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Intrinsically disordered region of influenza A NP regulates viral genome packaging via interactions with viral RNA and host PI(4,5)P_2

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ABSTRACT

To be incorporated into progeny virions, the viral genome must be transported to the inner leaflet of the plasma membrane (PM) and accumulate there. Some viruses utilize lipid components to assemble at the PM. For example, simian virus 40 (SV40) targets the ganglioside GM1 and human immunodeficiency virus type 1 (HIV-1) utilizes phosphatidylinositol (4,5) bisphosphate [PI(4,5)P2]. Recent studies clearly indicate that Rab11-mediated recycling endosomes are required for influenza A virus (IAV) trafficking of vRNPs to the PM but it remains unclear how IAV vRNP localized or accumulate underneath the PM for viral genome incorporation into progeny virions. In this study, we found that the second intrinsically disordered region (IDR2) of NP regulates two binding steps involved in viral genome packaging. First, IDR2 facilitates NP oligomer binding to viral RNA to form vRNP. Secondly, vRNP assemble by interacting with PI(4,5)P2 at the PM via IDR2. These findings suggest that PI(4,5)P2 functions as the determinant of vRNP accumulation at the PM.

1. Introduction

Intrinsically disordered proteins (IDPs) and proteins with intrinsically disordered regions (IDRs) are characterized by their biased sequences, which largely consist of charged and hydrophilic amino acids but not bulky hydrophobic acids (Uversky, 2011). These amino acid compositions prevent stable folding and cause the proteins to exist in a flexible state. Upon binding to cellular targets, IDPs and IDRs undergo a disordered-to-ordered transition, but the ordered complexes they form with their targets are often short-lived because of their inherent flexibility. These features facilitate rapid exchange of binding partners and allow for a degree of promiscuity in interactions with various binding partners with different sequences (Uversky, 2011). For example, the C-terminal regulatory domain of p53, which is disordered in its free form, adopts one of four distinct structures upon binding to four different binding partners (Uversky, 2011). The majority of IDPs and IDRs are involved in signaling and regulation of key cellular processes, and thus these proteins are intimately associated with the onset of disease. Consequently, IDPs and IDRs have been identified as potential drug targets, and several small compounds that target these disorder-based interactions already been identified (Metallo, 2010). In addition to eukaryotic proteins, IDRs are abundant in viral proteins, and are commonly used by viruses to hijack host factors and facilitate adaptation to hostile host environments (Xue et al., 2012).

Influenza A nucleoprotein (NP) is a highly basic protein covered with many positively charged residues that bind negatively charged RNA. NP is the primary structural backbone of viral ribonucleoprotein (vRNP), which consists of three polymerase subunits (PB2, PB1, and PA) and viral RNA (vRNA) (Ye et al., 2006; Arranz et al., 2012; Moeller et al., 2012). In addition to its structural role as a major component of vRNP, NP is involved in multiple processes, including viral genome transcription/replication, nuclear import and export of vRNP complexes, and viral genome packaging (Eisfeld et al., 2015). X-ray crystallographic analysis of the structure of influenza A NP has revealed three different regions that are not represented by electron densities and are therefore regarded as IDRs (Uversky, 2011; Ye et al., 2006). All three of these IDRs are involved in the regulation of NP functions including nuclear import, RNA binding, and NP–NP interaction (Uversky, 2011; Eisfeld et al., 2015). The second of these IDRs (IDR2;72-DERRNKYLEEHPSAGKDPKKT-92), which contains abundant positively charged residues, is located in an RNA-binding groove and exhibits the highest conservation ratio among human, swine, and avian influenza A viruses (IAVs). However, the biological significance and role of each conserved amino acid are not well defined.

In this study, we revealed that IDR2 targets two different

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binding partners. One is vRNA, and the other is phosphatidylinositol (4,5) bisphosphate [PI(4,5)P_2], a component of lipid rafts on the plasma membrane (PM) (DiNitto et al., 2003). The steps regulated by IDR2 cooperate to efficiently transport NP/vRNP to the PM and deliver viral genomes into progeny virions. Taken together, our results indicate that IDR2 plays an essential role in the regulation of vRNP function, and that PI(4,5)P_2 is a key host factor in viral genome packaging.

2. Materials and methods

2.1. Materials

Madin-Darby canine kidney (MDCK) cells and human embryonic kidney 293T (HEK293T) cells from our laboratory's repository were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing Pen-Strep Glutamine (PSG, Gibco) and 10% fetal bovine serum (Sigma). Cells were cultured at 37 °C in 5% CO_2. Transfections were performed using FuGENE HD (Roche Diagnostics) or Lipofectamine 2000 and 3000 (Invitrogen). Anti-WSN serum was kind gifts from Dr. Kazufumi Shimizu (Nihon University). Acti-stain 670 fluorescent phalloidin and cytochalasin D were purchased from Cytoskeleton and Sigma, respectively. NP proteins and GST-PLCδpH were expressed using the GST gene fusion system in *Escherichia coli* strain BL21 CodonPlus (DE3)-RIL (Stratagene) and purified using the Glutathione Sepharose 4FF bead system (GE Healthcare) as previously described (Kakisaka et al., 2015). For removal of GST-tag, GST-NP proteins were digested with PreScission Protease (GE Healthcare). The influenza A/WSN/1933 (H1N1) was propagated in MDCK cells at 37 °C for 48 h in 5% CO_2.

2.2. Antibodies

All antibodies were purchased from the indicated suppliers. Primary antibodies: Anti-NP monoclonal antibody (MAb) (Abcam), anti-HA MAb (GeneTex), anti-caveolin-1 polyclonal antibody (Santa Cruz Biotechnology), anti-PI(4,5)P_2 MAb (Santa Cruz Biotechnology), and anti-β-actin MAb (Sigma).

Secondary antibodies: horseradish-peroxidase (HRP)-conjugated goat anti-mouse IgG (1:3000 in PBS; Amersham Bioscience), HRP-conjugated goat anti-rabbit IgG (1:3000 in PBS; Amersham Bioscience), goat anti-mouse IgG Alexa Fluor 405 (1:500 in PBS; Invitrogen), and goat anti-mouse IgG Alexa Fluor 594 (1:500 in PBS; Invitrogen).

2.3. Plasmid construction

Plasmids NP/pHH21 and NP/pCAGGS harboring site-specific mutations (D72A, E73A, R74A, R75A, K77A, Y78A, E80A, P83A, G86A, K87A, D88A, P89A, K90A, K91A, and T92A) were generated using Prime STAR Max DNA Polymerase (Takara Bio) as described previously (Kakisaka et al., 2015). Plasmids NP/pHH21 and NP/pCAGGS were kind gifts from Dr. Yoshihiro Kawaoka (University of Tokyo). Plasmid HA/pCAGGS harboring site-specific mutations in the transmembrane domain (S534A, T535A, S538A, S539A) was also created by the protocol mentioned above. PB2-mRFP/pHH21 and NA-EGFP/pHH21plasmids express a virus-like RNA in which the region encoding mRFP and EGFP are flanked by the packaging signals of the PB2 and NA segments, respectively. These plasmids were generated by replacing the PB2 and NA ORF sequences with the mRFP and EGFP ORF sequences, as described previously (Li et al., 2009). Plasmids encoding 5-phosphat phosphatidylinositol polyphosphate 5 phosphatase type IV fused to the AcGFP-tag (AcGFP-5ptaseIV) were generated as follows. The coding sequence of 5ptaseIV was obtained from HeLa cell cDNA by PCR amplification using KOD-Plus- (Toyobo). These amplified sequences were cloned into vector pAcGFP1 (Takara Bio). The plasmids encoding PLCδPH fused to GST-tag were generated as follows. The coding sequence for PLCδPH was obtained from mCherry-PLCδPH plasmid by PCR amplification using KOD-Plus- (Toyobo). The amplified sequence was cloned into vector pGEX6p-3 (GE Healthcare). All sequences cloned into expression vector were confirmed by DNA sequencing.

2.4. Prediction of intrinsically disordered regions

The sequence of NP was derived from PDB (PDB code: 2IQH). Subsequent analysis of the disorder probability of the NP protein was performed using the DISOPRED server (Ward et al., 2004).

2.5. Replicon assay

HEK293T cells were cultured in 24-well plates at 37 °C for 24 h. Cells were transfected with plasmids encoding the components of viral ribonucleoprotein complex (PB2/pCAGGS, PB1/pCAGGS, PA/pCAGGS, and NP/pCAGGS) and PB2-mRFP/pHH21 or NA-EGFP/pHH21 RNA expression plasmid. Plasmids expressing mutant NP proteins were used as a substitute for the WT NP plasmid. After 48 h incubation, mRFP or EGFP expression was measured using a FACSCalibur instrument (Becton-Dickinson) with the CELLQuest software (Becton-Dickinson). The transcriptional activity of each NP mutant was calculated by counting the population of cells expressing the reporter gene.

2.6. Generation of recombinant viruses

Recombinant viruses were generated by DNA transfection as described previously (Kakisaka et al., 2015). Briefly, a co-culture of MDCK (4×10^5) or HEK-293T (6×10^5) cells was transfected with the eight viral genome-expressing plasmids and four viral protein-expressing plasmids. Plasmids expressing mutant NP proteins and RNA were used as substitutes for the WT NP plasmid. At 72 h post-transfection, recombinant viruses were harvested, and the viral titer in the cell supernatants was determined in a plaque assay.

2.7. Viral genome packaging assay

To evaluate viral genome packaging efficiency of each NP mutant, recombinant viruses were generated by DNA transfection as described previously (Li et al., 2009; Neumann et al., 2000). Briefly, HEK-293T (1×10^6) cells were transfected with the eight viral genome-expressing plasmids (PB2/pHH21, PB1/pHH21, PA/pHH21, HA/pHH21, NP/pHH21, NA/pHH21, M/pHH21, and NS/pHH21) and nine viral protein-expressing plasmids (PB1/pCAGGS, PB2/pCAGGS, PA/pCAGGS, NP/pCAGGS, NA/pCAGGS, HA/pCAGGS, NS2/pCAGGS, M1/pCAGGS, and M2/pCAGGS). Plasmids expressing mutant NP proteins were used as substitutes for the WT NP plasmid. In this expression system, vRNA expression plasmid encoding PB2-mRFP or NA-EGFP was used as a substitute for the correspondent RNA expression plasmid encoding the PB2 or NA gene, and the start codon of the NP gene encoded in the vRNA expression plasmid was replaced by a stop codon to avoid expression of wild-type NP protein. vRNAs encoding the PB2-mRFP and NA-EGFP reporter genes were used to monitor vRNA incorporation efficiency, because these reporter genes are flanked by the packaging signals of the PB2 and NA segments, respectively, and can be incorporated into recombinant viruses. The supernatants of transfected cells were harvested 48 h post-transfection and used to inoculate MDCK cells along with the A/WSN/1933 virus, which provided a functional polymerase complex