Desmet, E.A., Bussey, K.A., Stone, R., Takimoto, T. Identification of the N-terminal domain of the influenza virus PA responsible for the suppression of host protein synthesis

(2013) Journal of Virology, 87 (6), pp. 3108-3118.

Identification of the N-terminal domain of influenza PA responsible for the suppression of host protein synthesis

Emily A. Desmet[1], Kendra A. Bussey[2], Raychel Stone and Toru Takimoto*

Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642 USA

*Corresponding Author

1)Address: Department of Microbiology and Immunology, University of Rochester Medical Center, Box 672, 601 Elmwood Avenue, Rochester, NY 14642.

Tel: (585)273-2856

Fax: (585)473-9573

Email: toru_takimoto@urmc.rochester.edu

[1]Current address: Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Hungerford Hill Road, Ithaca, NY 14853.

[2]Current address: Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany.

Abstract

Cellular protein synthesis is suppressed during influenza virus infection, allowing for preferential production of viral proteins. To explore the impact of polymerase subunits on protein synthesis, we co-expressed eGFP or luciferase together with each polymerase component or NS1 of A/California/04/2009 (Cal) and found that PA has a significant impact on the expression of eGFP and luciferase. Comparison of the suppressive activity on co-expressed proteins between various strains revealed that avian virus or avian-origin PAs have much stronger activity than human-origin PAs, such as the one from A/WSN/33 (WSN). Protein synthesis data suggested that reduced expression of co-expressed proteins is not due to PA's reported proteolytic activity. A recombinant WSN containing Cal PA showed enhanced host protein synthesis shutoff and induction of apoptosis. Further characterization of the PA fragment indicates that the N-terminal domain (PANt), which includes the endonuclease active site, is sufficient to suppress co-transfected gene expression. By characterizing various chimeric PANt, we found that multiple regions of PA, mainly the helix α4 and the flexible loop of amino acids 51-74, affect the activity. The suppressive effect of PANt cDNA was mainly due to PA-X, which was expressed by ribosomal frameshifting. In both Cal and WSN viruses, PA-X showed a stronger effect than the corresponding PANt, suggesting that the unique C-terminal sequences of PA-X also play a role in suppressing co-transfected gene expression. Our data indicate strain variations in PA gene products, which play a major role in suppression of host protein synthesis.

Keywords: Influenza virus; Polymerase; PA; Shutoff; Protein synthesis; Apoptosis; Pathogenesis**Introduction**

Influenza A viruses remain a significant public health threat. During a typical year, seasonal epidemics result in at least 36,000 deaths in the United States alone (33). Typically, infection with seasonal influenza often presents as a mild infection. Periodically, however, pandemics arise, resulting in severe morbidity and mortality within the population. In the case of highly pathogenic influenza A viruses, such as avian H5N1, infection is often associated with severe tissue destruction and dysregulated immune responses (22). Factors that contribute to increased pathogenicity following human infection with avian viruses are not well understood.

Influenza infection results in the rapid decline of host protein synthesis, a process referred to as shutoff (14, 15). Inhibition of cellular protein synthesis is expected to aid in dampening the anti-viral response, which could be a major factor for efficient viral spread and pathogenicity. Therefore, it is no surprise that influenza viruses have adopted exquisitely complex methods for inducing shutoff during infection. It is not known which viral proteins are responsible for host protein synthesis shutoff. Several groups have shown that NS1 inhibits IFN-α/β synthesis (6, 35) and interferes with nuclear export and stability of cellular mRNAs (4, 24, 25), while enhancing the efficiency with which viral mRNAs are translated (1, 2). However, analysis of influenza-induced shutoff using a virus lacking NS1 expression showed that NS1 was not required for the inhibition of host protein synthesis, suggesting that other viral factors are involved in shutoff (28). More recently, several observations in the field have suggested a role for the polymerase complex in host protein synthesis shutoff, based upon the observation that cellular RNA polymerase II (RNAPII) is degraded following infection. The viral polymerase complex binds to the C-terminal domain, in particular the active phosphoserine form, of the large subunit of

RNAPII (3). Artificial expression of the polymerase complex is associated with degradation of the inactive unphosphorylated form of RNAPII. Inactive RNAPII is similarly degraded in cells expressing the PA subunit in the absence of PB1 and PB2, indicating a PA-dependent activity is responsible for the observed degradation. However, ubiquitination of the accumulating transcriptionally active form of RNAPII is dependent on the binding of PB2 to RNAPII, suggesting that the full polymerase complex is required for the inhibition of host transcription that is associated with a loss of a functional RNAPII (27, 37). Other studies have suggested a contribution of PA to shutoff based on the ability of PA to induce proteolysis. PA expression was reported to induce the degradation of co-expressed proteins (29). However, it is unclear if the proteolytic activity of PA is sufficient for shutoff, as the PA T157A mutation which reduces proteolysis has no effect on viral induction of shutoff (12, 27). This suggests that other functions of PA, or other targets, contribute to host protein synthesis shutoff. Interestingly, a new viral protein PA-X was recently found to be produced from the PA gene by ribosomal frameshifting (13). PA-X was shown to repress cellular gene expression and modulate viral virulence and global host response, suggesting that an additional PA gene product contributes to the regulation of host response upon viral infection.

In this study, we evaluated the impact of polymerase subunits on influenza induced host protein synthesis shutoff. We show that expression of influenza PA, but not PB1, PB2, or NS1, resulted in decreased protein expression from co-transfected cDNAs. Interestingly, PA genes of avian virus origin, such as A/California/04/2009 (Cal) were more potent in shutoff of protein synthesis than those from the human viruses tested including A/WSN/33 (WSN). The enhancement phenotype associated with avian origin PA was also observed during viral infection with a recombinant WSN virus expressing the PA gene from Cal. The efficient shutoff observed

in the presence of avian origin PA correlated with a sharp increase in apoptotic cell death. Interestingly, expression of the N-terminal 257 residues was sufficient to induce shutoff and further analysis using chimeric constructs identified a region in the N-terminal domain responsible for the different shutoff activity. Finally, we detected a similar difference in the shutoff activity of the recently identified PA-X proteins of WSN and Cal. Taken together, these observations implicate a major involvement of PA gene products in non-viral protein synthesis shutoff, which may contribute to viral evasion of the host's antiviral activity.

Materials and Methods

Viruses and cultured cells. A/California/04/2009 (H1N1) (Cal) and A/chicken/Nanchang/3-120/01 (H3N2) (Nan) were generously provided by R. Webster and R. Webby (St. Jude Children's Research Hospital, Memphis, TN). A/New Caledonia/20/99 (H1N1) (NC) was generously provided by A. Sant (University of Rochester). A/WSN/33 (H1N1) (WSN) was rescued from cDNAs generously provided by Y. Kawaoka (University of Wisconsin, Madison). All viruses were propagated in MDCK cells. 293T, A549 and MDCK cells were maintained at 37°C and DF-1 cells were maintained at 39°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (FCS).

Antibodies. Anti-Flag M2 (200472) and anti-β-actin (AC-15) antibodies were purchased from Agilent Technologies and Sigma-Aldrich, respectively. The anti-influenza A virus PA concentrated culture supernatant (clone 2C3-C10) was generated in the lab (19).

Plasmids. Cal PA, PB1, PB2, NP, and NS1, as well as NC and Nan PA genes were cloned by reverse transcription-PCR (RT-PCR) using total RNA extracted from infected cells and first subcloned to pCMV-Tag4a (Stratagene) to add an in-frame Flag-tag before final subcloning to

pCAGGSmcs (16, 20) for expression. pCAGGS-WSNPA, -WSNPB1, -WSNPB2 and -WSNNP were obtained from Y. Kawaoka. The eGFP cDNA was subcloned from pEGFP-N1 (Clontech) into pCMV-Tag4A, followed by further subcloning of eGFP-Flag into pCAGGSmcs. The pRL-SV40 vector (Promega) expresses Renilla luciferase under the control of the Simian virus 40 promoter. pCAGGS-Luc expressing firefly luciferase was constructed by subcloning the luciferase gene from pPolI-NP-Luc, which was provided by T. Wolff (Robert-Koch Institute, Berlin, Germany). Chimeric PA genes were constructed using PCR for gene splicing by overlap extension (11) or using PCR amplification of individual fragments, followed by recombination of multiple fragments and vector using the In-Fusion HD cloning system (Clontech). The full-length Cal PA gene was cloned into the pPolI vector for the rescue of WSN-CalPA using the reverse genetics system described below. pCAGGS cDNAs containing the PA N-terminal domain (resides 1-257), or C-terminal domain (residues 258-716) were produced by cloning the PCR products amplified using specific primers. Cal and WSN PA-X cDNAs were constructed using QuickChange II site-directed mutagenesis kit (Agilent Technologies). Cytosine 598 was removed from the PA cDNA. The coding region of PA-X was then amplified by PCR using primers containing unique restriction enzyme sites and the Flag sequence and cloned into pCAGGSmcs. CalPANtFS, which contains mutations at the frameshift motif in Cal PANt from UCCUUUCGU to AGCUUCAGA was constructed by site-directed mutagenesis,

as described above. All constructs were sequenced for confirmation.

Luciferase assay. 293T cells were transfected with the indicated pCAGGS expression vector together with pRL-SV40 or pCAGGS-Luc for 18-24 h using Lipofectamine 2000 (Invitrogen). Luciferase production was measured using reagents in the dual-luciferase reporter assay system

(Promega). All results are an average with standard deviations from three or four independent experiments.

Radioimmunoprecipitation Assay. To determine the impact of PA on the synthesis of co-expressed proteins, 293T cells were transfected with either empty pCAGGSmcs, pCAGGS-Cal PA-Flag, or pCAGGS-WSN PA-Flag together with pCAGGS-eGFP-Flag. Thirty min prior to the indicated time points, cells were radiolabeled and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing 9.25 mg/ml iodoacetamide, 1.7 mg/ml aprotinin and 10 mM phenylmethylsulfonyl fluoride. Flag-tagged proteins in cell lysates were immunoprecipitated using Dynabeads (Invitrogen) complexed with 5 μg anti-Flag M2 antibody. Samples were resuspended in NuPAGE LDS sample buffer and resolved on NuPAGE-Novex Bis-Tris gels (Invitrogen). Dried gels were exposed to a Phosphor Screen and visualized using a Biorad Personal Molecular Imager (Biorad). Percent adjusted volume was determined using Quantity One 1-D Analysis Software (Biorad).

Virus rescue. WSN-CalPA was rescued using the 12-plasmid rescue system developed by Neumann et al (20). Briefly, a 6-well plate was seeded with a 293T/MDCK co-culture. Cells were transfected with 0.1 μg of each pPolI plasmid (from WSN, except pPolI-Cal PA) and 0.4 μg each of pCAGGS-CalPA, pCAGGS-WSNPB1, pCAGGS-WSNPB2 and pCAGGS-WSNNP using Lipofectamine 2000 (Invitrogen) in Opti-MEM. Medium was changed to DMEM supplemented with 1% FCS after 24 h. Rescued virus was plaque purified and stock virus was prepared in MDCK cells. The PA sequence of the virus was confirmed. The titer of WSN-CalPA from MDCK cells was determined using the method of Reed and Muench (26).

Virus growth curves. A549 cells were infected at a multiplicity of infection (MOI) of 0.01 for 1 h, washed once with Dulbecco's phosphate-buffered saline (PBS) with magnesium and calcium

(Invitrogen), and then cultured at 37°C in DMEM containing 0.15% bovine serum albumin (BSA) and tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin at 1 μg/ml. At the indicated time points, 10% of the culture supernatant was harvested and viral titers in MDCK cells were determined as described above. Results shown represent mean and standard error of mean (SEM) from at least 3 independent experiments.

Metabolic labeling. To measure the impact of infection on protein synthesis, A549 cells were mock infected or infected with WSN or WSN-CalPA at a MOI of 3 for 1 h at 37°C. At 4, 6 and 8 hpi, cells were washed and labeled with 50 μCi [^{35}S]Met/Cys for 30 min in medium lacking methionine and cysteine. Cells were then lysed in RIPA buffer as above. Samples were resolved by SDS-PAGE. Dried gels were exposed to a Phosphor Screen and visualized using a Biorad Personal Molecular Imager. To compare the sum of intensities of pixels across lanes, volume analysis was performed using Quantity One 1-D Analysis Software. In brief, the volume of a selected band or region was compared across all lanes. These values are depicted as percent adjusted volume, or the volume expressed as a percentage of all of the volumes for a particular band or region of the gel.

Viability assays. Trypan blue assay was used to determine the percent viability of both infected and transfected cells (36). Briefly, A549 cells infected at a MOI of 3 or 293T cells transfected with 1.6 μg of cDNAs were collected after trypsin-EDTA treatment and incubated with trypan blue for 5 min before live/dead counts. Additionally, viability was measured using the Cell Lab ApoScreen Annexin V-FITC Apoptosis Kit (Beckman Coulter) according to the manufacturer's instructions.

Statistical analysis. Statistical significance was evaluated using the non-parametric unpaired t-test when comparing appropriate groups. A *P* value of less than 0.05 was considered significant.

Results

Pandemic H1N1 PA contributes to the suppression of protein expression from co-transfected cDNAs. The influenza polymerase complex is thought to be involved in the shutoff of host protein synthesis, as expression of the complex results in the ubiquitination and degradation of RNAPII (37). To further determine the contribution of each component to influenza-induced shutoff, we generated cDNA expression vectors containing Flag-tagged viral polymerase subunits from the pandemic A/California/04/09 strain (Cal). In addition, as previous studies suggested that NS1 inhibited cellular mRNA translation (4, 18), we chose to also include NS1 in our initial evaluation. 293T cells were co-transfected for 24 h with pCMV-eGFP-Flag and individual plasmids to direct the expression of the indicated Cal genes. We observed that, in the presence of Cal PB1, PB2, or NS1, eGFP expression was found to be similar to that in cells expressing empty vector. However, eGFP expression was markedly decreased in cells co-transfected with Cal PA (Fig. 1A). To validate these results quantitatively, we next measured the effect of individual viral protein expression on the production of luciferase from transfected cDNA. As observed with eGFP, co-expression of PA significantly reduced luciferase expression by 25-fold compared to empty vector, in sharp contrast to the 3-, 2-, or 6-fold reduction compared to empty vector in the presence of PB1, PB2, and NS1, respectively (Fig. 1B). These results suggest that PA has the most significant effect on suppression of co-expressed proteins.

During influenza infection, the majority of PA is incorporated into the polymerase complex. To address whether the shutoff function of PA is retained both when PA is expressed by itself, as well as when it is expressed as part of the polymerase complex, 293T cells were co-transfected with luciferase and either PA alone, or PA in conjunction with PB1, PB2, and NP. The

amount of PA cDNA was adjusted so that the same amount of PA was expressed across samples, which was confirmed by Western blot analysis (Fig. 1C, lower panel). Quantification of the luciferase level revealed a similar level of reduction in cells expressing PA alone or PA together with other nucleocapsid components, showing that both forms of PA can suppress co-transfected luciferase expression (Fig. 1C).

Avian origin PA is more efficient than human origin PA at suppressing protein expression. The PA gene from the 2009 pandemic strain originated from an avian virus (8). To determine the impact of gene origin on shutoff of exogenous protein expression, we compared the activity between avian and human virus PAs in suppressing non-viral protein synthesis using Cal and A/chicken/Nanchang/3-120/01 (Nan) PAs as avian-like or avian PA, and A/New Caledonia/20/99 (NC) and A/WSN/33 (WSN) PAs as human virus PA. Following co-transfection of eGFP cDNA together with the PA of Cal, Nan, NC, and WSN, we detected that eGFP expression was significantly reduced in the presence of Cal or Nan PA, which are of avian origin, compared to those of human virus origin (Fig. 2A). Similarly, luciferase expression was greatly reduced in the presence of avian origin PA, 32-fold with Cal and 53-fold with Nan, compared to the reduction seen in human origin PA, 5-fold with NC and 3-fold with WSN (Fig. 2B). Although the level of PA expression differed between strains, these differences did not correlate with reduced luciferase expression. For example, Nan PA was detected minimally in transfected cells; however, it reduced the level of co-expressed luciferase the most effectively. Similarly, although a large amount of WSN PA was expressed, it showed a very limited effect on luciferase expression (Fig. 2B). In order to evaluate if this difference of suppression was host cell specific, we performed the same luciferase assay in chicken fibroblast DF-1 cells. Avian origin PA most

effectively suppressed luciferase expression, suggesting that the difference in shutoff between viruses is not host cell specific (Fig. 2C).

Avian origin PA significantly affects the synthesis of co-expressed proteins. Our data shown above clearly indicate that Cal PA strongly suppresses expression of co-expressed proteins. This effect could also affect the level of PA itself, if PA affects cellular machinery for protein synthesis. To address this, we compared the synthesis of eGFP and PA at various times after transfection. 293T cells were co-transfected with PA and eGFP both tagged with Flag, and at 6, 10 and 14 h post transfection, cells were labeled with [^{35}S]Met/Cys for 30 min. Newly synthesized proteins were immunoprecipitated by anti-Flag antibody and analyzed by SDS-PAGE (Fig. 3A). In cells expressing eGFP and WSN PA, synthesis of both proteins increased over time. However, in cells expressing eGFP and Cal PA, Cal PA production was increased over time, while eGFP synthesis was strongly suppressed from early (6 h) to late (14 h) time points (Fig. 3B). It should be noted that the increase in Cal PA expression itself was slower than that observed for WSN PA or eGFP when expressed alone. This suggests that Cal PA negatively affects its own expression, although the suppressive effect was significantly stronger in the case of co-expressed eGFP. The Cal and WSN PA cDNA constructs do not contain the non-coding sequence upstream of the start codon that has previously been shown to enable preferential translation of viral mRNAs (7). Therefore, preferential expression of Cal PA in concert with the dramatic shutoff of non-viral proteins is not likely due to the intrinsic structural features of the non-coding sequence of the viral gene.

Growth of the WSN-CalPA virus is attenuated. Due to the significant difference in shutoff of non-viral protein synthesis between avian origin PA and human origin PA, we further evaluated this difference in the context of viral infection. To address this, we generated a recombinant

WSN virus containing Cal PA (WSN-CalPA) using the 12-plasmid rescue system (20). The rescued virus was plaque purified and subsequently amplified in MDCK cells. The origin of the PA gene of WSN-CalPA was confirmed by sequencing the RT-PCR product of the gene. First, we compared growth kinetics of WSN and WSN-CalPA in human lung carcinoma A549 cells. At early time points following infection, both WSN and WSN-CalPA had similar growth kinetics, while at later time points, the WSN-CalPA titer in the culture medium was reduced compared to WSN (Fig. 4A), possibly due to increased cytopathic effect observed in WSN-CalPA-infected cells. Plaque phenotypes for both viruses were similar, suggesting no major defects in viral replication or cell-to-cell spread (data not shown).

Protein synthesis is reduced to a greater extent during infection with WSN-CalPA. Reduced protein expression in the presence of PA could be the result of protein synthesis suppression or protein degradation due to the reported proteolysis function of PA. No studies to date have evaluated the impact of PA on protein synthesis in an infection model. To determine the impact of PA on protein synthesis, A549 cells were either left uninfected, or infected with WSN or WSN-CalPA at a MOI of 3, and at various time points after infection, cells were radiolabelled with [^{35}S]Met/Cys for 30 min to determine the overall protein synthesis in infected cells. At early times (4 h) following infection, cellular protein levels were similar in all three instances. However, as infection ensued, cellular protein synthesis was reduced in the presence of virus, and this was more efficient in cells infected with WSN-CalPA (Fig. 4B). Quantification of cellular proteins with a molecular weight of greater than 100 kDa (shown as CP1) or 50 kDa (CP2) confirmed that cellular protein synthesis was strongly inhibited by WSN-CalPA infection at 8 h (Fig. 4B and C). In contrast, viral protein synthesis increased with time and was more significant in WSN-CalPA infected cells. This difference is likely to reflect efficient host protein

synthesis shutoff by Cal PA and/or enhanced virus replication kinetics. Together, these data suggest that in the course of infection, Cal PA has a greater impact on cellular protein synthesis shutoff and viral protein production than WSN PA.

Increased apoptosis in cells infected with WSN-CalPA. Examination of cells transfected with PA cDNA revealed increased cytopathic effect in the presence of avian origin PA at 24 h after transfection (data not shown). We also detected reduced virus growth of WSN-CalPA at late time points, which could be explained by enhanced cell death (Fig. 4A). Our failure to establish A549 cell lines constitutively expressing Cal PA (data not shown) further suggested that Cal PA induces strong cytopathogenicity in cells. To determine if the presence of Cal PA affects cell viability, and enhances apoptotic cell death, A549 cells were infected with both WSN and WSN-CalPA at a MOI of 3, and at various time points after infection, cells were harvested and live cells were detected and counted by trypan blue exclusion assay. As early as 9 h after infection, the percent viable cells was reduced in cells infected with WSN-CalPA, and WSN-CalPA induced more cell death throughout infection as measured up to 48 h (Fig 5A). To determine if infected cells were undergoing apoptosis, we performed immunofluorescence for annexin V, an early marker of apoptosis, and propidium iodide, indicative of late apoptosis. At both 12 h and 24 h post infection, the percent of cells undergoing apoptosis was substantially higher in cells infected with WSN-CalPA as compared to WSN-infected cells (Fig. 5B). Taken together, these results suggest that the PA gene of influenza also contributes to the induction of apoptotic cell death, potentially as a result of host protein synthesis shutoff.

The N-terminal domain of PA is responsible for the suppression of protein synthesis. Structural studies of PA suggest that PA is composed of two domains, an N-terminal domain consisting of resides 1-257, and a C-terminal domain composed of residues 258-716. Taking

advantage of the difference in activity between Cal and WSN PA, we created two PA chimeras composed of WSN or Cal PA domains (Fig. 6A). The chimeric, as well as wt PA genes, were tagged with Flag for direct comparison of expressed protein levels. We expressed the PA proteins together with luciferase and determined the level of luciferase expression in co-transfected cells. As shown in Fig. 6B, chimera CW PA suppressed luciferase expression as efficiently as wt Cal PA, suggesting that the difference in activity between Cal and WSN is determined by the PA N-terminal domain.

To determine if the N-terminal domain by itself suppresses host protein synthesis as reported previously (30), we created cDNAs that express the N-terminal domains (residues 1-257) of either Cal and WSN or the C-terminal domain (residues 258-716) of Cal. For an unknown reason, we were unable to stably express the C-terminal domain of WSN. We determined the effect of these domains on the suppression of co-transfected luciferase gene products. As shown in Fig. 7, co-expression of the C-terminal domain of Cal had no effect on the luciferase level, while the N-terminal domain of Cal strongly suppressed the expression of co-transfected luciferase. In fact, the effect of the N-terminal domain was even stronger than the full size PA molecule. These results indicate that the N-terminal domain containing the endonuclease active site is responsible for the suppression of co-transfected gene product expression.

Identification of the residues that affect protein expression. Cal and WSN PA contain 15 amino acid differences in the N-terminal fragment (Fig. 8A). To identify the residues that differentiate the suppressive activity between Cal and WSN PA, we constructed 8 chimeric PA N-terminal fragments (Fig. 8A) and determined their ability to inhibit co-expressed luciferase expression (Fig. 8B). All of the chimeric PA N-terminal domains were expressed at a similar level as determined by Western blotting using an anti-Flag antibody (Fig. 8C). Comparison of the

level of luciferase coexpressed with chimeras 1 and 2 indicated that residues within the PA N-terminal 186 amino acids are responsible for the strong inhibition of co-expressed protein. Chimeras 8 and 5, which contain only residues 57-65 or 85-114 from Cal PA, suppressed luciferase expression 5.5- and 2.4-fold, respectively. Expression of chimera 7, which contains residues 57-114 of Cal PA, resulted in the greatest suppression (17-fold) of luciferase activity. Within the crystal structure of the PA N-terminal fragment, residues 57-65 are in a flexible loop region of unknown structure (38). Residues 85-91 are in the helix α4, and residue 100 is located in the loop between α4 and β2 (Fig. 8D). Interestingly, the region most strongly affecting shutoff activity (residues 57-114) is proximally located to an area containing a flexible loop and the helix α4, suggesting the overall structure of the region plays a major role in shutoff activity. In addition to this region, we noticed that residue 186 also contributes to the inhibitory effect. Comparisons of the shutoff activity between chimeras 1 and 3, as well as between chimeras 4 and 5, which differ only at residue 186, indicate that a glycine at position 186 confers greater shutoff activity than a serine at this position. Nan PA, which has a stronger inhibitory activity than Cal PA, contains a glycine at 186 (data not shown), potentially explaining the difference in activity between Cal and Nan (Fig. 2). Overall, characterization of chimeric PA N-terminal fragments suggests that multiple regions of PA, mainly the helix α4 and flexible loop at 51-74, are involved in shutoff activity.

PA-X strongly suppresses protein expression. Recently, a new viral protein, termed PA-X, has been identified to be expressed from the PA gene by ribosomal frameshifting (13). Cal and WSN PA-X are composed of 232 and 252 residues containing PA-X unique sequences in residues 192-232 or 192-252, respectively (Fig. 9A). It is possible that PA-X expressed by frameshifting from PANt cDNA is responsible for the reduction in protein expression. To address this, we

constructed a cDNA that expresses only PA-X by deleting cytosine 598, and a CalPANtFS cDNA containing mutations at the frameshift motif, which reduces the expression of PA-X (13). Co-transfection of these cDNAs with luciferase cDNA clearly indicates that PA-X expression has a strong impact on suppressive effects (Fig. 9B). This result indicates that the unique C-terminal sequence in PA-X plays a major role in the suppression of protein expression. Interestingly, WSN PA-X is less active than Cal PA-X at a similar level observed for PA N-terminal fragments (Fig. 8), suggesting that the regions shown in Fig. 8D are responsible for the difference in shutoff activity between Cal and WSN PA-X proteins.

Structural and functional analysis revealed that PA possesses an endonuclease site in the N-terminal domain with a putative $P_{107}D_{108}X_{10}E_{119}K_{134}$ active motif (38). It has been shown that D108A and K134A mutations completely inhibit endonuclease activity *in vitro* (10). *Jagger et al.* showed that mutations at residue 108 resulted in elimination of the shutoff activity of PA-X (13). To determine if endonuclease activity is required for shutoff activity, we created and analyzed the shutoff activity of Cal PA or Cal PA-X containing the K134A mutation. As shown in Fig. 9C, a single mutation at residue 134 completely abolished the repressive activity in co-transfected luciferase expression, supporting the idea that endonuclease activity is required for shutoff activity (13).

Discussion

Virally induced inhibition of cellular protein synthesis, or shutoff, is a process in which the virus hijacks cellular machinery for its own benefit. Viruses rely on host cell machinery for replication and production of progeny virions. Therefore, viral control of cellular transcription/translation machinery for preferential production of viral proteins is beneficial for

the virus. Inhibition of host protein synthesis also prevents the cellular anti-viral response, which can contribute to efficient virus production and spread. In the present study, we identified the domain within the PA subunit of the influenza polymerase complex that critically affects expression of exogenous protein as confirmed by co-transfection experiments. We also showed that PA suppresses cellular protein synthesis, and has an effect on the induction of apoptosis in virus-infected cells. Our results indicate an additional role for PA gene products in the control of host cell protein synthesis machinery, which is likely to affect viral production and cytopathogenicity of infected cells.

Influenza virus is known to shutoff host cell protein synthesis following infection (14, 15). It is highly likely that multiple viral proteins and their functions contribute to the overall magnitude of host protein synthesis shutoff. In fact, some viral proteins have been identified to have functions that contribute to shutoff. NS1 blocks nuclear export of mRNA and inhibits mRNA splicing (4, 31). The viral polymerase complex removes 5' methyl caps from host cell pre-mRNA for the synthesis of viral mRNA (23) and also degrades cellular RNA polymerase II (27, 37). These viral protein functions likely alter the steady-state levels of cellular mRNAs. However, analysis of mRNA levels using a cDNA microarray assay indicated that influenza infection only partially affected mRNA levels, whereas a significant down-regulation of cellular mRNAs, which would explain the profound inhibition of host protein synthesis, was not observed in infected cells (9). The data suggest that down-regulation of cellular transcription, or transcripts may not be the major factor that mediates host protein synthesis shutoff, but that there may be an additional mechanism(s) involved in the control of host cell protein synthesis in infected cells.

Our data presented here clearly indicate that PA gene products have a significant impact on host protein synthesis shutoff. Comparison of protein synthesis shutoff by each polymerase component, as well as by NS1, clearly implicates PA as the major factor for host protein synthesis shutoff (Fig. 1). A role for PA in shutoff of host protein synthesis is also supported by our failure and the inability of others, to generate cell lines that stably express PA (34). The molecular mechanism by which PA induces shutoff is unclear at this stage. However, previous reports implicate the proteolytic activity of PA in shutoff of both cellular and viral proteins (29, 30). In these studies, the impact of PA expression on the steady state levels of co-expressed proteins was evaluated by pulse-chase experiments, which suggest that the expression of PA affects the half-lives of co-expressed proteins. However, the degradation rate of co-expressed proteins in this report was not fast enough to explain the level of reduction we observed in cells labeled for 30 min without chase (Fig. 3). Furthermore, a study of the crystal structure of the PA N-terminal domain found no obvious protease active site, and an additional *in vitro* protease assay supported no detectable proteolytic activity in the N-terminal domain of PA (38). Together with our data, we anticipate that PA contributes to shutoff not through the proteolytic degradation of existing proteins, but rather by inhibiting the synthesis of new protein. Co-expression of eGFP and Cal PA, both tagged with the same Flag tag, resulted in suppression of eGFP synthesis (Fig. 3). PA expression was also suppressed, although it was not as significant as eGFP. This result may indicate that, even in the absence of the 5' untranslated region, there might be a mechanism that allows for viral proteins to escape the suppressive effect. Also, in infected cells, viral protein synthesis increases while cellular protein production is strongly blocked (Fig. 4). It is unclear how viral proteins are selectively produced; however, it is possible that PA is involved in selective shutoff of non-viral protein synthesis.

Interestingly, there was a difference in the activity of host protein synthesis shutoff between the avian and human virus PA proteins examined. PAs from an avian virus (Nan) or the avian origin pH1N1 (Cal) demonstrated much more efficient shutoff of host protein synthesis than those from a human virus (NC) or a mouse-adapted human virus (WSN) (Fig. 2). By characterizing the shutoff effect of Cal/WSN chimeric PA N-terminal fragments, we identified regions responsible for the difference in the activity, which include a flexible loop and the helix α4 (Fig. 8). To determine if these residues were conserved among avian or human isolates, we analyzed the PA sequences of 5,643 avian and 4,782 human influenza viruses. The results indicate that most of the avian, but not human viruses contain residues 57R, 62I, 65S and 100V (Table 1), which enhanced the reduction in protein expression from co-transfected cDNAs (Fig. 8). Therefore, the sequence data support the idea that avian influenza virus contains a PA gene that is more effective in shutoff activity. It is not clear if this difference in PA activity has significance for host-specific virus growth, although it is conceivable that strong host protein shutoff by avian virus PA gene products could be an important factor in preventing the anti-viral response in avian hosts. In mammalian hosts, a critical function of NS1 in suppressing the innate immune response is well established (5, 17, 32). The *in vivo* role of avian virus NS1 in chickens has not yet been studied in detail. However, a recent study using NS1 mutant viruses suggest that NS1 does not suppress IFN gene expression efficiently *in vivo*. It was suggested that, in chickens, other functions of NS1, such as its ability to inhibit apoptosis, might be more critical for maintaining the virus in an avian host (21). It is unknown how much of an effect PA has in antagonizing the innate immune response in avian hosts. However, it is possible that influenza viruses develop different mechanisms to escape the anti-viral response to achieve the best transmission efficiency in specific hosts.

Expression of an N-terminal domain of PA comprised of 257 residues was sufficient to inhibit protein synthesis (Fig. 7). This is consistent with a previous report using deletion mutants which shows that the N-terminal 247 residues are sufficient for reduced accumulation of co-expressed proteins (30). However, our data with chimeric PA N-terminal fragments uncovered the presence of a particular domain that determines the activity, located at the helix α4 and the flexible loop of amino acids 51-7 (Fig. 8). The fact that these regions are proximally located in the crystal structure suggests that a possible interaction with cellular protein(s) through the helix/loop domain is required for the suppressive activity of PA.

Recently, an additional PA gene product, termed PA-X has been reported (13). PA-X contains the region which reflects the difference in reducing protein synthesis between WSN and Cal. In fact, Cal PA-X repressed expression of co-transfected gene products more efficiently than WSN PA-X (Fig. 9B), supporting our findings that the flexible loop (residues 51-74) and helix α4 determine the difference in the activity between WSN and Cal. In addition, both Cal and WSN PA-X showed stronger suppressive activity than PA N-terminal fragments, indicating that unique sequences in the C-terminal region of PA-X also play an important role in reducing protein expression (Fig. 9A). Although the mechanism is unclear at this stage, it is highly likely that mRNA degradation is a key process in reducing protein expression as mutations at the endonuclease active sites (D108A and K134A) completely abolished the repressive activity (Fig. 9C and ref (13)). Further studies into the mechanism of how PA-X efficiently suppresses protein synthesis are required to unveil its role in virus replication and pathogenesis.

Acknowledgements

This work was supported by the New York Influenza Center of Excellence (NYICE), a member of the NIAID CEIRS network, under NIH contract HHSN266200700008C and National Institutes of Health Training Grant T32 AI007362 (to EAD). We thank Leslie MacDonald for technical assistance.

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Figure Legends

Fig. 1. PA reduces the expression of co-transfected proteins. 293T cells were co-transfected with expression vectors containing eGFP or luciferase genes together with the indicated Cal genes. A) eGFP expression at 24 h post transfection. B) Luciferase expression from pRL-SV40 in cells co-transfected with the indicated Cal genes in pCAGGS. Expressed viral proteins were determined by Western blot analysis using an anti-Flag Ab. Anti-β-actin Ab was used for a loading control. C) Luciferase expression in cells co-transfected with the indicated Cal polymerase subunits. Cells were transfected with 0.4 μg of each subunit (supplemented with empty vector) to total 1.6 μg. PA expression was determined by Western blot analysis using anti-PA antibody. *, $P<0.05$.

Fig. 2. Avian origin PAs are more active in suppressing co-expressed proteins. 293T cells were co-transfected with expression vectors containing eGFP or luciferase genes together with the PA genes of indicated viruses. A) eGFP expression as measured at 24 h post transfection. B) Luciferase expression in 293T cells as measured in cells transfected with the indicated genes in pCAGGS together with pRL-SV40. Expressed PA was determined by Western blot analysis using anti-Flag Ab. C) Suppression of luciferase expression by various PA in DF-1 cells. * $P<0.05$.

Fig. 3. Cal PA strongly suppresses production of co-expressed non-viral proteins. 293T cells were co-transfected with expression vectors containing eGFP and either WSN PA, Cal PA, or empty vector. At the indicated time points following transfection, cells were labeled with [^{35}S]Met/Cys for 30 min, and eGFP and PA were immunoprecipitated using an anti-Flag Ab. B)

The relative volume of eGFP and PA proteins at each time point were quantified using Quantity One 1-D Analysis Software (Bio-Rad).

Fig. 4. Virus growth and suppression of cellular protein synthesis. A) Reduced growth of WSN-CalPA in A549 cells. A549 cells were infected with either WSN or WSN-CalPA at a MOI of 0.01 and cultured in the presence of trypsin at 1μg/ml for 96 h. Virus titers in the culture supernatants were measured. B and C) WSN-CalPA inhibits cellular protein synthesis more efficiently than WSN. A549 cells were either left uninfected or infected with WSN or WSN-CalPA at a MOI of 3. At the indicated times after infection, cells were labeled with [^{35}S]Met/Cys for 30 min and total cell lysates were resolved by SDS-PAGE. (B). Representative cellular proteins above 100 kDa (CP1) and a 50 kDa band (CP2) as well as viral proteins (NP and NS1) were quantified using Quantity One 1-D Analysis Software (C).

Fig. 5. Enhanced apoptosis induction by WSN-CalPA. A549 cells were infected with either WSN or WSN-CalPA at a MOI of 3 and cell viability and apoptotic state were determined. A) Cell viability was determined by trypan blue staining. B) Apoptotic cells were identified at 12 and 24 h post infection. Annexin V (green) and propidium idodide (PI, red) signal was visualized using a fluorescence microscope.

Fig. 6. N-terminal domain of PA determines shutoff activity. A) Schematic diagram of the chimeric PA constructs. B) Luciferase expression from pRL-SV40 in cells co-transfected with the indicated chimeric PA genes in pCAGGS. C) Expression of PA proteins was determined by Western blot analysis using an anti-Flag Ab. Anti-β-actin Ab was used for a loading control.

Fig. 7. N-terminal 257 residues are sufficient to induce shutoff. A) Luciferase expression from pRL-SV40 in cells co-transfected with the indicated PA genes in pCAGGS. PANt indicates a protein containing the PA N-terminal 257 residues, and PACt indicates a protein containing the PA C-terminal residues 258 to 716. B) Expression of the PA fragments was determined by Western blot analysis using an anti-Flag Ab. Anti-β-actin Ab was used as a loading control.

Fig. 8. Identification of the residues required for shutoff. A) Sequence differences between the Cal and WSN PA N-terminal region (1-257). The amino acid differences in the Cal/WSN chimeric PA N-terminal constructs are also shown. B) Luciferase expression from pCAGGS-Luc in cells co-transfected with the indicated chimeric PANt genes in pCAGGS. C) Expression of chimeica PANt was determined by Western blot analysis using an anti-Flag Ab. Anti-β-actin Ab was used as loading control. D) Crystal structure of the PA N-terminal domain highlighting the locations of residues that affect shutoff activity (blue). Residues in the flexible domain with an undetermined structure are also shown in black. The putative endonuclease active site ($P_{107}D_{108}X_{10}E_{119}K_{134}$) (38) is shown in yellow.

Fig. 9. Shutoff activity of PA-X. A) Sequence differences in the unique region between the Cal PA N-terminal fragment and PA-X (top). The unique C-terminal region of PA-X is highly homologous between Cal and WSN, except that WSN contains an additional 20 residues (bottom). B) Luciferase expression from pCAGGS-Luc in cells co-transfected with the indicated PANt and PA-X genes in pCAGGS. Cells were cultured for 20 h after transfection. C) Shutoff

activity of CalPA134A and CalPA-X134A was determined as described in B) except that transfected cells were cultured for 24 h.

Table 1. Amino acid differences between avian and human PA proteins.

PA residue	Cal	WSN	Avian viruses		Human viruses	
57	R	Q	**R** (98.1)	Q (1.6)	R (4.4)	**Q** (95.4)
62	I	V	**I** (97.8)	V (1.9)	I (29.0)	**V** (65.4)
65	S	L	**S** (97.7)	L (0.1)	S (4.5)	**L** (76.0)
85	I	T	I (0.3)	**T** (97.5)	I (0.1)	**T** (99.2)
86	M	I	**M** (97.9)	I (1.2)	**M** (99.3)	I (0.4)
91	V	I	**V** (99.3)	I (0.6)	**V** (99.7)	I (0.2)
100	V	A	**V** (95.9)	A (2.6)	V (4.3)	**A** (94.7)
114	E	K	**E** (99.7)	K (0.2)	**E** (99.8)	K (0.1)
186	S	G	S (1.1)	**G** (98.8)	S (0.1)	**G** (99.7)

Percentage of the viruses containing indicated residues are shown. Sequence data of 5,643 avian isolates, and 4,782 human isolates (except pH1N1 strains) collected from Influenza Research Database were analyzed.