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Biophysical characterization of sites of host adaptive mutation in the influenza A virus RNA polymerase PB2 RNA-binding domain

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ABSTRACT

Influenza RNA polymerase is composed of three subunits, PA, PB1, and PB2, which interact with each other for transcription and replication of the viral RNA genome in the nucleus of infected cells. PB2 RNA-binding 627-domain (residues 535–693), located in the C-terminus, presents a highly basic surface around residue lysine 627 and has been proposed to interact with viral or cellular factors, resulting in host adaptation. However, the function of this domain is not yet characterized in detail. In this study, we identified RNA-binding activity and RNA-binding surfaces in both the N-terminal and basic C-terminal regions of PB2 627-domain using NMR experiments. Through mutagenesis studies, we confirmed which residues directly interact with RNA and mapped their locations on the RNA-binding surface. In addition, by luciferase activity assays, we showed that influenza virus polymerase activity may correlate with the interaction between PB2 and RNA. Representative host adaptive mutations (residues 591 and 627) were found to be located on the RNA-binding surface and were confirmed to directly interact with RNA and to affect polymerase activity. From these results, we suggest that influenza virus polymerase activity may be regulated through the interaction between PB2 627-domain and RNA and that consequently host adaptation of the virus may be influenced.

1. Introduction

Influenza A viruses are viral pathogens that cause both seasonal influenza epidemics and global pandemics, threatening human health. Influenza A viruses contain a genome composed of eight segments of single-stranded, negative-sense RNA that encode 10 major and several auxiliary proteins including the RNA-dependent RNA polymerase (RdRp) (Muramoto et al., 2013). Influenza RNA polymerase is a heterotrimer consisting of three subunits – PA, PB1, and PB2 – and plays a crucial role in replication and transcription of the viral genome in the nucleus of infected cells. During replication, viral RNA (vRNA) is first converted to complementary RNA (cRNA) and then cRNA is used as a template to replicate vRNA. Viral transcription follows the cap-snatching mechanism in which a capped RNA primer, cleaved from host pre-mRNA, is created, and then the PB1 subunit synthesizes viral mRNA using the capped RNA primer (Dias et al., 2009; Guilligay et al., 2008; Yuan et al., 2009).

Many structures of influenza RNA polymerase subunits have been determined, with the exception of the PB1 subunit, which is highly insoluble. The PB2 subunit of the RNA polymerase has been especially well structurally characterized (Fig. 1A) (Guilligay et al., 2008; Kuzuhara et al., 2009; Sugiyama et al., 2009; Tarendeau et al., 2007, 2008). Furthermore, PB2 is known to contain many host adaptive mutations, which are related to the adaptation of avian influenza virus to humans and the enhancement of viral replication in mammalian cells (Boivin et al., 2010; Naffakh et al., 2008; Reperant et al., 2012). In particular, PB2 627-domain (residues 535–693) includes residues Q591K/R and E627K, which are known as major host adaptive mutations and have been extensively studied (Hatta et al., 2001; Liu et al., 2012; Steel et al., 2009). However, the molecular mechanism of host adaptation is not clear. Whereas residue 627 is Glu (E) in avian influenza virus, Lys (K) is mainly identified at this position in the human influenza virus. And Lys (K) substitution of Glu (E) appears to increase viral polymerase activity in mammalian cells (Subbarao et al., 1993). However, the 2009 pandemic H1N1 strain (Fig. 1B, 2009-H1N1 (human)) had enhanced

Abbreviations: ATCC, American Type Culture Collection; EMSA, electrophoretic mobility shift assays; FAM, fluorescein amidite; FLuc, firefly luciferase; HEK, human embryonic kidney; NMR, nuclear magnetic resonance; RdRp, RNA-dependent RNA polymerase; RLuc, *Renilla* luciferase.

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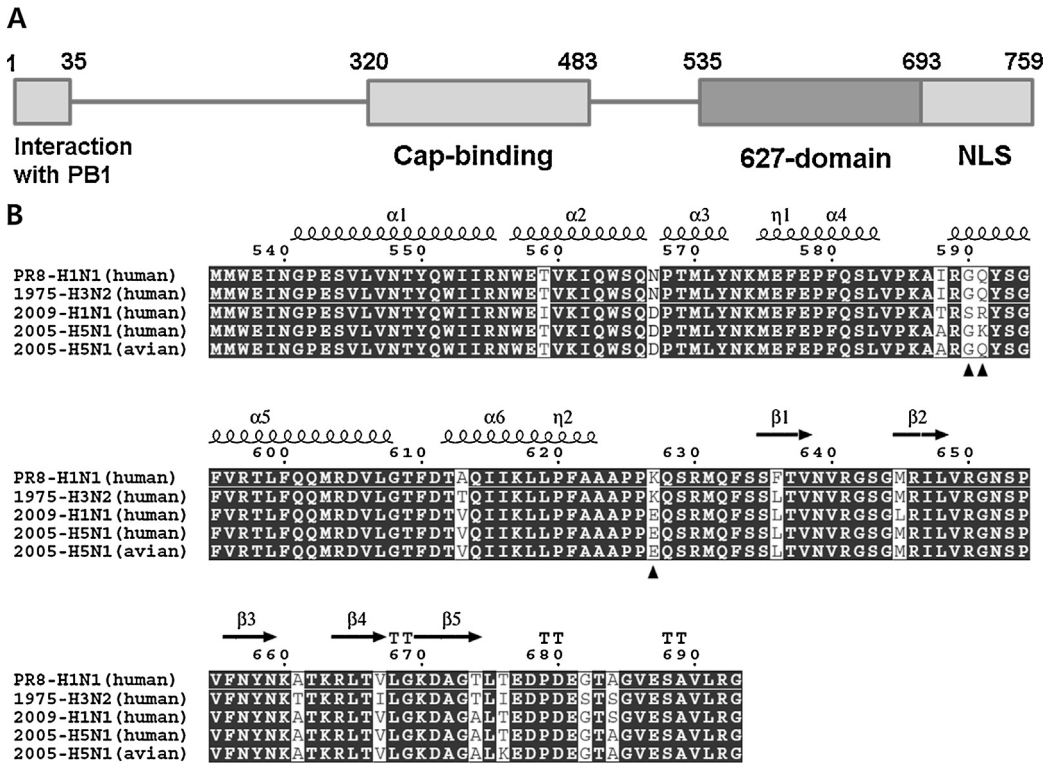

Fig. 1. Modular domains and structural elements of PB2. (A) Schematic diagram of full-length PB2. PB2 627-domain is located on the C-terminus of PB2, following cap-binding domain, and contains host adaptive mutations such as residues 590, 591, and 627. (B) Sequence alignment of the PB2 627-domain from various strains of influenza A virus using ESPript program (Gouet et al., 1999). The strain used in this study is PR8-H1N1. The residues known as host adaptive mutations are indicated by triangles (▲). Secondary structure elements are shown on the top of the sequence alignment. The secondary structure elements are from H3N2-PB2 627-domain (PDB: 2VY7). α = α-helix, β = β-strand, $\eta = 3_{10}$ helix, and TT = β-turn.

polymerase activity in mammalian cells, even though it retained a Glu at position 627. Extensive studies on this isotype showed that a basic amino acid at residue 591 rescues polymerase activity by neutralizing E627 (Mehle and Doudna, 2009; Yamada et al., 2010). Thus host adaptive mutations play a critical role in viral replication and host adaptation in mammals.

In spite of this importance and the well-characterized structure of PB2 627-domain (Kuzuhara et al., 2009; Tarendeau et al., 2008), the function of this domain is not yet characterized in detail. The 627-domain has been thought to interact with an RNA primer (Kuzuhara et al., 2009), on account of the basic surface of this domain including the residue K627, or proposed to interact with unknown cellular factors (Hudjetz and Gabriel, 2012; Mehle and Doudna, 2008). In this study, we characterized the RNA-binding activity and identified the RNA-binding surface of the PB2 627-domain using nuclear magnetic resonance (NMR) experiments. We also confirmed the direct interaction between this domain and RNA by site-directed mutagenesis and electrophoretic mobility shift assays (EMSAs). The physiological role of their interaction was investigated by an influenza virus polymerase activity assay. Finally, we observed that the major host adaptive mutations, such as Q591K/R and E627K in the PB2 627-domain, are involved in RNA-binding and affect influenza virus polymerase activity.

2. Materials and methods

2.1. Protein purification

The gene encoding PB2 627-domain (residues 535–693, 18 kDa) was amplified by PCR using cDNA (influenza A/Puerto Rico/8/1934, H1N1) in pET14b. The product was cloned into the NdeI and BamHI sites of the pET15b expression system. This expression plasmid was transformed into the BL21(DE3) CodonPlus RIL strain. Uniform isotope labeling was obtained by growing the cells in M9 minimal medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl). For backbone resonance assignments, the minimal medium was supplemented with 1 g/L of $^{15}NH_4Cl$ and 2 g/L of ^{13}C-glucose and using D_2O instead of H_2O. Protein expression was induced by addition of 0.4 mM IPTG at 0.7–0.8 OD_{600}. After 18 h further incubation at 20 °C, the cells were harvested by centrifugation. Harvested cells were resuspended in Ni-NTA binding buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole) and lysed by sonication. Cell debris was removed by centrifugation at 15,000 rpm for 1 h. The supernatant was loaded onto a Ni-NTA column (Qiagen). After washing, the protein was eluted by elution buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 125 mM imidazole). The His tag was removed with 100 U thrombin for 12 h at 20 °C, and the protein was dialyzed against SP-buffer (20 mM Tris–HCl, pH 7.0, 50 mM NaCl) and loaded onto an SP-sepharose column (GE-Healthcare). The peak fraction was eluted by a 50–1000 mM linear gradient of NaCl. After that, the protein was further purified by Superdex-75 gel filtration chromatography. The purity and homogeneity of the sample were assessed using SDS-PAGE.

2.2. Electrophoretic mobility shift assay (EMSA)

RNAs and various mutants of PB2 627-domain were incubated in 20 μl binding buffer (10 mM HEPES/KOH, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.25 mM DTT, 0.1 mM EDTA, 10% glycerol). The RNA substrates were single strand RNAs (5′vRNA; 5′-AGUAGAAACAAGG-3′, 3′vRNA; 5′-CCUGCUUUUGCU-3′, polyAC RNA; 5′-ACACACACACACA-3′ and polyGU RNA; 5-GUGUGUGUGUGUG-3′) and were synthesized by Integrated DNA Technologies. Reaction mixtures were incubated at room