Acta virologica **66**: 263 – 274, 2022 doi: 10.4149/av_2022_311

Generation and characterization of interferon-beta-resistant H1N1 influenza A virus

Brady T. Hickerson[1], Simone E. Adams[1], Nicolai V. Bovin[2], Raymond P. Donnelly[1], Natalia A. Ilyushina[1*]

[1]Division of Biotechnology Review and Research II, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD 20993, United States; [2]Laboratory of Carbohydrates, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow 117997, Russia

Received January 11, 2022; revised May 23, 2022; accepted June 20, 2022

Summary. – Interferons (IFNs) mediate innate antiviral activity against many types of viruses, including influenza viruses. In light of their potential use as anti-influenza agents, we examined whether resistance to these host antiviral proteins can develop. We generated IFN-β-resistant variants of the A/California/04/09 (H1N1) virus by serial passage in a human airway epithelial cell line, Calu-3, under IFN-β selective pressure. The combination of specific mutations (*i.e.*, L373I in PB1, $K154E_1$, $D222G_1$, $I56V_2$, and $V122I_2$ in HA, and M269I in NA) correlated with decreased ability of the virus to induce expression of IFN (*IFNB1*, *IFNL1*, and *IFNL2/3*) and IFN-stimulated genes (*IFIT1*, *IFIT3*, *OAS1*, *IRF7*, and *MX1*) by target respiratory epithelial cells. In addition, the IFN-induced mutations were associated with decreased HA binding affinity to α2,6 sialyl receptors, reduced NA enzyme catalytic activity, and decreased polymerase transcription activity. Our findings demonstrate that the mutations in the influenza HA, NA, and PB1 proteins induced by IFN-β selective pressure significantly increase viral ability to productively infect and replicate in host cells.

Keywords: influenza A virus; interferon β; lung epithelial cells; interferon response

Introduction

Influenza A viruses are respiratory tract pathogens that cocirculate in the human population and pose major health burdens, especially for young children, immunocompromised individuals, and the elderly. Influenza A viruses cause annual outbreaks and occasional pandemics, which have a huge impact on public health (Wright, 2013). Three classes of antiviral drugs are currently approved for clinical use against influenza: neuraminidase inhibitors (NAIs, oseltamivir, zanamivir, laninamivir, and peramivir), M2 ion-channel inhibitors (amantadine, rimantadine), and a polymerase acidic (PA) endonuclease inhibitor (baloxavir marboxil) (Beigel *et al.,* 2019). A major challenge in the treatment of influenza virus infections is the development of viral resistance to antivirals through mutation (Hussain *et al.,* 2017). Circulating influenza viruses are now largely resistant to the M2 inhibitors and their use is currently not recommended (Dong *et al.,* 2015). Moreover, the antiviral potency of the NAIs is relatively weak (Nicholson *et al.,* 2000; Treanor *et al.,* 2000), and the emergence of influenza viruses resistant to NAIs is a continuing public health concern. In fact, an epidemic of NAI-resistant influenza strains was experienced as recently as 2009 (Sheu *et al.*, 2008; Hurt *et al.*, 2015) and NAI-resistant viruses were also observed in

*Corresponding author. E-mail: Natalia.Ilyushina@fda.hhs.gov; phone: +1-240-402-9600.

Abbreviations: EGFP = enhanced green fluorescent protein; IFN(s) = interferon(s); ISG = IFN-stimulated gene; ISGF3 = interferon-stimulated gene factor 3; JAK = janus-kinase; MAVS = mitochondrial antiviral-signaling protein; MDA-5 = melanoma differentiation-associated gene 5; MEM: minimal essential medium; NAI = neuraminidase inhibitor; NS1 = nonstructural protein 1; PA = polymerase acidic, RIG-I = retinoic acid-inducible gene I; RNP = ribonucleoprotein; STAT = signal transducer and activator of transcription protein; SOCS = suppressor of cytokine signaling

immunocompromised patients more recently (Kim *et al.,* 2020). Therefore, additional effective antiviral agents with novel mechanisms of action are needed for the prevention and treatment of influenza virus infection, particularly in the event of widespread resistance to other classes of antivirals.

The human type I interferon (IFN) system includes several different IFN-α subtypes and a single form of IFN-β, which mediate early innate host defense against influenza virus infections. During influenza virus infection, the cytosolic retinoic acid-inducible gene I (RIG-I) and the RIG-I-like receptor, melanoma differentiation-associated gene 5 (MDA-5), detect influenza ribonucleoprotein (RNP) complexes as a pathogen-associated molecular pattern (Loo *et al.,* 2008; Rehwinkel *et al.,* 2010). Following detection of RNPs, RIG-I interacts with TRIM25 and is recruited to mitochondria by the scaffold mitochondrial antiviral-signaling protein (MAVS), finally leading to the activation of the TBK-1/IRF-3 IFN-β induction pathway (Yoneyama *et al*., 2002; Takaoka *et al*., 2006). As a secondary response, released type I IFN binds to its cognate receptor (IFN-α/βR) to activate the Janus kinase (JAK)/signal transducer and activator of transcription protein (STAT) signaling pathway. This in turn leads to phosphorylation of STAT1 and STAT2 and formation of active IFN-stimulated gene factor 3 (ISGF3) complexes, which then induce transcriptional activation of a large set of IFN-stimulated genes (ISGs) (Ehrhardt *et al.,* 2010; Garcia-Sastre, 2011). These ISGs amplify the host defense response and lead to the establishment of an antiviral state (Durbin *et al.,* 2013; Xiao *et al.,* 2013).

Numerous studies have shown that type I IFNs exert antiviral activity against influenza virus infection (Wu *et al*., 2020). Both seasonal and 2009 pandemic H1N1 influenza A viruses were sensitive to the antiviral actions of IFN-α/β in human monocyte-derived dendritic cells and macrophages (Osterlund *et al.,* 2010). Pretreatment with IFN-α/β significantly inhibited influenza virus replication, including highly pathogenic H5N1 avian influenza viruses, both *in vitro* and in a mouse animal model (Beilharz *et al*., 2007; Szretter *et al*., 2009; Scagnolari *et al*., 2011). In a guinea pig model, intranasal administration of human recombinant IFN-α significantly reduced lung and nasal wash titers of both reconstructed 1918 pandemic H1N1 virus and H5N1 strain (Van Hoeven *et al.,* 2009). Prophylactic use of IFN-α showed promise in preventing severe influenza in humans (Bennett *et al.,* 2013). Furthermore, IFN-β alone or in combination with the neuraminidase inhibitor, oseltamivir carboxylate, induced significant antiviral activity against influenza A viruses *in vitro* (Ilyushina *et al*., 2014).

In view of the critical antiviral action of type I IFNs and their potential use as anti-influenza agents, it is important to understand whether resistance to these host proteins can develop. To date, no published studies have directly examined the potential emergence of IFN-β–resistant influenza viruses. To gain insight into the probability of emergence of IFN-β resistance, we generated an IFN-β-resistant variant of the A/California/04/09 (CA/04, H1N1) virus by serial passage in a human airway epithelial cell line, Calu-3, under IFN-β selective pressure. We then characterized the acquired viral genomic mutations, growth potential, and levels of resistance to IFN-β of the selected mutant. We observed that a combination of mutations in the PB1, HA, and NA proteins is associated with markedly increased resistance to the antiviral activity of IFN-β.

Materials and Methods

Cells, viruses, and reagents. The Madin-Darby canine kidney (MDCK) cell line, human embryonic kidney cell line (293T), and the human lung epithelial cell line (Calu-3) were obtained from the American Type Culture Collection (Manassas, VA) and maintained as described previously (Ilyushina *et al*., 2014). Human influenza CA/04 (H1N1) virus was kindly provided by Dr. Robert G. Webster (St. Jude Children's Research Hospital, Memphis, TN). Stock virus was grown in the allantoic cavities of 10-day-old embryonated chicken eggs for 48 h at 37°C, and aliquots were stored at -80°C until use. All experimental work was performed in a biosafety level-2 (BSL-2) laboratory approved by the U.S. Department of Agriculture and the U.S. Centers for Disease Control and Prevention. Human recombinant IFN-β protein was obtained from R&D Systems, Inc. (Minneapolis, MN), and diluted in RPMI-1640 medium plus 10% fetal bovine serum (Hyclone, Logan, UT). Oseltamivir carboxylate was provided by Roche Diagnostics GmbH (Mannheim, Germany). Zanamivir was obtained from Sigma-Aldrich (St. Louis, MO). Laninamivir was purchased from Carbosynth (Compton, UK).

Infectivity of H1N1 influenza viruses. The infectivity of the H1N1 viruses was determined by plaque assay (Hayden *et al*., 1980). Briefly, MDCK cells were incubated at 37°C for 1 h with 10-fold serial dilutions of each virus. The cells were then overlaid with minimal essential medium (MEM) containing 0.3% bovine serum albumin, 0.9% Bacto agar, and 1 μg/ml l-(tosylamido-2-phenyl)ethylchloromethylketone (TPCK)-treated trypsin. After 3 days of incubation at 37°C, the cells were stained with 0.1% crystal violet in 10% formaldehyde solution, and the number of plaque-forming units (PFU) per milliliter and plaque size of any 10 plaques were determined using a Finescale magnifying comparator.

Virus yield reduction assay. The virus yield reduction assay was performed as described previously in 24-well plates containing confluent Calu-3 cells (Ilyushina *et al*., 2014). The concentrations of IFN-β ranged from 0.001 to 10 ng/ml (the 50%

cytotoxic concentration for IFN-β is > 1000 ng/ml; (Ilyushina *et al.*, 2014)) and IFN-β was added to the 24-well plates for 24 h. After pretreatment, the cells were overlaid with 2× drug-containing medium (100 µl/well), infected with influenza virus at a MOI of 0.01 PFU/cell, and incubated for 24 h at 37°C. Virus yields were determined as the number of PFU/ml in MDCK cells. The drug concentration that caused a 50% decrease in the PFU titer in comparison to control wells without drug was defined as 50% effective concentration (EC_{50}). The results of two independent experiments were averaged.

Viral replication kinetics. To determine multistep growth curves for each virus, Calu-3 cells were infected with the H1N1 viruses at the MOI of 0.001 PFU/cell. After incubation for 1 h, the cells were overlaid with MEM medium containing 0.3% bovine serum albumin and 1 µg/ml TPCK-treated trypsin. The supernatants were collected at 6, 24, 48, 72, and 96 h post-infection and stored at -80°C until titration.

Virus sequence analysis. Viral RNAs isolated from virus-containing cell culture fluid were reverse transcribed and analyzed by PCR using universal primers as described previously (Hoffmann *et al.,* 2001). Sequencing was performed by the Research Central Facility for Biotechnology Resources at the U.S. Food and Drug Administration, Silver Spring, MD. DNA sequences were completed and edited by using a Lasergene sequence analysis software package (DNASTAR, Madison, WI).

Receptor-binding assay. The affinity for biotinylated 3'-and 6'-glycans (chemical structures are shown in Supplementary Table 1) was measured in a direct binding assay as described previously (Ilyushina *et al.,* 2017). Briefly, plates pre-coated with each virus were incubated with each biotinylated sialyl-glycopolymer. Plates were then washed and incubated with streptavidin-peroxidase (Sigma-Aldrich, St. Louis, MO). After washing, tetramethylbenzidine (TMB) substrate solution (KPL, Gaithersburg, MD) was added, and the reaction was stopped with TMB stop solution (KPL). Optical density was determined at 450 nm with a Synergy 2 multimode microplate reader (BioTek Instruments, Winooski, VT). The association constant (K_A, 1/µM sialic acid) values were determined by fitting the data to the "one site-total binding" equation by using nonlinear regression in Prism 9.0 software (GraphPad Software Inc., San Diego, CA). The reported data represent the mean of at least four individual and independent experiments for each virus.

NA enzyme inhibition assay and NA enzyme activity. Influenza viruses were standardized to equivalent NA activity and incubated for 30 min at 37°C with NAIs (oseltamivir carboxylate, zanamivir, or laninamivir) at concentrations of 0.0001 to 5 µM and with MUNANA (Sigma-Aldrich) as a substrate for 1 h at 37°C. The reaction was then terminated by adding 14 mM NaOH and fluorescence was quantified in a Synergy 2 multimode microplate reader (BioTek Instruments). The half maximal inhibitory concentration (IC_{50}) of each NA inhibitor was determined by plotting the dose-response curve of inhibition of NA activity as a function of the compound concentration.

The NA activity of influenza viruses standardized to an equivalent NA protein content of 0.15 ng/µl was measured by a fluorescence-based assay using MUNANA (Sigma-Aldrich) as described previously (Adams *et al.,* 2020). The kinetic parameters Michaelis-Menten constant (K_M), maximum velocity of substrate conversion (V_{max}), and catalytic efficiency (k_{cat}/K_M) of the NAs were calculated by fitting the data to the appropriate Michaelis-Menten equations by using nonlinear regression analysis in Prism 9.0 software (GraphPad Software Inc.). Values represent the means of at least three individual and independent determinations.

Minigenome assay for polymerase activity. Subconfluent monolayers of 293T cells were transfected with the luciferase reporter plasmid (enhanced green fluorescent protein [EGFP] open reading frame in pHW72-EGFP replaced with the firefly luciferase gene) (Salomon *et al.,* 2006) and a mix of PB2, PB1, PA, and nucleoprotein (NP) expression plasmids (CA/04 or mutated) in quantities of 1, 1, 1, and 2 µg, respectively. The plasmid pGL4.75(*hRluc*/CMV), which expresses Renilla luciferase (Promega, Madison, USA), was used as an internal control for a dual-luciferase assay. As a negative control, 293T cells were transfected with the same plasmids, with the exception of the NP expression plasmid. After 24 h of incubation at 33°C or 37°C, cell extracts were harvested and lysed, and luciferase levels were assayed with a dual-luciferase assay system (Promega) and a Synergy 2 multimode microplate reader (BioTek Instruments). Experiments were performed in triplicate.

Quantitative real-time polymerase-chain reaction (qPCR) of IFN, IFN-stimulated, and viral matrix (M1) genes. Quantification of changes in gene expression was carried out by qPCR analyses of individual IFNs and ISGs using RT² qPCR primer assays (Qiagen, Germantown, MD). Viral *M1* gene copy numbers were assayed by using Taqman gene expression assay primer/probe sets and master mix (Life Technologies, Carlsbad, CA) and values were determined by running respective standard curves. Total cellular RNA was isolated from virus-infected Calu-3 cells (MOI = 1) pretreated with or without IFN-β (1 ng/ml) for 8 h using RNeasy Minikit (Qiagen) and 150 ng of the purified RNA was reverse-transcribed to cDNA with Quantiscript reverse transcriptase (Qiagen). The cDNA was mixed with RT² SYBR® green qPCR Mastermix (Qiagen) and qPCR was performed using the ViiA™7 system (Applied Biosystems, Beverly, MA). The results expressed as the mean-fold increase relative to the control gene expression levels of the housekeeping gene, *GAPDH*, were further normalized to viral *M1* gene copies in each reaction. Graphing and statistical analysis of qPCR results were performed using Prism 9.0 (GraphPad Software Inc.). Values are the means of three independent determinations.

Enzyme-linked immunosorbent assay (ELISA). The secreted levels of IFN-β from Calu-3 cell culture supernatants were analyzed using ELISA kit supplied by PBL Biomedical Laboratories (Piscataway Township, NJ). The IFN-β levels from cell culture

supernatants isolated from cells infected with CA/04 and selected variants were analyzed in one experiment.

Statistical analysis. The EC_{50} values, virus yield, plaque size and number, binding to sialyl receptors, NA enzyme kinetic parameters (K_M, V_{max}, and k_{cat}/K_M), polymerase activities of RNP complexes, secreted levels of IFN-β, and IFN and ISG gene expression values induced by CA/04 and selected variants were compared by one-way analysis of variance (ANOVA) or unpaired *t*-test. Probability values £ 0.05 indicate statistically significant differences.

Results

Generation of IFN-β-resistant variants in Calu-3 cells and assessment of IFN sensitivity and growth characteristics of two selected variants

We first measured the sensitivity of influenza CA/04 virus to treatment with recombinant IFN-β by virus reduction assay in Calu-3 cells (Fig. 1a). The parental CA/04 virus was very sensitive to inhibition by IFN-β: mean EC_{50} = 0.01 ± 0.004 ng/ml, which was consistent with our previous findings (Ilyushina *et al.*, 2014). The EC_{50} value (*i.e.*, 0.01 ng/ml) was selected as the initial passage concentration of IFN-β to obtain drug-resistant H1N1 variants. We then serially passaged the CA/04 strain 30 times in Calu-3 cells in the presence of increasing concentrations of IFN-β to provide an opportunity for selection of an IFN-β-resistant viral phenotype (Fig. 1b). Virus yields were measured by plaque assay using MDCK cells after each passage. Analysis of the CA/04 virus following culture in the presence of increasing concentrations of IFN-β showed the largest decrease in plaque number at passage 27 (2.0 ± 0.6 log_{10}PFU/ml). The plaque number rebounded at passage 28 (4.3 ± 0.4 log_{10}PFU/ml) and this increase could be associated with the acquisition of IFN-β-induced mutation(s). Furthermore, a mixed viral population was detected by plaque morphology analysis of culture supernatants at passage 23 (data not shown).

After 30 serial passages in the presence of IFN-β, two variants, CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2, were isolated by random selection of single plaques followed by plaque purification in MDCK cells. We then tested their sensitivities to the antiviral activity of IFN-β by virus yield reduction assay. As shown in Fig. 1a, the CA/04$^{+IFN-β}$-1 variant exhibited significantly reduced sensitivity to IFN-β compared to the wild-type CA/04 virus (EC_{50} = 0.4 ± 0.2 ng/ml). In contrast, CA/04$^{+IFN-β}$-2 remained sensitive to IFN-β (EC_{50} = 0.02 ± 0.01 ng/ml). A similar pattern of IFN-β dose-dependent reduction of viral *M1* gene copies was observed by qRT-PCR analysis (data not shown). Our findings indicated that the CA/04$^{+IFN-β}$-1 variant may have acquired one or more mutation(s) that decreased its sensitivity to the antiviral activity of IFN-β.

We next examined plaque morphology and replication of the wild-type, CA/04$^{+IFN-β}$-1, and CA/04$^{+IFN-β}$-2 viruses in MDCK cells. The two passaged variants formed significantly larger plaques than the wild-type virus (P <0.01, Table 1). To evaluate the replicative ability of the selected variants, we assayed their virus yield in comparison to that of the parental strain after multiple replication cycles in Calu-3 cells. As shown in Fig. 1c, the CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2 viruses grew to significantly higher titers than the wild-

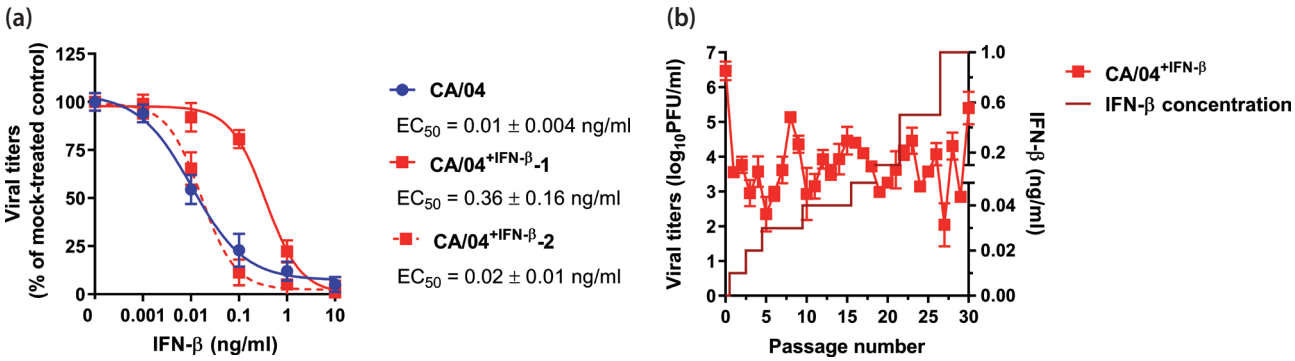


Fig. 1

Generation of IFN-β-resistant variants in Calu-3 cells and assessment of IFN sensitivity and growth characteristics of two selected variants

(a) Antiviral effect of IFN-β (ng/ml) against the CA/04, CA/04$^{+IFN-β}$-1, and CA/04$^{+IFN-β}$-2 viruses in Calu-3 cells as measured by the virus yield reduction assay. Mean EC_{50} values and standard deviations are calculated from at least two independent experiments. **(b)** Generation of influenza A virus with decreased sensitivity to IFN-β. CA/04 virus was passaged in Calu-3 cells in the presence (red line) of increasing concentrations of IFN-β (brown line). **(c)** Replication of the CA/04, CA/04$^{+IFN-β}$-1, and CA/04$^{+IFN-β}$-2 viruses in Calu-3 cells. The results are expressed as log_{10}PFU/ml from three independent experiments. °P <0.01, compared to the values for the wild-type virus by one-way ANOVA.

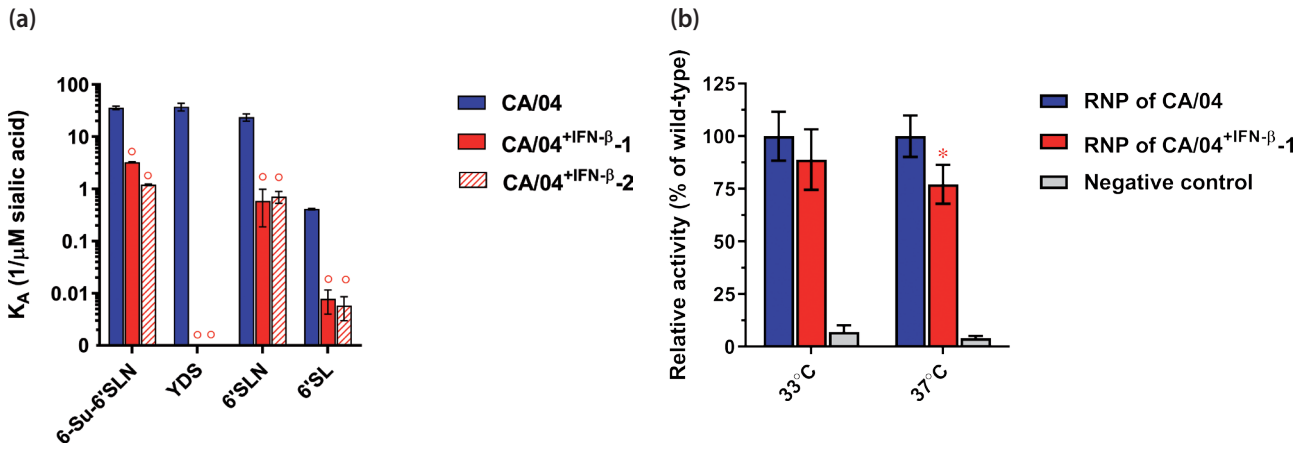


Fig. 2

Effects of HA1 and PB1 mutations on receptor specificity and viral polymerase activity

(a) Receptor specificity of CA/04, CA/04$^{+IFN-β}$-1, and CA/04$^{+IFN-β}$-2 viruses. °P <0.01, compared to the values for the wild-type virus by one-way ANOVA. **(b)** Polymerase activity of RNP complexes of wild-type and CA/04$^{+IFN-β}$-1 containing PB1 L373I mutation at 33°C and 37°C. The values represent the mean ± standard deviation of activity of the mutated RNP complex relative to that of CA/04 virus. *P <0.01, compared to the values for the CA/04 virus by unpaired t-test.

type CA/04 virus at 72 and 24 h post-infection, respectively (↑~1.7 log, P <0.01). These findings indicated that sequential passage of the parental H1N1 influenza virus in Calu-3 cells in the presence of IFN-β promoted selection of variants with enhanced replication potential.

Sequence analysis of selected variants

To identify potential amino acid changes in the H1N1 influenza virus after culture in the presence of IFN-β, we sequenced the complete genome of the selected variants, CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2, at the end of the passaging protocol. Sequence analysis revealed five substitutions in HA (K153E$_1$, K154E$_1$, D222G$_1$, I56V$_2$, and V122I$_2$), one substitution in NA (M269I), and one amino acid substitution (L373I) in the PB1 protein (Table 2, Supplementary Fig. 1). Notably, the two selected variants differed by only one HA mutation (*i.e.*, K153E$_1$ *vs* K154E$_1$). To determine if the amino acid changes identified in our CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2 variants might also occur in other pandemic 2009 H1N1 isolates, we analyzed ~52,900 H1N1 genomic sequences deposited in the Influenza Research Database (data were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) database: www.fludb.org). Two of the amino acid substitutions that we identified in our study, D222G$_1$ and V122I$_2$ in HA, were also present in ~1.0% of the related H1N1 strains in the Influenza Research Database. The remaining five substitutions (L373I in PB1, K153E$_1$, K154E$_1$, and I56V$_2$ in HA, and M269I in NA) were found in ≤0.2% of contemporary H1N1 isolates (Supplementary Table 2).

Effects of HA1, NA, and PB1 mutations on receptor specificity, NA sensitivity/activity, and viral polymerase activity

To determine if the HA1 mutations that we identified affect the HA affinity for sialyl receptors, we examined the

Table 1. Growth characteristics of wild-type, CA/04$^{+IFN-β}$-1, and CA/04$^{+IFN-β}$-2 influenza viruses

Viruses	Virus yield (log_{10}PFU/ml)[a]	Plaque size (mm)[b]
CA/04	6.8 ± 0.3	0.2 ± 0.1
CA/04$^{+IFN-β}$-1	6.8 ± 0.1	1.7 ± 0.3°
CA/04$^{+IFN-β}$-2	6.4 ± 0.3	2.0 ± 0.2°

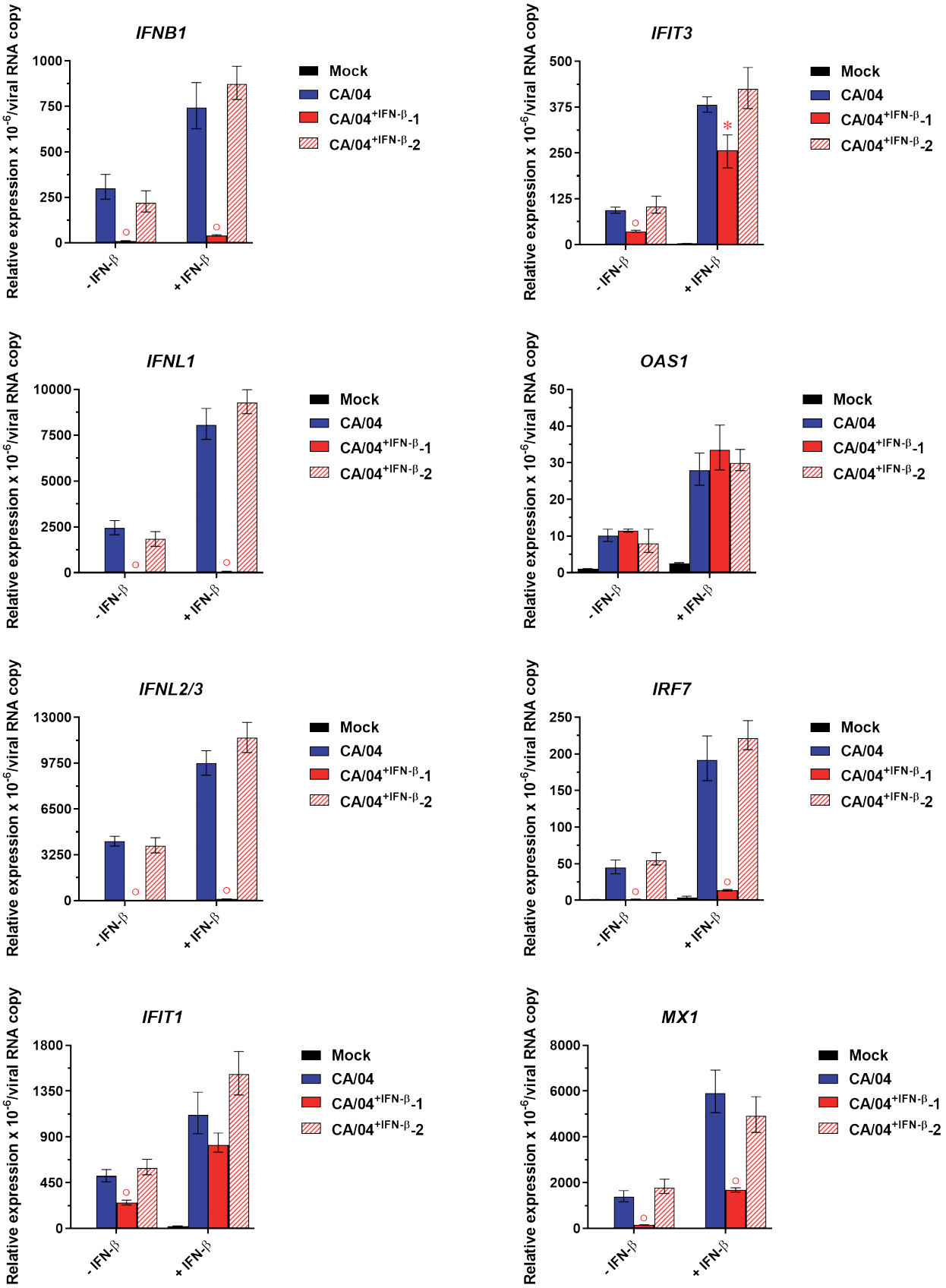
[a]Values represent the mean log_{10}PFU/ml ± standard deviations from three independent determinations. The number of PFU in MDCK cells was measured by plaque assay after incubation at 37°C for 3 days with 10-fold serial dilutions of virus (Hayden *et al.*, 1980). [b]Values represent the mean plaque diameter (mm ± standard deviations) as measured by use of a Finescale comparator. P <0.01, compared to the value for the wild-type virus by one-way ANOVA.

Table 2. Amino acid substitutions identified in CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2 viruses

Proteins	CA/04$^{+IFN-β}$-1	CA/04$^{+IFN-β}$-2
PB1	L373I	L373I
HA1[a]	K154E, D222G	K153E, D222G
HA2	I56V, V122I	I56V, V122I
NA[a]	M269I	M269I

[a]HA1 and NA1 numbering.

(a)


IFNB1
IFIT3
IFNL1
OAS1
IFNL2/3
IRF7
IFIT1
MX1
Relative expression x 10-6/viral RNA copy
- IFN-β
+ IFN-β
Mock
CA/04
CA/04+IFN-β-1
CA/04+IFN-β-2

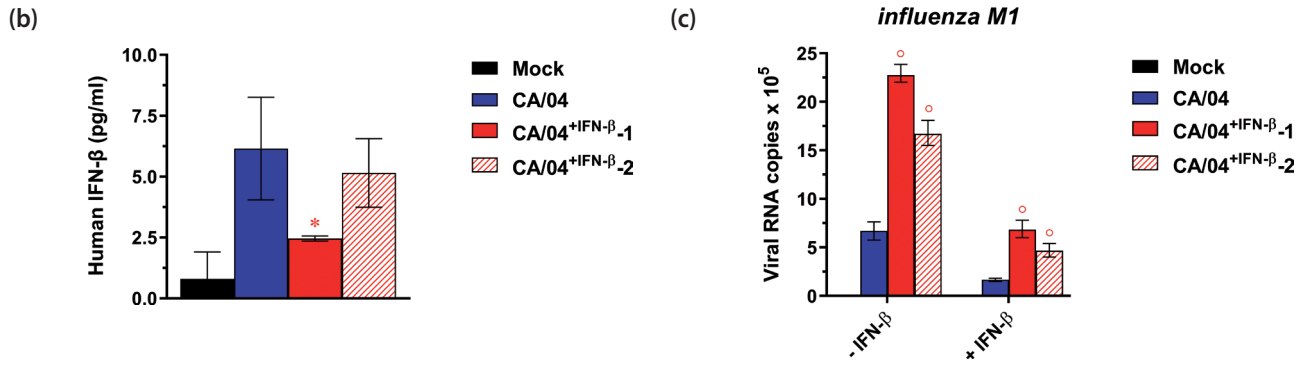


Fig. 3

IFN and ISG gene expression and IFN-β protein expression induced by CA/04, CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2

(a) Influenza-induced IFN and ISG gene expression levels in Calu-3 cells. Cells were pretreated with or without IFN-β (1 ng/ml) for 8 h, infected with the indicated viruses (MOI = 1), and the levels of IFNs and ISGs were quantified by qPCR at 24 h post-infection. Results are expressed as the mean-fold increase relative to the control gene expression levels of the housekeeping gene, *GAPDH*, which are further normalized to the viral *M1* gene copies in each reaction shown in Figure 3b. **P* <0.05; °*P* <0.01, compared to the values for the CA/04 virus (one-way ANOVA). **(b)** IFN-β protein production in Calu-3 cells. Cells were infected with the indicated viruses (MOI = 1). Supernatants were collected at 24 h post-infection and the levels of secreted IFN-β protein were determined by ELISA. **P* <0.05, compared to the values for the CA/04 virus by one-way ANOVA. **(c)** Comparison of the viral *M1* gene RNA copies in influenza-infected Calu-3 cells. Cells were pretreated with and without of IFN-β (1 ng/ml) for 8 h, infected with the indicated viruses (MOI = 1), and the levels of *M1* RNA copies were quantified by qPCR at 24 h post-infection. Values were determined by comparison to standard curves for the influenza *M1* gene and the results are expressed as RNA copy numbers. °*P* <0.01, compared to the values for the CA/04 virus by one-way ANOVA.

receptor specificity of the selected variants, CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2, in comparison to the wild-type virus. The binding level to the "avian-type" receptor (3'SL/N) was negligible among all three viruses (data not shown). However, based on their K_A values (Fig. 2a), CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2 demonstrated a statistically significant decrease in binding affinity to 6-Su-6'SLN and 6'SL/N glycans as compared to the parental CA/04 virus (~39.6-fold, $P < 0.01$). Moreover, the CA/04$^{+IFN-β}$-2 variant exhibited a 2.7-fold lower binding to 6-Su-6'SLN as compared to CA/04$^{+IFN-β}$-1. Binding of both viruses to YDS glycan was completely abolished (Fig. 2a).

We next examined the effect of the NA mutation found in our selected variants on susceptibility to three NA inhibitors: oseltamivir carboxylate, zanamivir, and laninamivir, using an enzyme-based NA inhibition assay. The CA/04$^{+IFN-β}$-1 and CA/04$^{+IFN-β}$-2 variants remained susceptible to all three of the NA inhibitors despite the presence of the M269I NA mutation (Table 3). To further evaluate the effect of the M269I NA mutation on NA enzymatic activity, a fluorescence-based assay based on MUNANA catalyzation was used to measure changes in the relative enzymatic activity (V_{max}), Michaelis-Menten constant (K_M), and catalytic efficiency (k_{cat}/K_M) of the mutant NA compared to wild-type CA/04 NA. We observed that although the M269I NA mutation had no effect on the K_M or k_{cat}/K_M values, NA protein containing this mutation exhibited significantly decreased NA enzyme activity (V_{max} ratio relative to the wild-type virus = 0.6, Table 3).

Table 3. NA inhibitor sensitivity[a] and NA enzyme kinetics properties of H1N1 viruses

Viruses	Oseltamivir carboxylate, IC_{50} (nM)	Zanamivir, IC_{50} (nM)	Laninamivir, IC_{50} (nM)	V_{max} ratio[b]	K_M (μM)[c]	k_{cat}/K_M (μM*sec)$^{-1}$
CA/04	1.3 ± 0.2	0.9 ± 0.1	0.2 ± 0.1	1.0	214.3 ± 74.9	0.1 ± 0.05
CA/04$^{+IFN-β}$-1	1.8 ± 0.2	1.2 ± 0.2	0.2 ± 0.1	0.6	163.7 ± 32.0	0.1 ± 0.04
CA/04$^{+IFN-β}$-2	1.6 ± 0.2	1.0 ± 0.2	0.1 ± 0.1	0.6	182.1 ± 44.0	0.1 ± 0.02

[a]Viral sensitivities were determined in NA inhibition assays. The values were fit to nonlinear regression curves with the variable slope model to determine the IC_{50}, using GraphPad Prism. Values are means ± standard deviation from two or more independent experiments. [b]The V_{max} was calculated using a nonlinear regression of the curve according to the Michaelis-Menten equation and then the ratio of the CA/04$^{+IFN-β}$ virus' NA V_{max} to the V_{max} of the wild-type CA/04 NA was determined. [c]The K_M represents the Michaelis-Menten constant, at which the reaction rate is half of V_{max}. The enzyme kinetic data were fit to the Michaelis-Menten equation using GraphPad Prism. Values are the means ± 95% confidence interval from 3 independent determinations.

To determine if the observed amino acid substitution L373I in the PB1 protein of the selected variants altered their viral transcription activity, we analyzed reconstituted RNP complexes in 293T cells using a luciferase mini-genome reporter assay (Fig. 2b). We found that the single point mutation, PB1 L373I, significantly decreased polymerase activity of the CA/04 polymerase complex to ~77.2% at 37°C ($P < 0.05$). To investigate whether the impact of the PB1 L373I substitution is temperature-dependent, we measured the transcription activity of the RNPs at 33°C and we observed that this substitution did not alter RNP activity at this temperature (Fig. 2b).

IFN and ISG gene expression and IFN-β protein expression induced by CA/04, CA/04$^{+IFN\text{-}\beta}$-1 and CA/04$^{+IFN\text{-}\beta}$-2

We compared the ability of the wild-type, CA/04$^{+IFN\text{-}\beta}$-1, and CA/04$^{+IFN\text{-}\beta}$-2 viruses to induce IFN (*IFNB1*, *IFNL1*, and *IFNL2/3*) and ISG (*IFIT1*, *IFIT3*, *OAS1*, *IRF7*, and *MX1*) gene expression in Calu-3 cells with and without IFN-β pretreatment (1 ng/ml). As shown in Fig. 3a, the CA/04$^{+IFN\text{-}\beta}$-1 virus induced significantly lower levels of *IFNB1*, *IFNL1*, *IFNL2/3*, *IFIT3*, *IRF7*, and *MX1* gene expression at 24 h post-infection independent of IFN-β pretreatment (↓~38.6-fold, $P < 0.05$). In contrast, infection of Calu-3 cells with CA/04$^{+IFN\text{-}\beta}$-2 induced levels of IFNs and ISGs similar to those induced after infection with the parental CA/04 strain. Moreover, the levels of IFN-β protein (Fig. 3b) correlated well with the levels of *IFNB1* gene expression (Fig. 3a) in the absence of IFN-β pretreatment.

We also examined if there is a difference in expression of the viral *M1* gene RNA copies after infection with the CA/04, CA/04$^{+IFN\text{-}\beta}$-1, or CA/04$^{+IFN\text{-}\beta}$-2 viruses. We observed higher expression levels of the *M1* gene after infection with either of the variants compared to the parental CA/04 strain at 24 h post-infection independent of IFN-β Fig. 3C pretreatment (↑3.2-fold, $P < 0.01$, Fig. 3c). These findings indicate that the CA/04$^{+IFN\text{-}\beta}$-1 variant induced significantly lower levels of IFN and ISG gene expression compared to CA/04 and CA/04$^{+IFN\text{-}\beta}$-2. The decreased levels of IFN and ISG expression correlated with higher levels of viral RNA expression suggesting that the CA/04$^{+IFN\text{-}\beta}$-1 variant was able to inhibit the IFN-β signaling as compared to the wild-type and CA/04$^{+IFN\text{-}\beta}$-2 variant.

Discussion

In the present study, we generated an IFN-β-resistant mutant, CA/04$^{+IFN\text{-}\beta}$-1, by serially passaging the parental virus in the presence of increasing IFN-β concentrations for a total of 30 passages. The selective pressure mediated by IFN-β induced the acquisition of several viral genomic mutations in the PB1, HA, and NA proteins. The specific combination of these selected mutations (*i.e.*, L373I in PB1, K154E$_1$, D222G$_1$, I56V$_2$, and V122I$_2$ in HA, and M269I in NA) correlated with decreased ability of the virus to induce expression of IFN (*IFNB1*, *IFNL1*, and *IFNL2/3*) and ISG (*IFIT1*, *IFIT3*, *OAS1*, *IRF7*, and *MX1*) genes by target respiratory epithelial cells. Moreover, the reduced levels of IFN and ISG expression correlated well with decreased expression of IFN-β protein in Calu-3 cells.

To ensure efficient replication and spread, influenza viruses have evolved several strategies to suppress the IFN antiviral response. The nonstructural protein 1 (NS1) inhibits induction of the type I IFN response via its interaction with RIG-I (Guo *et al.,* 2007; Mibayashi *et al.,* 2007) and TRIM25 (Gack *et al.,* 2009). NS1 has been shown to bind dsRNA and mask viral RNA species from recognition by the host cells (Wang *et al.,* 1999; Donelan *et al.,* 2003). NS1 can also interact with PKR and inhibit its activation (Bergmann *et al.,* 2000; Li *et al.,* 2006). NS1 also inhibits IFN-induced signaling by inhibiting expression of the type I IFN receptor subunit IFNAR1 and inducing expression of suppressor of cytokine (SOCS) 3 (Jia *et al.,* 2010). Moreover, NS1, PB2 and PB1-F2 were demonstrated to be type I IFN antagonists that inhibit IFN-β induction by interfering with the MAVS protein without affecting viral replication (Graef *et al.,* 2010; Iwai *et al.,* 2010; Varga *et al.,* 2011). A specific motif within the influenza PB1 and PA subunits (PB1 398E/524S/563I and PA 351E – referred as ESIE) was found to be sensed by RIG-I and to suppress the type I IFN response (Liedmann *et al.,* 2014). In addition, Xia *et al.,* 2015, demonstrated that the influenza surface glycoprotein, HA, promotes IFNAR1 ubiquitination and degradation (Xia *et al.,* 2015). This results in a reduction of the levels of IFNAR1 expression and marked suppression of cellular responsiveness to type I IFNs. A previous study by Grimm *et al.,* 2007, showed that influenza NA protein contributes to high virulence of a A/Puerto Rico/8/34 (H1N1) variant, that allows the virus to evade innate immune control due to the high speed of virus growth and early viral gene expression (Grimm *et al.,* 2007).

In two previous studies, we evaluated the potential emergence of amino acid changes associated with adaptation of the CA/04 virus to growth in Calu-3 cells by passaging the parental virus without any selective pressure (Ilyushina *et al.,* 2017; Adams *et al.,* 2020). We observed that three substitutions in the HA protein, G155E$_1$, S183P$_1$, and M257I$_1$, one amino acid substitution in the PA protein, V14I, and one substitution in the M2 protein, E70K, were associated with adaptation of the pandemic 2009 isolate to growth in Calu-3 cells (Ilyushina *et al.,* 2017). More recently, we found that two substitutions in the HA protein, D127E$_1$ and G155E$_1$, and one substitution in the NA protein, Y56C, are associated with adaptation of the paren-

tal CA/04 virus to growth in respiratory epithelial cells (Adams *et al.,* 2020). Since the combination of mutations selected without any selective pressure differed from that seen in the present study, we believe that at least some of the changes (most likely their combination) acquired under IFN-β selective pressure (*i.e.*, PB1 L373I, HA K154E$_1$, D222G$_1$, I56V$_2$, or V122I$_2$, or NA M269I) play a role not only in Calu-3 cell adaptation but also in inhibiting IFN-β signaling. Additionally, since the two variants, CA/04$^{+IFN\text{-}\beta}$-1 and CA/04$^{+IFN\text{-}\beta}$-2, selected in the presence of IFN-β differed by sensitivity to IFN-β and by one HA1 mutation (*i.e.*, K154E$_1$ *vs* K153E$_1$), it appears that the K154E$_1$ must be present in the combination of mutations for the CA/04 virus to acquire significantly increased IFN-β resistance.

We found that passaging of the CA/04 strain in the presence of IFN-β resulted in the development of five mutations in HA (K153E$_1$, K154E$_1$, D222G$_1$, I56V$_2$, and V122I$_2$). The D222G$_1$ mutation, located in the HA receptor binding site and mapped to the antigenic site Ca (Xu *et al.,* 2010), was previously shown to be associated with higher viral titers in Calu-3 cells (Ilyushina *et al.,* 2019), adaptation to mice (Ilyushina *et al.,* 2010; Song *et al.,* 2013), more prolonged hospitalization of infected patients, and a higher frequency of severe and fatal human cases (Chan *et al.,* 2011). This HA1 substitution conferred enhanced virulence to the parental virus both *in vitro* and *in vivo* via increased binding affinity to α2,3 sialyl receptors while maintaining α2,6 specificity (Chutinimitkul *et al.,* 2010). Since the HA D222G$_1$ mutation was previously shown to arise after serial passaging in Calu-3 (Adams *et al.,* 2020), it is logical to conclude that this HA1 change contributes to the increased ability of the parental virus to infect and spread in mammalian respiratory epithelial cells.

Our characterization of the receptor specificity of the CA/04$^{+IFN\text{-}\beta}$-1 and CA/04$^{+IFN\text{-}\beta}$-2 variants revealed their significant decrease in binding affinity to 6-Su-6'SLN and 6'SL/N glycans as compared to the CA/04 virus. It is very likely that K153E$_1$ and K154E$_1$, which are located near the HA receptor-binding site and map to the antigenic site Sa (Xu *et al.,* 2010), contribute to the decreased binding to the "human-type" receptors. Moreover, K153E$_1$ led to a significant decrease in binding of the CA/04$^{+IFN\text{-}\beta}$-2 variant to 6-Su-6'SLN as compared to the other viruses. Our findings correlate with a previous study by others, in which a lysine residue at position 153 was shown to provide additional and optimal contact with the α2,6 glycan and to affect receptor binding specificity by decreasing affinity for α2,6 sialyl receptors (Soundararajan *et al.,* 2009).

We found that one amino acid change located near the active site of the NA protein, M269I, was selected in the CA/04 virus after extended passaging in Calu-3 cells in the presence of IFN-β. The CA/04$^{IFN\text{-}\beta}$-1 variant exhibited reduced NA enzyme catalytic activity due to the M269I substitution, but this change was not associated with a reduction in viral replication in Calu-3 cells. Interestingly, this finding correlates with our previously reported data showing that decreased NA activity is necessary for the adaptation of the CA/04 virus for growth in Calu-3 cells under type III IFN-λ1 selective pressure (Ilyushina *et al.,* 2017). Taking into account the balance between the functions of the HA and NA glycoproteins (Wagner *et al.,* 2002), we surmise that the NA M269I substitution emerged together with concomitant HA mutations, including K153E$_1$ and K154E$_1$, that modify virus receptor-binding affinity. These HA1 changes result in increased efficiency of viral release from infected cells and decreased dependence on the reduced NA function. Indeed, the K153E$_1$ mutation was previously shown to have a potential association with the NA substitution that confers decreased NA enzymatic activity. Namely, the K153E$_1$ mutation was associated with the NA H274Y mutation in the CA/04 virus background in a mouse model and resulted in more rapid weight loss and higher mortality than the parental virus (Song *et al.,* 2013). Moreover, the decrease in NA activity might involve the acquisition of the compensating K154E$_1$ mutation by the CA/04$^{IFN\text{-}\beta}$-1 variant to modify both virus binding to its cognate cell surface receptors and responsiveness to IFN-β.

In this study, we found that mutation at one site in the RNP complex, namely PB1 L373I, evolved after serial passaging of the parental virus in the presence of increasing concentrations of IFN-β. This single amino acid substitution was sufficient to significantly decrease the transcription activity of the polymerase complex of the A/California/04/09 virus at 37°C ($P < 0.05$), but not at 33°C. It is possible that the PB1 L373I mutation is associated with the IFN-β antagonistic activity. However, this substitution might also be necessary for optimal interaction of the viral polymerase with human host proteins to enable the CA/04$^{+IFN\text{-}\beta}$-1 and CA/04$^{+IFN\text{-}\beta}$-2 variants to replicate efficiently in Calu-3 cells in the presence of IFN-β.

To our knowledge, this is the first study to evaluate the emergence of resistance by the CA/04 virus to the anti-viral activity of IFN-β. We found that a combination of changes in the HA, NA, and/or PB1 proteins provided a structural and/or functional means by which the CA/04$^{IFN\text{-}\beta}$-1 virus could acquire reduced anti-viral responsiveness to IFN-β. The observed mutations optimized HA receptor specificity, NA enzyme catalytic activity, and most likely, interaction of RNP with host cell factors. Although the functions of the HA, NA, and PB1 proteins in influenza virus replication have been widely studied, only rudimentary knowledge exists regarding the functions of these proteins in inhibiting IFN-β signaling. Our current findings indicate that the IFN-β-induced mutations in the HA, NA, and/or PB1 proteins mediate IFN-β antagonistic functions to enhance viral virulence. Further

characterization of the effects of the acquired mutations on responsiveness to host type I IFN activity is warranted.

Acknowledgments. We are grateful to Dr. Robert G. Webster (St. Jude Children's Research Hospital, Memphis, TN) for providing influenza A/California/04/09 (H1N1) virus. We thank Dr. Vladimir Y. Lugovtsev (CBER, FDA, Silver Spring, MD) for assistance with NA enzyme activity assay. Brady T. Hickerson and Simone E. Adams were supported in part by an appointment to the Research Participation Program in the Office of Biotechnology Products, Center for Drug Evaluation and Research at the U.S. Food and Drug Administration (FDA) administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the FDA and the U.S. Department of Energy.

Supplementary information is available in the online version of the paper.

References

Adams SE, Lugovtsev VY, Kan A, Bovin NV, Donnelly RP, Ilyushina NA (2020): Laninamivir-Interferon Lambda 1 Combination Treatment Promotes Resistance by Influenza A Virus More Rapidly than Laninamivir Alone. Antimicrob. Agents Chemother. 64(7), e00301–20. https://doi.org/10.1128/AAC.00301-20

Beigel JH, Nam HH, Adams PL, Krafft A, Ince WL, El-Kamary SS, Sims AC (2019): Advances in respiratory virus therapeutics – A meeting report from the 6th isirv Antiviral Group conference. Antiviral Res. 167, 45–67 https://doi.org/10.1016/j.antiviral.2019.04.006

Beilharz MW, Cummins JM, Bennett AL (2007): Protection from lethal influenza virus challenge by oral type 1 interferon. Biochem. Biophys. Res. Commun. 355, 740–744. https://doi.org/10.1016/j.bbrc.2007.02.019

Bennett AL, Smith DW, Cummins MJ, Jacoby PA, Cummins JM, Beilharz MW (2013): Low-dose oral interferon alpha as prophylaxis against viral respiratory illness: a double-blind, parallel controlled trial during an influenza pandemic year. Influenza Other Respir. Viruses 7, 854–862. https://doi.org/10.1111/irv.12094

Bergmann M, Garcia-Sastre A, Carnero E, Pehamberger H, Wolff K, Palese P, Muster T (2000): Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. J. Virol. 74, 6203–6206. https://doi.org/10.1128/jvi.74.13.6203–6206.2000 https://doi.org/10.1128/JVI.74.13.6203-6206.2000

Chan PK, Lee N, Joynt GM, Choi KW, Cheung JL, Yeung AC, Lam P, Wong R, Leung BW, So HY, Lam WY, Hui DC (2011): Clinical and virological course of infection with haemagglutinin D222G mutant strain of 2009 pandemic influenza A (H1N1) virus. J. Clin. Virol. 50, 320–324. https://doi.org/10.1016/j.jcv.2011.01.013

Chutinimitkul S, Herfst S, Steel J, Lowen AC, Ye J, van Riel D, Schrauwen EJ, Bestebroer TM, Koel B, Burke DF, Sutherland-Cash KH, Whittleston CS, Russell CA, Wales DJ, Smith DJ, Jonges M, Meijer A, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Garcia-Sastre A, Perez DR, Fouchier RA (2010): Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. J. Virol. 84, 11802–11813. https://doi.org/10.1128/JVI.01136-10

Donelan NR, Basler CF, Garcia-Sastre A (2003): A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. J. Virol. 77, 13257–13266. https://doi.org/10.1128/JVI.77.24.13257-13266.2003

Dong G, Peng C, Luo J, Wang C, Han L, Wu B, Ji G, He H (2015): Adamantane-resistant influenza A viruses in the world (1902–2013): frequency and distribution of M2 gene mutations. PLoS One. 10(3), e0119115. https://doi.org/10.1371/journal.pone.0119115

Durbin RK, Kotenko SV, Durbin JE (2013): Interferon induction and function at the mucosal surface. Immunol. Rev. 255, 25–39. https://doi.org/10.1111/imr.12101

Ehrhardt C, Seyer R, Hrincius ER, Eierhoff T, Wolff T, Ludwig S (2010): Interplay between influenza A virus and the innate immune signaling. Microbes Infect. 12, 81–87. https://doi.org/10.1016/j.micinf.2009.09.007

Gack MU, Albrecht RA, Urano T, Inn KS, Huang IC, Carnero E, Farzan M, Inoue S, Jung JU, Garcia-Sastre A (2009): Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. Cell Host Microbe 5, 439–449. https://doi.org/10.1016/j.chom.2009.04.006

Garcia-Sastre A (2011): Induction and evasion of type I interferon responses by influenza viruses. Virus Res. 162, 12–18. https://doi.org/10.1016/j.virusres.2011.10.017

Graef KM, Vreede FT, Lau YF, McCall AW, Carr SM, Subbarao K, Fodor E (2010): The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon. J. Virol. 84, 8433–8445. https://doi.org/10.1128/JVI.00879-10

Grimm D, Staeheli P, Hufbauer M, Koerner I, Martinez-Sobrido L, Solorzano A, Garcia-Sastre A, Haller O, Kochs G (2007): Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene. Proc. Natl. Acad. Sci. U.S.A. 104, 6806–6811. https://doi.org/10.1073/pnas.0701849104

Guo Z, Chen LM, Zeng H, Gomez JA, Plowden J, Fujita T, Katz JM, Donis RO, Sambhara S (2007): NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. Am. J. Respir. Cell Mol. Biol. 36, 263–269. https://doi.org/10.1165/rcmb.2006-0283RC

Hayden FG, Cote KM, Douglas RG Jr. (1980): Plaque inhibition assay for drug susceptibility testing of influenza viruses. Antimicrob. Agents Chemother. 17, 865–870. https://doi.org/10.1128/AAC.17.5.865

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001): Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289. https://doi.org/10.1007/s007050170002

Hurt AC, Hui DS, Hay A, Hayden FG (2015): Overview of the 3rd isirv-Antiviral Group Conference-advances in clinical management. Influenza Other Respir. Viruses 9, 20–31. https://doi.org/10.1111/irv.12293

Hussain M, Galvin HD, Haw TY, Nutsford AN, Husain M (2017): Drug resistance in influenza A virus: the epidemiology and management. Infect. Drug Resist. 10, 121–134. https://doi.org/10.2147/IDR.S105473

Ilyushina NA, Donnelly RP (2014): In vitro anti-influenza A activity of interferon (IFN)-lambda1 combined with IFN-beta or oseltamivir carboxylate. Antiviral Res. 111, 112–120. https://doi.org/10.1016/j.antiviral.2014.09.008

Ilyushina NA, Khalenkov AM, Seiler JP, Forrest HL, Bovin NV, Marjuki H, Barman S, Webster RG, Webby RJ (2010): Adaptation of pandemic H1N1 influenza viruses in mice. J. Virol. 84, 8607–8616. https://doi.org/10.1128/JVI.00159-10

Ilyushina NA, Komatsu TE, Ince WL, Donaldson EF, Lee N, O'Rear JJ, Donnelly RP (2019): Influenza A virus hemagglutinin mutations associated with use of neuraminidase inhibitors correlate with decreased inhibition by anti-influenza antibodies. Virol. J. 16, 149. https://doi.org/10.1186/s12985-019-1258-x

Ilyushina NA, Lugovtsev VY, Samsonova AP, Sheikh FG, Bovin NV, Donnelly RP (2017): Generation and characterization of interferon-lambda 1-resistant H1N1 influenza A viruses. PLoS One 12, e0181999. https://doi.org/10.1371/journal.pone.0181999

Iwai A, Shiozaki T, Kawai T, Akira S, Kawaoka Y, Takada A, Kida H, Miyazaki, T (2010): Influenza A virus polymerase inhibits type I interferon induction by binding to interferon beta promoter stimulator 1. J. Biol. Chem. 285, 32064–32074. https://doi.org/10.1074/jbc.M110.112458

Jia D, Rahbar R, Chan RW, Lee SM, Chan MC, Wang BX, Baker DP, Sun B, Peiris JS, Nicholls JM, Fish EN (2010): Influenza virus non-structural protein 1 (NS1) disrupts interferon signaling. PLoS One 5, e13927. https://doi.org/10.1371/journal.pone.0013927

Kim HM, Lee N, Kim MS, Kang C, Chung YS (2020): Characterization of neuraminidase inhibitor-resistant influenza virus isolates from immunocompromised patients in the Republic of Korea. Virol. J. 17, 94. https://doi.org/10.1186/s12985-020-01375-1

Li S, Min JY, Krug RM, Sen GC (2006): Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virology 349, 13–21. https://doi.org/10.1016/j.virol.2006.01.005

Liedmann S, Hrincius ER, Anhlan D, McCullers JA, Ludwig S, Ehrhardt C (2014): New virulence determinants contribute to the enhanced immune response and reduced virulence of an influenza A virus A/PR8/34 variant. J. Infect. Dis. 209, 532–541. https://doi.org/10.1093/infdis/jit463

Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcia-Sastre A, Katze MG, Gale M Jr. (2008): Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J. Virol. 82, 335–345. https://doi.org/10.1128/JVI.01080-07

Marathe BM, Leveque V, Klumpp K, Webster RG, Govorkova EA (2013): Determination of neuraminidase kinetic constants using whole influenza virus preparations and correction for spectroscopic interference by a fluorogenic substrate. PLoS One 8, e71401. https://doi.org/10.1371/journal.pone.0071401

Mibayashi M, Martinez-Sobrido L, Loo YM, Cardenas WB, Gale M Jr, Garcia-Sastre A (2007): Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J. Virol. 81, 514–524. https://doi.org/10.1128/JVI.01265-06

Nicholson KG, Aoki FY, Osterhaus AD, Trottier S, Carewicz O, Mercier CH, Rode A, Kinnersley N, Ward P (2000): Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group. Lancet. 355, 1845–1850. https://doi.org/10.1016/S0140-6736(00)02288-1

Osterlund P, Pirhonen J, Ikonen N, Ronkko E, Strengell M, Makela SM, Broman M, Hamming OJ, Hartmann R, Ziegler T, Julkunen I (2010): Pandemic H1N1 2009 influenza A virus induces weak cytokine responses in human macrophages and dendritic cells and is highly sensitive to the antiviral actions of interferons. J. Virol. 84, 1414–1422. https://doi.org/10.1128/JVI.01619-09

Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB (1979): Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N-acetylneuraminate) substrate. Anal. Biochem. 94, 287–296. https://doi.org/10.1016/0003-2697(79)90362-2

Rehwinkel J, Tan CP, Goubau D, Schulz O, Pichlmair A, Bier K, Robb N, Vreede F, Barclay W, Fodor E, Reis e Sousa C (2010): RIG-I detects viral genomic RNA during negative-strand RNA virus infection. Cell. 140, 397–408. https://doi.org/10.1016/j.cell.2010.01.020

Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, Hulse-Post DJ, Humberd J, Trichet M, Rehg JE, Webby RJ, Webster RG, Hoffmann E (2006): The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J. Exp. Med. 203, 689–697. https://doi.org/10.1084/jem.20051938

Scagnolari C, Trombetti S, Solda A, Selvaggi C, Monteleone K, Spano L, Pierangeli A, Clementi M, Turriziani O, Antonelli G (2011): Pandemic 2009 H1N1 influenza virus is resistant to the antiviral activity of several interferon alpha subtypes. J. Interferon Cytokine Res. 31, 475–479. https://doi.org/10.1089/jir.2010.0125

Sheu TG, Deyde VM, Okomo-Adhiambo M, Garten RJ, Xu X, Bright RA, Butler EN, Wallis TR, Klimov AI, Gubareva LV (2008): Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob. Agents Chemother. 52, 3284–3292. https://doi.org/10.1128/AAC.00555-08

Song MS, Hee Baek Y, Kim EH, Park SJ, Kim S, Lim GJ, Kwon HI, Pascua PN, Decano AG, Lee BJ, Kim YI, Webby RJ, Choi YK (2013): Increased virulence of neuraminidase

inhibitor-resistant pandemic H1N1 virus in mice: potential emergence of drug-resistant and virulent variants. Virulence. 4, 489–493. https://doi.org/10.4161/viru.25952

Soundararajan V, Tharakaraman K, Raman R, Raguram S, Shriver Z, Sasisekharan V, Sasisekharan R (2009): Extrapolating from sequence--the 2009 H1N1 ‚swine' influenza virus. Nat. Biotechnol. 27, 510–513. https://doi.org/10.1038/nbt0609-510

Szretter KJ, Gangappa S, Belser JA, Zeng H, Chen H, Matsuoka Y, Sambhara S, Swayne DE, Tumpey TM, Katz JM (2009): Early control of H5N1 influenza virus replication by the type I interferon response in mice. J. Virol. 83, 5825–5834. https://doi.org/10.1128/JVI.02144-08

Takaoka A, Yanai H (2006): Interferon signalling network in innate defence. Cell Microbiol. 8, 907–922. https://doi.org/10.1111/j.1462-5822.2006.00716.x

Treanor JJ, Hayden FG, Vrooman PS, Barbarash R, Bettis R, Riff D, Singh S, Kinnersley N, Ward P, Mills RG (2000): Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. US Oral Neuraminidase Study Group. JAMA. 283, 1016–1024. https://doi.org/10.1001/jama.283.8.1016

Van Hoeven N, Belser JA, Szretter KJ, Zeng H, Staeheli P, Swayne DE, Katz JM, Tumpey TM (2009): Pathogenesis of 1918 pandemic and H5N1 influenza virus infections in a guinea pig model: antiviral potential of exogenous alpha interferon to reduce virus shedding. J. Virol. 83, 2851–2861. https://doi.org/10.1128/JVI.02174-08

Varga ZT, Ramos I, Hai R, Schmolke M, Garcia-Sastre A, Fernandez-Sesma A, Palese P (2011): The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein. PLoS Pathog. 7, e1002067. https://doi.org/10.1371/journal.ppat.1002067

Wagner R, Matrosovich M, Klenk HD (2002): Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev. Med. Virol. 12, 159–166. https://doi.org/10.1002/rmv.352

Wang W, Riedel K, Lynch P, Chien CY, Montelione GT, Krug RM (1999): RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. RNA. 5, 195–205. https://doi.org/10.1017/S1355838299981621

Wright PF, Neumann G, Kawaoka Y (2013): Orthomyxoviruses: Fields Virology. Lippincott Willimas and Wilkins, pp. 1186–1243.

Wu W, Metcalf JP (2020): The Role of Type I IFNs in Influenza: Antiviral Superheroes or Immunopathogenic Villains? J. Innate Immun. 12, 437–447. https://doi.org/10.1159/000508379

Xia C, Vijayan M, Pritzl CJ, Fuchs SY, McDermott AB, Hahm B (2015): Hemagglutinin of Influenza A Virus Antagonizes Type I Interferon (IFN) Responses by Inducing Degradation of Type I IFN Receptor 1. J. Virol. 90, 2403–2417. https://doi.org/10.1128/JVI.02749-15

Xiao H, Killip MJ, Staeheli P, Randall RE, Jackson D (2013): The human interferon-induced MxA protein inhibits early stages of influenza A virus infection by retaining the incoming viral genome in the cytoplasm. J. Virol. 87, 13053–13058. https://doi.org/10.1128/JVI.02220-13

Xu R, Ekiert DC, Krause JC, Hai R, Crowe JE Jr, Wilson IA (2010): Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. Science 328, 357–360. https://doi.org/10.1126/science.1186430

Yoneyama M, Suhara W, Fujita T (2002): Control of IRF-3 activation by phosphorylation. J. Interferon Cytokine Res. 22, 73–76. https://doi.org/10.1089/107999002753452674

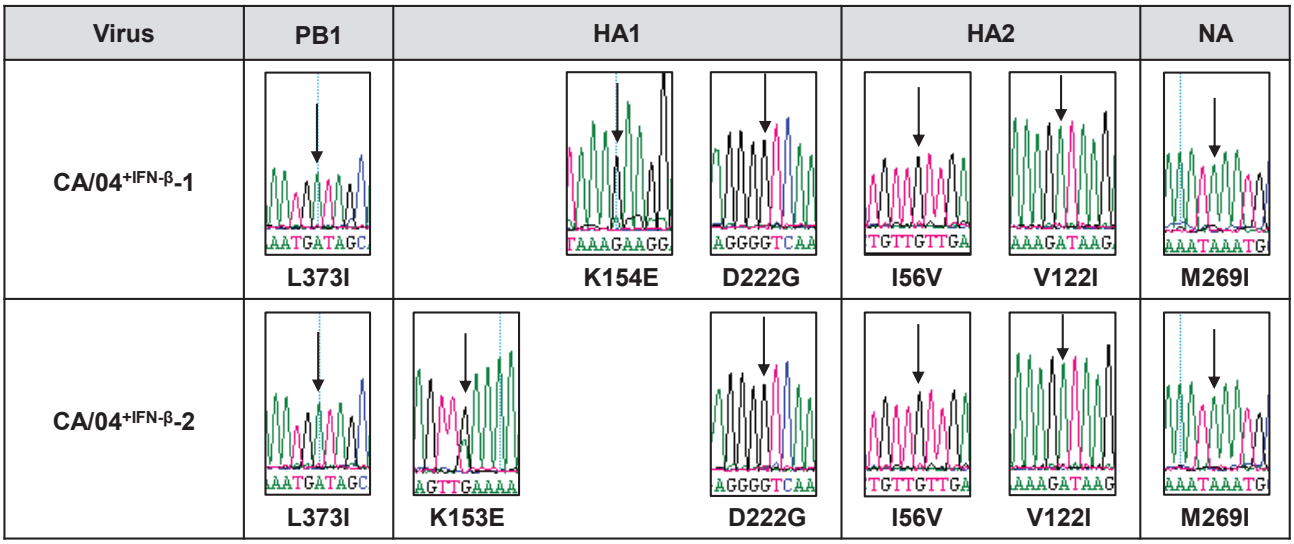
Acta virologica **66**: 263 – 274, 2022 doi: 10.4149/av_2022_311

SUPPLEMENTARY INFORMATION

Generation and characterization of interferon-beta-resistant H1N1 influenza A virus

Brady T. Hickerson[1], Simone E. Adams[1], Nicolai V. Bovin[2], Raymond P. Donnelly[1], Natalia A. Ilyushina[1*]

[1]Division of Biotechnology Review and Research II, Center for Drug Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD 20993, United States; [2]Laboratory of Carbohydrates, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow 117997, Russia



Supplementary Fig. 1

Sequencing chromatograms for the nucleic acid changes observed in the CA/04$^{+IFN-\beta}$-1 and CA/04$^{+IFN-\beta}$-2 variants

*Corresponding author. E-mail: Natalia.Ilyushina@fda.hhs.gov; phone: +1-240-402-9600.