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Phosphorylation and dephosphorylation of threonine 188 in nucleoprotein is crucial for the replication of influenza A virus

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ABSTRACT

Nucleoprotein (NP) is a major component of the viral ribonucleoprotein (vRNP) complex that is responsible for viral replication, transcription and packaging of influenza A virus. Phosphorylation of NP plays an important role during viral infection. In the present study, we identified threonine 188 (T188) as a novel phosphorylated residue in the NP of influenza A virus by using mass spectrometry. T188 is located within nuclear export signal 2 (NES2) which is chromosome region maintenance 1 (CRM1)-independent. We observed that the phosphorylation and dephosphorylation of residue T188 regulated viral replication by controlling NES2-dependent NP nuclear export and the polymerase activity of the vRNP complex. Our findings provide further insights for understanding the replication of influenza A virus.

1. Introduction

Influenza A viruses have large host reservoirs in animals, including humans (Liu et al., 2014; Wu et al., 2014). Influenza A viruses are widespread human pathogens that are responsible for seasonal flu epidemics and occasional severe pandemics of respiratory disease, with significant morbidity and mortality (Chen et al., 2013; Horimoto and Kawaoka, 2005).

Influenza A virus is a member of the Orthomyxoviridae family. The genome of influenza A virus contains eight negative-sense single-stranded RNA segments that encode viral proteins including viral polymerase proteins (PB1, PB2, and PA), nucleoprotein (NP), matrix1 protein (M1), transmembrane proteins (HA, NA, and M2), non-structural protein 1 (NS1), and viral nuclear export protein (NEP) (Chen et al., 2001; Jagger et al., 2012; Lamb and Choppin, 1983; Muramoto et al., 2013; Wise et al., 2009). NP, viral RNA (vRNA), and three polymerase subunits (PB1, PB2 and PA) form the major components of the viral ribonucleoprotein (vRNP) complex (Coloma et al., 2009; Marklund et al., 2012), which is responsible for viral replication, transcription, and packaging (Arranz et al., 2012; Klumpp et al., 1997; Matsuoka et al., 2013).

NP is an abundant protein in the virus particle and plays essential roles in the virus replication cycle (Lamb and Choppin, 1983) via intracellular trafficking (Cros et al., 2005; Neumann et al., 1997; Ozawa et al., 2007), oligomerization (Elton et al., 1999a; Ye et al., 2006), and NP-RNA binding for the vRNP complex (Elton et al., 1999b; Portela and Digard, 2002). Nuclear export signals (NESs) and nuclear localization signals (NLSs) play important roles in the nuclear-cytoplasmic shuttling of NP during viral infection (Chutiwitoonchai et al., 2014; Cros et al., 2005; Luo et al., 2018). Our previous study demonstrates that NP contains two CRM1-independent NESs (NES1 and NES2) and a CRM1-dependent NES (NES3) (Yu et al., 2012). The phosphorylation of NP regulates its functions, which is essential for viral polymerase activity and virus replication (Cohen, 2000). Several phosphorylated residues in the NP of influenza A virus have been reported. The phosphorylation and dephosphorylation of S9, Y10, and Y296 regulate the nuclear-cytoplasmic shuttling of NP and corresponding vRNP (Hutchinson et al., 2012; Zheng et al., 2015). The phosphorylation of S165, S407, and S486 affect the oligomerization of NP and polymerase activity, which are important for viral replication (Mondal et al., 2015; Turrell et al., 2015).

In the present study, we identified T188 as a novel phosphorylated

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residue in the NP of influenza A virus by using mass spectrometry. T188 is located within NES2. We found that the phosphorylation of T188 inhibited NES2-dependent NP nuclear export, decreased the polymerase activity of the vRNP complex, and thus affected influenza virus replication.

2. Materials and methods

2.1. Cells and viruses

293 T, MDCK and A549 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C and 5% CO_2. The influenza virus used in this study was the A/WSN/1933(H1N1) strain and was rescued from cDNAs and propagated in the allantoic cavities of 10-day-old specific pathogen-free embryonated chicken eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd, China).

2.2. Antibodies and reagents

Antibodies and reagents used in this study were obtained from the following sources: Complete protease inhibitor cocktail tablets was purchased from Roche (USA). Acrylamide-pendant phosphate-tag (Phos-tag acrylamide) was purchased from Wako (Japan). Sodium chloride (NaCl), HEPES, EDTA, Triton X-100, Phosphatase inhibitor tablets (PhosSTOP), $MnCl_2$, tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin, Cycloheximide, mouse anti-FLAG antibody, FLAG M2 agarose beads and protein G agarose beads were purchased from Sigma-Aldrich (USA). Rabbit anti-NP polyclonal antibody and mice anti-M1 monoclonal antibody were generated as previously described (Koestler et al., 1984; Liu et al., 2009). Mouse anti-β-actin antibody, anti-GAPDH antibody, anti-c-MYC antibody, anti-p-Thr antibody and rabbit anti-c-MYC antibody were purchased from Santa Cruz Biotechnology, Inc (UK and Ireland). Lipofectamine reagent and TRIzol were purchased from Invitrogen (USA). Bovine Serum Albumin (BSA) was purchased from Bovogen Biologicals (Australia). Paraformaldehyde was purchased from Sinopharm Chemical Reagent (China). 4',6-diamidino-2-phenylindole (DAPI) was purchased from Thermo Fisher Scientific (USA). All secondary antibodies were obtained from Bai Hui Zhong Yuan Biotechnology (China). Glycerol was purchased from Beijing Chemical Works (China).

2.3. Plasmid construction

The plasmid expressing NES2 was constructed by annealing double-stranded oligonucleotides into the pEGFP-C1 expression vector as previously described (Yu et al., 2012). For virus rescue assays, western blotting, luciferase assays, and indirect immunofluorescence assays (IFAs), amino acid mutations of the full-length NP from WSN in pHH21, pcDNA4/TO, pcDNA3.0-Flag, and pCMV-MYC were generated using a site-directed mutagenesis kit (New-pep, China).

2.4. Phosphate-affinity SDS-PAGE and preparation for nano-LC-MS/MS analysis

293 T cells infected with A/WSN/1933(H1N1) were lysed in lysis buffer (150 mM NaCl, 20 mM HEPES [pH 7.4], 1 mM EDTA, 10% glycerol, and 1% Triton X-100) supplemented with Complete protease inhibitor cocktail and alkaline phosphatase (ALP) inhibitor PhosSTOP. The lysates were incubated with rabbit anti-NP antibody for 4 h at 4 °C and purified with protein G agarose beads. The precipitated proteins were separated by 15% Mn^{2+}-Phos-tag SDS-PAGE as described previously (Kinoshita et al., 2009; Wang et al., 2013). Briefly, normal polyacrylamide gel electrophoresis was conducted according to the TaKaRa protocol, with 50 μM Phos-tag acrylamide and 0.1 mM $MnCl_2$ added to the separating gel before polymerization. The gel was silver stained, and the separated phosphorylated bands were detected by nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) identification at the Technological Platform of the Institute of Microbiology, Chinese Academy of Sciences (LCQ Deca XP Plus, Thermo Fisher Scientific, USA).

2.5. Computer modeling and statistical analyses

The three-dimensional (3D) crystal structure of NP was manipulated with the program PyMOL (Schrödinger). The sequence alignment for conservation analysis of influenza virus subtypes was performed using the multiple sequence alignment tool at the Influenza Virus Database of NCBI (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go%04database). Sequence alignment for site-specific mutations of plasmid constructs was performed using MegAlign software (DNAstar Software).

2.6. IFA

IFAs were performed with a Leica SP8 confocal laser scanning microscope. The 293 T cells were transfected with plasmids for different time periods, then treated with cycloheximide (CHX, 100 μg/mL) for 3 h, followed by washing with PBS and fixing with 4% paraformaldehyde. Cells were then blocked with 4% BSA, subjected to antibody-treatment with anti-NP antibody, and stained with DAPI (5 μg/mL). Secondary antibodies were tetramethyl rhodamine isocyanate (TRITC)-conjugated anti-rabbit IgG.

2.7. Luciferase assay of influenza virus polymerase activity

293 T cells were transfected with the PA, PB1, PB2, and NP (WT or mutants) expression plasmids, as well as luciferase reporter plasmids (pHH21-NS-Luc and pcDNA-β-gal) as described (Li et al., 2009). As a negative control, 293 T cells were transfected with the same plasmids, excluding the NP expression plasmid. The transfected cells were incubated at 37 °C for 33 h, and then the amount of the luciferase activity in the cells was measured and normalized to the amount of galactosidase activity, as tested using standard kits (Promega, USA).

2.8. RNA extraction, DNA synthesis, and real-time quantitative PCR

Total RNA was extracted from 293 T cells with TRIzol. RNA samples were subjected to reverse transcription PCR (RT-PCR) by using the following primers: mRNA, Oligo(dT); vRNA, 5'-AGCAAAAGCAGG-3'; and cRNA, 5'-AGTAGAAACAAGG-3'. Target gene PCR used the following primers: M1 forward and reverse, 5'-TCTGATCCTCTCGTCATT GCAGCAA-3' and 5'-AATGACCATCGTCAACATCCACAGC-3'. The housekeeping gene, GAPDH, was used as an internal control with the following PCR primers: forward and reverse, 5'-GGTGGTCTCCTCTGA CTTCAACA-3' and 5'-GTTGCTGTAGCCAAATTCGTTGT-3'. The blank control contained RNase-free H_20 into the reaction mixture. Real-time quantitative PCR was performed on an ABI 7300 following the manufacturer's instructions and the following cycling conditions: initial denaturation step at 95 °C for 30 s and 40 two-step cycles of 95 °C for 5 s and 60 °C for 31 s. All reactions were performed in triplicate. The relative gene expression datas were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.9. Western blotting analysis

Cells were lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM HEPES, 10% glycerol, and 1 mM EDTA (pH 7.4) supplemented with Complete protease inhibitor cocktail with an incubation period of 30 min at 4 °C. Insoluble components were removed by centrifugation at 12,000 rpm for 10 min. The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (Immobilon-P)