Fitness costs limit influenza A virus hemagglutinin glycosylation as an immune evasion strategy

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Here, we address the question of why the influenza A virus hemagglutinin (HA) does not escape immunity by hyperglycosylation. Uniquely among dozens of monoclonal antibodies specific for A/Puerto Rico/8/34, escape from H28-A2 neutralization requires substitutions introducing N-linked glycosylation at residue 131 or 144 in the globular domain. This escape decreases viral binding to cellular receptors, which must be compensated for by additional substitutions in HA or neuraminidase that enable viral replication. Sequence analysis of circulating H1 influenza viruses confirms the in vivo relevance of our findings: natural occurrence of glycosylation at residue 131 is always accompanied by a compensatory mutation known to increase HA receptor avidity. In vaccinated mice challenged with WT vs. H28-A2 escape mutants, the selective advantage conferred by glycan-mediated global reduction in antigenicity is trumped by the costs of diminished receptor avidity. These findings show that, although N-linked glycosylation can broadly diminish HA antigenicity, fitness costs restrict its deployment in immune evasion.

antigenic drift | viral evolution

Influenza A virus (IAV) remains an important human pathogen, largely because of its ability to evade the humoral immune response. Immune evasion is primarily based on the protean capacity of the HA glycoprotein to accommodate amino acid substitutions that modify antigenicity and modulate receptor avidity. HA is a homotrimer that attaches virus to terminal sialic acid residues on target cells to initiate the infectious cycle (1). Antibodies (Abs) that interact with the globular HA domain sterically block attachment and neutralize viral infectivity (2).

Sequencing RNA of escape mutants resistant to neutralization by individual monoclonal Abs (mAbs) revealed that mutants typically possess a single nucleotide change generating an amino acid substitution in the globular domain (3). Localization of the amino acid alterations in the crystal structure of the HA of A/Puerto Rico/8/34 (PR8) clearly shows the presence of five distinct antigenic sites: Sa and Sb are located at the tip of the globular domain, and Ca1, Ca2, and Cb are located more toward the stem of H1 HA (3). This method of epitope mapping, although indirect, is much more rapid and robust than other methods, and it is still capable of providing a reasonable physical definition of the relevant epitope (4).

The frequency of mutants that escape individual neutralizing anti-HA mAbs is typically in the range of 10^{-4}–10^{-6} (5). Variants capable of escaping selection mAbs specific for nonoverlapping epitopes occur at a frequency of $\sim 10^{-10}$, consistent with two independent point mutations (6). The low frequency of mutants with multiple amino acid substitutions raises the question of how IAVs evolve antigenically in man. Do individuals make biased antibody responses enabling selection of single point mutants, or do rare, multiply substituted mutants arise based on the very large aggregate virus populations among infected humans.?

Acquisition of N-linked glycosylation sites near antigenic sites represents another potential mechanism for IAV to escape antibody neutralization, because the large size of oligosaccharides can sterically prevent Ab access to its epitope. HIV gp160 provides a clear example of hyperglycosylation as an effective immune escape mechanism (7). Interestingly, although the H3 HA has gradually gained glycosylation sites in the globular region, H1 HA circulating for a similar period in a similarly large population has acquired far fewer sites (8). Moreover, the H2 HA during its 10 y of evolution in humans maintained a lone glycosylation site in the globular domain.

The limited addition of glycosylation sites suggests a high selection cost. Here, we provide compelling evidence for this conclusion by studying the ability of PR8 to escape neutralization of the H28-A2 mAb [designated Cx8 in the work by Gerhard et al. (9)]. This IgM mAb, generated from a BALB/c mouse immunized with infectious influenza, shows a number of unique properties, including the ability to select viruses that only escape neutralization through acquisition of an N-linked glycosylation site in the globular domain.

Results

H28-A2: A Unique mAb. H28-A2, one of hundreds of PR8 HA-specific mAbs generated by the Gerhard laboratory, has a number of unique properties. First, unlike dozens of other anti-PR8 mAbs tested, the H28-A2 mAb selects escape mutants at a frequency expected (5) for a double simultaneous mutation ($<10^{-9.12}$) (Table 1). Second, consistent with this property, H28-A2 alone among hundreds of neutralizing mAbs shows little change in binding affinity to a panel of more than 40 escape mutants with amino acid substitutions distributed among the five antigenic sites (3). Third, when one of the H28-A2 (termed Ab O in the Gerhard laboratory's escape variant nomenclature, where mutants were named after their selecting Ab) escape mutants selected in the allantois on shell system (OV1) was tested for binding to a 220-member panel of HA-specific mAbs, it paradoxically showed reduced binding to a remarkably large fraction of mAbs (71%), including many mAbs that map to each of the four antigenic sites in the globular domain (Table 2).

The unique properties of H28-A2 are not related to unusual gene use (10) or unusually high affinity. Affinity measurement by ELISA performed with intact virus revealed a typical affinity observed for HA-specific mAbs, with a K_A of 1.1×10^9 (Table 3).

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Table 1. Frequencies of H28-A2 escape mutants

Virus + antibody	Variant frequency	Mutations in HA
WT + 1 mAb	10^{-4}–10^{-6}	One substitution
WT + mix of 2 mAb	10^{-9}–10^{-10}	Two substitutions
WT + H28-A2 (egg)	$10^{-9.12}$	K144N + D225G (OV1) K144N N193K (OV2)
WT + H28-A2 (MDCK)	$10^{-10.66}$	K144N + P186S (OV3)
D225G + H28-A2 (MDCK)	$10^{-5.56}$	K144N or N133T
N193K + H28-A2 (MDCK)	$\sim10^{-7.2}$	K144N
P186S + H28-A2 (MDCK)	$\sim10^{-4.21}$	K144N

The frequency of escape mutants was determined as described (5).

Escape mutants showed a decrease of affinity of >10,000-fold to H28-A2 (Table 3).

H28-A2–Resistant Mutants Exhibit a Unique Escape Strategy. RNA sequencing of two H28-A2 escape mutants (OV1 and OV2) immediately explained their low frequency and high degree of escape. Each mutant possessed a common nucleotide mutation encoding a predicted substitution of K to N at position 144 (H3 numbering system based on PR8 HA crystal structure 1RVX) and generating a potential N-linked glycosylation site at position 144, because position 146 encodes S. Importantly, each variant had an additional mutation encoding an amino acid substitution: D225G for OV1 and N193K for OV2 (Fig. 1).

We extended these findings to the Madin Darby canine kidney (MDCK) system of escape mutant selection. Selection with H28-A2 generated an escape mutant (OV3) at an extremely low frequency ($10^{-10.66}$). RNA sequencing once again revealed two nonsynonymous mutations encoding the common K144N substitution accompanied by a P186S substitution.

Like virtually all HAs, the PR8 HA possesses glycosylation sites at or near stem residues 15, 26, 289, 483, and 542 (8). Although most contemporary H1 HAs possess glycosylation sites at 91, 129, or 162, PR8 lacks glycosylation sites in the globular domain. Locating residue 144 in the H1 HA structure reveals that it is present on the solvent-exposed surface, where it could easily accommodate the addition of an oligosaccharide (Fig. 1).

To validate glycosylation at the introduced site, we purified WT PR8, OV1, OV2, and OV3 and single escape mutants with identical alterations to the second site mutations observed in the OV variants (D225G, N193K, and P186S) (Fig. 2*A*). A mobility shift in the HA1 chain consistent with additional glycosylation at N144 was observed in SYPRO Ruby-stained reducing gels and through immunoblotting with the anti-HA1 mAb CM-1.

After removal of oligosaccharides with PNGase F, all HA1 chains migrated with the same mobility, clearly showing that the oligosaccharide attachment site generated by the K144N substitution is used (Fig. 2*B*).

Table 2. Antigenic mapping of OV1

	OV1 (%)
Sa (23 mAbs)	56.4 (13)
Sb (90 mAbs)	55.5 (34.4)
Ca (1 + 2; 43 mAbs)	93 (83.7)
Cb (64 mAbs)	63.4 (30.1)
Total (220 mAbs)	71.5 (44.5)

A large panel (220 mAbs) of mAbs with defined epitopes as indicated was used to determine the antigenicity of OV1 through radioimmunoassay as described (9). The percent of mAbs specific for each site showing major (more than fourfold) or moderate (two- to fourfold) decreases in avidity is given by top and bottom (in parentheses) values, respectively.

Glycosylation at Position 144 Requires Compensatory Substitutions. The bulk of an N-linked oligosaccharide, in conjunction with the central location of the 144 residue amid the antigenic sites in the globular domain (Fig. 1), provides an obvious steric explanation for the dramatically diminished antigenicity of H28-A2 escape mutants. It seemed likely, therefore, that the second mutations present in the escape mutants are necessary to compensate for functional deficits caused by glycosylation at position 144.

We confirmed this hypothesis by generating K144N recombinant virus by the eight-plasmid reverse genetic system. Immediate sequencing of virus from 293T culture supernatants post-transfection showed only the intended K144N substitution. However, as soon as we amplified this virus in eggs or MDCK cells, compensatory mutations appeared. Sequencing cloned egg virus revealed D225G or I194L substitutions. MDCK-propagated viruses revealed three types of substitutions: (*i*) alterations of N144 to Y or S, removing the glycosylation site, (*ii*) compensatory changes in HA, P186S, or G156E, and most remarkably, (*iii*) single or double substitutions in NA (L22P, G27R, P139S, P231L, and V273M with or without N434T; PR8 NA numbering). The latter result confirmed our recent finding that antigenic escape mutants in HA that limit fitness can be rescued by NA amino acid substitutions (11).

We confirmed the compensatory nature of second site substitutions (D225G, N193K, and P186S) by using H28-A2 in the MDCK cell system to select escape mutants from PR8 mutants with substitutions in the defined compensatory sites. Now, variant frequencies occurred within the range typical of single point mutants: $10^{-5.56}$ for D225G and $10^{-4.21}$ for P186S. H28-A2 escape mutants from N193K mutants occurred at a lower fre-

Table 3. Interaction of mAbs with mutants

	H28-A2		
	K_A (cell staining)	K_A (ELISA)	HI (Turkey RBCs)
WT	1.9×10^9	1.1×10^9	25,600
K144N, D225G	$<8.3 \times 10^6$	$<8.3 \times 10^6$	<20
K144N, N193K	$<8.3 \times 10^6$	$<8.3 \times 10^6$	<20
K144N, P186S	$<8.3 \times 10^6$	$<8.3 \times 10^6$	<20
P186S	1.4×10^9	1×10^9	25,600
N193K	4.3×10^8	2.7×10^8	12,800
D225G	2.4×10^8	2.3×10^8	6,400
K144N	$<8.3 \times 10^6$	$<8.3 \times 10^6$	<20
K144N, E156G	$<8.3 \times 10^6$	$<8.3 \times 10^6$	<20
K144Y	6.3×10^8	5.5×10^8	6,400
K144S	5.1×10^7	1.9×10^8	6,400
K144E, D225G	$<8.3 \times 10^6$	$<8.3 \times 10^6$	<20
N133T, D225G	$<8.3 \times 10^6$	$<8.3 \times 10^6$	<20
K144N, I194L	$<8.3 \times 10^6$	$<8.3 \times 10^6$	<20
N133S	$<8.3 \times 10^6$	$<8.3 \times 10^6$	1,600

Avidity values were determined either by staining 5 h postinfected cells or ELISA using intact virus coated on plates.

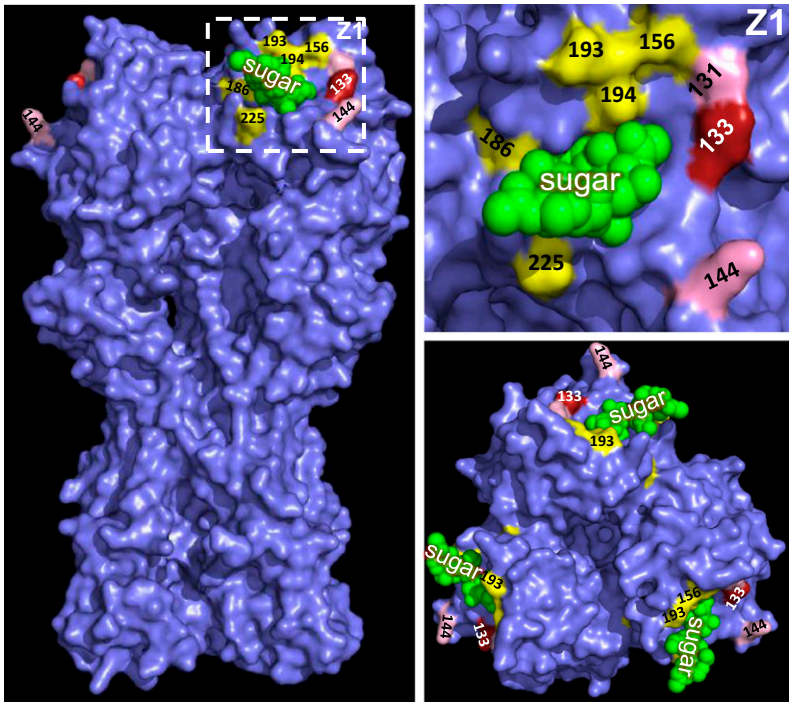

Fig. 1. Locating amino acid substitutions in H28-A2 escape mutants. 3D model of HA rendered by PyMol software as a solid surface looking at the top and side of the trimeric molecule (using PR8 HA crystal structure 1RVX). Amino acid substitutions in escape mutants are indicated by color and label. In Z1, which zooms in on the receptor binding site (RBS), the two glycosylation sites created by mutations, located at residues 144 (direct introduction of N) and 131 (mutation at residue 133 (red) creates a site for existing N) are shown in pink. Glycosylation at these residues causes global changes in antigenicity and also incurs fitness costs that must be compensated for by epistatic substitutions (at the yellow residues) for virus survival.

quency ($10^{-7.2}$), likely attributable to poor adaptation of these double mutants to MDCK cells because of fitness differences between the various compensatory substitutions.

Sequencing the sequentially selected viruses revealed the expected but also a surprise. Although all of the mutants obtained from N193K or P186S stocks possessed the expected K144N substitution, only 50% of D225G mutants possessed this substitution. Remarkably, the other 50% possessed an N133T substitution, now creating a glycosylation site at N131, located just north of position 144 and very close to the receptor binding site on the HA solvent-accessible surface (Fig. 1). Biochemical analysis of a selected D225G N133T double mutant confirmed glycosylation at position 131 (Fig. 2).

Together, these findings clearly show that escape from H28-A2 neutralization requires two events: introduction of a glycosylation site at residue 144 or 131 and substitution at residue 186, 193, or 225 (or various NA residues) to compensate for negative effects of glycosylation on viral fitness.

Effect of Escape Mutations on H28-A2 Affinity. All escape mutants with introduced glycans at HA residue 144 or 131 exhibit at least 1,000-fold decreased avidity for binding to H28-A2 determined either by ELISA with virus as immunoabsorbent or flow cytometry of infected cells (Table 3), showing that glycosylation alone in this region enables immune escape. Either N193K or D225G alone diminishes H28-A2 avidity, and the combination of K144E and D225G decreases affinity more, supporting the contribution of both of these residues to binding. The importance of residue 144 is also shown by the moderate effect of K144Y and K144S substitutions on H28-A2 avidity. HI titers paralleled the ELISA data, showing the functional consequences of diminished binding (Table 3).

Together, these findings show that H28-A2 binding is only completely blocked by glycosylation in the region near the receptor binding site (RBS) and that substitutions in residues surrounding the RBS have modest but clear effects on binding, and they are likely to define the epitope.

HA Glycosylation Decreases Receptor Binding. Residues 144 and 131 are in relatively close proximity to the RBS, and it is expected based on numerous studies that glycosylation at these residues will reduce HA receptor avidity (12–14). Indeed, we reported that the compensatory amino acids observed in the OV variants increase HA receptor avidity (15, 16).

To measure HA receptor avidity, we determined the capacity of viruses to agglutinate human or turkey erythrocytes treated with increasing amounts of soluble bacterial neuraminidase [receptor-destroying enzyme (RDE)] (Fig. 3). This finding confirmed the ability of D225G, N193K, and P186S, all located in close proximity to the RBS (Fig. 1), to increase viral receptor avidity. Importantly, for each of these substitutions, glycosylation at residue 144 or 131 reduced the avidity to lower than WT levels. Also, glycosylation alone at K144N (with compensatory mutations in NA that do not affect the RDE assay) or N133S (which also creates a predicted glycosylation site at residue 131) reduces HA receptor avidity.

From these findings, we infer that glycosylation near the RBS interferes with receptor binding to the extent that compensatory mutations that increase avidity or modulate NA function are required to restore viral fitness.

Evidence of HA Compensatory Mutations with Additional Glycosylation in Circulating Viruses. To relate these findings to natural IAV evolution in humans, we analyzed 1,640 full-length H1 HA sequences from human viruses downloaded from the National Center for Biotechnology Information (NCBI) influenza virus resource. The NetNGlyc prediction of glycosylation sites (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro) in the

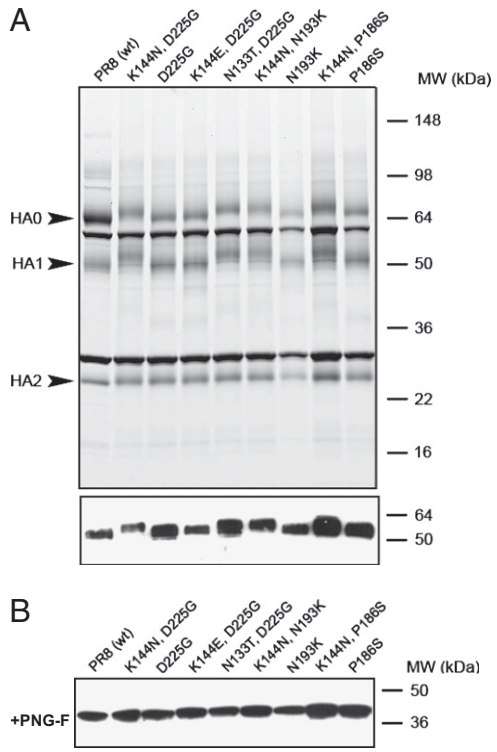

Fig. 2. Biochemical confirmation of N-linked glycosylation at introduced sites. Egg-grown viruses were purified and analyzed by SDS/PAGE. (*A*) SYPRO Ruby staining or immunoblotting by anti-HA antibody (CM1) shows a shift in HA mobility because of additional glycosylation, which was confirmed by near identical mobilities following PNGase F deglycosylation (*B*).

globular domain reveals the nonrandom distribution of probable glycosylation sites at nine locations. None of the isolates possess a glycosylation site at position 144, confirming the selection costs of glycosylation at this position in natural H1 evolution in humans.

Ninety-eight isolates, however, are predicted to be glycosylated at position 131 (Table S1). Yearwise distribution of glycosylation at position 131 showed a nonrandom distribution, showing that it was not immediately supplanted by other strains (Fig. S1). All of the isolates with glycosylation sites at position 131 possess the P186S substitution (Table S1), which increases receptor avidity and has minimal effects on HA antigenicity (15).

Taken together, these findings support the conclusion that glycosylation at position 144 incurs enormous fitness costs that have yet to be surmounted in H1 evolution, whereas glycosylation at position 131 is disfavored but can exist in circulating strains with compensatory mutations that restore HA receptor avidity.

HA Avidity Trumps Antigenicity in Escape from Polyclonal Abs. Given the dramatic effect of position 144 glycosylation on HA antigenicity (Table 1), it would be expected that the H28-A2 escape mutants would show robust escape from a polyclonal Ab. In fact, when tested in hemagglutination inhibition (HI) assays using pooled sera from outbred Swiss mice immunized with PR8, glycosylation had either a minor positive effect on escape (D225G vs. K144N, D225G or N133T, D225G) or remarkably, the opposite effect on escape (N193K vs. K144N, N193K; P186S vs. K144N, P186S) (Fig. 4*A*).

We extended these findings to an in vivo infection model by immunizing mice with PR8, challenging them with WT, P186S, or K144N, P186S viruses, and measuring viral lung titers 2 d postinfection. In naïve mice, all three viruses replicated to similar titers (Fig. 4*B*). Vaccination completely prevented replication of WT virus but only reduced replication of P186S by 10-fold, similar to what we previously reported (16). By contrast, mice were completely protected against K144N, P186S infection, despite the enormous antigenic escape associated with glycosylation at position 144.

These findings point to the important conclusion that receptor avidity can be a more important factor than antigenicity in escaping from neutralizing antibodies, which also limits the use of glycosylation as a means of HA immune escape because of its attendant reduction in avidity.

Discussion

The resistance of H28-A2 to immune escape by single amino acid substitutions in a highly variable HA domain capable of accepting numerous different substitutions is, to our knowledge, unprecedented among anti-HA mAbs. H28-A2 derives from a BALB/c mouse immunized i.p. with infectious PR8 and boosted i.v. 1.5 y later with PR8 3 d before fusion. Three other mAbs characterized in detail from the same fusion are specific for the Sb site, which is likely immunodominant in mice (16, 17). DNA sequencing of H28-A2 heavy and light chains revealed that it is closely related to six other mAbs derived from four individual BALB/c mice, all of which share VH 7183 and Vk 24 family genes (10). H28-A2 and three of six mAbs share the unusual property of binding with high affinity to the Denver 1957 H1 isolate (18). Examination of the reactivity patterns of the

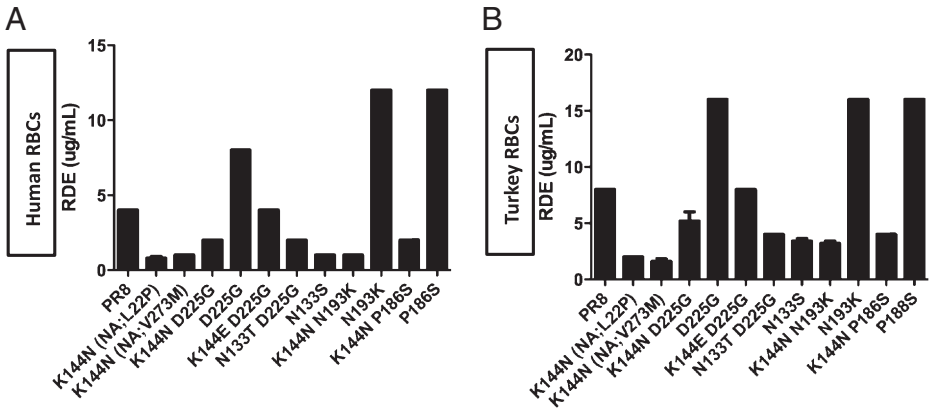

Fig. 3. Glycosylation diminishes receptor binding avidity. (*A*) Human or (*B*) turkey RBCs were treated with graded concentrations of receptor-destroying enzymes (RDEs) before the addition of viruses indicated to determine the relative amounts of terminal sialic acid required for agglutination.

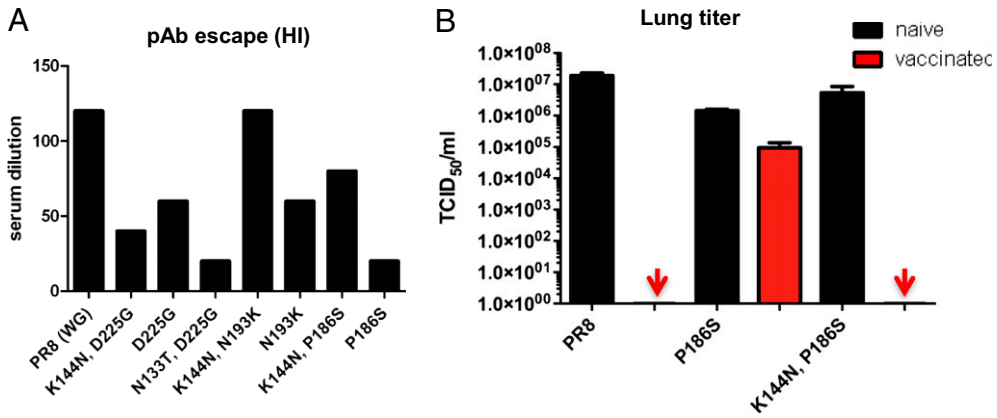

Fig. 4. Receptor binding modulates immune escape in vitro and in vivo. HI assays (*A*) using pooled mouse primary anti-PR8 antisera show that introduction of a glycosylation site at residue 144 diminishes HI titer. Each experiment was repeated three times with quadruple samples, and one representative experiment is presented here. Similarly, in mouse infections (*B*), addition of a glycosylation site at residue 144 to the adsorptive mutant S186P abrogates escape from anti-HA Abs induced by vaccination as shown by recovery of infectious virus from lungs. In each group, five mice were used per experiment, and representative data are presented of three separate experiments.

Denver-reactive VH7183/Vk24+ mAbs reveals large affinity losses to OV1 and mutants with amino acid substitutions at 193 and 145. These residues along with 225, where a D to G substitution reduces H28-A2 avidity by fivefold (Table 3), define a binding footprint consistent with the known interaction surface of antibodies with HA (Fig. 1). This proposed location of the H28-A2 epitope would explain the predicted steric effects of glycosylation at position 144 or 131 on H28-A2 binding. Interestingly, H28-A2 sits squarely atop the sialic acid binding site, which could be the key to its resistance to single amino acid escape.

The central location of residue 144 on the HA also provides a ready explanation for how glycosylation of this residue can reduce the binding of mAbs specific for each of the antigenic sites. This finding extends previous findings that glycosylation modulates HA antigenicity (12, 13, 19) and immunogenicity (20).

The low frequency of H28-A2 escape mutants, in conjunction with their special nature in generating a glycosylation site, conclusively indicates that HA is incapable of generating viable mutants with single amino acid substitutions that enable escape from H28-A2. Explanation for this remarkable property will likely require crystallographic analysis of H28-A2 complexed with HA, perhaps compared with the Denver-reactive VH7183/Vk24+ Abs, which likely define a highly similar epitope but are sensitive to individual side chain substitutions. Until then, we can speculate that structural limitations in the epitope region combine with unusual features in H28-A2 to limit escape. Understanding the features of antibodies that resist antigenic drift is of obvious practical importance in devising vaccines for viruses like IAV and HIV, where antigenic variation greatly impacts vaccine effectiveness. It is important to note that there might be significant differences between mouse and human anti-HA Ab repertoires because of differences in germ-line Ab genes, somatic mutation tendencies, or frequent reexposure of humans to evolving IAV strains.

Glycosylation at residue 144 is not observed among H1 isolates, confirming its fitness costs in the real world evolution of IAV in humans. By contrast, residue 131 is likely glycosylated in a number of strains, including A/Melbourne/35, which shows greatly reduced binding of H28-A2 (9). The glycosylation site in Mel is created by insertion of a triplet between residues 132 and 133 in PR8, and it is accompanied by 15 other amino acid substitutions in the globular domain and 18 other HA substitutions. This finding shows that, for some glycosylation sites that protect antigenic regions, the H1 HA is fully capable of acquiring compensatory mutations that mitigate the functional consequences of glycosylation. However, in over 60 y of circulation in human populations, the H1 HA maximally encodes only three potential glycosylation sites in the globular domain (8). Over a similar time frame, the H3 HA maximally encodes six sites, indicative of the difficulty of the H1 HA to maintain function with increasing glycosylation. It will be of interest to observe the evolution in man of the swine origin IAV, which possesses but a single glycosylation site in the globular domain. Notably, H2 viruses maintained a single glycosylation site in their 11-y circulation from 1957 to 1968, pointing to a high cost of glycosylation, which was shown in the work by Tsuchiya et al. (13).

Glycosylation at position 144 reduced HA receptor affinity, consistent with prior studies documenting a deleterious effect of glycosylation on HA binding to sialic acid receptors (12–14). The effect of position 144 glycosylation on receptor avidity allowed us to test our recent findings regarding the importance of HA receptor avidity in antigenic drift (16). Despite the enormous effect of glycosylation on overall antigenicity as measured by a significant decrease in binding affinity of 71% of a large and diverse mAb panel, the decrease in receptor affinity incurred by glycosylation at position 144 offset the evolutionary advantage conferred by antigenic escape.

This finding hammers home the point that receptor affinity can play a dominant role in escape from neutralizing antibodies. HA glycosylation is highly likely to interfere with receptor binding in a manner that must be compensated for by additional mutations, creating a fitness barrier to accumulating glycosylation sites and providing a ready explanation for the paucity of oligosaccharides on HA compared with other viral receptor proteins (e.g., HIV gp160).

This discussion is relevant to the recent description of mAbs that bind highly conserved epitopes in the stem region of HA, which allows them to neutralize nearly all isolates within the H1 subtype and even between H1, H2, and H5 subtypes (21). Binding to H3 HA, however, is likely blocked by the presence of an N-linked glycan absent on H1/H2/H5 HAs (22, 23), raising the possibility of glycan-based escape from immunization strategies aimed at inducing stem-reactive mAbs. Our findings suggest that the costs of HA glycosylation can be sufficient to delay, if not preclude, its deployment as an immune escape strategy.

Experimental Procedures

Virus, Antibodies, and Mice. WT and escape mutants of PR8 (H1N1; Mt. Sinai strain) were propagated in 10-d embryonated chicken eggs (CBT Farms).

Hybridoma anti-HA antibodies were produced and characterized as previously described (9).

Swiss mice were purchased from Charles River, and C57BL/6 and BALB/c mice were purchased from Taconic. Mice were maintained under specific pathogen-free conditions, and all procedures involving mice were approved by the National Institute of Allergy and Infectious Disease animal care and use committee.

Avidity Measurements. ***Virus ELISA.*** Ninety-six–well plates (4HBX; Immunlon) were coated overnight at 4 °C with saturating amounts of virus in allantoic fluid, washed three times with PBS containing 0.05% vol/vol Tween-20, and blocked with PBS with 7.5% FBS for 1 h. Antibodies present in culture fluid or ascites fluids over a complete range of dilutions from nondetectable to saturating binding were added for 2 h at room temperature. After washing, 100 μL TMB substrate (KPL) were added. The reaction was stopped by the addition of 50 μL 0.1 N HCl, and the amount of product was determined by an ELISA plate reader. Antibody concentrations were determined by competition ELISA using purified mAbs as standards. Antibody avidities were determined using Prism software, and all avidities reported showed excellent fit for one-site binding with Hill slope curve fitting (R^2 values > 0.98).
Cell staining. MDCK cells were infected at a multiplicity of infection of five with either WT or mutant viruses for 5 h in suspension. Cells were washed with BSS-PBS supplemented with bovine serum albumin and incubated at 4 °C with mAbs over a range of dilutions from background to saturating binding for 1 h, which was followed by FITC-labeled secondary Abs. Cells were analyzed live at 4 °C by flow cytometry, and mean fluorescent intensities for each concentration of mAbs were used to calculate avidities using Prism software.

HA, HI, and Virus Neutralization Assays. HA, HI, and virus neutralization assays were performed as described (16). Each assay was repeated at least three times with three to five replicates per each assay.
Variant selection. Variants frequencies were determined either as described (5) or using a modified protocol with MDCK cells. MDCK cells were seeded in 96-well plates (50,000 cells/well) and cultured for 16–24 h. Virus was serially diluted 10-fold (10^{-1}–10^{-8}) at 50 μL/well in a parallel 96-well plate. An equal volume of an overneutralizing concentration of mAb was added to each well, and the mixture was incubated at room temperature for 1 h before adding to MDCK cells. After 2 h at 37 °C, cells were washed with PBS and replenished with 200 μL media supplemented with 1 μg/mL TPCK trypsin and the selecting mAb. Cytopathic effect (CPE) was monitored 72–96 h post-infection. Supernatants from CPE-positive wells (endpoint titer) were isolated for additional amplification and characterization.
RNA sequencing. Viral RNA was isolated from MDCK supernatant (endpoint titrated viruses and stocks) or allantoic fluid using QiAmp Viral RNA Mini Kit (Qiagen) using the manufacturer's protocol. cDNA was synthesized using a one-step reverse transcriptase kit (Origene Technologies). Gene-specific primers (PR8 HA-F, 5′-ATGAAGGCAAACCTACTGGTCCTG-3′; PR8 HA1-R, 5′-CTGCATAGCCTGATCCCTGTT-3′) amplified HA1, and the PCR products were purified with a QIAQuick PCR purification kit (Qiagen); sequencing was performed through a third-party sequencing service (MacrogenUSA) using dye terminator cycle sequencing system with an ABI sequencer (Perkin-Elmer).
In vivo challenge experiment. For vaccine stocks, allantoic fluid was treated with paraformaldehyde (1:864 dilution) for 2 d at 4 °C. Mice were injected intraperitoneally with ∼100–500 hemagglutination units inactivated IVA; 10 d after vaccination, mice were retroorbitally bled, and the erythrocytes were removed using serum gel tubes (Sarstedt). Serum was inactivated by incubating at 56 °C for 30 min and tested for anti-IAV antibodies by HI. Naïve and vaccinated mice (five animals per group repeated three times) were anesthetized with isoflurane and infected intranasally with 10^5 tissue culture 50% infectious dose ($TCID_{50}$) units of WT and mutant viruses diluted in 50 μL PBS. Lungs isolated 2 d after infection were homogenized, and viral titers were determined by endpoint dilution in MDCK cells.

Source of Sequences, Prediction of N-Glycosylation Sites, and Multiple Sequence Alignment. A set of 1,640 full-length H1N1 HA sequences (human hosts from all geographic origins) were downloaded on June 26, 2009, from the influenza virus resource at NCBI (http://www.ncbi.nlm.nih.gov/genomes/FLU) (8). The NetNGlyc 1.0 web server (http://www.cbs.dtu.dk/services/NetNGlyc) was used to predict N-glycosylation sites (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro) of all HA sequences; a positive was scored when the jury returned a + score. According to NetNGlcy, 76% of positive-scored sequons are modified by N-glycans, with a bias to Thr-containing sequons. All HA sequences of H1N1 were aligned in a single common alignment using the program Muscle with default parameters (8).

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