Influenza viruses with receptor-binding neuraminidases

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Abstract

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For the vast majority of influenza viruses characterized to date, the two main surface proteins have opposing and mutually exclusive functions. Hemagglutinin (HA) is the viral entry protein, mediating attachment of the virus to the host cell via sialic acid-receptors and fusion of the viral and endosomal membranes. In contrast, neuraminidase (NA) is the viral exit protein, and is a sialidase that cleaves receptors from the surface of the producing cell to facilitate viral release. In the last few years, a growing body of evidence has shown that the receptor binding and receptor cleaving activities need not be mutually exclusive, and that single amino-acid mutations can allow NA to act as the receptor-binding protein. These mutations are D151G in N2 subtype NAs and G147R in N1 subtype NAs.

Here I describe the characterization of the G147R N1 NA mutation in Chapter II which was serendipitously discovered as a tissue-culture adaptation mutation. In Chapter III, I examine the effect of the G147R mutation in the background of naturally circulating viruses from three different viral lineages. I find that the receptor-binding NA mutation has no effect on the fitness of a pandemic human H1N1 virus in cell culture or in a mouse model of pathogenesis. The presence of the receptor-binding NA also does not affect sensitivity of the virus to neutralization
by an anti-HA IgG, although it does slightly protect against neutralization by the Fab domain of the antibody. The physiological relevance of escape from anti-HA Fab, however, remains unclear.

Overall, the identification of NA receptor-binding mutations represents a new aspect of influenza virus biology which warrants further investigation. The first NA-binding mutation was discovered when viruses failed to behave as expected in traditional hemagglutination inhibition assays. The acquisition of this D151G mutation by many recent H3N2 strains has remained a problem for the determination of vaccine efficacy in anti-sera hemagglutination inhibition assays. My work extended the finding that receptor-binding mutations can occur in N2 subtype NAs to the N1 subtype, as well. This suggests that the overall spectrum of NA-binding mutations may be much greater than is currently understood. Furthermore, because the N1 binding mutation does not significantly compromise the fitness of a recent human virus, my work also suggests that NA-binding mutations may have the potential to become more widespread in the future.
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List of Abbreviations

HA: hemagglutinin
MUNANA: 2’-(4-Methylumbelliferyl)-alpha-D-N-acetylace
NA: neuraminidase
pdmH1N1: human pandemic H1N1 influenza virus
RBC: red blood cell
RDE: receptor-destroying enzyme
VLP: virus-like particle
WT: wild-type
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Chapter I

Introduction

Influenza A viruses cause annual epidemics and occasional pandemics in humans (1). Epidemics are characterized by the circulation of viruses that represent slight variations on viruses that were present in the human population the previous season (2). For any given year, it is estimated that up to 10% of the world’s population may become infected (3). In contrast, pandemics occur when a new subtype of virus enters the human population from an animal reservoir, typically birds or pigs (4). These pandemic viruses sweep through the population very rapidly due to the absence of strong humoral immunity to the new viral variant. A pandemic can infect hundreds of millions of people, and in the case of the 1918 H1N1 pandemic, can cause millions of deaths (5). The morbidity, mortality, and massive socioeconomic burden of influenza has resulted in many decades of research on the virus (6), yet despite intense scrutiny, new intricacies are being discovered all the time.

Influenza virus structure and proteins

It has been well known for many years that influenza is a negative-sense RNA virus with a segmented genome. The 8 gene segments code for at least 10 functional proteins: PB1, PB2, PA, NP, M1 and M2, NS1 and NS2, HA and NA. Four of these proteins, PB1, PB2, PA, and NP, form the polymerase complex of the virus which both transcribe positive-sense RNA from which proteins are translated, and also complementary negative-sense RNA which serves as new viral genomes (7). NP also serves as a scaffold for the packaging of viral genome segments into new virions (8). M1 and M2 are the matrix proteins of the virus. M1 forms the cage-like structure under the viral membrane that gives viruses their shape and binds to the NP-coated genome
segments for proper packaging (9). M2 inserts into the membrane and acts as an ion channel to acidify the inside of the virus following endocytosis (10). This serves to dissociate the M1 matrix so that the viral genome can be released into the host cell. NS1 and NS2 are non-structural proteins that play critical roles in modulating the immune response against the virus (11) and mediating the nuclear import and export of viral nucleic acids (12), respectively.

The remaining two proteins, hemagglutinin (HA) and neuraminidase (NA), are the main surface proteins of the virus which protrude from the viral membrane. Because they are the most exposed proteins on the viral surface, HA and NA are the primary targets of antibodies against the virus (13). The ability of antibodies raised against one virus to recognize another viral variant forms the basis upon which viruses are classified into subtypes (14).

**Influenza subtypes**

Currently, there have been 18 subtypes of HA and 11 subtypes of NA identified (15). The vast majority of these subtypes occur in different combinations in wild waterfowl, while two, H17N10 and H18N11, have been found exclusively in bats (16, 17). There are currently only two subtypes which routinely infect humans, H1N1 and H3N2. Both entered the human population as pandemic viruses in 2009 and 1968, respectively. Since the early 20th century there have been 4 such pandemics in humans: H1N1 in 1918, H2N2 in 1957, H3N2 in 1968, and a novel reassortment of H1N1 again in 2009.

**Structure and function of HA**

HA is present in the viral membrane as a trimer (Figure 1.1). Each monomer is composed of two separate peptide chains, HA1 and HA2. HA is initially made as a single polypeptide, designated HA0, which is later proteolytically cleaved into its two subunits by host proteases (18). This cleavage event primes HA to perform fusion of the viral and endosomal membranes
following endocytosis (19). Prior to endocytosis, HA is also responsible for attaching the virus to a host cell by binding to cell-surface glycans with terminal sialic acids (20). One key aspect of a given virus’ ability to infect a host is its ability to bind to the type of sialic acid receptors present on target cells.
Figure 1.1. Structure of the HA trimer (PDB 4HMG). One monomer of the trimer is shown in blue. The protein has two functional domains that perform different functions. The stalk region, made primarily of the long and short α-helices, mediates fusion of the viral and endosomal membranes. The globular head domain binds to cell-surface sialic acid, shown as purple spheres. The trimer is anchored into the viral membrane which would be located at the bottom of the structure.
Receptor diversity and distribution

While nearly all characterized influenza viruses bind glycans with a terminal sialic acid moiety, these glycans are incredibly diverse as shown in Figure 1.2 (21). Even within a given host, the type of receptors can vary widely in different sections of the target organ (22, 23). The HAs of viruses that efficiently infect birds preferentially bind to sialic acid with an α2,3 linkage to the next sugar in the glycan chain (24). These types of glycans are highly prevalent in the avian intestinal tract, and it is known that influenza virus transmission in wild waterfowl is through the fecal-oral route (25). In contrast, viruses that have successfully jumped into the human population and cause widespread disease often preferentially bind sialic acid with an α2,6 linkage to the next sugar (26). These types of glycans are found in the upper airways of the human lung (27), and thus transmission between humans occurs through contact with aerosols containing virus (28). The human lung also has α2,3 linked sialic acids lower in the airways which may be why sporadic infections with avian viruses such as H5N1 and H7N9 cause much more severe infections due to a replication site deeper in the lung tissue.

Intriguingly, pigs represent an extremely likely intermediate host for the virus between birds and humans because the porcine airways contain a mix of α2,3 and α2,6 sialic acids (29). As such, a virus could infect a pig by binding to α2,3 sialic acids and then acquire mutations in HA which now allow the protein to bind α2,6 linked sialic acids (30). This switch in receptor preference often involves as few as one amino acid substitution, so a change in receptor preference can happen in just a single viral generation (31, 32).
Glycans are a broad class of molecules composed of many different types of sugars linked together with different chemical bonds. This produces an array of structures which can be long or short and branched or linear depending on the sugars and bonds involved.
Structure and function of NA

While HA typically performs the binding and fusion events necessary for viral entry, NA is responsible for viral egress by cleaving the cell-surface receptors that HA binds to (34). This serves both to release virions from the surface of the producing cell, and also to prevent the formation of viral aggregates because the HA and NA proteins themselves can be glycosylated (35). There is evidence that NA’s enzymatic activity may be necessary to initiate infection in vivo by cutting through mucins in the airway (36), but this function is completely (35), or nearly completely (37), expendable for viral growth in cell culture. As a result, NA has long been considered unimportant for initiation of infection, particularly in cell culture where no mucins are present on the target cells.
Figure 1.3. Structure of the NA tetramer (PDB 2HU4). Each monomer of NA is colored in a shade of blue or gray. The stalk region of NA is not part of the crystal structure, but similar to HA, it would extend down from the head region to anchor NA into the viral membrane. Each monomer of NA contains one active site that cleaves sialic acid. Oseltamivir, a sialic acid mimic which inhibits NA’s activity, is modeled in the active site of one monomer as shown by the green spheres.
Receptor-binding ability of N2 NAs

Recently, a growing body literature has challenged the notion that HA and NA have completely partitioned functions during viral entry and exit. The first example was a report on the appearance of a mutation that allows NA to function as the receptor-binding protein in hemagglutination assays (38). This study found that the NAs from recent human H3N2 viruses acquire the amino acid mutation D151G which allows NA to assume the receptor-binding function in the presence of weak HA-mediated sialic acid binding. Viruses with the D151G mutation were originally described to have retained their sialidase function, although this was later disproved when purified proteins rather than whole viruses were used for enzymatic assays (39). This difference in findings is likely attributed to low levels of D151 NA proteins present in the viral population which skewed the results of the assay.

This second study, which also solved the crystal structures of D151 and G151 NA, showed that the D151G mutation removes a sidechain critical for catalysis of sialic acid cleavage. In this way, NA is converted from an enzyme, to a very strong substrate-binding protein. This suggests that for viruses with poor-binding HAs, it is advantageous for the NAs to trade receptor-cleaving for receptor-binding ability. This mechanism of decreasing NA activity to compensate for poor HA binding for viruses replicating in vitro has been previously described (40). Indeed, for the D151G H3N2 viruses, the NA mutation appears to be the result of tissue-culture adaptation (41), and so it remains unknown if the mutation is of any physiological consequence in the human population.

Receptor-binding ability of N1 NAs

Shortly after these reports, we serendipitously isolated a similar mutation, this time in the N1 NA background. For another series of experiments, we generated a mutant HA that had been
heavily mutated to ablate receptor-binding. This was accomplished by introducing three point mutations, a loop deletion, and several potential glycosylation sites near the receptor-binding pocket of HA, all of which had been individually shown to reduce sialic acid binding. In one rare instance, the attempted rescue of this HA (derived from the X-31 H3N2 laboratory strain of virus) with all of the remaining genes from the A/WSN/33 H1N1 lab strain led to a fully infectious virus. Sequencing revealed no mutations or reversions in HA, but a single amino acid change, G147R, in NA. We hypothesized that this mutation had allowed NA to take on the receptor-binding role in the absence of HA-mediated binding similar to D151G N2 NAs. Further passage of this virus with the mutant NA eventually led to a mutation in the HA stalk, K62E, which improved growth, although it seemed unlikely that this mutation affects HA’s receptor-binding function since it is not located near the canonical receptor-binding pocket.

HA stalk mutation

Looking at the crystal structure of the parental version of the mutant HA reveals that the K62 sidechain normally packs between two negatively-charged side chains on the adjacent HA monomer (42). By changing this positively-charged sidechain to one with a negative charge, we hypothesize that the HA trimer may have become destabilized such that the conformational changes needed for fusion can now occur at a higher pH (43). A similar mutation in the HA stalk has recently been described to do exactly that (44). This would result in fusion taking place in an earlier endosome structure than for the wild-type virus and would shorten the generation time, thus giving the K62E mutant a growth advantage over the original mutant HA. This hypothesis, however, remains purely speculative, as we have not rigorously tested the fusion pH of both the parental and mutant HAs.
Goals of this dissertation

These preliminary results then became the basis of my dissertation research: to test whether the G147R mutant NA was in fact functioning to bind to cellular receptors to mediate viral entry. I also wanted to determine if this mutation knocks out the sialidase activity as is seen with the D151G N2 NA mutation. I then looked for the G147R mutation in a database of influenza sequences to see if it was present in any naturally occurring strains, and found it at low levels in three lineages of viruses with N1 neuraminidase. I next tested the ability of one representative sequence from each of these lineages to also act as the receptor-binding protein. I focused the remainder of my studies on the mutation in the human pandemic H1N1 background to examine its effects on in vitro and in vivo replication and pathogenesis. Finally, I tested the hypothesis that a virus might acquire an NA-binding mutation as a way to escape from neutralization by anti-HA antibodies.
Chapter II

A mutant influenza virus that uses neuraminidase as the receptor-binding protein


INTRODUCTION

Influenza expresses two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The classical view is that HA is a receptor-binding and fusion protein that is essential for viral entry (45), while NA is a receptor-cleaving protein that facilitates viral release but is expendable for viral entry (35). Specifically, HA binds to sialic acid on the cell surface which leads to viral endocytosis and pH-triggered membrane fusion (46), and blocking either HA receptor binding (47) or fusion activity (48-51) neutralizes viral infection. NA promotes the release of newly formed virions by enzymatically cleaving sialic acid from the cell surface – in the absence of NA’s sialidase activity, budding virions aggregate on the cell surface due to the binding of HA to cell-surface sialic acid (35, 52). While NA may aid in viral infection in vivo by cleaving mucins found in the airways (53), NA activity is completely (35) or nearly completely (37) expendable for viral entry in standard tissue-culture systems.

While this view of HA as the entry protein and NA as the release protein is almost certainly correct for the vast majority of influenza strains, several recent studies have suggested that NA can also acquire receptor-binding activity. In 2010, Lin et al. reported that some recent human H3N2 isolates contained an NA mutation (D151G near the active site) that enables them
to bind red blood cells by a mechanism that can be blocked by the NA inhibitor oseltamivir or by anti-NA antibodies (38). Zhu et al. subsequently crystallized an N2 NA with the D151G mutation, and showed that this mutant NA could indeed bind with high avidity to some sialylated glycans (39). Gulati et al. reported that oseltamivir blocked the binding to α2-3-linked sialic acids of human H3N2 isolates with D151G (54). For some of these isolates, oseltamivir also neutralized viral infectivity, suggesting that this mutant NA plays a role in viral entry. However, these viruses still retain the ability to bind to α2-6 linked sialic acids via HA (54), making it unclear if NA is the primary receptor-binding protein.

Here we report the discovery of a new mutation (G147R) that enables an N1 NA to completely co-opt the receptor-binding function normally performed by HA. Viruses with this mutation infect cells in an NA-dependent fashion even after the introduction of multiple mutations and a deletion to highly conserved residues in the HA receptor-binding pocket. We did not isolate the G147R mutation from a naturally occurring virus – rather, it arose de novo in a lab-generated chimeric virus during our studies. However, the reported NA sequences of several recent H1N1 and H5N1 isolates do contain G147R. Overall, our study demonstrates the completeness and evolutionary ease with which influenza can switch the receptor-binding function between its two glycoproteins.

MATERIALS AND METHODS

Viral strains/genes

All HA sequences were derived from the A/Hong Kong/2/1968 (X-31) H3N2 strain. Mutations to add potential glycosylation sites (Table 2.1) were first introduced into the parental X-31 HA through site-directed mutagenesis. This glycosylated HA variant is referred to as “WT”
throughout the manuscript. Receptor-binding site mutations (Table 2.2) were then introduced through site-directed mutagenesis to the WT variant to create the “BindMut HA.” A third variant, named “PassMut HA” also has the additional HA-stalk mutation, K62E in HA2, introduced through site-directed mutagenesis. All NA sequences were derived from the A/WSN/33 (WSN) H1N1 strain. The G147R point mutation was introduced through site-directed mutagenesis. The other viral genes (PB1, PB2, PA, NP, M, NS) were also from the A/WSN/33 strain.

**Plasmids**

All HA and NA variants generated during this study were cloned into the bidirectional pHW2000 backbone for reverse-genetics viral rescue (55). The other viral genes were expressed from previously described bidirectional WSN reverse-genetics plasmids (55), which were a kind gift from Robert Webster of St. Jude Children’s Research Hospital. For viral rescue experiments, we used a previously described GFP-based system where the coding region of PB1 is replaced by the coding region of GFP (56). This plasmid is referred to as “PB1flank-eGFP.” For some of the experiments, HA and NA were also cloned into an expression plasmid (HDM) which places the gene under the control of a CMV promoter followed by an IRES-GFP and the beta-globin polyA element.

**Cells**

Viruses carrying GFP in the PB1 segment were grown in previously described 293T and MDCK-SIAT1 cell lines that constitutively express PB1 under control of a CMV promoter (56). These cell lines are named 293T-CMV-PB1 and MDCK-SIAT1-CMV-PB1, respectively.
Viral rescue

Co-cultures of 293T-CMV-PB1 and MDCK-SIAT1-CMV-PB1 cells were transfected with eight reverse-genetics plasmids encoding PB2, PA, NP, M, NS, HA, NA, and PB1flank-eGFP. Cells were plated at a density of $2 \times 10^5$ 293T-CMV-PB1 and $0.5 \times 10^5$ MDCK-SIAT1-CMV-PB1 cells per well in 6-well dishes in D10 (DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.) The next day, 250 ng of each plasmid was transfected into the cells using the BioT transfection reagent (Bioland B01-02). At 12-18 hours post-transfection the cells were washed with PBS and the media changed to Influenza Growth Media (IGM) (OptiMEM supplemented with 0.01% heat-inactivated FBS, 0.3% BSA, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 100 μg/ml calcium chloride). TPCK-trypsin was added to IGM at 3 μg/ml immediately before use. Viral supernatants were collected 72 hours post-transfection and titered.

Viral titering

The titer of the PB1flank-eGFP viruses was determined by flow cytometry. Briefly, MDCK-SIAT1-CMV-PB1 cells were plated at $10^5$ per well in 12-well dishes in IGM and infected 4 hours later with 1 μl, 10 μl, and 100 μl of viral supernatant. 16 hours post-infection, wells with approximately 1-10% GFP positive cells were analyzed by flow cytometry to determine the fraction of cells that were GFP positive. The Poisson equation was used to convert this fraction to the initial MOI, allowing determination of the number of infectious particles in the original inoculum.

HA surface expression

The G1E point mutation in HA2 was introduced into WT and PassMut HA by site-directed mutagenesis, and the mutated genes were cloned into the HDM plasmid. 293T cells were
transfected with plasmid encoding each of the HA variants with and without the G1E mutation in triplicate. At 20 hours post-transfection, the cells were collected and resuspended in MOPS buffered saline (MBS) (15 mM MOPS, 145 mM sodium chloride, 2.7 mM potassium chloride, and 4.0 mM calcium chloride, adjusted to pH 7.4, 2% heat-inactivated FBS added immediately before use). Heat-inactivated polyclonal serum from influenza-infected mice at a 1:200 dilution was used as the primary antibody to stain for surface HA molecules, and a goat-anti-mouse TriColor antibody (Caltag Laboratories M32006) at a 1:100 dilution was used as the secondary antibody. Cells were analyzed by flow cytometry to determine the mean fluorescent intensity (MFI) of TriColor (APC channel) among the GFP positive (transfected) cells. Reported values for each G1E mutant are normalized to the respective wild-type.

NA surface expression

A C-terminal V5 epitope tag was added to both WT and G147R NA. Both genes were then cloned into the HDM plasmid, and used to transfect 293T cells. At 20 hours post-transfection, cells were collected and stained with an anti-V5 AF647-conjugated antibody (Invitrogen 45-1098) at a 1:200 dilution. Cells were analyzed by flow cytometry to determine the MFI of AF647 (APC channel) among GFP positive (transfected) cells. Reported values were normalized to the WT NA.

MUNANA activity assay

NA activity was assayed using the fluorogenic 2’-(4-Methylumbelliferyl)-alpha-D-N-acetylneuraminic acid (MUNANA) substrate (Sigma M8639). 293T cells were transfected with HDM plasmid encoding each NA variant in triplicate. At 20 hours post-transfection, cells were collected and diluted 1:40 in a 96-well plate such that each row contained one NA variant. Serial two-fold dilutions of MUNANA were made across each row of a Costar black flat-bottom 96-
well plate. Both plates were pre-warmed to 37°C for 20 minutes. Cells were then quickly resuspended by pipetting and added to the MUNANA plate. Fluorescent readings were taken every minute for 1 hour at an excitation wavelength of 360 nm and an emission wavelength of 448 nm. Fluorescence above background was then plotted versus time for each MUNANA concentration to determine the reaction rate. Reaction rate was then plotted against MUANANA concentration and the $K_M$ and $V_{max}$ determined by fitting Michaelis-Menten kinetics curves in GraphPad Prism 5.

**Oseltamivir inhibition assay**

293T cells were transfected with HDM plasmids encoding WT and G147R NA in triplicate. At 20 hours post-transfection, cells were collected, diluted, and then incubated with decreasing concentrations of oseltamivir carboxylate (kindly provided by Roche) at 37°C for 30 minutes to allow for oseltamivir binding. MUNANA was added to 300 µM and incubation continued for 45 minutes. The reaction was quenched by adding a solution of 0.153 M NaOH in 81.5% ethanol, and the signal was read as described above. Values were normalized to a no-oseltamivir control for each NA variant to determine the percent remaining activity.

**Mouse infections**

Serum for neutralization assays, hemagglutination inhibition assays, and cell-surface staining was obtained from influenza-infected mice. Mice were intranasally infected with replication-competent virus after being anesthetized with 2 mg ketamine and 0.2 mg xylazine per mouse. At three weeks post-infection, a booster infection was done using the same protocol. Mice were then euthanized and bled by cardiac puncture 4 weeks after initial infection, or 1 week after the booster. For neutralization assays, mouse serum was heat inactivated at 56°C for 40 minutes prior to use. For hemagglutination inhibition assays, serum was heat inactivated, then antibodies
were purified by Protein A column (Thermo Scientific 89952) and concentrated to the original volume prior to use. This animal work was approved under FHCRC IACUC protocol 1893.

Neutralization assays

Neutralization assays were performed using the PB1flank-eGFP viruses. To reduce the background media auto-fluorescence in the GFP channel, we developed a Neutralization Assay Media (NAM) consisting of Medium 199 supplemented with 0.01% heat-inactivated FBS, 0.3% BSA, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 100 µg/ml calcium chloride and 25 mM HEPES. Polyclonal serum or oseltamivir was diluted down the columns of a 96-well plate in NAM and virus was added at a multiplicity of infection (MOI) that ranged from 0.1 to 0.8 for the different viruses. Plates were incubated at 37°C for 1 hour to allow oseltamivir or antibody binding, then $4 \times 10^4$ MDCK-SIAT1-CMV-PB1 cells were added per well. A no-serum or no-oseltamivir control row for each virus was included to give a maximum infectivity value, and a no-virus control row was included to give the background fluorescence. After an 18-hour incubation, GFP fluorescence intensity was measured using an excitation wavelength of 485 nm and an emission wavelength of 515 nm (12 nm slit widths). The signal above background for each well was normalized to its respective no-oseltamivir or no-serum control; values are reported as percent infectivity remaining averaged over triplicate measurements.

Hemagglutination inhibition assays

Hemagglutination inhibition assays were performed using turkey (Lampire Biological Laboratories 7249409), chicken (Innovative Research IC05-0810), or guinea pig (Innovative Research IC05-0910) red blood cells (RBCs) diluted to 0.5% in PBS. The hemagglutination titer for each virus and blood cell type was determined, then 8 HAU used for inhibition assays. 10 µl containing 8 HAU of virus was pre-incubated at 37°C with 40 µl serum or oseltamivir for 1 hour
in U-bottom plates, then 50 µl of RBCs were added. Plates were scored after 1 hour incubation at room temperature.

**VLP production**

To produce virus-like particles (VLPs) expressing NA but not HA on their surface, 293T cells in D10 were transfected with an HDM plasmid expressing M1 and M2 from the A/PR/8/34 (H1N1) strain separated by a T2A linker, and an HDM plasmid expressing either WT or G147R NA. The media was changed to IGM at 24 hours post-transfection, and the VLP supernatant was collected at 72 hours post-transfection. Supernatants were clarified at 2000 × g for 5 minutes to pellet cell debris. The clarified supernatant was then concentrated with a 100 kDa cut-off centrifugal concentrator. MUNANA activity of the collected VLPs was determined for equal volumes of concentrated supernatants.

**Viral growth in the presence of RDE**

MDCK-SIAT1-CMV-PB1 cells were plated in 6-well dishes at a density of 5 × 10^4 cells per well in D10. After 18 hours, the media was changed to IGM with 4 µg/ml TPCK-trypsin after a PBS wash. Half of the wells also contained the bacterial sialidase RDE (Sigma C8772, 1 vial resuspended in 5 ml sterile water) added at 5 µl/ml. Plates were incubated at 37°C for one hour to allow for RDE cleavage, then infected at an MOI of 0.05. Beginning at 24 hours post-infection, supernatant was collected and titered every 12 hours as previously described.

**Analysis of G147R mutation in naturally occurring sequences**

To examine the occurrence of G147R in naturally occurring sequences, we downloaded all N1 protein sequences in the Influenza Virus Resource (57) as of July 3, 2013. These sequences were pairwise aligned with the WSN G147R NA to identify all sequences with an R at position 147 – these sequences are listed in Table 2.3. To examine whether the sequences with G147R formed
phylogenetic clusters, an initial tree was built using RAxML (58) on all G147R sequences and a subset of the other sequences. Based on visual inspection of this tree, three different clusters of at least three G147R sequences were identified. Comprehensive phylogenetic trees of sequences with high identity to these apparent clusters were then built using BEAST (59) with date-stamped sequences using the JTT (60) model of substitution. Maximum clade credibility trees were computed and visualized using FigTree to give the images shown in Fig. 2.8.

RESULTS

Loss of HA receptor-binding is compensated by a mutation in NA

We created a mutant of the HA from the A/Hong Kong/2/1968 (H3N2) strain that we expected to be unable to bind to its sialic-acid receptor. This mutant contained three point mutations previously shown to individually nearly abolish HA receptor binding (61) and a loop deletion near the receptor binding pocket (62). In the H3 numbering scheme, these mutations are Y98F, H183F, L194A, and deletion of amino acids 221 to 228 (Figure 2.1A and B; Table 2.2). We also added seven N-linked glycosylation site motifs at positions where glycosylation is found in contemporary human H3N2 HAs (potentially glycosylated asparagines at residues 45, 63, 122, 126, 133, 144, and 246 in H3 numbering; Table 2.1), as glycosylation of HA has been shown to reduce receptor avidity (63). This presumed binding-deficient mutant HA is henceforth referred to as BindMut HA.

The BindMut HA was used as a negative control during rescue of viruses by reverse genetics (56) for a series of other experiments. We did not expect to see growth of virus containing the BindMut HA due to its presumed lack of receptor-binding ability. To our surprise, in one rare instance we isolated a virus with the BindMut HA that grew to moderate titers in
tissue culture. The isolated virus contained the BindMut HA and all of the other genes from the A/WSN/33 (H1N1) strain. We sequenced this isolate and found no mutations or reversions in HA, but we discovered a single point mutation, G147R (N2 numbering), in NA. This mutation is located somewhat above the NA active site in the NA crystal structure (Figure 2.1C). Further passage of the virus yielded a variant that grew to increased titers. This virus had retained the G147R NA mutation, and had also acquired a stalk mutation, K62E in HA2, (H3 numbering) (Figure 2.1A). This HA mutant, which contains all of the original receptor-binding site mutations and glycosylation sites plus the K62E stalk mutation, is henceforth referred to as PassMut HA (Passage Mutant HA).

<table>
<thead>
<tr>
<th>Sequential numbering</th>
<th>H3 numbering</th>
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<th>Glycosylation site change</th>
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<td>+</td>
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<tr>
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<td>D63N</td>
<td>79/63</td>
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<td>T83K</td>
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<td>G124S</td>
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<td>+</td>
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<td>T126N</td>
<td>142/126</td>
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<td>G135T</td>
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<tr>
<td>N264T</td>
<td>N248T</td>
<td>262/246</td>
<td>+</td>
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Table 2.1. Sequential and H3 numbering of glycosylation site mutations added to the HA used in this study.

<table>
<thead>
<tr>
<th>Sequential numbering</th>
<th>H3 numbering</th>
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<td>L→A</td>
</tr>
<tr>
<td>237–244</td>
<td>221–228</td>
<td>Deletion</td>
</tr>
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Table 2.2. Sequential and H3 numbering of receptor-binding site mutations made in BindMut HA.
Figure 2.1. Attempted growth of a virus with extensive mutations in the HA receptor-binding pocket selects for a mutation near the active site of NA. (A) Crystal structure (PDB 4HMG) of an HA monomer with a sialic-acid analogue (purple spheres) bound in the receptor-binding pocket. The sites of the binding-pocket mutations are shown in colors other than gray, and the site of stalk mutation K62E in HA2 is also indicated. (B) Zoomed-in image of the receptor-binding pocket of the HA structure shown in (A). (C) Crystal structure (PDB 2HU4) of an NA monomer with oseltamivir (green spheres) in the active site and the site of the passage-derived G147R mutation shown in red.
To determine whether the HA and NA mutations were responsible for the growth phenotypes observed, we created reverse-genetics plasmids for all HA and NA variants. Three HA variants were made: a variant we will term wild-type (WT) HA which has the seven glycosylation sites added but none of the receptor-binding site mutations, the BindMut HA, and the PassMut HA. Two NA variants were created: Wild-type A/WSN/33 NA (WT NA), and WSN NA with the G147R mutation (G147R NA). We then attempted to rescue viruses containing all combinations of these HAs and NAs in the WSN background.

As expected, we were able to efficiently rescue virus carrying the WT HA paired with either WT or G147R NA (Figure 2.2). Also as expected, we were unable to rescue virus when the presumed binding-deficient BindMut or PassMut HAs were paired with WT NA. However, we could rescue moderate levels of virus carrying the BindMut HA and G147R NA, suggesting that G147R compensated for the loss of HA receptor binding. Virus containing the PassMut HA and G147R NA grew to levels nearly as high as WT virus. These results led us to hypothesize that the G147R mutation had enabled NA to acquire the receptor-binding function normally performed by HA.
Figure 2.2. Viruses with the HA receptor-binding mutations can only be rescued with the mutant G147R NA. Shown are viral titers in the supernatant 72 hours after attempted rescue of the indicated viruses by reverse genetics. Virus containing the BindMut HA can only be rescued in combination with the G147R NA. Further passage of this BindMut HA / G147R NA virus selected for the additional K62E mutation in HA2. The PassMut HA (which contains this HA2 mutation) also can only be rescued in combination with the G147R NA. Shown are the mean and standard errors for three replicates.
**HA is still required for viral fusion**

Although we hypothesized that NA was functioning as the receptor-binding protein in our mutant viruses, we wished to determine if HA was still needed to mediate membrane fusion. To test this, we introduced a point mutation that has been shown to abolish the fusion activity of HA, G1E in HA2 (64). The G1E mutation was introduced into both the WT and PassMut HA. To confirm that G1E did not affect HA levels at the cell surface, we used cell-surface staining with polyclonal anti-HA serum and flow cytometry to quantify cell-surface protein levels. Serum from mice infected with WT HA virus was used to stain WT and WT-G1E expressing cells, while serum from mice infected with PassMut HA virus was used to stain PassMut and PassMut-G1E expressing cells. In both cases, expression of the G1E mutant was greater than 90% that of the matched parent HA (Figure 2.3A and B), indicating that G1E does not substantially impair HA folding or trafficking to the cell surface.

We then attempted to rescue virus containing the G1E HAs with either WT or G147R NA. We were unable to rescue any G1E-containing viruses, indicating that abolishing HA’s fusion function ablates viral growth (Figure 2.3C).

To further confirm the requirement for HA-mediated fusion, we performed neutralization assays with the anti-fusion antibody FI6v3 (65). This broadly neutralizing antibody locks HA into the pre-fusion conformation. All viruses were neutralized by FI6v3 at similar concentrations, regardless of their HA and NA composition (Figure 2.3D). Taken together, these data show that HA is required for fusion regardless of whether or not the virus has NA with the G147R mutation.
Figure 2.3. HA is still required for viral membrane fusion. (A) Introduction of the fusion-blocking G1E mutation into WT HA does not substantially impact HA surface expression, as quantified by antibody staining and flow cytometry of transfected 293T cells. (B) Introduction of the G1E mutation into PassMut HA also does not substantially impact HA surface expression. (C) G1E completely blocks the rescue of infectious virus by reverse genetics, regardless of the NA used. Shown are the viral titers in the supernatant 70 hours after attempted rescue of the indicated viruses by reverse genetics. (D) Infectivity of all viral variants is neutralized by the fusion-inhibiting antibody FI6v3, regardless of which glycoprotein the virus uses to bind to the receptor. In all panels, data represent the mean and standard errors of three replicates.
G147R NA is still an active sialidase that is expressed at the cell surface

We next tested whether G147R altered NA’s surface expression or enzymatic activity as a sialidase. To assay for NA surface expression, a C-terminal V5 tag was added to both the WT and G147R variants, then 293T cells were transfected and surface stained for flow cytometry. G147R NA was found to be expressed on the cell surface at 70% the level of WT NA (Figure 2.4A).

We then measured the kinetics of cleavage of the NA surrogate substrate MUNANA by 293T cells transfected with both NA variants (Figure 2.4B and C). The maximum reaction rate, $V_{\text{max}}$, for G147R NA was about 55% that of WT NA, but when normalized to account for G147R’s lower surface expression, the reduction in G147R NA’s per-enzyme catalytic rate is only about 20%. G147R had about a two-fold higher substrate affinity than WT as indicated by a two-fold lower $K_M$. While these enzymatic parameters are not identical, the differences are sufficiently small that they seem unlikely to fully explain G147R’s dramatic acquisition of the capability to serve as the primary receptor-binding protein. However, it is important to note that our assays were performed using the single surrogate substrate MUNANA, and the results may not be representative of NA’s binding and cleavage of all possible sialic-acid variants. It is possible that cells express some other sialic-acid structure that is bound by the G147R NA, but is cleaved with much slower kinetics than those observed for the MUNANA substrate.
Figure 2.4. The G147R NA is an active sialidase that is inhibited by oseltamivir. (A) Surface expression of WT and G147R NA with C-terminal V5 epitope tags in transfected 293T cells. Expression of G147R NA is approximately 70% that of WT. (B) Rate of MUNANA cleavage at increasing substrate concentrations. Michaelis-Menten kinetics curves were fit to determine $K_M$ and $V_{\text{max}}$. (C) Enzyme kinetics for WT and G147R NA. $V_{\text{max}}$ is also normalized to expression levels in (A) to give a value proportional to $k_{\text{cat}}$. (D) NA activity at increasing concentrations of oseltamivir. Both NAs are inhibited at similar concentrations. The y-axis shows the percent remaining activity relative to the same NA variant in the absence of oseltamivir. For all panels, data represent the mean and standard error of three replicates.
Oseltamivir and anti-NA antibodies inhibit infection and hemagglutination by viruses dependent on the G147R NA

We next tested the ability of oseltamivir to inhibit enzyme activity for both the WT and G147R NA (Figure 2.4D). Both variants were inhibited by oseltamivir at similar concentrations, indicating that oseltamivir can still bind to the active site of the G147R NA. We therefore decided to test if oseltamivir could also inhibit the receptor binding of viruses dependent on the G147R NA.

We tested oseltamivir’s ability to block infectivity of three viruses: WT HA / WT NA, WT HA / G147R NA, and PassMut HA / G147R NA (Figure 2.5A). WT HA / WT NA virus was uninhibited at all concentrations tested, consistent with the prevailing belief that NA activity is not crucial for viral entry (35). However, PassMut HA / G147R NA was strongly neutralized at low nanomolar oseltamivir concentrations, consistent with the hypothesis that NA is the viral attachment protein for this virus. WT HA / G147R NA showed an intermediate phenotype, likely because oseltamivir inhibits NA-mediated but not HA-mediated receptor binding by this virus.

We also tested whether polyclonal mouse serum with NA-specific antibodies could block infectivity. Serum was obtained from mice infected with a virus containing G147R NA, but an H1 subtype HA. Because the WT and G147R NAs differ at only a single site, this polyclonal serum should substantially react with both NAs, but should not recognize the H3 subtype HA present in all three viruses tested. The degree of neutralization of the three viruses by this serum was similar to that seen for oseltamivir (Figure 2.5B). PassMut HA / G147R NA was strongly neutralized, WT HA / WT NA was completely uninhibited, and WT HA / G147R NA showed an intermediate phenotype.
To directly test if oseltamivir blocks viral attachment to cells, we performed hemagglutination-inhibition assays. All red blood cell (RBC) types tested (turkey, chicken, and guinea pig), were effectively agglutinated by the PassMut HA / G147R NA virus, but in all cases this agglutination was inhibited down to an oseltamivir concentration of 1.5 nM. In contrast, the WT HA / WT NA and WT HA / G147R NA were uninhibited at all oseltamivir concentrations tested (Figure 2.5C).

A hemagglutination-inhibition assay was also performed in the presence of purified polyclonal anti-NA antibodies from mouse serum. PassMut HA / G147R NA was potently inhibited, while WT HA / WT NA and WT HA / G147R NA were much more resistant (Figure 2.5D). Taken together, these data show that infectivity and cell binding of PassMut HA / G147R NA virus are inhibited by blocking NA with either a small molecule inhibitor or polyclonal antibodies. These results strongly suggest that the PassMut HA / G147R NA viruses are using NA as the sole receptor-binding protein.
FIG. 2.5. Oseltamivir neutralizes and inhibits hemagglutination by viruses that utilize G147R NA as the receptor-binding protein. (A) The extent of virus neutralization by oseltamivir depends on the degree to which NA is utilized as the receptor-binding protein. PassMut HA / G147R NA uses NA as the receptor-binding protein, and is nearly completely neutralized by oseltamivir. WT HA / G147R NA uses both HA and NA as receptor-binding proteins, and is partially neutralized by oseltamivir. WT HA / WT NA uses HA as the receptor-binding protein, and is resistant to neutralization by oseltamivir. (B) Similar effects as in (A) are seen when viral infectivity is inhibited with polyclonal anti-NA antibodies from mouse serum. The plots show neutralization by serum from mice infected with virus carrying the G147R NA, or mock infected with PBS. Both (A) and (B) represent the mean and standard error of three replicates. (C) Agglutination of red blood cells (RBCs) by PassMut HA / G147R NA is inhibited by oseltamivir while WT HA / WT NA and WT HA / G147R NA are resistant to inhibition at all concentrations tested. RBCs from the indicated species were incubated with 8 HA units of virus pre-treated with the indicated amount of oseltamivir. (D) Agglutination of turkey RBCs by PassMut HA / G147R NA is inhibited at low concentrations of polyclonal anti-NA antibodies from mouse serum. WT HA / WT NA and WT HA / G147R NA are much more resistant to inhibition. Values are reported as the reciprocal of the dilution factor for which complete inhibition was seen.
NA-only VLPs can agglutinate red blood cells, and agglutination is reversibly blocked by oseltamivir.

To conclusively show that the cell binding of PassMut HA / G147R NA is completely independent of HA, we produced virus-like particles (VLPs) that expressed NA but no HA. We did this by transfecting 293T cells with plasmids expressing M1 and M2 and either WT or G147R NA, as NA alone has previously been shown to be sufficient for VLP production with M1 slightly enhancing VLP release (66), and M2 is known to promote membrane scission (67). The total NA activity in the G147R VLP supernatant was 77% that of WT NA VLP supernatant, consistent with the slightly reduced activity of G147R NA reported in Figure 2.4.

We used concentrated VLP supernatants to perform a hemagglutination assay with turkey RBCs. Figure 2.6A shows images of the assay taken every 20 minutes. The WT NA-only VLPs slightly increased the speed of RBC settling relative to the PBS control, suggesting that removal of cell-surface sialic acid might promote the settling of RBCs, possibly by removing negative charges from the cell surface. At high concentrations, the G147R NA-only VLPs initially slightly agglutinated the RBCs, but this agglutination soon disappeared and the RBCs settled to the bottom of the plate. But at moderate concentrations (the 1:8 and 1:16 dilutions), the G147R NA-only VLPs potently agglutinated the RBCs over the full 60-minute time course. After 60 minutes, we added oseltamivir to all wells at a high concentration. Oseltamivir reversed the agglutination by the G147R VLPs, consistent with the idea that oseltamivir can elute the VLPs off the RBCs by competitively binding to the G147R NA.

Overall, the results in Figure 2.6A show that the G147R NA can bind VLPs to RBCs in a reversible manner. The eventual disappearance of agglutination at high G147R NA-only VLP concentrations suggests that G147R NA might slowly cleave the same receptor to which it
initially binds. In this scenario, at high VLP concentrations the G147R NA eventually removes all of the receptor, making the RBCs resistant to continued agglutination. At moderate VLP concentrations, the rate of receptor removal is lower and so long-term agglutination is observed.

We next performed a hemagglutination-inhibition assay in the presence of increasing dilutions of oseltamivir and a G147R NA-only VLP concentration that caused long-term agglutination. Oseltamivir inhibited agglutination by the G147R NA-only VLPs down to concentrations of 0.12 nM. At lower oseltamivir concentrations, agglutination did occur, but it could again be reversed by the addition of high concentrations of oseltamivir after one hour (Figure 2.6B). Taken together these data show that G147R NA is sufficient for agglutination in the complete absence of HA.
Figure 2.6. G147R NA-only virus-like particles (VLPs) agglutinate red blood cells, and agglutination is inhibited by oseltamivir. (A) A hemagglutination assay was performed using WT and G147R NA VLPs. VLPs were serially diluted two-fold across a U-bottom plate, turkey red blood cells (RBCs) were added, and the plate was imaged every 20 minutes. At 60 minutes, oseltamivir was added to all wells to a final concentration of 10 nM. The plate was imaged again 20 minutes later by which time agglutination by G147R VLPs had been reversed. (B) A hemagglutination inhibition assay was performed using serial three-fold dilutions of oseltamivir across a U-bottom plate. VLPs from HA assay were added at a concentration corresponding to the 1:8 dilution in (A). The plate was imaged 60 minutes after the addition of turkey RBCs. Oseltamivir was then added to all wells at a final concentration of 1.6 nM and plate was imaged 20 minutes later.
Sialidase treatment only partially inhibits infection by G147R NA viruses

We wished to determine if the G147R mutant NA still binds to the canonical sialic-acid receptor recognized by HA. To test this, cells were pre-treated with a broad-spectrum bacterial sialidase (receptor destroying enzyme, RDE) for one hour, and then infected with WT HA / WT NA, WT HA / G147R NA, and PassMut HA / G147R NA viruses at an MOI of 0.05. The viral supernatant was titered every 12 hours beginning at 36 hours post-infection. RDE treatment nearly completed inhibited growth of WT HA / WT NA except for low levels of viral growth at late time points (Figure 2.7). However, the WT HA / G147R NA and PassMut HA / G147R NA viruses were substantially less inhibited by RDE treatment of the cells (Figure 2.7), although their growth was still clearly reduced. These results suggest that the receptor for the G147R NA is more refractory to RDE cleavage than the receptor for HA. However, we are unable to ascertain whether the G147R NA recognizes a non-sialic acid receptor, or simply recognizes a class of sialic-acid moieties that is partially resistant to RDE cleavage since we lack a way to independently assess the extent to which all sialic acid has been removed by RDE. It seems quite plausible that G147R could recognize a sialic-acid structure that is incompletely removed by RDE.
Treatment with an exogenous bacterial sialidase (receptor-destroying enzyme, RDE) only partially inhibits infection by receptor-binding NA viruses. Cells both with and without RDE pre-treatment were infected with each viral variants at an MOI of 0.05. Supernatant was collected every 12 hours post-infection and viral titers determined. Data represent the mean and standard error of three replicates.
G147R NA mutation is present in some naturally occurring N1 isolates

To determine if G147R is present in naturally occurring influenza isolates, we examined all 19,528 N1 NA protein sequences in the Influenza Virus Resource (57) with an unambiguous identity at position 147. The vast majority of these sequences contain a G at position 147. However, 31 sequences contain the G147R mutation, and 24 contain the G147E mutation at this site. Table 2.3 gives a complete list of the sequences with G147R and their lineage. The majority of these sequences are from the human seasonal H1N1 lineage that circulated prior to the 2009 pandemic, but there are also several sequences from the 2009 swine-origin pandemic H1N1 lineage, and several from avian H5N1 lineages. All of these sequences have isolation dates after the year 2007 with the exception of one isolate from 2000 and two isolates from 2004.

We have no direct way to determine whether G147R was actually present in circulating viruses, whether it arose during tissue-culture adaptation prior to sequencing, or whether it was the result of a sequencing error. However, a phylogenetic analysis strongly suggests that a substantial number of the G147R sequences reflect the actual natural occurrence of this mutation. Specifically, as shown in Figure 2.8, there are two human seasonal H1N1 and one chicken H5N1 phylogenetic cluster of four or more sequences containing G147R. Since laboratory artifacts would likely occur fairly randomly among sequenced isolates, the existence of these phylogenetic clusters provides strong if circumstantial evidence that G147R has arisen naturally in N1 NAs several times during influenza’s evolution.
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Table 2.3 N1 sequences with R at position 147 (N2 numbering).
Figure 2.8. There are several phylogenetic clusters of N1 NA sequences containing G147R, suggesting that this mutation arose during natural evolution rather than as a laboratory artifact. (A) There are two clusters of sequences with G147R in human seasonal H1N1. (B) There is one cluster of sequences with G147R in chicken H5N1. In both trees, sequences with G147R have branches colored red and strain names shown, while sequences without G147R have branches colored black and lack sequence names. Clades without G147R are collapsed into triangles to shrink the size of the trees for visual display. Scale bars indicate a length equal to one year. In (A), there are two strains named A/Kentucky/08/2009 since there are two sequence variants for this strain in the Influenza Virus Resource.
DISCUSSION

We have described a surprising influenza mutant in which an N1 NA serves as the receptor-binding protein, enabling the virus to grow to high titers in the absence of HA receptor-binding function. HA is traditionally viewed as possessing two highly conserved functions: receptor binding and membrane fusion. While HA is still needed for fusion in our mutant viruses, it is remarkable to consider the evolutionary ease with which NA was able to co-opt the receptor-binding properties normally associated with HA. Specifically, the single amino-acid substitution G147R allows NA to mediate viral infection and red blood cell agglutination in a manner that can be reversibly blocked by NA inhibitors and anti-NA antibodies. Also surprisingly, the mutant G147R NA is still active as a sialidase with kinetics that are not dramatically different than those of the wild-type parental NA, at least on the single surrogate substrate for which we were able to obtain enzymatic parameters. Of course, it remains quite possible that the G147R NA binds but fails to cleave (or only cleaves very slowly) some other sialic-acid structure found on the cell surface. However, even in this case, our results show that it is possible for NA to evolve receptor-binding properties without completely sacrificing its sialidase activity.

Our results were unexpected because the receptor-binding and receptor-cleaving activities of influenza A are typically fully segregated between HA and NA. But several other viruses that use sialic acid as a receptor combine these activities within a single protein. For example, the single hemagglutinin-neuraminidase of human parainfluenza virus both binds and cleaves sialic-acid in a balanced fashion that enables effective viral infection and propagation (68). Similarly, the single hemagglutinin-esterase protein of influenza C binds to a sialic-acid structure that is then modified to a non-bindable form via the protein’s esterase activity (69). Our
findings indicate that a relatively small number of mutations are sufficient to allow influenza A to similarly combine the receptor-binding and receptor-destroying activities within NA, at least in tissue-culture settings.

The mutant G147R NA that we have described arose de novo during our experiments in the background of the NA from the lab-adapted WSN influenza strain, and was characterized in the context of a lab-generated chimeric virus. However, this same mutation is found in several natural clusters of human H1N1 and chicken H5N1 sequences, indicating that it also has arisen several times during natural evolution.

Our results are especially noteworthy because they come on the heels of several recent studies describing how a single mutation (D151G) has conferred receptor-binding activity on the NAs of some recent human H3N2 strains (38, 39, 54). It is possible that both the mutation that we have described and D151G are evolutionary oddities, with no real implications for influenza more generally. But given that the properties of both these mutations were only discovered by happenstance years after they began to appear in circulating influenza isolates, it is also worth considering that they might be indicative of an under-appreciated aspect of influenza biology. If receptor-binding NA variants turn out to be common, it would be interesting to examine their implications for influenza transmission, pathogenesis, and the effectiveness of humoral immunity that is typically envisioned as blocking HA-mediated attachment of viruses to target cells.
Influenza viruses with receptor-binding N1 neuraminidases occur sporadically in nature and show no attenuation in cell culture or mice


INTRODUCTION

The surface of an influenza virus contains about 400 trimers of hemagglutinin (HA) and about 100 tetramers of neuraminidase (NA) (70, 71). For decades, the understanding has been that the viral entry and release functions are partitioned neatly between these two proteins. Specifically, HA binds the virus to cell-surface sialic acids, and then after the virus is endocytosed, mediates fusion of the viral and host membranes (45, 46, 72). NA is a sialidase that cleaves the same cell-surface receptors that can be bound by HA, thereby facilitating release of free virions from host cells and viral aggregates (35). While enzymatic activity of NA likely contributes to the ability of influenza to penetrate airway mucins in vivo to reach target cells (53), NA is completely (35) or largely (37) expendable for entry in cell culture.

However the last few years have seen the characterization of several exceptions to this paradigm of neatly partitioned sialic-acid binding and sialic-acid cleaving activities. The most striking of these exceptions is a recently characterized lineage of influenza in bats, where HA lacks detectable sialic-acid binding activity and NA lacks detectable sialidase activity (73-76). But even in more canonical influenza lineages, a number of groups have described mutations that
confer receptor-binding activity on NA. The first such mutation to be characterized was D151G in NA of recent human H3N2 influenza (38). D151G alters a key residue in the NA active site, causing the protein to bind with increased affinity but then fail to cleave sialic acid moieties (39). A number of studies have presented evidence suggesting that D151G is not present in circulating viruses, but arises in cell culture because it provides NA receptor-binding activity that helps compensate for the reduced HA affinity of recent human H3N2 viruses for many types of sialic acid (38, 41, 54, 77, 78). According to this view, D151G is an interesting laboratory artifact that has little relevance for influenza in nature – and it certainly seems reasonable to suspect that D151G viruses that lack sialidase activity might be compromised in crucial aspects of their life cycle in natural settings.

We recently described another mutation that confers receptor-binding activity on NA, this time without greatly compromising sialidase activity (79). Specifically, in a series of experiments with heavily engineered viruses, we found that the mutation G147R confers receptor-binding activity on the NA from the lab-adapted H1N1 strain A/WSN/33 (79). We showed that the G147R A/WSN/33 NA was able to rescue growth of a virus in which the receptor-binding activity of HA had been eliminated by extensive mutagenesis, and that virus-like particles expressing this NA but lacking HA could agglutinate red blood cells (79). Interestingly, G147R is located above rather than directly in the NA active site, and this mutation only slightly decreases sialidase activity in the context of A/WSN/33 (79). These facts suggest that G147R might enable NA to bind receptors without severely compromising its other functions. In tentative support of this notion, we noted that G147R is present in a small number of reported sequences for human 2009 pandemic H1N1 (pdmH1N1), human seasonal H1N1, and chicken H5N1 lineages.
Here we characterize the effect of G147R on NAs from these three viral lineages. We show that in all three lineages, G147R enables NA to rescue growth of a virus with binding-deficient HA – yet in all cases the G147R NA retains substantial sialidase activity against the MUNANA substrate. We perform detailed characterization of the mutation’s effect in pdmH1N1 by reconstructing a virus that contains the HA and NA from a reported G147R isolate. We find that G147 and R147 variants of this virus are indistinguishable in their fitness in cell culture and pathogenesis in mice. However, G147R confers moderate resistance to neutralization by the Fab of a monoclonal antibody against the HA receptor-binding pocket. Overall, these results suggest that the G147R NA receptor-binding mutation occurs sporadically in circulating N1 lineages without greatly compromising replicative fitness.

METHODS AND MATERIALS

Analysis of N1 NA sequences and phylogenetic tree inference

N1 NA sequences in the Influenza Virus Resource (57) were analyzed for the occurrence of G147R. The input data, source code, detailed documentation, and a script enabling replication of the entire computational analysis are available at https://github.com/jbloom/NA_Mutation_Analysis for viewing and download.

Briefly, we downloaded all full-length NA coding sequences for pdmH1N, seasonal H1N1, and chicken H5N1. A total of 14,339 sequences were retrieved. We then aligned these sequences and counted the number of occurrences of each amino-acid identity at site 147 in each lineage.

To build phylogenetic trees, it was necessary to reduce the number of sequences to make the data set computationally tractable. We therefore retained only seasonal H1N1 sequences isolated in 2007 or later (with one exception, all G147R isolates fall into this range), only chicken H5N1
sequences isolated in 2004 or later (all G147R isolates fall into this range), and all pdmH1N1 sequences. For all lineages, we retained all G147R isolates in the date ranges. For pdmH1N1, we also retained the six unique non-G147R sequences with highest identity to each G147R isolate plus 10 randomly chosen sequences per year. For seasonal H1N1, we also retained the two unique non-G147R sequences with highest identity to each G147R isolate plus six randomly chosen sequences per year. For chicken H5N1, we also retained the four unique non-G147R sequences with highest identity to each G147R isolate plus 10 randomly chosen sequences per year.

After assembling these sequence sets, we inferred phylogenetic trees with codonPhyML (80) using the Goldman-Yang 1994 codon substitution model (81) with CF3x4 equilibrium frequencies (82), a single transition-transversion ratio estimated by maximum likelihood, and a single omega estimated by maximum likelihood. Branch supports were calculated using the SH-aLRT method (83). The trees were rooted using Path-O-Gen (http://tree.bio.ed.ac.uk/software/pathogen/) and visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). For compact visual display, many of the non-G147R clades are collapsed in the figure shown in this paper.

Reverse-genetics plasmids

We selected three G147R N1 NA sequences from the Influenza Virus Resource (57) for cloning into the pHW2000 reverse genetics plasmid (55). The three sequences are those reported for A/Finland/614/2009 (pdmH1N1), A/Texas/74/2007 (seasonal H1N1), and A/chicken/Bangladesh/12VIR-7140-3/2012 (chicken H5N1). These sequences were created by either sequential site-directed mutagenesis of existing plasmids or gene synthesis. We also created the G147 variant of each NA gene. Additionally, we used site-directed mutagenesis of an
existing plasmid to clone the HA sequence reported in the Influenza Virus Resource (57) for A/Finland/614/2009 (pdmH1N1) into pHW2000.

For some of our experiments, we used a binding-deficient HA and the binding-competent HA from which it was derived. Both of these HAs have been described previously (79). Briefly, the binding-deficient HA is that of the H3N2 strain X-31 with the following modifications (in H3 sequence numbering): N-linked glycosylation sites added at sites 45, 63, 126, 133, 144, and 246; mutations Y98F, H183F, and L194A at receptor-binding residues; deletion of the receptor-binding proximal loop spanning residues 221-228; and mutation K62E in the HA2 stalk (this is the variant referred to as PassMut in (79)). The paired binding-competent HA used here has all of the added glycosylation sites, but does not have the other point mutations or the loop deletion.

For some of our experiments, we generated viruses that packaged GFP in the PB1 segment and had all other internal genes from A/WSN/33. The plasmids for the internal genes for these viruses were pHH-PB1flank-eGFP (56), and pHW181-PB2, pHW183-PA, pHW185-NP, pHW187-M, and pHW188-NS (55).

For some of our experiments, we generated viruses containing the internal genes from the pdmH1N1 strain A/California/04/2009 (H1N1). The reverse genetics plasmids for these genes have been described previously (84).

Viral rescue and titering

To test whether the G147 and R147 NAs were able to rescue growth of virus with a binding-deficient HA (or its binding-competent counterpart), we generated viruses carrying this HA, the indicated NA, PB1 packaging GFP, and all other genes from A/WSN/33. These viruses were rescued and propagated in the PB1-expressing 293T and MDCK-SIAT1 cells described in (56) using the protocol described in (79). The viruses were titered by flow cytometry as described in
Briefly, the transfection supernatants were collected at 72 hours post-transfection, then used to infect $1 \times 10^5$ MDCK-SIAT1-CMV-PB1 cells. The following day, cells were collected and analyzed by flow cytometry to determine the percent GFP-positive. The amount of virus in the original supernatant was then back-calculated using the Poisson equation. Viruses containing the binding-competent HA with pdmH1N1 G147 and R147 NA, as well as virus with the binding-deficient HA and pdmH1N1 R147 NA packaging GFP also were used for oseltamivir neutralization assays as described in (79).

To measure the growth kinetics and final titer for the pdmH1N1 virus with either G147 or R147 NA, viruses containing the A/Finland/614/2009 HA and either R147 or G147 pdmH1N1 NA along with the internal genes from A/California/04/2009 were rescued in co-cultures of 293T and MDCK-SIAT1 cells, as previously described. 72 hours post-transfection, viral supernatants were collected and titered using the TCID50 assay. The Reed-Muench formula (https://github.com/jbloom/reedmuenchcalculator) was used to calculate the titer in TCID$_{50}$ units/$\mu$l.

**Measurement of NA surface expression, sialidase activity, and oseltamivir inhibition**

The NA genes were cloned into an expression vector under control of a CMV promoter with a C-terminal V5 epitope tag, followed by an internal ribosome entry site (IRES) driving the expression of GFP to quantify transfection efficiency. All assays were performed in transfected 293T cells using the procedures described in (79).

**Monoclonal antibody and Fab against HA**

The IgG of the human anti-HA monoclonal antibody 5J8 was produced as described in (85). The concentration was determined using Protein Determination Reagent (Hoefer, Inc. product number GR133-500). Full-length purified IgG was cleaved into Fab fragments using the Pierce
Fab Preparation kit (Thermo Scientific product number 44985). Fab fragments were buffer exchanged into PBS using Slide-A-Lyzer MINI Dialysis Devices, 20K MWCO (Thermo Scientific product number 88405) prior to neutralization assays.

**GFP-based viral neutralization assays**

All neutralization assays were performed using the viruses expressing GFP in the PB1 segment to quantify viral infection. Assays were read after 18 hours before secondary infection caused spread of GFP fluorescence. The procedures are described in detail in (79).

**Viral growth curves**

To determine the growth kinetics of pdmH1N1 variants with the R147 and G147 NAs, MDCK-SIAT1 cells were plated in 6-well dishes at a density of 2×10^5 cells/well in influenza growth medium (79) with 4 µg/ml TPCK-trypsin. Cells were allowed to adhere for 4 hours at 37°C then inoculated at an MOI of 0.01. Every 24 hours, an aliquot of the supernatant was collected and frozen at to -70°C and the volume removed was replaced with an equal amount of fresh medium. All aliquots for a given time point then were thawed at the same time and titered by TCID_{50}. For growth kinetics in A549 cells (ATCC CCL-185), 2×10^5 cells/well were plated in 6-well dishes in D10. The plates were placed in a 37°C incubator for 6 hours to allow cells to adhere. The media was then changed to influenza growth media with 4µg/ml TPCK-trypsin following a wash with 1ml PBS. The cells were then infected at an MOI of 0.1. Aliquot collection and titering was done as described for MDCK-SIAT1 cells.

**Mouse experiments**

Female BALB/cJ mice 8-weeks of age were purchased from The Jackson Laboratory. Mice were anesthetized by intraperitoneal injection of 2 mg of ketamine and 0.2 mg of xylazine in final volume of 0.2 ml per mouse, and then inoculated intranasally with a 20 µl volume containing 10^4
or $10^5$ TCID50 units of pdmH1N1 carrying either the R147 or G147 NA in the pdmH1N1 background. Control mice were inoculated in a similar fashion with PBS. Each treatment group consisted of 3 animals. The weight of each infected animal was monitored for 14 days after infection, and reported as a percentage of the starting weight on the day of infection. Animal work was approved under FHCRC IACUC protocol 1893.

RESULTS

NA$s$ with G147R are found in multiple N1 viral lineages

We have described previously how G147R confers receptor-binding activity on the NA of the lab-adapted H1N1 strain A/WSN/33 strain (79). In that previous work, we also noted that G147R is present in some reported human seasonal H1N1, pdmH1N1, and chicken H5N1 sequences.

To examine this issue more systematically, we analyzed all $\approx14,000$ NA sequences in the Influenza Virus Resource (57) from seasonal H1N1, pdmH1N1, and chicken H5N1. Table 3.1 shows that the vast majority of sequences in all three lineages contain G147; however, we did find sequences with G147R and G147E in all lineages.

We next sought to assess how the G147R sequences were related to each other and to sequences lacking this mutation. In the previous chapter, we reported that phylogenetic trees inferred using NA protein sequences indicate that many of the G147R sequences are evolutionarily related (79) – however, protein-based (and nucleotide-based) phylogenetic trees are less accurate than those inferred using codon sequences (86). To infer evolutionary relationships with maximal accuracy, we therefore used codonPhyML (80) to construct phylogenetic trees with a codon model of substitution (81). These trees are shown in Figure 3.1.
The G147R sequences are not monophyletic in any of the lineages, indicating that the occurrences of this mutation were not all due to a single evolutionary event. However there are clusters of G147R sequences in both seasonal H1N1 and chicken H5N1, suggesting that related viruses with this mutation may have persisted in natural populations for a sufficient duration to be isolated multiple times.

Overall, these computational analyses support the notion that viruses with G147R NAs occur sporadically in multiple influenza lineages. However, computational analyses alone cannot determine whether this mutation confers receptor-binding activity on these NAs in the same way that it does for the lab-adapted A/WSN/33 virus, nor can they ascertain definitively whether G147R was really present in circulating viruses or simply arose as a lab or sequencing artifact. We therefore used our computational results to design the experiments described in the rest of this paper.

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<td>Chicken H5N1</td>
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Table 3.1 Counts of different amino acids at NA position 147 in seasonal H1N1, pdmH1N1, and chicken H5N1 sequences in the Influenza Virus Resource.
Figure 3.1. Phylogenetic trees showing sequences with G147R (red) in (A) pdmH1N1, (B) seasonal H1N1, and (C) chicken H5N1. Branch supports are shown for key nodes; scale bars are in codon substitutions per site. The code and data to generate these trees are at https://github.com/jbloom/NA_Mutation_Analysis.
G147R NAs from seasonal H1N1, pdmH1N1, and chicken H5N1 rescue growth of virus with a binding-deficient HA

Previously, we showed that G147R enabled the NA of A/WSN/33 to rescue growth of a virus in which the receptor-binding activity of a HA had been obliterated by a combination of engineered point mutations and a deletion (79). To test if G147R NAs from pdmH1N1, seasonal H1N1, and chicken H5N1 possessed similar activity, we selected one representative R147 NA from each lineage in Figure 3.1. We selected A/Finland/614/2009 from pdmH1N1, A/Texas/74/2007 from seasonal H1N1, and A/chicken/Bangladesh/12VIR-7140-3/2012 from chicken H5N1. We cloned these R147 NAs into reverse-genetics plasmids (55), and also created an otherwise isogenic G147 version of each NA.

We then attempted to rescue by reverse genetics viruses with each NA paired with either our binding-deficient HA or the binding-competent HA from which it is derived (79). We efficiently rescued viruses with the binding-competent HA paired with either the R147 or G147 NA for all three lineages that replicated to high titers (Figure 3.2). However, we could only efficiently rescue viruses with the binding-deficient HA paired with the R147 NA – the binding-deficient HA did not support appreciable viral growth when paired with the G147 NA (Figure 3.2). These results suggest that G147R confers receptor-binding activity on NAs of pdmH1N1, seasonal H1N1, and chicken H5N1 much as it does for A/WSN/33.
Figure 3.2. G147R NAs from all three viral lineages rescue growth of virus with a binding-deficient HA. We used reverse genetics (55) to attempt to rescue viruses with either a binding-competent (Bind) or binding-deficient (ΔBind) HA (79), the indicated G147 or R147 NA, and internal genes from A/WSN/33 with GFP packaged in the PB1 segment (56). Shown are the viral titers in the supernatant at 72 hours post-transfection as determined by flow cytometry for GFP-positive cells. Virus with the binding-competent HA can be rescued efficiently with either the G147 or R147 NA, but virus with the binding-deficient HA is only rescued efficiently with the R147 NA. Shown are the mean and standard error of three independent replicates.
All of the G147R NAs are active sialidases that are inhibited by oseltamivir

One way that a mutation might confer receptor-binding activity on NA is by inhibiting its sialidase activity, so that the NA protein binds sialic-acid moieties in its active site but then fails to cleave them. This receptor-binding mechanism is used by D151G, which appears to arise in the NAs of recent human H3N2 NAs upon passage in cell culture (38, 41, 54, 77, 78). D151G N2 NAs have a markedly increased affinity for sialic acid (a ≈30-fold lower Michaelis-Menten $K_M$) and a dramatically decreased rate of substrate cleavage (a >100-fold lower Michaelis-Menten $k_{cat}$) (39). In contrast, G147R has only modest effects (less than two-fold) on the Michaelis-Menten enzymatic parameters for the A/WSN/33 NA on the surrogate substrate MUNANA.

To test the effect of G147R on the NAs of seasonal H1N1, pdmH1N1, or chicken H5N1, we cloned the NA coding sequences for the R147 and G147 variants into an expression plasmid with a C-terminal V5 epitope tag. We then quantified the effect of G147R on both the cell-surface NA protein expression and NA-mediated cleavage of the MUNANA substrate in transfected 293T cells (Figure 3.3A,B). In all three lineages, G147R had only a small effect (less than 1.5-fold) on NA surface expression. In pdmH1N1 and seasonal H1N1, G147R also had only a small effect (less than 2-fold) on $K_M$ and $k_{cat}$. In chicken H5N1, G147R did cause a more substantial (about 4-fold) decrease in enzymatic activity – but this effect still remains small compared to that observed for D151G in N2 NAs (39). Therefore, G147R only has a modest effect on the sialidase activity of NA on the MUNANA substrate for all three viral lineages.

We also tested whether G147R affected the inhibition of NA activity by the small-molecule drug oseltamivir (Figure 3.3C). All of the G147 and R147 NAs were potently inhibited by oseltamivir, demonstrating that G147R is not an oseltamivir-resistance mutation.
Figure 3.3. G147R NAs are active sialidases that are inhibited by oseltamivir. (A) Cell-surface expression, (B) sialidase activity, and (C) sialidase inhibition by oseltamivir for each R147 NA and its G147 counterpart. The NAs were cloned into an expression plasmid with a C-terminal V5 epitope tag and transfected into 293T cells, which were analyzed 20-hours post-transfection for cell-surface NA expression and sialidase activity. G147R had little effect on cell-surface expression or sialidase inhibition by oseltamivir. G147R did cause a modest decrease in sialidase activity for pdmH1N1 and a fairly sizable decrease for chicken H5N1, but in all cases NA retained substantial enzymatic activity. Shown are the mean and standard error of three independent replicates.
Oseltamivir inhibits infectivity of virus that depends on pdmH1N1 G147R NA for attachment

We focused the remainder of our studies on G147R in the NA of pdmH1N1, since this viral lineage is currently circulating in humans. In our previous work, we showed that the G147R NA of the A/WSN/33 strain mediated receptor binding via a mechanism that was dependent on the NA active site. To confirm that this same mechanism also was used by the G147R NA of pdmH1N1, we grew viruses containing our binding-deficient HA (79) and the receptor-binding R147 NA, or viruses containing the matched binding-competent HA and either the R147 or G147 NA. We then tested whether oseltamivir inhibited the infectivity of these viruses.

The infectivity of viruses with a binding-competent HA and the G147 NA was unaffected by oseltamivir (Figure 3.4), a finding consistent with the accepted view that oseltamivir does not affect the cell-culture infectivity of viruses that attach to cells via HA (35). However, oseltamivir ablated the infectivity of viruses with the binding-deficient HA and the R147 NA (Figure 3.4), consistent with the idea that these viruses attach to cells via a mechanism that involves the NA active site. The infectivity of viruses with a binding-competent HA and the G147 NA was inhibited partially by oseltamivir (Figure 3.4), consistent with the idea that these viruses can attach to cells via HA or NA.
Figure 3.4. Oseltamivir inhibits infectivity of virus dependent on the pdmH1N1 G147R NA for receptor binding. We used reverse genetics (55) to create viruses with a binding-competent (Bind) HA and either the R147 or the G147 pdmH1N1 NA, or with a binding-deficient (ΔBind) HA and the R147 pdmH1N1 NA (virus with the binding-deficient HA and the G147 NA cannot be created, see Fig. 2). The internal genes were from A/WSN/33, with GFP packaged in the PB1 segment (56). We inoculated target cells with these viruses in the presence of increasing concentrations of oseltamivir and quantified the amount of infection by GFP fluorescence. Shown are the mean and standard error of three independent replicates.
G147 and R147 NA pdmH1N1 viruses grow similarly in cell culture

We next examined the effect of G147R in the authentic background of pdmH1N1 viruses. There are pdmH1N1 strains in the Influenza Virus Resource that lack G147R but have HA and NA protein sequences that are otherwise identical to those of A/Finland/614/2009. We used reverse genetics to generate viruses that had the A/Finland/614/2009 HA, either the R147 A/Finland/614/2009 NA or its G147 counterpart, and the internal genes from the closely related pdmH1N1 strain A/California/04/2009.

To test the growth of these viruses in cell culture, we inoculated MDCK-SIAT1 cells at a low multiplicity of infection (MOI) and then monitored viral titers in the supernatant (Figure 3.5A). The growth kinetics and peak titers of the G147 and R147 variants were nearly indistinguishable.

We next performed similar growth curves in the human lung epithelial cell line, A549 (Figure 3.5B). Although the R147 NA variant appears to grows slightly better than the G147 NA variant, this result was not statistically significant when a t-test was applied with cutoff of p<0.05. Taken together, these results show that, at least in cell culture at the conditions tested, G147R does not have a strong effect on viral fitness.
Figure 3.5. Variants of pdmH1N1 with G147 and R147 NAs exhibit indistinguishable growth kinetics in cell culture. Viruses with the HA from A/Finland/614/2009 and either the R147 NA of this strain or its G147 counterpart were rescued by reverse genetics with the internal genes from A/California/04/2009. (A) MDCK-SIAT1 cells or (B) A549 cells were then inoculated at an MOI of 0.01 or 0.1, respectively, and viral titers in the supernatant were determined at 24 and 48 hours post-inoculation by TCID50. Shown are mean and standard error of three independent replicates. None of the differences between the G147 and R147 data points are significant at a p-value cutoff of 0.05 according to a t-test.
G147 and R147 pdmH1N1 variants cause similar pathogenesis in mice

To test if G147R affects viral pathogenesis in vivo, we inoculated mice with $10^4$ or $10^5$ TCID$_{50}$ of either the G147 or R147 pdmH1N1 viruses and monitored them for weight loss over 14 days (Figure 3.6). At both doses, both viral variants caused acute weight loss that peaked at three days post-infection, with all animals largely recovering within 14 days. There was no significant difference in weight loss between animals infected with the two viral variants, indicating that G147R does not strongly affect pathogenesis in mice.

Figure 3.6. Variants of pdmH1N1 with G147 and R147 NAs cause similar pathogenesis in mice. Mice were inoculated intranasally with (A) 104 or (B) 105 TCID50 of virus, or PBS. Animal weights were recorded for two weeks following infection. Each treatment group consisted of 3 animals, and the plots show the mean and standard error within each group. None of the differences between the G147 and R147 groups are significant at a $P$-value cutoff of 0.05 according to a t-test.
G147R-mediated NA receptor binding increases resistance to neutralization by an anti-HA Fab but not a full-length IgG

The dominant form of protective anti-influenza immunity is thought to be antibodies against the globular head of HA (13, 87). A major mechanism by which such antibodies neutralize influenza is by sterically blocking the binding of HA to cellular receptors (88-90). In principle, receptor-binding G147R NAs could enable influenza to evade such antibodies by providing an alternative mechanism of cellular attachment.

To test this idea, we performed neutralization assays using the anti-HA human monoclonal antibody 5J8, which inserts its complementarity determining region 3 loop into the receptor-binding pocket of the pdmH1N1 HA (85). The full-length 5J8 IgG neutralized both G147 and R147 NA variants of pdmH1N1 with equal potency (Figure 3.7A). We speculated that the inability of the R147 NA to provide resistance to neutralization by 5J8 might be because binding of this antibody to HA sterically inhibited receptor binding by NA. We therefore cleaved the full-length IgG to produce antigen-binding Fab domains, which have a smaller steric footprint. Indeed, the R147 variant exhibited a modest (about 5-fold) increase in Fab neutralization resistance relative to its G147 counterpart (Figure 3.7B), indicating that the receptor-binding NA provided a partially redundant mechanism of attachment after Fab binding to HA.

To confirm that this effect was real and reproducible, we repeated the neutralization assays with a new preparation of antibody and Fab. The R147 variant again was about 5-fold more resistant to the Fab (Figure 3.7C,D), confirming that the increased neutralization resistance is a genuine if modest effect.
If the increased resistance of the R147 variant to Fab neutralization is due to NA-mediated attachment, this effect should be abrogated by adding oseltamivir to block NA receptor binding. To test this idea, we performed neutralization assays in the presence of 100 nM oseltamivir, a concentration well above that which we showed in Fig. 3.4 is sufficient to completely inhibit infectivity of R147 NA viruses with a binding-deficient HA. As expected, oseltamivir increased the sensitivity of the R147 pdmH1N1 to the anti-HA Fab to a level comparable to that of its G147 counterpart (Figure 3.7D). Since oseltamivir alone did not neutralize pdmH1N1 variants with either G147 or R147 (Figure 3.7E), this result indicates that R147 increases resistance to the anti-HA Fab by enabling NA to provide a partially redundant mechanism of viral attachment.
Figure 3.7. A pdmH1N1 virus with the receptor-binding G147R NA has modestly increased resistance to neutralization by the Fab of an antibody that targets the HA receptor-binding pocket, but no change in sensitivity to the full-length IgG. (A) The full-length IgG neutralizes R147 and G147 variants of pdmH1N1 with equal potency. (B) However, the variant with the receptor-binding R147 NA has increased resistant to the antibody Fab. (C), (D) These results were repeatable with a second independent preparation of antibody. Oseltamivir abrogates the increased neutralization resistance of the R147 NA variant to the Fab (D), but (E) oseltamivir alone has no neutralizing activity.
DISCUSSION

Prior work has clearly demonstrated that single amino-acid mutations are sufficient to transform NA into a receptor-binding protein \((38, 39, 54, 79)\). The question that we have attempted to address here is whether viruses with such receptor-binding NAs have the potential to be anything more than interesting artifacts that can arise occasionally when influenza viruses are passaged in the lab.

We focused our studies on the NA receptor-binding mutation G147R, which is found at low frequency in the reported sequences of viruses from several lineages with N1 NAs. The fact that a few of the occurrences of this mutation involve small groups of sequences that cluster on phylogenetic trees weakly suggests that this mutation might sometimes persist long enough in natural settings to be transmitted among several hosts. However, it also remains possible that the G147R sequences in public databases represent sequencing errors or variants selected during lab passage prior to sequence analysis. Because we do not have access to the un-passaged samples for any of these reported G147R isolates, we instead investigated this mutation by experimentally characterizing its effect.

We found that G147R confers receptor-binding activity on NAs from three lineages: pdmH1N1, seasonal H1N1, and chicken H5N1. In all cases, the G147R NAs were active sialidases, recapitulating our earlier findings with the lab-adapted A/WSN/33 strain that G147R receptor binding does not come at the cost of enzymatic function \((79)\). The exact mechanism of G147R-mediated receptor binding therefore remains unclear. G147R receptor binding clearly involves the NA active site since it is inhibited by oseltamivir, but it is not due to the simple elimination of catalytic activity that underlies the receptor-binding mechanism of the D151G mutation to N2 NAs \((39)\). As discussed in our previous work \((79)\), we hypothesize that G147R
causes NA to bind to some specific glycan structure that it then cleaves inefficiently, thereby enabling the protein to promote viral attachment while at the same time cleaving its normal sialic-acid substrates. However, rigorous testing of this hypothesis will require new experimental approaches that can provide more than the circumstantial evidence presented in our current and previous (79) work.

To assess whether G147R affects viral fitness, we reconstructed a pdmH1N1 virus with the HA and NA of an isolate reported to carry this mutation. We did not detect an effect of G147R on viral replication in cell culture or on pathogenesis in mice – indeed, the only phenotype of G147R that we were able to detect was a modest increase in resistance to neutralization by an anti-HA Fab. We did not test the effect of G147R on virus transmission, so it is possible that G147R still exerts a deleterious effect there. Nonetheless, the fact that G147R is compatible with efficient viral replication supports the idea that this mutation may authentically occur in some naturally occurring influenza strains. However, G147R does not appear to play an essential role in the cellular attachment of such viruses, since the HA of the G147R isolate that we characterized is fully sufficient to support viral growth without this NA receptor-binding mutation.

If NA receptor binding mutations do indeed occur in circulating viruses, perhaps the most biomedically relevant question is whether they alter viral sensitivity to anti-HA antibodies. Our results suggest that any increased resistance to anti-HA antibodies in viruses with G147R is modest. The G147R pdmH1N1 variant is about five-fold more resistant to an anti-HA Fab, but it has no additional resistance to the full-length IgG. The most likely explanation is that large antibodies bound to HA sterically inhibit NA-mediated cellular attachment; such antibodies might also impair viral fusion or promote intracellular neutralization. In any case, our results
suggest that NA receptor-binding mutations are unlikely to cause wholesale changes in the
effectiveness of anti-HA humoral immunity, although they might lead to mild changes in
sensitivity to certain antibodies.
Chapter IV

Implication of Influenza viruses with receptor-binding neuraminidases

The studies undertaken in this dissertation have clearly demonstrated that a single amino acid mutation is sufficient to allow NAs of the N1 subtype to act as the receptor-binding protein in the absence of HA-mediated binding. This finding comes on the heels of the discovery of another single amino acid mutation in the N2 subtype NA that also allows those NAs to act as the receptor-binding protein. However, the mechanism by which these two subtypes of NA achieve a common function appears to be dramatically different.

In the case of N2 NAs with the D151G mutation, the protein has traded one function for another. That is to say, the mutation “breaks” the enzymatic function of the protein by drastically reducing the $k_{cat}$ of the sialic acid cleavage reaction (39). The $K_M$ of the enzyme for its substrate is also significantly reduced (39), indicating that the protein has now shifted from an enzyme to a substrate-binding protein. The mutant NA is thus able to bind sialic acid with high affinity which likely allows time for endocytosis of the virus to occur. It is less clear how these viruses are then able to exit cells, although this mutation appears to be a result of tissue culture selection where the need for strong NA enzymatic activity may be greatly reduced (41). It has also been recently reported that another tissue culture adaptation mutation, T148I, may also allow N2 NAs to act as the receptor-binding protein (91). This raises some interesting questions as to what the entire collection of NA binding mutations for a given subtype of NA might be.

In contrast, the G147R mutation in the N1 NA background does not appear to greatly compromise the enzymatic function, at least against the surrogate substrate used for my assays (79). For all four N1 NAs tested, the change in enzyme kinetic parameters were about two-fold (92), a small change compared to those seen for the D151G N2 NAs. This suggests that these
proteins have retained their enzymatic activity while also acquiring a new ability to bind to an unidentified cellular receptor. I hypothesize that there is likely a specific glycan structure that the mutant NAs can now bind tightly, but cleave either very slowly or not at all. However, they appear to retain the ability to cleave many common sialic acid moieties to allow for efficient release. In this way, by binding to one set of receptors and cleaving another, the same protein can perform both functions to allow for efficient viral growth.

This mechanism is in fact that which is used by another family of RNA viruses, the parainfluenza viruses. These viruses also have two coat proteins which perform the same basic functions of receptor binding, membrane fusion, and receptor cleavage. The first surface protein, HN, performs the receptor binding and cleavage functions while the second main surface protein F, performs the fusion of the viral and endosomal membranes (93). The mutant NA viruses with the ΔBind HA now have a very similar partition of functions where NA is both the receptor-binding and receptor-cleaving protein, while HA is the fusion protein.

The mechanism of the G147R mutant NA binding some sialic acid structures it does not cleave remains purely a hypothesis. However, the tools with which to probe this mechanism are rapidly improving. Notably, arrays in which glycans are spotted on a glass slide (33) similar to conventional DNA or RNA microarrays, are becoming commonly used by many in the field to determine what specific structures a given viral variant can recognize. Fluorescently-labelled protein or virus can be flowed across the surface of the array and a detection probe used to determine which spots on the array the virus/protein binds to. Presently, these arrays contain 610 target glycans, with 166 of them terminating in sialic acid moieties (94). The arrays have been successfully used to determine the nature of D151G N2-mediated binding and so, in principle, the same approach could be applied to the G147R N1 NAs.
By performing the arrays at 4°C, one can determine which sialic acid structures the virus can bind, then by raising the temperature to 37°C, the virus will elute off of the spots on the array for which NA is active in cleaving that particular glycan structure. For our mutant G147R NAs, we would expect the virus to elute off a subset of spots, but to remain bound to others. Analysis of the binding and cleaving patterns might be expected to produce a consensus among glycans with a particular linkage or branching pattern for which NA can cleave and a separate consensus for where it remains bound. Together with data about the types of glycans present in human airways, this may help shed significant light on both the mechanism of NA-mediated entry for human viruses, as well as potential physiological implications.

Another area of NA-binding viruses which remains to be explored is the effect of this mutation on transmission of the virus. Transmission studies involve the use of ferrets as a model system since ferrets are capable of spreading the virus both through direct contact (sharing of a housing cage) as well as through aerosols (sharing of adjacent housing cages) similar to the spread of the virus through the human population. While I did not find a viral growth defect \textit{in vitro} or an effect on pathogenicity \textit{in vivo} for the pdmH1N1 virus, I hypothesize that this mutation may not be more widespread in nature because it causes a defect during the transmission stage of the viral life-cycle. When both entering and exiting a particular host, the virus must be able to avoid becoming trapped in the mucins in the airways which are rich in sialic acids (53). It is plausible that a virus which has two surface proteins capable of binding sialic acid may not be able to effectively navigate through the extensive mucin network. Thus measuring the ability of G147 versus R147 NA pdmH1N1 virus to transmit between ferrets may help provide an explanation for the relatively low frequency of this mutation in natural isolates.
Finally, I feel there are still open questions as to whether the NA-binding mutation might allow for escape from anti-HA immunity. In my studies with a single monoclonal anti-HA antibody, the answer appears to be no. However, I think that in the presence of a sub-optimal immune response, it may be possible. To test this, it would be interesting to look, for example, at the protection from neutralization by antibodies raised against a slightly different strain of virus. This is the situation the virus might encounter if an individual was infected or vaccinated with influenza several seasons ago, but the virus has now drifted to mutate the epitopes strongly recognized by antibodies. In this case where antibody binding might be of weaker affinity or overall reduced for the number of antibodies that can bind a given viral particle, NA-mediated binding may allow for viral infection to occur. Following initial infection, many copies of the viral RNA will be generated which will introduce errors at the rate of approximately one base pair per genome per round of replication. If these errors are introduced into HA epitopes, the progeny virus will be more effective at escaping anti-HA antibodies and that variant will quickly be selected for. Subsequent rounds of infection can then drive further antibody escape mutations until the virus drifts sufficiently far to no longer be recognized by antibodies.

The time scale of such evolution is likely to occur over many rounds of infection in different hosts which will all impose different selective pressures based on the antibody repertoire of any given person. In this way, maintenance of the NA-binding mutation may promote evolvability of the virus by relieving the necessity for HA to retain its receptor-binding capability while acquiring antibody-escape mutations. The epitopes targeted by anti-HA antibodies are often clustered around the receptor-binding pocket. Thus, mutations which may promote antibody escape may also decrease sialic acid binding by HA. In the absence of NA-mediated binding, these mutations are likely to make viral progeny less fit and will be purged.
from the population. However if the virus also carries the NA-binding mutantation, this effect may not be as detrimental and thus that variant will remain viable in the population.

My studies of the N1 mutant NAs have also led me to questions about the mechanism of N2 NAs. The first paper in this field reported that they were able to grow pure populations of virus carrying either the D151 NA variant or the G151 NA variant and that both proteins retained their sialidase activity. However, later studies showed that the G151 mutant is almost completely deficient as an enzyme. Later studies would also lead the authors to conclude, “We and others have been unable to clone a population with 100% [G151] mutation, presumably because some NA activity is required for virus propagation” (94) so it seems likely that the G151 populations grown in the original paper in fact contained at least low levels of the G151D revertant.

This caught my attention because it makes intuitive sense that a virus that is completely lacking in NA enzymatic activity cannot grow efficiently. I then wondered if perhaps this meant the virus was instead existing and acting as a quasispecies. In other words, is the virus making use of both NA variants for different functions: the G151 for binding and viral entry and the D151 for cleaving and viral release? The problem this mechanism presents is that the virus is effectively haploid and can carry only one NA gene variant. However, if a cell were co-infected with viral particles each carrying a different genotype, both versions of the protein would be made by that cell. Budding viruses would then have essentially equal chances of carrying the gene segment for one variant or the other, while retaining both versions of the protein on their surface. So long as infection continued to occur by viruses carrying both gene segments, this viral quasispecies could be maintained.

As a preliminary test of this hypothesis, I attempted viral rescue via transfection of 293T-CMV-PB1 and MDCK-SIAT1-CMV-PB1 co-cultures as has been previously described. The HA
and NA genes used were from an H3N2 strain reported in the Influenza Virus Resources database as containing the D151G NA mutation. All other internal genes are from the A/WSN/1933 lab-adapted strain of virus. Both the D151 and G151 variants of NA were separately cloned into the reverse genetics backbone. I then did transfections with either a pure population of D151 plasmid, a pure population of G151 plasmid, or equal molar ratios of both plasmids. Results are shown in Figure 4.1. I find that, as expected, the D151 variant rescues to moderate viral titers while the G151 does not efficiently produce virus (and in fact, “virus” which contains only HA and no NA protein also rescues to low levels). In line with my hypothesis about both NA variants being needed for optimal growth, I find that the co-transfection of both plasmids leads to higher levels of viral growth than either NA alone.
Figure 4.1. H3N2 virus with both D151 and G151 NA is rescued to higher titers than either NA variant alone. Viral rescue was attempted with the HA gene from A/Hanoi/Q118/2007, either the D151 or G151 NA variant from the same virus, and internal genes from A/WSN/1933 where PB1 was replaced with PB1-flank-eGFP (56). Viral rescue was also attempted by mixing equal plasmid proportions of D151 and G151 NA. Virus with D151 NA rescues to moderate levels while virus with G151 NA does not rescue efficiently. Virus derived from transfection with both NA variants rescues more than an order of magnitude better than either variant alone.
This finding provides a basic proof-of-principle for the idea that both NA variants lead to more efficient viral replication. Current studies are now focused on producing pure populations of D151 and G151 viruses which will be then be used to co-infect cells at different ratios of D151 to G151 virus. We hypothesize that there may be an optimal ratio of D151:G151 which leads to the highest level of viral replication. By deep-sequencing the population of virus derived from these co-infections, we can determine the ratio of D151 to G151 genotype to see if initial infections ultimately converge on a single ratio. This would provide the first evidence, to the best of our knowledge, that in fact two influenza virus variants are needed for optimal growth of the entire population. Such a quasispecies phenomenon has previously been demonstrated for another RNA virus, poliovirus, but this finding has yet to be expanded into other families of viruses (95).

Overall, these studies demonstrate that the neat partitioning of entry and exit functions between the two surface proteins of influenza does not always occur. In the case of H3N2 viruses, receptor-binding by NA is becoming ever more prevalent, at least when the viruses are passaged in tissue culture prior to analysis. For H1N1 and H5N1 viruses, it seems that receptor-binding NAs may appear occasionally in naturally circulating viruses and may persist long enough to be transmitted between at least 2 hosts. The receptor-binding function of NA needs to be taken into consideration not only for performing in vitro receptor-binding and antigenicity assays, but potentially also when designing vaccines which focus on HA as the primary immunogen. What remains to be seen is the extent to which NA receptor binding will become more prevalent in the future and what effect this will have from a public health standpoint. Regardless, it is worth remembering that in many ways, viruses with short replication times and vast reproductive numbers are always one step ahead of our current understanding.
References

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92. **Hooper, K. A., J. E. Crowe, Jr., and J. D. Bloom.** 2015. Influenza viruses with receptor-binding n1 neuraminidases occur sporadically in several lineages and show no attenuation in cell culture or mice. Journal of virology 89:3737-3745.


Curriculum Vitae
Kathryn A. Hooper

Education

**Doctor of Philosophy**, Molecular and Cellular Biology
University of Washington  Seattle, WA  07/2011 – 06/2015

**Bachelor of Science**, Biochemistry and Microbiology

**Associate of Arts and Science with Honors**

Research Experience

**Graduate Research Assistant**
Fred Hutchinson Cancer Research Center  Seattle, WA  07/2011 – 06/2015
- Advisor: Dr. Jesse Bloom
- Committee members: Drs. Daniel Stetson, Julie Overbaugh, Roland Strong, and Adam Geballe
- **Dissertation: Influenza viruses with receptor-binding neuraminidases**
  - I have identified and characterized a novel mutation that allows neuraminidase to co-opt the receptor-binding function normally performed by hemagglutinin.
  - This work involved the development and optimization of several novel assays now widely used by the Bloom Lab as well as other influenza labs across the country.
  - I successfully earned a spot on the highly-competitive Cell and Molecular Biology Training Grant which has supported me for years three and four of my dissertation research.
  - I was selected as one of 44 students out of over 1,800 applicants to present my work at the St. Jude Children’s Research Hospital National Graduate Student Symposium in April 2015
  - As a graduate student I also mentored and supervised several undergraduates in the lab performing various molecular cloning projects, as well as tissue-culture viral growth experiments.
  - Primary techniques utilized include: tissue culture transfection and transduction, viral rescue and growth experiments, murine models.

**Undergraduate Research Assistant**
Fred Hutchinson Cancer Research Center  Seattle, WA  03/2010 – 06/2011
- Advisor: Dr. Michael Emerman
- **Project: The evolution of the HIV Nef accessory protein in cross-species transmission of HIV**
  - My work focused on mapping the interacting domains of chimeric HIV/SIV Nef proteins with the chimpanzee restriction factor, tetherin.
  - For my efforts on this project, I was named a Mary Gates Scholar and awarded a $4,000 scholarship.
  - Primary techniques utilized include: Western blots, luciferase assays, flow cytometry.
Science and Engineering Education Intern
- Advisor: Dr. Cheryl Baird
- **Project: Isolation and characterization of scFvs from a yeast surface-display library**
  - My work involved the use of a yeast-surface display library to isolate novel scFvs derived from both the human and mouse antibody repertoire, followed by optimization of affinity and specificity.
  - Primary techniques utilized include: FACS and MACS cell sorting, molecular cloning, antibody isolation and purification.

- **Publications**

  Hallstrand TS, Lai Y, **Hooper KA**, et al. Endogenous secreted phospholipase A2 group X regulates cysteinyl leukotrienes synthesis by human eosinophils. *In press, Journal of Allergy and Clinical Immunology.*

  **Hooper KA**, Crowe JE, and Bloom JD. Influenza viruses with receptor-binding N1 neuraminidases occur sporadically in several lineages and show no attenuation in cell culture or mice. Journal of Virology. 2015 Apr; 89(7):3737-3745.


  **Hooper KA** and Bloom JD. A mutant influenza virus that uses an N1 neuraminidase as the receptor-binding protein. Journal of Virology. 2013 Dec; 87(23):12531-40.
  - Selected as an *Article of Significant Interest* by the Editors

- **Technical Expertise**

  **Assay development**
  - Influenza GFP-based neutralization assay
    - I developed and optimized a GFP-based neutralization assay for influenza that can be used to test the neutralization potency of antibodies and small molecules. This approach greatly reduces both the time and cost traditionally associated with high-throughput neutralization assays. The assay has been successfully used by our lab for a number of projects and is now also in use by labs at numerous institutions.
  - Cell-based MUNANA assay
    - I developed a cell-based assay in which 293T cells are transfected with a plasmid encoding the influenza neuraminidase (NA) protein which is folded and trafficked to the cell surface through normal cellular pathways. This method circumvents the traditional need to purify either large amounts of protein which is both cost and time intensive, or live influenza virus, the production of which can give rise to NA mutants that may skew the results.
  - Human airway epithelial models
    - In order to assess influenza fitness in a more clinically relevant setting, I am developing an *in vitro* human airway epithelial system in an air-liquid interface. I am using this model both to measure the viral titer obtained from human epithelial cells and also to perform immunohistochemistry staining.
Cell and viral culture (BSL1 and BSL 2+)
Maintenance, transfection and transduction of numerous immortalized cell culture lines, production of influenza viruses by reverse genetics, TCID$_{50}$ assays, plaque assays, neutralization assays

Molecular biology
RNA isolation, PCR and RT-PCR, molecular cloning in E. coli, SDS-PAGE protein gels, Western blots, ELISAs

Protein production and purification
Antibody isolation from mouse serum and cell culture by Protein A column, purification by size exclusion chromatography, production of Fab and Fab’2 fragments from purified antibodies

Flow cytometry
Cell-surface and intracellular antigen staining, FlowJo data analysis, instrumentation: BD FACS Aria, Canto 2, Miltenyi autoMACS

Murine models
Intramuscular immunizations under isoflurane, intranasal influenza inoculation of anesthetized mice, cardiac puncture blood retrieval

Grants, Scholarships, and Awards

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Meetings and Presentations

Hooper KA and Bloom JD. Influenza viruses with receptor-binding N1 neuraminidases. St. Jude Children’s Research Hospital National Graduate Student Symposium; poster and oral presentation. April 2015.


Hooper KA and Bloom JD. Influenza viruses with receptor-binding N1 neuraminidases. NW American Society for Microbiology Meeting; poster presentation. October 2014.
Teaching Experience

Teaching Assistant for Laboratory Courses, University of Washington
- Microbiology 431: Recombinant DNA Techniques, Winter quarter 2013
  o Supervised 18 students throughout the 10 week laboratory course to ensure safety and procedural protocols were followed
  o Instructed students on background material and specific techniques such as DNA isolation and cloning, PCR and RT-PCR, Western blots, etc.
  o Wrote quiz and exam material, proctored quizzes and exams, and graded quizzes, exams, and lab notebooks
- Microbiology 302: General Microbiology Laboratory, Spring quarter 2011
  o Supervised 24 students throughout the 10 week laboratory course to ensure safety and procedural protocols were followed
  o Instructed students on background material and specific techniques such as aseptic technique, isolation and identification of bacteria from clinical and environmental samples, lytic vs. lysogenic phage infection, etc.
  o Proctored and graded weekly quizzes, as well as mid-terms and final exams, and graded lab notebooks

Teaching Assistant for Lecture Courses, University of Washington
- Biochemistry 442: Biochemistry and Molecular Biology, Spring quarter 2013
  o One of the Top Two TAs in the University of Washington School of Medicine based on student evaluations
  o Presented new material and clarify concepts in two weekly small group sections related primarily to cell biology and cancer
  o Wrote material for weekly quizzes, as well as mid-term and final exams
  o Held weekly office hours to provide students with additional assistance on an individual basis
- Biochemistry 441: Biochemistry and Molecular Biology, Winter quarter 2011
  o Led two weekly discussion sections to clarify concepts taught in lecture section related primarily to metabolism
  o Assisted in proctoring and grading weekly quizzes, mid-term and final exams