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Involvement of the N-terminal portion of influenza virus RNA polymerase subunit PB1 in nucleotide recognition

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ABSTRACT

The influenza virus PB1 protein functions as a catalytic subunit of the viral RNA-dependent RNA polymerase and contains the highly conserved motifs of RNA-dependent RNA polymerases together with putative nucleotide-binding sites. PB1 also binds to viral genomic RNAs and its replicative intermediates through the promoter regions. The detail function and interplay between functional domains are not clarified although a part of structures and functions of PB1 have been clarified. In this study, we analyzed the function of PB1 subunit in the sense of nucleotide recognition using ribavirin, which is a nucleoside analog and inhibits viral RNA synthesis of many RNA viruses including influenza virus. We screened ribavirin-resistant PB1 mutants from randomly mutated PB1 cDNA library using a mini-replicon assay, and we identified a single mutation at the amino acid position 27 of PB1 as an important residue for the nucleotide recognition.

1. Introduction

Influenza A virus belongs to the family of *Orthomyxoviridae*. Its genome consists of eight-segmented and single-stranded RNAs of negative polarity (vRNA). Each segment is encapsidated by nucleoprotein (NP) and associated with viral RNA polymerases to form viral ribonucleoprotein (vRNP) complexes. The vRNP complex is a basic unit for both transcription and replication [1]. The viral mRNA transcription is initiated using capped oligonucleotide as a primer. The elongation of mRNA chain proceeds until the viral polymerase reaches oligo U sequence present near the 5′-terminus of vRNA, and then the poly A tail is added by the viral RNA polymerase. In the viral genome replication, full-length cRNA (complementary RNA to vRNA) is generated from vRNA in a primer-independent manner, and progeny vRNAs are amplified from cRNA by the viral RNA polymerase. The viral RNA polymerase consists of PB1, PB2, and PA. PB1 functions as a catalytic subunit and the assembly core of the viral RNA polymerase [2–7]. PA is genetically found to be involved in the replication process and the polymerase assembly [8] and have the endonuclease activity [9–12]. PB2 is responsible for the recognition and binding of the cap structure [1,13–16].

The 14 amino acids residues from the N-terminus of PB1 interact with PA [4–7,17–20], while the C-terminal region of PB1 between amino acid (a.a.) positions 678–757 interacts with PB2 [4–6,21,22]. PB1 contains the motifs highly conserved among RNA-dependent RNA polymerases [2]. There are two putative nucleotide-binding sites between a.a. positions 179–297 and 458–519 [23,24]. Moreover, the N-terminal (a.a. positions 1–83) and C-terminal (a.a. positions 494–757) regions of PB1 are suggested to interact with the vRNA promoter [25]. In addition, the a.a. positions 249–254 of PB1 is important for the vRNA binding, and Phe251 (when the number indicates the amino acid position) and Phe254 are essential for this binding [26]. It is also reported that the regions between a.a. positions 1–139 and 267–493 bind to the cRNA promoter [27].

Ribavirin (1-β-D-ribofusanosyl-1,2,4-triazole-3-carboxamide, also known as Virazole) is a synthetic purine nucleoside analogue first synthesized by Sidwell et al. in 1972 [28]. It is phosphorylated by cellular adenosine kinases into ribavirin monophosphate, diphosphate, and triphosphate (RMP, RDP, and RTP, respectively) [29,30]. Ribavirin inhibits various RNA-dependent RNA polymerases such as those from influenza virus [31], vesicular stomatitis virus [32], La Crosse virus [33], Hantaan virus [34], Foot and mouth disease virus [35], West Nile virus [36], Andes virus [37], and Hepatitis C virus [38]. In contrast, ribavirin does not inhibit cellular RNA polymerase I, RNA polymerase II, and poly (A) polymerase [39]. Ribavirin inhibits the inosine monophosphate dehydrogenase, so that the *de novo* synthesis of purine nucleosides is interrupted [40]. Further, it

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is proposed that ribavirin inhibits the RNA capping and RNA polymerization by virus-encoded enzymes [40]. It is also known that since ribavirin forms hydrogen bonds with cytidine and uridine, the incorporation of ribavirin into viral genomic RNA induces G to A transition leading to the lethal mutations [29].

In this study, to elucidate functional residues required for the PB1 activity, we tried to isolate PB1 mutants which are resistant to ribavirin. We found that the amino acid position 27 of PB1 is important for nucleotide recognition.

2. Materials and methods

2.1. Biological materials

Monolayer cultures of 293T and MDCK cells were maintained at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) and minimal essential medium (MEM) (Nissui), respectively, supplemented with 10% fetal bovine serum (Bovogen). Influenza virus strain A/WSN/33 was prepared as previously described [8]. Ribavirin (Sigma) was dissolved in water to make stock of 100 μM.

2.2. PB1 random mutagenesis

For construction of a mammalian expression vector for PB1 containing random mutations, we used a PCR-based cloning strategy in the presence of $MnCl_2$. cDNA corresponding to the full-length PB1 was amplified with specific primers 5′-CCCCAAGCTTGCCGCC ACCATGGATGTCAATCCGACCTT-3′ and 5′-CATGCGGCCGCCTATTT TTGCCGTCTGAGCTCTT-3′. The PCR product was then cloned into the *Hind* III and *Not* I sites of pEGFP-N1 and replaced *EGFP* gene with mutated *PB1* cDNA. The mutation rate of the plasmid library was confirmed by sequencing randomly selected 20 clones using specific primer 5′-GGAAGGCTCATAGACTTCCTTA-3′, which is corresponding to the nucleotide position from 560 to 1050 of segment 2. The plasmid library was then used to analyze the influenza virus RNA polymerase activity in a mini-replicon assay system.

2.3. Mini-replicon assay system

293T cells were transfected with plasmids for the expression of viral proteins, PB1 (wild-type or mutants), PB2, PA, and NP, and a plasmid for the expression of artificial influenza virus genome containing either *EGFP* gene (for screening) or the *firely luciferase* gene (for luciferase assay) of negative polarity, which is synthesized in cells by the human DNA-dependent RNA polymerase I [41]. The mRNAs encoding either *EGFP* or *luciferase* genes are transcribed in a viral RNA polymerase-dependent manner. For the screening, ribavirin was added (0 or 75 μM) after 3 h post transfection (hpt), and the fluorescence of EGFP was observed at 15 hpt. For the luciferase assay, ribavirin was added in the medium at various concentrations after 3 hpt, incubated at 37 °C for 15 h, and then the luciferase activity was determined using commercially available reagents (Promega) according to the manufacturer's protocol. The relative luminescence intensity was measured with a luminometer for 20 s. A plasmid for the expression of *Renilla* luciferase driven by the simian virus 40 (SV40) promoter was used as an internal control for the dual-luciferase assay. As a negative control, 293T cells were transfected with the same plasmids, except for the PB1 expression plasmid.

3. Results

3.1. Screening of ribavirin-resistant PB1 mutants

To determine the 50% inhibitory concentration (IC_{50}) of ribavirin, we carried out plaque assays with WSN-infected MDCK cells in the presence of various concentrations of ribavirin (Fig. 1A). Based on the results, we determined that IC_{50} and IC_{90} of ribavirin on influenza virus were 20 and 75 μM, respectively.

To make mutated cDNA library of PB1, random mutagenesis was carried out by PCR in the presence of 0.1 mM of Mn^{2+} and 1.5 mM of Mg^{2+} as described in Section 2. To know the mutation frequency of this library, we transformed the library into *Escherichia coli* DH5α high competent cells and obtained 3×10^4 colonies. To evaluate the mutation frequency, plasmids were isolated from 20 independent colonies and sequenced between the nucleotide positions 560 and 1055 of *PB1* gene. The results of sequencing showed that approximately 4.7 mutations were introduced in 2,274 nucleotides of *PB1* gene on average (approximately 1–2 a.a./PB1 protein). Based on this in hand, we started screening of ribavirin-resistant PB1 from the mutated cDNA library as shown in Fig. 1B. At first, this library was divided into 10 groups (Group 1–10), and mini-replicon assays were performed in the presence of ribavirin at IC_{90} (Fig. 1C), and thereby EGFP-positive cells were hardly found in wild-type PB1 transfected cells. In contrast, in the case of cDNA library-transfected cells, approximately 10–30% of EGFP-positive cells were found. Among them, 34% of one of groups, Group 4-transfected cells were resistant to ribavirin on average. Thus, Group 4 was further divided into additional 10 groups and subjected to the mini-replicon assays. After enrichment by 5 time-repetitions of this cycle, we could isolate a single clone showing the resistance to ribavirin. Even in the presence of IC_{90} of ribavirin, 69% of the isolated clone-transfected cells were EGFP positive (Fig. 2A). By sequencing of the isolated clone, we found one nucleotide substitution from G to A at nucleotide position of 103 (where the 5′ terminal nucleotide of cRNA is referred to as nucleotide position 1). This nucleotide change leads to an amino acid change from Asp to Asn at amino acid position 27 (Fig. 2B).

3.2. Characterization of D27N mutant

To quantitatively measure the influenza virus RNA polymerase activity, the mini-replicon assays with the artificial genome containing *luciferase* gene was carried out in the presence of 12.5, 25, and 50 μM of ribavirin, respectively. The luciferase activity of D27N mutant remained even in the presence of ribavirin compared with that of wild type. IC_{50} of D27N to ribavirin was about 18 μM, while that of wild-type was around 10 μM (Fig. 2C). Furthermore, the expression level of D27N was confirmed by Western blot analysis. The expression level of D27N was unchanged compared with that of wild-type even in the absence or presence of 50 μM ribavirin (Fig. 2D).

To further characterize this mutant, we used methotrexate (MTX). MTX is an inhibitor for purine biosynthesis, resulting in decrease of intracellular purine concentration. The mini-replicon assays were performed in the presence of various concentrations of MTX. The viral polymerase activity of D27N was significantly more than that of wild-type even in the presence of MTX (Fig. 3).

4. Discussion

PB1 functions as a catalytic subunit of viral RNA polymerase [2–7] and contains the highly conserved motifs of RNA-dependent RNA polymerases [2]. Putative nucleotide-binding sites have been expected adjacent to the conserved motifs of RNA-dependent RNA polymerases [23,24]. PB1 also binds to the vRNA and cRNA promoters [25,27]. In this study, to elucidate the functional domain of PB1 involved in nucleotide recognition, we isolated ribavirin-resistant mutants. Ribavirin inhibits the *de novo* synthesis of purine nucleosides and thus blocks viral RNA synthesis. In addition, it has been proposed that ribavirin also inhibits directly the

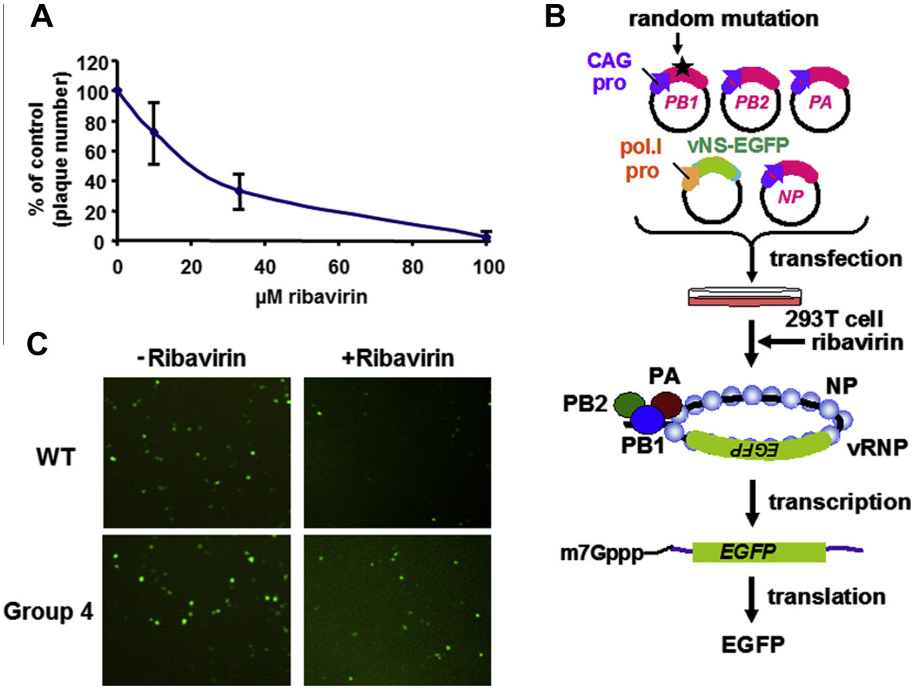

Fig. 1. Screening of ribavirin-resistant PB1 mutant. (A) Plaque assays were carried out with MDCK cells-infected WSN at MOI of 0.25 × 10^{-4} in the presence of ribavirin (0, 10, 33, 100, and 300 μM). The results are averages from three independent experiments with standard deviations. (B) Assay system for screening by mini-replicon assay. 293T cells were transfected with plasmids for the expression of viral proteins, PB1 (wild-type or mutant), PB2, PA, and NP, and a plasmid for the expression of artificial influenza virus genome containing *EGFP* gene of negative polarity. (C) At 3 hpt, ribavirin was added (0 or 75 μM), and the fluorescence of EGFP was observed at 15 hpt.

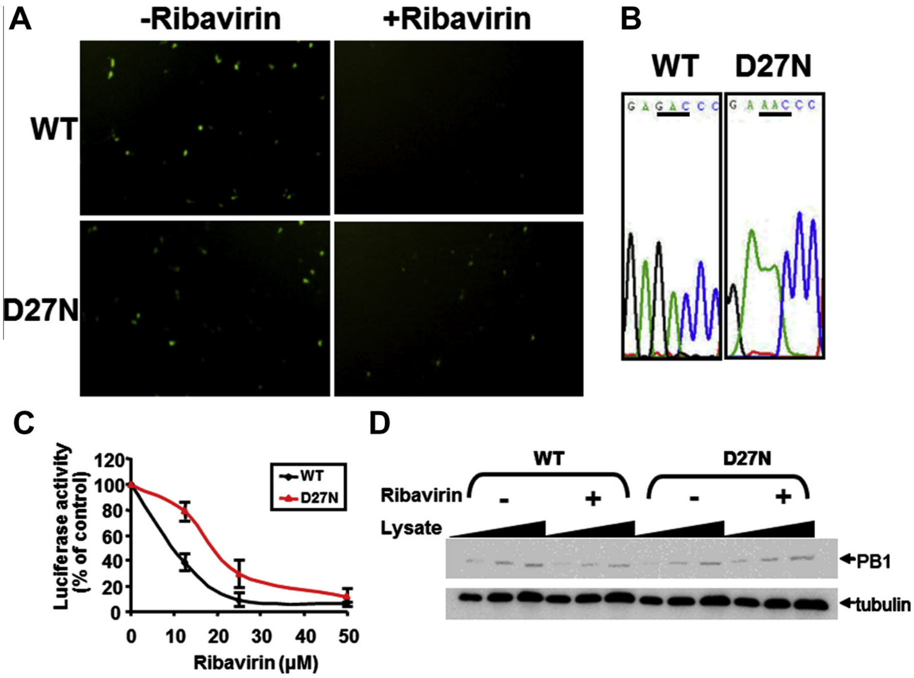

Fig. 2. Ribavirin-resistance of D27N mutant by mini-replicon assay system. (A) Mini-replicon assay using *EGFP* gene as a reporter gene. At 3 hpt, ribavirin was added (0 or 75 μM), and the fluorescence of EGFP was observed at 15 hpt. (B) Sequence of ribavirin-resistant PB1 mutant. (C) Mini-replicon assay using *luciferase* gene as a reporter gene was carried out. At 3 hpt, different concentrations (0, 12.5, 25, 50, and 100 μM) of ribavirin were added, and the luciferase activity was measured at 15 hpt. The vertical axis represents the percentage of the luciferase activity from ribavirin-treated cells relative to that from ribavirin-untreated cells. The results are averages from three independent experiments with standard deviations. (D) Effect of D27N mutation on assembly of PB1 subunit. Mini-replicon assay using *luciferase* gene as a reporter gene was carried out. At 3 hpt, ribavirin was added (0 or 50 μM). At 15 hpt, cells were lysed, and the lysates were subjected to Western blot analysis using anti-PB1 antibody and antibody against β-tubulin.

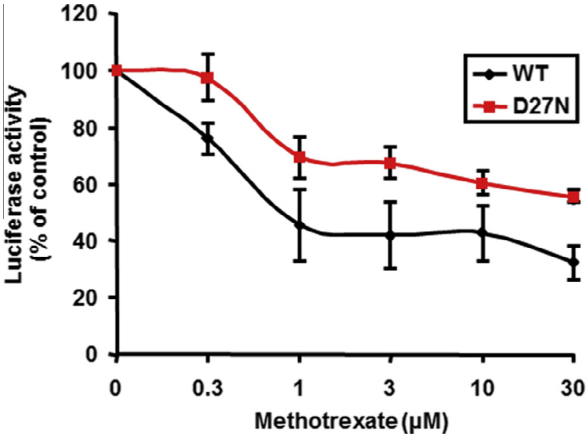

Fig. 3. Methotrexate-resistance of D27N mutant by mini-replicon assay system. The mini-replicon assay using *luciferase* gene as a reporter gene was carried out. At 12 hpt, different concentrations (0, 0.3, 1, 3, 10, and 30 μM) of methotrexate were added, and luciferase activity was measured at 22 hpt. The vertical axis represents the percentage of the luciferase activity from methotrexate-treated cells relative to that from methotrexate-untreated cells. The results are averages from three independent experiments with standard deviations.

viral RNA synthesis. We found that the viral polymerase activity of D27N was higher than that of wild type in the presence of ribavirin (Fig. 2C). Further, the viral polymerase activity of D27N was also resistant to MTX treatment compared with that of wild type (Fig. 3). These strongly suggest that D27 N mutant can polymerize the nascent RNA chains with the low concentrations of nucleotide. Therefore, it is expected that Asp27 is involved in the nucleotide recognition.

The Asp27 of PB1 is conserved over 99.9% of 7259 sequences of PB1 deposited in the NCBI Influenza Sequence Database. It has been reported that Asp27 is located upstream of the nucleotide binding site of PB1 but not in the catalytic active site. Similarly, the ribavirin-resistant mutant of poliovirus has a mutation in a domain out of the catalytic active site of the viral polymerase [42]. D27N is present within putative vRNA and cRNA promoter binding sites [25,27]. It is shown by mutants in the promoters of vRNA and cRNA that the RNA synthesis activity, cleavage of the cap structure, and the polyadenylation by viral polymerase are regulated through the promoter structure [43–45]. Further, the viral polymerase is stabilized by the interaction with its viral promoter [46]. Based on previous reports and our findings, the interaction between Asp27 of PB1 with vRNA and/or cRNA promoters may lead to the regulation of viral polymerase activity through the nucleotide recognition activity of PB1. This finding could useful for further studies about the mechanism of nucleotide recognition of the influenza viral RNA polymerase.

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