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Endonuclease substrate selectivity characterized with full-length PA of influenza A virus polymerase

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ABSTRACT

The influenza A polymerase is a heterotrimer which transcribes viral mRNAs and replicates the viral genome. To initiate synthesis of mRNA, the polymerase binds a host pre-mRNA and cleaves a short primer downstream of the 5′ end cap structure. The N-terminal domain of PA has been demonstrated to have endonuclease activity *in vitro*. Here we sought to better understand the biochemical nature of the PA endonuclease by developing an improved assay using full-length PA protein. This full-length protein is active against both RNA and DNA in a cap-independent manner and can use several different divalent cations as cofactors, which affects the secondary structure of the full-length PA. Our *in vitro* assay was also able to demonstrate the minimal substrate size and sequence selectivity of the PA protein, which is crucial information for inhibitor design. Finally, we confirmed the observed endonuclease activity of the full-length PA with a FRET-based assay.

Introduction

Influenza A virus encodes an RNA-dependent RNA-polymerase responsible for transcription of viral mRNAs and replication of the genomic RNA segments within the host cell nucleus. The viral polymerase is a heterotrimer composed of two basic proteins, PB1 and PB2, as well as the acidic protein PA. Genome replication occurs through primer-independent initiation of (+) strand RNA synthesis from the (−) strand viral genomic segments, which are then used as a template for production of new (−) strand genomic RNA. This process occurs mainly through the active site of the PB1 subunit, which contains the conserved motifs of an RNA polymerase (Biswas and Nayak, 1994; Poch et al., 1989). Viral mRNAs, however, must contain both a 5′ cap structure and a poly(A) tail in order to be recognized by host translational machinery. The polymerase creates a poly(A) tail through a stuttering mechanism (Poon et al., 1999), but the cap structure of viral mRNAs must be derived from cellular pre-mRNAs in a process known as cap-snatching (Beaton and Krug, 1981; Bouloy et al., 1978). The cellular mRNA is cleaved 10–15 nucleotides from the cap structure and is then used as a primer for transcription of the viral mRNA (Plotch et al., 1981).

The endonuclease activity involved in cap snatching is a novel viral process, making it an attractive target for small molecule inhibition. The cleavage of the host mRNA is catalyzed by endonuclease activity contained within the polymerase complex. This endonuclease active site has been previously reported to be in either the PB1 or the PB2 subunit but was not conclusively demonstrated (Li et al., 2001; Shi et al., 1995). This uncertainty was resolved when crystal structures of the N-terminal domain of the PA subunit revealed an endonuclease active site with similarity to type II restriction endonucleases such as *Sda* I (Dias et al., 2009; Yuan et al., 2009). The endonuclease activity of the PA subunit was biochemically confirmed using the purified N-terminal truncation (Crepin et al., 2010; Dias et al., 2009). The endonuclease domain of PA has been shown to be active against both single stranded RNA and DNA and is dependent on the binding of divalent cations (Dias et al., 2009). Maximal activity is seen using manganese, but magnesium has been argued to be more biologically relevant (Crepin et al., 2010; Dias et al., 2009; Doan et al., 1999). The active site is highly conserved among influenza viruses with only a single amino acid difference among influenza B and influenza C viruses (Yuan et al., 2009). A similar viral endonuclease has been identified in the L protein of viruses in the *Arenaviridae* and *Bunyaviridae* families, though the active site and substrate specificity are distinct from that of influenza PA (Morin et al., 2010; Reguera et al., 2010).

Previous studies of the influenza A endonuclease have used either viral ribonucleoparticles isolated from mammalian cells or the truncated N-terminal domain, which can be purified from *E. coli*. Both of these systems have limitations that make them unsuitable for in-depth enzymology and, ultimately, screening for

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small molecule inhibitors. Purification of protein from infected mammalian cells is not readily scaled up, making the large-scale production necessary for biochemical studies and high throughput screening not feasible. Assays using these viral ribonucleoprotein particles have also needed to use a capped substrate and the addition of the viral promoter RNA in order to observe endonuclease activity (Hagen et al., 1994). Protein purification from *E. coli* is amenable to large-scale production of protein, but the use of a truncated enzyme has several drawbacks. It was noted by Yuan et al. that, in their studies, PA N-terminal domain endonuclease activity could not be determined due to nuclease contamination (Yuan et al., 2009). Dias et al. were able to observe endonuclease activity using the truncated PA protein, but only in the presence of manganese and not with any other divalent cation tested (Dias et al., 2009). More recently, Crepin et al. demonstrated that the truncated PA subunit had endonuclease activity in the presence of both magnesium and manganese, but this activity was low and their assay used long incubations (up to 6 h) and did not quantify activity (Crepin et al., 2010). Given that cell-derived ribonucleoprotein particles have endonuclease activity with several metal ions in addition to magnesium and manganese (Doan et al., 1999), the truncated PA protein seems to behave differently than the full trimeric polymerase. We sought to develop an improved assay for characterizing the endonuclease activity of the influenza A protein using the full-length PA protein. We hypothesize that this intact protein will be more active as well as more biologically relevant than the truncated PA N-terminal domain previously used.

Here we demonstrate that enzymatically active intact PA can be purified from insect cells. Among the strains tested, the avian Nanchang strain was shown to have the highest *in vitro* endonuclease activity. This *in vitro* assay was used to show the minimal substrate size and sequence specificity. We also demonstrate that the divalent cation cofactor affects the cleavage pattern and secondary structure of the protein as well as the overall activity. The endonuclease activity of the PA subunit is comparable to that of the full trimeric polymerase but is expressed at much higher levels using our system. Given the difficultly in purifying the polymerase complex, our *in vitro* assay using the intact PA subunit is an attractive model for studying the endonuclease activity of the viral polymerase and is also a valuable tool for development of new antivirals.

Results

Comparison of PA endonuclease activities from different strains

Previous work has used purified PA from a single influenza A strain and no comparison of endonuclease activity across different strains has been performed. We purified PA from a variety of strains in order to determine the protein with the highest endonuclease activity. Using TAP-tagged constructs (shown in Fig. S1A), we were able to express PA from four different strains: the lab adapted H1N1 WSN (WSN), the pandemic 1918 H1N1 (1918), a high pathogenicity avian H5N1 strain isolated from a human (H5N1), and a low pathogenicity avian strain isolated from a chicken (Nanchang). The PA subunit from the 2009 pandemic strain expressed at very low levels (data not shown) despite being codon optimized, which precluded its use in our study. The intact PA subunits from the four different influenza A strains were purified from Tni insect cells and diluted to equal concentrations (Fig. S1B). To measure endonuclease activity, the proteins were incubated with a 5′ end labeled 33mer RNA. Manganese was added to the reactions, as maximal activity has been reported with this metal cofactor (Crepin et al., 2010). We tested activity at temperatures ranging from 30 to 42 °C (data not shown) and found that all of the strains had maximal endonuclease activity at 37 °C. All further experiments were carried out at 37 °C. After 1 h incubation, products were resolved using 20% urea PAGE. As shown in Fig. 1, distinct cleavage products were observed using all four PA proteins, although the levels of endonuclease activity varied between the strains. The pattern of cleavage is likely to be partially determined by the structured nature of this RNA substrate (predicted by RNAstructure (Reuter and Mathews, 2010)), as the PA endonuclease is only able to cleave single stranded regions (Dias et al., 2009). An additional caveat is that only products retaining the 5′ end label can be visualized on the gel. The smallest product (Fig. 1, *) results from the cleavage of the 5′ terminal G from the RNA (confirmed with size markers, data not shown). Gels in subsequent figures are cropped for presentation purposes, but this cleavage product was observed in all reactions. The presence of cleavage products near both ends of the RNA suggests that there is little directionality with PA binding given this long substrate. In order to cleave very close to the 5′ end, the protein must be binding the substrate in the opposite orientation as compared to when cleaving a capped

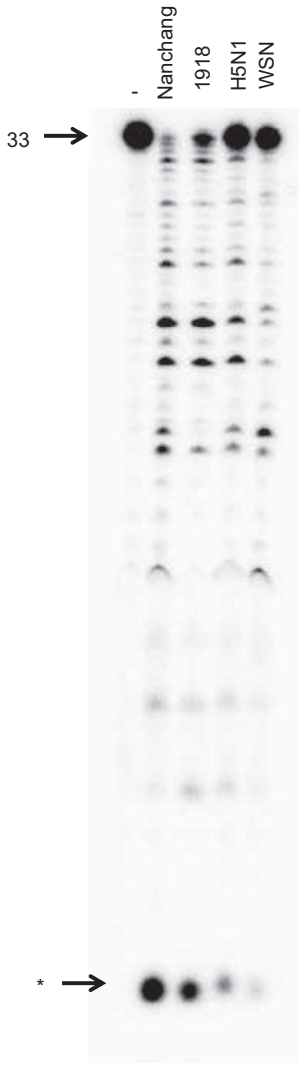

Fig. 1. Full-length purified PA protein from insect cells has endonuclease activity. Comparison of PA activity among strains. Protein was incubated with a 5′ ^{32}P-labeled 33mer RNA for 60 min at 37 °C in the presence of 1 mM $MnCl_2$. A control sample (-) was incubated without protein. Products were resolved using 20% urea PAGE. The full-length substrate is indicated with an arrow (33mer) and the smallest product is indicated (*).