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The Effect of Some Interferon-related Proteins on Influenza A Viruses RNA Polymerase Activity

İnfluenza A Virüslerinin RNA Polimeraz Aktivitesi Üzerine İnterferon İlişkili Bazı Proteinlerin Etkisi

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Abstract
Background/aim: Interferons (IFNs) are one of the most important components of innate immunity against viruses especially those carrying the RNA genomes such as influenza viruses. Upon viral infection, the IFNs are rapidly secreted, inducing the expression of several genes in the target cells and establishing an antiviral state. In this study, the effects of proteins encoded by some interferon-related genes on influenza A virus RNA dependent RNA polymerase enzyme were investigated. The importance of these proteins in the pathogenesis of different influenza A virus types was evaluated.
Materials and Methods: The interferon-related genes were amplified by PCR from the HEK293 cDNA library and cloned into pCHA expression vector. The expression of genes and subcellular localizations of the proteins were determined by Western blotting and immunofluorescence staining, respectively. The effects of interferons-related proteins on virus RdRP enzyme were determined with influenza A virus mini-replicons.
Results: The study revealed that the influenza A virus infections significantly altered the transcript level of the interferon-related CCL5, IFIT1, IFIT3, IFITM3, and OAS1 genes in HEK293 cells. It was determined that the alteration of the gene expression was also related to the virus type. The mini-replicon assays showed that the transient expression of CCL5, IFI27, OAS1, IFITM3, IFIT1, and IFIT3 have inhibitory effects on WSN and/or DkPen type virus RdRP enzymes. It was observed that the proteins except OAS1 inhibited WSN type RdRP enzyme at a higher level than that of DkPen enzyme.
Conclusions:
It was concluded that influenza A virus infection significantly alters the interferon-related gene expression in the cells. Most of the proteins encoded from these genes showed an

inhibitory effect on the virus RdRP enzymes in the HEK293 cells. The inhibition of the influenza virus RdRP with interferon-related proteins may be the result of direct or indirect interactions between the host proteins and the viral enzyme subunits.
Keywords: influenza A viruses, interferon response, PCR array, influenza RdRP

ÖZET
GİRİŞ ve AMAÇ: İnterferonlar (IFN), özellikle influenza virüsleri gibi RNA genomlarını taşıyan virüslere karşı doğuştan gelen bağışıklığın en önemli bileşenlerinden biridir Viral enfeksiyon esnasında, interferonlar hızla salgılanır, hedef hücrelerde birçok genin ekspresyonunu indükler ve antiviral bir durum oluşturur. Bu çalışmada, interferon ile ilişkili bazı genler tarafından kodlanan proteinlerin influenza A virüsü RNA bağımlı RNA polimeraz (RdRP) enzimi üzerindeki etkileri araştırıldı. Bu proteinlerin farklı influenza A virüs tiplerinin patogenezindeki önemi değerlendirildi.
YÖNTEM ve GEREÇLER: İnterferonla ilişkili genler, HEK293 cDNA kitaplığından PCR ile çoğaltıldı ve pCHA memeli ekspresyon vektörüne klonlandı. Genlerin ekspresyonu Western blotlama ve proteinlerin hücredeki lokalizasyonları immünofloresan boyama ile belirlendi. İnterferonlarla ilişkili proteinlerin virüs RdRP enzimi üzerindeki etkileri influenza A virüsü mini-replikonları deneyleri ile belirlendi.
BULGULAR: Çalışma, influenza A virüsü enfeksiyonlarının, HEK293 hücrelerinde interferonla ilişkili CCL5, IFIT1, IFIT3, IFITM3 ve OAS1 genlerinin transkript seviyesini önemli ölçüde değiştirdiğini ortaya koydu. Gen ekspresyonundaki değişikliğin virüs tipi ile de ilişkili olduğu belirlendi. Mini-replikon deneylerinde, CCL5, IFI27, OAS1, IFITM3, IFIT1 ve IFIT3'ün geçici ekspresyonunun hem WSN hem de DkPen tipi virüs RdRP enzimleri üzerinde inhibitör etkileri olduğunu göstermiştir. OAS1 dışındaki proteinlerin WSN tipi RdRP enzimini DkPen enziminden daha yüksek düzeyde inhibe ettiği gözlendi.
TARTIŞMA ve SONUÇ: İnfluenza A virüsü enfeksiyonunun, konakçı hücrelerde interferonla ilişkili gen ekspresyonunu önemli ölçüde değiştirdiği sonucuna varılmıştır. Bu genlerden kodlanan proteinlerin çoğu, HEK293 hücrelerinde virüs RdRP enzimleri üzerinde inhibitör etki göstermiştir. İnfluenza virüsü RdRP'nin interferonla ilgili proteinlerle inhibisyonu, konakçı protein ve viral enzim alt birimleri arasındaki doğrudan veya dolaylı etkileşimlerin sonucu olabilir.
Anahtar Kelimeler: İnfluenza A virüsleri, İnterferon yanıtı, PCR dizini, İnfluenza RdRP

INTRODUCTION

Influenza A viruses are enveloped viruses classified in the *Orthomyxoviridae* family. These viruses have a genome consisting of single-stranded, negative-sense RNA molecules. Influenza A viruses can be easily transmitted from person to person and cause upper respiratory diseases. The high mutation rate and reassortment of the viral genome make it necessary to develop new prevention and treatment methods.[1] In this context, it is critical to identify the viral and cellular protein factors that have a role in virus-host interactions. On the other hand, innate and acquired immunities are of great importance in terms of the severity of influenza A virus infections. Interferons are among the most important actors of innate immunity.[2,3] The interferons bind to specific receptors on the target cells, triggering several biochemical reactions within the cell and obstructing or completely inhibiting the virus replication. This occurs as a result of interactions between interferon-related host proteins and viral replication processes. The interferons secreted from the infected cells stimulate the transcription of the genes carrying the ISRE control elements by transmitting signals to other cells through both paracrine and endocrine pathways. These proteins, which are synthesized as a result of the stimulation of interferons, act as barriers to prevent viral replication in the cells by different mechanisms.

The influenza A virus RNA polymerase enzyme, consisting of PB2, PB1, and PA subunits, is the target of some host cellular proteins stimulated by interferons. Among these proteins, Mx proteins inhibit influenza A virus RNA polymerase enzymes by direct interaction or indirect mechanisms.[4,5] In addition, the viral RNA polymerase enzyme plays an important role in controlling the host's anti-viral mechanisms by binding to cellular proteins such as IPS-1 or inhibiting IFNβ production.[6] In this study, how the expression levels of some genes related to the interferon response were affected in cells infected with influenza A viruses was evaluated. The effects of the proteins encoded by these genes on the viral RdRP enzyme activity were investigated with a mini-replicon assay.[5]

MATERIAL AND METHODS

Cells

Human cervical cancer cells (HeLa) and human embryonic kidney cells (HEK293) were used for viral infections or transient transfection assays. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% of heat-inactivated fetal calf serum (Gibco Laboratories, Gaithersburg, MD, USA, #11573397), 100 IU/mL penicillin, 100 µg / mL streptomycin, and 2 mM glutamine. The cultures were incubated at 37°C in an atmosphere of >95% humidity and 5% carbon dioxide.

Viruses

Influenza A/WSN/33/H1N1 (WSN) and low pathogenic avian influenza A/Duck/Pennsylvania/10,218/84/H5N2 (DkPen) viruses were used to infect the HEK293 cells. The viruses were obtained from the infectious biology (Virology) laboratory of Tsukuba University (Japan). Viruses were propagated in Madin-Darby canine kidney cells and titrated with a standard plaque formation assay.[7]

Plasmid Vectors

Some of the plasmids were constructed within the scope of this work while others were constructed in previous studies. For protein expression in transiently transfected HEK293 and HeLa cells, the interferon-related chemokine (C-C motif) ligand 5 (CCL5), interferon, alpha-inducible protein 27 (IFI27), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), interferon-induced transmembrane protein 3 (IFITM3), and 2'-5'-Oligoadenylate Synthetase 1 (OAS1) genes were cloned in pCHA plasmid[8] derived from pCAGGS[9]. The cDNAs were prepared with reverse transcription of the HEK293 cells RNA. A total 500 ng of RNA and 50 pmol oligo-dT primer were added to a 1.5 ml plastic tube, and the volume was increased to 12.5 µl with nuclease free-water. After denaturation of the RNA molecules at 65°C for 10 minutes, 4 µl 5x reaction buffer, 0.5 µl (40U/µl) RNase inhibitor (Biotechrabbit GmbH, Hennigsdorf, Germany, #BR0400901), 2 µl (10 mM) dNTP mixture, and 1 µl (200U/ µl) reverse transcriptase enzyme (Biotechrabbit GmbH, Hennigsdorf, Germany, #BR0400601) were added to the samples. The reaction was carried out at 45°C for 60 minutes and then held at 80 °C for 10 minutes to inactivate the reaction. The cDNAs of the CCL5, IFI27, IFIT1, IFIT3, IFITM3, and OAS1 genes were amplified with polymerase chain reaction by using gene specific primer pairs phosphorylated with T4 polynucleotide kinase (New England Biolabs Ltd., Hitchin, UK, #M0201S). The primers given in Table 1 were designed from reference gene sequences of the genes (CCL5/NCBI Reference Sequence: NM_002985.3, IFI27-v.1/NCBI Reference Sequence: NM_001130080.3, IFIT-v.1/NCBI Reference Sequence: NM_001548.5, IFIT3/NCBI Reference Sequence: NM_001549.6, IFITM3 -v.1/NCBI Reference Sequence, and OAS1/NCBI Reference Sequence: NM_016816.4). The PCR-amplified cDNAs were cloned into pCHA plasmid digested by EcoRV (New England Biolabs

Ltd., Hitchin, UK, #R095S), and the resultant plasmid vectors were designated as pCHA-CCL5, pCHA-IFI27, pCHA-IFIT1, pCHA-IFIT3, pCHA-IFITM3, and pCHA-OAS1.
The construction of plasmids sets of the influenza A viruses mini-replicon (pCAGGS-PB2/WSN, pCAGGS-PB1/WSN, pCAGGS-PA/WSN, pCAGGS-NP/WSN, pCAGGS-PB2/DkPen, pCAGGS-PB1/DkPen and pCAGGS-PA/DkPen, pCAGGS-NP/DkPen and pHH21-vNS-Luc) have been described previously.[5,10] For normalization of firefly luciferase, the pRL (Promega, Madison, USA, #E289B) plasmid encoding *Renilla reniformis* luciferase enzyme was used.

Infections of HEK293 Cell with Viruses and Total RNA Extraction
The HEK293 cells were seeded in 12-well plates (5 x10^5 cells/well) and incubated in standard culture conditions (37°C, 5% CO_2, and >95% humidity) for 24 hours. After incubation, the media of the cultures were removed, and the cells were washed with serum-free DMEM medium. 200 µl of influenza A virus (WSN or DkPen) samples diluted at an average of 1 moi in 1% BSA were added to the cell monolayers. Plates were kept for 30 minutes at 37°C with gentle mixing at 5 minute intervals for virus attachment. The virus suspensions were removed, and 1 ml of OPTI-MEM (Gibco Laboratories, Gaithersburg, MD, USA, #31600-083) was added to the cells. Infected cultures were incubated for 8 hours, and then the RNA was extracted from the cells. The total RNA extraction was carried out with a commercial RNA extraction kit (New England Biolabs Ltd., Hitchin, UK, #T2010S), according to the manufacturer's instructions.

Quantification of Interferon-related Gene Transcripts in the Virus Infected Cells with qPCR
The cDNAs of interferon-related genes from the total RNA of virus-infected cells were prepared as described above. The 96-well "RT² Profiler PCR Array" (PAHS-016ZD-6) plates developed by Qiagen (Qiagen, Hilden, Germany) was used for quantitation of CCL5, IFI27, IFIT1, IFIT3, IFITM3 and OAS1 gene transcripts in the cDNAs with quantitative real-time polymerase chain reaction (qPCR). Equal amounts of ×2 SYBR Green Master Mix (Roche, Mannheim, Germany, #4673484001) were added to each PCR tube containing 2.5 µl cDNA and 5 µl primer mix. The cycle conditions were applied as an initial denaturation step at 95°C for 10 min, followed by 45 cycles of amplification for 15 s at 95 °C and 1 min at 60°C. The relative quantities of the transcripts were normalized by the amount of STAT3 (signal transducer and activator of transcription 3) transcript level. The relative expression values of each gene were revealed, and the results were given as bar graph and heat map.

Transfection
Polyethyleneimine (PEI) was used for transfection of plasmid DNAs into the HEK293 cells.[11] The cells were seeded in 12-well culture plates (1x10^5 cells/well) and incubated at standard culture conditions for 24 hours. The plasmid DNAs diluted in Opti-MEM (Gibco, USA, #31600-083) at a concentration of 20 ng/µl were mixed with equal volumes of the PEI solution (50 ng/µl). The mixtures were kept at room temperature for 5-10 minutes for complex formation and added to the cultures. Forty-eight hours after transfection, the cells were harvested for Western blotting or luciferase enzyme assays.

Western Blotting
The expression of human *CCL5, IFI27, IFIT1, IFIT3, IFITM3* and *OAS1* genes in transiently transfected HEK293 cells were analyzed by Western blotting. Plasmid DNAs (1-2 µg) were transfected into HEK293 cells as described above. After 48 hours, the cells were harvested in an SDS-sample loading buffer. The proteins in the cell lysates were separated with SDS-PAGE and transferred to the polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk, the membrane was first treated with mouse monoclonal anti-HA (Santa

Cruz, #sc-7392) followed by horseradish peroxidase-conjugated second antibody [anti-mouse IgG-HRP (Invitrogen, #31420). The proteins were visualized with an ECL detection kit (GE Healthcare, Italy, #RPN3004).

Immunofluorescence Assay

The HeLa cells were seeded on coverslips in a 12-well palate ($7.5x10^4$ cells /well) and incubated in standard culture conditions for 24 hours. Then the cells were transfected with 1.5 µg of the plasmid DNA encoding CCL5, IFI27, IFIT1, IFIT3, IFITM3, or OAS1 proteins as described above. After 40 hours transfection, the cells were rinsed in PBS and fixed with 3% paraformaldehyde. The cells were permeabilized with 0.1% NP-40, washed in PBS, treated with 1% skim milk for 30 min, and incubated with mouse monoclonal anti-HA for 60 min. After washing with PBS, the cells were treated with Alexa-488-conjugated goat anti-mouse IgG (at 1:300 dilutions in 1% skim milk) for 60 min. The nuclei of the cells were stained with DAPI. The coverslips were mounted in a media (0.1% p- phenylenediamine and 80% glycerol), and the cells were visualized with a fluorescence microscope.

RdRP Activity Assay

The effects of interferons-related proteins on the influenza A virus RdRP enzyme were investigated with the influenza A virus mini-replicon system.[5] The HEK293 cells were seeded in 24-well plates ($5x10^4$ cells/well) and incubated under standard culture conditions for 24 hours. The plasmids encoding interferons-associated proteins were mixed with a set of mini-replicon plasmids (pHH21-vNS-luc, pCAGGS-PB1, pCAGGS-PB2, pCAGGS-PA, and pCAGGS-NP). The pRL plasmid was used for normalization. The total amount of plasmid DNAs for each well was adjusted to 250 ng with pCAGGS plasmid. Then the plasmid DNAs were transfected with PEI as described above. After 48 hours transfection, the cells were lysed in a lysis buffer (Promega, Madison, USA, # E1531), and luciferase activities were detected with commercial kits (for firefly luciferase, #E1483; for Renilla luciferase, #Z3051, Promega, Madison, USA) according to the manufacturer's instructions.

Statistical Analysis

The statistical significance of differences between experimental groups was evaluated using analysis of variance (one-way ANOVA with the Newman-Keuls post-test) in the SPSS. P values less than 0.05 were considered statistically significant.

RESULTS

Quantifications of Interferon-related Gene Transcript in Virus-Infected Cells

In our previous study, the expression profiles of 84 interferon and interferon-related genes were determined in the cells infected with influenza A viruses.[12] Here, the *CCL5, IFI27, IFIT1, IFIT3, IFITM3* and *OAS1* genes, which are thought to be important in viral replication, and the effects of proteins encoded by these genes on influenza A viral RdRP enzymes were evaluated. The transcript levels of the genes in HEK293 cells infected with two types of influenza A virus were determined by the qPCR technique (Figure 1).

The results showed that *CCL5* gene expression is up-regulated in the HEK293 cells infected with both influenza A virus types ($p<0,01$). It was observed that up-regulation was more pronounced in the cells infected with WSN type viruses compared to the DkPen type viruses. No significant change was detected in the *IFI27* gene transcript level in the virus-infected cells. The profiles of the *IFIT1* and *OAS1* gene transcripts in the virus-infected cells were found to be similar to the changes in the *CCL5* gene transcript levels. A significant increase in the transcript level of these two genes was detected in the cells infected with WSN type viruses compared to uninfected cells ($p<0,01$). In contrast, the *IFIT3* gene was up-regulated in WSN-type virus-infected cells while it was significantly down-regulated in the cells infected with DkPen type viruses. While some decrease of *IFITM3* gene transcript was observed in

DkPen type virus infected cells, there was no statistically significant change in the *IFITM3* gene transcript level in WSN virus infected cells.

The Expression and Subcellular Localization of the Interferon-related Proteins

To identify the effects of proteins encoded by the interferon-related genes on the influenza A virus RdRP enzymes, the genes were cloned into the pCHA mammalian expression vector. Expression of the genes and subcellular localization of the encoded proteins were determined by Western blotting and immunofluorescence techniques, respectively (Figures 2 and 3). Western blot analysis showed that the genes cloned into pCHA plasmid were expressed at different levels in the HEK293 cells. The expected band sizes were detected based on the size of the protein: ~46 kDa for OAS1, ~55 kDa for IFIT1, ~56 kDa for IFIT3, ~15 kDa for IFITM3, and ~12 kDa for IFI27 (Figure 2A). The CCL5 protein (~10 kDa) could not be detected with Western blotting in transiently transfected HEK393 cells. The expression level of OAS1, IFIT1, and IFIT3 proteins were found to be higher than that of IFITM3 and IFI27 proteins in transiently transfected HEK293 cells (Figure 2B).

We have examined the subcellular localizations of the interferon-related proteins in the HeLa cells with an immunofluorescence assay. HeLa cells were transiently transfected with plasmids coding the HA-tagged proteins. The cells were fixed 44 h after transfection and stained with mouse monoclonal anti-HA antibodies (Figure 3). The results showed that all genes cloned into pCHA plasmid express in the cells. It was observed that all proteins except the OAS1 were dominantly localized in the cytosol. The presence of CCL5 and IFITM3 proteins as granules in the cytoplasm indicated that these proteins were localized in the organelles. The OAS1 protein was observed more intensely in the cell nucleus. However, OAS1 protein was found to be present in significant amounts in the cytoplasm.

The Effects of Interferon-related Proteins on the Influenza A Virus RNA Polymerase Enzyme

The influenza A virus RdRP enzyme consists of three subunits–PB2, PB1, and PA–that perform transcription and replication of the viral genome. This enzyme, which is vital for virus replication, interacts with many cellular events and proteins. These interactions may be necessary for the functions of the RdRP enzyme or cause enzyme inhibition. Therefore, the effects of CCL5, IFI27, IFIT1, IFIT3, IFITM3, and OAS1 proteins, which are thought to be important for influenza A virus replication, were tested on the viral RdRP enzymes in the HEK293 cells by using the mini-replicon assays [5, 10]. The HEK293 cells were co-transfected with a certain amount of minireplicon plasmids and increasing amounts of plasmids encoding interferons-related proteins. The reporter luciferase activities normalized with *Renilla* luciferase in transfected HEK293 cells are given in Figure 4. The results showed that the proteins, except OAS1, negatively affected both types of influenza A virus RdRP enzymes at different levels. It was observed that the proteins had a higher inhibition on the WSN type virus RdRP enzyme than that of DkPen in the HEK293 cells. In contrast, the OAS1 protein did have not a significant effect on the WSN type virus RdRP in the cells while it had a negative effect on the DkPen type virus enzyme.

DISCUSSION

Among the host defense mechanisms against the viral infections, the interferon-related pathways of host cells are of great importance. Influenza infection causes rapid synthesis and secretion of type I interferons after the appearance of viral components in infected cells. The binding of these proteins to the specific receptors on the cell surface causes up-regulation of hundreds of interferon-stimulated genes (ISG) which creates an "antiviral state" that will limit the further proliferation and spread of the viruses.[13] In this study, the expression profiles of

the some genes related to the interferon pathway in virus-infected cells and the effects of the proteins encoded from these genes on the influenza A virus RNA polymerase enzyme were investigated. It is known that several proteins in the IFN pathways interact with influenza A viruses, some of which have negative regulatory effects on viral replication and some of which have stimulating effects.[5,14-19] However, the expression profile of various genes in the cells infected with the viruses are altered at different levels and in different directions. It was observed that IFIT3 gene expression was up-regulated in cells infected with WSN type virus but down-regulated in DkPen type virus-infected cells (Figure 1). The genes other than IFI27 were more up-regulated in the cells infected with WSN-type viruses than those of DkPen. The results showed that the transcript levels of genes except IFI27 were higher in the cells infected with WSN type viruses. In some studies, it has been reported that DkPen type viruses have a lower replication efficiency in the mammalian cells.[20-22] Although this seems to contradict the fact that the DkPen type virus infection up-regulates the expression of some interferon-related genes less than WSN type viruses; this may be a result of the DkPen virus RdRP enzyme inhibiting the host cell gene expression more than the WSN type virus enzyme.[12]

The influenza A virus RdRP enzymes are targets of some interferon-related proteins. In this study, the HEK293 cells were co-transfected with certain amounts of minireplicon plasmids and increasing amounts of the plasmids encoding interferon-related proteins, and the viral RdRP enzyme activities were measured via the luciferase reporter enzyme activity. The results showed that the expression of interferon-related proteins CCL5, IFI27, IFITM3, IFIT1, and IFIT3 have inhibitory effects on both WSN and DkPen type virus RdRP enzymes. The OAS1protein showed negative effect only on the DkPen virus RdRP (Figure 4). The CCL5 gene encodes a G-protein-coupled receptor protein carrying 7 transmembrane domains. The CCL5 protein, along with the CCL3 and CCL4 receptors, belongs to the C-C chemokine family.[23] It was reported that the CCL5 gene, whose transcript level is up-regulated in HEK293 cells infected with influenza A viruses, negatively affects the virus replication, and mutant mice with this gene were found to be very susceptible to virus infection.[24] In this study, it was determined that the CCL5 gene transcript level increased in HEK293 cells infected with both the WSN and DkPen type viruses. Furthermore, the CCL5 gene expression was increased approximately 5 times more with the WSN type virus infection compared to the DkPen viruses. This difference may be important for influenza A virus host specificity.

No significant change was detected in the transcript level of the IFI27 gene in virus-infected HEK293 cells. However, the high inhibition of influenza RdRP enzymes by the IFI27 protein in HEK293 cells reveals the importance of this protein for viral replication. The remarkable increases in the expression level of this gene in virus-infected organisms has been reported.[25] Tang et al.[26] demonstrated that IFI27 is an important diagnostic marker in the differentiating of influenza and bacterial infections.

One of the interferon-related genes whose transcript level was increased in HEK293 cells infected with the influenza A viruses is the OAS1. The OAS1 gene expression in WSN-infected cells was also found to be higher than that of DkPen viruses. The human OAS1 gene belongs to the OAS gene family which includes the OAS2, OAS3, and OASL genes.[27] These genes encode the 2'-5' oligoadenylate synthetase enzymes catalyzing the formation of 2'-5' oligoadenylate, which activates RNase L enzymes.[28,29] The active RNase L enzyme inhibits the protein synthesis by breaking down both viral and cellular RNA molecules.[30-32] Although there was a significant increase in the OAS1 transcript level in cells infected with influenza A viruses, it was observed that this protein did not cause an inhibition on the WSN type virus RdRP enzyme (Figure 4). In contrast, it was observed that the OAS1 expression partially inhibited the DkPen virus RdRP enzyme. The negative effect of OAS1 on the DkPen virus RdRP enzyme can be considered one of the reasons for the slow replication of this virus type in the mammalian cells.

IFITM3 belongs to the IFN-induced transmembrane protein family (IFITM). These proteins get the fusion of viral and endosomal membranes difficult and prevent the release of virus genomes inside the cell.[14,15,17] No significant change in the transcription of this gene was observed in the WSN virus-infected cells (Figure 1). However, both viral RdRP enzymes were strongly inhibited with IFITM3 expression (Figure 4).

It is known that IFIT proteins (IFIT1 and IFIT3) related to the interferons defense mechanism have negative regulatory effects on viral replication. It is suggested that these proteins specifically inhibit the viral replication by binding directly to the viral nucleic acids or disrupting the function of eukaryotic initiation factor 3 (eIF3).[33] Both of these proteins showed a negative effect on the influenza A virus RdRP enzyme. However, while the IFIT3 gene transcription was up-regulated in WSN virus-infected cells, the down-regulation of the cells infected with DkPen viruses remains to be elucidated.

In conclusion, the majority of interferon-related proteins focused on in this study showed inhibitory effects on the virus RdRP enzymes in the HEK293 cells. However, the possible interactions of these proteins with the RdRP enzyme subunits have not been considered. Therefore, it is not certain that these proteins inhibit influenza A virus RdRP enzymes in minireplicons by directly interaction with the subunits. The inhibition of the influenza virus RdRP enzymes with interferon-related proteins in transfected HEK293 cells may also be the result of the indirect interactions.

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Table 1
Oligonucleotide Primers Used in PCR Amplification of Human CCL5, IFI27, IFIT1, IFIT3, IFITM3, and OAS1 Genes

Primer name	Nucleotide sequence
CCL5 For	5'-ATCATGAAGGTCTCCGCGGCAGC- 3'
CCL5 Rev	5'- ATCTAGCTCATCTCCAAAGAGTTG- 3'
IFI27 For	5'-ATCATGGAGGCCTCTGCTCTCAC-3'
IFI27 Rev	5'- ATCTAGTAGAACCTCGCAATGAC-3'
IFIT1 For	5'- ATCATGAGTACAAATGGTGATGA-3'
IFIT1 Rev	5'- ATCATAAGGACCTTGTCTCACAG -3'
IFIT3 For	5'-ATCATGAGTGAGGTCACCAAGAATTC-3'
IFIT3 Rev	5'-ATCAGTTCAGTTGCTCTGAGTTAGAG-3'
IFITM3 For	5'-ATC ATGAATCACACTGTCCAAAC-3'
IFITM3 Rev	5'-ATCTATCCATAGGCCTGGAAGAT-3'
OAS1 For	5'-ATCATGATGGATCTCAGAAATACCCC-3'
OAS1 Rev	5'-ATCAGAGGATGGTGCAGGTCCAGT-3'

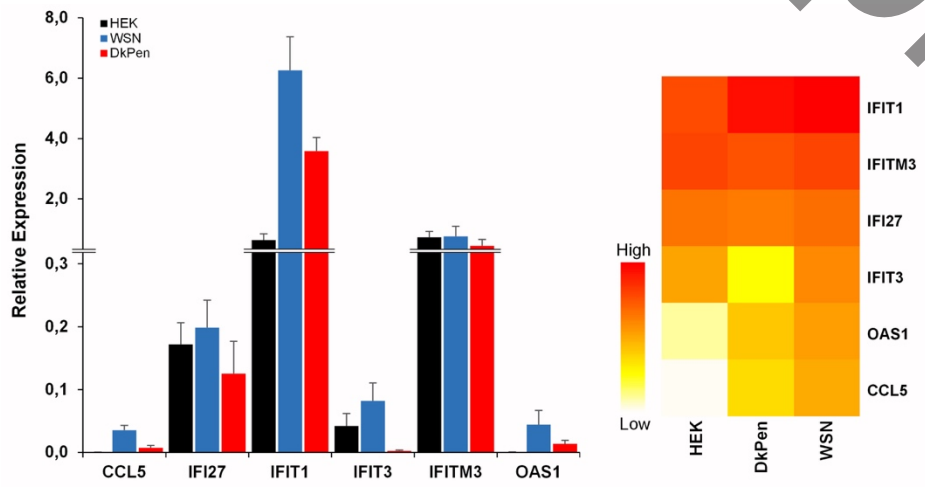
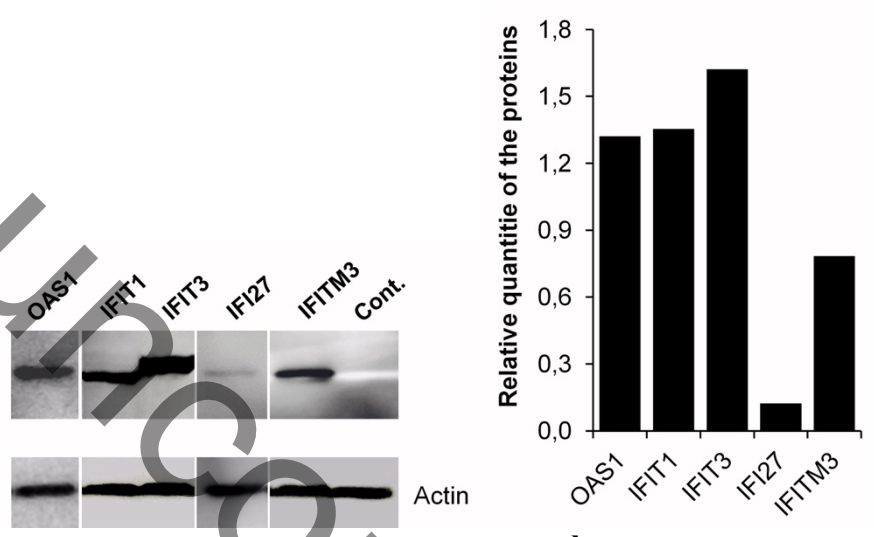


Figure 1

Expression Profiles of Interferon-related Genes in HEK293 Cells Infected with Influenza A Viruses (WSN or DkPen) and Uninfected HEK293 Cells



a **b**

Figure 2

Western Blot Analysis of the Interferon-related Proteins Encoded from the Plasmid Vectors in the HEK293 Cells Transiently Transfected. **A**. The bands of OAS1, IFIT1, IFIT3, IFI27, IFITM3 and Actin beta proteins. **B**. Relative quantity of the proteins compared to the actin beta. A total of 15 µl cell lysate was loaded into each well of 10% polyacrylamide gel. Electrophoresis was performed under a constant voltage of 40 V/gel for 15 minutes and then 80 V/gel for 75 minutes. The relative quantity of the proteins was determined by using ImageJ software. **Cont**. Untransfected-HEK293 cell lysate.

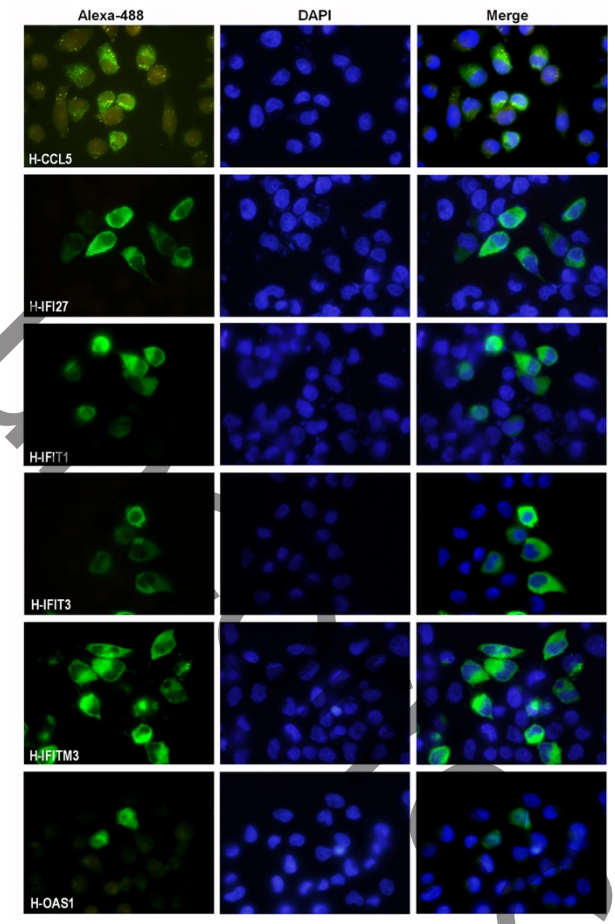


Figure 3

Subcellular Localizations of Interferon-related Proteins Encoded by Plasmids in HeLa Cells. HeLa cells were transiently transfected with plasmids encoding HA-tagged CCL5, IFI27, IFIT1, IFIT3, IFITM3, and OAS1 proteins. The cells were fixed 44 h after transfection and stained with mouse monoclonal anti-HA antibodies. Alexa 488-conjugated anti-mouse IgG antibody (1/300 dilution) was used as the secondary antibody. The cell nuclei were stained with DAPI

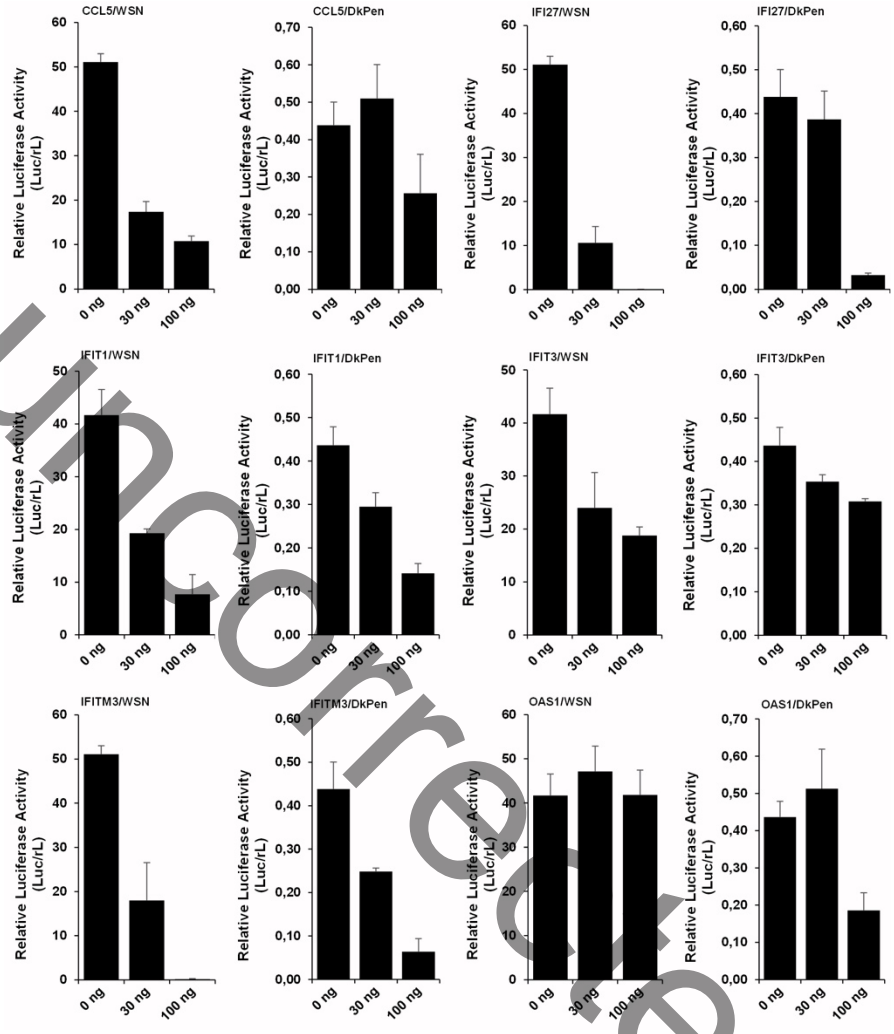


Figure 4

The Effects of Interferon-related CCL5, IFI27, IFIT1, IFIT3, IFITM3, and OAS1 Proteins on WSN and DkPen-type Virus RdRP Enzymes in the HEK-293 Cells The cells were grown in 24-well plates, and co-transfected with a protein coding-plasmid (30/well or 100 ng/well), and a certain amount of minireplicon plasmids [WSN: pCAGGS-PB2/W (3 ng/well), pCAGGS-PB1/W (3 ng/well), pCAGGS-PA/W (1 ng/well), pCAGGS-NP/W (10 ng/well), pHH21-vNS-luc (5 ng/well), pRL (2 ng/well), DkPen: pCAGGS-PB2/D (10 ng/well), pCAGGS-PB1/D (10 ng/well), pCAGGS-PA/D (3 ng/well), pCAGGS-NP/D (10 ng/well), pHH21-vNS-luc, (5 ng/well) pRL (2 ng/well)]. After 48 hours transfection, the cells were lysed, and reporter luciferase and Renilla luciferase activities were measured with luciferase assay kits.

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