The N-terminal fragment of PA subunit of the influenza A virus effectively inhibits ribonucleoprotein (RNP) activity via suppression of its RNP expression

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Running title: *PA fragment inhibits RNP activity*

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ABBREVIATIONS

RNP	Ribonucleaoprotein complex
RdRp	RNA dependent RNA polymerase
WSN	A/WSN/33 (H1N1)
NT	A/NorthernTerritory/60/68 (H3N2)
HK	A/HongKong/56/97 (H5N1)
VN	A/Vietnam/1194/2004 (H5N1)
SW	A/Kurume/K0910/2009 (H1N1)
vNA	Viral-like nuramidase gene
vLUC	Viral-like luciferase gene

ABSTRACT

The influenza RNP, which is formed from PB1, PB2, PA, NA subunits, and vRNA, is autonomously replicated and transcribed in the infected cell. The simplest method to inhibit RNP activity is to impair the formation of the RNP. Thereupon we confirmed whether the peptides/fragments mimicking one of RNP components can interfere with their formation. During the process of this inhibitory study we found interesting suppression of protein expression of the RNP components by the N-terminal fragment of PA subunit. Especially, we found two residues (D108 and K134) on the fragment that were critical for the suppression. Furthermore, we determined the combination of three amino acids (P28, M86 and E100) on the fragment that are important for the strong suppression, and identified the minimum essential region (residues from 1 to 188) of the PA subunit that allowed its suppression. Our findings indicate that the N-terminal fragment of PA subunit may become one of candidates for an effective inhibitor of influenza RNP activity.

The influenza A virus is a single-stranded negative-sense RNA virus belonging to the family *Orthomyxoviridae*. The influenza virus has RdRp, which is a heterotrimeric complex that includes three subunits: PB1, PB2 and PA. These subunits are assembled with nucleoproteins (NP) and viral RNA (vRNA), and form RNP [1,2].

PA, one of the RNP components, is known as a multi-functional protein, which provides RNA synthesis as well as proteolytic and endonuclease activity [3-8]. By Sanz-Ezquerro et. al., it was firstly observed that the full-length and N-terminal PA induced degradation of co-expressed proteins [3,4]. PA also is known to be involved in an endonuclease activity that cleaves a capped structure from host mRNA to provide a primer for its viral transcription [6-8]. More recently, it was established that endonuclease active sites of N-terminal PA subunit were required for suppression of protein expression by two groups [9,10].

On the other hand, we previously showed that

an incompatible combination of RNP components impaired own RNP activity, and indicated the importance of PA [11]. We had an idea that the simplest method to inhibit RNP activity is to impair the formation of the RNP. Accordingly, we focused on the PA, and investigated whether the fragment mimicking the PA subunit could be used as an inhibitor against influenza RNP activity. This unique study means that the influenza virus is inhibited by own proteins. During the process of this inhibitory study we found an interesting suppression of protein expression of influenza RNP components, which was actually correlated with its N-terminal region of PA, and not with its C-terminal region. We presumed that this mechanism of suppression against influenza RNP components might be correlated with proteolytic and/or endonuclease activities, which have previously been mentioned [3,4,9,10].

In the present study, we further characterized suppression of RNP expression by N-terminal PA fragment to obtain a more efficient inhibitor. Namely, we identified the minimum region, two essential and three important residues on the N-terminal fragment, which were related to its effective suppression of influenza RNP activity. The aim of this study is to obtain the peptides/fragment that can effectively inhibit influenza RNP activity.

MATERIALS AND METHODS

Strains and plasmids

cDNA clones isolated from the following influenza strains were used in this report: WSN, NT, HK, VN, and SW strains [6,12-14]. The PB1, PB2, PA, and NP expression plasmids of WSN, NT, HK, VN, and SW have previously been described [12-15]. The pPOLI/WSN/vNA for expressing a viral RNA of the influenza NA gene has also been described [16].

Construction of mutants and fragments

In order to construct the deleted PA and the point-mutants, site-directed mutagenesis was performed as previously described [11,13,14]. To avoid structural damage of the protein, the NT/PA was separated at amino acid position 212, which

was previously shown to be a sensitive site for trypsin [6], producing NT/PA/N212. To confirm these sequences, the open reading frames were fully sequenced by the outside order (FASMAQ co., Japan).

Luciferase reporter assay

To screen the RNP activity via the activity of luciferase, a pPOLI/vLUC vector was constructed by substituting the influenza NA sequence in a pPOLI/WSN/vNA with *firefly* luciferase sequence. A subconfluent monolayer of HEK 293T cells (human embryonic kidney cell) [6,12,17] in E-MEM medium with 10% fetal bovine serum in a 6-well plate was transfected with 0.2 μg each of WSN/PA, WSN/PB1 WSN/PB2, WSN/NP, and vLUC expression vector via Lipofectamin 2000 (Invitrogen). For the inhibition assay, 0.2 μg vector, such as the fragment of PA expression vector, was also co-transfected. In order to determine the dose-dependency of the fragment, various concentrations of VN/PA N212 vector (0.04 - 1.0 μg/well) were co-transfected with a constant concentration of WSN/RNP vectors (0.2 μg/well). After transfection, cells were lysed at 30 hours using Cell Culture Lysis Buffer (CCLB) (Promega). Luciferase activity was measured using a Luminometer Lumat LB 9507 (Berthold, Germany), and was calculated as a relative light unit (RLU).

RNA isolation and primer extension assay

To reconstitute the RNP of influenza A virus and analyze the RNP activity, a subconfluent monolayer of HEK 293T cells transfecting vectors of WSN/RNP components and PA fragment were prepared as described in the luciferase reporter assay. pPOLI/WSN/vNA was used as a vector expressing viral RNA in substitution for pPOLI/vLUC. WSN/RNP activities were analyzed via primer extension assay as descried in previous reports [6,12,17]. All transcripts were visualized by 6% polyacrylamide gel containing 7M urea in TBE buffer, and were detected by autoradiography.

Western blotting

To confirm the expression level of the protein, WSN/RNP was reconstituted with/without the PA fragments as described in the luciferase-reporter assays. The extracted lysate were confirmed by western blotting with each specific antibody against PB1, PB2, PA, NP [13,14], or beta-actin as an internal control using 12% SDS-PAGE.

RESULTS

Inhibitory effect of NT/PA subunits against a WSN/RNP

A recent study indicated that incompatible combinations of RNP, e.g., NT/PA, WSN/PB1, WSN/PB2, and WSN/NP, diminished its RNP activity [11]. Therefore, we first confirmed whether the full-length NT/PA inhibits WSN/RNP activity (Figure 1A). However, a significant difference in the inhibitory effect was not observed in the presence of full-length NT/PA compared with the empty vector (Figure 1B). We next confirmed whether the fragment mimicking NT/PA subunit relates to the inhibitory effect against WSN/RNP activity. Consequently, WSN/RNP activity was significantly reduced by the NT/PA/N212 (Figure 1B).
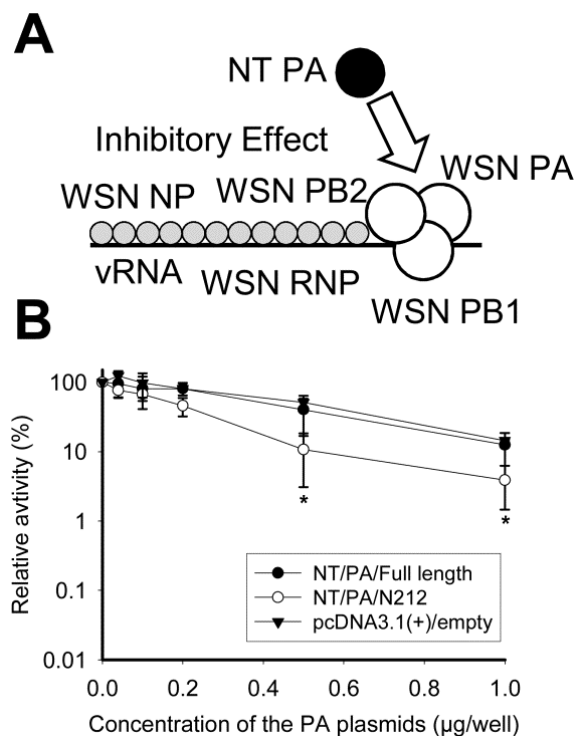
Inhibitory effect of N-terminal PA fragment from the other strains

To confirm whether the inhibitory effect is specific to this strain, the fragments derived from the other strains were additionally produced. Both SW/PA/N212 and VN/PA/N212 fragments showed the strongest inhibitory effect (Figure 2A). The same result was obtained from a primer extension assay (Figure 2B). For the following studies, the VN/PA/N212 fragment was chosen because it has the highest inhibitory effect and is a well-documented strain. The N-terminal fragment (VN/PA/N212) showed an exceedingly strong inhibitory effect from low concentration whereas the C-terminal fragment (VN/PA/C504) showed no inhibition (Figure 2C).

Confirming protein expression levels of RNP components and fragments

To investigate the mechanism behind the severe loss of RNP activity by the VN/PA/N212, the expression levels of the RNP and VN/PA/N212 were confirmed. The protein expressions of WSN/RNP components were severely impaired in the presence of the VN/PA/N212 (Figure 3A).

To confirm that each active site (D108, K134, or T157A) is involved in the reduction of the WSN/RNP expression, WSN/RNP activities (Figure 3B) and protein expression levels of WSN/RNP components (Figure 3C) were checked in the presence of fragments with inactivation of each active site. The strong inhibitory effect in the presence of VN/N212/T157A was still remained, whereas they were disappeared in both of the mutations of VN/N212/D108A and VN/N212/K134A.

Fig. 1 Confirming the inhibitory effect of the NT/PA subunit against WSN/RNP activity. **(A)** The model of the study for the inhibitory effect of the NT/PA subunit against WSN/RNP activity. **(B)** Dose-dependency of the inhibitory effects. The WSN/RNP activities were estimated by a luciferase reporter assay. The relative WSN/RNP activities without the inhibitor are expressed as 100% activity. The standard deviations and significant differences were calculated from three independent trials. * indicates statistically significant differences at < 0.05 based on a Student's t-test (n=3).
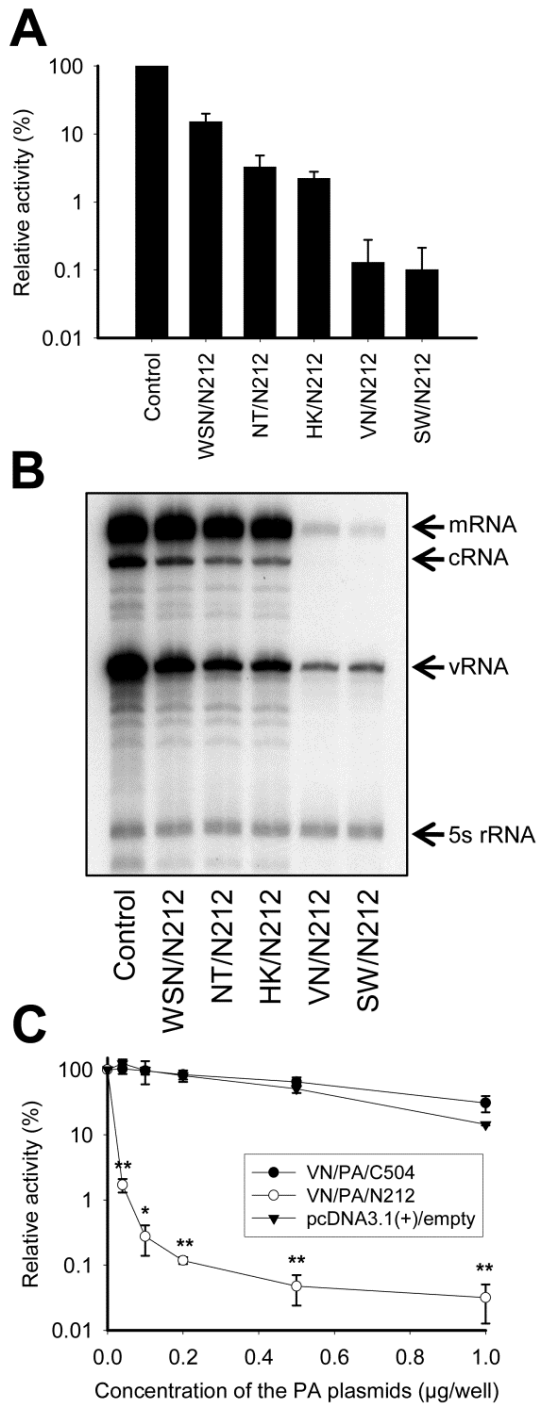
Fig. 2 Comparison of the inhibitory effects of the N-terminal fragments derived from PA subunits WSN, NT, HK, VN, and SW using a luciferase reporter assay **(A)** and a primer extension assay **(B)**. **(C)** Comparison of the inhibitory effects by N and C-terminal fragments derived from VN/PA subunit. The WSN/RNP activities were estimated by a luciferase reporter assay. The relative WSN/RNP activities without the inhibitor are expressed as 100% activity. The standard deviations and significant differences are calculated from three independent trials. * and ** indicate statistically significant differences at <0.05 and < 0.01, respectively, using a Student's t-test (n=3).
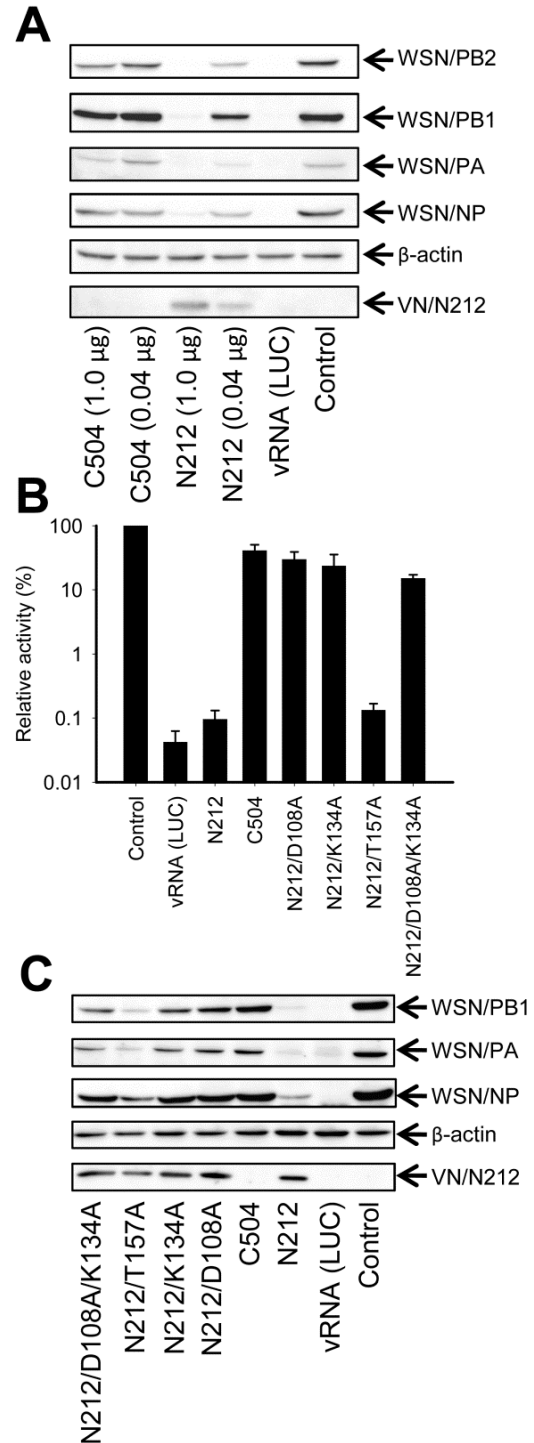
Fig. 3 Checking the protein expression levels of WSN/RNP and fragments. **(A)** Representative protein expression levels of the WSN/RNP components with either N- or C-terminal PA. **(B)** Determination of an important active site on the N-terminal fragment for the inhibitory effect. **(C)** Representative protein expression levels of WSN/RNP components in the presence of the fragments which either an endonuclease (D108 and/or K134) or proteolytic (T157) active site was substituted with an alanine. The vRNA (LUC) means that only luciferase vector was transfected to 293T cell. A reproduction of these results was confirmed by at least two independent trials.

Determination of the essential region for a strong inhibitory effect

To find the region that is essential to the strong suppression, WSN/RNP activity was measured in the presence of VN/PA/N212 that was further truncated (Figure 4A). However, the suppressions were all absent among fragments from nos.1 to 19. Next, the RNP activities were measured for fragments with deleted distal regions (Figure 4B). Dramatic changes were observed with regard to suppression when either N187 or N188 vector was co-transfected as inhibitor, although no reductions were obtained with fragments from N135 to N186.

In order to identify the importance of positions 187 and 188 on the fragment, they were substituted with the alanine on VN/PA/N212, and the inhibitory effects were measured (Figure 4C). The suppressions of these fragments were significantly attenuated, compared with that of VN/PA/N212 (wild type).

The amino acid on the PA fragment that is important for a strong suppression

Since a significant difference in the suppression was observed between VN/PA/N212 and WSN/PA/N212 (Figure 2A), we determined which of the amino acid are required for the strong suppression. By multiple alignments, we found 10 differences in the amino acids between VN/PA/N212 and WSN/PA/N212, and then these amino acids on VN/PA/N212 were serially substituted with those on WSN/PA/N212 (Figure 5A). The inhibitory effects against WSN/RNP activity were measured by fragments with each mutation via a luciferase-reporter (Figure 5B) and a primer-extension assay (Figure 5C). When the change in amino acid position 100 was accumulated in VN/PA/N212 (shown as Mut. 10), the suppression of WSN/RNP activity was almost disappeared (Figure 5B). Significant changes were also found with changes to amino acid positions 28, 86 and 100 on the VN/PA/N212 (Figures 5B and 5C). Thereupon, only these amino acids on the VN/PA/N212 were substituted with those of WSN. For a control of counterpart, three positions on the WSN/PA/N212 were also substituted with those on the VN. As shown in
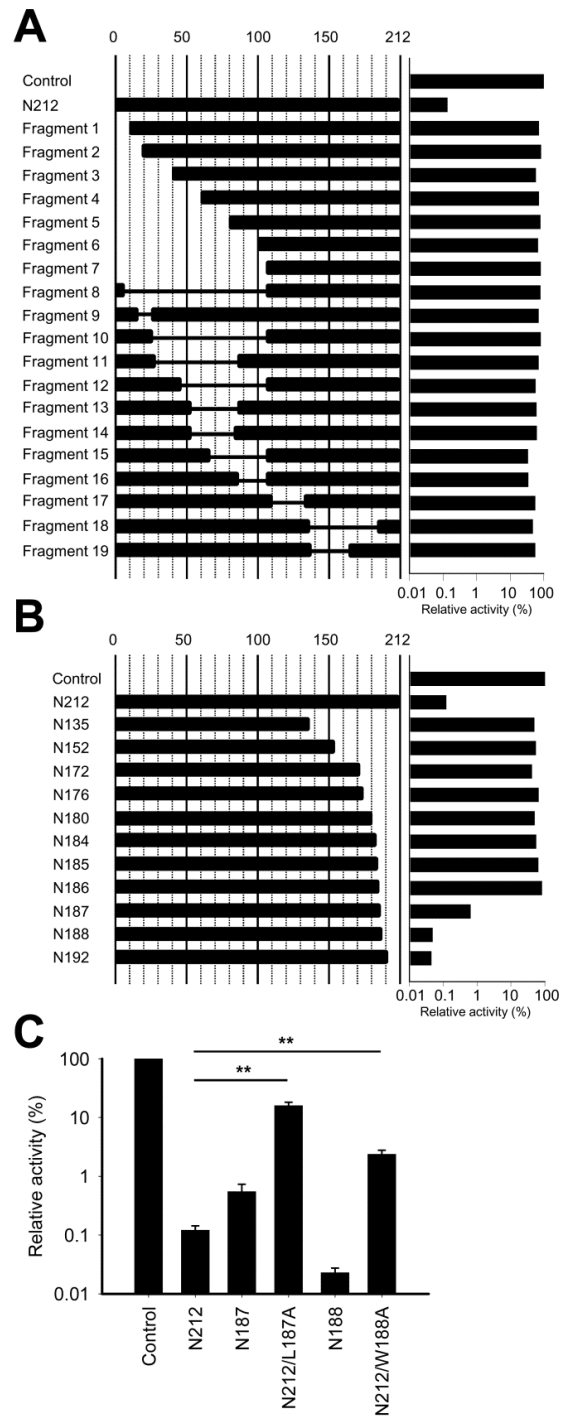

Fig. 4 Important region and amino acid of the VN/PA/N212 for an effective suppression. **(A)** Deleted mutants of the N or middle region of the VN/PA/N212. **(B)** Deleted mutants of the C-terminal region of the VN/PA/N212. Fragment numbers and deleted regions are indicated on the left side of the panel. The average was calculated from two independent trials, as indicated in the right side of the panel. **(C)** Fragments point-mutated at the position(s) 187 and/or 188. The relative RNP activities without the inhibitory PA fragment are expressed as 100% activity. The standard deviations and significant differences are calculated from three independent trials. ** indicates statistically significant differences at < 0.01, using a Student's t-test (n=3).

A

Mut	1	2	3	4	5	6	7	8	9	10
pos.	28	57	58	62	65	86	91	100	114	129
VN	P	R	S	I	S	M	V	V	E	T
WSN	L	Q	G	V	L	I	I	A	K	I

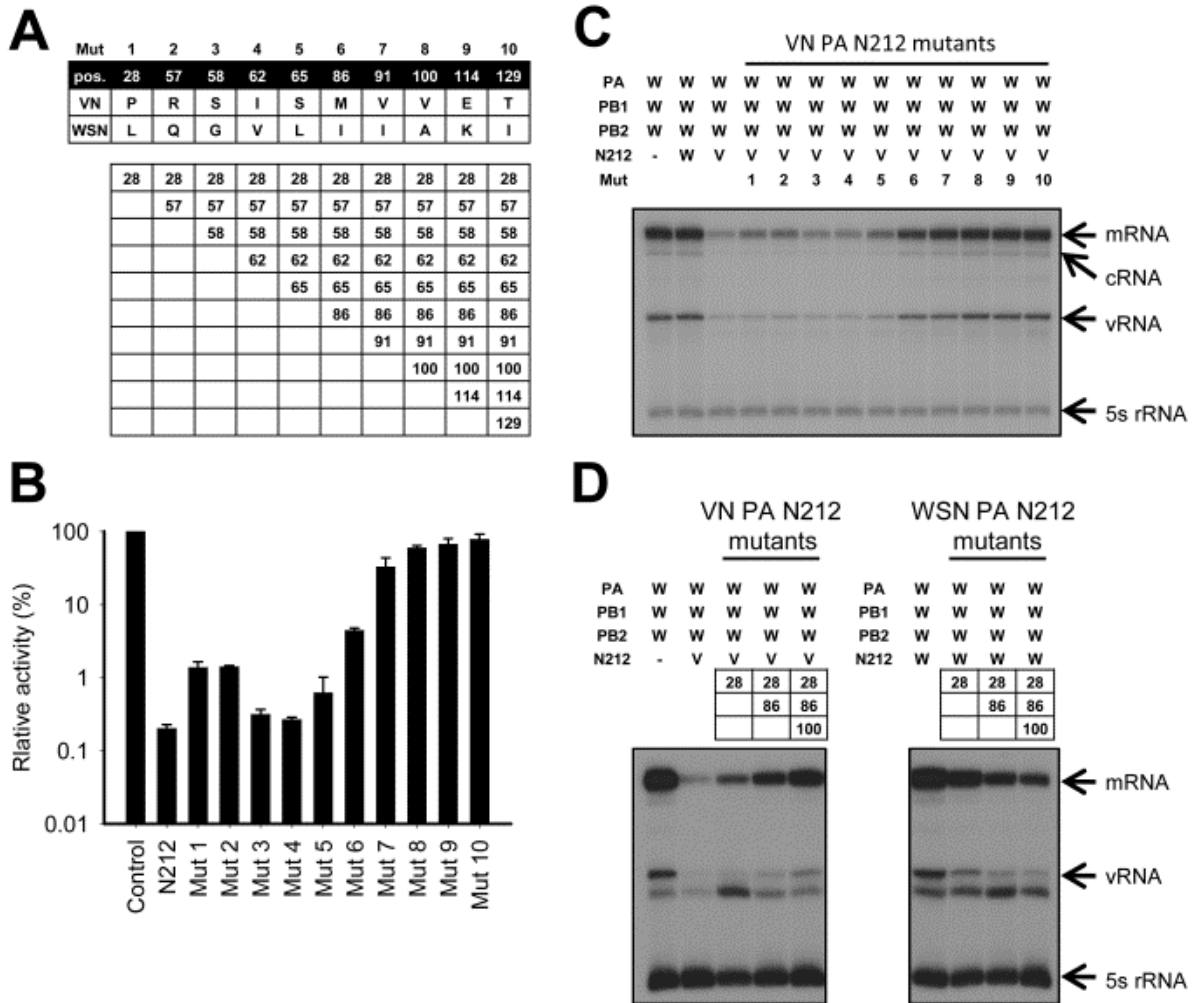| 28	28	28	28	28	28	28	28	28	28
	57	57	57	57	57	57	57	57	57
		58	58	58	58	58	58	58	58
			62	62	62	62	62	62	62
				65	65	65	65	65	65
					86	86	86	86	86
						91	91	91	91
							100	100	100
								114	114
									129

B

C

D

Fig. 4 Comparison of the inhibitory effect on WSN and VN/PA fragments. **(A)** Comparing the different amino acids on WSN and VN/PA subunits and the constructed fragments are indicated in the lower panel. Comparison of the inhibitory effect of each of the mutants derived from the VN/PA/N212 using a luciferase reporter assay **(B)** and a primer extension assay **(C)**. **(D)** Comparison of the inhibitory effect of the VN and WSN/PA/ where three points of amino acids (28, 86, and 100) were substituted. The numbers in the upper panel of **(D)** represent the substituted positions of an amino acid on the fragment. The standard deviations are calculated from three independent trials.

Figure 5D, three amino acids (28, 86 and 100) were determined to be important for strong suppression of WSN/RNP activity, because the suppression was complementarily changed by these substitutions.

DISCUSSION

We found interesting suppression of WSN/RNP activity by PA fragment, which actually correlated with the N-terminal PA subunit and not with the C-terminal region (Figure 1B and 2C). This result was very interesting, because we originally hypothesized that the PB1 binding site, which is part of C-terminal PA subunit, is required for a competitive inhibition against WSN/RNP activity, whereas the PB1 binding site was unimportant.

A very interesting result was also observed when protein expressions of WSN/RNP components were compared with and without inhibitory fragments (Figure 3A). Namely, all components of the WSN/RNP were severely decreased in the presence of VN/PA/N212, although beta-actin was not affected. Additionally, we confirmed that VN/PA/N212 didn't affect the *Renilla* luciferase activity as internal control (data not shown). Previously, some groups had also identified that the N-terminal functions of the PA subunit were required for the suppression of

protein expression [3,4,9,10]. These results indicate that the inhibition of WSN/RNP activity by VN/PA/N212 depends on the reduction of protein expression of WSN/RNP components. Moreover, the activity and protein expressions of WSN/RNP were rescued by inactivating its endonuclease catalytic sites (D108A and K134A) on the VN/PA/N212 (Figure 3B and 3C). These results suggest that the VN/PA/N212 impairs the protein expressions of RNP components via its endonuclease activity, although the detailed mechanism still remains unclear.

Our data indicated that the region comprised by amino acid positions 189 to 212 was not needed for suppression of WSN/RNP activity (Figure 4B). Structural studies have shown that the position 189 lies between the 6th and 7th alpha-helix of the endonuclease domain of PA subunit [7,8,18]. These results suggest that the regions from the 1st to the 6th alpha helixes are required, but that the 7th alpha helix is unimportant for suppression of RNP activity. Previously, Sanz-Ezquerro et. al. suggested that 1-247 residues were found to be important whereas 1-186 residues was not enough to induce the suppression of protein in their system [4]. Our data are consistent with their data, and we further identified that the minimum essential region for the suppression was that of 1-188 residues of the N-terminal PA subunit.

The amino acids at positions 28, 86, and 100 were found to be the amino acids that are important for strong suppression of WSN/RNP activity (Figures 5B and 5C). These amino acids are involved near the catalytic site of endonuclease [6-8], indicating again somehow the endonuclease activity on the fragment is involved in the suppression of RNP expression and that it is controlled by the combination of these three amino acids (positions 28, 86, and 100).

More recently, Jagger and Desmet et. al. showed how PA-X was important for the suppression of protein synthesis [9,10]. PA-X is a new protein that was found as a ribosomal frame-sifting protein, derived from the N-terminal PA cording region. Though the full-length of PA-X is not covered by our

essential region of the VN/PA/N188 that is needed for strong suppression of WSN/RNP (Figure 4B), the mechanism of the suppression may be involved into the PA-X functions.

During the process of the inhibitory study by PA subunit we found an interesting suppression of influenza RNP components, which was actually correlated with the endonuclease active sites (D108 and K134) of its N-terminal PA subunit. Furthermore, we newly identified the essential region (1-188 residues) and the combination of three amino acids (P28, M86 and E100) that was important for strong suppression. The natural target for the endonuclease of PA subunit is a host mRNA, which is a process that is referred to as cap-snatching. It remains unclear how the PA subunit can distinguish between host and viral mRNAs. In the present study, it is suggested that the PA subunit may lose control of its endonuclease activity and suppress the protein expression of WSN/RNP components by its fragmentation, although the direct mechanism is still unclear.

Our results indicate that the N-terminal fragment (1-188 residues) of PA subunit may become one of candidates for the inhibitor of influenza RNP activity via suppression of protein expression of the RNP components. This is very unique inhibition, because it means that influenza virus is inhibited by the fragment derived from own proteins. On the other hand, the cap-snatching mechanism is involved in the toxicity of the influenza virus, because this is what shuts off the host mRNA expression. Thus, our findings should help elucidate the molecular mechanism of the endonuclease activity on PA subunit, and should aid with developing new sights for the inhibition of the influenza A virus. Both of these could help reduce the influenza infection and toxicity in the future.

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CONFLICT OF INTEREST

The authors declare that they have no conflict and interest.

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