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# Analysis of Influenza Virus Hemagglutinin Receptor Binding Mutants with Limited Receptor Recognition Properties and Conditional Replication Characteristics▽

Konrad C. Bradley,[1] Summer E. Galloway,[1] Yi Lasanajak,[2] Xuezheng Song,[2] Jamie Heimburg-Molinaro,[2] Hai Yu,[3] Xi Chen,[3] Ganesh R. Talekar,[1] David F. Smith,[2] Richard D. Cummings,[2] and David A. Steinhauer[1]*

*Department of Microbiology and Immunology, Emory University School of Medicine, O. Wayne Rollins Research Center, Atlanta, Georgia 30322[1]; Department of Biochemistry and The Glycomics Center, Emory University School of Medicine, O. Wayne Rollins Research Center, Atlanta, Georgia 30322[2]; and Department of Chemistry, University of California, Davis, California 95616[3]*

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**To examine the range of selective processes that potentially operate when poorly binding influenza viruses adapt to replicate more efficiently in alternative environments, we passaged a virus containing an attenuating mutation in the hemagglutinin (HA) receptor binding site in mice and characterized the resulting mutants with respect to the structural locations of mutations selected, the replication phenotypes of the viruses, and their binding properties on glycan microarrays. The initial attenuated virus had a tyrosine-to-phenylalanine mutation at HA1 position 98 (Y98F), located in the receptor binding pocket, but viruses that were selected contained second-site pseudoreversion mutations in various structural locations that revealed a range of molecular mechanisms for modulating receptor binding that go beyond the scope that is generally mapped using receptor specificity mutants. A comparison of virus titers in the mouse respiratory tract versus MDCK cells in culture showed that the mutants displayed distinctive replication properties depending on the system, but all were less attenuated in mice than the Y98F virus. An analysis of receptor binding properties confirmed that the initial Y98F virus bound poorly to several different species of erythrocytes, while all mutants reacquired various degrees of hemagglutination activity. Interestingly, both the Y98F virus and pseudoreversion mutants were shown to bind very inefficiently to standard glycan microarrays containing an abundance of binding substrates for most influenza viruses that have been characterized to date, provided by the Consortium for Functional Glycomics. The viruses were also examined on a recently developed microarray containing glycans terminating in sialic acid derivatives, and limited binding to a potentially interesting subset of glycans was revealed. The results are discussed with respect to mechanisms for HA-mediated receptor binding, as well as regarding the species of molecules that may act as receptors for influenza virus on host cell surfaces.**

Influenza A viruses belong to the order *Orthomyxoviridae* and are responsible for significant annual morbidity and mortality. They are maintained enzootically in waterfowl species and are classified serologically based on the antigenic properties of their surface glycoproteins: the hemagglutinin (HA) and the neuraminidase (NA). In the environment, 16 HA subtypes and 9 NA subtypes have been identified in various HA-NA combinations in waterfowl. In these natural hosts, influenza A virus infections are typically asymptomatic, though a limited subset of H5 strains have been observed to cause disease (3, 23, 38, 59). With respect to human susceptibility, viruses of several of the HA subtypes, such as H5, H7, and H9, have been implicated in small-scale avian-to-human transmission (12, 34, 43, 48, 82). However, in the past century, only viruses of the H1N1, H2N2, and H3N2 subtypes have developed the capacity to be transmitted efficiently and circulate extensively in the human population. In addition, several subtypes of influenza A virus have also been observed to spread to other mammalian species, such as pigs, horses, seals, whales, dogs, and mink, and influenza A viruses are a perpetual problem for poultry worldwide (78).

The HA glycoprotein is responsible for the receptor binding and membrane fusion functions required for virus entry. It has generally been accepted that one major factor for virus host range and the capacity to cross species barriers involves the recognition of appropriate receptors on cells of mucosal surfaces at sites of infection. For influenza viruses of all types, data have accumulated over decades to show that cell surface glycans containing various forms of sialic acid at their termini constitute the principal attachment targets (21, 32, 39, 45, 51, 77, 79). Sialic acids are ubiquitous, nine-carbon, acidic sugars commonly expressed as the terminal sugar on mammalian glycans. In general, mammals express both the *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) derivatives of sialic acid, but humans synthesize only Neu5Ac, most likely due to evolutionary pressure exerted by pathogens (71). In addition, dozens of modified Neu5Ac species and other sialic acids are synthesized by mammals, such as 9-*O*-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Emory University School of Medicine, O. Wayne Rollins Research Center, 1510 Clifton Rd., Atlanta, GA 30322. Phone and fax (404) 712-8542. E-mail: dsteinh@emory.edu.

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lactoyl-acetylneuraminic acid (Neu5Ac9Lt) and 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (KDN; deaminated neuraminic acid), the latter of which is abundant on human erythrocytes, though their recognition by influenza viruses has not been examined in any detail.

Human influenza virus isolates bind Neu5Ac in either an α2,3 or α2,6 conformation, depending on the specificity of the HA protein expressed on the surface of the virion (9, 28, 51, 53, 61). Generally speaking, human and swine influenza virus isolates preferentially bind α2,6-linked sialic acid (5, 15, 33, 67), while avian isolates preferentially bind α2,3-linked sialic acid (14). This correlation with specificity corresponds, to a degree, with the availability and density of such glycans at the principle sites of infection in the host. For example, α2,3-linked glycans have been reported to predominate in the intestinal tract of ducks, which is where viral replication generally occurs in natural infection (2, 27, 31), while in humans, α2,6-linked glycans predominate in the upper respiratory tract (58). In swine species, which on occasion may provide the conduit for transmission of pandemic viruses from avian species to humans, both α2,3- and α2,6-linked glycans can be detected in the upper respiratory tract, where replication takes place (28, 70).

In addition to the linkage specificity of terminal sialic acid, other aspects of the chemistry of the carbohydrate component of receptor molecules appear to play a role in binding as well. Using glycan microarray technology, we and others noted that influenza viruses show enhanced binding to glycans containing internal sulfate or fucose groups, which seems to affect the binding efficiency among receptors that are otherwise similar or identical at the termini of the carbohydrate chains (5, 13, 33). In addition, proton nuclear magnetic resonance (NMR) studies of the binding affinities of receptor analogs that were chemically modified at particular positions of the sialic acid structure showed that bulkier functional groups are tolerated at particular positions and orientations and others are not (55), confirming the crystallographic data on how sialic acid is positioned in the HA binding pocket (11, 17, 54, 55, 77, 79). The significance of the role of modified sialic acid in natural binding is currently unclear, but it has been noted that equine influenza A viruses favor Neu5Gc rather than Neu5Ac forms of the terminal component of the carbohydrate chain (29, 52), and influenza C virus strains have a preference for 9-*O*-acetyl sialic acid species (52).

The region of HA responsible for receptor binding resides at the membrane-distal tip of each monomer of the HA trimer, and it has four main structural features, some of which are highlighted in Fig. 1A. The binding site is flanked by the 220 and 130 loops, which contain amino acids that interact with sialic acid or internal sugars of the glycan chain. The membrane-distal region of the site is formed by the 190 helix, which also includes residues with the potential to contact the receptor at either the sialic acid (residue 194) or internal glycans on the receptor (residues 190 and 193) (36, 53). The base of the site contains several highly conserved residues that form an extensive hydrogen bond network (55, 77, 79).

Reports identifying specific HA residues that can mediate discrimination between receptors with either α2,3- or α2,6-linked sialic acid have been limited to a selection of viral subtypes and strains, and for the most part, residues in the sialic acid binding region have been implicated. For example, with H2 and H3 viruses, amino acids in the 220 loop, at positions 226 and 228, have been linked closely to receptor binding specificity (53, 73). For some H1 subtype viruses, such as the H1N1 strains responsible for the 1918 and 2009 pandemics, residue 190 in the short helix and residues in the 220 loop have been demonstrated to affect specificity (17, 19, 36). Furthermore, studies on H5 subtype viruses have highlighted additional residues for their potential role in binding specificity, particularly at HA positions 186 and 193 and at position 158, where the presence or absence of a carbohydrate in combination with a mutation at position 227 has been notable for its effects on receptor binding (83, 84).

Our studies on a mutant HA that is inhibited in binding (Y98F mutant) and on pseudorevertant second-site mutant viruses derived from it suggest that a number of alternative structural mechanisms exist for adaptation of binding properties and also indicate that a broad range of HA positions throughout the structure may have the capacity to influence binding. HA1 position 98 is a highly conserved tyrosine at the base of the binding pocket (Fig. 1A), and mutation of this residue to phenylalanine resulted in a virus that bound very poorly to human, chicken, or turkey erythrocytes, yet this virus was generated rather easily by reverse genetics (37). The replication properties of the Y98F virus were found to be highly dependent upon the system used, as it reached titers comparable to those of wild-type (WT) virus in MDCK cells and embryonated chicken eggs but was highly attenuated in cells with limited sialic acid on the surface or in respiratory tracts of mice following intranasal infection. Upon passage of the Y98F virus in mice, we noted that in addition to viruses with a reversion at position 98, a large proportion of mutant viruses that were isolated were found to contain second-site pseudoreversion mutations and to also maintain the Y98F mutation (41). The locations of these second-site mutations included positions proximal to the binding site, positions that are likely to affect binding indirectly (referred to here as "second shell" positions), positions that may affect the location of carbohydrates originating on the adjacent monomer relative to the binding pocket, and positions that may affect monomer-monomer or subunit interfaces and alter binding from a distance. In the present study, we expanded the aforementioned studies by characterizing several of the pseudorevertant viruses isolated from mice as well as viruses isolated following passage of the Y98F virus in MDCK cells. Our results show that the second-site mutations significantly alter the binding properties of HA as well as the replication properties of the viruses in cell culture or in mice. Furthermore, we assessed binding of the mutant viruses by using two different types of glycan microarrays, including a novel array containing glycans modified to include derivatized sialic acids. For most of our pseudorevertant viruses, little or no high-efficiency binding was detected, indicating that the viruses may utilize a limited subset of attachment molecules not yet represented on the current arrays, and possibly suggesting a more complex mechanism for attachment and initiation of infection than is currently appreciated.

## MATERIALS AND METHODS

**Viruses and cells.** Virus stocks were prepared for analysis in MDCK cells by using standard protocols. Briefly, 85 to 90% confluent MDCK cells grown in T175 flasks were washed twice with phosphate-buffered saline (PBS), overlaid

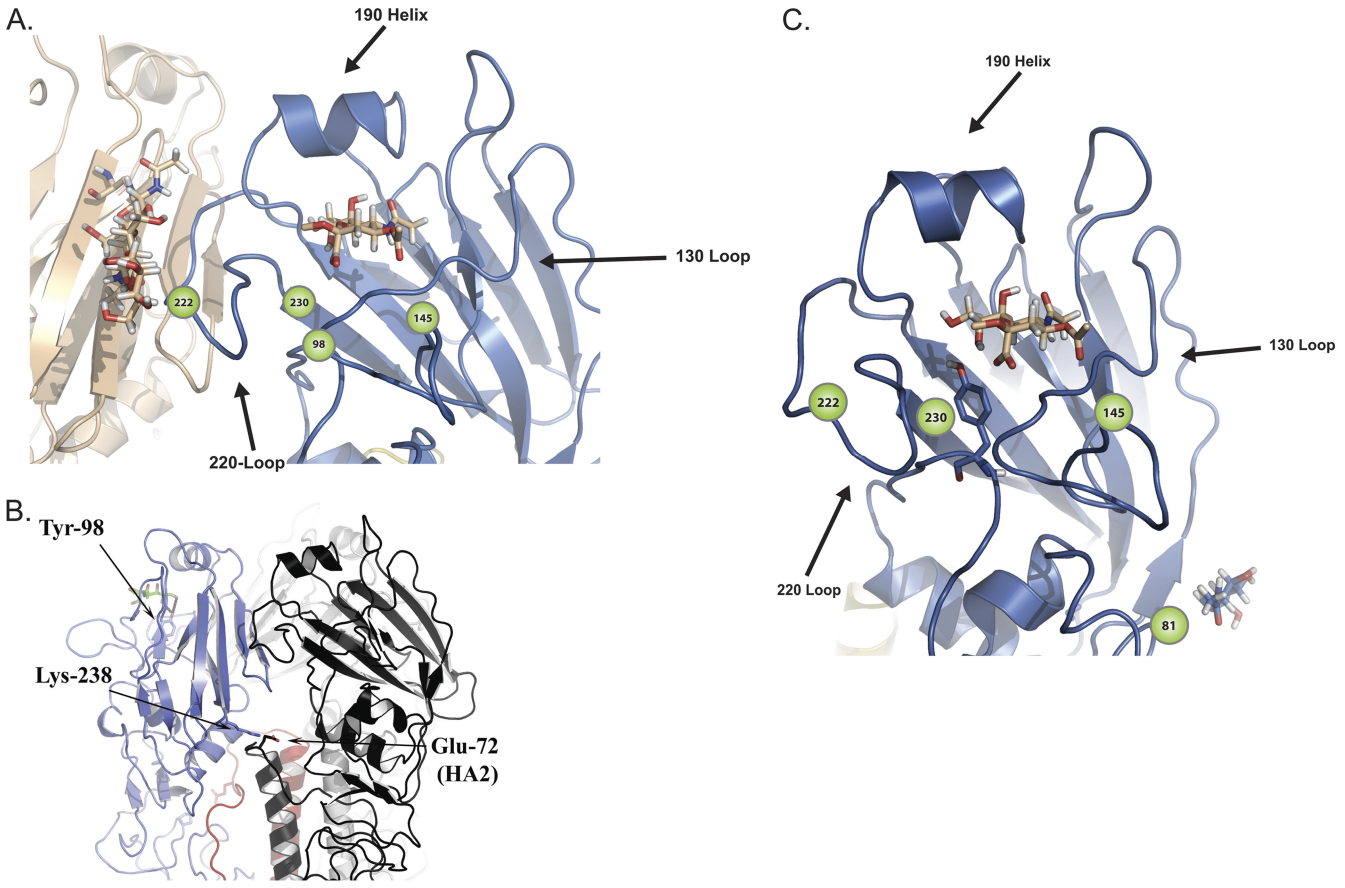


FIG. 1. Structural locations of mutations found in pseudorevertant viruses. (A) Locations of pseudorevertant mutations in the vicinity of the receptor binding site. The location of the sialic acid component of glycan receptors is visualized in the pocket just below the 190 helix, and positions of pseudoreversion mutations are indicated by green ball structures. The globular head of the adjacent monomer (beige) is also included to the left to illustrate its proximity to the 220 loop of the binding pocket and to show the location of the glycosylation site that originates from residue N165 of the adjacent monomer of A/Aichi/2/68 HA. (B) For the Y98F/K238N pseudorevertant, HA1 position 98, at the base of the receptor binding pocket, is quite distal (~40 Å) from HA1 position 238, which in WT HA forms an ion pair with the glutamic acid at HA2 position 72 of a neighboring monomer. Sialic acid is shown in green, the HA1 subunit is colored blue, and the HA2 subunit is shown in red, with the adjacent monomers shown in black and faded gray. (C) Alternative view of the binding site to illustrate the location of HA1 residue 81 and the glycosylation site that is lost in the Y98F/N81T mutant. HA is shown as a monomer, with sialic acid modeled in the receptor site, and the Y98F mutation is shown in the binding pocket. The pseudorevertant mutations are indicated as green ball structures, and the N-linked glycosylation at N81 present in the crystal structure is shown.

with 10 ml of serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin, and infected with a 1:1,000 dilution of the original virus stock. Cells were incubated with rocking for 1 h at ambient temperature, the inoculum was removed, and 30 ml of serum-free DMEM supplemented with 1 μg/ml TPCK trypsin was added. The flasks were incubated at 37°C for 2 to 3 days until monolayers were 80 to 90% destroyed. Virus was then harvested and frozen at −80°C until the purification procedure was carried out (see below).

MDCK cells were maintained using DMEM supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin. A549 cells were maintained using F-12 medium supplemented with 10% FBS and penicillin-streptomycin.

**Virus stock sequencing.** All virus stocks were sequenced prior to experimentation. Viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen) and converted to cDNA by using a Superscript III first-strand kit (Invitrogen), both according to the manufacturers' recommendations. PCRs to amplify the HA and NA genes were performed with Phusion High Fidelity PCR master mix (Finnzymes), using internal gene-specific primers designed to amplify the sequence in the HA gene from nucleotides 200 to 1540 and that in the NA gene from nucleotides 200 to 1360. Sequencing reactions were performed by MWG-Operon.

**Agglutination of erythrocytes.** Chicken, turkey, guinea pig, sheep, bovine, and horse erythrocytes were acquired from Lampire Biologicals as either washed or whole-blood preparations. Whole-blood preparations were washed two or three times with 1× PBS and diluted to 0.5% for hemagglutination experiments, which were performed using standard techniques. Briefly, 0.5% erythrocyte preparations were added to 2-fold serial dilutions of 50 μl of virus stock for 2 h to determine the HA titer. Viral elution was determined by additional incubation at 37°C, and hemagglutination units (HAU) were determined after 2-h and overnight incubations.

**Reverse genetics and viral rescue.** Recombinant influenza viruses were generated using a previously described 17-plasmid reverse genetics system (44). Briefly, 90% confluent 293T cells were transfected with seven plasmids encoding the viral RNA segments (PB1, PB2, PA, NP, NA, M2, and NS) of A/WSN/33 virus (H1N1), one plasmid encoding an A/Aichi/2/68 (H3N2) HA viral RNA segment with a wild-type or mutant sequence, and nine protein expression plasmids encoding the influenza A virus proteins needed to support the virus life cycle, using Lipofectamine (Invitrogen). Transfected cells were incubated at 37°C for 8 h, at which time the transfection medium was replaced with serum-free DMEM. Transfected 293T cells were incubated at 37°C for 24 to 48 h, and the monolayers were harvested and either frozen or passaged on 80% confluent MDCK cells. HA titers were determined using agglutination of chicken erythrocytes to ascertain whether samples were positive or negative for virus. Positive supernatants were passaged once more on MDCK cells, plaque purified, and

passaged two times in MDCK cells to create working stocks according to the protocol described above.

**Binding of fluorescently labeled virus to cell lines.** Binding of fluorescently labeled influenza virus was performed as previously described (5). Briefly, 100 to 200 μl of virus was incubated with 25 μg of Alexa 488 (Invitrogen) in 1 M $NaHCO_3$ (pH 9.0) for 1 h at room temperature. Labeled viruses were dialyzed against PBS containing 1 mM EDTA by using a 7,000-molecular-weight-cutoff (MWCO) Slide-A-Lyzer mini-dialysis unit (Thermo Scientific) overnight at 4°C. In all cases, labeled viruses were used in experiments the following day. To examine binding of labeled viruses to cell monolayers, confluent MDCK or A549 cells in 96-well plates were chilled at 4°C for 1 h to prevent endocytosis of the viral particles, and labeled viruses were bound at a multiplicity of infection (MOI) of 3. The cell monolayers were incubated with virus for 1 h at 4°C, washed three times with 1× PBS, and scanned using a Biotek Synergy 2 fluorimeter using a bottom optical position and excitation/emission at 485 nm/528 nm. As a control for NA-mediated elution, viral NA activity was assayed at both 4°C and 37°C, using the small substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA), and no activity was detected at 4°C.

**Purification of virus strains.** Purification of viruses was performed as described previously (5). Briefly, harvested virus was purified through a 25% sucrose cushion in NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA). Viruses were pelleted by centrifugation in an SW41Ti rotor at 28,000 rpm for 3 h, resuspended in 1,400 μl of NTE buffer, aliquoted, and frozen at −80°C. Frozen, purified virus strains were later thawed, and the HAU (see above) and PFU/ml titers were determined using standard techniques.

**Virus labeling and glycan microarray analysis.** Labeling of viruses for glycan microarray analysis was performed using the same protocol as that for our fluorescence binding experiments. After labeling of the purified viruses with 25 μg of Alexa 488 and overnight dialysis to remove excess Alexa 488, viruses were bound to a CFG array, version 4.1, at Core H of the Consortium for Functional Glycomics. Briefly, 70 μl of fluorescently labeled virus was incubated on a glycan microarray slide under a coverslip at 4°C for 1 h, washed to remove unbound virus, and scanned using a Perkin-Elmer ProScanArray instrument. Sialic acid derivative arrays were set up like the CFG array, except that 100 μl of labeled virus was used on slides fitted with a 16-chamber silicon grid and incubated without a coverslip.

## RESULTS

**Selection of mutant viruses and locations of amino acid substitutions in the HA structure.** We previously reported on the selection of viruses from mouse lungs following intranasal inoculation with the attenuated Y98F virus (41). Three of the viruses isolated during that study were selected for further examination: the Y98F/W222R, Y98F/I230M, and Y98F/K238N viruses. We also isolated two additional pseudorevertant viruses during passage of the Y98F mutant in MDCK cells (Y98F/N81T and Y98F/S145I viruses), and these were included in the current study. As mentioned previously, the Y98F virus replicates at the wild-type level in MDCK cells but does not agglutinate chicken erythrocytes. The Y98F/S145I mutant was isolated after repeated passage of the Y98F mutant in MDCK cells yielded a flask of virus that agglutinated chicken erythrocytes. The Y98F/N81T pseudorevertant was isolated following the second passage in MDCK cells of an independent rescue of the Y98F virus in 293T cells. Some of the virus populations at the T1C2 passage (one passage in 293T cells and two passages in MDCK cells) had gained the ability to agglutinate chicken erythrocytes, and plaques picked from the first passage in MDCK cells revealed that the N81T mutation arose early in the rescue protocol. The Y98F/N81T and Y98F/S145I viruses were subjected to additional passage in MDCK cells and were found to be genetically stable, and they were thus included in this study for further characterization.

The structural locations of HA1 position 98 and sites at which second-site mutations were selected are shown in Fig. 1. HA1 positions 98, 145, 222, and 230 are within or proximal to the pocket of conserved residues that bind the sialic acid component of receptor structures (Fig. 1A). Residue 238 resides approximately 40 Å from the binding site but may affect binding by altering subunit interfaces or the relative distances of subunits from one another (Fig. 1B) (41). The N81T mutation results in the loss of a consensus N-linked glycosylation motif (Asn-X-Ser/Thr). Electron density for this carbohydrate is present in the X-ray crystal structure of A/Aichi/2/68 HA (Fig. 1C) (81), and its presence was verified in a previous study (1). In the present study, using PAGE analysis, we examined the migration patterns of WT and Y98F/N81T HA proteins that were either treated or not treated with glycosidase to confirm that Y98F/N81T HA lacks this glycosylation site (data not shown).

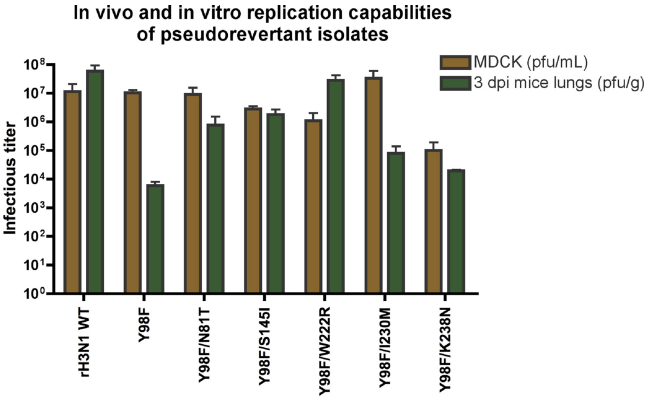


FIG. 2. *In vitro* and *in vivo* virus titers in MDCK cells and mouse lungs. MDCK cell infections were carried out at a low MOI as described in Materials and Methods. Mouse influenza virus infections were administered intranasally at $10^4$ PFU, and lungs were harvested at 3 days postinfection. Titers are expressed as PFU/ml.

**Replication of pseudorevertant viruses in MDCK cells and mice.** The Y98F virus is known to replicate well in MDCK cells but is highly attenuated in mice (37, 41). Therefore, we examined the *in vitro* and *in vivo* replication characteristics of the pseudorevertant viruses in order to determine whether the mutations are capable of conveying an easily observable selective growth advantage in each system, using yields of infectious virus as the readout. All mutant viruses utilized are variants of a recombinant H3N1 (rH3N1) laboratory strain which is a rescued reassortant with A/Aichi/2/68 HA (H3N2) in the genetic backbone of A/WSN/1933 virus (H1N1). To examine virus replication in MDCK cells, we infected cells at an MOI of 0.1 and determined the PFU titer at 48 h postinfection (hpi). To examine virus replication in the mouse lung, mice were infected intranasally with $10^4$ PFU and sacrificed at 3 days postinfection, and viral lung titers were determined by plaque assay on MDCK cells. As shown in Fig. 2, the rH3N1 WT and Y98F viruses reached comparable titers of approximately $10^7$ PFU/ml in MDCK cells, but the Y98F virus was attenuated by 3 to 4 log in the mouse lung, in agreement with our previous observations (37, 41). The viruses that were selected for in mice, the Y98F/W222R, Y98F/I230M, and Y98F/K238N viruses, all replicated to higher titers than the Y98F virus in mouse lungs, as might be expected. The most robust of the

TABLE 1. Agglutination of erythrocytes by pseudorevertant viruses[a]

| Erythrocytes[b] | Agglutination by virus (HAU/50 μl) | | | | | | |
|---|---|---|---|---|---|---|---|
| | H3N1 WT | Y98F | Y98F/N81T | Y98F/S145I | Y98F/W222R | Y98F/I230M | Y98F/K238N |
| cRBC | 128 | <2 | 8 | 256 | 512 | 128 | 256 |
| tRBC | 128 | <2 | 8 | 128 | 256 | 128 | 64 |
| gpRBC | 256 | <2 | 16 | 256 | 256 | 64 | 128 |
| sRBC | 32 | <2 | <2 | 16 | 4 | <2 | 4 |
| hoRBC | <2 | <2 | <2 | <2 | <2 | <2 | <2 |
| bRBC | <2 | <2 | <2 | <2 | <2 | <2 | <2 |
| Asialo RBC | <2 | <2 | <2 | <2 | <2 | <2 | <2 |

[a] Hemagglutination assays were performed using standard techniques.
[b] cRBC, chicken red blood cells (RBC); tRBC, turkey RBC; gpRBC, guinea pig RBC; sRBC, sheep RBC; hoRBC, horse RBC; bRBC, bovine RBC. Asialo chicken RBCs were created by enzymatically cleaving sialic acid with *Clostridium perfringens* prior to the agglutination assay.

three, the Y98F/W222R virus, reached titers that approached that of WT rH3N1 virus and were >3 log higher than that of Y98F virus.

When the replication properties of these mouse-selected mutants were determined in MDCK cells, two displayed reduced titers relative to the WT and Y98F viruses (Y98F/W222R and Y98F/K238N viruses), but the Y98F/I230M virus exhibited titers in excess of $10^7$ PFU/ml, the highest values for all viruses examined. The Y98F/N81T and Y98F/S145I viruses that were selected for in MDCK cells were slightly attenuated in mouse lungs relative to WT rH3N1 virus but were significantly greater than the Y98F virus titers in mouse lungs. Interestingly, neither mutant isolated by MDCK cell passage (Y98F/N81T or Y98F/S145I virus) displayed significantly higher titers than the Y98F virus in these cells. However, overall, these comparative studies demonstrate that certain receptor mutants have a distinct fitness advantage in one system versus the other. This is exemplified by comparing the results for the Y98F/W222R and Y98F/I230M viruses.

**Agglutination of erythrocytes by pseudorevertant viruses.** The results of the replicative fitness studies described above, in particular the observation that pseudorevertant viruses displayed higher titers than the parental Y98F virus in mouse lungs, suggest that the compensatory mutations lead to changes in the receptor binding properties of HA. As a first step toward examining this, we assayed the capacity of WT and mutant viruses to agglutinate erythrocytes from various species, which are known to differ in the density and distribution of cell surface glycan species that serve as potential receptors. Guinea pig, chicken, and turkey erythrocytes are known to contain a mixture of α2,3- and α2,6-linked sialic acids, whereas bovine, sheep, and equine erythrocytes contain primarily α2,3-linked sialic acids (29, 40). We utilized desialylated chicken erythrocytes as a negative control for binding. As shown in Table 1, the Y98F virus did not agglutinate erythrocytes from any species tested, consistent with previous findings with chicken and turkey cells (37, 41). However, all of the pseudorevertant viruses bound chicken, turkey, and guinea pig erythrocytes. Other than the Y98F/N81T virus, which displayed rather low HA titers, the HA titers of the pseudorevertant viruses were comparable to that of the WT, ranging from 64 to 512 for erythrocytes from chicken, turkey, and guinea pig species. None of the viruses bound to equine or bovine erythrocytes, but relatively low levels of agglutination with sheep erythrocytes were detected for the WT, Y98F/S145I, Y98F/W222R, and Y98F/K238N viruses. None of the viruses were able to agglutinate desialylated chicken erythrocytes.

These results show that the additional mutations acquired during replication of the Y98F virus in mouse lungs and MDCK cells yielded HAs that gained the ability to recognize receptors present on several species of erythrocytes. The erythrocytes known to contain higher densities of α2,6-linked sialic acids were agglutinated more efficiently, but these experiments do not rule out the possibility that α2,3-linked sialic acid glycoconjugates or alternative receptors may also be involved.

**Pseudorevertant virus NA activity toward erythrocyte receptors.** Influenza virus NA is responsible for cleaving sialic acid from cell surface glycoconjugates and facilitating virus release and dissemination (20, 21, 47). Several lines of evidence support the concept that HA binding activity and NA receptor-destroying activity evolve to seek an optimal functional balance for both infection and transmission (42, 74, 75); however, examples of mismatched HA and NA specificities also exist, such as the one involving recent H3N2 influenza virus strains (22). Therefore, we examined the NAs of the pseudorevertant viruses for the capacity to destroy their cognate receptors on chicken and guinea pig erythrocytes. Pseudorevertant viruses, WT rH3N1 virus, and an additional seasonal H1N1 control strain were allowed to agglutinate erythrocytes at 4°C and then incubated at 37°C for either 2 h or overnight to assay for NA-mediated elution of viruses from aggregates. As shown in Table 2, the H1N1 A/Pennsylvania/08/2008 control virus was

TABLE 2. Agglutination of chicken and guinea pig red blood cells by pseudorevertant viruses under different conditions[a]

| Virus | HAU | | | | | |
|---|---|---|---|---|---|---|
| | cRBC | | | gpRBC | | |
| | 4°C, 2 h | 4°C, 2 h, and 37°C, 2 h | 37°C overnight | 4°C, 2 h | 4°C, 2 h, and 37°C, 2 h | 37°C overnight |
| H3N1 WT | 256 | 128 | 128 | 256 | 64 | 64 |
| A/PA/08/2008 | 256 | 0 | 0 | 1,024 | 128 | 0 |
| Y98F/N81T | 64 | 32 | 32 | 128 | 0 | 0 |
| Y98F/S145I | 256 | 128 | 128 | 512 | 256 | 0 |
| Y98F/W222R | 256 | 128 | 128 | 512 | 256 | 256 |
| Y98F/I230M | 256 | 128 | 128 | 256 | 128 | 0 |
| Y98F/K238N | 256 | 128 | 128 | 256 | 0 | 0 |
| PBS | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Chicken and guinea pig red blood cells (cRBC and gpRBC, respectively) were incubated with virus for 2 h at 4°C and then placed at 37°C. HAU were recorded after 2 h at 37°C and then again after an overnight incubation.

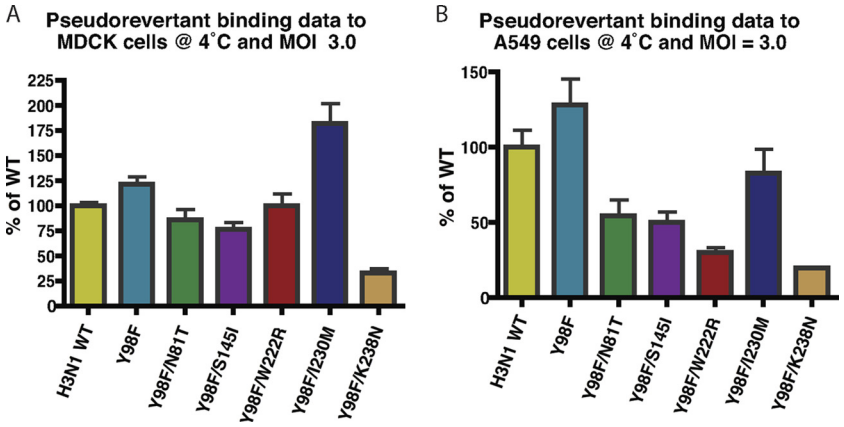


FIG. 3. Fluorescence binding assays of pseudorevertant viruses. Fluorescently labeled pseudorevertant strains were bound to chilled 96-well plates at an MOI of 3.0 and incubated at 4°C for 1 h. Binding is expressed as a percentage of the WT rH3N1 strain binding level. (A) Binding to MDCK cells. (B) Binding to A549 cells.

eluted from both chicken and guinea pig erythrocytes. The WT rH3N1 virus was also eluted from both chicken and guinea pig erythrocytes, though less efficiently, as in neither case were receptors completely removed, even following overnight incubation. For the pseudorevertant viruses, the results using chicken erythrocytes were very similar to those with rH3N1 virus. However, the data obtained with guinea pig erythrocytes were more interesting. All pseudorevertant viruses, with the exception of the Y98F/W222R virus, were able to completely destroy the receptors on these cells following overnight incubation. These data suggest either that Y98F/W222R HA has a higher affinity for a common receptor or that an alternative ligand that is resistant to the NA protein of this virus is recognized by this mutant.

Because HA and NA of influenza virus are known to operate in a delicate balance due to their opposing functions, we sequenced the NA gene of each pseudorevertant virus to determine if any of the observed phenotypes could be related to changes in NA enzymatic activity. The only NA mutation detected was in the Y98F/N81T virus, which contained a valine-to-methionine substitution at position 172, located on a solvent-accessible surface at the base of the NA globular head domains distal to the catalytic site. Though distal effects cannot be ruled out, there is no apparent reason to suspect that this mutation affects NA activity.

**Binding of fluorescently labeled viruses to MDCK and A549 cells.** To complement the erythrocyte binding experiments, studies were carried out to assess the binding of mutant viruses to two cell lines routinely used for influenza virus studies: MDCK and A549 cells. Although both lines have been reported to express glycans with α2,3- and α2,6-linked sialic acids, we reasoned that they were likely to contain different spectra and distributions of potential receptor species on their cell surfaces. We used a fluorescence-based assay to examine the binding of our pseudorevertant viruses. Briefly, Alexa Fluor 488-labeled influenza virus was adsorbed to confluent MDCK or A549 cells at 4°C for 1 h, followed by extensive washing, and bound virus was detected by fluorimetry (see Materials and Methods). The results shown in Fig. 3A and B indicate that all pseudorevertant viruses have the capacity to bind both MDCK and A549 cells. The Y98F/I230M virus bound to MDCK cells most efficiently, displaying values approximately 2-fold higher than those for the WT and most mutants, while the binding of Y98F/K238N virus was reduced by over 50% compared to that of the WT and the Y98F parental virus (Fig. 3A). Binding of all pseudorevertant viruses to A549 cells was reduced by various degrees relative to that of the WT and Y98F viruses, with the Y98F/K238N virus again displaying the least activity.

The results indicate that clearly detectable differences in binding capacity for each cell line exist among mutants and that the results can vary from one cell type to another. In conjunction with the erythrocyte studies and virus replication analyses, the data suggest that the mutant viruses either bind to ubiquitously occurring receptors with various degrees of efficiency or recognize alternative receptor species on the surfaces of these cells or that both of these phenomena come into play.

**Receptor binding characteristics of pseudorevertants on CFG arrays.** To examine the specific glycans that might serve as potential receptors for the WT and pseudorevertant viruses, we utilized glycan microarray technology. The glycan microarrays provided by the Consortium for Functional Glycomics consist of hundreds of different glycans covalently presented on *N*-hydroxysuccinimide (NHS)-activated glass slides. Glycans on these microarrays can be bound by viruses, bacteria, or plant lectins to explore their receptor specificities (4, 5, 35, 62, 84). Briefly, Alexa Fluor 488-labeled viruses were bound to slides for 1 h at 4°C and then washed, and the levels of bound virus were determined by fluorimetry. The WT rH3N1 virus was included during each replicate of the binding experiments, as we have extensively characterized the glycan array binding properties of this virus. As shown in Fig. 4A, the WT rH3N1 virus bound to numerous molecular species. These included glycans with α2,6- as well as α2,3-linked sialic acids, with a preference displayed toward branched α2,6-linked glycans, as we have detailed previously (5). Interestingly, as shown in Fig. 4B to G, neither the Y98F virus nor any of the pseudorevertants recognized any of the glycans with the high efficiency associated with WT rH3N1 virus or any other natural strains that we have examined previously (5). Among the pseudo-

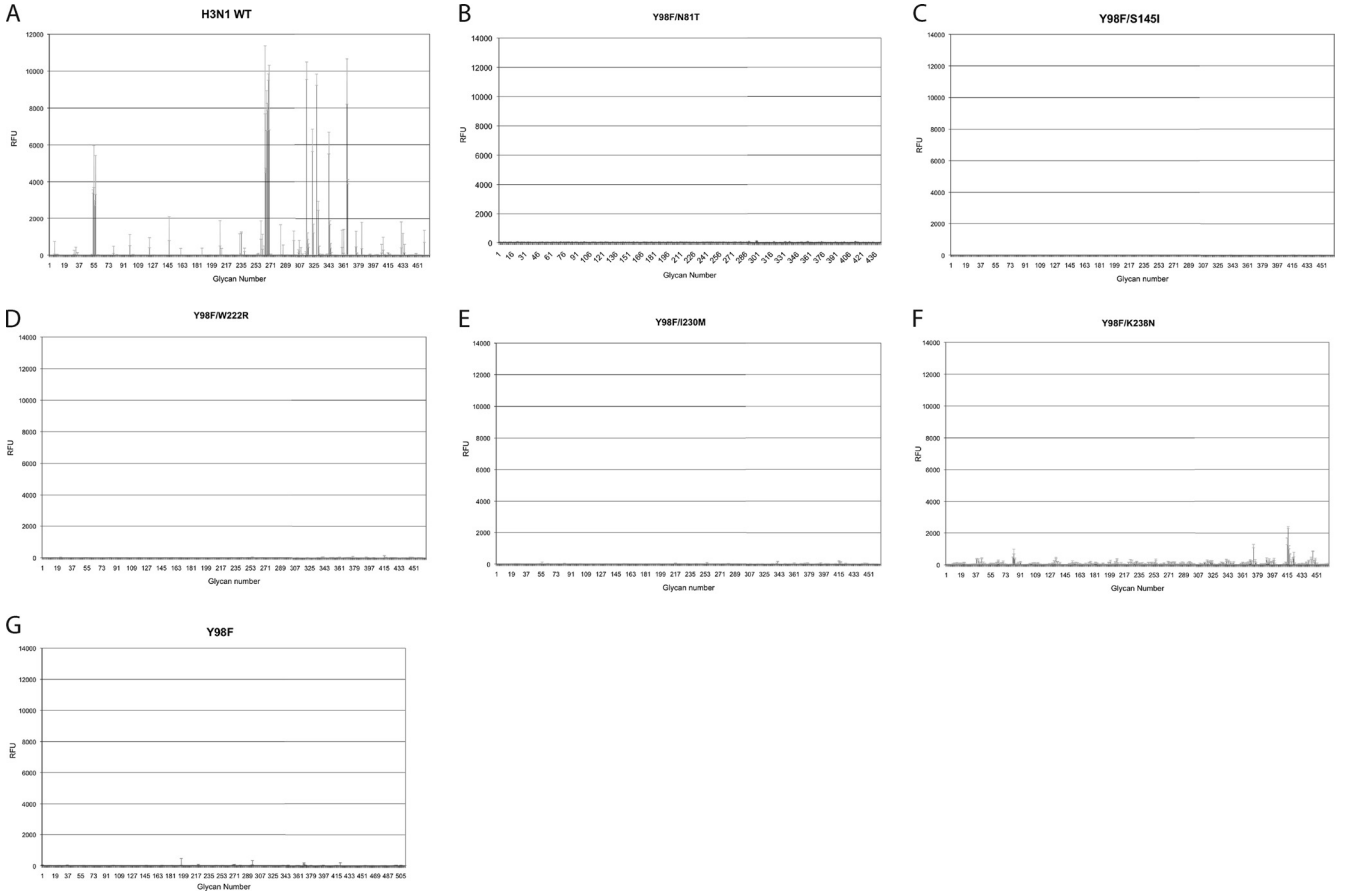


FIG. 4. Receptor binding to glycan arrays provided by the Consortium for Functional Glycomics (CFG arrays, version 4.1). Fluorescently labeled pseudorevertants were incubated on CFG arrays at 4°C for 1 h, washed, and then scanned, always with a WT rH3N1 control. (A) WT rH3N1 virus; (B) Y98F/N81T virus; (C) Y98F/S145I virus; (D) Y98F/W222R virus; (E) Y98F/I230M virus; (F) Y98F/K238 virus; (G) Y98F virus.

revertant viruses, only the Y98F/K238N virus displayed any binding activity, with limited binding detected for several highly fucosylated asialo-glycans (Fig. 4F and 5). Though highly fucosylated glycans were bound very poorly relative to the binding of the WT strain, the levels were clearly above background, with a reliable coefficient of variation (%CV) (defined as standard deviation/average). These results were quite unexpected, as we and others have examined numerous natural and laboratory strains of influenza viruses on previous occasions and have shown that all viruses consistently display the capacity to recognize multiple sialoside compounds on the arrays. A subsequent experiment using the substrate MUNANA revealed little to no NA activity at 4°C, indicating that the lack of binding was not due to sialic acid cleavage (data not shown). Since our mutant viruses bind to erythrocytes and laboratory cell lines and replicate in cell culture and in mice, the data suggest that they may recognize a subset of sialic acid-containing receptors that are not present among the >500 glycans presented on the current version of the Consortium glycan microarrays or that they recognize more unconventional receptors.

**Receptor binding to glycan microarrays terminating in derivatives of sialic acid.** Although most influenza virus receptor binding research has focused on Neu5Ac and Neu5Gc glycans, sialic acid on cells is expressed with a highly diverse group of modifications (7). In an effort to address the stereochemical nature of potential receptor species for our viruses, we utilized a recently developed glycan microarray that contains chemically derived sialic acid species (65). This array presents 77 sialic acid glycans with 16 different sialic acid modifications in α2,3 and α2,6 linkages to 3 different precursor glycans. Viruses were labeled with Alexa Fluor 488, and assays were carried out at 4°C as described in Materials and Methods. Consistent with the CFG array results, the WT rH3N1 virus bound to compounds containing branched α2,6-linked sialic acid and also to α2,3-linked species (Fig. 6A and Table 3). In addition, the WT strain was also observed to bind several sialic acid derivatives not present on the CFG glycan microarrays, which included 9-*O*-lactoyl-acetylneuraminic acid (Neu5Ac9Lt) and a Neu5Gc containing an additional methyl group (Neu5OMeGc). Structures of bound sialic acid derivatives and how they relate to Neu5Ac are portrayed in Fig. 6D.

In general, the pseudorevertant mutants did not display highly prominent binding to any structures on the sialic acid derivative array. However, two pseudorevertant viruses, the Y98F/S145I and Y98F/K238N viruses, demonstrated low but detectable levels of binding to a number of glycans that were considered sufficient enough for further discussion (Fig. 6B

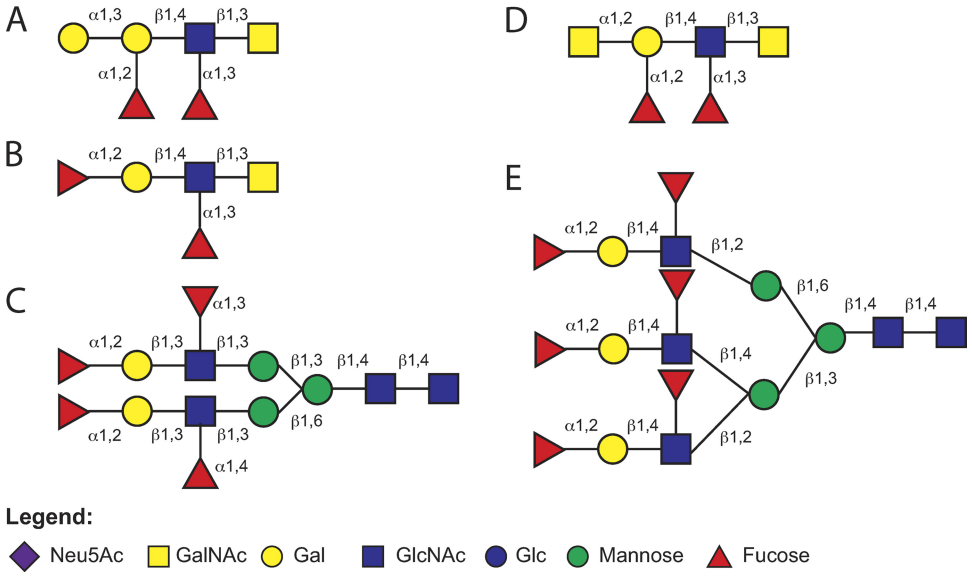


FIG. 5. Structures of the five glycans with the highest binding levels with Y98F/K238N HA. Glycan linkages are shown between glycan symbols. All glycans shown had a %CV of <50% and were considered to be above the level of background, which was 2 times the average for all glycans on the array.

and C and Table 3). The Y98F/S145I virus bound to a methylated KDN structure as well as to a branched α2,3-linked sialic acid. The Y98F/K238N virus bound to numerous structures, including two different KDN glycans as well as methylated Neu5Ac and a 5,9-*N*-acetylneuraminic acid ($Neu5,9Ac_2$) structure which contains an additional acetyl group off the 9th carbon of sialic acid. KDN is structurally identical to Neu5Ac, except that it lacks the *N*-acetyl group on carbon 5.

## DISCUSSION

Studies of influenza virus HA residues that affect receptor binding have been fairly narrow in scope, due in part to the restricted number of strains and subtypes examined and to limitations of the various individual techniques that have been utilized to study binding. The majority of HA residues that have been implicated in binding affinity and specificity are located in proximity to the sialic acid binding pocket, at positions in HA1 such as positions 186, 190, 193, 225, 226, 227, and 228 (H3 numbering), most of which are in the 220 loop at the "left" side of the site or in the small alpha helix at the membrane-distal edge of the site (Fig. 1). While the importance of these sites in contributing to receptor specificity, and possibly host range, is not in question, our current studies suggest that a range of residues at various structural locations in HA have the potential to affect binding properties and that several mechanisms may exist to modify binding affinity or influence the capacity of HA to recognize alternative receptor ligands.

Our previous studies with the Y98F HA substitution mutant showed that it was unable to agglutinate human and chicken erythrocytes, and though it could replicate to nearly wild-type levels in MDCK cells, it was severely debilitated for replication in mice. Our current study focused on the characterization of several second-site pseudorevertant viruses selected from the Y98F virus following passage in mice or, in two examples, following passage in MDCK cells. In all cases, the viruses with additional point mutations had a restored capacity to agglutinate erythrocytes of selected species and had greater fitness for replication in the mouse lung.

The locations of second-site mutations selected upon passage of the Y98F virus are shown in Fig. 1, and for each mutant, the significance of these second-site mutations and structural interpretations of how they modulate binding are discussed below.

**(i) Y98F/S145I mutant.** Residue 145 is located near the receptor binding site within antigenic domain A of HA and may interact directly with sialic acid (Fig. 1A) (80, 81). S145 has been associated with adaptation of H3 strains during egg passage (16, 30), as well as being reported to be a target for glycosylation of H9 and H3 HA proteins (49). The S145I mutation could alter the residues in the 130 loop and perturb interactions with the HA receptor.

**(ii) Y98F/N81T mutant.** The N81T mutation was interesting due to its distance from the receptor binding site, approximately 28 Å, as well as the fact that it results in the loss of a glycosylation site (Fig. 1C and data not shown). The glycosylation site at N81 was present in the strains that initiated the H3N2 pandemic, such as A/Aichi/2/68, but was lost over time and has not been observed in sequences of human H3N2 viruses since 1974 (56). Though the loss of the site has been attributed to antigenic drift by seasonal isolates, the more typical selection pattern is for glycosylation sites to be added, rather than removed, in response to immune pressure (1). The acquisition of the N81T mutation or the loss of the glycosylation site after passage of the Y98F virus in MDCK cells results in the ability of the Y98F/N81T virus to agglutinate red blood cells and suggests the presence of selective pressure against the reduced receptor binding capability of Y98F HA. Depending on the location and/or type of N-linked glycosylation, such modifications of HA are capable of affecting the receptor bind-

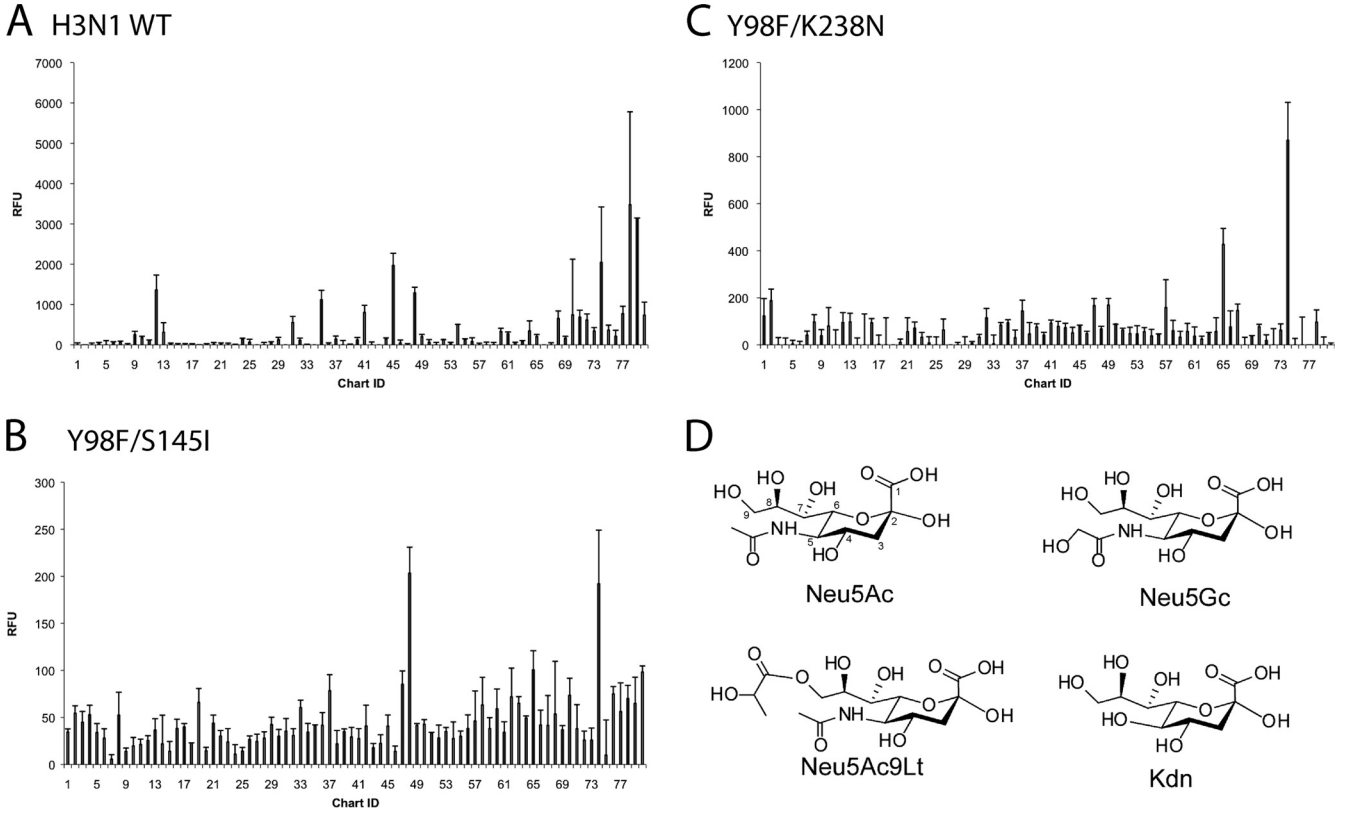


FIG. 6. Binding to sialo-derivative arrays by pseudorevertant viruses. Fluorescently labeled influenza virus was incubated for 1 h at 4°C, washed, and then scanned. The *y* axis is different for each panel to best represent peaks considered above background levels. Pseudorevertant strains that did not bind significantly to any structures are not shown. The structures of significant glycans can be seen in Table 3. A glycan was considered to be significant if the average number of relative fluorescence units (RFU) for a particular peak was >2 times the average for all glycans on the plate and the %CV was less than 50%. (A) WT rH3N1 virus; (B) Y98F/S145I virus; (C) Y98F/K238N virus. (D) Structures of sialyl derivatives bound by viruses in this study. Numbers present on the Neu5Ac structure denote the carbon number.

ing affinity and specificity of influenza virus HA (46, 66, 75, 76, 85). Referencing the crystal structure, the N81 side chain makes only localized contacts with L118 and Y120 on a nearby β-sheet, and based on its distance from the binding pocket, the mechanism by which the N81T mutation influences the receptor binding capacity is difficult to discern (81).

**(iii) Y98F/W222R mutant.** HA residue 222 is located in the 220 loop at the left side of the binding site when the protein is viewed from the orientation depicted in Fig. 1A. In H1 subtypes, this position is often occupied by a K residue, which forms hydrogen bonds with Gal-2 of both α2,3- and α2,6-linked receptor analogs (17). By extrapolation, the side chain intro-

TABLE 3. Glycans considered to be bound significantly by pseudorevertant viruses on sialo-derivative glycan arrays[a]

| Virus and print ID | Glycan structure | Mean | SD | %CV |
|---|---|---|---|---|
| WT rH3N1 | | | | |
| 79 | Galβ4GlcNAcβ2Manα3(Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB[b] | 3,124 | 24 | 1 |
| 45 | Neu5Ac9Ltα6Galβ4GlcNAcβ2Manα3(Neu5Ac9Ltα6Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB | 1,971 | 302 | 15 |
| 12 | Neu5Ac9Ltα6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB | 1,364 | 365 | 27 |
| 48 | Neu5Acα3Galβ4GlcNAcβ2Manα3(Neu5Acα3Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB | 1,292 | 139 | 11 |
| 35 | Neu5Acα6Galβ4GlcNAcβ2Manα3(Neu5Acα6Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB | 1,122 | 233 | 21 |
| Y98F/S145I | | | | |
| 48 | Neu5Acα3Galβ4GlcNAcβ2Manα3(Neu5Acα3Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB | 204 | 28 | 14 |
| 74 | Neu5,9Ac2α3Galβ3GlcNAcβ3Galβ4Glcitol-AEAB | 192 | 57 | 30 |
| 65 | Kdn5Meα6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB | 101 | 20 | 20 |
| Y98F/K238N | | | | |
| 74 | Kdn5Meα6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB | 871 | 161 | 18 |
| 65 | Neu5,9Ac2α3Galβ3GlcNAcβ3Galβ4Glcitol-AEAB | 427 | 68 | 16 |
| 2 | Neu5Ac8Meα6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB | 188 | 49 | 26 |
| 49 | Neu5Ac8Meα3Galβ4GlcNAcβ2Manα3(Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB | 169 | 28 | 17 |

[a] Significant binding was considered to be 2 times the background average, with a %CV of <50%.
[b] AEAB, 2-amino-*N*-(2-aminoethyl)benzamide.

duced by the W222R mutation in our A/Aichi/2/68 (H3) HA protein may also have the ability to form a hydrogen bond with Gal-2 of the receptor. In addition, W222 of WT A/Aichi/2/68 HA packs against a carbohydrate that originates from N165 of the adjacent monomer, as shown in Fig. 1. The presence of R at position 222 could influence the conformation or mobility of the N165 carbohydrate and could alter access to the binding site by certain receptor species.

**(iv) Y98F/I230M mutant.** I230 does not form direct contacts with the receptor; however, it does pack against residues Y98 and W153, which are components of the conserved hydrogen bond network made up of Y98, H183, Y195, and W153, which form the base of the binding pocket. As such, the I230M mutation can be considered a "second shell" mutation. The tighter hydrophobic packing of M230 with F98 and W153 in the HA protein of the pseudorevertant virus could help to stabilize the site, which might compensate for the loss of the H bond between F98 and H183. Interestingly, a survey of >800 full-length HA sequences in the Influenza Research Database (www.fludb.org) shows that human seasonal H3N2 and H1N1 strains that circulated prior to 2009 contained I230, whereas novel human H1N1 strains have M230. Among North American H1N1 swine strains that circulated prior to the 2009 pandemic, M230 predominated, with infrequent examples of I230 present. For H3 strains, changes at I230 have been rare.

**(v) Y98F/K238N mutant.** The K238N mutation is interesting because it resides approximately 40 Å from the sialic acid binding pocket. In WT HA, K238 forms an ion pair with HA2 residue E72 of the neighboring monomer which would be lost with the K238N mutation. Presumably, this could disrupt subunit interfaces and affect the position and orientation of one monomer relative to another. Mutations such as this have the potential to disrupt the binding region from a distance, particularly if strains contain carbohydrates on adjacent monomers (such as the N165 carbohydrate described above) that might interact with receptors.

The evidence that these second-site mutations alter receptor binding properties derives in part from the results of hemagglutination assays, which demonstrated that all of the pseudorevertant viruses had a restored capacity, to various degrees, to agglutinate chicken, turkey, and guinea pig erythrocytes. Binding to horse, sheep, and bovine erythrocytes was not detected for most of the viruses. This binding disparity might suggest that the pseudorevertant viruses retained the preference for α2,6-linked sialic acids of the parental A/Aichi/2/68 HA, as opposed to the α2,3 linkage preference that might be expected for those selected in the mouse respiratory tract, which has been shown to be rich in α2,3-linked glycans (18, 24). However, as discussed in more detail below, the results obtained with the glycan microarrays indicate that receptor specificity for the pseudorevertant viruses does not appear to involve acquisition of binding to conventional α2,3- or α2,6-linked glycans.

Our assessment of the virus replication phenotypes of each pseudorevertant virus in cell culture and in mice shows that the compensatory mutations lead to altered replication properties. As expected, all mutants that were selected in the mouse respiratory tract displayed higher 3-day lung titers than the Y98F virus. In some cases, the reversion from the highly attenuated phenotype of the Y98F virus was partial, and in others, lung titers were comparable to that of the WT, or even slightly higher, as in the case of the I230M virus (Fig. 2). In MDCK cells, some mutants replicated to levels comparable to that of the WT, while others were consistently lower. In addition, a comparison of virus yields obtained from each system for the Y98F/I230M and Y98F/W222R viruses demonstrates that the effects on replication are specific to the system. The Y98F/I230M virus titers were approximately 2 log greater in MDCK cells than in mouse lungs, and the Y98F/W222R virus grew to a 1-log higher titer in mice than in MDCK cells. These differences might be a direct reflection of the affinity for or availability of specific receptors for the mutant HAs, but particularly for the Y98F/W222R virus, they could reflect a functional imbalance between the HA and NA proteins. The lack of elution from erythrocyte aggregates mediated by the Y98F/W222R virus was unique among our collection of mutants in that the N1 subtype NA common to all of our mutants was unable to destroy the receptor recognized by the Y98F/W222R mutant, even following overnight incubation. Perhaps this quality inhibited dissemination of the Y98F/W222R mutant during virus replication in MDCK cells, leading to its lower titer than those obtained in mouse lungs.

Glycan microarrays have been used in numerous studies to examine the receptor binding specificities of pandemic, seasonal, and recombinant influenza viruses (4–6, 33, 67, 68), and the current CFG array contains >500 glycans. Despite the large number of sialic acid-containing glycans, with numerous modifications and linkers, none of the pseudorevertant viruses bound to the array at levels remotely comparable to those of our WT rH3N1 strain, and in most cases, binding above background levels was not detected. These results were surprising given that the pseudorevertant viruses bound erythrocytes (Table 1) and cellular monolayers (Fig. 3) at or near WT levels. However, it should be noted that the complete structures of all glycans or the complete glycome of these cells is not known. Discrepancies in receptor binding characteristics are not unprecedented. Recently, an H13 strain was shown to bind guinea pig erythrocytes but did not show binding to human airway epithelial (HAE) cultures, despite the presence of ciliated α2,3-linked sialic acids which were bound by other avian species (57). These results are further evidence that receptor binding by influenza virus is highly complex and may involve unknown receptor specificities.

The only virus to bind glycans on the CFG array of mammalian cell glycans above background levels was the Y98F/K238N virus (Fig. 4F and 5). The glycans recognized by the Y98F/K238N virus were generally branched asialo-glycans containing a Galβ1,4(Fucα1,2)GlcNAc-R motif in the glycan structure. Binding to asialo-glycans is rather atypical, but this observation may offer insight into how mutations that affect the monomer-monomer interface alter receptor binding patterns. If changes to the interactions in the globular head domain serve to relax restrictions on the receptor binding capabilities of the pseudorevertant virus, it may be possible that the three available -OH groups found in fucose could increase interaction with the receptor site enough to promote binding. Interestingly, the glycans with the highest binding levels all contained at least two fucose residues, either branched off the main glycan chain or at the terminus of the chain.

While the CFG array has >500 glycans, the overall glycome is likely to be much larger and could number well into the thousands of glycans (10). In terms of influenza virus receptor binding, the most commonly studied sialic acid derivatives are the *N*-acetyl-

and *N*-glycolylneuraminic acids (Neu5Ac and Neu5Gc). Both glycans are present on the current CFG array, but a plethora of sialic acid modifications exist in nature which are not found on the current CFG array (72). Therefore, we examined binding of the pseudorevertant viruses to a recently generated modified sialic acid glycan microarray (65). The rH3N1 WT, Y98F/S145I, and Y98F/K238N viruses all bound to sialic acid derivatives, albeit very poorly to some. One of the derivatives bound by the WT rH3N1 virus, but not the pseudorevertants, was Neu5Ac9Lt (Fig. 6A). Neu5Ac9Lt contains a lactoyl group extending off the ninth carbon of sialic acid and is known to be present in higher mammals, including being present in human serum and erythrocytes (60). The Y98F/K238N and Y98F/S145I pseudorevertant viruses did not bind Neu5Ac9Lt glycans but did bind a group of sialic acid derivatives referred to as KDN (deaminated neuraminic acid) (Fig. 6B and C). KDN is similar in structure to Neu5Ac, except that a hydroxyl group replaces the acetamido group on the fifth carbon (Fig. 6D). The amine in the acetamido group of Neu5Ac is predicted to form a hydrogen bond with residue 135; however, whether this bond would be lost during binding of KDN by influenza virus HA is difficult to determine. KDN expression has been found in A549 cells (25), human erythrocytes, and ascites cells from ovarian cancer patients, albeit at much lower concentrations than those of Neu5Ac (26). Notably, the second and third sugars in the glycan chain for all bound sialo-derivatives were Galβ1,3/4GlcNAc, which has been observed to contact residues in the HA receptor binding site in complexes with receptor analogs containing sialic acid in the α2,3- or α2,6-linked conformation (54). As observed previously (55), the position and orientation of sialic acid modifications also factor into the effects on binding. As shown here, relatively bulky derivatives such as Neu5Ac9Lt can be bound efficiently. In other cases, relatively small differences may be significant, as exemplified by the broad binding exhibited by human HAs to Neu5Ac but not Neu5Gc glycans.

The results presented here are not in conflict with the established concept that influenza virus HA binds principally to glycans with terminal sialic acids or with the basic generalization that α2,3 and α2,6 linkage specificity plays a role in host range. However, they do raise questions regarding influenza virus receptor usage and whether more stringent discrimination exists for specific subsets of glycans (or other molecules) under certain circumstances. Recent evidence regarding influenza virus binding to macrophages, as well as evidence examining infection of desialylated cells and of mice lacking particular sialotransferases, has suggested that properties beyond linkage specificity may be at play in influenza virus receptor binding in some situations (18, 24, 50, 69), and the possibilities involving alternative or "second" receptors have been discussed (69). Further evidence that shows binding but not infection of cells lacking N-linked glycoproteins suggests that while sialic acid is responsible for the initial attachment to cells, it may not be sufficient for endocytosis of viral particles (8). While our results do not require us to invoke a "second receptor" or nonglycan entity for attachment or virus entry, they suggest that these propositions warrant further examination, at least for some virus-host environments. The pseudorevertant viruses can obviously replicate rather efficiently in cell culture and *in vivo* but do not recognize any of the glycans present on CFG arrays. A rather simple explanation is that the viruses recognize glycan receptor species that are not present among the >500 glycans on CFG microarrays or on modified sialic acid glycan microarrays. With the advent of natural glycan arrays and "shotgun glycomics" methods that are being developed (63, 64), it may be possible to address these questions in the near future. Should we find that specific molecules among the vast collection of glycans serve as natural receptors for strains or mutants of influenza virus, it may become possible to utilize mutants such as the Y98F virus to select variants in particular cell types or host species and then to determine whether they can recognize subsets of natural receptor molecules that may be tissue or species specific.

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