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The influenza A virus hemagglutinin glycosylation state affects receptor-binding specificity

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ABSTRACT

In this study we evaluated the receptor-binding properties of recombinant soluble hemagglutinin (HA) trimers (subtype H2 and H7) produced in insect S2 cells, human HEK293T or HEK293S GnTI(-) cells, which produce proteins with paucimannose, complex or high-mannose N-linked glycans, respectively. The results show that HA proteins that only differ in their glycosylation status possess different receptor fine specificities. HEK293T cell-produced HA displayed a very narrow receptor specificity. However, when treated with neuraminidase this HA was able to bind more glycans with similar specificity as HEK293S GnTI(-) cell-produced HA. Insect cell-produced HA demonstrated decreased receptor specificity. As a consequence, differences in HA fine receptor specificities could not be observed with the insect cell-, but were readily detected with the HEK293S GnTI(-) cell-produced HAs.

Introduction

Influenza A viruses are enveloped, negative-strand RNA viruses with a segmented genome. They cause acute viral disease that affects a large variety of animal species, including humans, pigs, horses, and birds. Influenza A virus classification is based on the antigenic properties of their two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). All subtypes of HA (H1–H16) have been identified in avian influenza A viruses. Birds are therefore considered to be the reservoir from which all influenza A viruses in other species originate (Webster et al., 1992). The HA protein is the most important determinant of virulence and host switching as it binds to sialic acid-containing cell surface receptors on epithelial cells (de Wit et al., 2009; Shinya et al., 2006; yora-Talavera et al., 2009; Nicholls et al., 2008). While avian viruses preferentially bind to sialic acids attached to the vicinal galactose via a α2-3 linkage (Connor et al., 1994), human viruses prefer the α2–6 linkage (Rogers et al., 1985; Rogers and D'Souza, 1989).

HA is a homotrimeric type I transmembrane protein with an ectodomain composed of a globular head and a stem region (Ha et al., 2001; Wilson et al., 1981). The sialic acid moieties of the viral receptors bind to a shallow depression at the top of the HA protein (Chandrasekaran et al., 2008; Stevens et al., 2004; Wilson et al., 1981). HA receptor-binding affinity was found to be affected by oligosaccharide linkages adjacent to the receptor-binding site (Ohuchi et al., 1997b; Ohuchi et al., 1997a; Gunther et al., 1993). In addition, a recent study demonstrated that enzymatic truncation of the N-glycan structures on HA increased receptor-binding affinity, while decreasing specificity towards disparate sialic acid ligands (Wang et al., 2009).

Cross-species transmission is an important aspect of the epidemiology of influenza A viruses. Although the requirements for and mechanisms of influenza A viruses to cross the species barrier are still poorly understood mutations in HA are known to be critical (Tumpey et al., 2007). At the present time, the specificity of HA–receptor interactions is most conveniently analyzed using glycan array technology (Stevens et al., 2006a). Glycan array analyses can either be performed using intact virus preparations or recombinant soluble HA proteins. Intact virus preparations have obvious biosafety issues when dealing with unknown viruses, while the growth of viruses in cell culture or eggs may result in adaptive mutations in HA. Recombinant soluble proteins therefore provide an attractive alternative (Stevens et al., 2006b). The production of soluble HA proteins, the trimeric state of which is essential for effective binding to sialic acid moieties can be relatively easily achieved once the sequence of the particular viral genome has been determined.

With our research we aim to determine HA–receptor interactions for various HAs, using recombinant soluble HA trimers. These trimers can be produced in different cell types, such as insect cells, by using among others the baculovirus expression system (Chandrasekaran et

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al., 2008), or mammalian cells (Wei et al., 2008). These different cell types produce, however, glycoproteins that carry different oligosaccharide side chains. Although insect cells are able to produce N-linked glycoproteins, the cells fail to elongate the trimmed N-glycan precursors to produce complex terminally galactosidated and/or sialylated N-glycans that are produced in mammalian cells (Kost et al., 2005). Rather, the insect cells produce glycoproteins that carry paucimannose N-glycans, containing three mannoses. As differences in the glycosylation status of the recombinant HAs might interfere with HA–receptor interactions, we first specifically investigated this aspect. Thus, we produced soluble avian influenza A virus H2 and H7 trimers, which are predicted to contain 7 and 4 glycan side chains, respectively, using insect and mammalian cell expression systems. Two types of mammalian cells were used: HEK293T and HEK293S GnTI(-) cells (Reeves et al., 2002). While the former produce glycoproteins carrying complex N-glycans, glycoproteins produced in the latter contain high mannose glycans as these cells lack a functional *N*-acetylglucosaminyltransferase I. The purified HA trimers were subsequently analyzed for their ability to bind sialic acid receptors using different assays. The results show that HA–receptor interactions depend on the specific expression system used. HA trimers produced in the insect cells bind sialic acid ligands with decreased specificity when compared to the trimers produced in the HEK293S GnTI(-) cells. HEK293T cell-produced HA displayed the most narrow receptor specificity, which broadened after treatment of the protein with neuraminidase to resemble the specificity of the HEK293S GnTI(-) cell-produced HA. Differences in receptor specificity between H2 and H7 proteins, which are derived from influenza A viruses from different species of birds, could be demonstrated using recombinant HA proteins produced in HEK293S GnTI(-) cells, but not with the HA proteins made in the insect cells.

Results

Expression, purification, and characterization of the HA trimers

As we aim to study HA–receptor interactions for a large panel of recombinant HAs, we first analyzed to what extent the specific expression system used would affect this interaction, by using H2[N8] (A/Herring Gull/DE/677/88) as a model. In order to express a soluble, trimeric HA ectodomain in either insect or mammalian cells, the H2 ectodomain-coding sequence was first cloned into the appropriate expression vectors. In both plasmids used, the HA-sequence was preceded by a signal peptide-encoding sequence and followed by sequences coding for the GCN4 isoleucine-zipper trimerizaton motif (Harbury et al., 1993) and the Strep-tag II, the latter for purification purposes (Fig. 1A). Expression of the HA ectodomain was achieved by transient transfection of the appropriate plasmid into either HEK293T cells or HEK293S GnTI(-) cells or by the generation of stably transformed *Drosophila* Schneider S2 cells. HA proteins secreted into the culture media were purified using the Strep-tag technology and subjected to gel electrophoresis followed by western blotting using an antibody directed against *Strep*-tag II (Fig. 1B). The results show that the HA ectodomains differ in their electrophoretic mobility depending on the expression system used. The HA protein migrated as a discrete band after expression in the insect cells or in the HEK293S GnTI(-) cells, with the insect cell-produced HA migrating slightly faster. This result is in agreement with the expected oligosaccharide modifications of the proteins, as glycoproteins expressed in the GnTI(-) or the insect cells will be homogenously modified by high mannose or paucimannose N-glycans, respectively. In contrast, the HA protein produced in the HEK293T cells, which appeared as a fuzzy band, migrating slightly slower in the gel consistent with its more heterogeneous and complex N-linked glycans. However, when the HA proteins derived from the different expression systems were treated with N-Glycosidase F (PNGase F) to remove all N-linked glycans, they ran at the same position in the gel in agreement with the HA proteins, expressed in the different cells, having identical protein backbones and only differing in their N-linked glycosylation. The positions of the glycan side chains were modeled into the structure of a homologous H2 trimer (A/duck/Ontario/77; Protein Data Bank ID 2WR3) (Liu et al., 2009), which showed that 1 N-linked glycan is present at the tip of H2 (Fig. 1E).

Next, the oligomerization state of the different HA proteins was analyzed by gel filtration column chromatography (Fig. 1C). In all three cases the bulk of the HA protein eluted with the velocity of an oligomer, presumably a trimer, while only a minor fraction was found as aggregates in the void volume. The mammalian cell-derived HA oligomers were larger than those from the insect cells, which is in agreement with their different extent of N-glycosylation, although the increase in size was greater than expected. Similar differences in gel filtration column chromatography for HAs produced in either mammalian or insect cells were also reported by Wei et al. (2008). To confirm their trimeric nature, the HA preparations were subjected to blue-native gel electrophoresis followed by western blotting (Fig. 1D). When the insect-derived HA protein was heat-denatured prior to electrophoresis, three protein species appeared which very likely correspond to HA monomers, dimers and trimers, of which the fastest migrating monomeric form was most abundant. When the HA preparations were analyzed directly, i.e. without heating, the major part of the protein migrated at the position of the trimer. In conclusion, soluble trimeric HA ectodomains, differing only in their oligosaccharide modification, were expressed in the three expression systems and easily purified.

Biological activity of the HA ectodomain trimers

The biological activity of the different HA preparations was studied using hemagglutination and solid phase-binding assays. These studies were performed with recombinant HA proteins precomplexed with anti-Strep-tag antibody as detailed in the Materials and methods section. The results of the hemagglutination assay are shown in Fig. 2A. Clearly, the HA protein produced in the HEK293T cells was much less capable of hemagglutinating chicken red blood cells than the insect cell-derived (Fig. 2A) and the HEK293S GnTI(-)-produced HA protein. No hemagglutination was observed when the red blood cells had been treated with *Vibrio Cholera* derived neuraminidase (VCNA), when the HA proteins had not been precomplexed with the antibody directed against *Strep*-tag II, or after addition of only the antibody (data not shown). HA proteins were also evaluated in a bovine fetuin solid phase-binding assay. Fetuin is a blood glycoprotein that contains 3 *N*-linked and 3 *O*-linked sialylated glycan side chains. Binding of HA was measured by means of the horseradish peroxidase (HRP) conjugated to the anti-Strep-tag II antibody as detailed in the Materials and methods section. No binding was observed when the HA proteins had been produced without a GCN4-trimerization tag, when the proteins had not been precomplexed with antibody directed against the purification tag, when antibody was applied alone (data not shown), or when fetuin had been treated with VCNA (Fig. 2B). All trimeric HA proteins demonstrated a concentration dependent binding to fetuin. The HA proteins derived from the insect or the HEK293S GnTI(-) cells displayed a similar, much higher activity than the HA protein produced in the HEK 293T cells. Essentially similar results were obtained when the HA proteins were used to bind to wells coated with either human A549 cells or chicken DF-1 cells (Fig. 2C and D). The reduced ability of the HEK293T cell-produced HA protein to bind to sialylated substrates is in agreement with previous studies (Ohuchi et al., 1995; Wei et al., 2008; Uhlendorff et al., 2009) which showed that removal of sialic acids from the HA protein is important for efficient substrate binding. As shown in Fig. 2E, this was also the case in our experimental setup. When the HA protein produced in the HEK293T cells was treated with VCNA prior to elution from the Strep-Tactin beads it acquired the

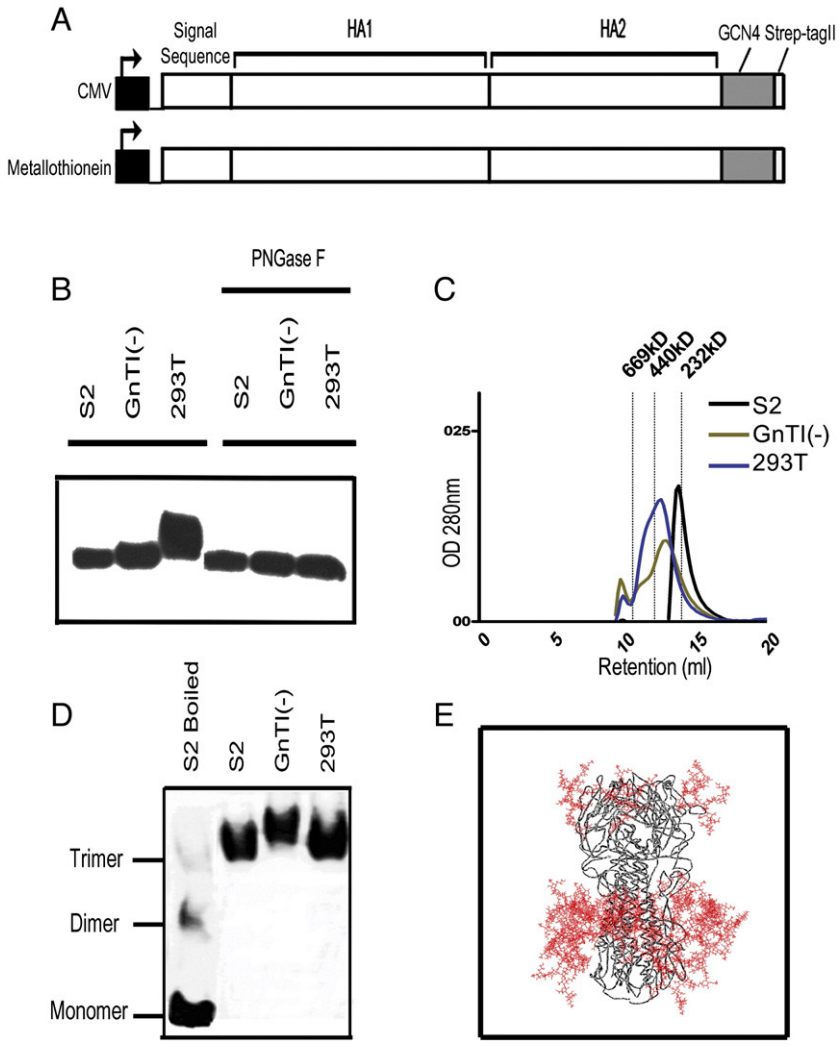


Fig. 1. Expression of recombinant trimeric HA proteins. (A) Schematic representation of the HA expression cassettes used. The HA ectodomain encoding sequence was cloned in frame with DNA sequences coding for a signal sequence, the GCN4 isoleucine zipper trimerization motif and the Strep-tag II under the control of either a CMV or a methallothionein promoter for expression in mammalian or insect cells, respectively. (B) HA ectodomains expressed in insect (S2), HEK293S GnTI(-) cells [GnTI(-)] or HEK293T (293T) cells and purified from the culture media were analyzed by SDS–PAGE followed by western blotting. The recombinant proteins were detected using a mouse anti-Strep-tag antibody. When indicated samples were treated with PNGase F prior to electrophoresis. (C) Analysis of purified recombinant HA proteins by gel filtration. The elution profiles of the different HA proteins using a Superdex200GL 10-300 column are shown. The elution of a 232 kDa catalase control is indicated by the dotted line. (D) Blue native-PAGE analysis of the recombinant HA proteins. The position in the gel of the momomeric, dimeric and trimeric ectodomain species observed after heating of the HA sample prior to electrophoresis is indicated. HAs subjected to gel electrophoresis without prior heating migrated at the position of the trimer. (E) Ribbon representation of the structure of H2 (A/dk/Ontario/77; Protein Data Bank ID 2WR3), N-linked glycans are displayed in red. All N-glycans are modeled by GlyProt (Bohne-Lang and von der Lieth, 2005), while the graphics are generated by Swiss-PdbViewer (http://spdbv.vital-it.ch/).

ability to bind to sialylated substrates with an efficiency comparable to that of HA protein synthesized in the HEK293S GnTI(-) cells (Fig. 2E and data not shown). Our results demonstrate that HA trimers produced in all three expression systems are biologically active.

Sialic acid receptor-binding specificities of the different HA trimers: glycan array analysis

HA proteins produced in the different expression systems were characterized for their oligosaccharide binding specificity by performing glycan array analyses in collaboration with the Consortium for Functional Glycomics. To this end, the different HA trimers, precomplexed with mouse antibodies directed against the *Strep*-tag II (2:1 molar ratio), were subjected to an array containing approximately 400 different glycans. Glycan binding by the HA trimers was monitored using an FITC-conjugated secondary anti-mouse IgG antibody. The glycan array data, including the glycan structures, can be accessed via the Web site of the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/index.shtml; see the legends of Figs. 4 and 5 for direct links to the raw data). A summary of the results is shown in Fig. 3. The HA protein produced in the HEK293T cells exhibited considerably less glycan binding activity than its counterparts produced in the other expression systems (compare Figs. 3A with 3B and 3C and see Fig. 3E), consistent with our earlier binding assays (Fig. 2). This 293T cell-produced HA protein showed a preference for glycan structures containing 2 sialic acids. In addition to these glycans the HA trimer produced in the HEK293S GnTI(-) cells recognized many other glycans containing α2-3-linked sialic acids (Fig. 3B and E), as did the HA trimer produced in the insect cells. This latter trimer additionally bound to a variety of other glycans containing α2-3-linked sialic acids, not recognized by the HEK293S GnTI(-) derived HA (Fig. 3C and E).

To investigate the contribution of the terminal sialic acids to the binding of complex glycosylated HA trimers, HA protein produced in HEK293T cells was treated with VCNA before applying it to the glycan array. The results are shown in Fig. 3D and E. Although

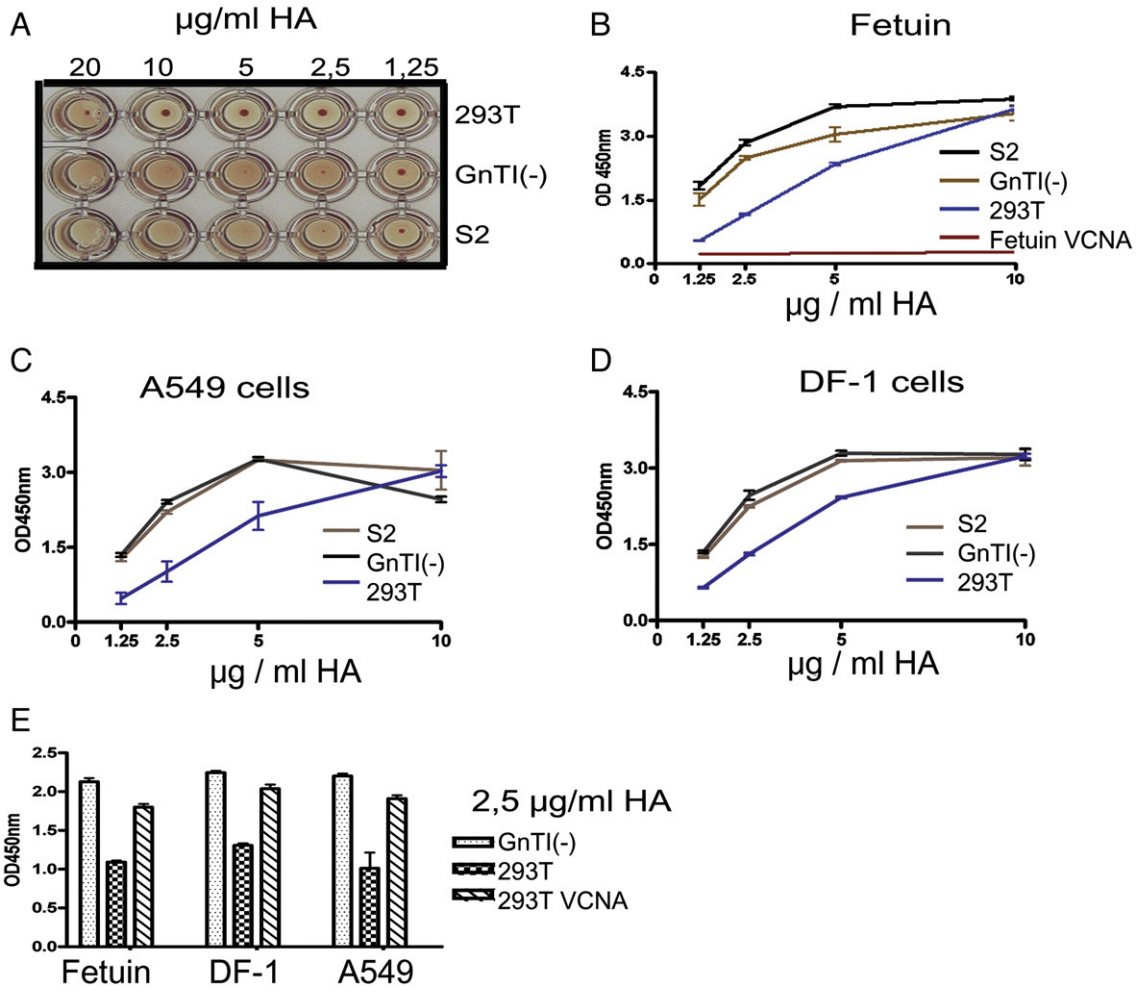


Fig. 2. Binding of recombinant trimeric HA proteins to various sialic acid containing substrates. Recombinant soluble HA trimers were complexed with a HRP-conjugated, mouse antibody directed against the Strep-tag prior to their application in the different binding assays. (A) Hemagglutination of chicken red blood cells by HA protein expressed in insect (S2), HEK293S GnTI(-) [GnTI(-)] or HEK293T (293T) cells is shown at different HA concentrations as indicated. Binding of the HAs to fetuin (B, E), fetuin treated with VCNA (B), A549 cells (C, E) or DF-1 (D, E) cells was detected using TMB substrate by reading the OD at 450 nm. When indicated recombinant HA protein expressed in HEK293T cells was treated with VCNA prior to the binding assay (E).

generally lower signal intensities were measured for the VCNA-treated HA trimers than for the HEK293S GnTI(-) derived trimers, almost all glycan structures bound by the latter HA were also bound by the desialylated HA (Fig. 3E). In summary, our results show that trimeric HA proteins that are produced in different expression systems and hence differ only in the length of their N-glycans exhibit different receptor fine specificities. The specificity of HA–receptor binding appears to decrease with the HA protein itself carrying shorter glycan side chains (S2-HA<HEK293S GnTI(-)-HA<HEK293T-HA). HA produced in the HEK293S GnTI(-) cells demonstrated a specificity similar to that of the VCNA-treated HA protein produced in the HEK293T cells.

Receptor-binding specificities of different HA subtypes

Finally, we studied whether HA proteins produced in the different expression systems are suitable to distinguish between HA–receptor-binding fine specificities. Therefore, we compared the glycan array binding profile of the H2[N8] (A/Herring Gull/DE/677/88), which we used so far, with that of another HA subtype derived from an influenza A virus isolated from a different bird species: H7[N2] (A/turkey/NY/4450-5/94). Also this HA protein carries a N-glycan at the tip of the molecule. H2 and H7 are expected to have similar but different receptor fine specificities. The soluble trimeric H7 ectodomain was expressed in HEK293S GnTI(-) and insect S2 cells and purified as described above. The trimeric nature of the soluble ectodomain was confirmed by gel filtration and blue native gel electrophoresis, while its ability to bind sialylated substrates was confirmed by solid phase-binding assays (data not shown). Next, glycan array binding profiles were generated, the detailed results of which can be found on the Web site of the Consortium for Functional Glycomics (http://www.functionalglycomics.org/static/index.shtml). The results are compiled in Figs. 4 and 5. As for the H2 protein, more sialic acid-containing ligands were recognized by the H7 protein produced in the insect cells than by that derived from the HEK293S GnTI(-) cells (compare Fig. 4A with B and C with D).

Comparison of the receptor fine specificities of the two different HA proteins revealed that all glycans recognized by H2 were also bound by H7, provided the proteins had been produced in the insect cells (compare Fig. 4A with C and see Fig. 4E). However, when produced in the HEK293S GnTI(-) cells, the two proteins had clearly different receptor fine specificities, though many ligands were still bound by both HAs (compare Fig. 4B with D and see Fig. 4F). In general, it appeared that H2 produced in HEK293S GnTI(-) cells preferred binding to type 1 chain glycans [Gal(β1→3)GlcNAc] containing α2-3-linked sialic acids, except when these glycans were fucosylated, while HEK293S GnTI(-) cell-produced H7 preferred binding to α2-3-sialylated type 2 chain glycans [Gal(β1→4)GlcNAc].

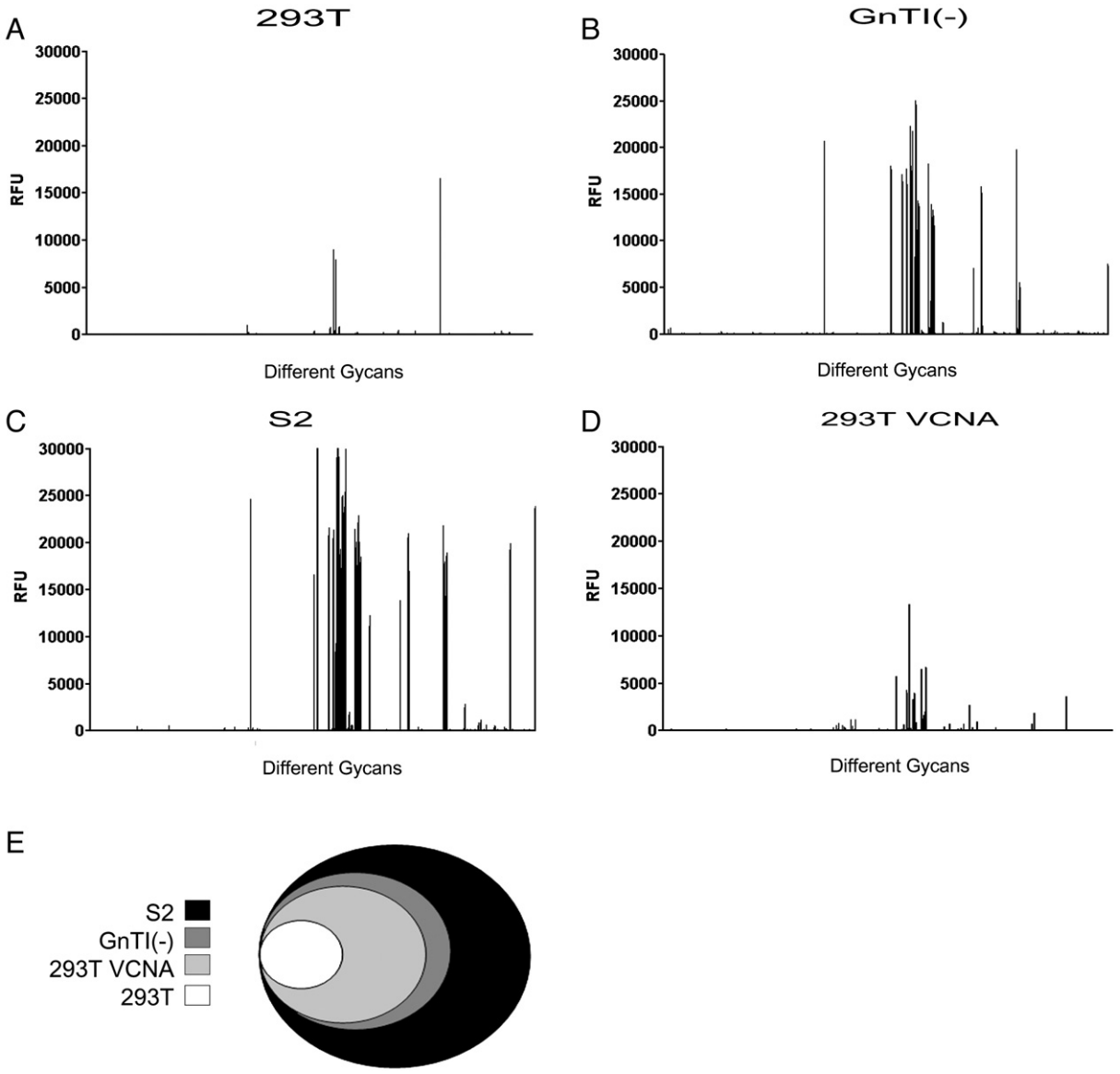


Fig. 3. Glycan array analysis of HA trimers produced in the different expression systems. Glycan array analyses are shown for the HA proteins produced in HEK293T cells (293T; A and D), HEK293S GnTI(-) cells [GnTI(-); B] and insect cells (S2; C). (E) The HA protein was treated with VCNA prior to the analysis (293T VCNA). Glycan array 4.0 (A, B, and C) or array 4.1 (D) was used. The raw data and the glycan structures can be accessed at the following Web sites: A; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2622, B; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2621, C; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2623, D; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2725. Binding of HA to different glycans was measured in relative fluorescence units (RFU) as indicated on the *Y*-axis. The order of the different glycans arranged on the *X*-axis of graphs A–D is identical. (E) A Venn diagram demonstrates the overlaps observed in the different HA–receptor-binding profiles. The surfaces of each of the ovals correspond with the number of glycans bound by the different HAs.

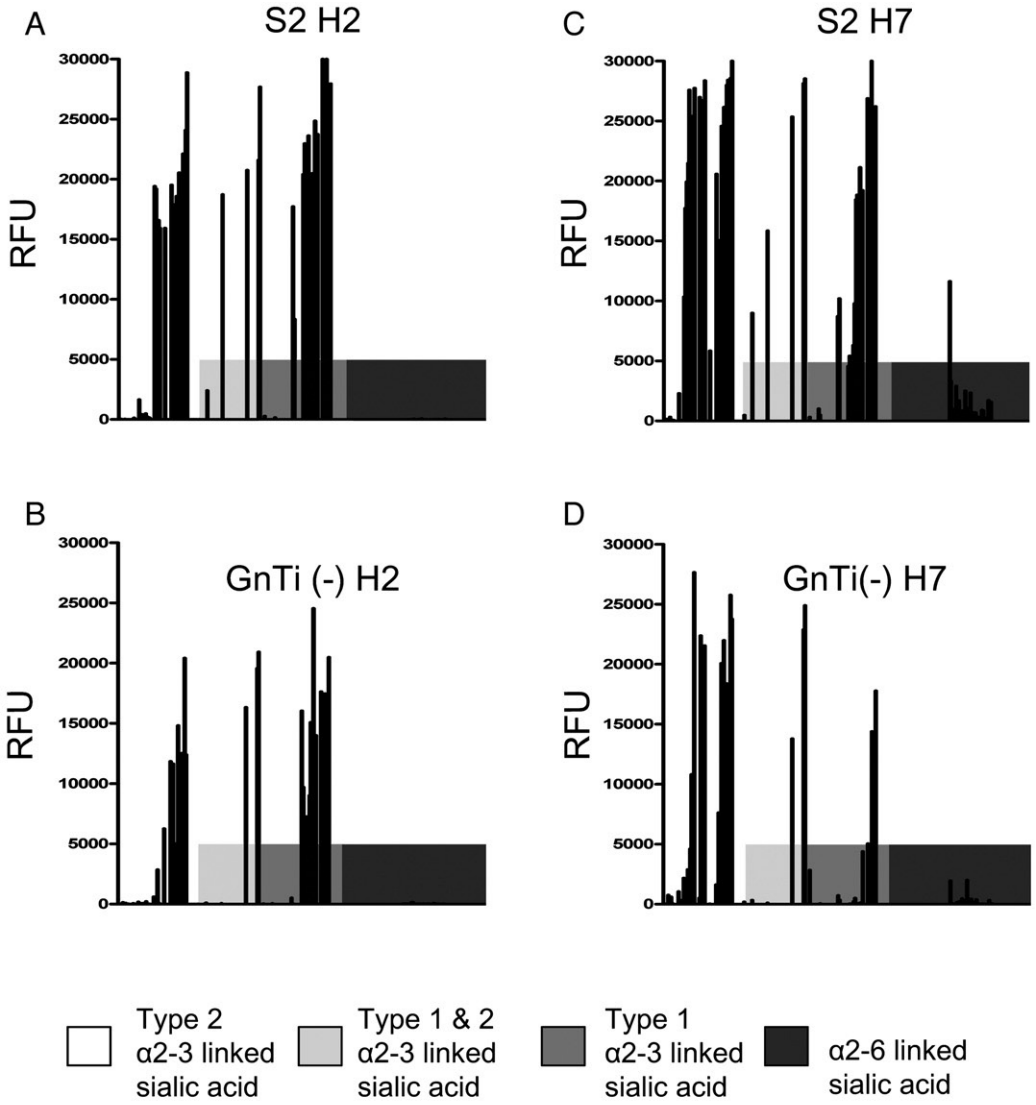
The core glycan structure of type 1 and 2 chain glycans, containing α2-3-linked sialic acid, is shown in Fig. 4G.

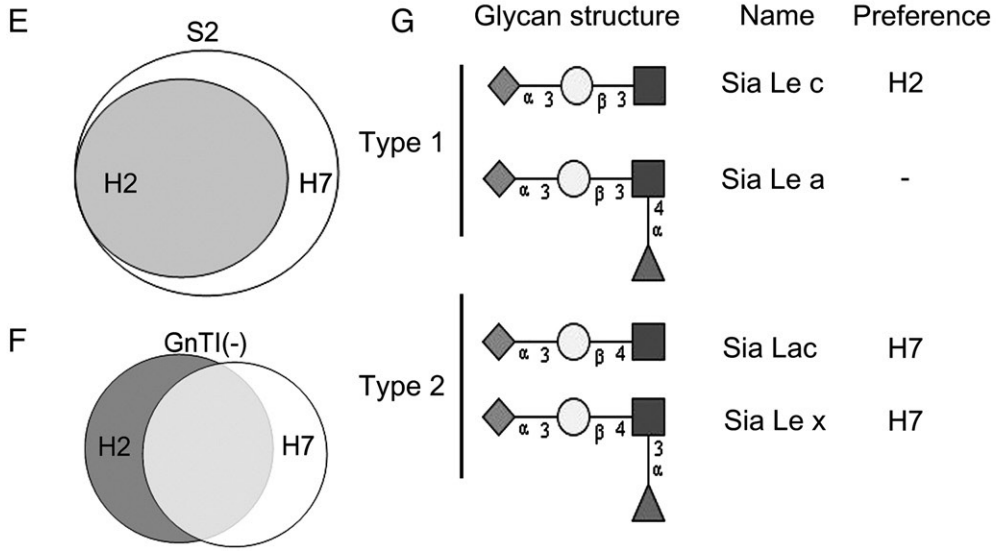
This observation was confirmed by comparing the relative binding strength of H2 and H7 produced in HEK293S GnTI(-) cells to type 1 and type 2 glycans. To this end the ratio of the fluorescence intensities of H2 and H7 (measured in RFU), was determined and plotted as in Fig. 5A. The results show that type 1 chain glycans were preferentially bound by H2 (value>1), while H7 preferred binding to type 2 chain glycans (value<1). When the same procedure was applied to the insect cell-derived HA proteins carrying paucimannose glycans (Fig. 5B), the large majority of the type 1 or 2 chain glycans appeared to be bound by H2 and H7 with similar efficiency. However, as only a single concentration of the HAs was applied to the glycan arrays, we cannot exclude that at lower concentrations similar differences in receptor fine specificities can be observed for the insect cell-produced H2 and H7. In summary, differences in receptor fine specificities between different HAs could most readily be observed with recombinant soluble trimeric HA ectodomains produced in HEK293S GnTI(-) cells.

Discussion

One of the key factors in the epidemiology of influenza A viruses is their adaptability to different sialic acid receptors occurring at the cell surfaces of different species. It is now well established that this adaptive potential most often is caused by subtle mutations in the viral glycoproteins, providing the virus with improved binding properties in its new host. Much less is known about the possible influence on these binding properties of the glycans attached to the viral surface glycoproteins, though previous reports support the significance of such effects (Ohuchi et al., 1995b; Wang et al., 2009d; Wei et al., 2008b). Influenza A virus HAs, which constitute the

receptor-binding structures, generally carry some 2 to 8 oligosaccharides side chains distributed across the trimeric ectodomain complex (Vigerust et al., 2007), some of them located close to the sialic acid receptor-binding surface depression. With the aim to study the influence of the HA glycosylation states on receptor binding and to develop tools for convenient analysis of binding specificities of





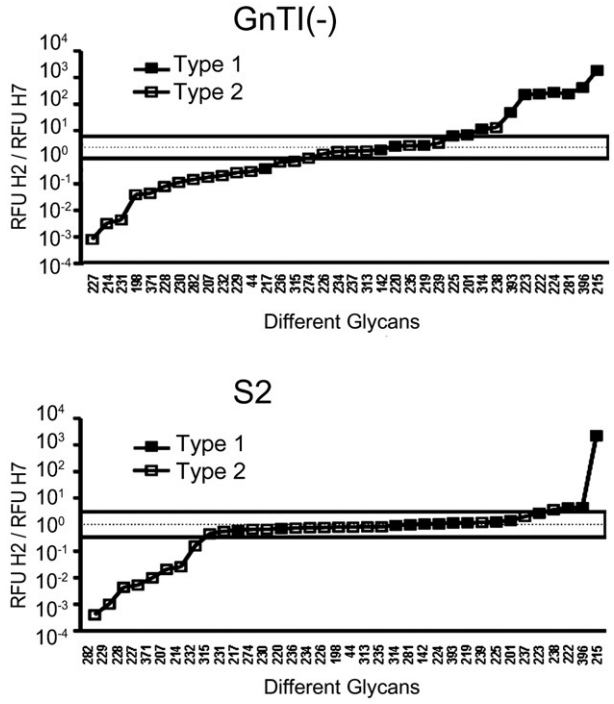


Fig. 5. Relative binding of H2 and H7 to type 1 and type 2 chain glycans. The ratio of the relative fluorescence units (RFU), which are a measure for the binding of the HAs to the different sugars, of H2 and H7 for type 1 and 2 chain glycans (on the *X*-axis) is plotted. Only those type 1 and type 2 chain glycans were included that showed significant binding by either insect cell-produced H2 or H7 (>4000 RFU). Type 1 and 2 chain glycans are indicated by black and white boxes, respectively. A ratio higher than 1 indicates preferred binding by H2, while a ratio smaller than 1 indicates preferred binding by H7. The large rectangles mark the ratios ranging between values 3 and 0.33. The dotted line indicates a ratio of 1. The ratios are shown for HA proteins expressed in HEK293S GnTI(-) (A) or insect S2 (B) cells.

different HAs, we established expression systems for the production of soluble, variously glycosylated trimeric HAs. The resulting HAs, identical in their protein structure but differing in their glycan makeup, allowed us to compare the receptor-binding specificities of HAs with 4 different glycosylation states.

The HA proteins produced in HEK293T cells demonstrated only very limited receptor binding as demonstrated in the hemagglutination assay, solid phase-binding assays and the glycan array profiling. In agreement with previous studies (Ohuchi et al., 1995; Wang et al., 2009; Wei et al., 2008), removal of sialic acids from the HA protein itself was important for efficient receptor binding. The sialic acid-containing oligosaccharide may interfere with the accessibility of the receptor-binding site by steric hindrance or by electrostatic repulsion. Alternatively, HA-bound sialic acid may completely fill the receptor pocket of another HA molecule (HA–HA interaction). Indeed, both the H2 and the H7 subtypes used in this study carry an N-linked glycan at their tip, which may be bound to or interfere with the accessibility of the receptor-binding site of the neighboring monomer. Either way, the HA proteins containing sialylated N-glycans were only able to bind to di-sialylated glycan structures (Fig. 3 and data not shown). Apparently, only di-sialylated glycan structures are able to obviate the steric hindrance or the electric repulsion caused by the sialylated glycans on HA itself, or alternatively to effectively compete with the HA glycans for binding with the receptor pocket.

Influenza A viruses normally contain HA proteins that carry complex glycans that are desialylated by the viral neuraminidase. To mimic this HA glycosylation status, we removed the sialic acids from the HA protein produced in HEK293T cells. This desialylated HA protein revealed a similar receptor specificity as when the HA protein was produced in the HEK293S GnTI(-) cells. These observations are consistent with a recent study (Wang et al., 2009), which demonstrated that the association constants and calculated free energy changes of H5, expressed either in HEK293S GnTI(-) or in HEK293T cells followed by neuraminidase treatment, were very similar when binding to sialylated substrates. From these results we assume that HA produced in the HEK293S GnTI(-) cells display similar receptor specificity as HA expressed in virus-infected cells, although desialylated HA probably mimics the natural situation most.

Our results show that receptor-binding specificity of influenza A virus HA is not only affected by the presence or absence of sialylated glycans, but also by glycan length. HAs produced in the insect cells, which carry paucimannose glycans exhibited decreased receptor specificity, when compared to the proteins expressed in the HEK293S GnTI(-) cells, which contain high mannose oligosaccharides. Consistent with our results, enzymatic trimming of N-glycan structures on HA was shown to increase receptor affinity, while decreasing the specificity (Wang et al., 2009). Complete elimination of the glycan side chains either by treatment with PNGase F or by disruption of the N-linked glycosylation consensus sequence increased the affinity of HA for its receptor to such an extent that release by neuraminidase was severely impeded (Ohuchi et al., 1997a). The different HAs used in these studies (Ohuchi et al., 1997a; Wang et al., 2009; this study) all carry N-linked glycosylation sites at the top of the HA molecule. Therefore, the length of the HA glycans is likely to also affect the receptor-binding specificity of other HAs with N-linked side chains at their tip.

The decreased specificity of the insect cell-produced HA probably makes this protein less suitable to study receptor fine specificities than HA produced in the GnTI-lacking cells. This notion was supported by comparing the glycan array binding profiles of H2 and H7 derived from influenza A virus from herring gull and turkey, respectively. These HAs were expected to exhibit comparable, but different receptor-binding specificities. While both HAs demonstrated clear preference for binding to sialic acids attached to the vicinal galactose via a α2-3 linkage (Neu5Acα2-3Gal), regardless of the expression system used, differences in fine receptor specificity could not be observed with the insect cell-, but were readily detected with the HEK293S GnTI(-) cell-produced HA.

The comparison of the H2 and H7 glycan array profiles demonstrated that binding of HA is not only determined by the linkage of the terminal sialic acid to the vicinal galactose, but is also affected by the linkage of the galactose to N-acetylglucosamine. Thus, while H2, derived from a herring gull-virus, preferred binding to type I chain glycans (Neu5Acα2-3Galβ1-3GlcNAc), H7, derived from a turkey-virus, more readily bound to type 2 chain glycans (Neu5Acα2-3Galβ1-4GlcNAc). Furthermore, H2 did not bind fucosylated type I chain glycans, which might be explained by steric hindrance of the fucose moiety. Our results are consistent with the results of

Fig. 4. Fine specificity analysis of insect and mammalian cell-produced HA proteins. Glycan array analyses are shown for the H2 (A and B) and H7 (C and D) proteins produced in insect cells (S2; A and C) or HEK293S GnTI(-) cells [GnTI(-); B and D]. The raw data for H7 and the structures of the bound glycans can be accessed at the following Web sites: C; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2618 and D; http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_2620. Graphs A–D show binding of HA to sialylated glycans on Glycan array 4.0 (in relative fluorescence units [RFU]). On the *X*-axis the sialylated glycans are grouped according to the α2-3 or α2-6 linkage of the sialic acid to the vicinal galactose. Glycans containing α2-3-linked sialic acids are divided further into type 1 or type 2 chain glycans. Glycans that contain both type 1 and type 2 chains are also indicated. (E–F) Venn diagrams demonstrate the overlap observed in the HA glycan array 4.0 binding profiles of H2 and H7 expressed either in insect S2 cells (E) or in HEK293S GnTI(-) cells (F). (G) Schematic structures of the type I chain glycans Sialyl Lewis c (Sia Le c) and Siayl Lewis a (Sia Le a) and the type II chain glycans Sialyl Lewis x (Sia Le x) and Sialyl lactosamine (Sia Lac) are shown. H2 or H7 indicates the preferred binding of these HAs to these different sugars, while – indicates the absence of HA binding. ■, ○, ◆, and ▲ represent N-acetylglucosamine, galactose, sialic acid and fucose, respectively.

Gambaryan and coworkers who showed that influenza viruses isolated from distinct species of aquatic and terrestrial birds may differ in their fine receptor-binding specificity by recognizing the structure of the inner parts of the Neu5Acα2-3Gal-terminating receptors (Gambaryan et al., 2005; Gambaryan et al., 2008).

Successful cross-species transmission of influenza A virus usually involves alterations in the HA receptor specificity, allowing the virus to replicate efficiently within and spread efficiently between its new host (Chandrasekaran et al., 2008; Connor et al., 1994; Kuiken et al., 2006). Glycan array technologies may aid in the surveillance of animal viruses by rapidly assessing HA receptor specificity in fine detail (Childs et al., 2009; Tumpey et al., 2007). The production of recombinant HAs may be very useful for these analyses, as it obviates the use of potentially dangerous viruses, while in addition receptor adaptation of cell culture- or egg-grown viruses is prevented (Wang et al., 2006; Widjaja et al., 2006). In this study we evaluated the effect of producing recombinant HAs in different expression systems on receptor-binding specificity. Our results show that the expression system used, and hence the glycosylation status of the recombinant protein, affects the receptor fine specificity of HA.

Material and methods

Genes and expression vectors

pCIneo plasmid containing full-length HA of A/Herring Gull/DE/677/88 H2 (GenBank accession no. AAA43096) and A/Turkey/NY/4450/94 H7 (GenBank accession no. AAD26925) were a kind gift of Dr. D.L. Suarez (Lee et al., 2006). Based on H3 numbering (Wilson et al.), cDNAs corresponding to residues 18 to 523 were produced by PCR, isolated and cloned into the pGEMTeasy vector (Promega, USA). The HA ectodomain encoding cDNAs were subsequently cloned into the pCD5 vector for efficient expression in mammalian cells (Zeng et al., 2008) or into the pMT-Bip vector (Invitrogen) for expression in Schneider S2 cells (Kim et al., 2009). Both vectors were modified such that the HA-encoding cDNAs were cloned in frame with DNA sequences coding for a signal sequence, an artificial GCN4 isoleucine zipper trimerization motif (RMKQIEDKIEEIESKQKKIENEIARIKK) (Harbury et al., 1993) and the Strep-tag II (WSHPQFEK; IBA, Germany).

Protein expression and purification

pCD5 expression vectors containing the HA ectodomain-encoding sequence were transfected into HEK293T and HEK293S GnTI(-)cells (Reeves et al., 2002) using polyethyleneimine I (PEI) in a 1:5 ratio (μg DNA:μg PEI). At 6 h post transfection, the transfection mixture was replaced by 293 SFM II expression medium (Invitrogen), supplemented with sodium bicarbonate (3.7 g/l), glucose (2.0 g/l), Primatone RL-UF (3.0 g/l), penicillin (100 units/ml), Streptomycin (100 μg/ml), glutaMAX (Gibco), and 1.5% DMSO. Tissue culture supernatants were harvested 5–6 days post transfection. HA proteins were purified using Strep-Tactin sepharose beads according to the manufacturer's instructions (IBA, Germany). When indicated, HA trimers bound to Strep-Tactin beads were treated with VCNA for 3 h at 37 °C (2 μU/ml), followed by three washing steps prior to elution of the protein from the beads. *Drosophila* Schneider S2 cells were co-transfected with pMT-Bip vector encoding the HA ectodomain and pCoBlast using Cellfectin in a 19:1 ratio, and stable cell lines were selected according to manufacturer's protocols (Invitrogen), using blasticidine selection. Cells were kept under blasticidine pressure and cultured in serum-free Insect Xpress Medium (Lonza, Belgium) at 28 °C. Protein expression was induced with CuSO4 (500 μM). Culture supernatants were harvested after 10 days. HA proteins were purified as described above. HA protein expression and purification were confirmed by SDS–PAGE followed by western blotting using a mouse anti-Strep-tag antibody (IBA, Germany). When indicated, the HA proteins were treated with PNGase F (NEB) prior to electrophoresis according to the manufacturer's procedures in order to remove N-linked oligosaccharides. Oligomerization status of the HA proteins was determined by analyzing the elution profile using a Superdex200GL 10-300 column (GE Healthcare) and by blue native-PAGE analysis.

Biological characterization of recombinant HAs

Functionality of the HAs was assessed using different solid phase assays as well as by analyzing the ability of the recombinant HAs to hemagglutinate chicken erythrocytes. For the fetuin binding assay, 100 μg/ml fetuin per well was used to coat 96-well nunc maxisorp plates. Recombinant HA was precomplexed with HRP-linked anti-Strep-tag antibody (2:1 molar ratio) for 30 min at 0 °C prior to incubation of limiting dilutions on the fetuin-coated plates (60 min, room temperature [RT]). HA binding was subsequently detected using tetramethylbenzidine substrate (BioFX) in an ELISA reader (EL-808 [BioTEK]), reading the OD at 450 nm. In the CellELISA assays, essentially the same protocol was used, with the exception that DF-1 and A549 (10^5 cells per well) were cultured in a 96 well plate, for 16 h at 37 °C and fixed with 4% paraformaldehyde solution in phosphate buffered saline (PBS) rather than coating the wells with fetuin. The hemagglutination assay was performed according to standard methods, briefly; 2-fold dilutions of the HA-anti-Strep-tag antibody complex were incubated with 0.5% chicken erythrocytes for 30 min, at RT.

Glycan array analysis

Microarrays were printed as described (Blixt et al., 2004). The glycan array analysis of the HA proteins was performed as previously described (Stevens et al., 2006a). Briefly 200 μg/ml recombinant HA was precomplexed with HRP-linked anti-Strep-tag antibody (2:1 molar ratio) for 30 min at 0 °C prior to incubation for 60 min on the microarray slide under a microscope cover-glass in a humidified chamber at RT. Microarray slides were subsequently washed by successive rinses in PBS with 0.05%Tween and overlaid with a AlexaFluor 488 anti-Mouse Ab. After repeated washes with PBS with 0.05%Tween, PBS, and deionized water the slides were immediately subjected to imaging as described (Blixt et al., 2004).

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