Published in final edited form as:

J Proteome Res. 2012 January 1; 11(1): 217–223. doi:10.1021/pr200894t.

Inhibition of Type I Interferon Production via Suppressing IKK-Gamma Expression: A New Strategy for Counteracting Host Antiviral Defense by Influenza A Viruses?

Yimeng Wang, Jianhong Zhou, Chuanmin Ruan, and Yuchun Du*

Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas, United States

Abstract

Blockage of the induction of type I interferons (IFNs) is essential for the success of influenza virus proliferation in host cells. Several molecular mechanisms by which influenza viruses inhibit IFN induction have been characterized. Here we report a potentially new strategy influenza viruses employ to inhibit IFN production during viral infection. Through a two-dimensional gel electrophoresis based proteomic approach, we found that the expression of I κ B kinase-gamma (IKK γ) was suppressed by influenza A virus infection in human lung epithelial A549 cells. Silencing of cellular IKK γ by small interfering RNA led to enhanced replication of influenza viruses. Concomitantly, overexpression of IKK γ resulted in increased production of IFN α/β , whereas influenza virus infection completely eliminated the IKK γ -overexpression-induced production of IFN α/β . Our results suggest that IKK γ and influenza virus are mutually inhibitory, and influenza viruses may inhibit IFN production through suppressing the expression of IKK γ during viral infection.

Keywords

Influenza virus; H1N1; interferon; IKK γ ; NF- κ B; 2-DE; proteomics; protein expression; LC-MS/MS

INTRODUCTION

NF- κ B is an important transcription factor and plays a critical role in antiviral defense^{1–3}. NF- κ B normally binds to its inhibitor, I κ B and is localized in the cytosol in its inactivated form. Upon virus infection, the virus-activated I κ B kinase (IKK) phosphorylates I κ B, resulting in its degradation through the ubiquitin-dependent pathway. The freed NF- κ B then translocates to the nucleus and initiates the transcription of antiviral cytokines including type I interferons (IFNs), which are major components of host innate antiviral defense^{4,5}. IKK is a trimeric protein complex consisting of two catalytic subunits, IKK α and IKK β , and a regulatory subunit, IKK γ . IKK γ (also termed NEMO or IKBKG) regulates the kinase activity of IKK α/β ⁶. IKK γ -deficient cells lack the ability to activate NF- κ B in response to multiple stimuli⁷.

Influenza A viruses, belonging to the Orthomyxoviridae family with 8 segmented genes⁸, continue to be a threat to human health. It has been well established that influenza viral protein NS1 plays a vital role in suppressing IFN production^{9–11}. In this regard, one important host antiviral factor is protein kinase R (PKR), which is a serine/threonine protein

*To whom correspondence should be addressed: Yuchun Du, Department of Biological Sciences, SCEN 601, University of Arkansas, Fayetteville, AR 72701, USA. ydu@uark.edu. Tel: +1-479-575-6944, Fax: +1-479-575-4010.

kinase functioning upstream of IKK in activating NF- κ B. PKR is activated by binding to dsRNA, and the activated PKR in turn activates the IKK complex through physically binding to IKK β ⁴. Viral protein NS1 is known to suppress the activation of NF- κ B through either competitively binding to dsRNA¹², or directly interacting with PKR to block its activation^{13–15}. Another important antiviral factor is interferon regulatory factor 3 (IRF-3), which is a key regulator of IFN gene expression¹⁶. dsRNA-bound NS1 was reported to prevent retinoic acid-inducible gene I (RIG-I)-mediated activation of IRF-3¹⁷. Furthermore, NS1 protein can bind to a 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF) to mediate the inhibition of posttranscriptional processing of cellular mRNAs, resulting in blockage of the nuclear export of newly synthesized cellular mRNAs including IFNs and IFN-stimulated genes^{18–20}. However, NS1 from some influenza virus strains, including the A/PR/8/34 (H1N1) strain, may have lost the CPSF binding capability^{21, 22}.

In the present study, through a two-dimensional gel electrophoresis (2-DE) based comparative proteomic approach, we found that the expression of IKK γ was suppressed by influenza virus during viral infection. Functional validation experiments demonstrated that IKK γ and influenza virus were mutually inhibitory. Our results suggest that influenza viruses may inhibit IFN production via suppressing the expression of IKK γ during viral infection.

EXPERIMENTAL PROCEDURES

Cell Culture and Virus Infection

Human embryonic kidney 293T cells, human lung epithelial A549 cells and Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) were cultivated in Dulbecco modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and 1% penicillin and streptomycin. Influenza A/PR/8/34 H1N1 viruses (ATCC, Manassas, VA) were propagated and titrated in MDCK cells as described²³. For virus infection, cells at 90–95% confluency were washed twice with phosphate buffered saline without Mg²⁺ and Ca²⁺ (DPBS) followed by incubation with viruses at the indicated multiplicity of infection (MOI) for 1 hour in a humidified incubator at 37°C with 5% CO₂. The virus solution was then aspirated, and cells were incubated with virus growth medium [DMEM with 0.2% BSA, 25 mM HEPES, 2 mM L-glutamine, sodium pyruvate, 2 μ g/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin and antibiotics] at 37 °C in a 5% CO₂ incubator. For control, the same amount of virus growth medium was used in place of virus solution. Other procedures were the same as the procedures for the virus infection.

2-DE

Ten hours postinfection, the mock- and virus-infected A549 cells were harvested, washed twice with isotonic buffer (10 mM Tris-HCL, pH 7.5 and 250 mM sucrose), and lysed with rehydration buffer (8 M urea, 2% w/v CHAPS, 50 mM DTT, 0.2% w/v Bio-Lyte and 0.002% w/v bromophenol blue). After centrifugation at 50,000 g for 30 minutes at room temperature, the supernatant was collected, and the protein concentration was determined using a RC DC protein assay kit (BioRad, Hercules, CA) for 2-DE fractionation. Briefly, 450 μ g of protein was loaded onto a 17 cm ReadyStrip IPG strip (pH 3–10 or 4–7), which was in turn kept at room temperature overnight. Isoelectric focusing was carried out with a Protean IEF Cell using the following conditions: 250 V for 20 minutes with a linear ramp, 10,000 V for 1 hour with a linear ramp, and 10,000 V for a total of 50,000 V/h with a rapid ramp. Other procedures were performed according to the manufacturer's instructions (BioRad, Hercules, CA).

Mass Spectrometry (MS) Analysis and Database Search

In-gel digestion was performed as described previously^{24–26}, and liquid chromatography – tandem mass spectrometry (LC-MS/MS) analysis was carried out using a LTQ-XL mass spectrometer (Thermo, San Jose, CA) in the Proteomic Facility at the University of Arkansas for Medical Sciences (Little Rock, AR). Briefly, proteins were in-gel-digested with trypsin (Promega, Madison, WI) overnight at 37 °C, and the resulting peptides were dissolved in 20 µl 0.1% formic acid for LC-MS/MS analysis. In the MS analysis, peptides were separated by an IntegraFrit column (10 cm × 50 µm ID; New Objective, Woburn, MA). Solvent A was 0.5% acetonitrile and 0.1% formic acid, and solvent B was 75% acetonitrile and 0.1% formic acid. The gradient started with a mixing of A:B = 95:5 and increased to A:B = 60:40 over 30 min. The flow rate was 500 nl/min. The LTQ-XL was operated in ESI positive-ion mode with the following settings: collision-induced dissociation (CID) fragmentation, data-dependent acquisition, and centroid mode for both MS and MS/MS spectrum recordings. MASCOT (Version 2.2; Matrix Science, Boston, MA) was used to search against a target-decoy²⁷ International Protein Index (IPI) human protein database (version 3.68) or Swiss-Prot database taxonomic field for virus (version 51.6) using LC-MS/MS data as described^{24–26}. The parameters for database searching were as follows: (i) 2.0 Da mass error tolerance for MS and 0.65 Da for MS/MS, (ii) a maximum of one missed cleavage, and (iii) variable modifications: acetylation at peptide N terminus, phosphorylation on tyrosine/serine/threonine and oxidation on methionine. Proteins with two or more peptides with a score of more than 44 ($p < 0.05$) were considered as positive identification. Search results were further processed by Scaffold software (version 2_06_00; Proteome Software, Portland, OR) for viewing protein and peptide identification information. In the Scaffold analysis, protein identification probability with at least two peptides was set to 99% and the peptide identification probability was set to 95%. For the target-decoy database search, the false-positive rate for peptide identification was <5%.

Plasmid DNA Construction and Transfection

For the generation of NS1 expression plasmid, NS1 cDNA (GenBank accession no: CY021961) was inserted into the *Bam*H I and *Xho* I sites of pcDNA3.1 vector (Invitrogen, Carlsbad, CA) to generate pcDNA3.1-NS1. IKK γ (GenBank accession no: NM_003639.3) was amplified from a human cDNA library using primers with *Bam*H I and *Xho* I sites (forward: 5'-GGATCCACCATGAATAGGCACCTCTGGAAG-3' and reverse: 5'-CTCGAGCTACTCAATGCACTCCATGAC-3') and inserted into a pcDNA3.1 vector. All expression plasmids were verified by DNA sequencing. Expression plasmid was transiently transfected into 293T cells with the standard calcium phosphate method.

Western Blotting

Western blotting was performed as described previously^{24, 25, 28}. Mouse monoclonal anti-NS1 antibody was a gift from Dr. Stephan Ludwig at the University of Muenster (Muenster, Germany). Rabbit polyclonal anti-IKK γ antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA).

RNA Interference (RNAi)

siRNA fragment (5'-GAGAAUCAAGAGCUCCGAGAUGCUU-3') targeting IKK γ was designed using a tool from the Whitehead Institute (<http://www.whitehead.mit.edu/index.html>). A randomized siRNA sequence (Shanghai GenePharma Co., Ltd., Shanghai, China) was used as the control. The siRNA as well as control oligos were transfected into A549 cells with LipofectamineTM RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, A549 cells were seeded in a 6-well plate (3×10⁵ cells/well) in DMEM with 10% FBS one day before

transfection. Five μ l LipofectamineTM RNAiMAX reagent and 120 pmol siRNA were diluted in 250 μ l Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA) respectively, followed by mixing the diluted siRNA with the diluted reagent. After incubation at room temperature for 20 minutes, the mixture was added to the cells with a pipette. The cells were then incubated at 37 °C in an incubator with 5% CO₂ for 48 hours. The IKK γ -silenced as well as control cells were either harvested for analysis or infected by viruses for further treatments.

Plaque Assay

For influenza A/PR/8/34 virus plaque assay, 95% confluent MDCK cells in each well of the six-well plate were washed twice with warm DPBS and then incubated with 200 μ l 10-fold serially diluted viruses for 1 hour at a 35 °C incubator with 5% CO₂. The plates were swirled every 15 minutes. Cells were then overlaid with 3 ml overlay medium containing 1% agarose and 2 μ g/ml TPCK-trypsin in diluted DMEM (diluted by 30% with DPBS). After incubation in a 35 °C incubator with 5% CO₂ for 96 hours, the agarose was removed and 2 ml of 70% ethanol was added to each well, followed by incubation of the plates at room temperature for 20 minutes. Cells were then stained with 0.3% crystal violet solution for 10 minutes for visualization of the plaques. Virus titer was expressed as PFU/ml determined by dilution factors and plaque numbers.

Quantitative Real Time PCR (qRT-PCR)

RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA), and the first strand of cDNA was synthesized from 1 μ g of RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA), according to the manufacturer's instructions. The IFN α/β and reference (actin) transcripts were amplified with BioRad CFX detection system as described²⁹. mRNA abundance was measured using SYBR Green Supermix (Invitrogen, Carlsbad, CA) from three independent sample preparations. Relative gene expression of IFN α/β was calculated in the traditional $2^{-\Delta\Delta C_t}$ method³⁰.

Statistical Analysis

Statistical analysis was performed using an independent-sample T test by Systat 13 (SPSS 13). A *p*-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Identification of the Proteins Whose Expression is Affected by Influenza Viral Infection

In order to identify the proteins whose expression was affected by influenza viral infection, we infected human lung epithelial A549 cells with influenza A/PR/8/34 (H1N1) viruses at an MOI of 1. Ten hours postinfection, mock- and virus-infected cells were harvested and analyzed by 2-DE. We analyzed the proteins using 2-DE with IPG strips of both pH 4–7 and 3–10 to increase the chance of protein identification (Fig. 1). After 2-DE, the differences in protein spot intensity between the control gel (the gel that resolved the proteins from mock-treated cells) and the “virus” gel (the gel that resolved the proteins from virus-infected cells) were quantified by PDquest (BioRad, Hercules, CA). Protein spots with a more than 2-fold change in intensity were excised for LC-MS/MS analysis. Table 1 lists the identified proteins, which can be classified into several different biologically functional areas. Most of the identified proteins were in the expected size and pH ranges on the 2D-PAGE gel. However, a few of the proteins were identified in unexpected locations on the 2D-PAGE gel. For example, the full length heat shock cognate 71 kDa protein (HSPA8) has a theoretical molecular weight of 71 kDa, but the protein was identified in spot 3, which was close to the molecular weight marker of 25 kDa (Fig. 1). When we examined the peptides

identified by MS, we found that the 15 unique peptides detected by MS all matched to the first 236 residues at the N-terminal end of HSPA8 (data not shown), suggesting that the HSPA8 in spot 3 was a truncated but not the full length version of the protein. One issue in protein identification that has some uncertainties is that several proteins were identified from same protein spot (Table 1). Further expression validation tests are needed to examine the actual levels of changes in the expression of those proteins in the IAV infected cells.

Among the identified proteins, some have been previously reported to be related to influenza virus infections. For example, the expressions of enoyl-CoA hydratase (mitochondrial precursor) and glutathione S-transferase were reported to be altered by avian influenza H9N2 and H5N1 viruses, respectively^{31, 32}. The expression of vimentin was found to be up-regulated by influenza H1N1 viruses³³. Identification of those proteins that have been reported from other research groups to be related to influenza viruses serves as a good validation of our current experimental approach. In addition to the proteins that have been reported previously, we also identified multiple proteins that have not been reported previously, such as eukaryotic translation initiation factor (EIF) (different isoforms), eukaryotic elongation factor (different isoforms) and IKK γ . EIFs are involved in initiating protein synthesis. It is known that host cellular protein synthesis in influenza virus infected cells is shutdown by viral elements, leaving the exclusive translation of viral mRNAs^{34, 35}. Specifically, viral protein NS1 recruits EIF4GI (the large subunit of the cap-binding complex EIF4F) to the 5' untranslated region of the viral mRNA to facilitate the preferential translation of the viral mRNA^{36, 37}. The highly confident identification of EIF4H (5 unique peptides and 21% sequence coverage) in the present study suggests that EIF4H may also play an important role in regulating viral and host protein expression in virus infected cells.

IKK γ Expression Is Suppressed by Influenza Viruses

One more protein that was identified in this study but has not been reported to be related to influenza viral infection was IKK γ , whose expression was found to be suppressed by viral infection (Fig. 1). The protein was identified by LC-MS/MS with high confidence, having 5 unique peptides and a 12% protein sequence coverage (Fig. 2). We decided to choose this protein for further analysis because IKK γ is known to play an important role in regulating the NF- κ B pathway⁶, a pathway that determines the production of IFNs^{4, 12}. Fig. 3 shows results of a Western blot analysis of mock- and virus-infected A549 cells. Consistent with the 2-DE results (Table 1 and Fig. 1), Western blot analysis demonstrated that IKK γ expression was indeed suppressed by influenza viral infection (Fig. 3).

IKK γ Inhibits Influenza Virus Replication

In order to test whether the alteration in IKK γ expression affects influenza viral replication, we used an RNAi technique to suppress the expression of endogenous IKK γ in A549 cells and examined its effect on viral replication. We first transfected the A549 cells with siRNA oligos targeting the IKK γ sequence. Western blot analysis demonstrated that the expression of IKK γ was suppressed by 80% (judged by image analysis with ImageJ) by the siRNA (Fig. 4A; upper panel). We then infected the mock-treated (non-silenced) and the IKK γ -silenced A549 cells with influenza A/PR/8/34 at an MOI of 0.5, followed by 30 hours of incubation. Western blot analysis indicated that IKK γ silencing enhanced viral replication, as more viral protein NS1 was produced in the IKK γ -silenced cells than the mock-treated cells (Fig. 4A; middle panel). We also harvested the viruses in the supernatants of the control and the IKK γ -silenced cells for plaque assay. Consistent with the Western blot results (Fig. 4A; middle panel), the plaque assay demonstrated that silencing of endogenous IKK γ significantly raised virus titers ($p < 0.05$) (Fig. 4B and C). Results from this and previous sections (Figs. 1, 3 and 4) suggest that endogenous IKK γ plays an inhibitory role in

influenza viral replication in the infected host cells, and influenza viruses counteract the inhibitory effect of IKK γ by suppressing its expression.

Influenza Viruses may Inhibit IFN α/β Production through Suppressing IKK γ Expression

It is well-known that IKK γ is an important immune regulatory factor in activating NF- κ B, which can promote the transcription of anti-apoptosis factors and immune cytokines, such as IFN α/β ^{4,5}. Previous studies have established that influenza viruses can inhibit IFN α/β production in viral infected cells through several molecular mechanisms as described in the previous sections, but none of those reported mechanisms involves direct action on IKK γ . Results from our 2-DE based proteomic analysis (Table 1, Figs. 1 and 3) implied that the expression of IKK γ may be under direct influence of influenza viral infection. To further confirm our proteomic results, we examined the effect of overexpression of IKK γ on IFN α/β production. Since IKK γ can indirectly initiate the transcription of IFNs via activating NF- κ B, we expected that overexpression of IKK γ can lead to the increased expression of IFN α/β . As expected, when IKK γ was overexpressed in human 293T cells (Fig. 5A), qRT-PCR analysis demonstrated that the levels of IFN α/β mRNA increased more than three-fold (Fig. 5B). We then challenged the control cells that expressed an endogenous IKK γ and the IKK γ overexpressing cells with influenza viruses and compared IFN α/β production between the two types of cells that differed only in IKK γ expression. As shown in Fig. 5C, after cells were infected with influenza viruses, the cells overexpressing IKK γ had slightly but significantly reduced levels of IFN α/β mRNA compared with the control cells expressing an endogenous IKK γ . This result contrasted sharply with what was observed in non-virus-infected cells shown in Fig. 5B, which demonstrated that overexpression of IKK γ increased IFN α/β mRNA levels. In other words, the results in Fig. 5B and C demonstrated that influenza viral infection completely eliminated the IKK γ -overexpression-induced increases in production of IFN α/β in the infected cells, suggesting that influenza viruses may block IFN α/β production by affecting the expression of IKK γ .

The NF- κ B pathway is one of the most important mechanisms underlying the suppression of IFN α/β production by influenza viruses ¹. IKK γ is the only regulatory factor in the IKK complex and is located upstream of the NF- κ B signaling pathway. IKK γ is essential for NF- κ B activation ^{6,7}. The influenza virus must have evolved certain mechanism(s) to block IFN α/β production through directly or indirectly regulating this essential component. Viral protein NS1 has been shown to play a vital role in suppressing IFN α/β production via inhibiting NF- κ B activation. Because NS1 exerts its effect on NF- κ B activation in several previously reported mechanisms largely through physically binding to the target molecules such as dsRNA and PKR ¹³⁻¹⁵, we performed coimmunoprecipitation to examine whether NS1 was also associated with IKK γ during influenza viral infection. The result was negative, suggesting that NS1 may not be directly involved in the suppression of IKK γ expression during influenza viral infection (data not shown). However, the result does not exclude the possibility that NS1 may contribute to the influenza virus infection-induced suppression of IKK γ expression in an indirect way. On the other hand, because the NS1 protein in the A/PR/8/34 virus strain cannot inhibit host cellular gene expression through binding to CPSF ²², it was unlikely that the decreased expression of IKK γ in the virus infected cells resulted from influenza viral NS1-mediated host cellular gene expression inhibition. Therefore, one possibility is that the reduced expression of IKK γ in the influenza virus infected cells resulted from NS1-induced inhibition of NF- κ B or IRF-3. Alternatively, it is also possible that Influenza viruses have evolved a novel mechanism that has yet to be characterized to suppress the IKK γ expression during viral infection. The exact molecular mechanism underlying the suppression of IKK γ during influenza viral infection remains to be investigated.

Acknowledgments

We thank Dr. Stephan Ludwig (University of Muenster, Muenster, Germany) for kindly providing the mouse monoclonal anti-NS1 antibody. This work was supported by part of NIH P20RR015569/3P20RR015569-10S2 (Y. Du).

References

1. Santoro MG, Rossi A, Amici C. New embo member's review - NF-kappa B and virus infection: who controls whom. *Embo J.* 2003; 22(11):2552–2560. [PubMed: 12773372]
2. Munir M, Zohari S, Berg M. Non-structural protein 1 of avian influenza A viruses differentially inhibit NF-kappaB promoter activation. *Virology.* 2011; 8(1):383. [PubMed: 21810221]
3. Balachandran S, Basagoudanavar SH, Thapa RJ, Nogusa S, Wang JM, Beg AA. Distinct Roles for the NF-kappa B RelA Subunit during Antiviral Innate Immune Responses. *J Virol.* 2011; 85(6):2599–2610. [PubMed: 21209118]
4. Bonnet MC, Weil R, Dam E, Hovanessian AG, Meurs EF. PKR stimulates NF-kappa B irrespective of its kinase function by interacting with the I kappa B kinase complex. *Mol Cell Biol.* 2000; 20(13):4532–4542. [PubMed: 10848580]
5. Bernasconi D, Amici C, La Frazia S, Ianaro A, Santoro MG. The I kappa B kinase is a key factor in triggering influenza A virus-induced inflammatory cytokine production in airway epithelial cells. *J Biol Chem.* 2005; 280(25):24127–24134. [PubMed: 15837793]
6. Rothwarf DM, Zandi E, Natoli G, Karin M. IKK-gamma is an essential regulatory subunit of the I kappa B kinase complex. *Nature.* 1998; 395(6699):297–300. [PubMed: 9751060]
7. Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, Kirk HE, Kay RJ, Israel A. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell.* 1998; 93(7):1231–40. [PubMed: 9657155]
8. Bouvier NM, Palese P. The biology of influenza viruses. *Vaccine.* 2008; 26 (Suppl 4):D49–53. [PubMed: 19230160]
9. Garcia-Sastre A. Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. *Virology.* 2001; 279(2):375–384. [PubMed: 11162793]
10. Geiss GK, Salvatore M, Tumpey TM, Carter VS, Wang X, Basler CF, Taubenberger JK, Bumgarner RE, Palese P, Katze MG, Garcia-Sastre A. Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. *Proc Natl Acad Sci U S A.* 2002; 99(16):10736–41. [PubMed: 12149435]
11. Jia DL, Rahbar R, Chan RWY, Lee SMY, Chan MCW, Wang BXH, Baker DP, Sun B, Peiris JSM, Nicholls JM, Fish EN. Influenza Virus Non-Structural Protein 1 (NS1) Disrupts Interferon Signaling. *Plos One.* 2010; 5(11)
12. Wang X, Li M, Zheng H, Muster T, Palese P, Beg AA, Garcia-Sastre A. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. *J Virol.* 2000; 74(24):11566–73. [PubMed: 11090154]
13. Tan SL, Katze MG. Biochemical and genetic evidence for complex formation between the influenza A virus NS1 protein and the interferon-induced PKR protein kinase. *J Interferon Cytokine Res.* 1998; 18(9):757–766. [PubMed: 9781815]
14. Sen GC, Li SD, Min JY, Krug RM. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology.* 2006; 349(1):13–21. [PubMed: 16466763]
15. Krug RM, Min JY, Li SD, Sen GC. A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. *Virology.* 2007; 363(1):236–243. [PubMed: 17320139]
16. Au WC, Moore PA, Lowther W, Juang YT, Pitha PM. Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. *Proc Natl Acad Sci U S A.* 1995; 92(25):11657–61. [PubMed: 8524823]

17. Garcia-Sastre A, Mibayashi M, Martinez-Sobrido L, Loo YM, Cardenas WB, Gale M. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. *J Virol*. 2007; 81(2):514–524. [PubMed: 17079289]
18. Kim MJ, Latham AG, Krug RM. Human influenza viruses activate an interferon-independent transcription of cellular antiviral genes: outcome with influenza A virus is unique. *Proc Natl Acad Sci U S A*. 2002; 99(15):10096–101. [PubMed: 12114540]
19. Das K, Ma LC, Xiao R, Radvansky B, Aramini J, Zhao L, Marklund J, Kuo RL, Twu KY, Arnold E, Krug RM, Montelione GT. Structural basis for suppression of a host antiviral response by influenza A virus. *Proc Natl Acad Sci U S A*. 2008; 105(35):13093–8. [PubMed: 18725644]
20. Krug RM, Yuan WM, Noah DL, Latham AG. Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology*. 2003; 309(2):181–189. [PubMed: 12758165]
21. Krug RM, Kuo RL. Influenza A Virus Polymerase Is an Integral Component of the CPSF30-NS1A Protein Complex in Infected Cells. *J Virol*. 2009; 83(4):1611–1616. [PubMed: 19052083]
22. Martinez-Sobrido L, Kochs G, Garcia-Sastre A. Multiple anti-interferon actions of the influenza A virus NS1 protein. *J Virol*. 2007; 81(13):7011–7021. [PubMed: 17442719]
23. Coico, R. *Current protocols in microbiology*. Wiley; Hoboken, N.J.: 2006.
24. Du Y, Zhou J, Fan J, Shen Z, Chen X. Streamline proteomic approach for characterizing protein-protein interaction network in a RAD52 protein complex. *J Proteome Res*. 2009; 8(5):2211–7. [PubMed: 19338310]
25. Du Y, Gu S, Zhou J, Wang T, Cai H, Macinnes MA, Bradbury EM, Chen X. The dynamic alterations of H2AX complex during DNA repair detected by a proteomic approach reveal the critical roles of Ca(2+)/calmodulin in the ionizing radiation-induced cell cycle arrest. *Mol Cell Proteomics*. 2006; 5(6):1033–44. [PubMed: 16522924]
26. Gu S, Du Y, Chen J, Liu Z, Bradbury EM, Hu CA, Chen X. Large-scale quantitative proteomic study of PUMA-induced apoptosis using two-dimensional liquid chromatography-mass spectrometry coupled with amino acid-coded mass tagging. *J Proteome Res*. 2004; 3(6):1191–200. [PubMed: 15595728]
27. Elias JE, Haas W, Faherty BK, Gygi SP. Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. *Nat Methods*. 2005; 2(9):667–75. [PubMed: 16118637]
28. Liu Z, Lu H, Shi H, Du Y, Yu J, Gu S, Chen X, Liu KJ, Hu CA. PUMA overexpression induces reactive oxygen species generation and proteasome-mediated stathmin degradation in colorectal cancer cells. *Cancer Res*. 2005; 65(5):1647–54. [PubMed: 15753358]
29. Spann KM, Tran KC, Chi B, Rabin RL, Collins PL. Suppression of the induction of alpha, beta, and gamma interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages. *J Virol*. 2004; 78(8):4363–4369. [PubMed: 15047850]
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25(4):402–8. [PubMed: 11846609]
31. Liu N, Song W, Wang P, Lee K, Chan W, Chen H, Cai Z. Proteomics analysis of differential expression of cellular proteins in response to avian H9N2 virus infection in human cells. *Proteomics*. 2008; 8(9):1851–8. [PubMed: 18398875]
32. Zhang WP, Li HY, Cheng GF, Hu SS, Li ZL, Bi DG. Avian influenza virus infection induces differential expression of genes in chicken kidney. *Res Vet Sci*. 2008; 84(3):374–381. [PubMed: 17692877]
33. Coombs KM, Berard A, Xu W, Krokhin O, Meng X, Cortens JP, Kobasa D, Wilkins J, Brown EG. Quantitative proteomic analyses of influenza virus-infected cultured human lung cells. *J Virol*. 2010; 84(20):10888–906. [PubMed: 20702633]
34. Inglis SC. Inhibition of host protein synthesis and degradation of cellular mRNAs during infection by influenza and herpes simplex virus. *Mol Cell Biol*. 1982; 2(12):1644–8. [PubMed: 14582206]
35. Lyles DS. Cytopathogenesis and inhibition of host gene expression by RNA viruses. *Microbiol Mol Biol Rev*. 2000; 64(4):709–24. [PubMed: 11104816]

36. Aragon T, de la Luna S, Novoa I, Carrasco L, Ortin J, Nieto A. Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. *Mol Cell Biol.* 2000; 20(17):6259–6268. [PubMed: 10938102]
37. Burgui I, Aragon T, Ortin J, Nieto A. PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes. *J Gen Virol.* 2003; 84(Pt 12):3263–74. [PubMed: 14645908]

Fig. 1.

Comparative 2-DE of cellular proteins extracted from mock- (left panels) and influenza virus-infected cells (right panels). A549 cells at 90–95% confluency were mock-infected or infected by influenza A/PR/8/34 H1N1 viruses at an MOI of 1. At 10 hours postinfection, cells were harvested, lysed and separated on a pH 3–10 (A) or 4–7 (B) linear IPG strip, followed by an 8–16% gradient SDS-PAGE fractionation and coomassie blue staining. Protein spots with a more than 2-fold change in intensity were marked. Proteins identified by MS analysis from the marked spots are listed in Table 1. (C) An enlarged image of gel regions around spot 1, which contains IKK γ . When the wet gels were visually inspected, the IKK γ protein spot in the control gel was clearly visible but was very faint in the “virus” gel.

Fig. 2.
Identification of IKK γ by MS. (A) IKK γ was identified by LC-MS/MS with 5 unique peptides (highlighted). (B) MS spectrum of a representative peptide.

Fig. 3.

Validation of the suppression of IKK γ expression in the influenza virus infected cells with Western blotting. (A) A549 cells were infected by influenza A/PR/8/34 at an MOI of 0, 0.02, 0.075 and 0.3, followed by 24 hours of incubation. Total protein extracted from cells was analyzed by Western blotting with anti-IKK γ . β -actin was used as a loading control. (B) Quantitation of the intensity of IKK γ protein bands shown in (A). The quantitation was performed using software ImageJ.

Fig. 4. Silencing of endogenous IKK γ enhances influenza virus replication. A549 cells (3×10^5) were mock-treated or transfected with siRNA targeting IKK γ , followed by 48 hours of incubation. The cells were then infected with influenza A/PR/8/34 at an MOI of 0.5 and incubated for 30 hours. The resulting cells were harvested for Western blot analysis and the supernatants for virus plaque assay. (A) Silencing of endogenous IKK γ leads to increased NS1 expression. (B) Silencing of endogenous IKK γ results in more viral plaques. A representative plaque assay for control as well as siIKK γ -treated cells is shown. (C) Silencing of endogenous IKK γ results in higher virus titers. Values are the means $\pm$ standard errors of five independent titrations. * denotes $p < 0.05$. siIKK γ , IKK γ silencing by siRNA.

Fig. 5.

Overexpression of IKK γ enhances IFN α/β production and influenza viral infection eliminates IKK γ -overexpression-induced increases in IFN α/β production. (A) Overexpression of IKK γ in 293T cells. Whole cell lysates from 293T cells transiently transfected with IKK γ plasmid were analyzed by Western blotting with anti-IKK γ . (B) Overexpression of IKK γ results in higher levels of IFN α/β mRNA. IFN α/β mRNA extracted from IKK γ overexpression and control cells were determined by qRT-PCR. (C) Influenza viral infection eliminates IKK γ -overexpression-induced increased IFN α/β production. The cells prepared in the same way as in (B) were challenged with influenza viruses at an MOI of 0.5. Thirty hours postinfection, IFN α/β mRNA levels were analyzed by qRT-PCR. Actin was used as an endogenous control. Values are the means $\pm$ standard errors of three separate sample preparations. * denotes $p < 0.05$. IKK γ _{over}, IKK γ overexpression.

Table 1

List of proteins whose expression is altered by the infection of H1N1 virus in A549 cells

Category	Protein name	Spot no. ^a	Accession no.	No. unique peptides	No. assigned spectra	Sequence coverage (%) ^b	Mascot score	Fold change (Infected/control)
Immune response ATPase	IκB kinase-gamma	1	IPI00002411	5	5	12	122	< 0.2
	RuvB-like 2	1	IPI00009104	6	6	15	105	< 0.2
Translation factors	Eukaryotic translation initiation factor 4H	2	IPI00014263	5	5	21	119	2.5
	Elongation factor 1-delta	3	IPI00023048	7	8	35	179	2.2
Carbohydrate and energy metabolisms	Isoform 1 of Triosephosphate isomerase	4	IPI00465028	10	19	42	420	> 6.6
	Aldo-keto reductase family 1 member C1	2	IPI00029733	10	18	35	389	2.5
	Isoform alpha-enolase of Alpha-enolase	2	IPI00465248	4	6	12	185	2.5
	Glutathione S-transferase P	5	IPI00219757	10	35	61	797	2
Signal transduction	Enoyl-CoA hydratase, mitochondrial precursor	6	IPI00024993	10	28	37	591	< 0.2
	14-3-3 protein zeta/delta	3	IPI00021263	9	13	42	188	2.2
	14-3-3 protein beta/alpha	7	IPI00216318	4	7	32	174	2
	Vimentin	8	IPI00418471	12	14	29	204	3.5
Structure and chaperone proteins	Tubulin beta chain	9	IPI00011654	17	73	43	980	3.4
	Ezrin	10	P15311	12	24	17	645	0.2
	Heat shock cognate 71 kDa protein	3	IPI00003865	15	65	28	652	2.2
	Non-structural protein 1 (NS1)	4	P03496	11	49	55	1143	> 6.6
Viral protein								

^a Spot numbers correspond to those on Fig. 1.

^b Coverage of all peptide sequences matched to the identified protein sequence (%).