博士論文（要約）

Characterization of influenza A virus protein PA-X

(A 型インフルエンザウイルス蛋白質 PA-X の性状解析)

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CONTENTS

CHAPTER III

Identification of Amino Acid Residues of Influenza A Virus PA-X That Are Important for PA-X Shutoff Activity by Using Yeast

PREFACE

When viruses enter host cells to reproduce themselves, they commandeer host cell machineries (*1-4*). In infected individuals, the production of cytokines, chemokines, and anti-viral antibodies is stimulated as part of the host anti-viral response (*5-7*). Most viruses possess proteins that suppress this response to facilitate viral replication (*8-10*). As one strategy to counteract the host anti-viral response, some viruses suppress host protein expression via a process termed host shutoff (*11-13*). Viruses inhibit host protein expression at various steps, including transcription (*14*), mRNA processing (*15*), mRNA export from the nucleus (*16*), mRNA stability (*15, 17*), translation (*18*), and protein stability (*19, 20*).

Influenza A virus, which is a negative-strand RNA virus of the *Orthomyxoviridae* family, also inhibits host gene expression in infected cells. It encodes at least 18 viral proteins in its genome (*21, 22*), some of which are involved in host shutoff. The multifunctional protein nonstructural protein 1 (NS1) plays a central role in antagonism against the innate immune response of the host via type I interferon at multiple steps (*23-25*). NS1 has been reported to interact with CPSF30 (*26*), PABP II

(*16*), and the key components of the cellular mRNA export machinery (*27*). The binding of NS1 to CPSF30 inhibits cleavage and polyadenylation of host cell mRNAs at the 3' end (*26*). The interaction of NS1 with PABP II and components of the cellular mRNA export machinery blocks the nuclear export of cellular mRNAs (*16*). Host RNA polymerase II complex is specifically degraded in virus-infected cells by an uncharacterized mechanism (*28, 29*). The viral polymerase complex uses capped RNA derived from host RNAs, thereby reducing the availability of host transcripts (*30*). A subunit of the polymerase complex, PA, had long been thought to induce proteolysis in virus-infected cells. An N-terminal region of PA has been identified to be important for this activity (*31*). However, the crystal structure of the N-terminal regions of PA revealed that it functions as an endonuclease, not a protease (*32, 33*), since PA suppresses host protein expression by cleaving mRNA (*32*).

In 2012, Jagger et al. (*34*) identified a novel viral protein expressed from an mRNA encoding PA via a ribosomal frameshift. This novel protein consists of the N-terminal PA region and a unique C-terminal region. The authors named this protein PA-X, and characterized its high shutoff activity via mRNA cleavage. The shutoff

activity of PA-X is significantly higher than that of PA, indicating that PA-X plays a crucial role in shutoff in influenza virus-infected cells. To examine the importance of PA-X to viral replication and pathogenicity, several groups generated mutant viruses with reduced PA-X expression (*34, 35*). Analyses with these mutant viruses showed that PA-X could increase (*35, 36*) or decrease (*37*) viral replication in vitro, and enhanced (*36, 38*) or attenuated (*34, 37*) viral pathogenicity in mice. Moreover, the shutoff activity of PA-X was shown to disable anti-viral stress-induced translation arrest in infected cells by inhibiting stress granule formation (*39*).

PA-X selectively cleaves RNA transcribed from the cellular Pol II promotor, leading to the specific suppression of host protein expression in infected cells (*40*). Furthermore, an *in vitro* assay using recombinant PA-X revealed that PA-X digests both single- and double-stranded RNAs and requires a bivalent cation as a cofactor for its shutoff activity (*41*). The shutoff activity of PA-X is dependent on the endonuclease activity of the protein (*34*) since an alanine substitution at a key residue for the N-terminal endonuclease activity eliminates the shutoff activity (*34, 42*). Although PA has the N-terminal endonuclease domain, it has lower shutoff activity than PA-X (*42*),

suggesting that at least one other region of PA-X is also important for the shutoff activity. Although functional analysis of PA-X had been performed, the mechanism of PA-X shutoff, including whether host genes are involved, had not been elucidated. Therefore, the aim of my study was to identify amino acid residues and host genes that are required for the shutoff activity of PA-X.

In Chapter I, I attempted to identify the amino acid residues that are important for the shutoff activity of PA-X. For this purpose, I expressed PA-X in yeast to examine whether PA-X suppressed protein expression in other eukaryotes. Yeast transformed with a plasmid encoding wild-type PA-X formed barely any colonies, indicating that PA-X has shutoff activity even in yeast. From this result, I attempted to identify host genes involved in the shutoff activity of PA-X by screening against a yeast knockout library (Chapter II). During this screening, I obtained 29 false-positive colonies harboring plasmids that encoded unintentional mutations in PA-X. In Chapter III, I analyzed these clones and identified novel amino acid residues involved in the shutoff activity of PA-X.

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CHAPTER Ⅰ

Mapping of a Region of the PA-X Protein of Influenza A Virus That Is Important for Its Shutoff Activity

Abstract

The influenza A virus PA-X comprises an N-terminal PA endonuclease domain and a C-terminal PA-X-specific domain. PA-X reduces host and viral mRNA accumulation via its endonuclease function. Here, I found that the N-terminal 15 amino acids, particularly 6 basic amino acids, in the C-terminal PA-X-specific region are important for PA-X shutoff activity. These 6 basic amino acids enabled a PA deletion mutant to suppress protein expression at a level comparable to that of wild-type PA-X.

Introduction

The influenza A virus genome comprises eight negative-sense, segment viral RNAs (*1, 2*). Each viral segment encodes at least one viral protein (*3*). Recently, several novel viral proteins were identified including PB1-F2, PB1-N40, PA-N155, and PA-N182, which are translated from the PB1 or PA mRNA, respectively, via leaky ribosomal scanning (*4-6*). PB1-F2, which localizes to mitochondria to regulate apoptosis and innate immune response, has been found in many isolates of influenza A virus and is considered a virulence factor (*4, 7-9*). PB1-N40 plays an important role in maintaining the balance between PB1 and PB1-F2 expression (*5*). Loss if PA-N155 and PA-N182 has been shown to decrease viral growth kinetics in culture cells (*6*).

In 2012, Jagger et al. (*10*) reported that PA-X was expressed from the PA segment of influenza A viruses as an alternative product in infected cells (*10*). PA-X consists of an N-terminal endonuclease domain of PA (191 amino acids) and a C-terminal PA-X-specific domain (61 amino acids), which is encoded by an overlapping open reading frame generated by a ribosomal frameshift (*10, 11*). Jagger et al. also revealed that PA-X decreases host and viral mRNA accumulation via its shutoff activity, which is dependent on its endonuclease activity (*10, 12*).

Although PA has the N-terminal endonuclease domain, it has lower shutoff activity than PA-X (*12*), suggesting that other regions of PA-X are also important for the shutoff activity. In this study, I mapped such a novel region of PA-X. I also identified the amino acids in this region that are required for the shutoff activity. On the basis of my findings, I propose a detail shutoff mechanism involving PA-X.

Results

The C-terminal PA-X-specific region is important for the shutoff activity of PA-X.

To examine whether the C-terminal PA-X-specific region was important for the shutoff activity of PA-X, I constructed two C-terminal deletion mutants of PA (PA_191 and PA_252), which contained the N-terminal 191 and 252 amino acids of PA, respectively (Fig. 1A). The N-terminal 191 amino acids of PA are shared with PA-X, and PA_252 had the same number of amino acids as PA-X. 293 cells were transfected with pGL4.74[hRluc/TK], which encodes *Renilla* luciferase, together with an empty plasmid or plasmid encoding PA, PA-X, PA_191, or PA_252. *Renilla* luciferase activities were measured at 24 h post-transfection (Fig. 1B). *Renilla* luciferase activities were suppressed by PA (0.6 log-fold), PA-X (2.0 log-fold), PA_191 (1.3 log-fold), and PA_252 (0.7 log-fold). To confirm these results, I analyzed the expression levels of *Renilla* luciferase and the PA-X and PA variants by western blotting with an anti-*Renilla* Luciferase pAb (Fig. 1C) and an anti-PA monoclonal antibody (Fig. 1D).

Renilla luciferase was detected in the mock lane, but not in the PA-X lane. In the PA, PA_191, and PA_252 lanes, the *Renilla* luciferase expression levels were reduced compared with the mock lane (43.6%, 53.7%, and 52.7%, respectively). PA-X

was barely detected because of self-suppression, whereas PA, PA_191, and PA_252 were expressed to a similar extent. These results indicate that the shut-off activity of PA_191 and PA_252 is lower than that of wild-type PA-X, suggesting that the C-terminal PA-X-specific region is important for PA-X shutoff activity.

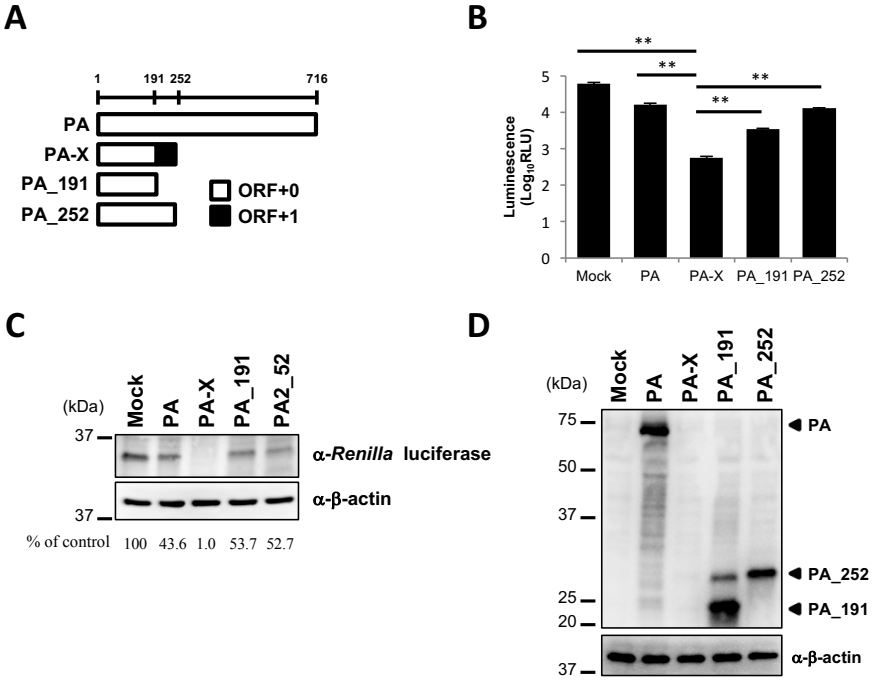

Figure 1. Comparisons of shutoff activities of wild-type PA, wild-type PA-X, and C-terminal deletion mutants of PA. (A) Schematic representation of PA, PA-X, and C-terminal deletion mutants of PA. PA-X is composed of the N-terminal 191 amino acids of PA and a C-terminal PA-X-specific region of 61 amino acids. PA_191 and PA_252 contained the N-terminal 191 and 252 amino acids of PA, respectively. (B–D) *Renilla* luciferase activity (B), expression of *Renilla* luciferase (C), and expression of each viral protein (D) in cells transfected with pGL4.74[hRluc/TK] together with an empty plasmid or a plasmid encoding wild-type PA, wild-type PA-X, or PA deletion mutants were evaluated by using a luciferase assay and western blotting with an anti-*Renilla* luciferase and an anti-PA monoclonal antibody. (B) Luminescence data are means ± SD (n = 3). **, $P < 0.01$, according to a one-way ANOVA, followed by a Bonferroni correction. (C) The intensities of the *Renilla* luciferase and β-actin bands were quantified and their ratios were calculated. The ratio of the mock lane was set to 100%. (C–D) β-actin served as a loading control.

The N-terminal 15 amino acids of the C-terminal PA-X-specific region are important for its shutoff activity. To narrow down the C-terminal PA-X-specific region that is important for the shutoff activity of PA-X, I prepared a series of C-terminal deletion mutants of PA-X (Fig. 2A) and examined their shutoff activities in the luciferase assay (Fig. 2B). Compared with wild-type PA-X (which exhibited a 1.8 log-fold reduction in shut-off activity compared with the mock sample), PA-X_X10, PA-X_X5, and PA_191 showed significantly lower shut-off activities (1.6, 1.1 and 1.1 log-fold reductions compared with the mock sample, respectively), whereas the PA-X_X15 shut-off activity was similar to that of wild-type PA-X. These results indicate that the N-terminal 15 amino acids in the C-terminal PA-X-specific region are important for the PA-X shut-off activity.

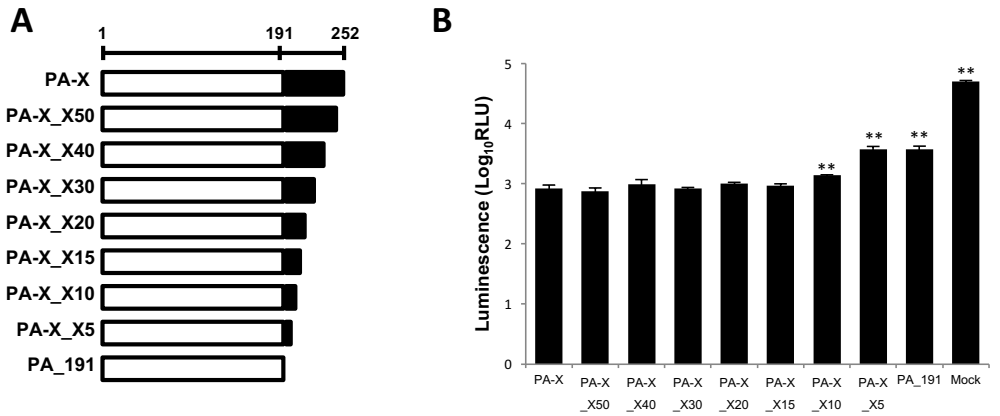

Figure 2. Importance of the N-terminal 15 amino acids in the PA-X-specific region for the shutoff activity of PA-X. (A) Schematic diagram of the C-terminal deletion mutants of PA-X. A series of C-terminal deletion mutants of PA-X was constructed to identify the region(s) important for the shutoff activity of PA-X. PA-X_X50, PA-X_X40, PA-X_X30, PA-X_X20, PA-X_X15, PA-X_X10, PA-X_X5, and PA_191 possessed 50, 40, 30, 20, 15, 10, 5, and no PA-X-specific amino acids, respectively. (B) Suppression of *Renilla* luciferase activity by each C-terminal deletion mutant of PA-X in 293 cells. *Renilla* luciferase activity in cells co-transfected with wild-type PA-X, a series of C-terminal deletion mutants, or PA_191 was measured by using a luciferase assay. Luminescence in the each C-terminal deletion mutant sample was compared with that in the wild-type PA-X sample. Luminescence data are means ± SD (n = 3). **, $P < 0.01$, according to a one-way ANOVA, followed by a Dunnett's test.

A cluster of 6 basic amino acids is required for the shutoff activity of PA-X. To pinpoint the amino acids among the N-terminal 15 amino acids that are required for the shutoff activity of PA-X, I compared the amino acid sequences of PA-X to an amino acid sequence from an identical region of PA that showed lower shutoff activity (Fig. 3A). I found a characteristic difference: 6 basic amino acids [lysine (K) and arginine (R)] in PA-X were acidic amino acids [glutamic acid (E)] in PA. To evaluate the significance of these 6 basic amino acids for the shut-off activity, I constructed PA-X mutants in which I substituted these 6 basic amino acids (K and R) with E (PA-X_6E), aspartic acid (D) (PA-X_6D), alanine (A) (PA-X_6A), R (PA-X_6R), K (PA-X_6K), or R and K (PA-X_KR) (Fig. 3B) and examined their shutoff activities (Fig. 3C). PA-X_6E, PA-X_6D, and PA-X_6A showed reduced *Renilla* luciferase activities compared with wild-type PA-X, whereas the *Renilla* luciferase activities of PA-X_6R, PA-X_6K, and PA-X_KR were similar to that of wild-type PA-X. The trend of expression levels of wild-type and mutant PA-X and related viral proteins (Fig. 3D) and the corresponding *Renilla* luciferase level (Fig. 3E) was similar with the exception of PA-X_6A; although PA-X_6A suppressed *Renilla* luciferase and its own expression

when analyzed by western blotting (Fig. 3D and 3E), in the luciferase assay, the suppression of *Renilla* luciferase activity by PA-X_6A (Fig. 3C) was limited for an unknown reason. To identify the essential amino acid for PA-X shut-off activity, I introduced single point mutations (K or R to E) at each of the 6 basic amino acids. These mutants shared similar shutoff activities all of which were slightly lower than that of wild-type PA-X (data not shown). These data imply that the basic amino acids at these 6 positions contribute equally to the shut-off activity of PA-X.

To confirm the importance of these 6 basic amino acids, I substituted all 6 E in PA_252 with K or R (PA_252_6KR) (Fig. 3F), and assessed the shut-off activity (Fig. 3G). PA_252_6KR showed high shutoff activity at a level comparable to that of wild-type PA-X. When I assessed the expression levels of *Renilla* luciferase and mutant and wild-type PA-X and related viral proteins (Fig. 3H and 3I), the results were similar to those obtained in the luciferase assay (Fig. 3G). These results demonstrate that the 6 basic amino acids at these positions are essential for the shutoff activity of PA-X.

I then asked whether these 6 basic amino acids in the PA-X-specific region were conserved among other influenza isolates deposited in the Influenza Research

Database in December 2014. PA-X of different viruses had substitutions between K and R at these 6 positions; however, basic amino acids (K or R) in these 6 positions were highly conserved among human (99.9% of 3417 isolates), avian (99.3% of 4047), swine (96.7% of 1030), and equine (100% of 6) isolates (Table 1). This information further supports the concept that these basic amino acids are required for the shutoff activity of PA-X.

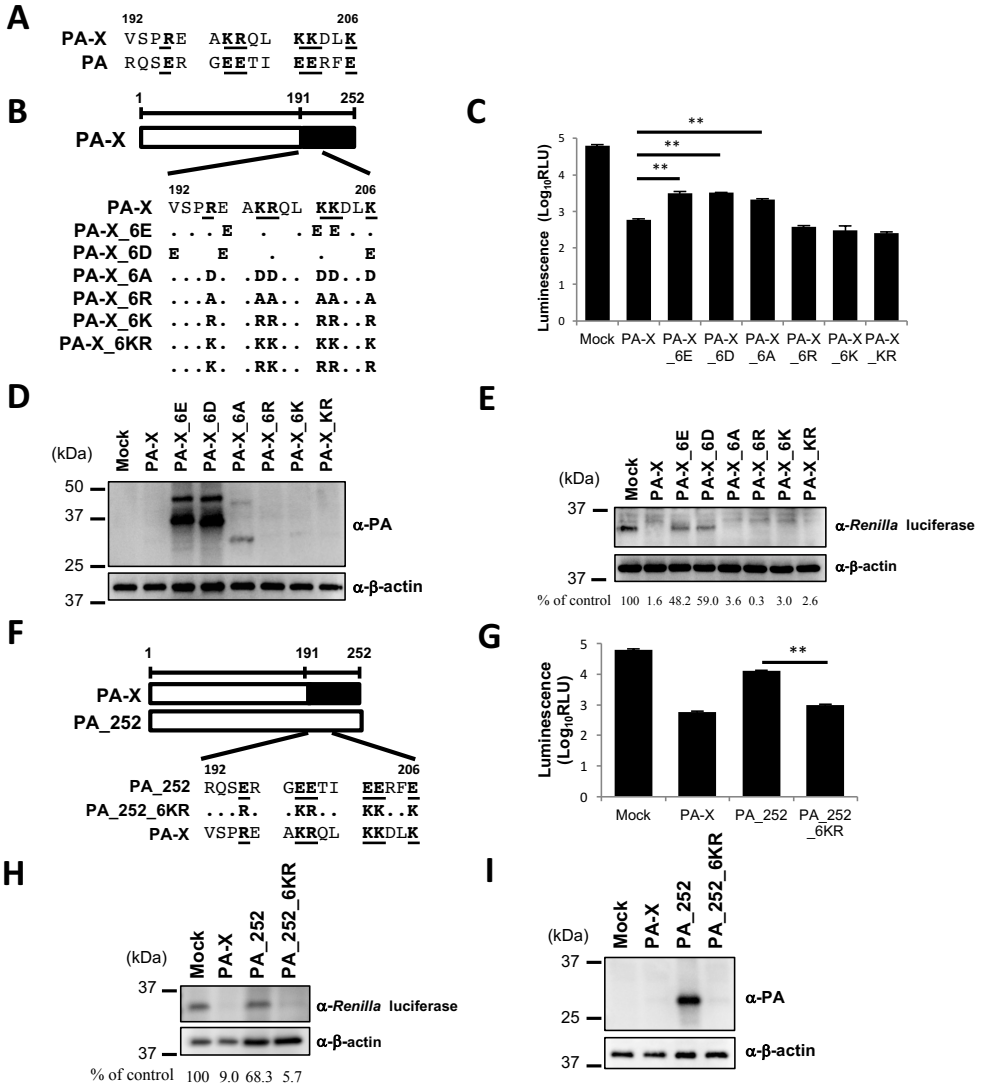

Figure 3. Identification of amino acids in the PA-X-specific region that are important for the shutoff activity of PA-X. (A) Comparison of the N-terminal PA-X-specific 15 amino acids of PA-X with the identical region of PA. All 6 basic amino acids, K or R, in PA-X were the acidic amino acid E in PA. The 6 amino acids are underlined. (B) Substitution of the 6 basic amino acids in the PA-X-specific region. Six basic amino acids of PA-X were substituted with acidic amino acids (PA-X_6E or PA-X_6D), a neutral amino acid (PA-X_6A), or basic amino acids (PA-X_6R, PA-X_6K, or PA-X_KR). (C–E) Shut-off activities of PA-X mutants. In 293 cells, *Renilla* luciferase was co-expressed with wild-type PA-X or each PA-X mutant. *Renilla* luciferase activity (C), expression levels of *Renilla* luciferase (E) and of wild-type PA-X or each PA-X mutant (D) were evaluated by means of a luciferase assay or western blotting. (F) Comparison of the N-terminal PA-X-specific 15 amino acids of PA-X with the identical region of PA_252. Six acidic amino acids in PA_252 (underlined) were substituted with those of PA-X (PA_252_6KR).
(G–I) Shutoff activities of PA_252_6KR. In 293 cells, *Renilla* luciferase was co-expressed without (mock) or with wild-type PA-X, PA_252, or PA_252_6KR. *Renilla* luciferase activity (G), and expression levels of *Renilla* luciferase (H) and of each viral protein (I) were evaluated by means of a luciferase assay or western blotting. (C and G) Luminescence data are means ± SD (n = 3). **, $P < 0.01$, followed by a Bonferroni's t-test. (E and H) The intensities of the *Renilla* luciferase and β-actin bands were quantified and their ratios were calculated. The ratio of the mock lane was set to 100%. (D, E, H, and I) β-actin served as a loading control

Table 1. Conservation of the basic amino acid residues at 6 positions of PA-X.

Host	Number of the basic amino acid residues at the 6 positions[a]						
	6	5	4	3	2	1	0
Human	3414[b] (99.9[c])	3 (0.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Avian	4020 (99.3)	27 (0.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Swine	996 (96.7)	33 (3.2)	0 (0)	1 (0.1)	0 (0)	0 (0)	0 (0)
Equine	6 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a]The 6 residues were at positions 195, 198, 199, 202, 203, and 206 of PA-X.

[b]The number of isolates that possessed basic amino acid residues at the 6 positions. Nucleotide sequences deposited in the Influenza Research Database were analyzed in December 2014.

[c]Percentage of isolates from each host.

Effect of the N-terminal 15 amino acids in the PA-X-specific region on the shutoff activity. The addition of a nuclear localization signal can rescue the activity of a functional motif-deficient mutant (*14*). To examine whether the N-terminal 20 amino acids in the PA-X-specific region could increase the shut-off activity of PA_252 or PA-X_6E, I prepared PA_252_X20 and PA-X_6E_X20 (Fig. 4A) and evaluated their shutoff activities (Fig. 4B). PA_252, PA_252_X20, PA-X_6E, and PA-X_6E_X20 showed lower shutoff activity than that of wild-type PA-X. I confirmed that the expression levels of *Renilla* luciferase and each viral protein were comparable by western blotting (Fig. 4C and 4D). These results indicate that the N-terminal 20 amino acids of the PA-X-specific region contribute to the shutoff activity only when they are located at their original position.

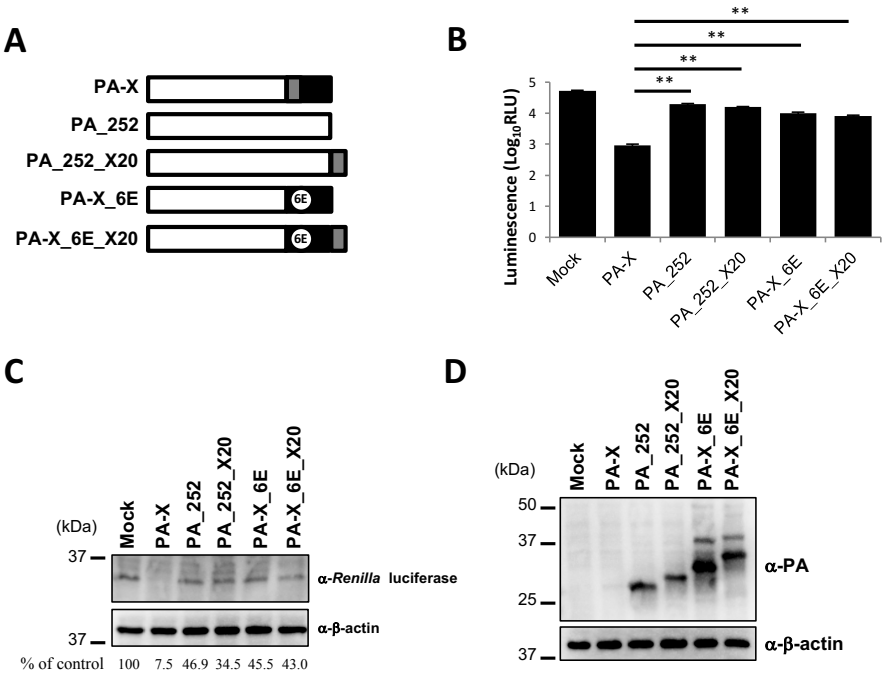

Figure 4. Effect of the N-terminal 20 amino acids in the PA-X-specific region on the shutoff activity of PA_252 and PA-X_6E. (A) Schematic diagram of PA_252_X20 and PA-X_6E_X20. These proteins contain the N-terminal 20 amino acids of the PA-X-specific region (gray) at the C-terminus of PA_252 or PA-X_6E, respectively. (B–D) Shutoff activities of PA_252_X20 and PA-X_6E_X20. In 293 cells, *Renilla* luciferase was co-expressed without (mock) or with wild-type PA-X, PA_252, PA_252_X20, PA-X_6E, or PA-X_6E_X20. *Renilla* luciferase activity (B), expression of *Renilla* luciferase (C) and of each viral protein (D) were evaluated by means of a luciferase assay or western blotting. (C) The intensities of the *Renilla* luciferase and β-actin bands were quantified and their ratios were calculated. The ratio of the mock lane was set to 100%. (C–D) β-actin served as a loading control.

Discussion

PA-X has a shutoff activity to reduce mRNA accumulation in infected cells and this shutoff activity is dependent on the N-terminal endonuclease activity of the protein (*10, 12, 13*). In this study, I found that the N-terminal 15 amino acids in the C-terminal PA-X-specific region are required for the full shut-off activity of PA-X. Most influenza A viruses, isolated from human, equine, and avian species, encode a PA-X protein that is identical in length to the PA-X of the WSN strain, which possesses 61 PA-X-specific amino acids at its C-terminus (*15*). However, most human H1N1pdm and swine viruses possess a stop codon upstream of the stop codon present in other PA-Xs, leading to a C-terminally truncated PA-X that possesses only 41 PA-X-specific amino acids (*15*). Despite this C-terminal truncation, these viral PA-Xs would have adequate shut-off activity because the N-terminal 15 amino acids in the PA-X-specific region are sufficient for full shutoff activity.

I also that 6 basic amino acids in the C-terminal PA-X-specific region of PA-X are important for the shutoff activity. This finding led me to speculate as to the function of this cluster of basic amino acids. First, I hypothesized that these 6 basic amino acids could function as nuclear localization signal (NLS) because NLSs are

known to be rich in basic amino acids (*14, 16*), For high shutoff activity, PA-X may be transported into nucleus to digest mRNAs, because I found that all of PA-X mutants that showed low shutoff activity mainly localized to the cytoplasm. Unfortunately, I was unable to determine the intracellular localization of wild-type PA-X or the PA-X mutants that showed high shutoff activity because of their low levels of expression. Although the possibility that these 6 basic amino acids function as an NLS remains, the addition of the N-terminal 20 amino acids of the PA-X-specific region, which included the 6 basic amino acids, to the C-terminus of PA_252 or PA-X_6E did not increase their shutoff activity (Fig. 4), indicating that these amino acids do not function as a repositionable NLS. However, the possibility that these 6 basic amino acids function as NLS remains because NLSs are not always repositionable (*17-19*). Accordingly, it is hard to prove this hypothesis. Second, I hypothesized that PA-X might bind mRNA via this cluster of 6 basic amino acids since RNA-binding domains are also known to be rich in basic amino acids (*16, 20*). The captured mRNA could then be digested by the N-terminal endonuclease activity of PA-X. Finally, these 6 basic amino acids might be important for interactions with as-yet unknown host proteins that facilitate or mediate

mRNA digestion by PA-X. Although in this study I could not determine the function of these 6 basic amino acids, further analyses are clearly warranted to dully characterize the PA-X shutoff mechanism.

Material and Methods

Cells. Human embryonic kidney 293 (HEK293) cells were maintained in DMEM (Sigma) supplemented with 10% FCS and penicillin-streptomycin (Wako).

Plasmids. The nucleotide sequences encoding PA-X or PA from influenza virus A/WSN/33 (H1N1) with a C-terminal FLAG tag was cloned into the mammalian expressing vector pCAGGS/MCS. Mutant PA and PA-X with the C-FLAG were constructed by PCR using appropriate primers, and then cloned into the same plasmids. All constructs were sequenced to confirm the nucleotide sequence.

Shutoff assay. 293 cells in 12- or 24- plates (Wako) were transfected with a plasmid encoding wild-type PA, wild-type PA-X, or their mutants together with pGL4.74[hRLuc/TK] (Progema), which expresses *Renilla* luciferase, by using Trans IT-293 (Minus Bio LLC) to analyze both *Renilla* luciferase activity and expression. The transfected cells in three of the 24-well plates were analyzed for *Renilla* luciferase activity by using the *Renill*a-Glo luciferase assay system (Promega) at 24 h post-transfection. Data are shown as the average of the relative shutoff activity ± standard deviation (n=3). To analyze the expression of PA, PA-X, or *Renilla* luciferase, the transfected cells in one of the 12-well plates were lysed in 2×SDS sample buffer at 24 h post-transfection. The cell lysates were sonicated, incubated for 10 min at 95 °C, and then loaded onto an Any KD Mini-PROTEAN TGX Gel (Bio-Rad). Separated proteins were transferred to Immobilon-P PVDF membrane (Millipore) and detected by

using anti-*Renilla* luciferase pAb (MBL), anti-DYKDDDDK (FLAG) tag antibody clone 1E6, or anti-β-actin antibody clone AC-74 (Sigma), followed by donkey anti-rabbit IgG-HRP (GE Healthcare) or sheep anti-mouse IgG-HRP (GE Healthcare).

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CHAPTER II

N-terminal Acetylation by NatB Is Required for the Shutoff Activity of Influenza A Virus PA-X

Chapter 2 はやむを得ない事由により除外しています。

CHAPTER Ⅲ

Identification of Amino Acid Residues of Influenza A Virus PA-X That Are Important for PA-X Shutoff Activity by Using Yeast

Abstract

The influenza A virus protein PA-X comprises an N-terminal PA region and a C-terminal PA-X-specific region. PA-X suppresses host gene expression, termed shutoff, via mRNA cleavage. Although the endonuclease active site in the N-terminal PA region of PA-X and basic amino acids in the C-terminal PA-X-specific region are known to be important for PA-X shutoff activity, other amino acids may also play a role. Here, I used yeast to identify novel amino acids of PA-X that are important for PA-X shutoff activity. Unlike wild-type PA-X, most PA-X mutants predominantly localized in the cytoplasm, indicating that these mutations decreased the shutoff activity of PA-X by affecting PA-X translocation to the nucleus. Mapping of the identified amino acids onto the N-terminal structure of PA revealed that some of them likely contribute to the formation of the endonuclease active site of PA.

Introduction

Influenza A virus suppresses host gene expression via its NS1 (*1*), viral polymerase complex (*2*), NP (*3*), and PA-X (*3*) proteins to prevent anti-viral host protein expression from infected cells (*4, 5*). PA-X suppresses protein expression by using its endonuclease activity to digest mRNA (*6, 8*).

PA-X comprises the N-terminal 191 amino acids of PA fused to the C-terminal 41 or 61 amino acids that result from the frameshift. Since the bivalent cation-binding residue D108 and the catalytic residue K134 are important for both the endonuclease activity of PA (*11*) and the shutoff activity of PA-X (*6*), the endonuclease domain of PA-X likely forms a similar structure to that of PA. At the C-terminal PA-X-specific region, the basic amino acid residues spanning the N-terminus of this region are also important for PA-X shutoff activity (*12*). Thus, although some residues important for PA-X shutoff activity have been identified, other amino acid residues may also be involved in the shutoff activity of PA-X. Here I identified novel amino acid residues involved in the shutoff activity of PA-X by using yeast.

Results

Emergence of unintentional PA-X mutants in yeast. It remained unclear whether PA-X suppressed protein expression in eukaryotes other than mammals. To this end, I expressed PA-X in *Saccharomyces cerevisiae* (yeast) and found that yeast that were transformed with a plasmid expressing wild-type PA-X formed very few colonies, indicating that PA-X has shutoff activity even in yeast. Based on this finding, I attempted to identify host genes involved in the shutoff activity of PA-X by screening a yeast knockout library (manuscript submitted). Briefly, I transformed ~4,700 knockout yeast with a plasmid encoding the C-terminally FLAG-tagged authentic PA-X (PA-X C-FLAG) and observed colony formation. From this screen, I obtained 29 colonies, which harbored plasmids encoding unintended PA-X mutants. Of these 29 clones, a single nucleotide mutation was detected in 27, whereas two nucleotide mutations were detected in the other two clones (Table). Three mutations, g322a, g372t, and c436t, were found in two clones and the a80g mutation was found in three clones. Since all of these identified nucleotide mutations led to amino acid changed, 24 PA-X mutants were

ultimately identified. I assumed that these amino acid substitutions affected the shutoff activity of PA-X.

Table. Unintended mutations in PA-X found in yeast colonies.

Amino acid mutations (Nucleotide mutation)	Number of clones obtained
F4S (t11c)	1
F9L (c27a)	1
Y24S (a71c)	1
D27G (a80g)	3
C39Y (g116a)	1
C45W (c135g)	1
A87V (c260t)	1
I94N (t281a)	1
L106P/S184I (t317c/g551t)	1
P107S (c319t)	1
D108N (g322a)	2
D108E (t324a)	1
E119N (g355a/a357t)	1
I120F (a358t)	1
T123I (c368t)	1
R124S (g372t)	2
R125K (g374a)	1
H146Y (c436t)	2
E154K (g460a)	1
E154A (a461c)	1
D160Y (g478t)	1
L163R (t488g)	1
R168M (g503t)	1
I171M (c513g)	1

Identified mutations reduce the shutoff activity of PA-X in mammalian cells. To evaluate the shutoff activity of the PA-X mutants in mammalian cells, I constructed plasmids encoding each mutant PA-X and conducted the shutoff assay using the luciferase system. The shutoff activities of all of the PA-X mutants were significantly lower than that of wild-type PA-X (Fig. 1A). Since PA-X suppresses its own expression via its shutoff activity (*12*), the expression of each PA-X mutant was assessed by western blotting to confirm findings of the shutoff assay. The expression of all of the PA-X mutants was higher than that of wild-type PA-X (Fig. 1B). These results demonstrate that these amino acid mutations reduced the shutoff activity of PA-X. Of note, although the shutoff activities of PA-X R124S and PA-X R125K were comparable to those of the other PA-X mutants (Fig. 1A), the expression of these two mutants was somewhat lower than that of the other mutants (Fig. 1B). This observation could indicate that these mutations may decrease the stability of PA-X as well as its shutoff activity.

To determine the contribution of each mutation in PA-X L106P/S184I to the shutoff activity, I prepared two PA-X mutants possessing single amino acid mutations

and compared their shutoff activities. The shutoff activity of PA-X L106P was lower than that of wild-type PA-X and comparable to that of PA-X L106P/S184I. PA-X S184I showed comparable shutoff activity to wild-type PA-X (Fig. 1C). The expression level of PA-X L106P was similar to that of PA-X L106P/S184I, whereas that of PA-X S184I was similar to that of wild-type PA-X (Fig. 1D). These data indicate that L106 is required for the shutoff activity of PA-X.

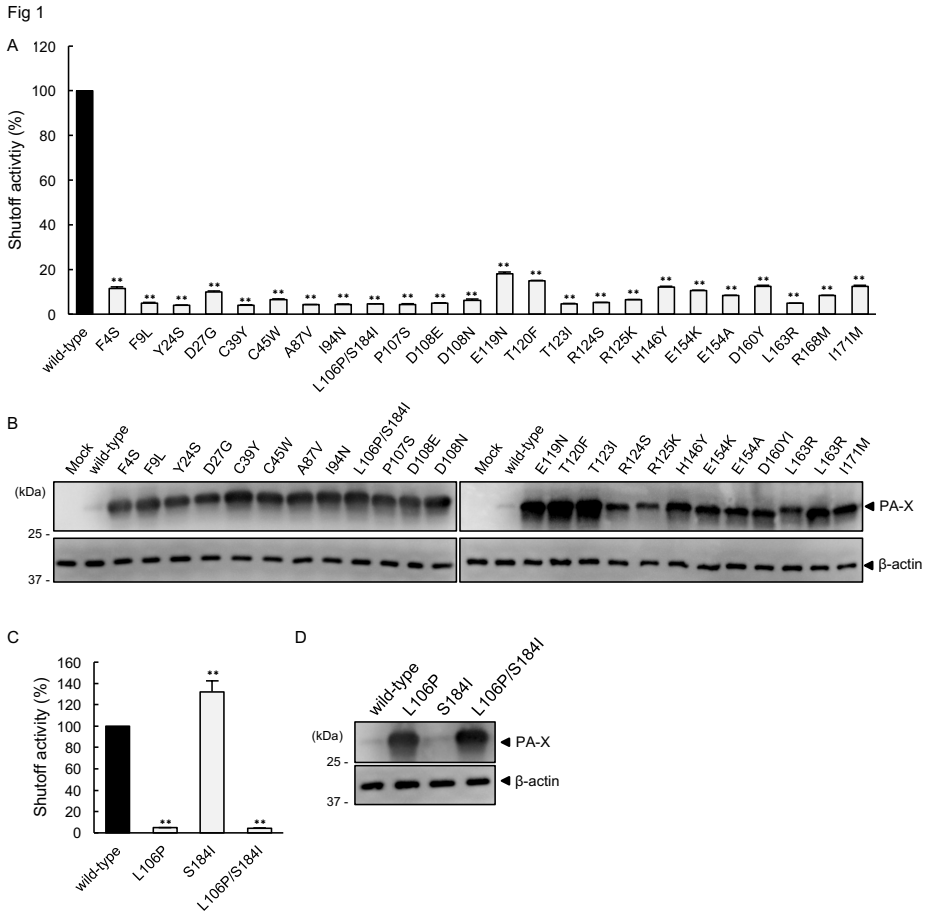

Figure 1. Identified mutations that reduced the shutoff activity of PA-X in mammalian cells. Shutoff activity (A and C) and expression (B and D) of wild-type PA-X or the indicated PA-X mutants in 293 cells were compared. (A and C) The shutoff activity of wild-type PA-X was set to 100%. The shutoff activities are presented as the mean values ± SD (n=3 technical replicates). Representative data from three individual experiments are shown. **, $P < 0.01$ (one-way ANOVA followed by Dunnett's test). (B and D) Expression of wild-type or mutant PA-X was analyzed by western blotting using a anti-FLAG antibody; β-actin served as a loading control.

Intracellular localization of the PA-X mutants. To examine whether the mutations that reduced the shutoff activity of PA-X affect its intracellular localization, the PA-X mutants were expressed in 293 cells by means of plasmid transfection. At 24 h post-transfection, I performed immunostaining with an anti-FLAG antibody to observe the intracellular localization of the PA-X mutants using confocal microscopy. Since wild-type PA-X robustly suppresses its own expression, I could not detect wild-type PA-X even in transfected cells, as described previously (*12*). PA-X Y24S, PA-X R124S, and PA-X R125K localized both in the nucleus and the cytoplasm, as did PA-X E119N albeit at lower levels than these other three PA-X mutants; the remaining PA-X mutants predominantly localized in the cytoplasm (Fig. 2). All of the PA-X mutants were detected as a punctual staining pattern in a limited number of cells. Since nuclear localization of PA-X is important for its shutoff activity (*10, 13*). I conclude that most of the identified mutations likely decrease the shutoff activity of PA-X by inhibiting its translocation to the nucleus.

Fig 2

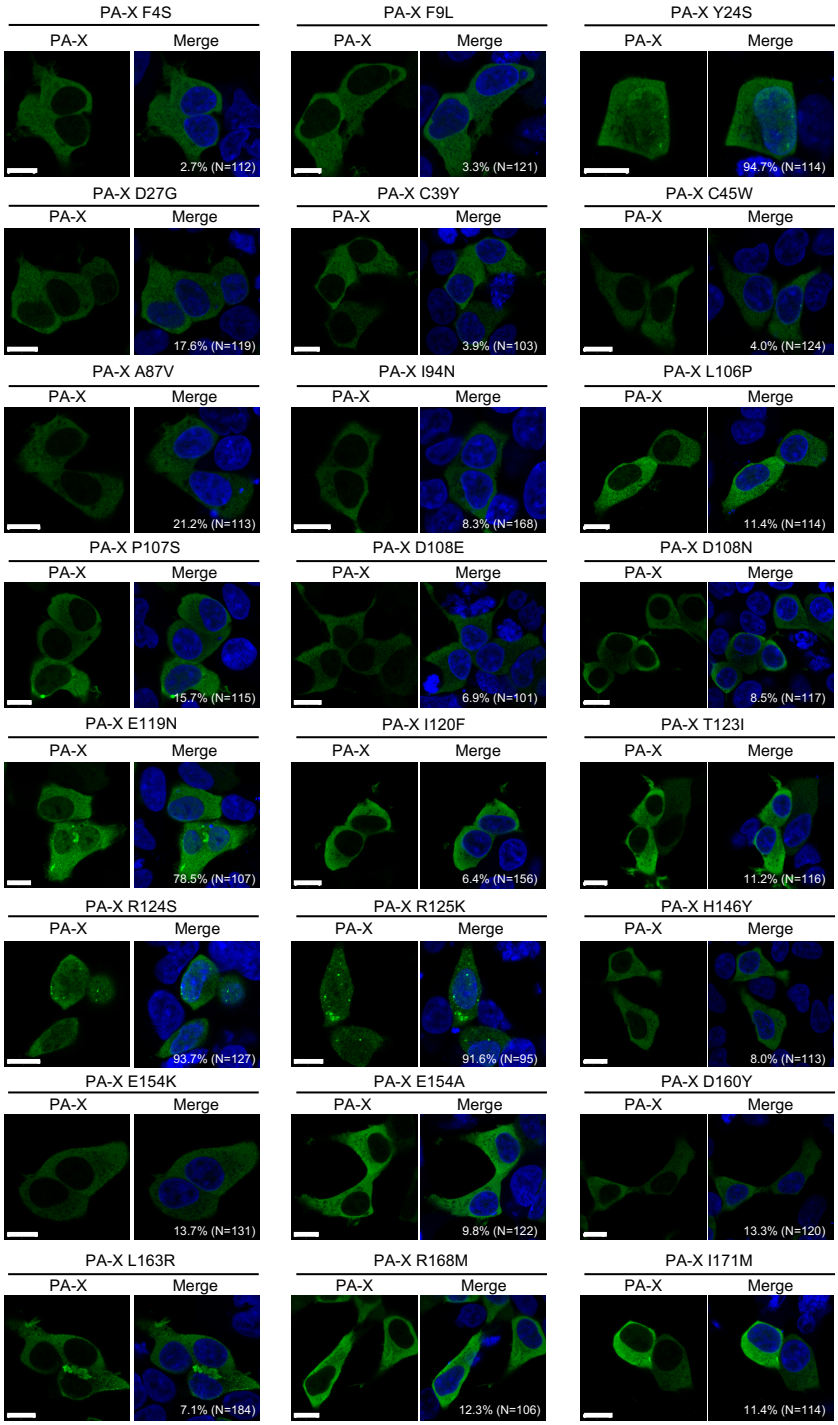

Figure 2. Intracellular localization of mutant PA-X. 293 cells were transfected with a plasmid encoding the mutant PA-X and fixed 24 h later. These cells were then stained with an anti-DYKDDDDK (FLAG) tag antibody (green). All images were obtained using confocal microscopy. The nuclei are stained with Hoechst 33342 (blue). The percentage of cells in which PA-X was localized both in the nucleus and the cytoplasm is indicated in each panel. The total number of counted cells is indicated in parentheses. Bars, 10 μm.

Discussion

Here, I identified 22 amino acids that were important for the shutoff activity of PA-X. Since PA-X shares its N-terminal 191 amino acids with PA (*6*), I mapped the identified 22 amino acids onto the N-terminal structure of PA, which includes a nuclease motif for mRNA cleavage (Fig. 3). Amino acid residues H41, E80, L106, P107, D108, E119, and K134 (Fig. 3; magenta) are known to be important for the nuclease activity of PA (*11, 14*). The former four residues bind to a bivalent cation, whereas K134 is the catalytic amino acid (*15*). These findings thus suggest that these residues are also important for the shutoff activity of PA-X. Of the seven residues, my study demonstrated the importance of L106, P107, D108, and E119 (Fig. 3, shown in magenta) to the shutoff activity of PA-X. Therefore, some of the other amino acids identified as important for the shutoff activity of PA-X may also be important for the nuclease activity of PA in the cap-snatching process. Around the nuclease active site, the side chains of Y24, C45, A87, I94, Y120, L163, and I171 (Fig. 3; cyan) extend toward the endonuclease active site, suggesting that these residues structurally support the formation of the nuclease active site. In contrast, F4, F9, D27, C39, T123, R124,

and R125 (Fig. 3; green) are located far from the endonuclease active site. Therefore, these amino acids are likely involved in other ways. Although amino acid residues in the C-terminal unique region of PA-X, especially the six basic amino acids, are important for the shutoff activity of PA-X (*12*), I did not obtain a PA-X mutant possessing a mutation in its C-terminal unique region.

PA-X possessing a mutation at Y24, E119, R124 or R125 localized both in the nucleus and the cytoplasm. This localization pattern is similar to that of wild-type PA-X (*10, 13*). Y24 and E119 are located near the endonuclease active site, whereas R124 and R125 are not. Therefore, there was no correlation between the position of the mutation and PA-X localization.

In summary, here I identified novel amino acid residues that are important for the shutoff activity of PA-X by expressing PA-X in yeast. Since some of these amino acids have been shown to be important for the nuclease activity of PA, these residues in PA-X are likely involved in mRNA cleavage. The roles of the other amino acid residues that I identified in this study remain unknown; structure–activity correlation analyses will be required as part of future studies to further characterize these residues.

Fig 3

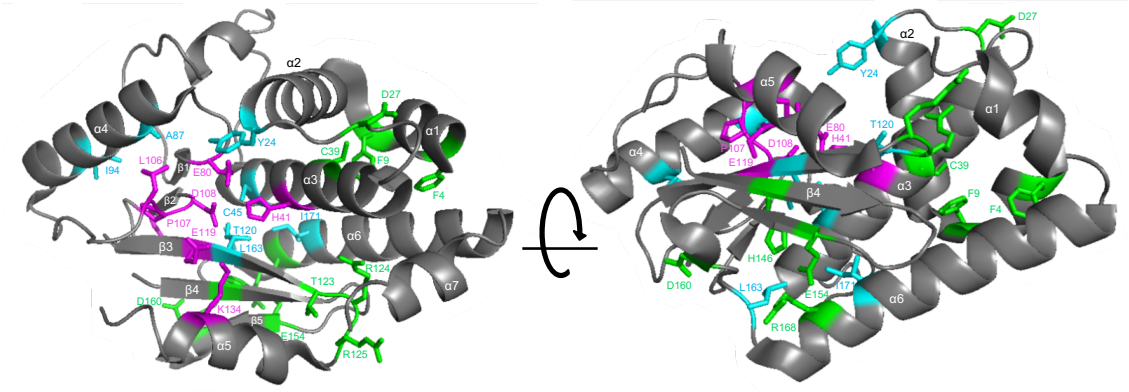

Figure 3. Structural positions of the identified amino acids that reduced the shutoff activity of PA-X. The identified amino acids are mapped onto a ribbon diagram of the structure of the N-terminal region of PA (2W69). Amino acid residues that are important for the endonuclease activity of PA are shown in magenta. Identified amino acid residues that locate near the endonuclease active site are shown in cyan. Other identified amino acids are shown in green.

Material and Methods

Cells. Human embryonic kidney 293 cells were maintained in DMEM (Sigma) supplemented with 10% FCS and penicillin-streptomycin.

Plasmids. Nucleotide sequences of PA-X derived from A/WSN/33 (H1N1) were cloned into a mammalian expression plasmid, pCAGGS/MCS, or a yeast protein expression plasmid, pKT10 (Ura) (*16*), with a C-terminal FLAG tag (C-FLAG). Mutant PA-X with the C-FLAG was amplified with primers possessing the desired substitution, and then cloned into pCAGGS/MCS. Primer sequences are available upon request. All constructs were sequenced to confirm the absence of unwanted mutations.

Yeast strains. Yeast strain BY4743 and Yeast Knockout Collection Homozygous Diploid (BY4743 background), which includes 4,653 knockout strains covering 96% of the yeast genome, were purchased from GE Healthcare Dharmacon.

Yeast transformation. Each knockout yeast strain was transformed with pKT10 (Ura) encoding PA-X with the C-FLAG or with an empty plasmid by using the *S. cerevisiae* Direct Transformation Kit Wako (Wako). Transformed yeast were plated onto SD/-Ura plates containing 200 μg/ml G418 and incubated for 3–5 days at 30 °C.

Plasmid extraction from yeast. Transformed yeast were picked from the SD/-Ura plate, grown in liquid SD/-Ura medium for 1–2 days at 30 °C, and then harvested by centrifugation. To disrupt the yeast, they were suspended in PBS and vigorously vortexed together with acid-washed glass beads (Sigma). Plasmids were extracted from the supernatant of the disrupted yeast by using Wizard *Plus* SV Minipreps DNA Purification Systems (Promega). By using the extracted plasmids as a template, an open reading frame of PA-X was amplified by PCR and directly sequenced. To exclude the possibility of PCR errors, the plasmids extracted from yeast were also used to transform *E. coli* (DH5α strain*)*. Plasmids extracted from *E. coli* were then resequenced.

Shutoff assay. 293 cells were transfected with a plasmid encoding firefly luciferase together with an empty plasmid, or a plasmid encoding wild-type or mutant PA-X with

a C-FLAG. The shutoff activity of PA-X was measured and calculated as described above. PA-X expression was determined by western blotting as described above by using anti-DYKDDDDK (FLAG) tag antibody clone 1E6, or anti-β-actin antibody clone AC-74 (Sigma), followed by donkey anti-rabbit IgG-HRP (GE Healthcare) or sheep anti-mouse IgG-HRP (GE Healthcare).

Immunofluorescent assay. 293 cells were transfected with a plasmid encoding the indicated PA-X mutant. At 24 h post-transfection, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were stained with the anti-DYKDDDDK (FLAG) tag antibody clone 1E6, followed by an Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen). Nuclei were stained with Hoechst 33342 (Invitrogen). Images were obtained by using a Zeiss LSM780 (Carl Zeiss).

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CONCLUDING REMARKS

Viruses induce inhibition of host gene expression to escape from the innate immune response of the host and promote their replication. The influenza A virus protein PA-X was identified in 2012 and shown to be important for host shutoff. Although several groups have reported on the importance of the shutoff activity of PA-X to virus replication and pathogenicity in cultured cells and mice, its mechanism remains unknown.

In Chapters I and III, I identified 6 basic amino acid residues in the C-terminal PA-X-specific region and 22 amino acid residues in the N-terminal PA region that are important for the shutoff activity of PA-X. Although I could not determine the function of these amino acids, my findings may be helpful in future studies designed to characterize the PA-X shutoff mechanism.

In Chapter II, I revealed that the host protein NatB, which comprises NAA20 and NAA25, is required for the shutoff activity of PA-X. Although other viruses possessing shutoff activity require host proteins for their activities, there had been no previous reports identifying host proteins involved in the shutoff activity of PA-X. NatB,

an N-terminal acetyltransferase, catalyzes the N-terminal acetylation of newly synthesized proteins. I revealed that N-terminal acetylation by NatB is required for the shutoff activity of PA-X, which is also N-terminally acetylated.

I focused on PA-X, which is one of the influenza virus proteins. Since the expression level of PA-X is very low, it is hard to determine the localization of PA-X, or to purify PA-X for in vitro analysis. This may explain why the shutoff mechanism of PA-X remains unknown even though PA-X plays roles in viral infection. In closing, even though I was unable to completely elucidate the shutoff mechanism of PA-X, I hope that my findings will contribute to future studies and clarify the shutoff mechanism of PA-X, which will ultimately help us to control influenza virus infections.

ACKNOWELEGMENTS

Foremost, I would like to express my deepest appreciation to Prof. Yoshihiro Kawaoka for his insightful suggestions and comments, and strict, but warm encouragement. I especially would like to offer my deepest gratitude to my supervisor, Dr. Seiya Yamayoshi, for his precise and intelligent advice, and strict, but warm encouragement. Because of their guidance, I gained so much valuable experience, and acquired a broad set of skills and knowledge base of biology.

I am also grateful for my collaboration with Drs. Masaaki Oyama and Hiroko Kozuka-Hata, which generated valuable advice and technical support. Thanks to their support and guidance, I obtain important data.

I am really grateful to Prof. Takeshi Noda, Dr. Shinya Yamada, Dr. Maki Kiso, Dr. Kyoko Iwatsuki-Horimoto, Dr. Yuko Sakai-Tagawa, Dr. Mutsumi Ito, Dr. Yuriko Tomita, for their helpful suggestions, guidance, and support. I would also like to offer my deepest appreciation to Dr. Tomomi Ando, Dr. Ryuta Uraki, Dr. Tomoko Kuwahara-Hayashi, Dr. Sumiho Nakatsu-Akimoto, Dr. Tadashi Maemura, Dr. Sarah Takasaki, Mr. Hiroki Ui, Mr. Atsuhiro Yasuhara, and Ms. Misako Konno for their

helpful support and discussions. I also thank Dr. Susan Watson for correcting my manuscripts many times. Finally, I would like to express my gratitude to all members of the Division of Virology, Institute of Medical Science, University of Tokyo, and my friends and family for their warm support.