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Nuclear Localization of Influenza B Polymerase Proteins and Their Binary Complexes

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Summary

The viral RNA-dependent RNA polymerases of influenza A and B are trimeric complexes of PA, PB1, and PB2 subunits that are crucial for both transcription and replication of the viral genome. Unlike the significant progress made recently in understanding nuclear transport and molecular assembly of influenza A polymerase, little is known about the influenza B polymerase, although influenza B viruses cause severe upper respiratory disease in humans. The aim of this study was to characterize nuclear localization of the influenza B RNA polymerase proteins and binary complexes. We demonstrated that each polymerase protein has a nuclear localization function, and among them, the PB2 protein exclusively locates to the nucleus while PA and PB1 proteins are associated with the cytoplasm and the nucleus. Furthermore, we show that pairwise binary complexes are formed among the influenza B subunits (PA-PB1, PA-PB2, and PB1-PB2) and both PB1-PB2 and PA-PB2 complexes are predominantly associated with the nucleus while the PA-PB1 complex exhibits both nuclear and cytoplasmic fluorescence signals. Results of our studies represent the first step toward the understanding of nuclear transport and molecular assembly within the influenza B polymerase complex.

1. INTRODUCTION

Influenza viruses belong to the family orthomyxoviridae and are classified as type A, B, and C in accordance with antigenic differences among their nucleoprotein and matrix protein components (Palese and Shaw, 2007). Influenza A and B viruses cause severe upper respiratory disease in humans, while influenza C virus normally causes a mild disease

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(Palese and Shaw, 2007). Among these viruses, influenza A and B are similar in their morphology, genome structure, and protein function. A common feature of influenza A and B viruses is a viral RNA-dependant polymerase complex that consists of three subunits, namely PA, PB1, and PB2. This complex is required for transcription and replication of the viral RNA genome (Palese and Shaw, 2007). Previous studies of influenza A (Flu A) virus replication indicated that (i) PB1 plays a central role in the RNA elongation activity of the polymerase complex (Biswas and Nayak, 1994; Biswas and Nayak, 1996; Li et al., 2001), (ii) PB2 has a cap-binding function and initiates transcription (Fechter et al., 2003; Fodor, 2002; Guilligay et al., 2008), and (iii) PA is an endonuclease for the cap-snatching activity exhibited during influenza viral replication (Dias et al., 2009; Yuan et al., 2009).

Each of the three polymerase proteins of influenza A virus can independently perform nuclear import function (Jones et al., 1986; Mukaigawa and Nayak, 1991; Nath and Nayak, 1990; Nieto et al., 1994; Nieto et al., 1992). It has been shown that PA or PB1, when expressed alone, is associated with both the cytoplasm and the nucleus while PB2 alone predominantly locates to the nucleus (Deng et al., 2005; Fodor and Smith, 2004; Naito et al., 2007). Despite protein-specific nuclear localization functions, several lines of evidence have indicated that influenza A polymerase subunits can enter the nucleus through binary complexes (Deng et al., 2005; Fodor and Smith, 2004; Hemerka et al., 2009; Naito et al., 2007). A prevailing nuclear import and assembly model concerning the heterotrimeric complex of influenza A polymerase in virus- infected cells suggests that PA and PB1 interact initially in the cytoplasm and form a PA-PB1 complex, prior to their subsequent nuclear import as a binary complex. PB2 translocates to the nucleus independently, where it then associates with PA-PB1 to form a functional heterotrimeric complex (Deng et al., 2005).

In comparison with Flu A, little is known about the nuclear transport and assembly of the influenza B (Flu B) polymerase complex. In this study, we sought to characterize nuclear location of all three proteins of the influenza B polymerase complex using EGFP-tagged proteins. We also performed bimolecular fluorescence complementation (BiFC) experiments to determine both the formation of any potential binary complexes amongst the three protein subunits and their nuclear location. The results presented in this study provide a framework to further characterize the nuclear import and assembly of the influenza B polymerase complex and to examine this machinery as a target for antiviral discovery.

2. Material & Methods

2.1 Plasmids

To express Flu B polymerase subunits, cDNA sequences encoding PA, PB1, and PB2 were individually amplified from a Flu B Lee/40 reverse genetic system (Dauber et al., 2004) by PCR, and subcloned into the eukaryotic expression vector pCAGGS (Matsuda and Cepko, 2004). To study the nuclear localization function of the influenza B polymerase subunit proteins, three EGFP fusion constructs (EGFP-B.PA, EGFP-B.PB1, and EGFP-B.PB2) were produced by fusing EGFP to the N-terminus of viral proteins. Additionally, plasmids for the BiFC assay (which is used to detect protein-protein interaction) (Hemerka et al., 2009; Wang et al., 2010) were constructed by fusing sequences encoding the amino (residues 1 to 173, VN) or carboxyl (residues 155 to 238, VC) fragments of the Venus Fluorescence Protein to the N terminus of each polymerase protein through an HA tag linker to produce: pVN-B.PA, pVC-B.PA, pVN-B.PB1, pVC-B.PB1, pVN-B.PB2, and pVC-B.PB2. To validate the specificity of BiFC in detecting PA-PB1 interactions, a mutant, designated VN-B.PB1 Δ N25 containing a deletion of the 25 N-terminal residues of PB1, was generated by a traditional PCR-overlapping-PCR mutagenesis approach as described previously (Hemerka et al., 2009; Jin et al., 2007).

2.2 Cells and antibodies

COS-1 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10 % fetal bovine serum. Antibodies used for this study were provided from the following sources: anti-GFP rabbit polyclonal antibody (Abcam), anti-RL rabbit polyclonal antibody (MBL), and Alexa Fluor 680/750 goat anti-rabbit secondary antibody (Li-Cor).

2.3 Odyssey Western Blot Assay

COS-1 cells grown on 12-well plates were transfected with various plasmids (1.0 µg) using *TransIT-LT1* according to the manufacturer's instructions (Mirus). At 24 or 48 h posttransfection, cellular protein extracts were prepared and analyzed by SDS-PAGE and Western blot as previously described (Hemerka et al., 2009; Wang et al., 2010).

2.4 Confocal microscopy

COS-1 cells grown on 12-well plates containing glass coverslips were transfected with EGFP-B.PA, EGFP-B.PB1, or EGFP-B.PB2 as indicated. At 24 or 48 h posttransfection, cells on coverslips were fixed with 2% paraformaldehyde in PBS followed by nuclei staining for 1 min with 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen). Confocal microscopic analysis of fluorescent imaging was done using a FluoView FV300 confocal system (Olympus) as described previously (Hemerka et al., 2009; Jin et al., 2007).

2.5 Bimolecular fluorescence complementation assay

Bimolecular fluorescence complementation assay (BiFC) coupled with confocal microscopy was used to characterize protein-protein interactions in living cells. COS-1 cells grown on coverslips in 12-well plates, were transfected with either previously selected combinations of BiFC plasmids or an individual BiFC plasmid (1.0 µg each plasmid). At 24 hours posttransfection, cell monolayers were fixed and stained with DAPI (Invitrogen). Image collection and processing was performed as described previously.

3. Results and Discussion

3.1 Nuclear Localization of influenza B polymerase protein subunits

We first characterized the cellular localization of the Flu B subunit proteins by transiently transfecting COS-1 cells with each EGFP fusion construct and measuring their nuclear location as a function of GFP expression in the cytoplasm and the nucleus (Fig. 1). We were interested in determining whether each subunit protein, like its counterpart in Flu A polymerase, had a nuclear localization function. A similar GFP-tagged fusion protein strategy has been used successfully in previous investigation of nuclear transport for Flu A polymerase protein subunits (Fodor and Smith, 2004). Our nuclear localization experiment generated a number of interesting observations concerning Flu B polymerase proteins (Fig. 1). First, each protein has a nuclear import function and encodes a nuclear localization signal (Fig. 1A). Secondly, PB2 protein locates almost, if not completely, exclusively to the nucleus while PA and PB1 proteins are always associated with both the cytoplasm and the nucleus (Figs. 1A and 1B). For example, at 48 h posttransfection, we observed (Fig. 1B) approximately 82% of counted EGFP-positive cells exhibited exclusively nuclear fluorescence signals in cells expressing EGFP-B.PB2, whereas only approximately 3% and 14% of EGFP-positive cells displaying the same pattern (i.e., exclusively nuclear localization) were observed in cells expressing EGFP-B.PA and EGFP-B.PB1, respectively. The result suggested that PB2 has a strong and predominant nuclear localization function by compared to PA and PB1 proteins. The cellular localization of Flu B polymerase subunits was similar to that observed for Flu A polymerase subunits (Fodor and Smith, 2004). It is

interesting to note that both the Flu A and Flu B PB2 protein exhibited an intrinsic property of nuclear accumulation during a steady state of protein expression (e.g., 48 h post transfection) (Fodor and Smith, 2004; Jones et al., 1986). A nuclear location for the Flu B PB2 protein was also observed in transfected COS-1 cells at 24 h posttransfection (data not shown).

3.2 Detection and nuclear localization of binary complexes of influenza B polymerase by bimolecular fluorescence complementation (BiFC)

Next, we used a previously developed BiFC assay to study Flu B polymerase (Hemerka et al., 2009). We were interested in determining (i) whether BiFC can effectively detect binary complexes that may exist among Flu B polymerase protein subunits and (ii) the cellular localization of binary complexes formed through BiFC. All BiFC fusion proteins were expressed well with expected sizes (Fig. 2A). Results of our BiFC experiments demonstrated pairwise protein-protein interactions (PA-PB1, PB1-PB2, and PA-PB2) of Flu B polymerase complex which is similar to those reported for Flu A polymerase using BiFC and yeast two-hybrid assays (Hemerka et al., 2009; Shapira et al., 2009). While we did not quantify the BiFC signal, qualitatively, the PA-PB1 interaction appears to be more robust compared to PA-PB2 or PB1-PB2 (Fig. 2B). Interestingly, combining PB1 with PB2 in various BiFC orientations did not reconstitute the levels of fluorescence complementation similar to those observed for Flu B PA-PB1 (Fig. 2B or data not shown). It should be noted that the BiFC assay, when used in Flu A virus polymerase proteins, revealed a PB1-PB2 interaction comparable to PA-PB1 (Hemerka et al., 2009), which differs substantially from our current observation of a weaker Flu B polymerase PB1-PB2 interaction. The reason for the observed weak interactions for PA-PB2 and PB1-PB2 is not clear but could be attributed to a low expression level of VN.B.PB2 fusion protein in comparison to its counterparts (Fig. 2A).

To validate the specificity of the BiFC assay, we took advantage of recent findings that Flu B PB1, like its counterpart in Flu A, utilizes its N-terminal domain to bind to PA (Wunderlich et al., 2010; Wunderlich et al., 2009). We generated a VN-B.PB1^{ΔN25} fusion construct in which 25 N-terminal residues of PB1 were deleted and fused to VN through a linker at its N-terminus in an identical manner to WT VN-PB1 (Fig. 2A). While COS-1 cells transfected with BiFC VN-B.PB1 or VN-B.PB1^{ΔN25} resulted in a similar level of protein expression (Fig. 2A), imaging analysis revealed no fluorescence complementation amongst COS-1 cells transfected with the BiFC VC-B.PA/VN-B.PB1^{ΔN25} combinations (Fig. 2B), indicating the specificity of BiFC assay in detecting PA-PB1 interaction. Similar experiments are needed to validate the observed PB1-PB2 and PA-PB2 interactions and rule out nonspecific effects that might be associated with the BiFC approach.

By analysis of the cellular localization, we noted that all three binary complexes exhibited nuclear location (Fig. 2B). At 48 h posttransfection, both PB1-PB2 and PA-PB2 complexes located to the nucleus while PA-PB1 complex was still distributed to both the cytoplasm and nucleus. Interestingly, the two binary complexes (PB1-PB2 and PA-PB2) involving PB2 exhibited the same pattern of nuclear location as PB2 alone. The observation may suggest that PB2 is superior to the other two proteins (PA and PB1) in driving protein accumulation in the nucleus of its own expression or its associated complex. This difference warrants future investigation of PB2's role in influenza B polymerase assembly and virus replication.

In summary, we demonstrated in this study that each polymerase protein has a nuclear localization function, and that an intrinsic property of nuclear accumulation is observed in PB2 but not the other two polymerase proteins. With the BiFC assay we observed that pairwise interactions occur between each of the three subunits of the influenza B polymerase in the absence of a third partner (e.g., PA/PB1 without PB2). The PA-PB1 interaction

appears to be the strongest, while the remaining pairs are much weaker. Cellular localization properties of influenza B polymerase complexes are similar to those demonstrated for the influenza A polymerase complex, which continues to support a theme that these two viruses are closely related with each other in terms of protein structure and function, although they differ substantially in infectious ecology (Palese and Shaw, 2007). Results of our studies represent the first step toward understanding of nuclear transport and molecular assembly of influenza B polymerase complex.

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REFERENCES

- Biswas SK, Nayak DP. Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1. *J Virol* 1994;68(3):1819–1826. [PubMed: 8107244]
- Biswas SK, Nayak DP. Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. *J Virol* 1996;70(10):6716–6722. [PubMed: 8794308]
- Dauber B, Heins G, Wolff T. The influenza B virus nonstructural NS1 protein is essential for efficient viral growth and antagonizes beta interferon induction. *J Virol* 2004;78(4):1865–1872. [PubMed: 14747551]
- Deng T, Sharps J, Fodor E, Brownlee GG. In vitro assembly of PB2 with a PB1-PA dimer supports a new model of assembly of influenza A virus polymerase subunits into a functional trimeric complex. *J Virol* 2005;79(13):8669–8674. [PubMed: 15956611]
- Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, Baudin F, Cusack S, Ruigrok RW. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature* 2009;458(7240):914–918. [PubMed: 19194459]
- Fechter P, Mingay L, Sharps J, Chambers A, Fodor E, Brownlee GG. Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. *J Biol Chem* 2003;278(22):20381–20388. [PubMed: 12646557]
- Fodor, E.; Brownlee, GG. Influenza virus replication. In: Potter, CW., editor. *Influenza*. New York, N.Y.: Elsevier; 2002. p. 1-29.
- Fodor E, Smith M. The PA subunit is required for efficient nuclear accumulation of the PB1 subunit of the influenza A virus RNA polymerase complex. *J Virol* 2004;78(17):9144–9153. [PubMed: 15308710]
- Guilligay D, Tarendeau F, Resa-Infante P, Coloma R, Crepin T, Sehr P, Lewis J, Ruigrok RW, Ortin J, Hart DJ, Cusack S. The structural basis for cap binding by influenza virus polymerase subunit PB2. *Nat Struct Mol Biol* 2008;15(5):500–506. [PubMed: 18454157]
- Hemerka JN, Wang D, Weng Y, Lu W, Kaushik RS, Jin J, Harmon AF, Li F. Detection and characterization of influenza A virus PA-PB2 interaction through a bimolecular fluorescence complementation assay. *J Virol* 2009;83(8):3944–3955. [PubMed: 19193801]
- Jin J, Sturgeon T, Chen C, Watkins SC, Weisz OA, Montelaro RC. Distinct intracellular trafficking of equine infectious anemia virus and human immunodeficiency virus type 1 Gag during viral assembly and budding revealed by bimolecular fluorescence complementation assays. *J Virol* 2007;81(20):11226–11235. [PubMed: 17686839]
- Jones IM, Reay PA, Philpott KL. Nuclear location of all three influenza polymerase proteins and a nuclear signal in polymerase PB2. *EMBO J* 1986;5(9):2371–2376. [PubMed: 3023071]

- Li ML, Rao P, Krug RM. The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. *EMBO J* 2001;20(8):2078–2086. [PubMed: 11296240]
- Matsuda T, Cepko CL. Electroporation and RNA interference in the rodent retina in vivo and in vitro. *Proc Natl Acad Sci U S A* 2004;101(1):16–22. [PubMed: 14603031]
- Mukaigawa J, Nayak DP. Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. *J Virol* 1991;65(1):245–253. [PubMed: 1985200]
- Naito T, Momose F, Kawaguchi A, Nagata K. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. *J Virol* 2007;81(3):1339–1349. [PubMed: 17121807]
- Nath ST, Nayak DP. Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1 N1). *Mol Cell Biol* 1990;10(8):4139–4145. [PubMed: 2196448]
- Nieto A, de la Luna S, Barcena J, Portela A, Ortin J. Complex structure of the nuclear translocation signal of influenza virus polymerase PA subunit. *J Gen Virol* 1994;75(Pt 1):29–36. [PubMed: 8113737]
- Nieto A, de la Luna S, Barcena J, Portela A, Valcarcel J, Melero JA, Ortin J. Nuclear transport of influenza virus polymerase PA protein. *Virus Res* 1992;24(1):65–75. [PubMed: 1320800]
- Palese, P.; Shaw, M. Orthomyxoviridae: The viruses and their replication. In: Fields, BN.; Knipe, DM.; Howley, PM., editors. *Field's Virology*. 5th ed.. Vol. 2. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007. p. 1647-1689.
- Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, Gupta PB, Hao T, Silver SJ, Root DE, Hill DE, Regev A, Hacohen N. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. *Cell* 2009;139(7):1255–1267. [PubMed: 20064372]
- Wang D, Harmon A, Jin J, Francis DH, Christopher-Hennings J, Nelson E, Montelaro RC, Li F. The lack of an inherent membrane targeting signal is responsible for the failure of the matrix (M1) protein of influenza A virus to bud into virus-like particles. *J Virol* 2010;84(9):4673–4681. [PubMed: 20181696]
- Wunderlich K, Juozapaitis M, Manz B, Mayer D, Gotz V, Zohner A, Wolff T, Schwemmle M, Martin A. Limited compatibility of polymerase subunit interactions in influenza A and B viruses. *J Biol Chem* 2010;285(22):16704–16712. [PubMed: 20363752]
- Wunderlich K, Mayer D, Ranadheera C, Holler AS, Manz B, Martin A, Chase G, Tegge W, Frank R, Kessler U, Schwemmle M. Identification of a PA-binding peptide with inhibitory activity against influenza A and B virus replication. *PLoS One* 2009;4(10):e7517. [PubMed: 19841738]
- Yuan P, Bartlam M, Lou Z, Chen S, Zhou J, He X, Lv Z, Ge R, Li X, Deng T, Fodor E, Rao Z, Liu Y. Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site. *Nature* 2009;458(7240):909–913. [PubMed: 19194458]

Figure 1. Nuclear localization of influenza B polymerase proteins

(A) Fluorescence microscopy of live cells expressing the indicated EGFP fusion proteins. At 48 h posttransfection, cells expressing EGFP fusion proteins were fixed, stained with DAPI (blue), and examined using confocal microscopy at a 100×magnification for intracellular localization. Imaging data shown is representative of six independent experiments. (B) Subcellular localization of influenza B polymerase proteins. At 48 h posttransfection, cells expressing EGFP fusion proteins were fixed, stained with DAPI (blue), counted, and examined for intracellular localization pattern (cytoplasm, cytoplasm + nucleus, and nucleus) of each EGFP fusion protein (EGFP-B.PA, EGFP-B.PB1, and EGFP-B.PB2) by fluorescence microscopy. The total number of cells expressing EGFP counted was 32 for EGFP-B.PA; 29 for EGFP-B.PB1, and 46 for EGFP-B.PB2, respectively. The percentage of cells expressing exclusive nuclear fluorescence signals relative to all the counted EGFP-positive cells was calculated, and was used to determine nuclear import function of Flu B polymerase proteins.

Figure 2. Visualization of influenza B polymerase protein binary complexes by BiFC

(A) Schematic representation of BiFC constructs and their protein expressions. At 48 h post transfection, cellular lysates were collected from COS-1 cells transfected with the indicated BiFC constructs, and were subject to analysis by Western-blotting with anti-EGFP antibody. Numbers on the right indicate the migration positions of molecular mass markers (in kilodaltons). (B) Fluorescence microscopy of live cells expressing the indicated fusion proteins. COS-1 cells grown on glass coverslips were transfected with indicated BiFC plasmids. At 48 h post transfection, cells were fixed, stained with DAPI, and imaged using confocal microscopy at a 100×magnification. The figures shown here are representative of at least six independent experiments.