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Natural Influenza Isolates and Pseudorevertant Mutants Have Species Specific Receptor Binding Profiles and Recognize a Subset of Sialic Acid Derivatives

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By

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B.S. Montana Tech of the University of Montana, 2005

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An abstract of
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Abstract

Influenza A virus is a single-stranded, negative-sense RNA virus, responsible for significant seasonal morbidity and mortality every year. Influenza encodes the surface hemagglutinin glycoprotein (HA), a multifunctional protein that is responsible for receptor binding, and closely linked to transmission, host adaptation, and pathogenicity. Binding to sialic acid is critical to viral infectivity, and can be affected by several well-characterized amino acids within the receptor binding site. We passaged a non-agglutinating mutant virus in mice and MDCK cells to isolate pseudorevertant viruses with mutations outside of the receptor binding site that significantly affected receptor binding. Despite being fully infectious in mice and MDCK cells, these mutant viruses did not recognize the classical sialic acid receptors present on glycan microarrays available from the Consortium for Functional Glycomics. Rather these mutants bound to a subset of sialic acid derivatives present in human serum and other mammalian tissues. Additionally, we sought to determine if the receptor binding patterns of early pandemic H1N1 isolates differed from both a prior seasonal strain and a related swine strain incapable of sustained human-to-human transmission. Interestingly, the strains bound highly similar receptors, indicating that many circulating swine strains might have the necessary receptor specificity to cause human infection, but additional factors are necessary for efficient transmission among humans. Together, these results utilize glycan microarray technology to broadly explore the influenza receptor binding of laboratory and mammalian species, and begin to describe binding to a new subset of biologically relevant sialic acid derivatives.

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INTRODUCTION

INFLUENZA BACKGROUND

Classification and Ecology

Influenza viruses belong to the family *Orthomyxoviridae*, which includes five genera: Influenza A, Influenza B, Influenza C, Thogotovirus, and Isavirus. Two genera, Influenza B and C, only infect humans, however Influenza A viruses are also able to infect many other mammalian species such as swine (275), guinea pigs (179), mice (274), ferrets (286), seals (102), minks (150), whales (119), and horses (293). Although influenza virus is a notable human pathogen, infecting between 5-20% of the human population per year (www.cdc.gov), the natural reservoir for Influenza A viruses is avian waterfowl, where all HA and NA subtypes have been isolated.

Influenza is made up of a single-stranded, negative sense, RNA genome. Depending on the virus genera, the genome contains 6-8 segments. Members of *Thogotovirus* encode six gene segments, while *Influenza C* viruses include seven segments, and *Influenza A* and *B* have eight segments. Unlike *Influenza A*, *Influenza C* viruses combine receptor binding, release, and membrane fusion activities into a single HEF (hemagglutinin-esterase-fusion) protein. In influenza A and B viruses, these functions are separated into two different proteins, termed the hemagglutinin (HA) and neuraminidase (NA) proteins.

Influenza A viruses consist of 16 HA and 9 NA subtypes, as determined by reactivity to antigenically distinct polyclonal sera (353), but no subtypes have been identified for *Influenza B* and *C*. As mentioned above, all HA and NA subtypes

circulate in waterfowl, which serves as the natural reservoir for *Influenza A* virus. In contrast, relatively few subtypes can efficiently infect humans, and to date only three HA (H1, H2, and H3) have paired with two NA (N1 and N2) to cause productive infection in humans.

Disease Course

Depending on the severity of the strain, seasonal influenza typically manifests itself as an upper respiratory infection with some lower lung involvement. After infection an asymptomatic phase is followed by the onset of fever, chills, malaise, myalgia, sore throat, and cough. Pneumonia is often associated with severe cases, and a recent study estimated the rate of pneumonia among seasonal cases between 1-2%, with the case fatality rate (CFR) of <0.1% (10). However, significant antigenic drift between seasonal strains can cause that rate to increase slightly, and pandemics can have a CFR of >0.4%. Viral shedding might occur as early as 1-2 days prior to disease onset, increasing the likelihood of human-to-human transmission (162). The onset of symptoms is very rapid, and correlates with peak viral titer around 2 days post infection (dpi). For seasonal strains, disease is generally more severe in children less than 5 y.o. and in those >65 y.o. In severe pandemic years, such as 1918, this trend changes with the greatest mortality burden among young and middle-aged adults (112, 172). Highly pathogenic viruses also follow this trend, which is believed to be due to a massive innate immune response that causes extreme tissue damage and fluid buildup in the lungs. This so-called “cytokine storm” is in essence an overreaction by the immune system in the lungs. It’s

characterized by excessive production of TNF-α, interferon, and other cytokines that ultimately lead to increased lung infiltrates and fulminate pneumonia (50). Increased viral tropism for α2,3 linked sialic acids, which are more prevalent in the lungs, as well as the presence of a polybasic cleavage site that permits systemic viral infection, both greatly contribute to increased pathology as well (359).

Early history

Influenza has been described in the human population for at least 500 years, and is hypothesized to have caused 14 different pandemics in that time period (319). In 1918 a influenza pandemic was responsible for 20-50 million deaths worldwide, but the causative agent was originally thought to be the bacterium *Pfeiffer's bacillus*, which was cultured from many fatal cases of the 1918 pandemic. The actual etiology was unknown until 1931 when Richard Shope published a series of landmark papers, which identified influenza virus as the causative agent of the 1918 pandemic as well as its establishment in swine (165, 275, 276). In these papers, Shope thoroughly described the pathology of swine influenza in pigs from 1928, established influenza as a filterable virus, and also described the relationship between the filterable influenza virus and *Pfeiffer's bacillus*, now know as *H. influenza*. Not long after, Dochez, Mills, and Kneeland discovered that influenza virus could be cultured in chick embryo tissue (and later successfully inoculated in human volunteers) (78). The relationship between swine and human influenza was examined when the Lancet reported Smith, Andrewes, and Laidlaw had successfully passaged influenza in ferrets, and that ferrets infected by the swine strain of

influenza were "solidly immune" to the human virus isolate indicating the two strains were closely related (286). The ferret model they established is still in use today, and is widely considered to be the best correlate of human pathogenicity and transmission. In 1941, Hirst determined that influenza agglutinated red blood cells, and that this could provide a method for viral quantitation (120, 121). In addition to the groups above, Burnet also significantly advanced the field of influenza research by discovering the sialidase activity of the NA, and revealing its action on mucins. He also observed differences in agglutination patterns of egg-passaged viruses versus their unadapted counterparts; an early indication of the role HA plays in host adaptation. Research from these groups, among many others, continued to significantly advance the field of influenza research for many years by establishing animal models (274), vaccines (90, 91, 259), characterizing viral immunology and neutralization (273, 285), among numerous other discoveries, together making influenza one of the most understood viruses at the time.

Pandemics

In contrast to antigenic drift, which is a slow, continuous mutation of the antigenic HA and NA proteins over time, antigenic shift involves the replacement of gene segments that creates an antigenically novel influenza virus capable of causing high levels of morbidity and mortality. Antigenic shifts associated with efficient human to human transmission often lead to pandemics where either the HA, NA, or both are antigenically novel to the immune system. Over the past 120 years there

have been six different pandemics of differing levels of severity, and these are detailed below.

1889 "Russian" influenza

Although it was not the first human influenza pandemic, the pandemic of 1889 is unique as it was the first pandemic to occur in a world highly connected by railroads and relatively quick transatlantic travel (329). It is also among the oldest pandemics to have significant data regarding morbidity and mortality rates available, possibly due to the birth of the field of epidemiology a half century earlier by John Snow. Journals such as *The Lancet* in Britain recorded the outbreak, symptoms, and recommended treatments for influenza. The pandemic first emerged in St. Petersburg around December 1st 1889, and appears to have arrived in London around the start of the New Year in 1890. It was noted to have had an extremely rapid transmission rate, with an initial increase in excessive deaths primarily among people between 20-60 years old (159). However, as the pandemic progressed evidence indicates that at the end of the pandemic, mortality rates were highest amongst the elderly (330). The pandemic virus reached the United States by the 12th of January, by which it was advancing at an astonishing 1015 km/week (329). Overall, the case fatality rate in the French, British, and German armies was not alarmingly high, and has been estimated between 0.1 and 0.28%, similar to the 1957 pandemic to be discussed later (329). Serological evidence from the 1889 pandemic indicates it is most likely the result of a H3 subtype strain, (186) although some evidence for H2 subtypes exists as well (206).

1918 "Spanish" Influenza

The H1N1 pandemic that emerged in 1918 is believed to have killed upwards of 50 million people worldwide (138). The origin of the 1918 influenza is still up for debate, but phylogenetic data suggests it was of wholly avian origin, similar to the "bird flu" strains circulating in Southeast Asia today. Research by Shope and Smith, Andrewes, and Laidlaw established that both human and swine influenza strains circulating in 1933 originated from the same parental strain, suggesting an avian origin (273, 286), but without avian influenza isolates from before 1918, it is difficult to be certain as to the origin of the 1918 influenza.

In general, influenza causes a U-shaped mortality curve, with the highest mortality seen in those less than 5 y.o. and greater than 65 y.o. However, the 1918 mortality rates were W-shaped, with an increase in mortality in younger adults (79). This has been attributed to a "cytokine storm," (232, 287) and is also associated with fatal H5N1 cases (72). In addition, recruitment of the protease plasmin by the 1918 neuraminidase protein increases pathogenicity in mice, due to the enhanced cleavage of the HA protein (41, 108, 109, 310). Finally, the pro-apoptotic PB1-F2 protein expressed in the 1918 virus is associated with increased bacterial secondary infections in mice, leading to greater respiratory involvement and pneumonia (194). After the initial outbreak and pandemic, the 1918 H1N1 influenza displaced the previously circulating seasonal influenza strain in humans, and also became established in swine.

1957 "Asian" Influenza

The 1957 pandemic emerged in Southern China, but did not cause the same level of mortality as the 1918 pandemic, which was estimated at ≈70,000 deaths (64). The virus was an H2N2 subtype, and was the result of a reassortment event that occurred in swine that replaced three gene segments from the circulating H1N1 virus (HA, NA, and PB1) with segments from an avian strain. It was the first example of a swine reassortant influenza virus leading to significant morbidity and mortality, and prompted swine to be labeled a "mixing vessel" for human and avian influenza viruses (140, 263). The H2N2 virus replaced the previously circulating H1N1 virus, and continued to circulate in humans for 11 years until 1968.

1968 "Hong Kong" Influenza

The 1968 pandemic was again the result of a reassortment involving the circulating H2N2 and the HA and PB1 of an avian influenza strain (140, 348). The resulting H3N2 strain replaced the H2N2 strain and caused an estimated 33,800 excess deaths in the United States alone (64). This virus continues to circulate in humans today and has been sporadically identified in swine, but has failed to become established in the swine population (228).

1977 "Russian" Influenza

The so-called "Russian" influenza pandemic was unique, in that it did not involve the introduction of a new strain of influenza. Rather the pandemic was caused by the reemergence of the H1N1 strain that had been displaced by the 1957

H2N2 strain (212). The strain largely infected people under the age of 25 years old, and bore a striking antigenic resemblance to the previously circulating H1N1 isolate, indicating it might be due to an accidental release of a laboratory strain. After the reintroduction of the 1977 H1N1 virus, it co-circulated in the human population until 2009, when it was displaced by the currently circulating 2009 novel H1N1 pandemic strain.

2009 Novel H1N1 Pandemic

At the start of 2009, there were two influenza virus subtypes circulating in the human population. The first was the 1977 H1N1 virus genetically related to the 1918 virus, and the second strain was the 1968 pandemic H3N2 virus. In April of 2009, a novel H1N1 virus was identified in Mexico (1, 40). The virus spread rapidly after its discovery, causing the WHO to raise the global pandemic alert to phase 5, characterized as efficient human-to-human transmission. Early reports out of Mexico City indicated that initial patients were an average of 40 years old, with a case fatality rate (CFR) of 0.4%, comparable to the 1957 pandemic (80, 92).

The virus that caused the outbreak was made up of a reassortant of two viruses, a swine triple-reassortant H1N1 (trH1N1) from North America, and a Eurasian swine strain of avian origin (100, 283). The Eurasian swine strain became established in the European swine population after the direct transmission of an avian influenza virus into swine sometime around 1979 (261). The trH1N1 strain was itself the result of a reassortment between a classical swine H1N1 strain (NP, M, NS, HA, NA), a human seasonal H3N2 strain (PB1), and an avian H1N1 virus (PB2

and PA). This trH1N1 virus was first identified in 1999, and although it was responsible for a few influenza-like illnesses in the Midwestern United States, there was no widespread transmission seen in any of those cases (271).

Where and when the reassortant event occurred between six segments of the trH1N1 virus (PA, PB1, PB2, HA, NP, and NS), and two segments from the Eurasian swine lineage (NA and M) is still a mystery. Phylogenetic evidence suggests that the strain first emerged at least ten years ago, but went undetected until it infected humans in Mexico in 2009 (283). The most likely location for reassortant is Southeast Asia, where all four major swine strains circulate, and a large number of reassortant strains have been identified (154).

The resultant pandemic reassortant influenza virus was estimated by the CDC to have resulted in approximately 270,000 hospitalizations in the US between April 2009 and August 2010 (37). Since then, the novel 2009 H1N1 virus has displaced the previously circulating H1N1 strain, and continues to co-circulate with the descendent lineage of the H3N2 virus that emerged in 1968 (38).

Virus Structure and Morphology

The influenza virion typically exists in one of two morphologies. Electron microscopy of virions isolated from nature generally shows the virus particles as filamentous (54), while tissue culture adapted strains are mostly spherical (51). Structural locations in the M1 protein are known to affect viral morphology, and mutations at those sites have dramatically altered virion structure (34). However, it is currently not understood why adapted lab strains transition from elongated to

spherical virus particles. The spherical virion is approximately 80-100 nM in diameter, but the length of filamentous particles tends to vary widely, even within the same strain (36, 54). Not all strains convert from filamentous to spherical when passaged in tissue culture, the A/Udorn/1972 strain is a classical filamentous laboratory strain that does not vary in morphology (22, 245).

The M1 protein is largely responsible for virion morphology, and is interior to the lipid bilayer that forms the envelope of the influenza virion (17, 216). Electron microscopy has revealed that eight viral RNPs associate with the M1 protein, clustering at one end of the virion after budding, while the remainder of the virion appears to be empty (36). In general, Influenza A virions are thought to specifically enclose one copy of all eight viral gene segments, although discrepancies in the literature exist, including a isolate from France lacking a neuraminidase gene, and a genetically modified virion known to contain nine segments (99, 200). A more thorough discussion on viral assembly and RNP packaging will be addressed later in the introduction.

VIRAL GENE SEGMENTS

PB1

The PB1 gene segment is thought to encode for three different viral proteins, the PB1, PB1-F2 and the N40. Little is known about the function of the recently discovered N40, which is an N-terminal truncation of the PB1 protein, but interactions with the PB2 gene have been discussed (358).

PB1-F2 is an 87-90 residue protein with a very short half-life that is translated using a novel ORF in the PB1 gene segment. The prevalence and function of the protein appear to be strain and cell line specific (195), but expression of PB1-F2 is associated with increased apoptosis in monocytes and has been hypothesized to selectively target immune cells (48). PB1-F2 traffics to the mitochondria, and is thought to induce apoptosis by permeabilizing the cell membrane, leading to the release of cytochrome c. A mutation at N66 is associated with highly pathogenic strains such as the 1918 influenza, and certain H5N1 influenza isolates (60, 194). PB1-F2 interacts with MAVS as well, acting as an interferon antagonist, preventing the cell from entering an anti-viral state (332).

PB1 protein forms one part of the RNP complex via interactions with PB2, PA, and vRNA segments (76). It contains domains consistent with previously identified RNA-dependent RNA-polymerase motifs, and is involved in the addition of nucleotides during RNA chain elongation (12, 24, 107, 167).

PB2

The PB2 protein, the second protein in the RNP complex, binds the 5' capped RNA of vRNA segments, and plays a critical role in the initiation of transcription. In addition, the N terminal end of PB2 must play a role in replication, as mutants have been identified that abolish RNA replication, but do not inhibit transcription (101). PB2 also has a role in host adaptation, as the two residues 627 and 701, affect virus temperature sensitivity. K627 promotes replication at 33˚C and 37˚C, the temperature of the human upper and lower respiratory tracts respectively (171,

188, 196). In contrast, E627, associated with avian influenza isolates, is attenuated at 33˚C, but fully functional at both 37˚C and 40˚C. This appears to be a significant barrier to interspecies transmission between the two species, and might explain why the PB2 of the 1957 and 1968 reassortant pandemic strains originated from humans (296, 307). The mechanism behind this temperature sensitivity is currently unknown.

PA

Relative to the other two polymerase proteins, much less is understood about PA protein functions, although site-directed mutations have suggested a role in transcription, as well as the endonuclease activity of the polymerase (87).

Hemagglutinin (HA)

The hemagglutinin protein of influenza is a 550 amino acid type I membrane glycoprotein that extends outward from the viral membrane. It consists of two obvious structural features, a stem domain and a globular head domain that form a 130 Å tall, non-covalently linked homotrimer on the viral membrane. It is predicted that there are approximately 500 HA trimers present on the surface of an influenza virion, compared to 100 NAs, which might be why the HA and NA are the primary antigenic component of influenza (256, 265, 322). Because of this, the HA is highly variable, particularly within the antigenic regions of the protein (355) and often adds glycosylation sites on the globular head region (309). The addition of

glycosylation sites serves to “shade” the antigenic sites from HA-specific antibodies, and is regarded as an important component of antigenic drift.

Influenza HA has two main functions, receptor binding and promoting fusion of the viral and endosomal membranes after endocytosis. Both of these functions will be discussed in much greater detail later, but are why the HA is so closely linked to pathogenicity (45, 56, 75, 145, 207, 233, 324), transmission (148, 183, 292, 294, 325), and host adaptation (57, 128, 131, 143, 192, 207, 213, 238). In order for a virion to be infectious, the precursor HA0 must first be cleaved by a protease and split into HA1 and HA2 components. The full extent of proteases that cleave HA is currently unknown, but trypsin and subtilisin-like proteases, as well as TMPRSS2, HAT, and Clara proteases have been implicated in the past. TMPRSS2, HAT, and Clara are generally found in the human URT, where influenza primarily infects. These proteases typically cleave HA either while bound to the cell membrane, or as a soluble proteases (19, 20, 144). HA cleavage often correlates to pathogenicity of a viral strain, in particular, highly pathogenic avian isolates have a series of basic amino acids near the cleavage site which serves to elongate the protruding loop containing the cleavage site (18). This polybasic cleavage site (PBCS) can then be cleaved by furin and subtilisin-like proteases, which are ubiquitously expressed and allow for the systemic spread of the virus (98, 123, 124, 141, 305, 342, 366). After cleavage, the HA1 and HA2 structures are linked by a single covalent bond between HA1 residue 14, and HA2 residue 137. Although the structures of the cleaved and uncleaved HA are very similar, these modifications are necessary for infectivity, and leave the HA in a conformation where it can promote fusion.

Nucleoprotein (NP)

The nucleoprotein is generally thought to have two functions. First, it forms the backbone of the RNP complex, and is required to maintain the RNP structure. The NP also contains up to three nuclear localization signals (NLS), which allow the entire RNP complex to enter the cell nucleus, most likely through the karyopherin α/β pathway (223, 345).

Neuraminidase (NA)

The neuraminidase is a homotetramer surface glycoprotein enzyme, responsible for cleaving terminal sialic acid from glycan chains upon viral budding. Gottschalk first identified the sialidase activity of influenza and *V. cholerae* in 1957, and the mechanism was elucidated in 1994 (110, 320). The length of NA widely varies, but is usually projects ≈ 60Å from the viral membrane, which makes it roughly half as tall as the HA protein (166). In avian strains, the NA stalk length is known to shorten, and can affect virulence (313), transmission (291), and the *in vitro* growth capabilities (6) of a virus strain.

In general, cleavage of sialic acid is a necessary step for productive viral infection; however, viruses have been isolated both in the lab (125, 126, 173), and in nature (201) that lack a functional neuraminidase. This suggests the sialidase activity of the NA is dispensable in some situations, most likely requiring an HA protein with very weak sialic acid binding affinity. The NA protein is also postulated to have a role in viral entry (193, 225), although this has been disputed elsewhere

(174). Several sialidases from other organisms are known to preferentially cleave sialic acids in a conformation specific manner (277). Influenza neuraminidase is thought to act similarly (8, 62), and dual conformation specific neuraminidases may be necessary to prevent a virus from becoming entrapped by respiratory mucins, which tend to contain vast quantities of sialic acids, and are known to be inhibitory to influenza viruses (35, 63).

Matrix protein (M)

The matrix gene segment encodes for two proteins, the M1 and M2 proteins. The M1 protein is the most abundant protein in the virion, and is known to be necessary and sufficient for VLP formation (106). M1 is a multifunctional protein, with roles in viral morphology (34, 85, 245, 281), nuclear export (30), trafficking (222, 365), assembly, and budding of influenza virions (106). These topics will be discussed in greater detail later.

The M2 gene encodes a tetrameric, integral membrane proton channel that is also a minor antigenic protein. The M2 protein transports hydrogen ions across the viral membrane after endocytosis, which acidifies the interior of the virus. This step is critical to the viral life cycle (316), and because of this, it has been the target of both antivirals (amantadine and rimantadine), and recent vaccine candidates (89, 288, 289). The M2 protein is also hypothesized to play a role in both release and assembly, both of which will be addressed in greater detail below.

Non-structural protein (NS)

The NS gene segment encodes for two proteins via a splicing event. The NS2/NEP protein is involved in the nuclear export of RNPs, and the NS1 protein acts as an interferon antagonist. Interferons are involved in the cellular response to a viral infection, and act in an autocrine and paracrine manner to prevent the spread of virions to other cells. The NS1 protein is thought to act as an antagonist to interferon and the subsequent anti-viral response by three methods:

1) NS1 prevents transcriptional activation of IFNβ, by inhibiting IRF3 and NF-κB (318, 346), therefore preventing interferon production.
2) NS1 sequesters CPSF30, a cellular posttranscriptional processing protein involved in processing the 3' ends of all cellular mRNA, including IFN β (69, 219).
3) NS1 also inhibits protein kinase R, a cellular protein whose activation leads to a general suppression of the cellular translational machinery. It does so by both sequestering dsRNA, the activator of PKR, and by binding to PKR itself (169).

These events result in a general knockdown of the cellular antiviral response, and allow influenza time to complete the replication cycle.

The NS2/NEP protein is equally critical to the influenza virus replication cycle, but does so using a very different method. NEP contains a nuclear export signal on the N-terminal end of the protein, and is responsible for the export of vRNPs from the cell nucleus (137, 217).

VIRUS LIFE CYCLE

Receptor binding

The initial step in the course of viral infection is the interaction of the influenza receptor binding protein, HA, with the viral receptor, sialic acid. Receptor binding has been shown to have a role in transmission, pathogenicity, and species specificity, among other traits. The receptor for influenza is sialic acid, a nine-carbon acidic monosaccharide, ubiquitously expressed throughout the body. HA binding to the receptor occurs at the receptor binding site, found at the viral membrane distal end of the HA protein (Figure 1).

When the structure of a bromelain released H3 HA protein was deciphered in 1981 (356), the location and structural aspects of the receptor binding site were also determined. The receptor binding site has four main structural characteristics, seen in Figure 1 below.

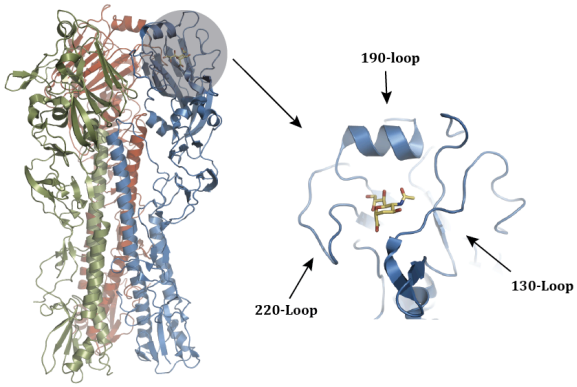

Figure 1 – Structure of A/Aichi/2/1968 hemagglutinin. The shaded site is the receptor binding site, distal to the viral membrane. On the right is an enlarged receptor binding site with helices and loops critical to receptor binding marked.

The base of the site is made up of several highly conserved residues that form a hydrogen bond network. These residues located at 98, 153, 183, and 195 interact with each other to form the base of the site, and in some occasions may interact with the sialic acid receptor as well (350). The receptor site is also flanked by two loops, the 220-loop and the 130-loop, both of which have extensive interaction with the receptor. On the 130-loop, the residue 135 interacts with the receptor at the 1-carbon of sialic acid, while the 136 and 137 interact with the N-acetyl group branching off of the 5-carbon. The 220 loop flanks the binding pocket on the "left" side. Residues in this location have extensive interaction with sialic acid and the internal glycans, and play major roles in determining receptor binding specificity. In human influenza strains, a methyl group in L226 interacts with both the base of the site (Y98) and the 130 loop (residue 138). This serves to widen the receptor binding site slightly, facilitating binding to α2,6 SA whereas a Q226 at this position shrinks the site and promotes direct interaction between the Q226 and an α2,3 receptor. The 228 residue is believed to affect the binding pocket in a similar manner, by drawing the 220 loop closer to Y98 in avian viruses, and also widening the site for human strains (113). These two residues on the 220-loop are most often associated with H3 receptor specificity, and more recently have been associated with receptor specificity in H2 strains as well (252, 337, 361), while the residue at 225 is known to affect H1 binding (56, 176). Additionally, 219, 227, 186, and 187 are thought to act in concert to form a network that influences binding to α2,6 SA. A disruption of this ionic network has been associated with the relatively weak binding affinity by early Novel 2009 H1N1 pandemic viruses (185). Finally, the 190

α-helix lies above the sialic acid binding site, and several residues are known to interact with the viral receptor, generally with the 3rd and 4th sugar on the receptor. The most notable residues involved in this interaction are 190 and 193, which are also important to the receptor binding specificity of H1 and H5 viruses (105, 302, 303, 325). Recently, a mutation at residue T200A that interacts with the 190 α-helix suggests the 190-helix can also be influenced by sites on the β-sheet behind the receptor binding site as well(74).

It is important to note that the receptor/HA interactions vary widely depending on the composition of critical residues within the site, as well as the virus host and subtypes. Although these sites are the best characterized locations that affect receptor binding specificity, they are by no means the only way specificity can be altered, and residues at 189, 193, 194, 216, 198, 211, and 222 have also been implicated (185, 303, 304). Additionally, in Chapter 3 we identified a number of mutations outside of the receptor binding site that affect receptor binding in novel ways.

Although all human influenza A and B viruses interact with receptors containing terminal sialic acids, they show remarkable specificity for the sialic acid linkage they prefer to bind. Influenza A and B both bind N-acetylneuraminic acid (Neu5Ac), the most common form of sialic acid in humans (31), but generally do not bind the highly similar N-glycolyneuraminic acid (Neu5Gc) which is absent in humans, but seen in most other mammals (295). These two sialic acid derivatives differ by a single –OH group off of the 5-carbon of sialic acid, as seen in Figure 2 below.

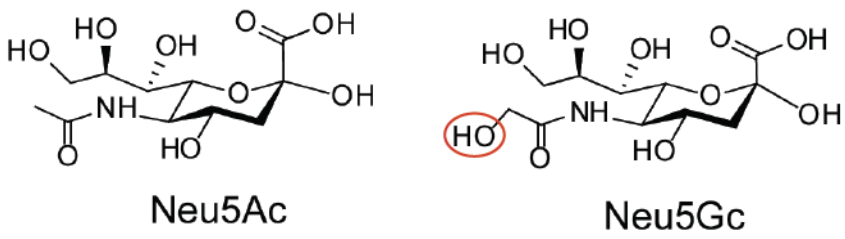

Figure 2 – Differences between N-acetyl neuraminic acid and N-glycolyl neuraminic acid.

Our group and others have noted swine influenza to bind both Neu5Ac and Neu5Gc but swine viruses transmitted to humans do not retain Neu5Gc binding (26, 311). The loss of Neu5Gc binding by human influenza strains is probably due to the lack of selective pressure to maintain binding to Neu5Gc sialic acids, since humans have not been able to produce Neu5Gc for millions of years, most likely due to evolutionary pressure from pathogens (52, 53, 333, 335). However, residues directly involved in promoting Neu5Gc receptor binding in conjunction with Neu5Ac binding are currently unknown.

Neu5Ac is present on the cell surface generally as the terminal glycan on a glycan chain that protrudes away from the cell membrane. Terminal sialic acid is generally linked to the second sugar molecule on a glycan chain in one of three ways: α2,3, α2,6, or α2,8. Influenza binds to either α2,3 or α2,6-linked sialic acid in a species specific manner, although some α2,8 linked sialic acid binding has been seen (360). Human viruses bind Neu5Ac in an α2,6 linked conformation (α2,6 SA),

while avian viruses prefer to bind sialic acid in an α2,3 linked conformation (26, 47, 61, 97, 129, 151, 175, 183, 257, 270, 302, 303, 361) (and Figure 3 below).

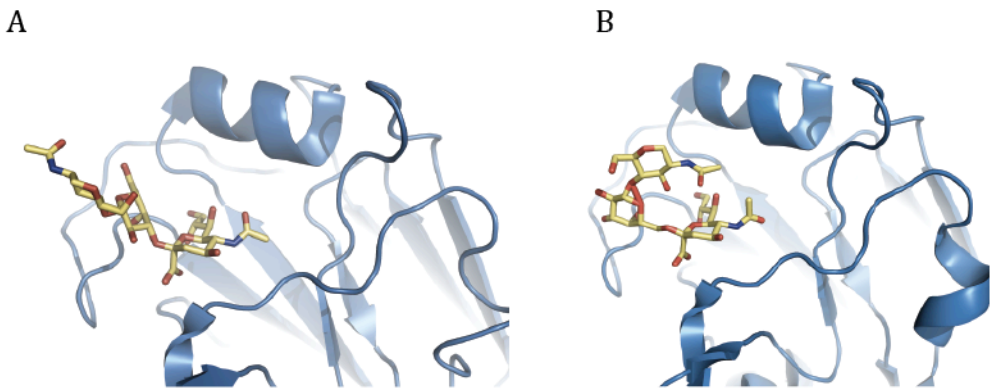

Figure 3 – Influenza interacts with the receptor sialic acid in one of two conformations, A) α2,3 linked sialic acid, and B) α2,6 linked sialic acid.

Not surprisingly, this binding specificity corresponds directly to the sialic acid specificity of the infection site of influenza for both humans and birds. In humans, the site of initial influenza infection, the upper respiratory tract, predominantly contains α2,6 SA (272), while in birds influenza largely infects the digestive tract, where the sialic acid preference mainly consists of α2,3 SA (146). This difference is thought to be one of the main barriers towards infection of humans by avian influenza isolates, and could be why avian influenza strains typically do not cause sustained human-human transmission. In general, it's believed that in order for an avian virus to become established in the human population, it must go through an intermediate host, although the 1918 pandemic is a notable exception. Swine are unique in that they express both α2,3 SA and α2,6 SA

in their respiratory tract, and are often described as a "mixing vessel" for human and avian influenza isolates (136, 215, 262). Interestingly, although swine contain both human and avian receptors, the receptor binding characteristics of swine isolates have generally been highly specific for α2,6 SA receptors (96, 249, 317). Recent evidence has suggested that swine have less α2,3 SA than α2,6 SA present in their respiratory tract, which is possibly why the dominant swine isolates prefer α2,6 SA (331).

Figure 3 shows how glycans with different sialic acid linkages are bound by the HA receptor binding site. Glycans with an α2,6 SA conformation sit in the receptor binding pocket and extend towards the 220-loop, before crossing back in front of the 190 α-helix. It's important to note that the distance between the helix and glycan can vary greatly depending on the virus subtype (257). Alternatively, glycans in an α2,3 SA conformation extend outward past the 220-loop, where it can make additional contacts with the 220 loop, but little is known about the conformation of the glycans past the third sugar, due to a loss of electron density (97). How the receptor interacts with the sialic acid receptor varies greatly depending on the subtype, species, and most likely the receptor itself, and might be critical in determining the pathogenic potential of swine and avian influenza isolates (97, 218).

Considering the seemingly high degree of receptor specificity by the HA protein, it might be thought that the affinity for the receptor would be high. However, Sauter et al revealed that HA has a surprisingly weak dissociation

constant in the millimolar range (260). The relative affinity for influenza HA towards the receptor can be affected in the different ways outlined below.

The level of glycosylation on the globular head of the HA varies as a pandemic strain becomes more adapted in humans, but not swine (29, 132). N-linked glycosylation sites are added throughout the HA, but are especially prevalent in the globular head region, where they can affect pathogenicity (242), receptor binding specificity (366), antigenicity (280, 355), and receptor affinity (341). The addition of glycosylation sites to the globular head of HA reduces the affinity for the sialic acid receptor, while the removal of these sites can increase the affinity for viral receptors to the point they are unable to elute effectively from cellular membranes (226, 340). The number of glycosylation sites influences receptor binding, but the length of N-linked glycosylation sites also can affect how the HA interacts with certain receptors (344). The removal of the terminal sialic acid from the N-linked glycan on HA could increase or decrease binding affinity to the same receptor, depending on the composition of the receptor glycan chain. As the N-linked glycans on the globular head decreased in length, the binding affinity generally increased, confirming the experiments performed by Wagner et al. These results have implications for the many different techniques used to determine receptor binding, which will be expanded upon in the discussion.

Viral entry and endocytosis

The ability of influenza to effectively infect cells despite a relatively low affinity for the sialic acid receptor seems to indicate that receptor binding requires a

high number of low level interactions to hold influenza at the cellular membrane for viral entry. Although a great deal is known about receptor binding, much less is understood about the exact mechanism for viral entry into the cell. Viruses have been shown binding to glycolipids (189, 191), but the productive infection of a glycolipid deficient cell line, GM95 (130), shows they are dispensable for viral infection and replication (2). In addition to glycolipids, the surface of the cell also contains N- and O- linked glycans, both of which may be sialylated. Little is known regarding the role of O-linked glycans on influenza binding and endocytosis. N-linked glycosylation is known to be necessary for infection, as the Lec1 cell line derived from CHO cells is unable to produce N-linked glycosylation is known to be resistant to virus infection and endocytosis, indicating the "trigger" that leads to endocytosis requires an N-linked glycan (55, 177). Direct binding of virus to macrophage surface proteins even suggested the receptor for macrophages might be proteinaceous in nature, as a virus overlay binding assay identified macrophage galactose-type lectin (MGL) and macrophage mannose receptor (MMR) as potential receptors (241, 328).

Endocytosis of pathogens into the cell can occur via five different routes:

1) Clathrin-mediated endocytosis
2) Caveolin mediated endocytosis
3) Macropinocytosis
4) Non-clathrin, non-caveolin mediated endocytosis
5) Phagocytosis

Currently, three of the five routes of infection have been determined to play a role in influenza infection: Clathrin-mediated, Macropinocytosis, and clathrin- and caveolin-independent endocytosis (73, 190, 235, 278). On one occasion, individual influenza virions were visualized entering a cell via two independent pathways in parallel, a clathrin mediated pathway and a clathrin- and caveolin-independent pathway (258), indicating there might be no single preferred method of entry for influenza virions. Interestingly, Rust et al noticed clathrin mediated endocytosis of influenza viruses seemed to involve the *de novo* initiation of clathrin-coated pits, and hypothesized it was receptor mediated.

Viral fusion and acidification

After endocytosis from the cellular membrane, the virion is exposed to lower and lower pH as it moves from the early to the late endosome. This leads to dramatic changes in the cleaved HA structure, and subsequent fusion of the viral and endosomal membranes. Concurrently, acidification of the endosome leads to an opening of the M2 proton channel and subsequent acidification of the virion, causing the release of RNP complexes from the M1 protein. Both will be discussed in greater detail below.

Unlike several other members of the order Mononegavirales (199, 282), membrane fusion occurs in the endosome, rather than the cell membrane. The fusion of the viral and endosomal membranes occurs in several steps, and has been well defined, thanks to the determination of the crystal structures of the precursor HA0, cleaved HA and the low-pH HA (32, 46, 357), as well as a plethora of studies

examining fusion mutants (68, 103, 114, 152, 170, 220, 230, 298, 299). The steps involved in membrane fusion are described below:

1) HA0 must be cleaved into HA1 and HA2 by cellular proteases. This causes the exposed N-terminus, which is made up of largely hydrophobic residues, to insert into a cavity near the cleavage site. This cavity is lined with ionizable residues, which are then shielded by the insertion of the highly conserved HA2 N-terminal end.
2) The virus binds to an N-linked glycan, and is endocytosed. The transition between an early and late endosome leads to a decrease in the pH of the endosome. Fusion occurs at a pH between 5.0-6.0, but varies depending on the composition of the fusion peptide (67, 298, 299).
3) After reaching the requisite pH, the heads of the HA detrimerize and can no longer be visualized by electron microscopy due to high electron disorder.
4) Detrimerization of the globular heads may affect the ionizable residues near the fusion peptide (which is generally considered to consist of ≈23 amino acids), and the fusion peptide is expelled from the cavity it filled after cleavage.
5) The long α-helix in HA2 is elongated as the extended chain transitions into an α-helix. This leads to the relocation of the fusion peptide toward the endosomal membrane.
6) The amino acids between residues 106-112 transition from an α-helix to a loop conformation, and in the process are inverted by 180 degrees. This

conformational change serves to force the membrane anchor domain to the endosomal membrane near the fusion peptide.

It should be stated the full details of these rearrangements and folding/refolding events are currently unknown, as is the order they occur, in part due to the lack of determined intermediary structures.

Along with HA fusion, acidification of the virion leads to dissociation of the NP and M1 proteins. Confocal microscopy experiments have visualized co-localization of these two proteins prior to entry into the cell, but they dissociate not long after penetration (187). This dissociation is required for the RNP to enter the nucleus, as M1 is directly responsible for interacting with exported vRNAs to prevent reimportation of the gene segments (352). Transport of the RNP to the nucleus is an active process aided by interaction of the NLS signal(s) on NP with importin α and β (21, 223, 224). The RNP complex enters the nucleus via nuclear pores, a process that is highly unusual for RNA viruses, as most complete their replication in the cytosol. Protein transcription and translation occurs immediately after the complex enters the nucleus, which will be discussed in greater detail with protein trafficking below. At some point during viral replication, a switch occurs where the RNP complex produces fewer mRNA segments and more vRNA segments, but exactly what triggers this shift is still uncertain.

Viral Genome Replication

The viral RNA (vRNA) replication of the influenza genome is a complicated process involving PB1 (24), PB2 (13, 326, 327), PA (87), NP (9, 122, 267), and

cellular RNA Polymerase-2 protein through cap snatching. Viral RNA is transcribed into either capped mRNA, which is translated into protein, or into complementary RNA (cRNA), which serves as a template for additional vRNA replication. At the beginning of viral replication, the majority of the vRNA is transcribed into mRNA for protein synthesis, however later in the infection, the dynamic shifts, and more cRNA is transcribed. The factors responsible for this switch are unknown, but convincing evidence suggests that small viral RNA's might be responsible (237). The expression of svRNAs seem to cause an increase in vRNA, and later, a bias in the RNA-dependent RNA-polymerase (RDRP) away from transcription and toward genome replication.

The synthesis of mRNA first requires a 5' cap before transcription can take place. Influenza acquires a 5' cap in a process known as "cap snatching," carried out by the PB1 and PB2 proteins (239). PB2 identifies and binds to the 5' cap of a cellular mRNA, and after it does so, the endonuclease function of PB1 removes the cap. The RNP complex carrying the 5' cap begins transcription using the 10-13 nucleotides remaining on the cap to act as a primer. Elongation is performed by the PB1 protein, and continues until reaching the poly(A) sequence marker at the 5' terminus of vRNA (24). The poly(A) tail is created by PB1, rather than a different viral or cellular polymerase. This is accomplished by a stuttering event that occurs when PB1 reaches the 5-7 "U" residues at the 5' end, and stutters to form the poly(A) tail (240). The mRNA templates can then be translated into protein.

Protein expression and trafficking

Newly synthesized vRNA/RNP complexes exit the nucleus with the aid of the NEP protein and the M1 protein, which prevents re-entry of the complex into the nucleus as described previously. The M1 is then believed to carry the RNP complex to the apical cell membrane, but how this occurs is still unclear.

Influenza contains eight gene segments that encode between 10 and 12 different proteins. The PB1-F2 protein is translated due to an alternate ORF found in the PB1 protein (48). Segments 7 and 8 both encode two proteins, and the translation of M2 and NS2/NEP proteins occur by spliced mRNA products (155, 156). After translation of viral proteins, the HA, NA and M2 protein traffic to the ER where they are folded, oligomerized (HA becomes a trimer, NA and M2 are tetramers), glycosylated (except for M2) and palmitoylated (except for NA).

Glycosylation of HA is important for everything from structural formation, trafficking, receptor binding, and immune evasion. The overall number of glycosylation sites varies dramatically depending on the strain and subtype, but only N-linked glycosylation is seen on the HA. N-linked glycosylation sites occur at consensus Ser/Thr-X-Asn sites, but not every site is glycosylated, depending on the availability of the site to various host glycosyltransferases. Human pandemic strains historically encode for fewer glycosylation sites than circulating seasonal strains, but increase that number in an attempt to "hide" antigenic sites from neutralizing antibodies (355). The addition of glycosylation sites to circulating strains over time might allow for immune escape, but recent work indicates that it may carry a fitness cost to the virus (336). The 1968 pandemic strains encoded only 6-7 N-linked glycosylation sites, but H3N2 viruses circulating today that are descendents of that

pandemic strain contain 10-14 glycans (370). Those additions typically occur in the head domain, rather than the HA stalk, as the number and location of stalk glycosylation sites is known to have an affect on folding and fusion (227, 244). Site directed mutagenesis reveals that at least three glycosylation sites are necessary for the proper transport of HA. The type of glycosylation present at a particular site varies from residue to residue, depending on the location and availability of the site to enzymatic processing (142, 198). For example, the glycosylation site at N65 is buried in the HA structure, and so the N-linked glycan consists of only an oligomannose oligosaccharide, whereas the glycosylation sites exposed near the receptor binding domain are complex glycans (142, 198). HA is also palmitoylated on three cystine residues in the golgi apparatus. These sites are critical for trafficking to lipid rafts on the cell membrane, and mutations at these sites lead to a decrease in efficient virus production (44).

The HA, NA, and M1 proteins can all traffic to the lipid rafts at the cell membrane without assistance, whereas M2 does not appear to do so (164, 315). At the lipid raft, the M1 protein associates with the cell membrane and is thought to attach to the NP protein of the RNP. There is also evidence that the M1 protein binds to the cytoplasmic tails of HA and NA, and might act as a master recruiter to the site of budding (43).

The mechanism of viral gene segment packaging is still controversial, primarily due to a number of conflicting results. Two models have been proposed, the “random incorporation” model, and the “selective incorporation” model. The selective incorporation model states that each virion encases one copy of each gene

segment, and is boosted by the fact that although there are varying levels of vRNA in the cell, there appear to be equimolar vRNA quantities in viral particles (284). There is also evidence that removal of a gene segment decreases the efficiency of virus particle formation (93, 211). Electron microscopy that gives a view of the interior of a virion appears to show only eight segments present inside the virion (221) The "random incorporation" model stipulates that more than eight gene segments are packaged, and the main evidence comes from viruses containing more or less than eight segments (86, 99, 200).

Discrepancies between VLP particles and influenza virions have made it difficult to accurately examine the mechanism of budding from the viral membrane. As mentioned earlier, both HA and NA traffic to lipid rafts, but M2 does not. Currently, four viral proteins are known to mediate viral budding: HA (43), NA (153), M1 (106), and M2 (254). However, of the four proteins involved in mediating viral budding, only the HA and NA seem capable of initiating the budding event. The scission event mechanism has not yet been fully deciphered, but is known to be ATP dependent, as Antimycin and CCCP were shown to inhibit budding (127). Evidence also shows scission does not require the ESCRT machinery (254), and therefore must require additional host machinery. The late expression of the M2 protein is thought to indicate a role in budding and scission (369), and a model has been proposed (255). Upon completion of scission, the virus may still be bound to the cell membrane via sialic acid receptors on the surface. The final step involves the cleavage of sialic acid by the NA protein, which may be concentrated at the scission end of the virion to aid in virus release (36).

Transmission/Host adaptation

The error prone RNA dependent, RNA-polymerase (RDRP) contributes greatly to the adaptability of influenza viruses in nature. The error rate is often cited as approximately 10^{-3} substitutions per site per year, although that estimation probably varies from gene to gene (33, 297). Several animal models have been developed to study transmission patterns of influenza strains. The ferret model is the oldest, and seems to be the most human-like in terms of pathogenicity and rate of transmission (286). Influenza strains are capable of infecting ferrets without adaptation, and reproduce many of the same symptoms as humans (sneezing, fever...etc) (117, 286, 371). More recently, a new model for influenza transmission has emerged. This model, first described by Lowen and Palese, utilizes guinea pigs (179), which are advantageous due to their size, and are capable of shedding high titers of influenza viruses without prior virus adaptation (179). Unfortunately, they do not display any signs of illness or symptoms after infection, making them suitable for only describing the transmission capabilities of strains, rather than pathogenicity. Mice are an ideal model to study the immunology, pathogenesis, and challenge studies, based on their size and the large number of immunological tools available. However, the sialic acid distribution drastically differs between humans and mice, and infected animals do not display the same disease symptoms as humans. Finally, mice are unsuitable for transmission studies as they do not effectively shed virus (179), and they display $CD8^{+}$ mediated heterosubtypic

immunity which calls into question their effectiveness as a model for human vaccine challenge studies (160).

Both the ferret and guinea pig models can distinguish between respiratory droplet (RD) transmission, direct contact transmission (DC), and fomite transmission. Most human and swine influenza strains are capable of transmitting via DC, but RD transmission is most likely the best indication of human-human transmission capabilities. Notably, the recent 2009 H1N1 pandemic isolates (pH1N1) were shown to spread between ferrets as efficiently as seasonal H1N1 strains using the direct contact method, but were notably less efficient at transmitting via respiratory droplet (185). Also, the closely related triple reassortant swine strains isolated from human patients efficiently transmitted in the DC ferret model, but were unable to transmit via respiratory droplets (11), which is consistent with the relatively poor human-human transmission seen with these strains (271). In addition avian viruses such as H5N1, generally display poor ferret-ferret transmission capabilities (184, 368). This seems to be due to the lack of α2,3 SA expression in the human upper respiratory tract, as well as inhibitory affects from human upper respiratory tract mucins (35, 303). Fomite transmission of influenza is generally regarded as poor, as work with guinea pigs has shown (203), but cannot be entirely ruled out as influenza related conjunctivitis with H7N7 (88) and H5N1 (42) viruses could be the result of contaminated fingers touching the mucosal surface of the eye.

Because influenza infections occur primarily in the winter months, it would seem logical that influenza transmission is more efficient in the winter, rather than

the summer. Early research confirmed that influenza transmits more efficiently during the winter months (264), but did not fully explain why this occurs. More recently research by Lowen and Palese revealed that viral transmission was much more efficient at 5˚C, in part due to an extended viral shedding time of 40 hours over the same virus at 20˚C. Decreasing the humidity also served to increase overall transmission as well, further explaining the increase in influenza cases during the winter (178).

In addition to the role played by sialic acid specificity and temperature described above, the temperature sensitivity of a virus also plays a role in virus adaptation. Influenza infects the avian enteric tract where the internal temperature is around 40˚C, while human upper and lower respiratory tract are closer to 32˚C and 37˚C respectively (3, 349). The significant variance between the temperature of the avian enteric tract, and the human upper respiratory tract suggest that for an avian influenza strain to successfully infect and transmit from person-person, adaptations to the polymerase are first necessary. Two amino acids at 627 and 701 in the PB2 protein are closely linked to temperature sensitivity, host adaptation, and transmission. Viruses isolated from avian species contain the E627 variant, while human influenza strains contain L627. This mutation was first determined to be a host restriction factor for growth in MDCK cells (307), and later was determined to increase polymerase activity (94). More recently, the residue at 627 was determined to be a factor in the temperature sensitivity (*ts*) of influenza strains (188), with E627 conferring productive replication at 37˚C, and K627 conferring replication at 33˚C and 37˚C.

As was alluded to earlier, the interaction of HA with its receptor sialic acid also plays a major role in adaptation to a new host. Sialic acid is bound by influenza in one of two conformations, α2,3 SA by avian strains, or α2,6 SA by human strains. Aside from differences in body temperature, humans, birds, and swine differ in the predominant type of sialic acid expressed on their cell surfaces. Influenza in avian species causes an intestinal infection, and the cells it infects predominantly express α2,3 SA (136, 147). On the other hand, the sialic acid expression in humans is slightly more complex. Cells in the upper respiratory tract (URT) predominantly express α2,6 SA except for mucin-secreting goblet cells (95, 272). However, as the URT turns into the lower respiratory tract (LRT), α2,3 SAs expression increases and is predominantly found on Alveolar type II cells. This sialic acid expression pattern might partly explain why the H5N1 viruses that infect humans often cause pneumonia, but do not transmit efficiently human-human. It has been suggested that avian strains must first be able to bind α2,6 SAs before they can become fully established in humans. Worryingly, some avian viruses circulating in Southeast Asia are purported to have an increased affinity for α2,6 SAs, possibly acquired during human infection (300), and there has been an increase in upper respiratory febrile illness since 2005, which may be another indication of a shift in sialic acid specificity (359). However, direct infection of humans by avian influenza isolates remains extremely rare, and it is generally thought that in order for an avian H5N1 virus to fully adapt to the human receptor, it must first infect an intermediate host such as swine, where both α2,3 and α2,6 SAs are expressed (331). Both human and avian isolates are known to propagate in swine (261), and are often described as "mixing

vessels" for avian and human isolates, which is appropriate considering the high amount of segment reassortment associated with swine influenza strains. Most of these strains appear to be evolutionary dead-ends, but their presence makes continual surveillance of circulating swine strains a high priority.

The receptor specificity of influenza isolates predictably mimics the dominant sialic acid expressed in the host. Therefore, human isolates preferentially bind α2,6 SA, and avian isolates preferentially bind α2,3 SA (61, 151, 249, 251, 303, 361, 362, 364), and numerous crystal structures of the HA bound to sialic acid in both conformations have been determined (84, 97, 113, 175, 257, 303, 357, 361). Interestingly, despite the presence of both α2,3 and α2,6 SA in the swine respiratory tract, evidence indicates that established swine isolates, including trH1N1 isolates, exclusively bind α2,6 SA (7, 26, 47, 96).

SIALIC ACID AS A RECEPTOR

Sialic acid is a ubiquitous, nine-carbon acidic monosaccharide, predominantly found in nature in one of two major forms, N-acetylneuraminic acid (Neu5Ac), and N-glycolyneuraminic acid (Neu5Gc). In general, both major forms of sialic acids are found in all organisms in the *Deuterosome* lineage (mammals and higher insects), as well as in some bacteria (5, 95, 334). Humans are a notable exception, in that they lack the ability to express Neu5Gc, due to a 92-bp deletion in the human copy of the CMP-Neu5Ac hydroxylase (53, 135) responsible for adding the –OH group onto the fifth carbon of Neu5Ac (C5) (204, 268, 269). Neu5Gc makes up less than 0.1% of all the sialic acid in the body (279), and anti-Neu5Gc antibodies

have been identified (118, 205). It is believed the loss of Neu5Gc synthesis occurred early during human evolution, most likely the result of evolutionary pressure by pathogens such as ancient malaria parasites (335). Interestingly, Neu5Gc is expressed at higher concentrations in tumors, which might suggest additional active suppression of Neu5Gc in normal human cells.

In addition to the Neu5Ac and Neu5Gc forms of sialic acid listed above, numerous other sialic acid derivatives exist. In general, these derivatives all share the common nine-carbon backbone associated with Neu5Ac and Neu5Gc, but carry additional modifications such as the addition of methyl, acetyl, hydroxyl, or lactyl groups among many others (334). The full importance of these sialyl-derivatives is unknown, but our group and others have recently published articles that showed binding to sialic acid derivatives, in particular KDN (deaminated neuraminic acid) and Neu5Ac9Lt (9-O-lactyl-5-N-acetyl-neuraminic acid) (25, 290). Both KDN and Neu5Ac9Lt are expressed in humans, albeit at lower levels. Neu5Ac9Lt comprises approximately 20% of the sialic acid in serum, and 1-8% of the sialic acid on the surface of erythrocytes, depending on blood type (27, 31). KDN was first isolated in fish eggs (209), and has more recently been found in human RBCs, fetal cord blood, A549 cells, and pig submaxillary glands among others (133, 134). The presence of these glycans in mammalian tissues seems to indicate that binding by influenza isolates is biologically relevant, but the role they play in influenza infection is still unclear.

Sialic acid has numerous functions in cellular biology and signaling. Most importantly, the presence of sialic acid often determines “self” from “non-self”, by

mediating cell-cell interactions via CD 22, which leads to modulation of the immune system (58, 81, 104, 111). The removal of SAs on dendritic cells is also linked to a decrease in endocytosis followed by an increase in MHC and costimulatory proteins, the overall effect being the promotion of a Th1 response (66).

Because of the vast abundance of sialic acids on the cell surface, they are often utilized by pathogens as receptors. As mentioned earlier, human influenza A and B both bind to Neu5Ac, and some strains of swine influenza viruses are believed to bind Neu5Gc as well (311). Influenza C virus binds exclusively 5,9 N-acetylneuraminic acid (9-O Acetyl or Neu5,9Ac_2), as do some human coronaviruses (250, 253, 338). Paramyxoviruses such as Newcastle disease virus, sendai virus, HPIV-1, and -3 are believed to preferentially bind α2,3 SAs (4, 236, 312). Sialic acid receptor binding is not limited to just viral pathogens, and many bacteria and a few parasites are known to bind it as well (182, 210, 229). Mucins secreted by goblet cells in the mammalian URT are an evolutionary response to sialic acid binding pathogens. They are encoded by several MUC genes, and generally consist of long carbohydrate chains terminating in sialic acid, often containing fucose (reviewed in (158)). Interestingly, the predominant linkage of sialic acid in mucins is often opposite of the predominant linkage found on cells of the URT. In humans, secreted mucins generally contain α2,3 SA (157), while the cells of the URT are predominantly α2,6 SA (272). However, in the murine and great ape respiratory tract, the linkage specificity of mucins is reversed, with mucins predominantly α2,6 SA, while the URT cells do not express α2,6 SA (95, 129).

VACCINES AND ANTIVIRALS

Vaccines

Currently, there are two licensed influenza vaccines available, the trivalent inactivated vaccine (TIV) and the live-attenuated vaccine (LAIV). The continuous antigenic drift of circulating influenza strains requires vaccine companies to update their vaccine stock strains on a regular basis. To do this quickly, both TIV and LAIV utilize a 6:2 reassortant strategy, meaning the two external glycoproteins are replaced as necessary while the other six segments make up the genetic backbone remain constant (246). In the case of LAIV, the genetic backbone (A/Ann Arbor/6/1960) contains the temperature sensitive components of the vaccine (181), which allows the HA and NA genes to be replaced upon antigenic drift (208). Because the internal genes do not change rapidly though antigenic drift, and also contain the primary T-cell epitopes, it is thought the LAIV strains can elicit both an antibody and cell-mediated immune response (161, 197). The TIV vaccine, on the other hand, appears to only elicit an antibody response. Randomized trials have put the influenza vaccine efficacy at 85-90%, but this likely changes from year to year depending on how well the vaccine and circulating strain are antigenically matched (28, 354).

Li and colleagues showed the 6:2 reassortant strategy could be used to prepare vaccine stocks for pandemic viruses after the 1997 H5N1 outbreak (168). This strategy has been utilized several times since then, with either the cold adapted Ann Arbor strain, or PR8 acting as the genetic backbone (49, 234, 308, 347). In general however, H5 vaccine candidates are poorly immunogenic, and an

inactivated subvirion vaccine only elicited a response in approximately half of recipients after two doses (323). Why the H5 strains are so poorly immunogenic is currently unknown, as influenza strains typically promote a robust immunological response (14, 65, 83, 321). Recently, a live attenuated H5N1 candidate was protective and efficacious in mice and ferrets, despite low antibody responses, but did not elicit neutralizing antibody responses when tested in healthy adult volunteers (139, 308). One issue could be a lack of replication by recent LAIV H5 candidates, since the magnitude of T-cell and antibody responses seem to correlate with LAIV replication (163).

Alternative vaccine strategies for influenza do exist, and are in ongoing experiments and clinical trials. Current vaccines are required to grow well in eggs in order for the vaccine to be ready in time for the yearly outbreaks. DNA vaccines circumvent this requirement, and can be manufactured without the use of eggs, which also gives them the advantage of being available to those with egg allergies. It was hoped that DNA viruses would prompt a cell-mediated immunity response to influenza; however, early results were not promising (180). More recently, a DNA vaccine containing the HA gene segment underwent phase I clinical trials, and proved to be safe, and effective at high plasmid dosages (82).

Other than DNA based vaccines, vector based vaccines are also under development. The M2 protein is generally conserved from strain to strain, but is poorly immunogenic. However, when the ectodomain is expressed with the hepatitis B core protein (which effectively acts as a VLP), the result is a particle that is highly protective in mice (214). Finally, another approach supplements the

inactivated influenza vaccine with an M2 expressing VLP, was shown to lead to long lasting, heterosubtypic immunity in mice.

Antivirals

Because vaccine production takes between 6-9 months to prepare, antivirals are the most likely first line of defense against any emerging pandemic. There are currently four licensed antiviral drugs on the market, which can be categorized into two classes, M2 inhibitors, and neuraminidase inhibitors. Amantadine and rimantadine comprise the M2 inhibitors. Amantadine was the first influenza antiviral drug designed (70), and acts similarly to rimantadine by filling the M2 proton channel, preventing acidification of the virion during entry. Resistance to the M2 inhibitors is currently widespread in circulating influenza strains (39), and is associated with four residues, V27, A30, S31, and G34 (116). A single mutation at any of these sites renders the virus resistant to amantadine, and does not appear to have any affect on viral fitness (314).

The second class of inhibitors, the neuraminidase inhibitors (NAI), is comprised of oseltamivir (Tamiflu) and zanamivir (Relenza). Both of these inhibitors act in a similar manner by using sialic acid analogs that bind the catalytic pocket of the neuraminidase, allowing them to inhibit both influenza A and B viruses (59, 339). Viruses treated with neuraminidase inhibitors have poor neuraminidase activity, and are therefore unable to release from the cell membrane upon viral exit. Resistance to NAIs was first noted in 2008, and is often associated with a single mutation at residue 274 in the neuraminidase (115). By 2009, resistance to NAIs

was prevalent in seasonal influenza isolates (77). The H274Y mutation and other identified resistance mutations, are noted to confer a fitness cost to the seasonal H3N2 by reducing aerosol transmission efficiency (23). More recently, pH1N1 oseltamivir resistant mutant viruses were shown to have no decrease in aerosol transmission efficiency, or pathogenicity in ferret and guinea pig models (266). This might indicate the pH1N1 strains contain a weakly binding HA that is less dependent on viral neuraminidase activity for release. This is consistent with our results in Chapter 2, where we examined the receptor binding patterns of early pandemic isolates, and noted relatively weak binding to the glycan micro arrays provided by the Consortium for Functional Glycomics (www.functionalglycomics.org) (26, 185).

In the following chapters, we attempt to detail the receptor binding of HA to sialic acid using glycan microarray technology, by examining currently circulating strains, as well as viruses with mutations outside of the receptor binding site. We use glycan microarray technology to provide a unique opportunity to explore the nuances of influenza interaction with the glycan receptor, including the role of fucose, sulfate, and other sialic acid derivatives. Specifically we were able to demonstrate changes in the recognition properties of seasonal strains immediately prior to a pandemic, early pandemic isolates, and swine strains with closely related HA proteins. In addition to our work on pandemic strains, we isolated and characterized mutants that do not bind to classical influenza receptors, indicating an alternative method in binding and entry is present. We hope the results below have

an impact on future receptor binding studies, viral entry, and pandemic surveillance of avian influenza and swine reassortant viruses.

CHAPTER 2

Comparison of the receptor binding properties of contemporary swine isolates and early human pandemic H1N1 isolates (novel 2009 H1N1)

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Title: Comparison of the receptor binding properties of contemporary swine isolates and early human swine origin influenza virus isolates (Novel H1N1)

Running Title: S-OIV Receptor Binding

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Abstract:

We have utilized glycan microarray technology to determine the receptor binding properties of early isolates from the recent human pandemic of H1N1 swine-origin influenza virus (S-OIV), and compared them to North American swine influenza isolates from the same year, as well as past seasonal H1N1 human isolates. We showed that the S-OIV strains, as well as the swine influenza isolates examined, bound almost exclusively to glycans with α2,6-linked sialic acid with little binding detected for α2,3-linked species. This is highlighted by pair-wise comparisons between compounds with identical glycan backbones, differing only in the chemistry of their terminal linkages. The overall similarities in receptor binding profiles displayed by S-OIV strains and swine isolates indicate that little or no adaptation appeared to be necessary in the binding component of HA for transmission from pig to human, and subsequent human to human spread.

Keywords: Influenza, Hemagglutinin, Receptor Binding, Glycan Microarray, Swine influenza

Introduction:

According to the World Health Organization, the viruses responsible for the recent human outbreak of the H1N1 subtype swine-origin influenza viruses (S-OIV) were first detected in North America in April of 2009, have since been isolated in more than 200 countries, and have caused greater than 18,000 deaths worldwide. The pandemic H1N1 strains are composed of six segments derived from a triple reassortant swine H1N1 strain (trH1N1), and two segments from the Eurasian "avian-like" swine H1N1 viruses (Garten et al., 2009). Initially, there were concerns that the new human strains were highly pathogenic, due a high mortality rate of infected patients with complications in a Mexico City hospital (Domínguez-Cherit et al., 2009; Fraser et al., 2009). However, as the virus spread through the United States, the majority of symptomatic infections were found to result in uncomplicated, upper respiratory tract illness with a relatively low mortality rate. The newly emergent H1N1 strains rapidly spread worldwide, and the S-OIV outbreak was declared a phase 6 pandemic by the WHO in June 2010 (Dawood et al., 2009). Despite the fact that pandemic H1N1 viruses (pdmH1N1) are not recognized as highly pathogenic in the classical sense, certain features of their disease profile appear to differ from the seasonal H1N1 viruses that have circulated in humans since 1977. They affected children and younger adults at much higher rates than older adults and the elderly, likely as a result of immunity in the population that had been exposed to H1N1 viruses that circulated prior to 1957 (Hancock et al., 2009). In addition, the disease they caused in children is often more serious than had been

observed with the previously circulating seasonal H1N1 strains (Bhat et al., 2005; CDC, 2009; Louie et al., 2009), and had significant effects in terms of years of lost life (Viboud et al., 2010). For these reasons, the characteristics of replication, transmission, and pathogenicity of pdmH1N1 viruses and related human and swine viruses warrant continued and detailed scrutiny.

The triple reassortant virus (trH1N1), which contributed 6 of the 8 gene segments in the pdmH1N1, is made up of segments from avian, human, and swine viruses. The trH1N1 viruses emerged in swine in the late 1990's, and were first reported in humans in 2005 (Olsen, 2002; Shinde et al., 2009). These strains periodically infected humans through 2009, typically resulting in mild illness, but did not become established in the human population (Shinde et al., 2009). Currently, it is unclear when or where the reassortant event that combined the six segments from the trH1N1 and the NA and M segments from the Eurasian H1N1 swine influenza viruses occurred, but phylogenetic analysis has suggested the pandemic reassortant could have been circulating undetected for several years (Smith et al., 2009). Numerous studies have characterized the pathogenicity and transmissibility of the current S-OIV strains in rodents, ferrets, and swine, as well as the susceptibility of different age groups to the new strain (Belser et al., 2010; Brookes et al., 2010; Ge et al.; Hancock et al., 2009; Itoh et al., 2009; Maines et al., 2009; Steel et al.), and in some experimental settings it seems that S-OIVs can replicate more efficiently in the lower respiratory tract compared to seasonal H1N1 strains. Such studies suggest that the receptor binding properties of pdmH1N1 may

have unusual features by comparison with other H1N1 viruses that have been characterized previously.

Influenza virus receptor binding is mediated by the hemagglutinin glycoprotein (HA), and is thought to play a major role in the ability of influenza to infect and propagate in a new species (Horimoto and Kawaoka, 2005; Webster et al., 1992). Influenza virus binds to cell-surface glycans containing N-acetylneuraminic acid (Neu5Ac) or its derivatives (termed sialic acids) at their termini (Gottschalk, 1959; Klenk et al., 1955). The linkage by which sialic acids are attached to the penultimate sugar of the glycan is thought to play a role in host range specificity of influenza viruses, often based on whether this glycosidic bond is present in an α2,3 or α2,6 conformation (Connor et al., 1994; Ito and Kawaoka, 2000; Rogers and D'Souza, 1989; Skehel and Wiley, 2000). Influenza viruses isolated from avian species commonly replicate in the gut, and preferentially bind α2,3-linked sialic acid (α2,3 SA), while influenza in humans is a respiratory disease, and tends to prefer α2,6-linked sialic acids (α2,6 SA). Swine contain an abundance of both α2,3 and α2,6 SA in their respiratory tract (Ito and Kawaoka, 2000; Nelli et al., 2010) and therefore are considered to be a potential "mixing vessel" for human, avian, and other swine influenza strains (Scholtissek et al., 1985). In previous studies using enzymatically modified erythrocytes, swine viruses have shown a preference for α2,6 SA binding (Gambaryan et al., 2005; Rogers and D'Souza, 1989; Takemae et al., 2010), and it is generally believed for an avian or swine influenza strains to infect humans and transmit efficiently from person to person, it must first be able to bind α2,6 SA (Horimoto and Kawaoka, 2005; Matrosovich et al., 2000).

Although there has been a selection of studies investigating the binding properties of pdmH1N1 viruses published recently (Childs et al., 2009; Maines et al., 2009; Yang et al., 2010), we have chosen to expand on these reports by conducting a comprehensive study of the binding characteristics of pdmH1N1 viruses using glycan microarrays. For comparative purposes, we include in these studies parallel analyses of H1N1 swine influenza viruses that were isolated from pigs during the same year of the pandemic outbreak, as well as seasonal H1N1 viruses that circulated in humans before 2009. In addition to HA receptor-binding characteristics, it is believed that a functional balance between HA binding and neuraminidase (NA) enzymatic activity may be critical for efficient transmission of influenza viruses(Banks et al., 2001; Matrosovich et al., 1997). The NA of pdmH1N1 viruses is derived from avian origins, but has been circulating in Eurasian swine for three decades. Therefore, to evaluate the functional balance between HA and NA, we have also examined the NA enzymatic activity of these viruses.

Results:

Viruses examined

The details regarding initial transmission of pdmH1N1 to humans have not been established, but the two strains of influenza virus that combined to create the quadruple reassortant pandemic H1N1 virus involved triple reassortant viruses (trH1N1) that had circulated in North American swine since the late 1990s and Eurasian “avian-like” H1N1 viruses, with HA genes being derived from the trH1N1

viruses (Garten et al., 2009). For these studies, we assessed the receptor binding properties of three 2009 human pandemic H1N1 strains, three contemporary strains of 2009 swine isolate trH1N1 viruses, and three seasonal human H1N1 strains that had circulated in previous years. We also examined the classical 1931 swine H1N1 isolate A/swine/Iowa/1976/31, and two H3N2 viruses for comparative purposes.

Influenza isolates are routinely amplified in embryonated chicken eggs; however, it is well established that viruses passaged in eggs have a propensity to select for mutations in the HA receptor binding region that may alter the HA affinity for various sialic acid containing cell surface receptors (Burnet and Bull, 1943; Gambaryan et al., 1999; Katz et al., 1990; Katz and Webster, 1988; Lugovtsev et al., 2009; Robertson et al., 1987; Robertson et al., 1985; Stevens et al., 2010). Therefore, we utilized minimal passage in MDCK cells to amplify viruses whenever possible, and the HA and NA genes for stock viruses were sequenced and compared to the sequences obtained from initial isolates as well as published sequences to ensure against laboratory-generated selection of viruses with altered binding characteristics. The A/Brisbane/59/2007 and A/New Caledonia/20/1999 human vaccine strains, as well as the 1931 swine isolate A/Swine/Iowa/1976/1931 were included in this study for comparative purposes, but their passage histories are not well documented, and almost certainly include propagation in eggs at some stage. The viruses and their sequences at HA positions known to be relevant for receptor binding properties are shown in Table 1. Figure 1 shows the structure of the H1 S-OIV A/California/04/2009 receptor binding site (Xu et al., 2010) and highlights the

locations of residues pertinent to this study. Figure 2 shows the phylogenetic relationships of viruses utilized in this study.

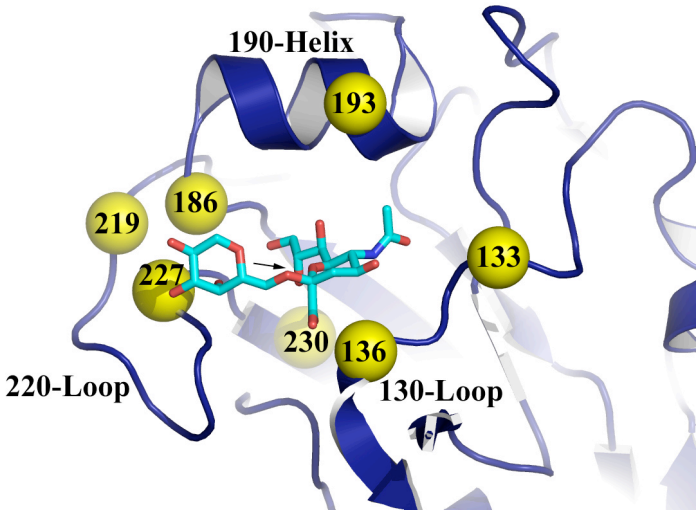


Figure 1 – Schematic diagram of the HA receptor binding site and major structural characteristics (dark blue), and the location of bound Neu5Aca2,6Gal (light blue) in the binding pocket. The arrow indicates the a2,6 glycosidic linkage, and the yellow balls show the locations of residues that differ among strains analyzed in this study and discussed in the text. All numbering based on H3 the subtype prototype HA from A/Aichi/2/68 virus.

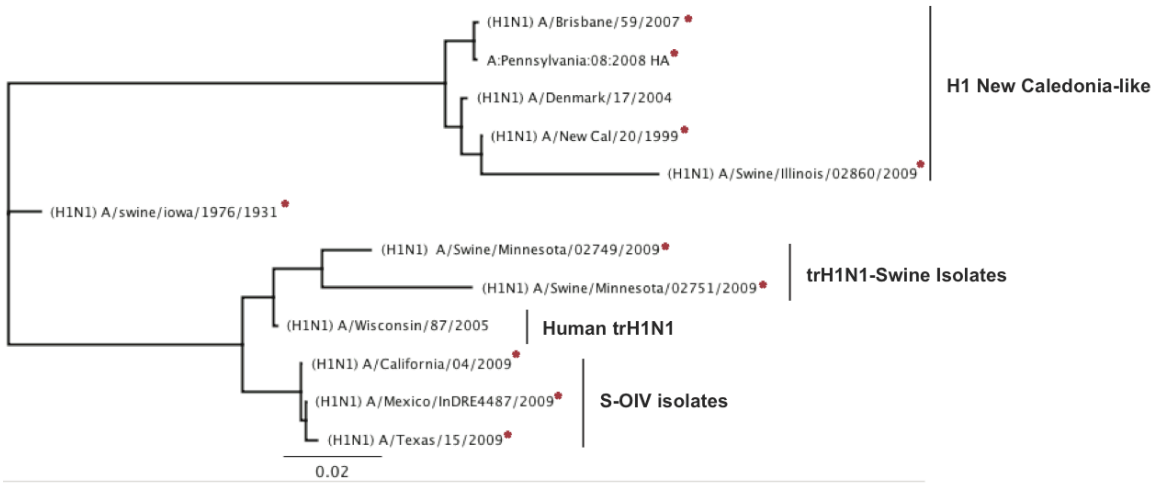


Figure 2 – Basic phylogenetic tree of related influenza isolates, as well as isolates utilized in this study. The tree was assembled using the genetic distance model HKY and the tree building neighbor-joining methodology with no outgroups. Strains marked with a red asterisk indicate the isolate was used in this study.

Table 1
Receptor binding site sequence alignment of strains examined by glycan array analysis (H3 numbering)

		Residue number													
		130 Loop								190 Helix					
Subtype	Strain	133	134	135	136	137	138	183	186	190	191	192	193	194	195
Seasonal H1N1	**A/Pennsylvania/08/2008**	**T**	**G**	**V**	**S**	**A**	**S**	**H**	**P**	**D**	**Q**	**K**	**T**	**L**	**Y**
	A/Brisbane/59/2007	T	G	V	S	A	S	H	N	N	Q	K	A	L	Y
	A/New Caledonia/20/1999	T	G	V	S	A	S	H	N	N	Q	R	A	L	Y
SOIV H1N1	A/California/04/2009	K	G	V	T	A	A	H	S	D	Q	Q	S	L	Y
	A/Mexico/INDRE4487/2009	K	G	V	T	A	A	H	S	D	Q	Q	S	L	Y
	A/Texas/15/2009	K	G	V	T	A	A	H	S	D	Q	Q	S	L	Y
Swine Isolates (H1N1)	A/Sw/Minnesota/02749/2009	R	G	V	T	A	A	H	S	D	Q	Q	S	L	Y
	A/Sw/Minnesota/02751/2009	K	G	V	T	A	A	H	P	D	Q	Q	T	L	Y
	A/Sw/Illinois/02860/2009	T	G	V	S	A	S	H	P	N	Q	R	T	L	Y
H3 subtype	H3N1 WT	N	G	G	S	N	A	H	S	E	Q	T	S	L	Y
	A/Sw/Minnesota/02719 (H3N2)	D	G	S	S	Y	A	H	G	D	Q	T	N	L	Y

		220 Loop													
Subtype	Strain	217	218	219	220	221	222	223	224	225	226	227	228	229	230
Seasonal H1N1	**A/Pennsylvania/08/2008**	**I**	**A**	**K**	**R**	**P**	**K**	**V**	**R**	**D**	**Q**	**E**	**G**	**R**	**I**
	A/Brisbane/59/2007	I	A	K	R	P	K	V	R	D	Q	E	G	R	I
	A/New Caledonia/20/1999	I	A	K	R	P	K	V	R	D	Q	E	G	R	I
SOIV H1N1	A/California/04/2009	I	A	I	R	P	K	V	R	D	Q	E	G	R	M
	A/Mexico/INDRE4487/2009	I	A	I	R	P	K	V	R	D	Q	E	G	R	M
	A/Texas/15/2009	I	A	I	R	P	K	V	R	D	Q	E	G	R	M
Swine Isolates (H1N1)	A/Sw/Minnesota/02749/2009	I	A	T	R	P	K	V	R	D	Q	A	G	R	M
	A/Sw/Minnesota/02751/2009	I	A	V	R	P	K	V	R	D	Q	A	G	R	M
	A/Sw/Illinois/02860/2009	I	T	K	R	P	K	V	R	D	Q	E	G	R	I
H3 subtype	H3N1 WT	I	G	S	R	P	W	V	R	G	L	S	S	R	I
	A/Sw/Minnesota/02719 (H3N2)	I	G	S	R	P	W	V	R	G	V	S	S	I	I

Residues which differ from the reference strain (A/Pennsylvania/08/2008) are labeled in red

Erythrocyte agglutination properties of pandemic H1N1 & swine isolates

The collection of viruses shown in Table 1 was first assessed for their capacity to agglutinate erythrocytes from different species, which are known to vary in their expression of cell-surface glycans with sialic acids in the α2,3 versus α2,6 composition (Ito et al., 1997). Chicken red blood cells (cRBCs), turkey red blood cells (tRBCs) and guinea pig red blood cells (gpRBCs) are commonly used to characterize influenza isolates and laboratory stocks, as these erythrocytes contain both α2,3- and α2,6-linked glycans, with the α2,6 conformation being predominant on gpRBCs (Medeiros et al., 2001). By contrast, horse red blood cells (hoRBCs) contain a predominance of cell surface glycans with sialic acids in α2,3 configuration (Ito et al., 1997). Nearly all of the viruses in our panel were found to agglutinate guinea pig erythrocytes with high efficiency, while agglutination using turkey and chicken erythrocytes was generally observed to have slightly lower HA titers (Table 2). In contrast, among the viruses in our panel, only the A/New Caledonia/20/1999 vaccine strain and the A/Pennsylvania/08/2008 human seasonal strain demonstrated detectable HA titers using horse erythrocytes, suggesting that these viruses may have the capacity to recognize receptors in α2,3 conformation.

Table 2
Agglutination of erythrocytes by purified influenza strains

Subtype	Virus	Erythrocytes			
		Chicken	Turkey	Horse	Guinea Pig
Seasonal H1N1	A/Brisbane/59/2007	1024	1024	0	2048
	A/New Caledonia/20/1999	512	256	16	2048
	A/Pennsylvania/08/2008	1024	1024	16	2048
SOIV H1N1	A/California/04/09	64	256	0	1024
	A/Texas/15/2009	16	64	0	256
	A/Mexico/INDRE4487/2009	32	128	0	512
Swine isolates (H1N1)	A/Swine Iowa/1976/1931	128	128	0	2048
	A/Swine/Minnesota/02749/2009	64	512	0	2048
	A/Swine/Minnesota/02751/2009	128	256	0	256
	A/Swine/Illinois/02860/2009	16	64	0	64
H3 Subtype	H3N1 WT	128	128	0	256
	A/Swine/Minnesota/02719/2009 (H3N2)	128	512	0	1024

HA assays were performed using 0.5% erythrocytes from each animal, as described in the Materials and Methods.

Glycan microarray analyses

For a more direct analysis of the receptor binding capabilities of the viruses shown in Table 1, we utilized glycan microarray analysis (Blixt et al., 2004). Purified viruses were labeled using the Alexafluor 488 amine reactive dye, as described in Materials and Methods, and bound to glycan microarrays provided by the Protein-Glycan Interaction Core (Core H) of the Consortium of Functional Glycomics. The plots of binding data are presented in histogram form in Figures 3 and 4. The binding results were sorted based on their glycan structure in order to group the glycans containing terminal sialic acids on the graph. A peak was considered to be significant if the relative fluorescence units (RFUs) were 2x higher than the average RFUs for all the glycans expressed on the microarray. The overall levels of binding were variable among samples if RFUs of maximum peaks are compared; therefore, the graphs shown in Figures 3 and 4 have variable ranges on the y-axis in order to

provide optimal visualization of the distribution of glycan species bound for individual samples. The structures of the top five glycans bound by each virus are presented in diagrammatic form in Figure 5. These structures do not include the spacers necessary for attaching a glycan to the glass slide.

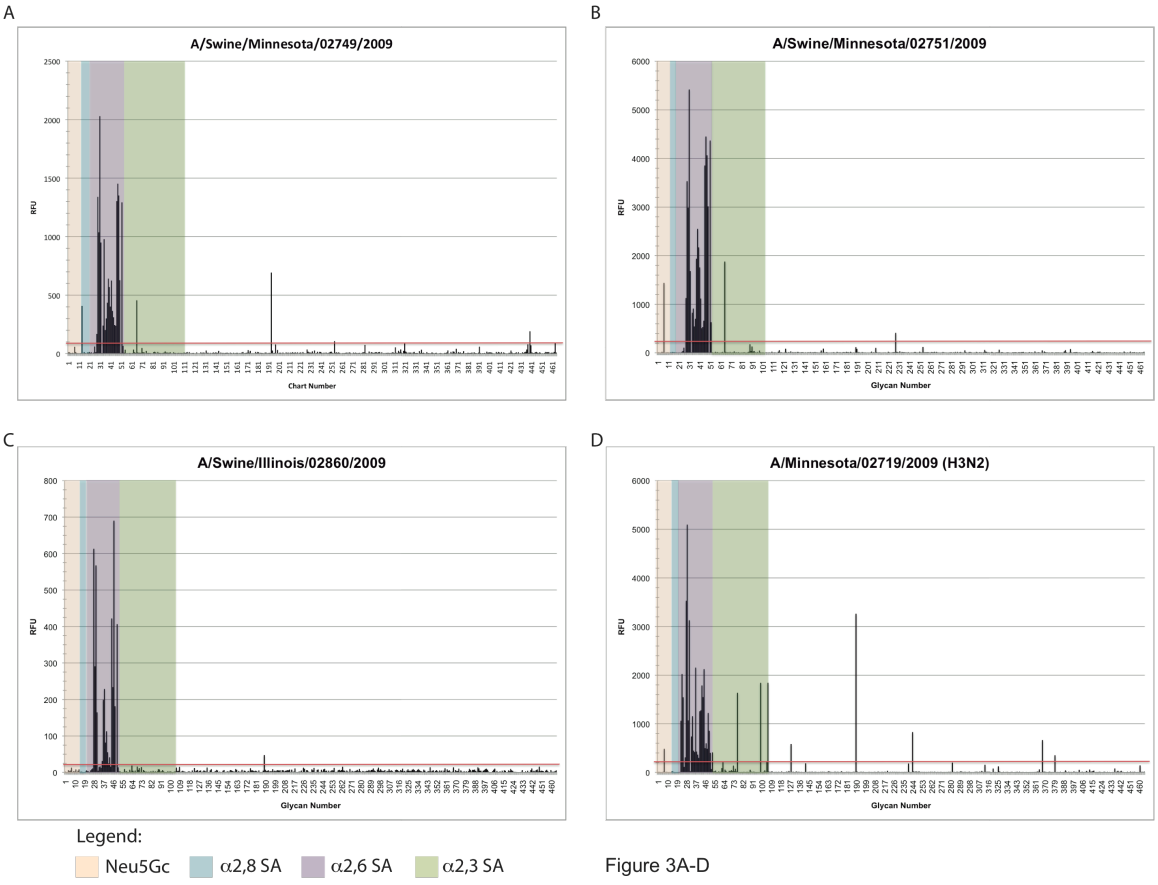


Figure 3A-D

Figure 3 – Binding to CFG glycan microarrays using fluorescently labeled virus shows a strict α2,6 specificity by contemporary swine isolates. Glycan microarray studies were performed at the Consortium for Functional Glycomics using CFG microarrays. Alexafluor488 labeled viruses were incubated for 1 hour at 4˚C prior to scanning. Glycans in the graph were sorted based on their glycan structure to group a2,6 and a2,3 SA. Background is indicated by the red line, and was determined by multiplying the average of all glycans on the microarray by two. A) A/Swine/Minnesota/02749/2009 (H1N1), B) A/Swine/Minnesota/02751/2009 (H1N1), C) A/Swine/lllinois/02860/2009 (H1N1) D) A/Swine/Minnesota/02719/2009 (H3N2).

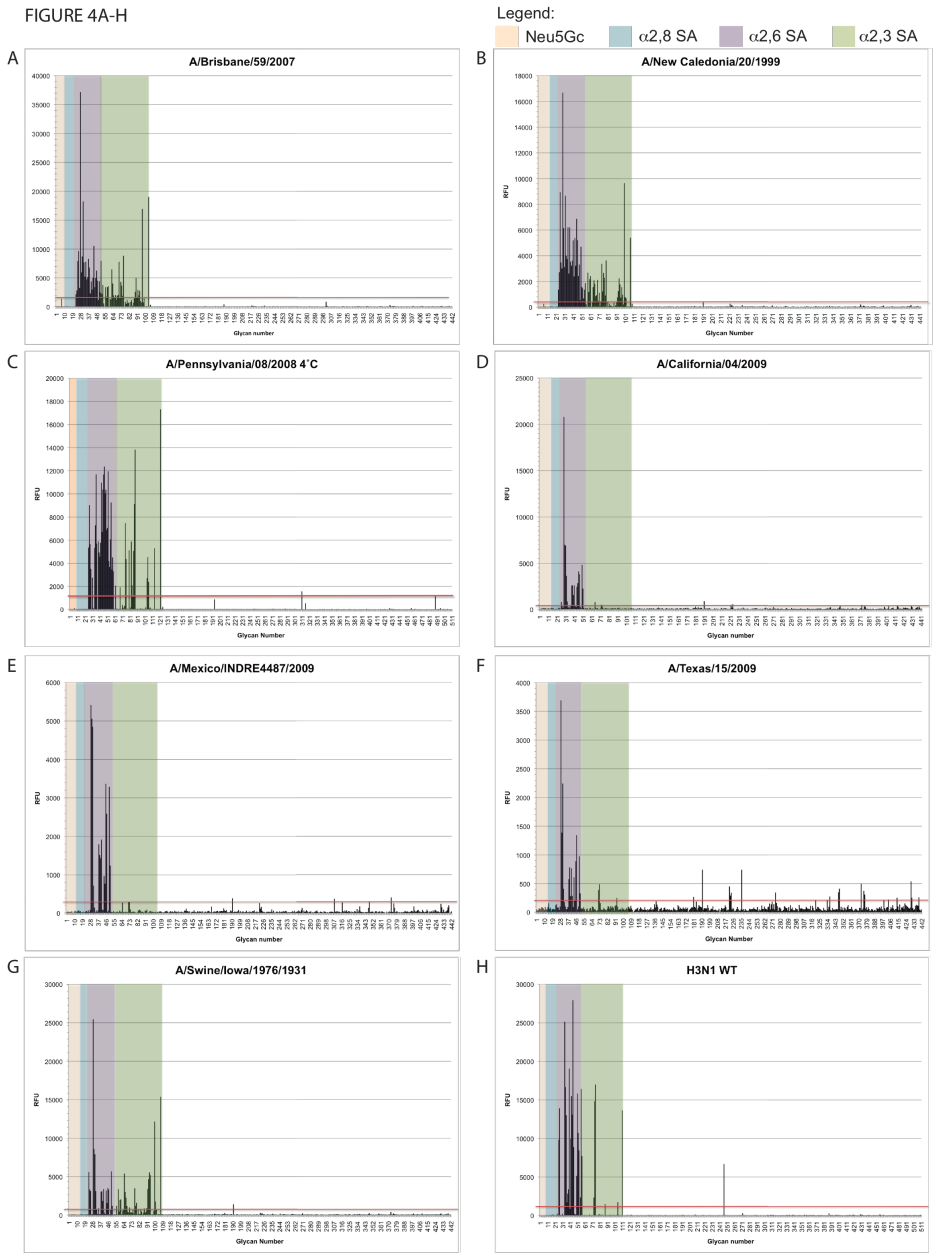


Figure 4 – Glycan microarray experiments were performed as indicated in Figure 3, and the Materials and Methods. A) A/Brisbane/59/2009 (H1N1) B) A/New Caledonia/20/1999 (H1N1) C) A/Pennsylvania/08/2008 (H1N1) D) A/California/04/2009, (H1N1) E) A/Mexico/INDRE4487/2009, (H1N1) F) A/Texas/15/2009, (H1N1) G) A/Swine/Iowa/1976/1931, (H1N1) H) H3N1 Aichi HA WT (H3N1).

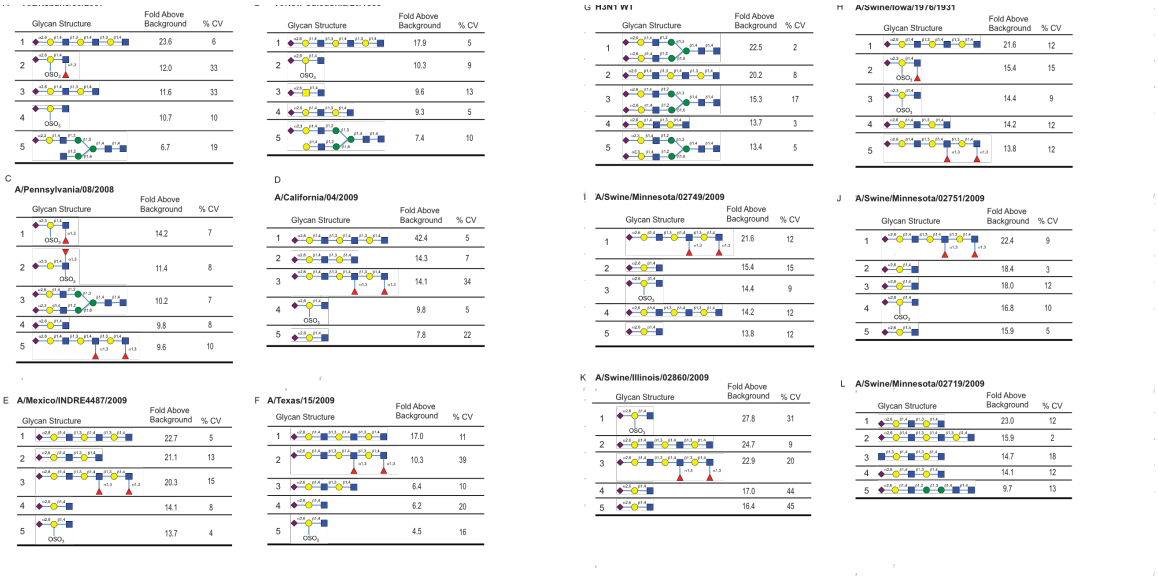
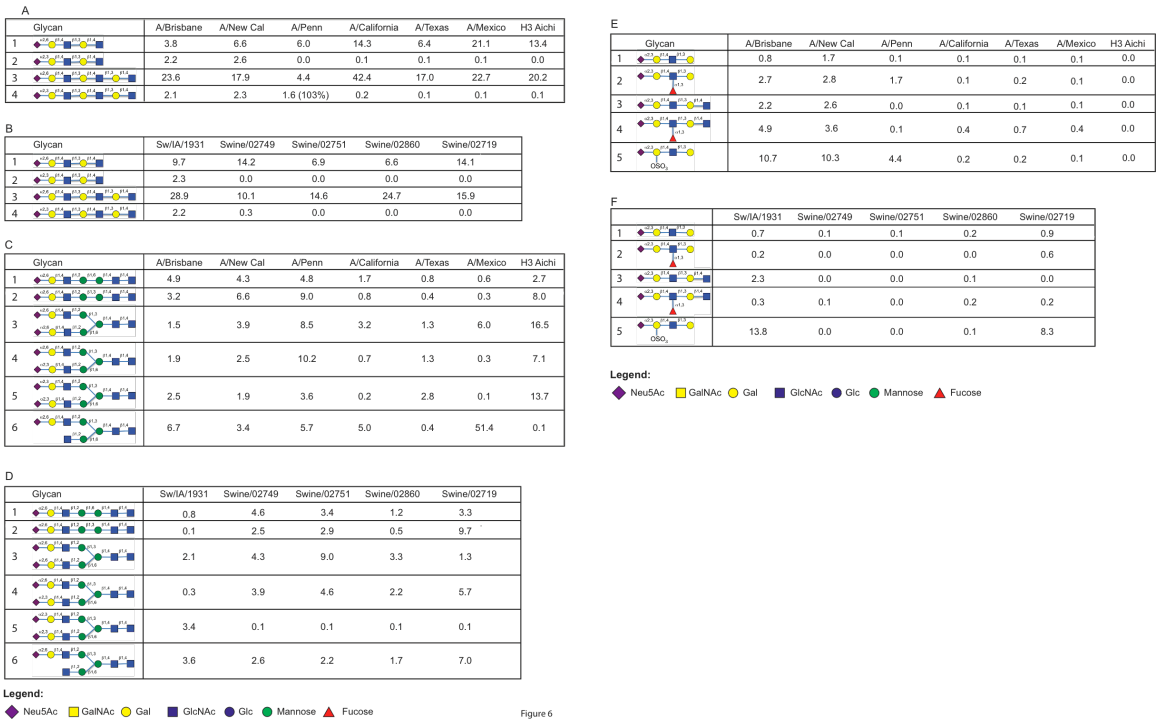


Figure 5 – Top five glycans bound by purified swine isolate viruses on the CFG microarray. The five highest binding glycans with %CV's less than 50% are listed without the spacer used to attach glycans to the consortium slides. The %CV shown is the number provided by glycan array analysis software. Identical structures are indicative of virus binding to glycans linked to the slide with different spacers. Nomenclature is as follows: purple diamond, Neu5Ac; Yellow circle, Galactose; Blue square, GlcNAc; Red triangle, fucose; OSO_3, Sulfate group. Linkages are indicated between sugar symbols. A) A/Brisbane/59/2007, B) A/New Caledonia/20/1999, C) A/Pennsylvania/08/2008, D) A/California/04/2009, E) A/Mexico/INDRE4487/2009, F) A/Texas/15/2009, G) H3N1 WT, H) A/Swine/Iowa/1976/1931, I) A/Swine/Minnnesota/02749/2009, J) A/Swine/Minnesota/02751/2009, K) A/Swine/Illinois/02860/2009, L) A/Swine/Minnesota/02719/2009.



A

	Glycan	A/Brisbane	A/New Cal	A/Penn	A/California	A/Texas	A/Mexico	H3 Aichi
1		3.8	6.6	6.0	14.3	6.4	21.1	13.4
2		2.2	2.6	0.0	0.1	0.1	0.1	0.0
3		23.6	17.9	4.4	42.4	17.0	22.7	20.2
4		2.1	2.3	1.6 (103%)	0.2	0.1	0.1	0.1

B

	Glycan	Sw/IA/1931	Swine/02749	Swine/02751	Swine/02860	Swine/02719
1		9.7	14.2	6.9	6.6	14.1
2		2.3	0.0	0.0	0.0	0.0
3		28.9	10.1	14.6	24.7	15.9
4		2.2	0.3	0.0	0.0	0.0

C

	Glycan	A/Brisbane	A/New Cal	A/Penn	A/California	A/Texas	A/Mexico	H3 Aichi
1		4.9	4.3	4.8	1.7	0.8	0.6	2.7
2		3.2	6.6	9.0	0.8	0.4	0.3	8.0
3		1.5	3.9	8.5	3.2	1.3	6.0	16.5
4		1.9	2.5	10.2	0.7	1.3	0.3	7.1
5		2.5	1.9	3.6	0.2	2.8	0.1	13.7
6		6.7	3.4	5.7	5.0	0.4	51.4	0.1

D

	Glycan	Sw/IA/1931	Swine/02749	Swine/02751	Swine/02860	Swine/02719
1		0.8	4.6	3.4	1.2	3.3
2		0.1	2.5	2.9	0.5	9.7
3		2.1	4.3	9.0	3.3	1.3
4		0.3	3.9	4.6	2.2	5.7
5		3.4	0.1	0.1	0.1	0.1
6		3.6	2.6	2.2	1.7	7.0

Legend: Neu5Ac, GalNAc, Gal, GlcNAc, Glc, Mannose, Fucose

Figure 6

E

	Glycan	A/Brisbane	A/New Cal	A/Penn	A/California	A/Texas	A/Mexico	H3 Aichi
1		0.8	1.7	0.1	0.1	0.1	0.1	0.0
2		2.7	2.8	1.7	0.1	0.2	0.1	0.0
3		2.2	2.6	0.0	0.1	0.1	0.1	0.0
4		4.9	3.6	0.1	0.4	0.7	0.4	0.0
5		10.7	10.3	4.4	0.2	0.2	0.1	0.0

F

		Sw/IA/1931	Swine/02749	Swine/02751	Swine/02860	Swine/02719
1		0.7	0.1	0.1	0.2	0.9
2		0.2	0.0	0.0	0.0	0.6
3		2.3	0.0	0.0	0.1	0.0
4		0.3	0.1	0.0	0.2	0.2
5		13.8	0.0	0.0	0.1	8.3

Legend: Neu5Ac, GalNAc, Gal, GlcNAc, Glc, Mannose, Fucose

Figure 6 – Comparison of glycans bound on CFG microarrays. The fold of background is indicated. In the instance where the %CV is greater than 50%, the %CV is indicated in parenthesis. Compared glycans are all bound to the same spacer that links the glycan to the CFG slide

Contemporary H1 swine isolates

To determine the receptor binding patterns of recent swine isolate strains, three 2009 isolates were examined. The first strain, A/swine/Minnesota/02749/2009 (Mn/02749), revealed a highly restricted binding phenotype, binding exclusively to α2,6 SA containing glycans, with no binding to glycans containing α2,3 SA that did not also contain a terminal α2,6 SA (Figure 3A and Figure 6D, glycans 3-5). A/swine/Minnesota/02751/2009 (Mn/02751) also preferentially bound α2,6 SA-containing glycans (Figure 3B), with greater binding to branched glycans over single chain glycans (Figure 6D, glycans 1 and 3), but the addition of a 6'-O-SO3 did not seem to increase binding over identical glycans lacking a sulfate (Figure 6F, glycans 1 and 5). The receptor binding domains of Mn/02749 and Mn/02751 are identical, with the exception of three residues at positions 133, 193, and 219. One difference observed for these strains was that Mn/02751 displayed significant binding to a single α2,3-linked N-glycolylneuraminic acid (Neu5Gc) present on the microarray, a Neu5Gcα2,3Galβ1,4(Fucα1,3)GlcNac glycan (Figure 3B). However, the full breadth of Neu5Gc binding for these two strains is difficult to ascertain due to the limited number of Neu5Gc glycans present on the microarray. We attempted to determine how the number of sugars in the glycan chain altered binding, but effects varied greatly depending on the type and conformation of internal sugars and therefore do not appear to be a major determinant of binding preference.

A/swine/Illinois/02860/2009 (Il/02860) virus is divergent from Mn/02749

and Mn/02751, differing at several positions in the receptor binding site (Table 1). Relative to the other swine isolates, it bound poorly to the microarrays, recognizing less than 2/3 of the glycans bound by both Minnesota strains (Figure 3C). It was also highly restricted to α2,6 SA, with preferential binding to an unbranched poly-N-acetyllactosamine glycan with a terminal α2,6 SA. In addition, Il/02860 bound to the same Neu5Gc glycan recognized by Mn/02751. However, any trends relating to length or additional charged sugars are difficult to determine due to the overall poor binding of Il/02860 to the microarray.

Analysis of S-OIV isolates

To compare the binding of trH1N1 swine isolates with early isolates from the S-OIV outbreak, we examined three prototype pdmH1N1 strains that were isolated from patients presenting with a representative spectrum of disease during the early stages of the outbreak. All three S-OIV strains bound primarily α2,6 SA-containing glycans (Figure 4D-F), with very limited binding to glycans with α2,3 SA. Overall, the strongest binding glycans bound by the 2009 S-OIV isolates were highly restricted to the same structure (Figure 5D-F).

A/California/04/2009 (Figure 4D) was isolated on 1 April from a 10 y.o. male patient, and was an early isolate of the pandemic. This virus displayed a highly restricted α2,6 SA binding preference. Although this virus showed slight binding to a long biantennary structure with both α2,3 SA and α2,6 SA, the relative binding was reduced relative to an identical glycan containing two terminal α2,6 SA (Figure 6C glycans 3 and 4). The addition of a sulfate (6'-O-SO_3) or fucose to a glycan did not

seem to affect binding of A/California to α2,3 SA- or α2,6 SA-containing glycans, either positively or negatively (Figure 6E glycans 1, 2 and 5). The glycans to which A/California bound were long, unbranched, poly-N-acetyllactosamine glycans, and there appeared to be a preference for structures containing Galβ1,4GlcNAc as the second and third sugar from the terminus in the glycan chain. A/California preferentially bound biantennary glycans present on the microarrays over identical monoantennary glycan chains (Figure 6, glycans 1 and 2), but curiously, the biantennary glycan chain did not require a terminal sialic acid on both chains to increase binding (Figure 6C, glycans 1, 3, and 6). This could indicate that the sialic acid on an unbound branch of the biantennary glycan does not increase avidity, but rather increases steric hindrance with the globular head of HA.

A/Mexico/INDRE4487/2009 (A/Mexico) was isolated from a 29 y.o. female with a severe URT infection (Belser et al., 2010). A/Mexico showed a similar overall binding phenotype to A/California with a few notable differences. Again, a high preference for glycans having terminal α2,6 SA, was observed, but in some cases, binding to monoantennary glycans with terminal α2,3 SA was visualized above background (Figure 4E). In each case where A/Mexico bound a monoantennary glycan having a terminal α2,3 SA, a fucose was branched off of the third sugar, GlcNAc (data not shown). Interestingly, the data indicated a preferential binding of A/Mexico to glycans having a Galβ1,4GlcNac linkage at the fourth and fifth sugar, as opposed to a Galβ1,3GlcNAc at the same location (data not shown).

The A/Texas/15/2009 virus (A/Texas) was isolated from a fatal influenza infection of a 1 y.o. pediatric patient (Belser et al., 2010). A/Texas bound similar

glycans as A/Mexico, but among the human S-OIV strains examined, it showed the greatest capacity for binding to α2,3 SA glycans, (Figure 4F). Similar to A/Mexico, all bound glycans with a terminal α2,3 SA also contained a branched fucose off of the third sugar, GlcNAc. Further, A/Texas bound a surprisingly large number of non-sialylated glycans present on the microarrays, most of which contained at least one fucose branched off of the third GlcNAc sugar, but the significance of this is unclear. Further studies will be needed with additional types of fucosylated glycans to explore this phenomenon.

Glycan Microarray Analysis of seasonal H1N1 vaccine strains

Three viruses were examined to compare the receptor binding patterns of S-OIV strains to those of H1N1 seasonal and vaccine strains from previous years. A/Pennsylvania/08/2008 (A/Penn) is a pre-pandemic seasonal H1N1 human isolate from October of the 2008-2009 influenza season, minimally passaged in MDCK cells. We also examined the receptor binding phenotype of vaccine strains A/Brisbane/59/2007 (A/Brisbane) and A/New Caledonia/20/1999 (A/New Cal), which were both vaccine strains for seasonal H1N1 strains in circulation prior to the S-OIV outbreak, but their passage history is undocumented.

The A/Penn human isolate preferentially bound glycans having α2,6 terminal SA, but displayed some interesting characteristics that included α2,3 SA binding as well. Notable among these were a number of short, sulfated glycans with α2,3 terminal SA (Figure 5C). In fact, eight out of twelve glycans having α2,3 terminal SA

that showed significant binding by A/Penn were either sulfated or fucosylated, while the other four glycans were long biantennary structures with either α2,3/α2,6 terminal SA combination or α2,3/α2,3 SA present at the glycan termini (data not shown). In general, A/Penn bound both branched and unbranched glycans equally, provided they contained at least one terminal α2,6 SA. Of note, the glycan with the second highest level of binding was a sialylactosamine (SLN) glycan containing a sulfate and fucose branched from the same GlcNAc (Figure 5C, glycan 2). This glycan was recently modeled into the H5 and H7 crystal structures and predicted to create additional contacts with residues K222 and K193 in H5 and H7 influenza subtypes, respectively (Nicholls et al., 2008). All of the S-OIV strains and human isolates utilized in our study contain a K at position 222, but there is significant variation at residue 193 between the S-OIV strains and vaccine strains (Table 1).

The highest peaks associated with A/Brisbane binding represented glycans having long, monoantennary, terminal α2,6 SA linked to poly-N-acetyllactosamine glycans. We compared virus binding of glycans having α2,3 or α2,6 SA, which revealed a clear preference for α2,6 SA (Figure 6A, glycans 1-4). In a comparison of the ability of A/Brisbane to bind biantennary versus monoantennary glycans, the data showed a preference for monoantennary counterparts (Figure 6C, glycans 1-4). This could indicate that long biantennary glycans create steric hinderance in and around the receptor binding pocket, causing reduced binding to the receptor. Generally, the highest binding α2,3 SA receptors contained either a fucose or a sulfate near the glycan terminus, and their absence lead to a reduction in binding (Figure 6E, glycans 1-5).

Similar to A/Brisbane, A/New Cal showed a preference for long, monoantennary glycans with a terminal α2,6 SA, and generally bound similar glycans as A/Brisbane (Figure 4B and 5B). The increased binding of A/New Cal to fucosylated glycans was less pronounced than for that observed for A/Brisbane (Figure 6E, glycans 1-4), but the addition of a sulfate to the sugar near a terminal α2,3 SA clearly lead to increased binding. Sequence analysis of the two strains indicated that the receptor binding site is highly conserved between the two viruses, except for a mutation, K192R, in the A/New Cal strain. Both strains contain the D190N mutation that is commonly associated with egg adaptation, and has been shown to increase binding to glycans with a terminal α2,3 SA.

A/Swine/Iowa/1976/1931 and H3N2 isolate glycan microarrays

We also determined the receptor binding phenotype of A/Swine/Iowa/1976/1931 (Sw/IA/1931), a classical swine H1N1 strain, which is a direct descendent of the 1918 pandemic strains that entered the North American swine population (Shope, 1936). Sw/IA/1931 bound to a mixture of glycans having α2,3 and α2,6 terminal SA (Figure 4G) with a clear preference for long poly-N-acetyllactosamine glycans having α2,6 terminal SA (Figure 6B, glycans 1 and 3), but did not bind well to monoantennary glycans containing mannose sugars (6D glycans 1 and 2). Because the passage history of this virus is unknown, these results are most likely representative of binding patterns acquired during multiple passages in animals, eggs and/or tissue culture (Francis, 1937; Horsfall et al., 1941).

Finally, we examined the receptor binding phenotype of a swine H3N2 influenza isolate, A/swine/Minnesota/02719/2009 (swine/02719). Similar to the H1N1 isolates, this strain also preferentially bound glycans having α2,6 terminal SA (Figure 3D). Interestingly, binding to a branched glycan having terminal α2,6/α2,6 SA was decreased relative to the binding observed for a monoantennary glycan with similar sugar composition having a terminal α2,6 SA; however, a branched glycan having α2,6/α2,3 SA at the termini did not exhibit a similar decrease in binding ability (Figure 6D, glycans 1-4). Significant binding to monoantennary glycans having α2,3 terminal SA were only visualized when a sulfate was present (Figure 6F, glycans 1 and 5). This is in contrast to binding of our H3 Aichi laboratory adapted strain, which bound primarily α2,6 linked sialic acid, but was still capable of binding biantennary glycans having α2,3/α2,3 or α2,3/α2,6 SA at their termini (Figure 4H and Figure 6C, glycans 4 and 5).

Binding of S-OIV strains to various cell monolayers

The type and distribution of cell-surface glycans varies from species to species, and is known to vary among tissue culture cell lines. As a complementary assay, we compared the relative binding of the Alexafluor 488 labeled S-OIV and swine isolates to two different cell lines commonly used to propagate influenza viruses: MDCK cells, a widely-utilized canine kidney cell line, and A549 cells, a human lung carcinoma cell line. Viruses were adsorbed to cell monolayers, washed, and binding was quantified by fluorimetry. Each strain was normalized to the WT level of binding, and is shown as a percentage of the lab strain, H3N1 WT. Figure 7A

shows that the laboratory-adapted H3N1 strain bound extremely well to MDCK cells. In contrast, H1N1 S-OIVs and the swine/02749 isolate bound to MDCK cells at less than 25% of WT levels (Figure 7A). Showing intermediate levels of binding were A/Brisbane and swine/02719 strains, which bound 64.6% and 65.5% of WT respectively. A comparison of relative binding to A549 cells shows a similar pattern as that observed on MDCK cells. The primary differences were that overall binding by all viruses was increased in A549 cells (data not shown) and that increased binding was observed for A/California and A/Texas strains. The fact that A/Brisbane bound both MDCK and A549 cells greater than any of the S-OIV isolates is largely consistent with the increased number of glycans bound on the glycan microarrays.

Relative NA activity of S-OIV strains

As mentioned previously, the HA/NA functional balance has been shown to be critical for viral fitness and propagation. In order to compare the relative neuraminidase activity of S-OIV strains, we utilized the fluorescent chemiluminescent substrate methylumbelliferone N-acetylneuraminic acid MUNANA, which fluoresces when cleaved by a neuraminidase. Ten thousand (10^4) PFU of virus was diluted with PBS and MUNANA substrate into cold 96 well plates. Plates were then incubated at 37ºC for 10, 20, or 30 minutes, at which time the reactions were stopped and assayed by fluorimetry. Interestingly, the A/Brisbane and H3N1 WT had increased levels of NA activity relative to the two S-OIV strains (Figure 7C). Sequence alignments indicated few mutations between the S-OIV NA

proteins, and all catalytic sites remained identical between S-OIV strains and A/Brisbane (Colman et al., 1983). However, a recent structural analysis of a 2009 H1N1 S-OIV isolate revealed that a V149I mutation in the NA of S-OIV isolates alters the 150 loop, and appears to form a smaller catalytic pocket. This alteration could affect the highly conserved catalytic residues D151 and R152 (Li et al., 2010), which would most likely affect NA activity.

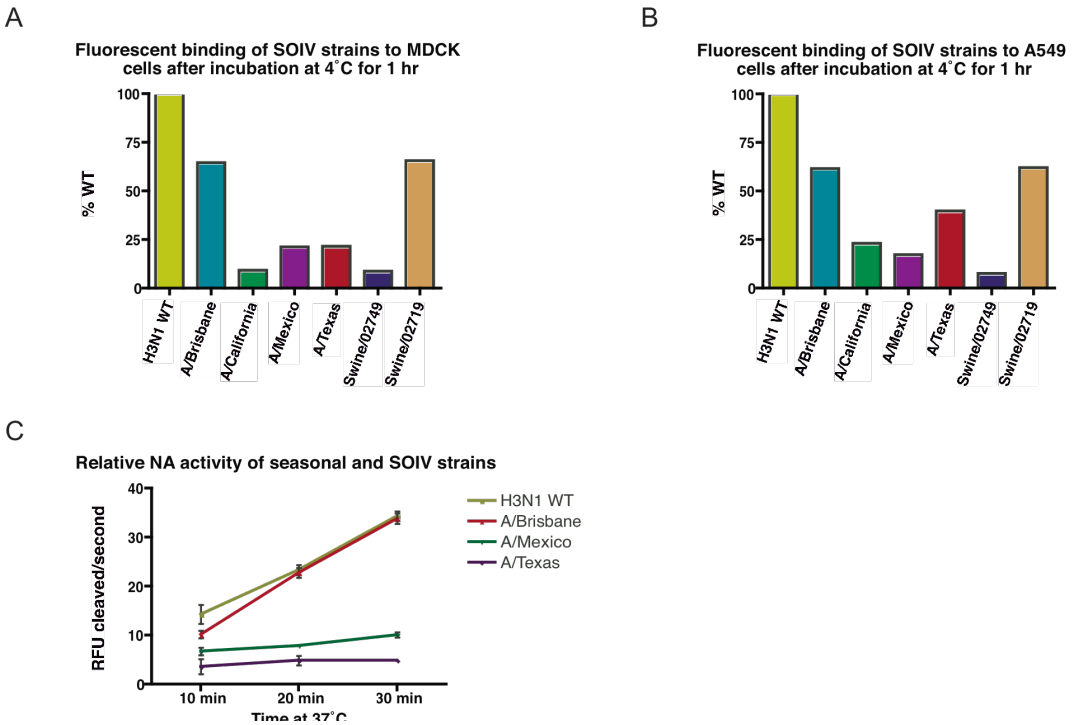


Figure 7 – Fluorescent binding and neuraminidase activity assays of influenza isolates. For the fluorescent binding assay, Alexa488 labeled influenza virus was incubated at an MOI = 3.0 for 1 hour at 4˚C, washed and scanned. Neuraminidase activity assays were performed using 0.01 mg MUNANA and 10^4 PFU of virus. Fluorescence was determined at 10 min, 20 min, and 30 minutes. Panels – Fluorescent binding results in Panel A) MDCK and B) A549. Panel C – Neuraminidase assay with influenza isolates.

Discussion

The 1997 emergence of highly pathogenic strains of avian H5N1 viruses and their sporadic transmission to humans, raised serious concerns over the genesis of a new human pandemic, but fortunately, these strains have yet to establish themselves in the human population, or transmit readily from person to person (Miller et al., 2009). By contrast, the current H1N1 S-OIV strains spread rapidly in the human population from the time they were first detected, and do not appear to be the result of multiple introduction events (Garten et al., 2009). Understanding the molecular determinants necessary for the adaptation of influenza viruses to humans is essential to improved pandemic preparedness. In particular, the role of HA-mediated receptor binding should always be examined, as this property is one of the first obstacles to overcome for adaptation to a new species. Here, we examined the receptor binding properties of H1N1 S-OIV strains currently circulating in the human population, and compared them to earlier seasonal H1N1 strains, as well as to trH1N1 swine influenza isolates. Swine are of particular interest to the ecology and emergence of influenza viruses, as that they are capable of propagating viruses of both human and avian origins (Scholtissek et al., 1983), which allows the segmented influenza genome to reassort in co-infected animals, potentially giving rise to novel influenza viruses.

The early S-OIV isolates examined almost exclusively recognized α2,6-linked sialic acids. For the A/California virus, the only example of binding to a glycan having α2,3 SA is with a glycan that also contained a terminal α2,6 SA (Figure 6C,

glycan 4 and data not shown). Binding of glycans having terminal α2,3 SA by both A/Texas and A/Mexico was highly correlated with the presence of fucose, since all monoantennary α2,3 SA glycans bound by A/Mexico and A/Texas were fucosylated. The 2008 H1N1 seasonal strain, A/Penn, showed a preference for glycans having a terminal α2,6 SA as well, but demonstrated a greater ability to recognize glycans with terminal α2,3 SA than the S-OIV strains that we examined. However, similar to A/Texas and A/Mexico, the majority of the glycans containing terminal α2,3 SA that were bound by A/Penn contained either a sulfate, fucose, or both.

The two glycans that were bound at the highest levels by the S-OIV isolates and the A/Penn isolate were a simple SLN and a seven-sugar, monoantennary, sialylated poly-N-acetyllactosamine glycan that contained two additional fucose residues at the fifth and seventh positions in the glycan, both having terminal α2,6 SA (Figure 5C glycan 5, 5D glycan 3, 5E glycan 3, and 5F glycan 2). Both of these glycans were recently shown to bind the majority of both egg-adapted and MDCK-passaged H3N2 isolates (Stevens et al., 2010). The possibility exists that if the S-OIV HA receptor binding affinity is relatively weak, as may be indicated by the relatively low binding to MDCK and A549 cells shown in Figure 7A and B, the addition of a fucose could lead to greater interaction with residues in the 220 loop, which has been visualized previously in modeling experiments (Nicholls et al., 2008).

Interestingly, the H3 subtype MN/02719 was the only swine isolate to not bind the monoantennary seven-sugar glycan with fucoses linked to the 5th and 7th sugar residues at levels above background (depicted in Figure 5I, glycan 1). The other H3 virus used in this study, the lab strain H3N1 WT, also did not bind the

structure, which could indicate a subtype-specific binding preference. Swine isolates generally had the same pattern of binding as S-OIV strains, consistently binding short SLN glycans (i.e. Figure 5J, glycans 2, 3, and 5). These isolates also preferred glycans with terminal α2,6 SA versus α2,3 SA (Figure 6B, compare glycans 1 and 2, 3 and 4). The near absence of α2,3 SA binding by the swine isolates utilized in this study is interesting considering the presence of both α2,3 and α2,6 SA in the swine respiratory tract. This could indicate that a reassortment event occurring in the swine respiratory tract could select for variants that preferentially bind α2,6 SA. However, we note that the small sample size tested on these glycan microarrays may not be representative of all circulating swine influenza isolates in North America or Europe.

Overall, the results confirm the hypothesis that human isolates generally adopt high specificity for α2,6-linked terminal SA, and that this is probably favored over mixed or duel α2,3 and α2,6 SA binding capacity. Previous glycan microarray binding experiments performed with seasonal H3N2 isolates have shown a marked preference for glycans with terminal α2,6 SA (Kumari et al., 2007), and seasonal non-egg-adapted H1N1 isolates bind α2,6 SLN over α2,3 sialyllactose (Gambaryan et al., 1999; Gambaryan et al., 1997). The vaccine strains, A/Brisbane and A/New Cal, bound to glycans having both α2,3 and α2,6 terminal SA, but this is likely due to repeated passage through eggs, rather than an accurate reflection of the binding preferences of the original strain. We obtained similar results for A/Swine/1976/1931 virus, the classical swine influenza isolate, and this also is likely to reflect an extensive laboratory passage history that included replication in

eggs. Although it has been known for decades (Burnet and Bull, 1943), these results emphasize the importance of passage history for any viruses to be analyzed for receptor binding properties. Even minimal passage in eggs is likely to select for mutants with altered binding profiles (Burnet and Bull, 1943; Gambaryan et al., 1999; Katz et al., 1990; Katz and Webster, 1988; Lugovtsev et al., 2009; Robertson et al., 1987; Robertson et al., 1985; Stevens et al., 2010). Although the use of MDCK cells may not be the ideal substitute for amplification of virus stocks, it would appear to be preferable to eggs for binding studies, at least for human and swine isolates (Stevens et al., 2010).

Data from numerous laboratory studies, and observations of evolving phenotypes of early strains in the 1957 and 1968 pandemics indicate that the HA and NA proteins operate in a reciprocal balance due to their opposing functions (Mitnaul et al., 2000; Wagner et al., 2002; Wagner et al., 2000). Viruses with a tightly binding HA coupled with a weak NA protein have difficulty releasing from cell membranes upon viral release, while poorly binding HA and a strongly active NA may prevent efficient binding to glycans present on the cell membrane. In experiments examining NA activity, we found S-OIV isolates had relatively weak neuraminidase activity, indicating that the HA and NA functions are fairly well matched, and indicative of the lengthy period of time the S-OIV strains have been predicted to be circulating undetected in nature (Smith et al., 2009). The role of the neuraminidase in viral infection has been linked to cleaving sialic acids present on the cell surface, as well as in releasing mucin bound virus. Human mucins have been shown to contain a abundance of glycans having α2,3 terminal SA, and are known to

be inhibitory to influenza strains (Burnet, 1948; Couceiro et al., 1993). This data, in combination with the predominance of α2,6 SA found in the upper respiratory tract of humans (Shinya et al., 2006), could indicate a selective advantage for strains that bind strictly to glycans containing terminal α2,6 SA over those with dual specificity. Presumably, virus strains that bind glycans containing either α2,3 or α2,6-linked terminal SA can evade the upper respiratory mucins with a weakly binding HA, but the lack of glycans containing α2,3 SA in the upper respiratory tract could lead to poor transmission capabilities (Maines et al., 2006; Shinya et al., 2006).

The overall binding levels observed for S-OIV strains and contemporary swine H1N1 viruses seemed to be low relative to H3N1 WT and vaccine isolates. This was reflected in our fluorescent binding assays, and we also observed high PFU:HA titer ratios for these viruses (data not shown). Glycosylation in the globular head of HA has been associated with weaker binding (Wagner et al., 2000), but in the S-OIV strains, no additional glycosylation is present that could cause steric hindrance with any potential receptor (Igarashi et al., 2010). Alternatively, the possibility exists that the strains are binding to host glycans that are not present on the microarray. The current glycan microarray consists of approximately 500 glycans, but the overall mammalian glycome is likely to be much larger with a larger set of glycan determinants (Cummings, 2009). In addition, swine, unlike humans, express Neu5Gc on their cell membranes, and there is evidence that swine, but not human influenza strains recognize Neu5Gc (Suzuki et al., 1997). Because the glycan microarrays used in this study contain fewer than ten Neu5Gc glycans, an absence of binding may not necessarily indicate an inability to bind. However, the weak

binding of S-OIVs observed in our studies may, in fact, be reflective of their inherent biology. Weak HA binding might confer a selective advantage with regard to transmission efficiency, as there are examples with other viruses of weaker binding allowing for greater dissemination, systemic spread, and increased pathogenicity within a host animal (Byrnes and Griffin, 2000; Stehle and Harrison, 1996).

The viruses examined here differ at several positions in and around the glycan receptor binding site (Table 1 and Figure 1), though it is not currently possible to directly correlate binding properties to the presence of particular residues at specific HA positions. For H1 viruses such as those responsible for the 1918 pandemic, mutations at positions 190 and 225 have been shown to heavily influence a strains ability to bind glycans having α2,3 versus α2,6 terminal SA; however, these residues are unchanged between the A/Penn, S-OIV, and swine isolates (Table 1). Residues in the receptor binding site that are altered include: 133, 136, 138, 192, 193, 219, 227, and 230 (H3 numbering). With the exception of 136 and 138, most of these residues do not contact sialic acid directly, but may interact with contact residues, or potentially interact with internal sugars of the glycan receptor. In the case of glycans with α2,6 SA, mutations at 219 and 227 are thought to interact with internal sugar residues in the glycan, as are residues 192 and 193, which were recently modeled to interact with a Glc5 sugar in glycans similar to those found in Figure 6A (Maines et al., 2009). Structural interpretations for binding to glycans with α2,3 SA are more difficult to determine, largely due to the reduced electron density for such receptors beyond the 3rd sugar residue in HA-receptor complexes (Gamblin et al., 2004). However, as discussed previously, a

sulfate or fucose on the 3rd sugar residue of a receptor could potentially interact with the residues at 222 or 193. Another of these mutations, I230M, has been observed previously by selection in mice during passage of an H3 Y98F mutant with reduced receptor binding activity (Meisner et al., 2008). This residue is considered to be a "second shell" mutation, in that it does not directly interact with sialic acid, but appears to interact with the Y98 residue, potentially altering the structure of the glycan binding pocket. It is interesting that the two strains associated with severe disease, A/Texas and A/Mexico, bound several glycans with terminal α2,3 SA. Although increased α2,3 binding has been associated with higher pathogenicity in the past (Hatta et al., 2001; Liu et al., 2010; Shinya et al., 2005), and has been observed to correlate in one case with changes at position 225 (Chen et al., 2010), differences in patient age, as well as unknown patient history, make concrete conclusions relating to pathogenicity difficult to determine.

Increased surveillance and improved techniques have vastly increased our ability to identify potential pandemic strains in swine and avian species. However, the combinations of conditions necessary for a strain to efficiently transmit to humans from another species are not well understood. Influenza infection and transmission in a new host species is clearly a multifactorial trait, but as the HA and NA are critical to the initial events of infection and transmission, it is important to examine the characteristics of early pandemic isolates and compare them to closely related strains. Such studies may make it possible to identify critical properties of viruses with a greater likelihood for initiating new pandemics.

Materials and Methods:

Viruses and Cells

Influenza virus stocks for glycan microarray analysis were grown in cell culture using standard methods. In brief, Madin-Darby Canine Kidney (MDCK) cells (ATCC, Manassas, VA) were seeded into 75 cm^2 or 175 cm^2 tissue culture flasks and grown in DMEM (Hyclone, Logan, UT), 5% FBS, 2 mM L-glutamine until approximately 90% confluent (~8 x 10^6 cells total). Cells were rinsed twice with 1X PBS and 5mls MEM (Hyclone) with TPCK-treated trypsin (10ug/ml, Worthington Biochemical, Lakewood, NJ) was added to each flask. Original stock virus was added at 1:100 dilution in 5 ml MEM/TPCK trypsin and incubated 2-4 hrs at 37oC, 5% CO2 before an additional 5 ml of MEM/TPCK trypsin was added. Infection continued for 48-72 hrs and was monitored for CPE. Supernatant was harvested and centrifuged at 1000 rpm, 5 minutes to clarify cell debris. Virus titer was evaluated by a hemagglutination (HA) and plaque assay and/or TCID50 on MDCK cells. Viral RNA was purified using a QiaAmp Viral RNA purification kit (Qiagen), and reverse transcribed using SuperScript III 1st strand synthesis (Invitrogen). The HA and NA genes were amplified using HA or NA specific primers designed according to published sequences on the Influenza Research Database (www.fludb.org).

The human influenza viruses used in these studies were kindly provided by Drs. Alexander Klimov and Xiyan Xu at the Centers for Disease Control and Prevention (Atlanta, GA): A/California/04/09, A/Mexico/INDRE4487/09, A/Texas/15/09, A/Brisbane/59/2007, A/New Caledonia/20/99, and A/Pennsylvania/08/2008. The recent swine influenza viruses used in this study

were isolated by the University of Minnesota Veterinary Diagnostic Laboratory (Saint Paul, MN): A/Swine/MN/02719/2009, A/Swine/MN/02749/2009, A/Swine/MN/0251/2009, A/Swine/IL/02861/2009. The classical swine H1N1 A/Swine/1976/31 was kindly provided by Dr. Richard Webby (St. Jude Children's research Hospital, Memphis, TN).

Virus purification

Harvested virus was purified through a 25% sucrose cushion in NTE buffer, consisting of 100mM NaCl, 10 mM Tris buffer, and 1 mM EDTA. Viruses were centrifuged for 3 hrs at 28,000 RPM, resuspended in NTE buffer, and kept frozen at -80˚C until needed.

S-OIV labeling

Purified influenza strains were labeled with the amine reactive dye Alexafluor488 (Invitrogen). Twenty-five µg of amine reactive Alexa 488 was incubated with 100 µl of virus and 1M $NaHCO_3$ (pH 9.0) for 1 hour. The labeled virus was dialyzed overnight in a 7000 MWCO Slide-A-Lyzer MINI dialysis unit (Thermo Scientific) against PBS + 1 mM EDTA, and binding experiments were carried out by members of the CFG Core H facility.

Glycan microarrays & analysis

Core H of the Consortium of Functional Glycomics performed glycan microarray binding experiments (www.functionalglycomics.org) using CFG array

version 4.0 or 4.1. Briefly, 70µl of fluorescently labeled virus was incubated on a glycan microarray slide at 4˚C for 1 hour, prior to scanning by a Perkin-Elmer ProScanAray. Significant peaks were determined by averaging the relative fluorescent units (RFU) of all glycans on the microarray, which was then multiplied by two to determine the background RFU level. Finally, any glycans above background with a %CV of greater than 50% were not considered to be significant.

Fluorescent binding assays

Live influenza virus were labeled in the same manner as strains prepared for glycan microarrays. Fully confluent MDCK, A549 and PT-K75 cell lines were chilled at 4˚C for 1 hour prior to influenza binding. 10^4 PFU of labeled virus was bound and incubated on chilled cell lines at 4˚C for 1 hour, washed 3 times with cold PBS, and scanned using a Biotek Synergy 2 fluorimeter using a bottom optics position and excitation/emission of 485/528.

NA activity assays

Cold 96 well plates were kept on ice while cold PBS, 104 PFU of virus, and 0.01 mg MUNANA (Invitrogen) were added to each well. Plates were placed in a 37˚C incubator and reactions stopped at 10, 20, and 30 min using 1M Glycine (pH 10.7). After the stop solution was added, plates were scanned using a Biotek Synergy 2 fluorimeter using an excitation/emission of 360/460 respectively. Each virus at each time point was performed in triplicate and the fluorescence from a mock well containing only PBS and 0.01 mg MUNANA was subtracted from the

mean fluorescence obtained from each condition tested. NA activity was determined by subtracting the mock well from the virus-treated well, and divided by the number of seconds passed since the plates had been place at 37˚C.

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CHAPTER 3

Comparison of the receptor binding properties of contemporary swine isolates and early human pandemic H1N1 isolates (novel 2009 H1N1)

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Title: Analysis of influenza HA receptor binding mutants with limited receptor recognition properties and conditional replication characteristics

Running Title: Influenza binding mutants

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ABSTRACT

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 in mice a virus containing an attenuating mutation in the hemagglutinin (HA) receptor binding site, and characterized the resulting mutants with respect to the structural locations of mutations selected, the replication phenotypes of the viruses, and their binding properties to glycan microarrays. The initial attenuated virus had a tyrosine to phenylalanine mutation at HA1 position 98 (Y98F) in the receptor binding pocket, but viruses that were selected contained second site pseudoreversion mutations in various structural locations that reveal a range of molecular mechanisms for modulating receptor binding that go beyond the scope that are 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 display distinctive replication properties depending on the system, but that all were less attenuated than Y98F in mice. An analysis of receptor binding properties confirmed that the initial Y98F virus binds poorly to several different species of erythrocytes, while all mutants re-acquire hemagglutination activity to varying degrees. Interestingly, both Y98F and the pseudoreversion mutants were shown to bind very inefficiently to standard glycan microarrays provided by the Consortium for Functional Glycomics, which contain an abundance of binding substrates for most influenza viruses that have been characterized to date. The viruses were also examined on a recently developed microarray of glycans terminating in sialic acid derivatives, where

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 the species of molecules that may act as receptors for influenza on host cell surfaces.

INTRODUCTION

Influenza A viruses belongs to the order Orthomyxoviridae and are responsible for significant annual morbidity and mortality. They are enzootically maintained 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 nine 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 those viruses of the H1N1, H2N2, and H3N2 subtypes have developed the capacity to transmit efficiently and circulate extensively in the human population. In addition, several subtypes of influenza A have also been observed to spread to other mammalian species such as pigs, horses, seals, whales, dogs, and mink, and they 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 has 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 and other sialic acids are synthesized by mammals, such as 9-O-lactoyl-acetylneuraminic acid (Neu5Ac9Lt) and 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid structure (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 isolates bind Neu5Ac in either 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 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 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 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 to 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 have 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 NMR studies on binding affinity of receptor analogs that were chemically modified at particular positions of the sialic acid structure, show 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 strains have a preference for 9-O-acetyl sialic acid species (52).

The region of the 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 Figure 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 the discrimination between receptors with either α2,3 or α2,6 sialic acid linkages 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 closely linked 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 of absence of a carbohydrate, in combination with a mutation at 227, has been notable for it's effects on receptor binding (83, 84).

Our studies on a mutant HA that is inhibited for binding, Y98F, and second site pseudorevertant 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.

Figure 1

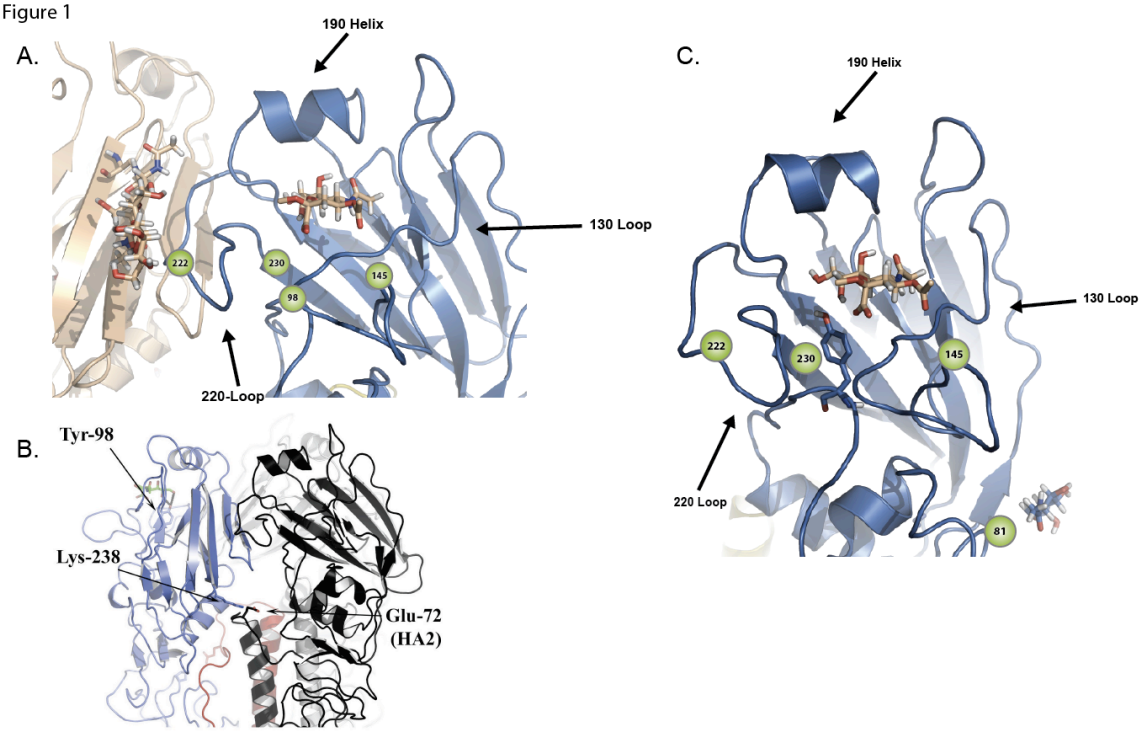

Figure 1 – Structural locations of mutations found in pseudorevertant viruses. A) Location of pseudorevertant mutations in the vicinity of the receptor-binding site. The location of the sialic acid component of glycan receptors is visualized in the pocket just below the 190 helix, and positions of pseudoreversion 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 show the location of the glycosylation site that originates from residue N165 of the adjacent monomer of the Aichi HA. B) This panel illustrates that for the Y98F/K238N pseudorevertant, HA1 position 98, at the base of the receptor binding pocket, is quite distal (~40Å) from HA1 position 238, which in the 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 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. The HA is shown as a monomer, with sialic acid modeled in the receptor site, and Y98F is shown in the binding pocket. The pseudorevertants are indicated as green ball structures, and the N-linked glycosylation at N81 present in the crystal structure is shown.

HA1 position 98 is a highly conserved tyrosine at the base of the binding pocket (Figure 1A), and mutation of this residue to phenylalanine resulted in a virus that bound very poorly to human, chicken, or turkey erythrocytes, and 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 WT virus in MDCK cells and embryonated chicken eggs, but was highly attenuated in cells with limited sialic acid on their surface or in the respiratory tract of mice following intranasal infection. Upon passage of the Y98F virus in mice, we noted that, in addition to isolating viruses with a reversion at position 98, a large proportion of mutant viruses that were isolated were found to contain second site pseudoreversion mutations that also maintained 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 (herein referred to 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 have 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 the HA as well as the replication properties of the viruses in cell culture or mice. Furthermore, we assess

binding of the mutant viruses 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 be utilizing 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 using standard protocols. Briefly, 85-90% confluent MDCK cells grown in T175 flasks were washed 2X with PBS, overlayed with 10 mL of serum-free DMEM supplemented with 1μg/mL TPCK trypsin, and infected with a 1:1000 dilution of the original virus stock. Cells were incubated with rocking for 1 hr at ambient temperature, the inoculum was removed, and 30 mL of serum-free DMEM supplemented 1μg/mL TPCK trypsin was added. The flasks were incubated at 37˚C for 2-3 days until monolayers were 80-90% destroyed. Virus was then harvested in and frozen at -80˚C until the purification procedure was carried out (see below).

MDCK cells were maintained using DMEM supplemented with 5% FBS and penicillin/streptomycin. A549 cells were maintained using F-12 media supplemented with 10% FBS and penicillin/streptomycin.

Virus stock sequencing

All virus stocks were sequenced prior to experimentation. Viral RNA was extracted using the QiAmp Viral RNA Mini Kit (Qiagen) and converted to cDNA using the First-strand Superscript III kit (Invitrogen), both according to the manufacturer's recommendations. PCR reactions to amplify the HA and NA were performed with Phusion High Fidelity PCR Master Mix (Finnzymes), using internal gene specific primers designed to amplify the sequence in HA from 200-1540 nucleotides, and 200-1360 in NA. 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 2-3x with 1X PBS, and diluted to 0.5% for hemagglutination experiments, which was performed using standard techniques. Briefly, 0.5% erythrocytes were added to two-fold serial dilutions of 50µl virus stock for 2 hours to determine HA titer. Viral elution was determined by additional incubation at 37˚C, and HAU was determined after 2 hours and overnight incubation.

Reverse genetics and viral rescue

Recombinant influenza viruses were generated using the 17-plasmid reverse genetics system previously described (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 the A/Aichi/2/68 (H3N2) HA viral RNA segment of wild-type or mutant sequence, and nine protein expression plasmids encoding the influenza A viral proteins to support the virus life cycle using Lipofectamine (Invitrogen). Transfected cells were incubated at 37ºC for 8 hrs at which time, the transfection medium was replaced with serum free DMEM. Transfected 293T cells were incubated at 37˚C for 24-48 hours, and the monolayers were harvested and either frozen or passaged onto 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 cell to create working stocks according to the protocol described above.

Fluorescent binding to cell lines

Binding of fluorescent labeled influenza virus was performed previously described (5). Briefly, 100-200µl of virus was incubated with 25 µg of Alexa488 (Invitrogen) in 1M $NaHCO_3$ (pH 9.0) for 1 hour at room temperature. Labeled viruses were dialyzed against PBS containing 1mM EDTA using a 7000 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 hr to prevent endocytosis of the viral particles and labeled

viruses were bound at a MOI of 3. The cell monolayers were incubated with virus for 1 hour at 4˚C, washed 3x with 1X PBS, and scanned using a Biotek Synergy 2 fluorimeter using a bottom optics position and an excitation/emission of 485/528. As a control for NA-mediated elution, viral NA activity was assayed at both 4ºC and 37ºC using the small substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA), and no activity was detected at 4ºC.

Purification of virus strains

Purification of viruses was performed as described previously (5). Briefly, harvested virus was purified through at 25% sucrose cushion in NTE buffer (100mM NaCl, 10mM Tris, 1mM EDTA). Viruses were pelleted by centrifugation in a SW41Ti rotor at 28,000 RPM for 3 hours, resuspended in 1400µl 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 for our fluorescent binding experiments. After labeling of the purified viruses with 25 µg of Alexa488 and overnight dialysis to remove excess Alexa488, virus was bound to the CFG array version 4.1 at the Core H of the Consortium for Functional Glycomics. Briefly, 70 µl of fluorescent-labeled virus was incubated on a glycan microarray slide under a cover slip at 4˚C for 1 hour, washed

to remove unbound virus and scanned using a Perkin-Elmer ProScanAray. Sialo-derivative arrays were performed as for the CFG array, except 100 µl of labeled virus was used on slides fitted with a 16-chamber silicon grid and incubated without a cover slip.

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: Y98F/W222R, Y98F/I230M, and Y98F/K238N. We also isolated two additional pseudorevertant viruses during passage of the Y98F mutant in MDCK cells (Y98F/N81T and Y98F/S145I), and these are included in the current study. As mentioned previously, the Y98F virus replicates at 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 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 Figure 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 (Figure 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 (Figure 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 Aichi HA (Figure 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 the WT and the Y98F/N81T HA that were either treated or not treated with glycosidase to confirm that the Y98F/N81T HA lacks this glycosylation site (data not shown).

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 laboratory strain recombinant H3N1 WT virus (rH3N1), which is a rescued reassortant with an A/Aichi/2/1968 HA (H3N2) on the genetic backbone of A/WSN/1933 virus (H1N1). To examine virus replication in MDCK cells, we infected cells at a MOI of 0.1 and determined the titer of plaque forming units (pfu) at 48 hpi. To examine virus replication in the mouse lung, mice were infected intranasally with 10^4 pfu, sacrificed three days post infection, and viral lung titers were determined by plaque assay on MDCK cells. As shown in Figure 2, the rH3N1 WT and Y98F virus reached comparable titers of approximately 107 pfu/ml in MDCK cells, but Y98F was attenuated by 3-4 logs in the mouse lung, in agreement with our previous observations (37, 41). The viruses that were selected for in mice, Y98F/W222R, Y98F/I230M, and Y98F/K238N, all replicated to higher titers than Y98F in mice lungs, as might be expected. The most robust of the three, the Y98F/W222R virus reached titers that approached those of rH3N1 WT, over 3 logs higher than Y98F virus.

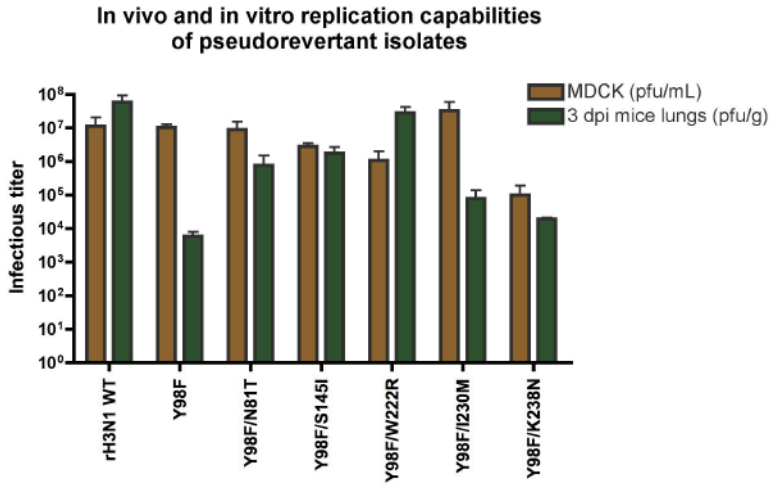

Figure 2 – In vitro and in vivo virus titers in MDCK cells and mouse lungs. MDCK cell infections were carried out at a low MOI as described in the Materials and Methods. Mouse influenza infections were administered intranasally at 10^4 pfu, and lungs were harvested 3 days post infection. Titers are expressed as pfu/mL.

When the replication properties of these mouse-selected mutants were determined in MDCK cells, two displayed reduced titers relative to WT and Y98F (Y98F/W222R and Y98F/K238N), 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 mice lungs relative to rH3N1 WT virus, but were significantly greater than the Y98F virus titers in mouse lung. Interestingly, neither mutant isolated by MDCK cell passage (Y98F/N81T or Y98F/S145I) displayed significantly higher titers than Y98F 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, and in particular, the observation that pseudorevertant viruses display higher titers than the parental Y98F virus in the mouse lung, suggest that the compensatory mutations lead to changes in the receptor binding properties of the 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 desialyated 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 WT, ranging from 64-512 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 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 lung 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 for erythrocyte receptors.

The influenza NA is responsible for cleaving sialic acid from cell surface glycoconjugates and facilitate 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 strains (22). Therefore, we examined the NAs of the pseudorevertant viruses for their capacity to destroy their cognate receptors on chicken and guinea pig erythrocytes. Pseudorevertant viruses, rH3N1 WT 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 hours 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 able to elute from both chicken and guinea pig erythrocytes. The rH3N1 WT virus was also able to elute 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 rH3N1. However, the data obtained with guinea pig erythrocytes was more interesting. All pseudorevertant viruses with the exception of Y98F/W222R were able to completely destroy the receptors following overnight incubation. These data suggest that the Y98F/W222R HA either has higher affinity for a common receptor, or that an alternative ligand that is resistant to the NA of this virus is being recognized by this mutant.

Table 1 - Agglutination of erythrocytes from various animal species by pseudorevertant viruses

	Virus						
Species	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 cRBC	<2	<2	<2	<2	<2	<2	<2

Agglutination of erythrocytes by pseudorevertant viruses. Hemagglutination assays were performed using standard techniques and expressed as HAU/50µl. Species used: chicken (cRBC), turkey (tRBC), guinea pig (gpRBC), sheep (sRBC), horse (hoRBC), and bovine (bRBC). Asialo RBC were created by enzymatically cleaving sialic acid with *C. perfringins* prior to the agglutination assay.

Table 2 - Receptor destroying capabilities of pseudorevertant viruses

	HAU					
	cRBC			gpRBC		
Virus	4˚C 2 hrs	4˚C 2hrs 37˚C 2hrs	37˚C o/n	4˚C 2 hrs	4˚C 2hrs 37˚C 2hrs	37˚C o/n
H3N1 WT	256	128	128	256	64	64
A/PA/08/2008	256	<2	<2	1024	128	<2
Y98F/N81T	64	32	32	128	<2	<2
Y98F/S145I	256	128	128	512	256	<2
Y98F/W222R	256	128	128	512	256	256
Y98F/I230M	256	128	128	256	128	<2
Y98F/K238N	256	128	128	256	<2	<2
PBS	<2	<2	<2	<2	<2	<2

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

Because HA and NA of influenza virus are known to operate in a delicate balance due to their opposing functions, we sequenced the NA genes 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 a different spectrum and distribution of potential receptor species on their cell surfaces. We used a fluorescence-based assay to examine the binding of our pseudorevertant viruses. Briefly, Alexafluor488-labeled influenza virus was adsorbed to confluent MDCK or A549 cells at 4˚C for 1 hour followed by extensive washing, and bound virus was detected by fluorimetry (see Materials and Methods). The results shown in Figure 3A and 3B 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 two-fold higher than WT and most mutants, while the Y98F/K238N

virus was reduced by over 50% compared to wild-type and the Y98F parental virus (Figure 3A). Binding of all pseudorevertant viruses to A549 cells was reduced by varying degrees relative to WT and the Y98F, with the Y98F/K238N virus again displaying the lowest activity.

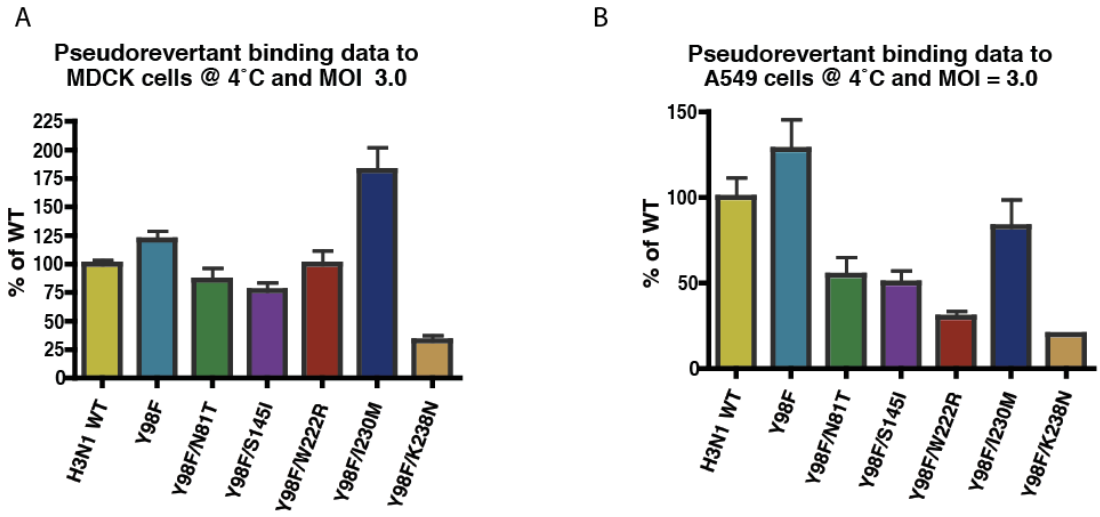

Figure 3 – Fluorescent binding assays of pseudorevertant viruses. Fluorescently labeled pseudorevertant strains were bound to chilled 96 well plates at an MOI = 3.0 and incubated at 4˚C for 1 hour. Binding is expressed as a percent of the rH3N1 WT strain. Panel A) shows binding to MDCK cells, and B) shows binding to A549 cells.

The results indicate that clearly detectable differences exist in the binding capacity among mutants to each cell line, and that the results can vary with one cell type relative to the other. In conjunction with the erythrocyte studies and virus replication analyses, the data suggest that either the mutant viruses bind to ubiquitously occurring receptors with varying degrees of efficiency, or recognize alternative receptor species on the surfaces of these cells, or that both of these phenomena are coming 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 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, Alexafluor488-labeled viruses were bound to slides for 1 hour at 4° C, washed, and the levels of bound virus were determined by fluorimetry. The rH3N1 WT 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 Figure 4A, the rH3N1 WT virus bound to numerous molecular species. These included glycans with α2,6 as well as α2,3 linked sialic acids, with a preference displayed towards branched α2,6 linked glycans as we have detailed previously (5). Interestingly, as shown in Figure 4B-4G, neither the Y98F virus, nor any of the pseudorevertants, recognized any of

the glycans with the high efficiency associated with rH3N1 WT virus or any other natural strains that we have examined previously (5). Amongst the pseudorevertant viruses, only the Y98F/K238N virus displayed any binding activity, with limited binding being detectable to several highly fucosylated, asialo glycans (Figure 4F and Figure 5). Though highly fucosylated glycans were bound very poorly relative to the WT strain, the levels are clearly above background, with a reliable %CV (coefficient of variation, 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 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 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 they may recognize a subset of sialic acid-containing receptors that are not present among the >500 glycans represented on the current version of the Consortium glycan microarrays, or they are recognizing more unconventional receptors.

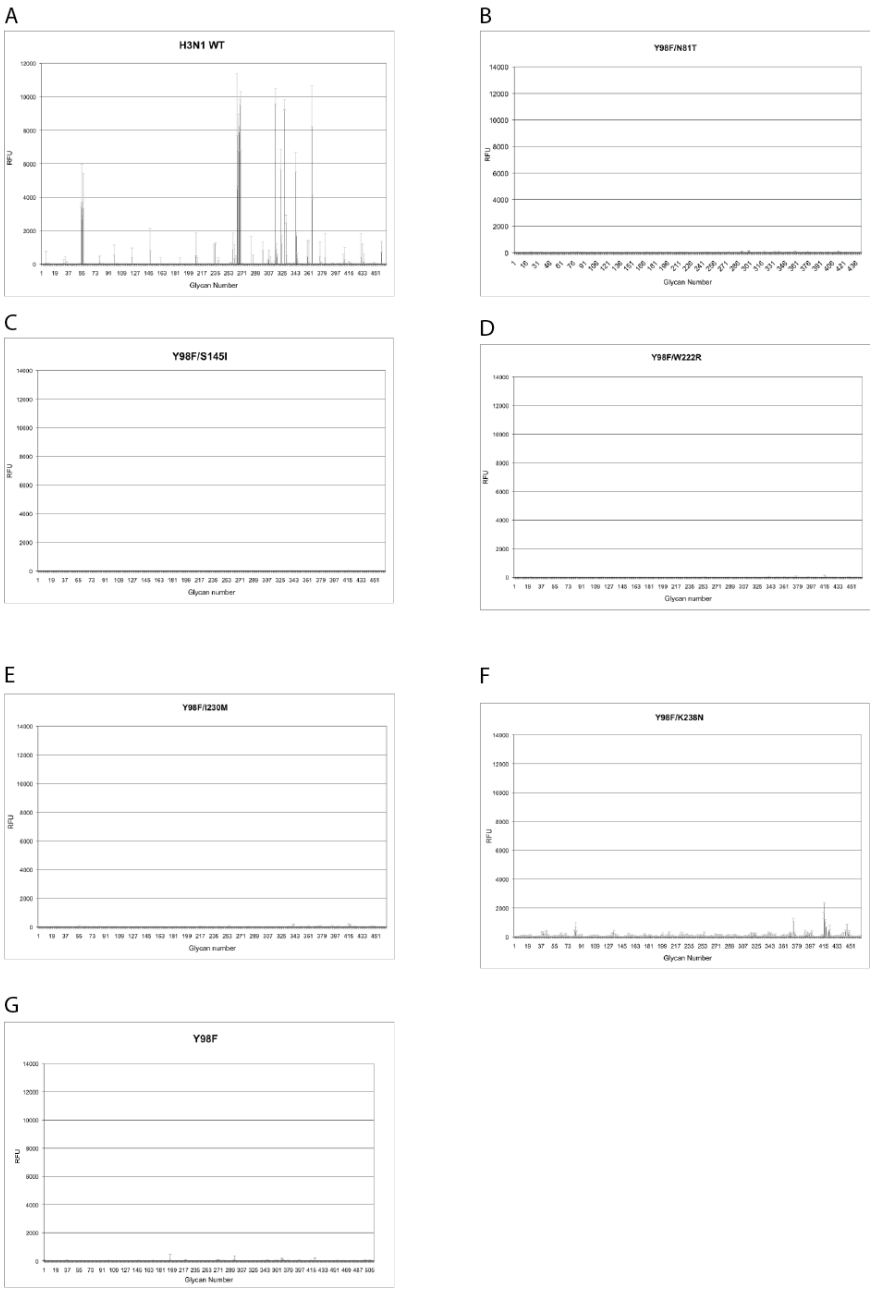

Figure 4 – Receptor binding to glycan arrays provided by the Consortium for Functional Glycomics (CFG), array version 4.1. Fluorescently labeled pseudorevertants were incubated on CFG arrays at 4˚C for 1 hour, then washed and scanned, always with an rH3N1 WT control. Panel A) rH3N1 WT; B) Y98F/N81T;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

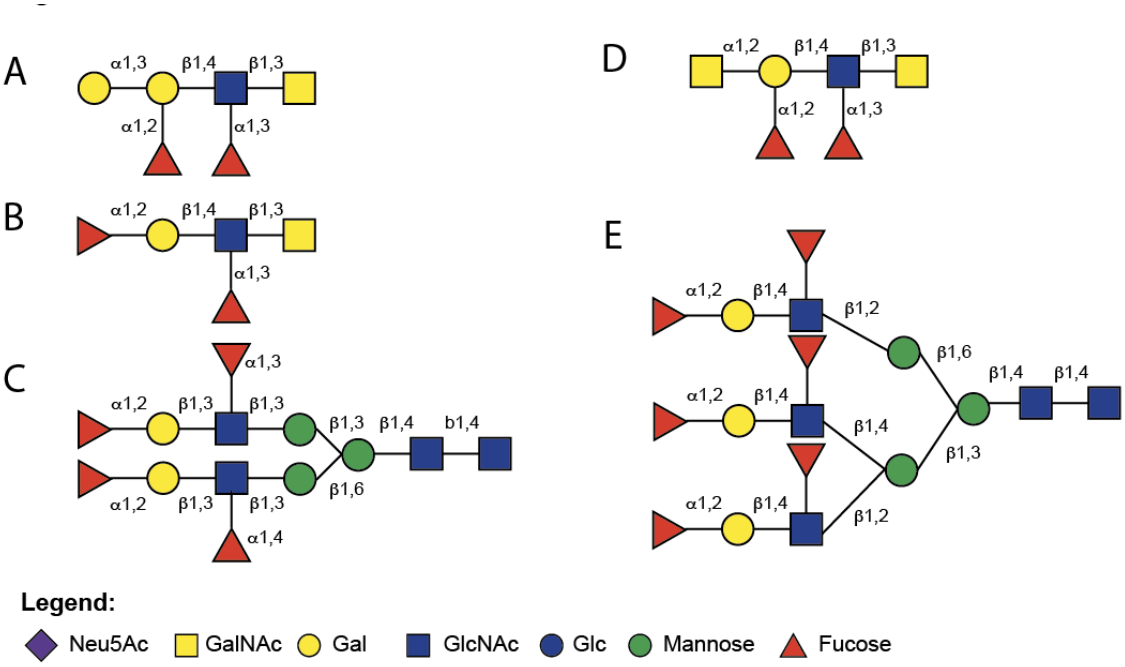

Figure 5 – Structure of the five highest binding glycans bound by Y98F/K238N. Glycan linkages are shown between glycan symbols. All glycans shown have a %CV of <50% and are considered to be above the level of background, which is two times the average of all glycans on the array.

Receptor binding to glycan microarrays terminating in derivatives of sialic acid

Although most influenza receptor binding research has focused on Neu5Ac and Neu5Gc glycans, sialic acid is expressed on cells with a highly diverse group of modifications (7). In an effort to address the steriochemical nature of potential receptor species for our viruses, we utilized a recently developed glycan microarray that contains chemically derived sialic acid species (65). These arrays present 77 sialic acid glycans with 16 different sialic acid modifications in α2-3 and 2-6 linkages to three different precursor glycans. Viruses were labeled with Alexafluor488 and assays were carried out at 4˚C as described in the Materials and Methods. Consistent with the CFG results, the rH3N1 WT bound to compounds containing branched α2,6 linked sialic acid, and also α2,3 linked species (Figure 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 Figure 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, Y98F/S145I and Y98F/K238N, demonstrated low, but detectable levels of binding to a number of the glycans that were considered sufficient enough for further discussion (Figure 6B-C and Table 3). Y98F/S145I bound to a methylated 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid structure

(KDN; Deaminated neuraminic acid), as well as a branched α2,3 linked sialic acid. The Y98F/K238N bound 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 of the 9th carbon of sialic acid. KDN is structurally identical to Neu5Ac, except it lacks the N-acetyl group on carbon-5.

Table 3 - Glycans bound by pseudorevertants on sialo-derivative arrays

Print ID	rH3N1 WT	mean	stdev	%CV
79	Galβ4GlcNAcβ2Manα3(Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB	3124	24	1
45	Neu5Ac9Ltα6Galβ4GlcNAcβ2Manα3(Neu5Ac9Ltα6Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB	1971	302	15
12	Neu5Ac9Ltα6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB	1364	365	27
48	Neu5Acα3Galβ4GlcNAcβ2Manα3(Neu5Acα3Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB	1292	139	11
35	Neu5Acα6Galβ4GlcNAcβ2Manα3(Neu5Acα6Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB	1122	233	21

Print ID	Y98F/S145I	mean	stdev	%CV
48	Neu5Acα3Galβ4GlcNAcβ2Manα3(Neu5Acα3Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB	204	28	14
74	Neu5,9Ac2α3Galβ3GlcNAcβ3Galβ4Glcitol-AEAB	192	57	30
65	Kdn5Meα6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB	101	20	20

Print ID	Y98F/K238N	mean	stdev	%CV
74	Kdn5Meα6Galβ3GlcNAcβ3Galβ4Glcitol-AEAB	871	161	18
65	Neu5,9Ac2α3Galβ3GlcNAcβ3Galβ4Glcitol-AEAB	427	68	16
2	Neu5Ac8Me α6Galβ4GlcNAcβ3Galβ4Glcitol-AEAB	188	49	26
49	Neu5Ac8Meα3Galβ4GlcNAcβ2Manα3(Galβ4GlcNAcβ2Manα6)Manβ4GlcNAcβ4GlcNAcitol-AEAB	169	28	17

Glycans considered to be significantly bound by pseudorevertant viruses on sialo-derivative glycan arrays. Significant binding is considered to be two times the background average, with a % CV of less than 50%.

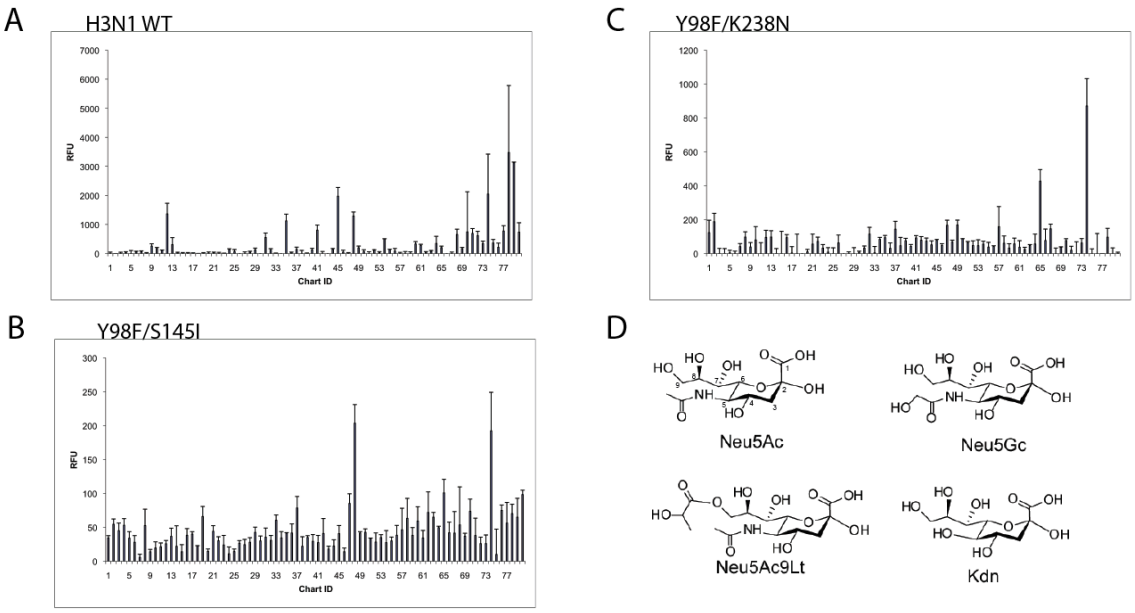

Figure 6 – Binding to sialo-derivative arrays by pseudorevertant viruses. Fluorescently labeled influenza virus was incubated for 1 hour at 4˚C, prior to washing and then scanned. The Y-axis is different for each panel to best represent peaks considered above background levels. Pseudorevertant strains that did not significantly bind to any structures are not shown. The structure of significant glycans can be seen in Table 3. A glycan is considered to be significant if the average RFU of a particular peak is greater than two times the average of all glycans on the plate, and the % CV is less than 50%. Panel A) rH3N1 WT; B) Y98F/S145I; C) Y98F/K238N.

DISCUSSION

Studies on influenza 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 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 (Figure 1). While the importance of these sites in contributing to receptor specificity and possibly host range is not in question, our current studies suggests that a range of residues at various structural locations in the 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 near wild-type levels in MDCK cells, it was severely debilitated for replication in mice. Our current study focuses 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 restored the capacity to agglutinate erythrocytes of selected species, and resulted in greater fitness for replication in the mouse lung.

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

1) Y98F/S145I – Residue 145 is located near the receptor binding site within antigenic domain A of HA and may directly interact with sialic acid (Figure 1A) (80, 81). The S145 has been associated with adaptation of H3 strains during egg passage (16, 30) as well as 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.

2) Y98F/N81T – 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 (Figure 1C and data not shown). The glycosylation site at N81 was present in the strains that initiated the H3N2 pandemic, such as A/Aichi/2/1968, 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 the 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 the Y98F HA. Depending on the location and/or type of N-linked glycosylation, such modifications of HA are capable of affecting the receptor binding affinity and specificity of influenza 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 the distance from the binding pocket, the mechanism by which the N81T mutation influences the receptor binding capacity is difficult to discern (81).

3) Y98F/W222R – HA residue 222 is located in the 220 loop at the "left" side of the binding site when viewed from the orientation depicted in Figure 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 introduced by the W222R mutation in our Aichi (H3) HA may also have the ability to form a hydrogen bond with Gal-2 of the receptor. In addition, W222 of the WT Aichi HA packs against a carbohydrate that originates from N165 of the adjacent monomer, as shown in Figure 1. The presence of R at 222 could influence the conformation or mobility of the N165 carbohydrate, and alter access to the binding site by certain receptor species.

4) Y98F/I230M – 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-W153 that form the base of the binding pocket. As such, I230M can be considered a "second shell" mutation.

The tighter hydrophobic packing of M230 with F98 and W153 in the HA 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 contain 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.

5) Y98F/K238N – The K238N mutation is interesting as 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 N165 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 demonstrate that all of the pseudorevertant viruses restored the capacity to agglutinate chicken, turkey, and guinea pig erythrocytes to varying degrees. 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 Aichi HA, as opposed to the α2,3 preference that might be expected for those selected for 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 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 for 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 three-day lung titers than the Y98F virus. In some cases, the reversion from the highly attenuated phenotype of Y98F was partial, and in others, lung titers were comparable to WT, or even slightly higher as in the case of I230M (Figure 2). In MDCK cells, some mutants replicated to levels comparable to 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 for the system. The Y98F/I230M virus titers were approximately two logs greater in MDCK cells than in mice lungs, and the Y98F/W222R grew to one log higher 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 relative to those obtained in mouse lungs.

Glycan microarrays have been used in numerous studies to examine the receptor binding specificity of influenza viruses 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 our rH3N1 WT strain, and in most cases the binding above background levels was not detected. These results were surprising given that the pseudorevertant viruses bound erythrocytes (Table 1) and cellular monolayers at or near WT levels (Figure 3). However, it should be noted that the complete structures of all glycans or the complete glycome of these cells are not known. Discrepancies in receptor binding characteristics are not unprecedented. Recently, a H13 strain was shown to bind guinea pig erythrocytes, but did not show binding to 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 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 (Figure 4F and Figure 5). The

glycans recognized by the Y98F/K238N virus were generally branched asialo glycans containing a Galß1,4(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 among 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 highest binding glycans all contained at least two fucose residues either branched off of the main glycan chain, or at the terminus of the chain.

While the 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 receptor binding, the most commonly studied sialic acid derivatives are the N-acetyl and N-glycolyl neuraminic 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 recently generated modified sialic acid glycan microarrays (65). The rH3N1 WT, Y98F/S145I, and Y98F/K238N viruses all bound to sialic acid derivatives, albeit some very poorly. One of the derivatives bound by the rH3N1 WT, but not the pseudorevertants was Neu5Ac9Lt (Figure 6A). Neu5Ac9Lt contains a lactyl group extending off the ninth carbon of sialic acid, and is known to be present in higher mammals, including 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) (Figure 6B and C). KDN is similar in structure to Neu5Ac, except that a hydroxyl group replaces the acetamido group on the fifth carbon (Figure 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 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 Neu5Ac (26). Notably, the second and third sugars in the glycan chain for all bound sialo-derivatives were Galß1,3/4GlcNAc, which have been observed to contact residues in the HA receptor binding site in complexes with receptor analogs containing sialic acid is in the α2,3 or α2,6 linked conformations (54). As observed previously (55), the position and orientation of sialic acid modifications will also feature 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 HA principally binds to glycans with terminal sialic acids, or with the basic generalizations that α2,3 and α2,6 linkage specificity plays a role in host range. However, they do raise questions regarding influenza receptor usage, and whether more stringent discrimination exists for specific subsets of glycans (or other

molecules) under certain circumstances. Recent evidence regarding influenza binding to macrophages, as well as evidence examining infection of desialylated cells, and mice lacking particular sialotransferases, have suggested that properties beyond linkage specificity may be at play in some situations for influenza receptor binding (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 non-glycan 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 receptors species that are not present among the >500 glycans on CFG microarrays or on the modified sialic acid glycan microarrays. With the advent of natural glycan arrays, and "shotgun glycomics" 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, it may become possible to utilize mutants such as Y98F to select variants in particular cell types or host species, and then determine whether they can recognize subsets of natural receptor molecules that may be tissue or species specific.

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CHAPTER 4 – Discussion and Future Directions

The emergence of highly pathogenic H5N1 isolates in 1997 highlighted the importance of understanding what facilitates adaptation of influenza to a new host. Research rapidly focused on the HA protein as a primary mediator of host adaptation and efficient transmission to humans. However, despite the continuing evolution of avian H5N1 viruses, numerous introduction events in humans, and the slow acquisition of human receptor binding capabilities, these strains have never exhibited efficient human-human transmission. Similarly, triple reassortant swine strains isolated in the Midwestern United States, as well as H7 and H9 isolates in Europe and elsewhere, have never truly become established in the human population (271). This is in stark contrast to the novel 2009 H1N1 pandemic strain, which replaced the NA and M gene segments of the trH1N1 swine strain with segments derived from a Eurasian swine lineage (71). Where this reassortant event occurred, and why it facilitates efficient human-human transmission is currently unclear. In the preceding chapters, we investigated whether a shift in receptor specificity had occurred between early pH1N1 strains and the closely related HA from trH1N1 isolates. We also sought to determine how mutations outside of the receptor binding site can affect the receptor binding properties of an HA protein, and noted binding to a subset of sialic acid derivatives.

In Chapter 2, we acquired a number of different human, swine, and vaccine strains from collaborators at the University of Georgia and University of Minnesota, in an attempt to compare the receptor binding patterns of sH1N1, pH1N1, and

trH1N1. Previous work had determined that many swine isolates agglutinated enzymatically modified erythrocytes expressing α2,6 linked sialic acids (α2,6 SA), rather than α2,3 SA (96, 249, 317), so it was understood that the primary receptor binding phenotype would skew towards greater α2,6 SA binding. First, we tested the agglutination of erythrocytes from animals with different sialic acid expression patterns, and none of the human or swine isolates agglutinated erythrocytes that express purely α2,3 SA (Chapter 2 Table 2). However, both human and avian influenza isolates are known to propagate efficiently in swine, in fact the predominant circulating strain of swine influenza circulating in Europe (eaH1N1) was caused by direct avian-swine transmission (29). It is believed avian strains can replicate efficiently in swine because the internal body temperature is around 39˚C, similar to avian species, and they express both avian (α2,3 SA) and human (α2,6 SA) receptors in their respiratory tract (215). This raised the possibility that although circulating swine strains preferentially bind α2,6 SA receptors, there might be a low level binding to α2,3 SA receptors as well, which facilitates avian influenza infection.

Glycan arrays provided by the Consortium for Functional Glycomics (CFG) (www.functionalglycomics.org) (15, 301) give the possibility of examining binding patterns with great sensitivity, due to the built-in redundancy of the glycans. The CFG arrays consist of an NHS activated slide, on which is printed a library of glycans that can be bound by bacteria, viruses, lectins and antibodies (16, 26, 47, 183, 300, 302-304, 343, 362, 363). We used the amine reactive fluorophore Alexa Fluor 488 to label purified, live virus stocks. In all cases, these viruses were then incubated on the glycan microarray slides at 4˚C to prevent the NA protein from cleaving the sialic

acid glycans on the slide. The inactivity of NA at 4˚C was confirmed in an experiment utilizing the substrate MUNANA ((2'-4-methylumbelliferyl)-α-D-N-acetylneuraminic acid), which fluoresces if it is cleaved by sialic acid. The results from the CFG microarray binding studies provided an abundance of data regarding binding of sialic acid, and allowed numerous comparisons between influenza strains.

Human isolates are known to preferentially bind the primary receptor found in the upper respiratory tract, α2,6 SA. But it was interesting to note, that the A/Pennsylvania/08/2008 virus, which was isolated just before the 2009 pandemic, also bound α2,3 SA with one of two characteristics. Either glycans were biantennary with one terminal α2,3 SA and one terminal α2,6 SA, or the glycan also contained a fucose and/or sulfate near the glycan terminus. Binding to fucosylated and sulfated α2,3 SA glycans was also seen in the course of collaborative experiments performed on mouse adapted strains with Dr. Compans group at Emory University, and with avian isolates with Dr. Tompkins group at the University of Georgia ((231) and Skountzou, et al. unpublished results). That these results are consistent between human, mouse-adapted, and avian isolates indicates they are not species specific, but might act to promote more efficient binding to α2,3 SAs. Structural modeling of the HA interaction with α2,3 SA offers hints in regards to the importance of the addition of these sugars to a glycan chain. Structures of human HAs co-crystalized with α2,3 glycans are often only determined out to the third sugar, due to a lack of electron density as opposed to structures crystallized with α2,6 SA glycans, which can often be visualized out to the 5th sugar (97). Increased

disorder of the glycan structure indicates a less stable receptor, which might be stabilized by the addition of a fucose or sulfate that could provide additional stability to facilitate α2,3 binding in a less than favorable situation. Modeling of these structures in H5 and H7 strains suggest an interaction at residues 222 and 193 occurs when a fucose or sulfate is present (218).

In contrast to A/Pennsylvania/08/2008, the three early pandemic H1N1 isolates (pH1N1) were highly specific in their preference for α2,6 SAs. Binding to long, monoantennary, α2,3 glycans was not tolerated (Chapter 2 Figure 6A), even when the glycans contained fucose or sulfate (Chapter 2, Figure 6E). Because the pH1N1 strains were derived very early in the pandemic, the strict α2,6 preference most likely indicated the strains had acquired the binding phenotype prior to human infection. This strict specificity may have given the early pandemic viruses an evolutionary advantage over competing circulating strains by reducing the possibility of capture and inactivation by mucins found in the upper respiratory tract (URT). As mentioned previously, mucins often consist of long carbohydrate chains terminated in sialic acid that often contain fucose. They are considered a part of the innate immune system and are known to inhibit influenza infection (35).

Interestingly, the trH1N1 and two other swine lineage strains were also highly specific for α2,6 SAs. This indicates that despite the presence of both α2,3 SA and α2,6 SA in the swine respiratory tract, circulating swine influenza HA remains specific for a single sialic acid conformation. The swine respiratory tract also produces mucins, which are known to have similar structure and conformation specificity as their human counterparts (Rana 1986). The receptor binding patterns

in swine indicate several things. 1) Many circulating swine strains might already encode HAs with human receptor specificity. 2) The evolution of swine influenza HA receptor binding might be driven by the presence of α2,3 containing mucins found in the swine upper respiratory tract. 3) Despite the seemingly correct receptor binding pattern, trH1N1 viruses are still unable to facilitate efficient human-to-human transmission, indicating additional factors are necessary. One factor to consider is that all three of our swine isolate HA proteins are derived from human influenza isolates, indicating they most likely had α2,6 SA receptor specificity when they first infected swine. However, as mentioned previously, the predominant Eurasian swine influenza strain originated from a direct avian-to-swine transmission event. That virus has been circulating in swine for a number of years, so it would be interesting to know if it retains the α2,3 SA receptor specificity of the parental virus, or if it has shifted to an α2,6 SA specificity. If it has shifted to α2,6 SA specificity, it might indicate the strength of the selective pressures faced by swine influenza by mucins in the URT, rather than antibodies as swine isolates do not add glycosylation sites in the globular head of HA to shield antigenic sites (29, 132, 309).

The strain responsible for the 2009 pandemic has yet to be identified in swine, and when or where the reassortment event took place is still a mystery, but genetic evidence suggests it has circulated for at least 10 years. Southeast Asia appears to be the most likely location for reassortment, as all of the major swine strains circulate there and multiple reassortant viruses can be isolated (154). Until the parental strain is identified, it is difficult to make definitive conclusions

regarding the mutations necessary to facilitate efficient transmission in humans. Several mutations exist between the trH1N1 and pdmH1N1 HA receptor binding site, notably at residues 186, 219, and 227 which may act in concert to affect receptor binding (185). However, the receptor binding patterns of trH1N1 and pdmH1N1 strains is remarkably similar. The replacement of the NA and M gene segments suggest any, or all, of the three proteins encoded by these segments might contribute to better transmission. The role of the M gene segment is still unknown, but recent work indicates that substitution of the NA protein in a swine H1N1 virus promotes efficient transmission in ferrets without affecting replication, possibly through increased NA activity (367).

Viral NA substrate specificity might also play a role in efficient transmission. Several viral and bacterial NAs are known to preferentially cleave either α2,3 or α2,6 SAs, and the specificity of the influenza NA is capable of adapting in a new host (62, 202, 248). For example, the N2 protein introduced in 1957 was highly specific for α2,3 SA, but was noted to acquire greater cleavage capability of α2,6 SA over time (8). The swine NAs tested by Yen et al, suggest the pandemic virus NA might have a greater preference of α2,3 SLN over α2,6 SLN than their swine counterparts (367). Our own research on the NA activity of an early pandemic isolate on MDCK cells also notes a more rapid decrease in MAA lectin binding (specific for α2,3 SA) over SNA lectin binding (specific for α2,6 SA), suggesting the increased preference for α2,3 SA by the NA protein is broader than glycans with sialyllactosamine backbones (Figure 1). How the Eurasian NA protein in the pandemic strains promotes efficient human-to-human transmission, while the trH1N1 NA does not, is

still largely a mystery. The pandemic NA differs from the NA of 2008 seasonal H1N1 (sH1N1) strains via a mutation at residue 149, which causes the loss of the 150 cavity in the NA catalytic site. This structural change could have mechanistic implications for the residues at 151 and 152, which play an integral role in the mechanism of cleavage of sialic acid (320).

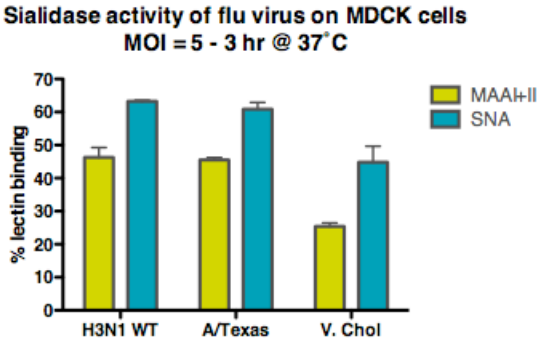


Figure 1 – Cleavage of sialic acids on fixed MDCK cells. Fixed cells were incubated for 3 hrs at 37˚C in the presence of a 5 MOI of either rH3N1 WT or A/Texas/15/2009. After 3 hours, cells were washed and incubated with either SNA or MAA I+II, for 1 hour to determine the level of NA activity. NA activity is expressed as a % of the original levels of bound lectin.

Overall, glycan arrays and neuraminidase assays show the pandemic viruses to have a strictly α2,6 SA binding HA protein with dual tropic neuraminidase protein that preferentially cleaves α2,3 SA. Although the NA specificity of swine H1N1 viruses is currently unknown, the high percentage of mucins containing α2,3 SA strongly suggests a neuraminidase with a strong α2,3 SA specificity. If this is true, it once again highlights the importance of swine as an intermediary "mixing

vessel" for influenza, as the HA and NA properties necessary for sustained transmission appear very similar between the two species.

Changes in the receptor binding domain between trH1N1 and pH1N1 strains might be responsible for changes in receptor specificity for those strains, in particular the mutations at 186, 219, and 227 could destabilize the ionization network associated with those residues. These residues and others within the receptor binding site have been explored numerous times before; however, in Chapter 3 we explored mutations outside of the receptor binding site that significantly altered the association of the receptor binding site with it's receptor.

Passage of a non-agglutinating Y98F mutant in either mice or MDCK cells lead to the rapid acquisition of compensating mutations that promoted erythrocyte agglutination. The majority of the pseudorevertant viruses agglutinated erythrocytes at approximately the same levels as our rH3N1 *wt* strain (Chapter 3 Table 1). The *wt* virus used is a rescued 7:1 reassortant virus with the A/Aichi/2/1968 HA in a WSN backbone, and is the parental virus to all of the pseudorevertant strains, making it an ideal control virus. Because the pseudorevertant viruses agglutinate erythrocytes, but the parental Y98F virus does not, it is logical to assume that the mutations acquired during passage of the Y98F virus alter receptor binding. This could be by either increasing the affinity for a receptor, or by facilitating binding to a new receptor. These pseuodrevertant viruses appear to affect receptor binding by one of four general mechanisms:

1) Direct interaction with the sialic acid receptor.

2) Interaction with a residue critical to sialic acid binding, referred to as a "second shell" mutation.
3) Perturbation of the HA monomer/monomer interface.
4) Removal of a glycosylation site.

Interestingly, the majority of mutants isolated affected receptor binding from afar, rather than by direct interaction with sialic acid. In particular, the mutants at Y98F/N81T and Y98F/K238N affect receptor binding from a distance of 28 Å and 40 Å respectively, apparently through two different mechanisms. Y98F/N81T most likely alters binding due to the removal of a consensus Asn-Xaa-Ser/Thr N-linked glycosylation site, as residue 81 only makes localized contacts at 118 and 120, which are unlikely to significantly alter the binding site structure. This site is visible in the A/Aichi/2/1968 structure, and we confirmed the loss of the site by comparing the migration patterns of the rH3N1 WT and Y98F/N81T mutant during PAGE analysis (Figure 2). How the removal of this site affects binding is still a mystery, since the composition and length of the N-linked glycan is unknown, as only the first sugar on the chain is ordered in the structure. Similar to Y98F/I230M and Y98F/S145I, none of these mutants bound glycans on the CFG microarrays (Chapter 3 figure 4).

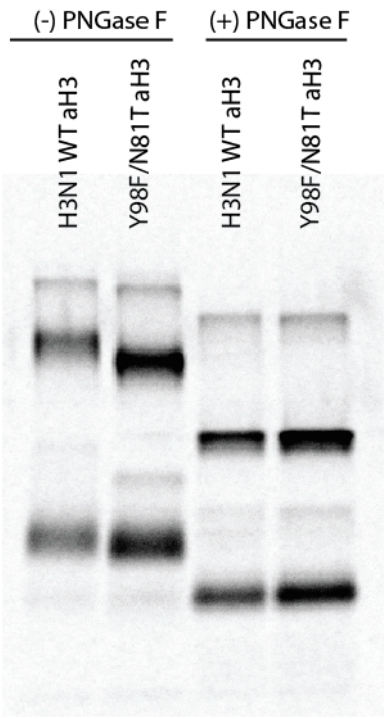


Figure 2 – Y98F/N81T mutation leads to a loss of an N-linked glycosylation site. Cells were infected with a low MOI of either rH3N1 *wt* or Y98F/N81T. Radioactive labeling was performed with S^{35}, and harvested lysates were treated ± PNGaseF. Virus growth was performed by KB, and PAGE analysis was carried out by Summer Galloway.

Y98F/K238N and Y98F/W222R both appear to affect receptor binding in the same manner, by perturbing the monomer-monomer interface. Structurally, we believe this serves to slightly pry apart the globular heads of HA which might facilitate binding to a different receptor. Y98F/W222R did not bind to the CFG array, however, Y98F/K238N did reveal low level binding to highly fucosylated asialo glycans (Chapter 3 Figure 5). It's difficult to structurally determine how binding to fucose might occur, but these glycans were bound above the background

level, and had a low % CV. It is possible that the opening of the globular heads drastically affects the structure of the binding site to the point that the three available –OH groups on the fucose monosaccharide can lead to low level binding by the virus. One unexplored avenue of Y98F/K238N and Y98F/W222R pseudorevertant viruses is the affect the compensating mutation has on the pH of fusion. Any disruption of the monomer-monomer interface could lead to an increase in fusion pH due to destabilization of the globular heads. We are planning to examine this possibility in the near future.

Because Y98F and the pseudorevertant viruses can all agglutinate erythrocytes, and bind and infect MDCK cells (Chapter 3 Table 1 and Figure 2 and 3), it is understood they are entering the cell in some manner. This suggests the pseudorevertant strains are binding an alternate receptor not present on the CFG glycan microarrays. A logical possibility is that the changes to the HA could promote binding to a slightly different form of sialic acid, such as the derivatives discussed in the introduction. To determine if the pseudorevertant viruses might be binding to a derivative of sialic acid, we incubated the pseudorevertant mutants on the new sialic acid derivative arrays (290). These arrays consist of 16 modified sialic acids printed as 77 different glycans on a glass slide. Derivatives such as Neu5Ac9Lt were previously reported to bind hPIV and influenza isolates, indicating sialic acid derivatives might present a potential receptor for the pseudorevertant viruses (290). Binding to these arrays was weak, but both Y98F/S145I and Y98F/K238N bound to glycans terminating in KDN. As mentioned in the introduction, KDN (deaminated neuraminic acid) is a derivative of sialic acid that replaces the

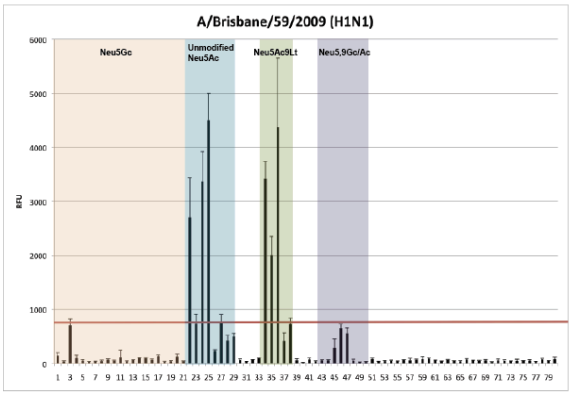
acetamido group off of the 5th carbon with a hydroxyl group (See Chapter 3 Figure 6D). KDN is unique among sialic acid derivatives in that cells specifically encode a KDNase, responsible for cleaving KDN, likely because KDN is not cleavable by most sialidases (149). The acetamido group is predicted to create a contact with residue 135, but it is unknown how this might affect HA/sialic acid interactions, or if the –OH group present on KDN can also make contact with the 130-loop. Binding to KDN is interesting because it has been isolated in several mammalian tissues, and might signify a biologically relevant alternative to Neu5Ac (133, 134). Unfortunately, the distribution of KDN in cell lines and mammalian tissues, along with most of the other sialic acid derivatives, is not well understood. However, tools do exist to examine sialic acid derivative binding, in particular a KDN monoclonal antibody, as well as the more common plant lectins MAA and SNA, which recognize KDN in addition to the better understood Neu5Ac receptors.

Although the pseudorevertant viruses bound to KDN, our rH3N1 *wt* mutant did not; rather it bound to a subset of Neu5Ac9Lt sialic acid derivatives. This structure, visualized in Chapter 3 Figure 6D, adds a lactyl group on the 9th carbon of sialic acid. Binding to this glycan derivative is quite surprising as it suggests the receptor binding site can tolerate a much larger receptor. As mentioned earlier, this receptor was also bound by hPIV isolates as well as an H1N1 influenza strain, indicating Neu5Ac9Lt might be a common derivative bound by other sialic acid recognizing viruses. By exploring the receptor binding site, it is difficult to imagine how this binding might occur in the receptor pocket, as the addition of the lactyl group would represent a bulky addition to a receptor binding pocket that appears

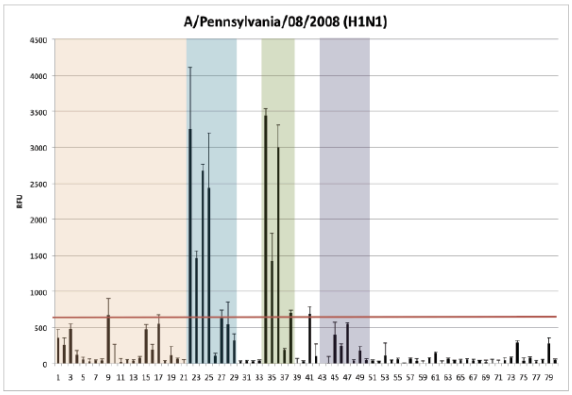
finely evolved to bind a nine carbon glycan. It is possible this binding is facilitated by a shift in how sialic acid fits in the pocket, although in structural studies, it is fairly stable generally only shifting by 1 Å with a minor rotation. A future aim of this work might be to crystallize an HA protein with a Neu5Ac9Lt glycan, in order to determine the interactions of these sialic acid derivatives in the pocket, which could open up new avenues for discussion on the type of glycans bound by influenza.

Binding to KDN and the Neu5Ac9Lt structures was a surprise, but because our rH3N1 virus is a laboratory adapted strain, it is possible that our *wt* and pseudorevertant viruses had evolved to recognize these receptors during passage in MDCK cells. If either KDN or Neu5Ac9Lt represented a true receptor for influenza, it would stand to reason that natural strains would also recognize these glycans. In order to determine this, we incubated a panel of recent human, swine, and vaccine strains used in Chapter 2 on the sialo-derivative arrays (Figure 3). Consistent with the binding results from the CFG array, all of the viruses bound to unmodified Neu5Ac glycans. To our surprise however, all seven strains tested also bound to

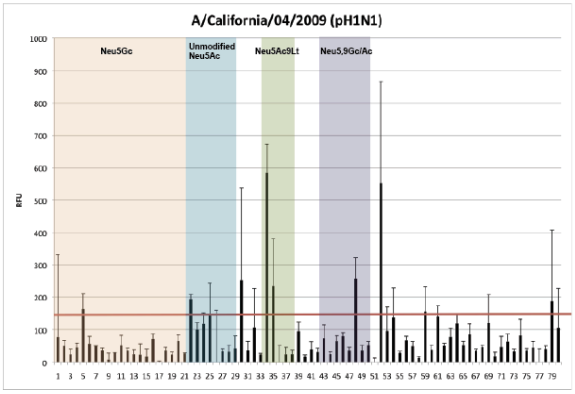
A


A/Brisbane/59/2009 (H1N1)
Neu5Gc
Unmodified Neu5Ac
Neu5Ac9Lt
Neu5,9Gc/Ac
RFU

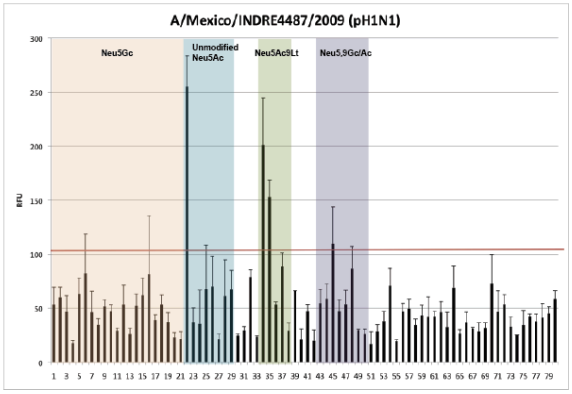
B


A/Pennsylvania/08/2008 (H1N1)
RFU

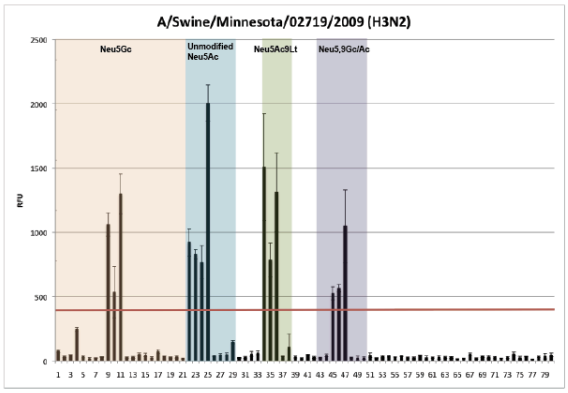
C


A/California/04/2009 (pH1N1)
Neu5Gc
Unmodified Neu5Ac
Neu5Ac9Lt
Neu5,9Gc/Ac
RFU

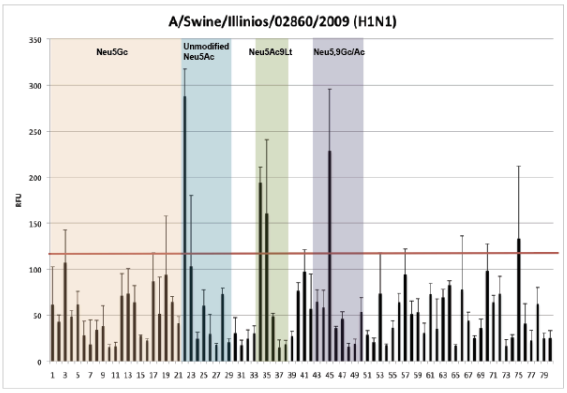
D


A/Mexico/INDRE4487/2009 (pH1N1)
Neu5Gc
Unmodified Neu5Ac
Neu5Ac9Lt
Neu5,9Gc/Ac
RFU

E


A/Swine/Minnesota/02719/2009 (H3N2)
Neu5Gc
Unmodified Neu5Ac
Neu5Ac9Lt
Neu5,9Gc/Ac
RFU

F


A/Swine/Illinios/02860/2009 (H1N1)
Neu5Gc
Unmodified Neu5Ac
Neu5Ac9Lt
Neu5,9Gc/Ac
RFU

G

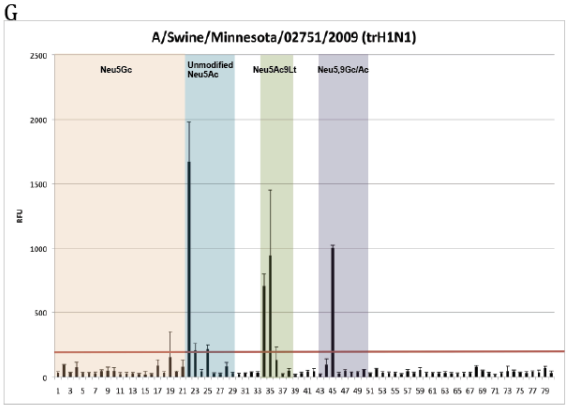

A/Swine/Minnesota/02751/2009 (trH1N1)
Neu5Gc
Unmodified Neu5Ac
Neu5Ac9Lt
Neu5,9Gc/Ac
RFU

Figure 3 – Binding of human and swine isolates to sialo-derivative glycan microarrays. The solid red line indicates the background level, defined as 2x the average of all 77 glycans. Glycans are sorted based on structure, and important structures are highlighted. Orange – Neu5Ac; Blue – Unmodified Neu5Ac; Green – Neu5Ac9Lt; Purple – Neu5,9Ac/Gc. Panel A) A/Brisbane/59/2007 (vH1N1); B) A/Pennsylvania/08/2008 (sH1N1); C) A/California/04/2009 (pH1N1); D) A/Mexico/INDRE4487/2009 (pH1N1); E) A/Swine/Minnesota/02719/2009 (H3N2); F) A/Swine/Minnesota/02860/2009 (H1N2-like swine/H1N1) G) A/Swine/Minnesota/02751/2009 (trH1N1)

Neu5Ac9Lt. These results might indicate that influenza HA is capable of utilizing sialic acid derivatives as alternative receptors to enter the cell. Also interesting was the recognition that conformation specificity of these isolates was maintained for sialic acid derivatives as well, in that the swine and early 2009 pandemic strains strictly bound α2,6 linked Neu5Ac9Lt receptors, while the vaccine strain A/Brisbane/59/2007 bound a mixture of α2,3 and α2,6 SAs. The recent evidence that an H13 virus was noted to agglutinate turkey erythrocytes, but did not display binding to human airway epithelial cells might be further evidence that influenza isolates can recognize derivatives in a species specific manner (270). Although we did not have avian isolates available to examine on the sialo-derivative microarrays, we recently received two strains from collaborators at UGA, and are currently

growing these strains in the lab. We hope to determine if these avian strains also bind to the sialic acid derivatives mentioned above. It is possible these avian isolates could bind to a different subset of sialic acid derivatives, which could further explain why circulating H5N1 isolates have yet to efficiently transmit in humans.

The HA binding to sialic acid derivatives led us to question whether these sialoderivatives could also be cleaved by the NA. Typically the HA/NA functions are well balanced, due to their opposing functions. Viruses grown in the presence of NAIs aggregate on the surface of a cell membrane, as they are unable to release from sialic acid expressed on the cell surface. In Figure 1 of the discussion we showed that early pH1N1 NAs appear to preferentially cleave α2,3 SA on MDCK cells, and others have seen this with past strains as well (8). We believe we can use the techniques learned from these experiments to examine the NA cleavage specificity of sialic acid derivatives as well. In preliminary experiments, we incubated *V. cholera* and *C. perfringins* sialidases on sialo-derivative microarrays, and noted an obvious preference for α2,3 SA, but more specifically a lack of cleavage of KDN and methylated Neu5Ac derivatives (Figure 4).

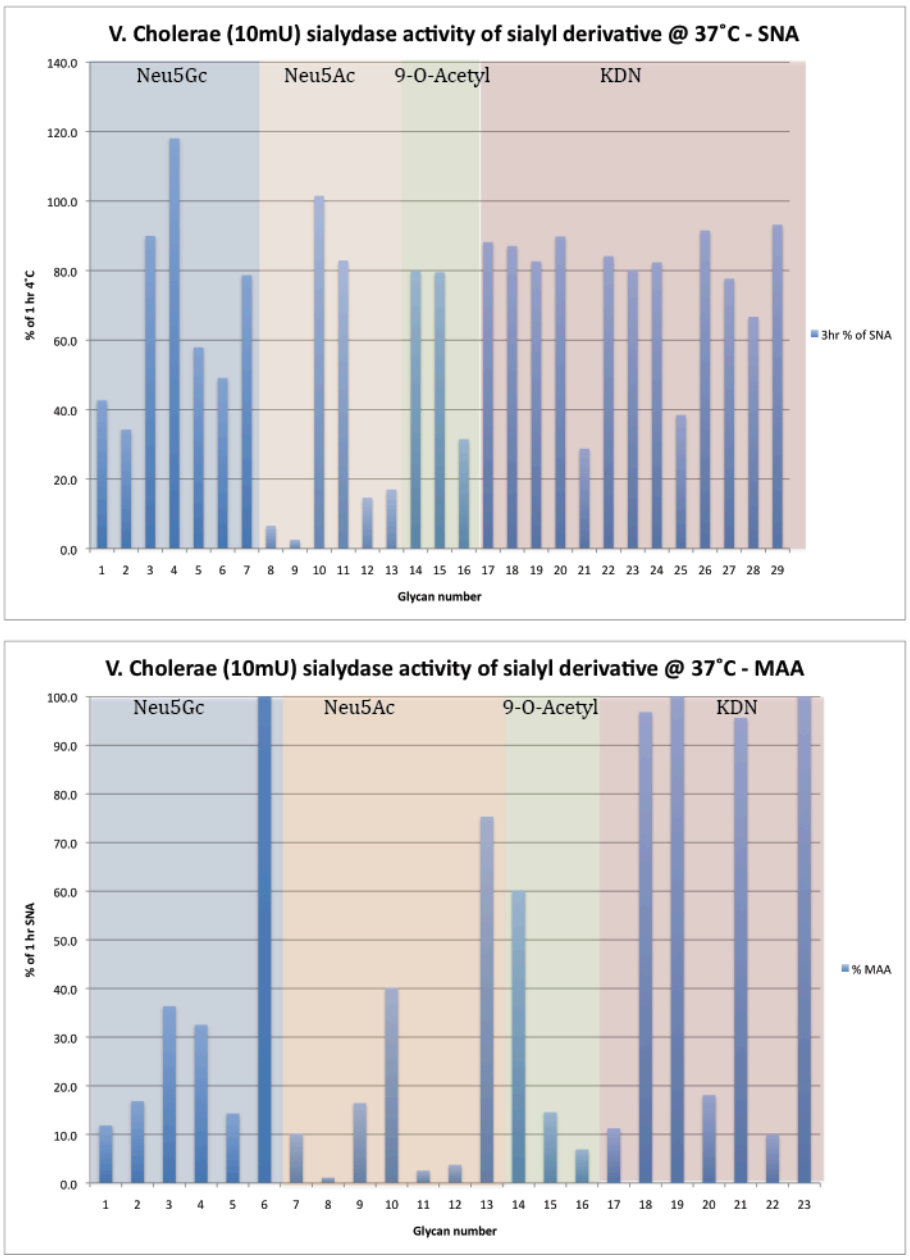


Figure 4 – Cleavage of sialic acid derivatives from sialo-derivative microarray by *V. cholera* sialidase. 10 mU of sialidase was incubated at 37˚C on sialo-derivative microarrays for 3 hours, washed and bound by either SNA (top panel) or MAA (bottom panel). Only glycans are shown that exhibited significant MAA & SNA binding, and sorted by glycan structure. Data is shown as a percentage of bound lectin without sialidase treatment.

This proof-of-concept experiment shows that bacterial neuraminidase activity appears to be lessened for certain derivatives, and additionally might explain why influenza has been shown to infect cell lines desialylated with bacterial NA (177, 231, 306, 328). It also sets the stage for us to use similar techniques for examining viral NA cleavage specificity. Reduced sialidase activity against derivative glycans bound by HA might indicate a route of infection, as readily cleaved glycans might be unable to "hold" a virion long enough to trigger endocytosis. It also might suggest an alternative route of infection due to HA irreversibly binding to a receptor and entering during endosomal recycling, which could also explain why influenza was visualized entering via two different mechanisms concurrently (258). These derivatives have been identified in both glycoprotein and glycolipid fractions (31, 243), although are predominantly found in glycoproteins, which appear to be necessary for influenza infection of CHO cells (55), and binding to the macrophage receptors macrophage mannose receptor (MMR) and macrophage galactose-type lectin (MGL). However, because the infection of macrophages does not lead to active viral production, it is uncertain whether these proteins represent realistic receptors (247, 351).

Although binding to sialic acid derivatives could represent a significant step forward in understanding HA specificity, the goal of this project is to ultimately determine the glycans bound by viruses on a cell membrane. This new type of microarray under development, termed "natural" arrays, seeks to isolate N- and O-linked glycans and glycolipids from a cell line or animal tissue. We have chosen a

panel of influenza strains that include human, avian, and swine isolates that represent a broad sample of circulating virus strains. In addition, these microarrays present a unique opportunity to determine the receptors involved in infection by our pseudorevertant strains. By using Alexa Fluor 488-labeled viruses, binding experiments should be able to identify glycans found on the cell membranes of MDCK cells, guinea pig erythrocytes, and even pig respiratory epithelium. Variations in binding patterns between seasonal and pandemic viruses could reveal how pandemic strains alter their receptor binding patterns over time, while the avian virus binding to swine respiratory tract arrays can begin to describe the initial receptors during host adaptation. In addition, by incubating Alexa Fluor 488-labeled virus on natural arrays at 37˚C and following with Cy-5 labeled MAA or SNA, we can determine how closely the neuraminidase activity on these cells mirrors the binding phenotype of hemagglutinin. We feel this will give important insight on the role both of these two proteins play in viral entry.

The discussion and experiments above offer insights into the receptor binding patterns of laboratory and circulating influenza isolates, and have introduced a new series of questions we are continuing to explore. Hopefully, these questions can set the stage for a greater understanding of virus receptor binding, cellular tropism, and virus adaptation to a new host in the future.

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