Targeting importin-α7 as a therapeutic approach against pandemic influenza viruses

Patricia Resa-Infantea, Duncan Patersonb, Jaume Bonetc*, Anna Ottea**, Baldo Olivac, Ervin Fodorb and Gülsah Gabriela,d§

aHeinrich Pette Institute, Leibniz Institute for Experimental Virology, 20251 Hamburg, Germany
bSir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom
cStructural Bioinformatics Lab (GRIB), Universitat Pompeu Fabra, Barcelona Research Park of Biomedicine (PRBB), Barcelona 08003, Spain.
dCenter for Cellular and Structural Biology in Medicine, University of Lübeck, 23562 Lübeck, Germany

Running title: Targeting importin-α7 against influenza

Word count Abstract: 138
Word count Text: 3827

§Address correspondence to Gülsah Gabriel, guelsah.gabriel@hpi.uni-hamburg.de.

Heinrich Pette Institute, Leibniz Institute for Experimental Virology. Martinistr. 52, 20251 Hamburg, Germany.

Present addresses: *Jaume Bonet, Laboratory of Protein Design & Immunoengineering, STI, EPFL, Lausanne, Switzerland. **Anna Otte, Institute of Biochemistry, Kiel University, Kiel, Germany.
Abstract

Viral drug resistance is believed to occur less likely if compounds are directed against cellular rather than viral proteins. In this study, we analyzed the feasibility of a crucial viral replication factor, namely importin-α7, as a potential cellular drug target to combat pandemic influenza. Surprisingly, only five viral lung-to-lung passages were required to achieve 100% lethality in importin-α7−/− mice that are otherwise resistant. Viral escape from importin-α7 requirement was mediated by five mutations in the viral ribonucleoprotein complex and the surface glycoproteins. Moreover, the importin-α7−/− mouse-adapted strain became even more virulent for wild-type mice compared to the parental strain. These studies show that targeting host proteins may still result in viral escape by alternative pathways eventually giving rise to even more virulent virus strains. Thus, therapeutic intervention strategies should consider a multi-target approach to reduce viral drug resistance.

Importance

Here, we investigated the longstanding hypothesis based on in vitro studies that viral drug resistance occurs less likely if compounds are directed against cellular rather than viral proteins. Here, we challenged this hypothesis by analyzing in an in vivo animal model the feasibility of targeting the cellular factor importin-α7 - that is crucial for human influenza virus replication and pathogenesis - as an efficient antiviral strategy against pandemic influenza viruses. In summary, our studies suggest that resistance against cellular factors is possible in vivo and the emergence of even more virulent viral escape variants calls for particular caution. Thus, therapeutic intervention strategies should consider a multi-target approach using compounds against viral as well as
cellular factors to reduce the risk of viral drug resistance and potentially increased virulence.

Key words

influenza, host factors, importin-α7, virus-host interactions, nuclear transport, virus adaptation, virus evolution, antiviral treatment, drug resistance.
INTRODUCTION

Influenza A viruses are responsible for acute respiratory diseases in humans posing a severe burden for health and economy worldwide. Vaccination is considered the best option for disease prophylaxis. However, production of novel vaccines in case of a pandemic requires at least six months. Thus, antiviral drugs are essential to bridge this gap. Two classes of antiviral agents are currently available against influenza: neuraminidase inhibitors and adamantanes (1). However, their efficiency is limited and several influenza virus strains have developed resistance to these virus-directed drugs (2). Hence, development of novel antiviral agents is urgently required.

Inhibitors developed against host cell proteins are considered an attractive strategy compared to drugs that target viral proteins because they are less prone to mutations than viral proteins. Thus, it is believed that targeting host factors would limit the emergence of drug resistant virus strains. Several compounds targeting cellular factors to halt influenza virus replication are currently under development (3-5). For example, inhibitors targeting the Raf/MEK/ERK signalling pathway, NF-κB signalling, the PI3K/Akt pathway and the PKC signalling cascade are already in an early phase of preclinical development (6).

For influenza, the nuclear import protein importin-α7 is a crucial pathogenicity factor that increases human-type influenza virus replication by a yet unknown mechanism beyond nuclear import. Mice with a deleted importin-α7 gene (α7−/−) are resistant to an otherwise lethal 2009 pandemic H1N1 influenza virus infection (7-9). Since deletion of the importin-α7 gene did not cause an obvious phenotype in adult α7−/− mice (10), its transient inhibition poses a very attractive target to combat human-type influenza viruses.
In this study we investigated whether targeting importin-α7 would provide an efficient antiviral strategy against pandemic influenza viruses reducing the risk of viral resistance. In particular, we employed a method of accelerated viral evolution thereby challenging viral escape in a resistant host.
MATERIAL AND METHODS

Ethical Statement

All animal experiments were approved by the relevant German authority (Behörde für Gesundheit und Verbraucherschutz, Hamburg) and performed according to the national guidelines of the animal protection law (Tierschutzgesetz; project number: 97/11) in Germany.

Animal experiments

Importin-α7−/− (α7−/−) mice (8, 10) and wild-type (WT) littermates in the C57BL/6J genetic background were bred and housed under specific-pathogen-free conditions at the animal facility of the Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology in Hamburg, Germany.

Mice were anaesthetized with ketamine/xylazine (70 mg/kg and 7 mg/kg, respectively) and inoculated intranasally with 50 µl of virus diluted in PBS. Survival and weight loss were monitored for 14 days. Mouse-lethal-dose-50 (MLD50) was determined using serial 10-fold virus dilutions (11). Mice were humanely euthanized upon >25 % weight loss.

Cells

Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (PAA, Austria) supplemented with fetal calf serum (FCS; PAA, Austria), L-glutamine (PAA, Austria), and penicillin-streptomycin (PAA, Austria). Human embryonic kidney 293T (HEK) and human alveolar adenocarcinoma (A549) cells were grown in DMEM (Dulbecco’s modified Eagle’s medium; PAA, Austria) supplemented with 10% FCS, L-glutamine and penicillin-streptomycin. WT and α7−/− murine embryonic fibroblasts
MEF) were grown in DMEM supplemented with 10 % FCS, penicillin/streptomycin, L-Glutamine, nonessential amino acids (PAA, Austria) and sodium pyruvate (PAA, Austria). MEFs were kindly provided by M. Bader (Max Delbrück Center for Molecular Medicine, Berlin, Germany) (12).

HEK and A549 cells were transduced with lentiviral vectors expressing short hairpin RNA (shRNA) anti-importin-α7 (clone ID is NM_012316.3-223s1c1; Sigma-Aldrich, Germany) to generate importin-α7 knockdown cell lines, named HEK-shα7 and A549-shα7. Transduced cells were selected and maintained in medium containing 2μg/ml puromycin (Calbiochem, USA). shRNA-mediated knockdown was confirmed by Western blot analysis using anti-importin-α5/α7 antibody (kindly provided by E. Hartmann Institute of Biology, Lübeck, Germany) (8). GAPDH expression was detected by an anti-GAPDH antibody (Cell Signaling Technology, Inc., USA).

Adaptation of pH1N1 influenza virus to importin-α7−/− mice

We used the 2009 pandemic H1N1 A/Hamburg/NY1580/09 virus strain (abbreviated as pH1N1) as a parental strain (8, 13, 14). Three α7−/− mice were intranasally infected with 10^5 p.f.u. of the pH1N1 strain. Lungs were harvested and homogenized 3 days post infection (p.i.). Subsequently, 50 μl of the pooled lung homogenate supernatant was used to intranasally infect the next group of three α7−/− mice. Serial passaging was stopped upon 100% lethality. One viral clone was isolated from the lung homogenates of the fifth passage by plaque purification from MDCK cells and named as pH1N1-MA7. All experiments were performed at the biosafety level 2 facilities of the Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology.
Plasmids and recombinant pH1N1 viruses

The gene segments of pH1N1 and pH1N1-MA7 viruses were sequenced as described (15). pH1N1 genes were cloned into the pHW2000 vector to generate recombinant influenza viruses by reverse genetics. pH1N1-MA7 specific mutations were introduced using the QuickChange Site-Directed Mutagenesis kit (Stratagene/Agilent Technologies, USA). Virus stocks were grown in MDCK cells and sequenced for verification.

PB1, PB2, PA and NP genes from pH1N1 or pH1N1-MA7 viruses were additionally cloned from pHW2000 vector into pcDNA3.1 vector to generate pC-HH15-PB1, pC-HH15-PB2, pC-HH15-PA, pC-HH15-NP, pC-HH15-PA_{MA7} and pC-HH15-NP_{MA7} expression plasmids.

Polymerase activity

HEK or HEK-shα7 cells were co-transfected with pC-HH15-PB1, pC-HH15-PB2, pC-HH15-PA, pC-HH15-NP, pC-HH15-PA_{MA7} or pC-HH15-NP_{MA7} to generate recombinant RNPs. Reporter constructs pPol-I-NP-Luc-human (encoding firefly luciferase in negative polarity flanked by the non-translated regions of influenza NP segment) (16) and pRL-TK (encoding Renilla luciferase; Promega, USA) were co-transfected. The transfection solution was prepared by incubating a mixture of DNA, polyethylenimine (PEI; Polysciences), and DMEM in a ratio of 1 μg DNA to 2.4 μg PEI to 600 μl DMEM for 20 min at room temperature. At 20 h post-transfection, luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega, USA). All experiments were performed in triplicates.
Co-immunoprecipitation assay

Immunoprecipitations were performed using EZview Red ANTI-FLAG M2 affinity gel (Sigma, USA) and eluted using a 3x FLAG peptide (Sigma, USA) according to the manufacturer’s instructions. Quantification of coimmunoprecipitation products was performed by Western blot analysis using mouse anti-FLAG (Sigma, USA) and rabbit anti-FPV serum (17) antibodies. The β-Actin antibody (Abcam, UK) was used for normalization of the total protein amount used in respective cell lysates. Immunoreactive bands were visualized with the Bioimager Image Quant LAS 4000 at non-saturated levels and quantified by densitometry with ImageJ software.

Analysis of virus replication

A549 and A549-shα7 cells were infected at MOI 0.1 with pH1N1 or pH1N1-MA7 viruses, respectively, in the presence of 0.25 μg/μl L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-trypsin. Supernatants were collected at 0, 24, 48 and 72h p.i. and virus titres were determined as plaque forming units (p.f.u.) on MDCK cells. WT and α7⁻/⁻ MEFs were infected at MOI 2 with pH1N1 recombinant viruses containing pH1N1-MA7 mutations as indicated. Total RNA was isolated at 24h p.i. by extraction with TRIzol reagent (Invitrogen, USA). Viral RNA accumulation was analysed by primer extension using specific primers for the viral NP gene (18). Primer PrE-NP- (5’-ATGATGAAAGTGCAAAGCC-3’) was used to detect vRNA and primer PrE-NP+ (5’-ATTCTGTGGCATCCTGGC-3’) to detect cRNA and mRNA. A primer detecting cellular 5S rRNA (5’-TCCCAGGGCGGTCTCCCATCC-3’) was used for normalization. Transcription products were analyzed on 6% polyacrylamide gels containing 7 M urea in TBE buffer, detected by autoradiography and quantified by densitometry of phosphorimages using AIDA software.
Modelling of pH1N1 protein structure domains.

The three-dimensional (3D) conformations of the proteins of interest from the parental pH1N1 strain were modelled to evaluate the effects of the mutations over their structural stability. The templates used to build these structures were their closest homologue with a known structure in the Protein Data Bank (PDB) (19, 20), found by sequence similarity through BLAST (21). In all cases, a template containing the region with the mutations was found with an e-value of 0, which indicates the probability of a protein-template assignation due to chance. Specifically, PA was modelled over 4AVL-chainD (22), NP over 4DYS-chainC, NA over 4B7R-chainD (23) and HA over 2WR0-chainB (24). Except for PA, the templates covered more than 80% of the protein length. 30 parental models were created for each protein through MODELLER (25).

Global and local energies were analysed with Prosa (26). Global energy (z-score) represents a normalized score of the stability of the protein. Local energy represents the individual stability of each residue in the structural conditions in a given 3D representation. Both values are further divided in surface, pair (structural) and combined (pair + surface) energy. As a rule, negative values represent stable conformations. A total of 30 variants for each possible mutant of interest were created with Prosa and analysed for both their global and local energies.

Statistical analysis

All data shown are presented as mean ± SEM. Mean, SEM, Student’s t test (unpaired, 2-tailed) and Mantel-Cox test were calculated with Prism GraphPad software (GraphPad Software, Inc., USA). Statistical significance was defined as \( p<0.05 \) (*\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \)).
RESULTS

Adaptation of a 2009 pandemic H1N1 influenza virus strain to importin-α7-/- mice.

The importin-α7 gene is crucial for replication and pathogenicity of human-type influenza A viruses in mammals. The lack of the importin-α7 gene leads to 100% survival in mice infected with several 2009 pandemic H1N1 influenza virus strains, whereas 100% of WT mice succumb to infection (8). Here, we addressed whether targeting importin-α7 would pose a promising antiviral strategy to combat human influenza viruses. To analyse whether viral escape would be possible using a cellular target, we challenged this approach by adapting a 2009 pandemic H1N1 strain (A/Hamburg/NY1580/09; pH1N1) to importin-α7-/- (α7-/-) mice by serial lung-to-lung passages. Interestingly, 100% lethality could be achieved after only 5 passages. We isolated one α7-/- mouse-adapted virus clone (pH1N1-MA7) by plaque assay and assessed its pathogenesis in WT and α7-/- mice, in comparison to its parental strain (Figure 1A). Consistent with previous findings, infection of α7-/- mice with the parental pH1N1 strain did not result in significant weight loss or lethality (8). However, the adapted pH1N1-MA7 strain was lethal for WT (MLD50: 102.4 p.f.u.) and α7-/- mice (MLD50: 102.6 p.f.u.). Notably, the pH1N1-MA7 strain was more virulent for WT mice than its non-adapted parental strain suggesting that its virulence increased during viral escape from importin-α7 requirement (Figure 1B). Sequencing of both viral genomes revealed two amino acid substitutions in the ribonucleoprotein (RNP) complex (PA D27E and NP G102R) and three substitutions in the viral surface glycoproteins, including two mutations in HA (K171E and D239G) and one mutation in NA (Y170H) (Figure 1C).
Overall, we could show that pH1N1 influenza viruses are able to escape the requirement for importin-α7 by acquiring adaptive mutations in the viral RNP and the surface glycoproteins. This escape rendered the virus even more virulent for WT mice.

**Identification of host adaptive sites responsible for overcoming viral restriction in importin-α7⁻ mice.**

In order to identify the relevant viral genes responsible for the evasion of viral restriction in α7⁻ mice, we generated single-gene reassortant (SGR) recombinant influenza viruses containing one exchanged gene segment of the pH1N1-MA7 strain in the parental pH1N1 virus background. In order to study the role of the RNP on viral pathogenesis, we also generated the double gene reassortant pH1N1-PA,NPMA7 virus that contains PA and NP genes of the pH1N1-MA7 strain.

We then assessed the pathogenic potential of these viruses in WT and α7⁻ mice (Figure 2). Infection with the recombinant pH1N1-MA7 (pH1N1-MA7 rec) virus containing all five adaptive mutations showed similar pathogenesis comparable to the isolated clone. Interestingly, none of the SGR viruses increased lethality in α7⁻ mice. However, pH1N1-NPMA7 significantly enhanced weight loss in α7⁻ mice, which was further increased when combined with the PA_{MA7} gene segment. This suggests a synergistic effect of PA_{MA7} and NP_{MA7} on viral pathogenesis in α7⁻ mice. In contrast, pH1N1-HA_{MA7} and pH1N1-NA_{MA7} SGR viruses did not cause any significant weight loss in α7⁻ mice. Conversely, in WT mice, adaptive mutations in HA and NA strongly enhanced pathogenicity in contrast to adaptive mutations in the RNP (Figure 2B).

These data suggest that mutations in the RNP are adaptations to the lack of importin-α7, while mutations in HA and NA seem to represent adaptations to the WT animal.
However, a combination of both, mutations in the RNP as well as in the glycoproteins are required to completely escape importin-α7 mediated restriction in mice.

Adaptive mutations are localized on the respective protein surface.

We then generated structural models to investigate the localization of the pH1N1-MA7 adaptive mutations (Figure 3). All adaptive mutations are located on the surface of the viral proteins. In particular, the PA$_{MA7}$ D27E mutation is located on the surface of the endonuclease domain. HA$_{MA7}$ D239G mutation is included in the 220-loop element of the receptor binding site and HA$_{MA7}$ K171E mutation is nearby this domain. NP$_{MA7}$ G102R and NA$_{MA7}$ Y170H mutations are located in domains without a specific function described at the moment (Figure 3A).

To evaluate potential energy changes, we mimicked the adaptive mutations and compared between the energies of the different mutants against their parental proteins (Figure 3B). The comparison showed no significant differences between the parental and the mutant structures; neither in global (z-score) nor in local energies. This suggests that these adaptive mutations do not alter the global stability of the protein. Although some local differences are observed, those are extremely small and focused in the surface energy. These results indicate that these mutations in PA, NP, HA and NA do not directly affect protein conformation but they might affect interaction with other viral and/or host factors which might affect protein functions.

Adaptive mutations increase RNP activity

To study the effect of the adaptive mutations in PA and NP genes on viral polymerase activity, we reconstituted viral RNPs in wild-type (HEK-WT) as well as in importin-α7 knockdown (HEK-shα7) cell lines (Figure 4). The pH1N1-MA7 polymerase activity
was increased up to three-fold compared to the non-adapted pH1N1 polymerase in HEK-WT cells. Interestingly, PA_{MA7} was mainly responsible for this increase in viral polymerase activity in HEK-WT cells while NP_{MA7} did not show significant effects. However, both pH1N1 as well as pH1N1-MA7 polymerase activity was restricted in HEK-shα7 compared to HEK-WT cells. Similarly, PA_{MA7} was the main mediator of elevated polymerase activity in HEK-shα7 cells.

Next, we addressed whether the adaptive mutation in NP_{MA7} might result in altered importin-α binding affecting vRNP activity. NP_{MA7} shows a generally reduced binding affinity to importin-α1, -α3 and -α7 isoforms relevant for influenza virus replication (27) compared to non-adapted NP, albeit statistically not significant (Figure 5).

These findings show that α7−/−-adaptive mutations in the RNP mediate partial escape from importin-α7 depletion in mice. However, a complete circumvention of the RNP from importin-α7 requirement could not be achieved. These data suggest that alternate pathways are utilized to circumvent the lack of importin-α7.

Adaptive mutations increase virus replication

We next analyzed the viral replication kinetics of pH1N1-MA7 in comparison to the parental pH1N1 virus in WT epithelial (A549-WT) and in importin-α7 knockdown (A549-shα7) cell lines (Figure 6). Consistent with previous reports, parental pH1N1 virus replication was strongly impaired in A549-shα7 cells by approximately 2 logs compared to A549-WT cells. In contrast, pH1N1-MA7 virus replication was only slightly reduced in A549-shα7 compared to WT cells.

Compared to the pH1N1 parental strain, pH1N1-MA7 mutations remarkably enhanced virus replication in both cell lines; by about 1 log in parental cells (A549-WT) and 3 logs in importin-α7 knockdown cell lines (A549-shα7). However, both parental
pH1N1 and adapted pH1N1-MA7 viruses replicated better when importin-α7 expression was not depleted. This suggests that alternative strategies are exploited to overcome importin-α7 requirement to restore viral replication efficiency in human cells.

**Importin-α7 dependency remains upon adaptation**

Then, we investigated the effect of these adaptive mutations on virus replication in cells where the entire importin-α7 gene is deleted in order to abolish the possibility that residual importin-α7 could still affect viral replication (Figure 7). Here, we studied virus RNA accumulation by primer extension analysis in monocycle infections using MEFs isolated from WT or α7⁻/⁻ mice (12). Adaptive mutations increased vRNA levels in WT and α7⁻/⁻ cells (Figure 7A, B). Specifically, vRNA accumulation was increased 220.7% in MEF-α7⁻/⁻ but 2360.9% in MEF-WT cells. However, importin-α7 dependency persisted with all recombinant viruses analysed. Accordingly, vRNA accumulation was remarkably reduced during infection of MEF-α7⁻/⁻ compared to MEF-WT cells (Figure 7C). These data show that importin-α7 dependency remains upon adaptation to α7⁻/⁻ mice. However, circumvention of the importin-α7 requirement seems to occur by an alternate pathway that results in an increased replicative fitness.
DISCUSSION

Antiviral drugs play a key role in mitigating influenza disease severity (28). Since most of the circulating influenza strains are becoming increasingly resistant against currently available drugs that are directed against viral proteins. Thus, the development of novel antiviral strategies is fundamental (29). Targeting cellular proteins required for viral replication is considered a promising strategy since cellular proteins are generally less prone to mutations than viral proteins (3).

Herein, host factors that are essential for viral infection but not for cellular viability, are considered excellent targets for therapeutic intervention (3). In this study, we have chosen importin-α7 that is crucial for human-type influenza virus replication as well as severe lung damage and virulence in mice (7, 9, 16). Most importantly, adult mice lacking the importin-α7 gene appear healthy (8, 10). Thus, this is a particularly attractive factor for transient inhibition to combat acute influenza virus infection. Additionally, recent reports highlight that importin-α belongs to the few host proteins that possess sufficient interactive surface area for virtual screening and host-directed drug discovery (3).

Moreover, antiviral compounds targeting importin-α proteins might pose a novel pan-viral strategy to combat various viral infections since different importin-α isoforms were reported to be utilized by many other viruses, such as HCV, HIV, SARS-CoV, Ebola, and dengue viruses (30-39). However, developing and evaluating novel antiviral candidates is often time and money consuming. Therefore, we sought to study the feasibility of importin-α7 as an attractive cellular target to combat pandemic influenza using an in vivo model. Here, we infected α7⁻/⁻ mice with a 2009 pH1N1 influenza virus
and challenged for potential viral escape by serial lung-to-lung passages. Surprisingly, only five passages were required until 100% lethality was reached in α7−/− mice. Importantly, pH1N1-MA7 became approximately 10 times more virulent during viral escape in α7−/− mice compared to the parental virus strain in WT animals. In contrast, viral variants that escape antiviral drugs such as oseltamivir were reported to be as virulent but not more virulent than the parental strain in mice (40). This finding calls for particular caution since it shows that viral escape may easily occur and furthermore lead to the emergence of potentially more virulent strains. This is not in line with previous reports where viral resistance could not be observed against cellular targets despite serial passaging in cell culture (41). Indeed, no resistance was observed when using the cellular MEK kinase as a target to inhibit influenza B virus replication. However, it should be noted that these studies were performed in cell culture experiments. Since influenza viruses infect several cell types besides epithelial cells, one might speculate that at least the number of cell types to produce potentially more virus variants in vivo would be higher than in cell culture experiments. Moreover, some influenza virus infections may lead to suppression of innate and adaptive immune responses that might further facilitate the emergence of novel virus variants. As stated above, the different selective pressure in cell culture and the whole organism highlights the need for an adequate animal model to evaluate the risk from newly emerging host-targeting drug resistant virus strains.

Interestingly, escape from importin-α7 requirement was mediated mainly by mutations in the RNP proteins. Thus, weight loss in α7−/− mice was only observed upon infection with recombinant viruses containing NP MA7 mutation and further enhanced when combined with PA MA7 mutation. However, combination with adaptive HA and NA
mutations were required for an entire circumvention of importin-α7 requirement.

However, we did not test the single surface protein mutations combined with vRNP adaptive sites which might have been sufficient for lethality in mice. Thus, several adaptive pathways may have been utilized by the surface glycoproteins and the RNP to overcome the lack of importin-α7.

The PA subunit possesses endonuclease activity that plays a key role in cap-snatching required to initiate viral transcription. The PA-D27E mutation is located in the endonuclease domain but outside of the active site (22, 42). Hence, this mutation could indirectly influence its endonuclease activity. The NP-G102R mutation is located opposite to the oligomerisation loop (43, 44). Moreover, the NP-G102R mutant showed a generally reduced binding affinity to other importin-α isoforms relevant for influenza virus replication and pathogenicity (27). This might suggest that an alternate nuclear import pathway might be preferred, such as the non-classical nuclear import pathway that is independent of importin-α, like RanBP5, shown to be utilized by PBI and PA (45). Future studies are required to analyse whether these RNP adaptive mutations represent adaptation to alternate nuclear import pathways or are representative of novel binding sites to viral or cellular proteins increasing virus replication.

Since full circumvention from importin-α7 requirement could only be achieved in combination of adaptive mutations in the RNP with those in HA and NA, drugs targeting crucial host factors might be combined with those preventing cell entry such as shown recently for SALPs (46) or with NA inhibitors such as oseltamivir inhibiting viral release to further reduce the risk of antiviral resistance. However, it should be noted that in this case five mutations in four different gene segments were required to
achieve resistance. This could be considered as a rather higher resistance barrier compared to the single mutation in NA, NA-H274Y that is sufficient to confer resistance towards neuraminidase inhibitors (47). However, the fact that only five passages in vivo were sufficient to overcome importin-α7 requirement seems to be on the other hand rather low. Clearly, future studies will be required to compare the selective pressure required in vitro and in vivo to overcome drug resistance.

The NA-Y170H mutation that occurred in this study, is located in the inner part of the NA tetramer and thus outside the active site of the enzyme (48). This mutation could affect the NA assembly into tetramers. The receptor binding site (RBS) of HA constitutes of three main structural elements: the 190-helix, the 220-loop and the 130-loop (49). While the HA-K171E mutation is located near the HA RBS, the HA-D239G mutation is included in the 220-loop. Moreover, the D239G substitution corresponds to D222G mutation (H1 numbering) that has been associated with altered receptor specificity observed in severe cases of 2009 pandemic H1N1 influenza virus infections in humans (50, 51). As a result, these HA mutations might facilitate receptor binding thereby increasing virulence in mice.

It should be noted that despite the successful circumvention of importin-α7 requirement, the pH1N1-MA7 virus still showed dependency suggesting that the alternate pathway is not entirely able to compensate for importin-α7 absence. This further highlights that importin-α7 still represents a strong target but viral escape is nevertheless rapidly possible if only one cellular target is chosen. Thus, future strategies should be directed towards targeting multiple viral and/or cellular factors to reduce the risk of antiviral
resistance. This is even more important considering the fact that escape variants from host factors might even be more virulent posing an increased risk for public health.
ACKNOWLEDGMENTS

This work was supported by grants from the European Union (FLUPHARM) (to GG), the Emmy-Noether Programme of the German Research Foundation (to GG) [GA 1575/1-1], the Medical Research Council (MRC) (to EF) [MR/K000241/1] and the Spanish Ministry of Science and Innovation (MICINN) (to BO) [FEDER BIO2011-22568, EU12009-04018]. PRI was funded by the Alexander von Humboldt Foundation [3.3SPA/1142463 STP-2] and with a Boehringer Ingelheim Fonds travel grant [BIF TG Infante]. DP was supported by a Polonsky Foundation Scholarship and Lincoln College, University of Oxford. JB was supported by a MICINN fellowship [FEDER BIO2008-0205]. The Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology is supported by the Free and Hanseatic City of Hamburg and the Federal Ministry of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank Gökhan Arman-Kalcek and Ilara Hudjetz for excellent technical assistance.
References


virus VP24 targets a unique NLS binding site on karyopherin alpha 5 to selectively compete with nuclear import of phosphorylated STAT1. Cell Host Microbe 16:187-200.


FIGURE LEGENDS

Figure 1
Adaptation of 2009 pH1N1 influenza virus to α7⁻/⁻ mice. (A) Pathogenicity of pH1N1-MA7 influenza virus. WT (black squares) or α7⁻/⁻ (open squares) mice were intranasally inoculated with 10⁵ p.f.u. of pH1N1-MA7 virus. As controls, a group of α7⁻/⁻ mice were infected with the parental strain 2009 pH1N1 (open circles) or received PBS only (grey circle). Weight loss was monitored for 14 days. Data shown represent means ± SEM (n = 5-13). (B) MLD₃₀ of pH1N1 and pH1N1-MA7 strains in WT and α7⁻/⁻ mice. (C) Amino acid sequence differences are shown for parental pH1N1 and adapted pH1N1-MA7 viruses.

Figure 2
Pathogenicity of pH1N1-MA7 recombinant viruses in WT and α7⁻/⁻ mice. (A) WT (black square) or α7⁻/⁻ (open square) mice were intranasally inoculated with recombinant viruses, i.e. 10⁴ p.f.u. of adapted pH1N1-MA7rec, 10⁵ p.f.u. of pH1N1-PA₁MA7, 10⁵ p.f.u. of pH1N1-NP₁MA7, 10⁵ p.f.u. of pH1N1-PA₁NP₁MA7, 10⁵ p.f.u. of pH1N1-HA₁MA7 and 10⁵ p.f.u. of pH1N1-NA₁MA7. Weight loss and survival was monitored for 14 days. Control mice received PBS only (grey circle). Data shown represent means ± SEM (n = 5-15). (B) Weight loss has been summarized representing the mean area under the curve (A.U.C). WT (black bars) or α7⁻/⁻ (white bars) mice were infected with 10⁶ p.f.u. of wild-type viruses (pH1N1 and pH1N1-MA7) and recombinant viruses (pH1N1-PA₁MA7, pH1N1-NP₁MA7, pH1N1-PA₁NP₁MA7, pH1N1-HA₁MA7 and pH1N1-NA₁MA7). Control mice received PBS (grey bar). Data shown represent means ± SEM (n = 5-15; *p<0.05, **p<0.01, ***p<0.001, by students t-test compared to control group).
Figure 3

Localization of the adaptive mutations in pH1N1 protein structure and their effect in the structural stability. (A) Structural models of the regions surrounding the adaptive mutations of pH1N1-MA7 with homologous structures. Adaptive mutations are indicated in blue. Relevant sites in the structures are indicated in orange (PA endonuclease active site; RNA binding site and oligomerisation domain of NP; HA receptor binding site and NA active site). (B) Global z-score (as boxplots) and local energy comparison between the mutants of pH1N1-MA7 and their respective wild types (pH1N1). The X-axis shows the corresponding amino acid position and the y-axis shows the energy change. Each set is composed of 30 structural models. Pair (red), surface (blue) and combined (black) energy are shown for each analysis. Dots placed above the z-score distributions of the pH1N1 models represent the score of the structural template.

Figure 4

Biological activity of recombinant RNPs with adaptive mutations in epithelial HEK and HEK-shα7 cell lines. (A) Human HEK-WT (black bars) and HEK-shα7 (white bars) cells were co-transfected with plasmids expressing PB1, PA, NP and PB2, as well as a plasmid encoding Renilla luciferase and a plasmid encoding firefly luciferase in negative polarity, flanked by the non-translated regions of influenza NP segment. Plasmid expressing PB1 was omitted as a negative control. Plasmids encoding PA_{MA7} and NP_{MA7} were used when indicated, or both in pH1N1-MA7. At 20 h post-transfection, luciferase accumulation was determined. Values were normalized to Renilla expression and the activity of the pH1N1 RNP in HEK-WT cells was set as
100% (means ± SEM; n = 9-18; ***p<0.001, by students t-test compared to pH1N1 RNP activity in HEK-WT cells). (B) Confirmation of importin-α7 knockdown in human HEK cells by Western blot analysis. Since importin-α7 antibody cross-reacts with importin-α5, the doublet represents importin-α5 (upper band) and importin-α7 (lower band). GAPDH was used as a loading control.

Figure 5

**Importin-α binding affinities to NP monomers with adaptive mutation.** (A) Human HEK-WT cells were co-transfected with plasmids encoding FLAG-tagged importins (α1, α3 or α7) and NP or NPMA7. NP-only transfected cells served as a control (cont). At 48 h post-transfection, importins were immunoprecipitated using the FLAG-tag and co-immunoprecipitated NP was determined by Western blot analysis. β-actin was used as a loading control in the input samples. (B) Quantification of NP (black bars) or NPMA7 (dashed bars) binding to over-expressed importins. Values of NP were normalized against precipitated importin-α levels and the relative amount of NP bound to importin-α1 was set to 100%. Data shown represent means ± SEM (n = 3).

Figure 6

**Replication kinetics in epithelial A549 and A549-shα7 cell lines infected with pH1N1-MA7 virus.** (A) Human A549-WT and A549-shα7 cells were infected at MOI of 0.1 with parental (pH1N1) or adapted (pH1N1-MA7) viruses. Virus titres of the supernatants were measured by plaque assay at indicated hours p.i.. Data are expressed as means ± SEM of viral titres (n = 8-12). (B) Confirmation of importin-α7 knockdown in human A549 cells by Western blot analysis. Since importin-α7 antibody cross-reacts
with importin-α5, the doublet represents importin-α5 (upper band) and importin-α7 (lower band). GAPDH was used as a loading control.

**Figure 7**

**Viral RNA accumulation in WT and α7-/- MEFs infected with pH1N1-MA7 virus.**

(A) WT and α7-/- MEFs were infected at MOI of 2 with parental (pH1N1), adapted (pH1N1-MA7) or SGR (pH1N1-PA<sub>MA7</sub>, pH1N1-NP<sub>MA7</sub>, pH1N1-PA,NP<sub>MA7</sub>, pH1N1-HA<sub>MA7</sub> and pH1N1-NA<sub>MA7</sub>) viruses. After 24h, total RNA was isolated and analyzed by NP gene-specific primer extension. A primer specific for 5S rRNA was used for normalization. (B) vRNA accumulation in MEF-WT (black bars) and MEF-α7-/- (white bars) infected with the parental and adapted viruses. Values were expressed relative to vRNA levels in MEF-WT infected with pH1N1 virus which was set to 100%. (C) vRNA accumulation was expressed relative to vRNA levels in MEF-WT which was set to 100% for every virus tested. Data shown represent means ± SEM (n = 9-12). (D) Importin-α7 expression in MEF cells was checked by Western blot analysis. Since importin-α7 antibody cross-reacts with importin-α5, the doublet represents importin-α5 (upper band) and importin-α7 (lower band). GAPDH was used as a loading control.
A.

![Graph showing weight loss over days p.i. for different conditions.]

B.

<table>
<thead>
<tr>
<th>MLD$_{50}$ (p.f.u.)</th>
<th>pH1N1</th>
<th>pH1N1-MA7</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$10^{3.5}$</td>
<td>$10^{2.4}$</td>
</tr>
<tr>
<td>$\alpha_7^{-/-}$</td>
<td>$&gt;10^5$</td>
<td>$10^{2.6}$</td>
</tr>
</tbody>
</table>

C.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Adaptive mutations in pH1N1-MA7</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>Asp 27 Glu</td>
</tr>
<tr>
<td>NP</td>
<td>Gly 102 Arg</td>
</tr>
<tr>
<td>HA</td>
<td>Lys 171 Glu</td>
</tr>
<tr>
<td></td>
<td>Asp 239 Gly</td>
</tr>
<tr>
<td>NA</td>
<td>Tyr 170 His</td>
</tr>
</tbody>
</table>