Introduction

The 20th century witnessed three influenza pandemics: the Spanish flu of 1918 (H1N1), the Asian flu of 1957-58 (H2N2) and the Hong Kong flu of 1967-68 (H3N2). Among these subtypes the H1N1 and H3N2 continue to circulate in the human population leading to epidemic outbreaks annually and the H1N1 subtype was responsible for the 2009 ‘swine flu’ pandemic (2009 H1N1). The H2N2 subtype had stopped circulating in humans by 1968. Strains of H2 subtype still continue to circulate in birds and occasionally pigs and could be reintroduced into the human population through antigenic drift or shift. Such an event is a potential global health concern because of the waning population immunity to H2 hemagglutinin (HA). The first step in such a cross-species transmission and human adaptation of influenza A virus is the ability for its surface glycoprotein HA to bind to glycan receptors expressed in the human upper respiratory epithelia. Recent structural and biochemical studies have focused on understanding the glycan receptor binding specificity of the 1957-58 pandemic H2N2 HA. However, there has been considerable HA sequence divergence in the recent avian-adapted H2 strains from the pandemic H2N2 strain. Using a combination of structural modeling, quantitative glycan binding and human respiratory tissue binding methods, we systematically identify mutations in the HA from a recent avian-adapted H2N2 strain (A/Chicken/PA/2004) that make its quantitative glycan receptor binding affinity (defined using an apparent binding constant) comparable to that of a prototypic pandemic H2N2 (A/Albany/6/58) HA.
efficiency of airborne viral transmission in the ferret animal model [19,21], which is an established model to evaluate viral transmissibility in humans [22,23,24,25,26]. Such a relationship has yet to be shown for the H2N2 subtype.

Previous structural and biochemical studies have provided insights into interactions of the receptor binding site (RBS) of HA with avian and human receptors for both wild type (WT) and mutant forms of HA derived from the 1957-58 H2N2 pandemic strains [27,28]. However, it has recently been demonstrated that changes in the interactions between amino acids within and proximal to the RBS, arising from substitutions due to antigenic drift or reassortment, have profound effects on HA-glycan interactions which in turn influences the glycan binding affinity of HA [19,20]. This observation is particularly relevant to HA from recent avian-H2 strains that have diverged considerably in sequence compared to the HA sequence of the pandemic H2N2 strains [29]. Therefore in order to monitor changes in the recent avian H2-subtype viruses that would possibly lead to their human-adaptation, it is important to understand the mutations in their HA that would confer human receptor-binding affinity that is quantitatively in the same range as that of HA from the 1957-58 human-adapted H2N2 pandemic viruses.

In this study, we have systematically analyzed the effects of mutations in the glycan RBS of pandemic and recent avian H2N2 HAs on their respective glycan-binding specificities. The HA from a representative 1957-58 pandemic H2N2 strain, A/Albany/6/58 (Alb58), was chosen as a reference human-adapted HA. The HA from a representative avian H2N2 virus, A/Chicken/Pennsylvania/2004 (CkPA04), which is among the most recent strains isolated from birds was also evaluated in this study [29]. We first characterized the glycan receptor-binding affinity and human respiratory tissue binding properties of these avian- and human-adapted H2N2 HAs. The glycan receptor-binding affinity of HA is quantitatively defined using an apparent binding constant \( K_d' \) that takes into account the cooperativity and avidity in the multivalent HA-glycan interactions as described previously [21]. Next, using homology-based structural models of Alb58 HA-human receptor and CkPA04 HA-avian receptor complexes we analyzed the RBS of these HAs and designed and evaluated mutations in CkPA04 HA that would make its human receptor binding affinity in the same range as that of Alb58 HA.

Results

Characterization of glycan receptor-binding specificity of Alb58 HA

We have previously developed a dose-dependent glycan array binding assay [10,21] to quantitatively characterize glycan receptor binding affinity of HA by calculating an apparent binding constant \( K_d' \). Alb58 HA was recombinantly expressed and analyzed using this assay. Alb58 HA bound with high affinity to the representative human receptors, 6'SLN (\( K_d' \sim 35 \) pM) and 6'SLN-LN (\( K_d' \sim 5 \) pM) (Figure 1A). Notably, the binding affinity of Alb58 HA to 6'SLN-LN is in the same range as that of the pandemic H1N1 (A/South Carolina/1/1918 or SC18) HA [21]. However unlike SC18 HA, Alb58 HA also showed substantial binding to the representative avian receptors 3'SLN-LN (\( K_d' \sim 1.5 \) nM) and 3'SLN-LN-LN (\( K_d' \sim 1 \) nM) on the glycan array (Figure 1A) and thus correlated with its high affinity binding to human receptors. Additionally, the substantial \( \alpha2\rightarrow3 \) sialylated glycan binding of Alb58 observed in the glycan array assay was also reflected in its binding to the human deep lung alveolar tissue (Figure 1B) that predominantly expresses these glycans [10,21].

Previous studies have pointed to the roles played by the amino acids in positions 226 and 228 in the RBS of H2N2 HAs in governing the glycan receptor binding specificity [27,28]. The observation includes the fact that HA from most human H2N2 isolates has Leu226 and Ser228 within its RBS, whereas HA from most avian H2 isolates has Gln226 and Gly228. To understand the roles of these residues on the quantitative glycan receptor binding affinity of Alb58 HA, three mutant forms of Alb58 were designed. Two of these mutants possessed a single amino acid change, Leu226→Gln (Alb58-QS mutant) and Ser228→Gly (Alb58-QG). The third mutant carried two amino acid changes, Leu226→Gln and Ser228→Gly (Alb58-QG). Alb58-QG mutant retained the human receptor binding specificity of the WT Alb58 HA but showed a complete loss in the avian receptor binding in the dose-dependent direct binding assay (Figure 2A). On the other hand, Alb58-QS mutant showed a complete loss in human receptor binding and but displayed a substantial binding to avian receptors in contrast to Alb58 HA (Figure 2B). Surprisingly, Alb58-QG mutant exhibited little to no
binding to either the avian or human glycan receptor (Figure 2C). Circular dichroism analysis of Alb58-QS ruled out the possibility of Alb58-QS being misfolded (data not shown). A homology-based structural model of the Alb58-QS mutant was constructed to investigate the molecular basis of the observed biochemical binding property. Analysis of the glycan receptor-binding site of this mutant in the model showed that Ser228 is positioned to form a hydrogen bond with Gln226 (Figure 2D). The interaction between Gln226 and Ser228 potentially disrupts the favorable positioning of Gln226 for optimal contact with avian receptor. This observation offers an explanation for the loss of avian receptor binding in the Alb58-QS mutant. Furthermore, the absence of contacts between Gln226 and human receptor could explain the loss of human receptor binding.

Mutations in RBS of CkPA04 and their effects on its glycan receptor binding specificity

The dose-dependent glycan array binding of CkPA04 HA showed high affinity binding to the representative avian receptors 3'SLN, 3'SLN-LN and 3'SLN-LN-LN with minimal binding to human receptors (Figure 3A). Furthermore, the glycan array binding property of CkPA04 correlated with its extensive binding to the human alveolar tissues and minimal binding to the apical side of the tracheal tissues (Figure 3B).

To understand the molecular aspects of the H2 HA-glycan receptor interaction, we constructed homology-based structural models of the CkPA04-avian (Figure 4A) and the Alb58-human receptor complexes (Figure 4B). Based on these structural models of CkPA04 and Alb58 HAs, the amino acids positioned to interact with the glycan receptors were compared (Table 1). In addition to the differences in 226 and 228 positions, there were differences in other positions including 137 and 193. The amino acids at positions 137 and 193 are oriented to interact with Neu5A-c2→6Gal motif as well as sugars beyond this motif in the context of the human receptor (and potentially play a role in antigenic variations among current strains of H2 viruses; see discussion). These differences potentially impinge on the human receptor binding of H2N2 HA. Notably, CkPA04 HA differs from earlier
avian-adapted H2N2 HAs in the 137 and 193 positions. Therefore, while the Gln226→Leu and Gly228→Ser substitutions would make the RBS of earlier avian-adapted H2N2 HAs almost identical to that of the pandemic Alb58 HA, additional amino acid changes are required in the more recent avian-adapted HAs, including CkPA04.

Based on the above analysis, three sets of mutations were progressively made on CkPA04 to improve its contacts with the human receptor. The first mutant comprised of the two amino acid change Gln226→Leu/Gly228→Ser (CkPA04-LS). The second mutant, CkPA04-TLS, included an additional Ala193→Thr amino acid change in the CkPA04-LS HA. The third mutant, CkPA04-RTLS, was generated by introducing an additional Gln137→Arg mutation in the CkPA04-TLS HA. These HA mutants were recombinitely expressed and characterized in terms of their quantitative glycan receptor binding affinity and human tissue binding properties.

CkPA04-LS showed decreased binding to avian receptors and substantial binding to human receptors in comparison with CkPA04 (Figure 5A). CkPA04-TLS showed substantially higher binding signals to both human and avian receptors when compared to CkPA04-LS (Figure 5C). CkPA04-RTLS on the other hand showed increased binding signals to human receptor and similar binding signals to avian receptor as compared to CkPA04-LS (Figure 5E). The human respiratory tissue binding of these mutant H2 HAs was in agreement with their observed glycan array binding (Figure 5B, 5D, 5F). The dose-dependant glycan binding data of the described HAs were used to calculate $K'_R$ and $n$ values ($n \sim 1.3$ for all the HAs) by fitting the binding data to the Hill equation (for multivalent binding) and this was then used to generate theoretical binding curves to clearly distinguish the relative binding affinities of WT and mutant H2 HAs to representative avian and human receptors (Figure 6). The human receptor binding affinity of CkPA04-LS [$K'_R \sim 50$ pM] was 10-fold lower than that of the Alb58 HA [$K'_R \sim 5$ pM]. On the other hand the human receptor binding affinity of both CkPA04-TLS [$K'_R \sim 3$ pM] and CkPA04-RTLS [$K'_R \sim 8$ pM] were several fold higher than that of CkPA04-LS and in the same range as that of Alb58 HA. The avian receptor binding affinity of CkPA04-TLS [$K'_R \sim 50$ pM] was the same range as that of the WT CkPA04-LS [K_R \sim 20 pM] and several fold higher than that of CkPA04-LS [K_R \sim 220 pM] and CkPA04-RTLS [$K'_R \sim 220$ pM]. Therefore, among the different mutants, CkPA04-RTLS was the closest to Alb58 HA in terms of its relative human to avian receptor binding affinity. Based on our structural understanding, this observation is consistent with the fact that the RBS of CkPA04-RTLS and Alb58 were very similar to each other, including extended range contacts with the glycan receptor beyond the Neu5Ac linkage.

Discussion

Our study highlights the importance of integrating a systematic sequence and structure analysis of HA-glycan molecular interactions and a quantitative binding assay to study the effects of these interactions on the biochemical glycan receptor binding affinity of HA.

Previous studies have focused on amino acid substitutions in 226 and 228 positions in the RBS of pandemic H2N2 HAs [28]. Recently the glycan receptor-binding properties of the Alb58 virus and the WT and mutant forms (with substitutions in 226 and 228 positions in HA) of a related pandemic H2N2 virus – A/El Salvador/2/57 (or ElSalv57) were characterized by analyzing these whole viruses in a dose dependent fashion on the glycan array platform [30]. The glycan receptor-binding properties of the recombinant Alb58 HA reported in the present study are in good agreement with those obtained using the whole viruses [30]. Our results further augment these observations by characterizing the effect of substitutions in the 226 and 228 position on the quantitative glycan receptor binding affinity of Alb58 HA.

In addition to the previously noted 226 and 228 positions [27,28], our systematic sequence and structural analysis of H2 HA-glycan complexes revealed key differences between CkPA04 and Alb58 HAs in other positions, such as 137 and 193. By progressively designing mutations in CkPA04 we demonstrate that substitutions at the 137 and 193 positions (in addition to those in 226 and 228 positions) considerably alter the glycan receptor binding affinity. In fact, introducing these additional amino acid changes (CkPA04-TLS and CkPA04-RTLS mutants) leads to a 10-fold increase in the human receptor binding affinity compared to that of the CkPA04-LS mutant and makes the affinity in the range of that observed for the pandemic H2N2 HA (Alb58). Therefore, monitoring the mutations in these additional positions in the RBS.
is important for understanding changes in glycan receptor binding affinity of the H2 HAs. Moreover, these additional positions are also a part of antigenic loops and hence are likely to undergo constant substitutions as a result of antigenic drift in the H2 viruses to escape antibody neutralization. Monitoring these mutations also have important implications in vaccine development should a scenario arise wherein recent avian or swine H2 viruses are able to gain a foothold in the human population.

Figure 4. Homology-based structural model of HA-glycan receptor complexes. A, stereo view of the RBS (shown as cartoon in cyan) of CkPA04 HA – avian receptor structural complex constructed using co-crystal structure of A/Chicken/NY/91-avian receptor (PRB ID: 2WR2) as a template. The resolved coordinates of the avian receptor (Neu5Ac$_2$R$_3$Gal$_b$1R$_3$GlcNAc) are shown using a stick representation (in green). B, stereo view of RBS (shown as cartoon in gray) of Alb58 HA – human receptor complex constructed using co-crystal structure of A/Singapore/1/57– human receptor (PDB ID: 2WR7) as the template. The resolved coordinates of the human receptor (Neu5Ac$_2$R$_6$Gal$_b$1R$_4$GlcNAc$_b$1R$_3$Gal) are shown using a stick representation (in orange). The side chains of the key residues involved in interaction with glycan receptor are shown and labeled. The residues in the RBS that differ between CkPA04 and Alb58 HA are labeled in red.

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Table 1. Comparison of key amino acids in the RBS of CkPA04 and Alb58 Has.

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The apparent binding constant $K_d'$ calculated in our study is used primarily to compare the relative binding affinities of different recombinant HAs by taking into account a defined spatial arrangement of HA (that is fixed for all the HAs) relative to the glycans. Among the various factors that influence the efficient viral transmissibility in humans we have shown in both the 1918 pandemic H1N1 and the recently declared 2009 pandemic H1N1 that the binding affinity to the human receptors (quantified using $K_d'$) correlates with the transmissibility of the virus via respiratory droplets in ferrets [19,21]. The human receptor binding affinity of $\text{Alb58 HA}$

**Figure 5. Glycan receptor-binding specificity of mutant forms of CkPA04 HA.** Shown in the figure is the dose-dependent glycan receptor binding (A, C, E) and human tissue binding (B, D, F) of CkPA04-LS, CkPA04-TLS and CkPA04-RTLS mutants respectively. All the mutants show substantial improvement in the human receptor binding and reduction in avian receptor binding in comparison to the WT CkPA04 HA as observed in both the glycan array tissue-binding experiments.

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being in the same range as that of the SC18 HA taken together with the efficient respiratory droplet transmission of the Alb58 virus [30] extends this correlation to the H2N2 viruses. Furthermore, given that Alb58 virus transmits efficiently via respiratory droplets in ferrets, our results underscore the fact that a complete switch from avian to human receptor binding is not the critical determinant for human adaptation of influenza A virus HAs. Both the quantitative glycan array binding and human tissue binding results of Alb58 HA show substantial avian receptor binding. Instead, it appears that the high affinity binding to human receptors is a common factor shared by H2 HA with that of other human-adapted virus subtypes (H1 and H3) [10,21] and therefore this property appears to be a necessary determinant for efficient human adaptation and transmission. In summary our studies offer important strategies to monitor the evolution of human-adaptive mutations in the HA of currently circulating avian H2 influenza A viruses.

Materials and Methods

Homology based modeling of CkPA04 HA- and Alb58 HA-glycan structural complexes
The co-crystal structures of A/Singapore/1/57 H2N2 HA – human receptor (PDB ID: 2WR7) and A/ck/NewYork/91- avian receptor (PDB ID: 2WR2) were used as templates to model the structural complexes of Alb58 – human receptor and CkPA04- avian receptor respectively. Homology modeling was performed using the SWISS-MODEL web-based program (URL: http://swissmodel.expasy.org/SWISS-MODEL.html).

Cloning, mutagenesis and expression of HA
The Alb58 and CkPA04 plasmids were gifts from Dr. Terrence Tumpey and Dr. Adolfo Garcia-Sastre respectively. The human and avian WT H2N2 HA genes were subcloned into a pAcGp67A vector to generate pAcGp67-Alb58-HA and pAcGp67-CkPA04-HA respectively for baculovirus expression in insect cells. Using pAcGp67-CkPA04-HA as a template the gene was mutated to yield pAcGp67-LS-HA [Gln137Arg, Ala193Thr, Gln226Leu, Gly228Ser], pAcGp67-TLS-HA [Gln137Arg, Ala193Thr, Gln226Leu, Gly228Ser] and pAcGp67-RTLS-HA [Gln137Arg, Ala193Thr, Gln226Leu, Gly228Ser]. The primers for mutagenesis were designed using PrimerX (http://bioinformatics.org/primers/) and synthesized by IDT DNA technologies (Coralville, IA). The mutagenesis reaction was carried out using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, CA) Alb58, CkPA04, CkPA04-LS, CkPA04-TLS and CkPA04-RTLS baculoviruses were created from their respective plasmids, using Baculogold system (BD Biosciences, CA) as per the manufacturer’s instructions. The baculoviruses were used to infect 300 ml suspension cultures of S9 cells (Invitrogen, Carlsbad, CA) cultured in SF-900 II SFM medium (Invitrogen, Carlsbad, CA). The infected cultures were monitored and harvested 4–5 days post-infection. The soluble trimeric form of HA was purified from the supernatant of infected cells using modification of the protocol described previously [31]. In brief, the supernatant was concentrated using Centricron Plus-70 centrifugal filters (Millipore, Billerica, MA) and the trimeric HA was recovered from the concentrated cell supernatant using affinity chromatography with columns packed with Ni-NTA beads (Qiagen, Valencia, CA). The fractions containing HA were pooled together and subjected to ultrafiltration using Amicon Ultra 100 K NMWL membrane filters (Millipore, Billerica, MA). The protein was reconstituted in PBS and concentrated. The purified protein concentration was determined using Bio-Rad’s protein assay (Bio-Rad, CA).

Dose dependent direct glycan array-binding assay
To investigate the multivalent HA-glycan interactions a streptavidin plate array comprising representative biotinylated α2→3 and α2→6 sialylated glycans as described previously [21]. The glycans 3’SLN, 3’SLN-LN, 3’SLN-LN-LN are representative avian receptors. 6’SLN and 6’SLN-LN are representative human receptors. LN corresponds to lactosamine (Galβ1-4GlcNAc) and 3’S LN and 6’SLN respectively correspond to Neu5Acα2→3 and Neu5Acα2→6 linked to LN. The biotinylated glycans were obtained from the Consortium of Functional Glycomics through their resource request program. Streptavidin-coated High Binding Capacity 384-well plates (Pierce) were loaded to the full capacity of each well by incubating the well with 50 µl of 2.4 µM of

Figure 6. Glycan receptor-binding affinities of the mutant forms of CkPA04 HA. A, shows the theoretical binding curves (with the apparent binding constant $K_{d}'$ observed) of WT and mutant H2N2 HAs to the avian receptor (3’S LN-LN). B, shows the theoretical binding curves (with the apparent binding constant $K_{d}'$ observed) of WT and mutant H2N2 HAs to the representative human receptor (6’S LN-LN). The range of $K_{d}'$ values (3–8 pM) is shown for CkPA04-TLS, Alb58 and CkPA04-RTLS that is contrasted with the $K_{d}'$ value of CkPA04-LS. The binding curves were generated by fitting to the Hill equation (see Methods) and plotting the theoretically calculated fractional saturation (y-axis) against HA concentration (x-axis). The n value for all the binding events is around 1.3.

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biotinylated glycans overnight at 4°C. Excess glycans were removed through extensive washing with PBS.

The trimeric HA unit comprises of three HA monomers (and hence three RBS, one for each monomer). The spatial arrangement of the biotinylated glycans in the wells of the streptavidin planar array favors binding to only one of the three HA monomers in the trimeric HA unit. Therefore in order to specifically enhance the multivalency in the HA-glycan interactions, the recombinant HA proteins were pre-complexed with the primary and secondary antibodies in the ratio of 4:2:1 (HA-primary;secondary). The identical arrangement of 4 trimeric HA units in the precomplex for all the HAs permits comparison between their glycan binding affinities.

A stock solution containing appropriate amounts of Histidine tagged HA protein, primary antibody (Mouse anti 6X His tag IgG) and secondary antibody (HRP conjugated goat anti Mous IgG (Santacruz Biotechnology) in the ratio 4:2:1 and incubated on ice for 20 min. Appropriate amounts of precomplexed stock HA were diluted to 250 µl with 1% BSA in PBS. 50 µl of this precomplexed HA was added to each of the glycan-coated wells and incubated at room temperature for 2 hrs followed by the above wash steps. The binding signal was determined based on HRP activity using Amplex Red Peroxidase Assay (Invitrogen, CA) according to the manufacturer’s instructions. The experiments were done in triplicate. Minimal binding signals were observed in the negative controls including binding of precomplexed unit to wells without glycans and binding of the antibodies alone to the wells with glycans. The binding parameters, cooperativity (n) and apparent binding constant (Kd’), for H2 HA-glycan binding were calculated by fitting the average signal value (from the triplicate analysis) and the HA concentration to the linearized form of the Hill equation: log(y/(1-y)) = n * log([HA]) - log(Kd’), where y is the fractional saturation (average binding signal/maximum observed binding signal). The theoretical y values calculated using the Hill equation y = [HA]^n / [HA]^n + Kd’ for the set of n and Kd’ parameters were plotted against the varying concentration of HA.

References