following PCR primers: mRNA primer, oligo(dT); cRNA primer, 5′-AGTAGAAACAAGG-3′; and vRNA primer, 5′- AGCGAAAGCAGG -3′. The analysis of relative M1 gene expression