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Avian influenza H5 hemagglutinin binds with high avidity to sialic acid on different *O*-linked core structures on mucin-type fusion proteins

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Abstract The interaction between P-selectin glycoprotein ligand-1/mouse IgG_{2b} (PSGL-1/mIgG$_{2b}$) fusion protein carrying multiple copies of the influenza hemagglutinin receptor Siaα2-3Gal on different *O*-glycan chains and recombinant human influenza H5N1 A/Vietnam/1203/04 hemagglutinin was investigated with a Biacore biosensor. The fusion protein was produced by stable cell lines in large scale cultures and purified with affinity- and gel filtration chromatography. The C-P55 and 293-P cell lines were established by transfecting the Chinese hamster ovary (CHO)-K1 and Human embryonic kidney (HEK)-293 cell lines with plasmids encoding the PSGL-1/mIgG$_{2b}$ fusion protein, while the C-PSLex cell line was engineered by transfecting CHO-K1 cells with the plasmids encoding the core 2 β1,6GnT-I and FUT-VII glycosyltransferases. Glycosylation was characterized by lectin Western blotting of the proteins and liquid chromatography - mass spectrometry of released non-derivatized *O*-glycans. Biacore experiments revealed that PSGL-1/mIgG$_{2b}$ is a good binding partner of H5. The binding curves displayed a slow dissociation indicating a multivalent binding. The H5 hemagglutinin binds with similar strength to PSGL-1/mIgG$_{2b}$ carrying mostly sialylated core 1 (clone C-P55), a mix of sialylated core 1 and sialylated lactosamine (clone 293-P) or mainly sialylated lactosamine (clone C-PSLex) *O*-glycans, indicating that this hemagglutinin is unable to discriminate between these structures. The potential use of the large, flexible PSGL-1/mIgG$_{2b}$ mucin-type fusion protein carrying Siaα2-3Gal as a multivalent inhibitor of influenza virus is discussed.

Keywords Mucins · Avian influenza · Hemagglutinin · Mass spectrometry · Biacore

Abbreviations

P-selectin glycoprotein ligand-1/mouse IgG_{2b}	PSGL-1/mIgG$_{2b}$
LC-MS	liquid chromatography mass spectrometry
HexNAc	*N*-acetylhexosamine
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
Hex	hexose
Gal	galactose
CHO	chinese hamster ovary cells
Neu5Ac	*N*-acetylneuraminic acid
Sia	sialic acid

Introduction

Influenza A virus is categorized by the two surface antigens it carries in the envelope, hemagglutinin (HA) and neuraminidase (NA). Sixteen HA subtypes (H1-H16) have been identified and nine NA subtypes (N1-N9) [1]. HA binds to sialic acid (Sia) on the cell surface and mediates membrane fusion and cellular uptake of the virus due to a conformational

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change of HA [2, 3]. NA hydrolyzes sialic acid (neuraminic acid) on the cell surface which is needed for release of budding virus particles [2]. HA is composed of a homotrimer in the virus envelope, but may form aggregates of several trimers, "rosettes", if produced in soluble form [4–6]. Only three influenza A subtypes have been adapted to humans, H1N1, H2N2 and H3N2 [7, 8]. Variants of these strains are responsible for the seasonal epidemics of influenza which cause 3–5 million clinical infections and 250 000–300 000 deaths annually [9]. Novel variants of influenza A are accountable for pandemic outbreaks with the 1918 H1N1 "Spanish flu" being the most severe with an estimated 50 million deaths [10]. The latest pandemic 2009 was caused by a novel influenza A H1N1 strain of swine origin [9].

Avian influenza A preferentially binds to sialic acid α2-3 linked to galactose, while human adapted influenza binds preferentially to sialic acid α2-6 linked to galactose [3, 11]. The distribution of these receptors in the avian and human hosts contribute to the different disease symptoms [12, 13]. In humans, Siaα2-3Gal is prevalent on secreted mucins while Siaα2-6Gal is common on glycoproteins present on the surface of cells in the respiratory tract [11, 14]. Avian influenza A virus thus have problems establishing an infection in the upper respiratory tract since the cell layer has low amounts of Siaα2-3Gal receptors and is covered with mucins that carry Siaα2-3 decoy receptors. This underscores the selection pressure of avian HA and NA to adapt to human receptors in order to be able to bind to the cell surface and to avoid binding to sequestered mucins [11]. The limited few cases of infections caused by avian influenza A virus with strict Siaα2-3Gal specificity may be explained by the presence of these receptors in the lower respiratory tract [15]. The location site of receptor expression may also contribute to the severity of these infections, but consequently human to human infections are scarce [9, 16]. Several adaptations of avian influenza A are necessary for efficient transmissibility in-between humans (*i.e.* air-borne transmission), including virus attachment to the upper respiratory tract, efficient replication in these tissues and release and aerosolization of single virus particles [17] [16]. The emerging avian influenza A strain H5N1 is responsible for occasional outbreaks with limited spread among humans, but with high death rates. It is therefore of great concern that H5N1 might adapt to humans and cause a high number of fatalities [18, 19]. Interestingly, in some outbreaks of avian influenza, the main clinical symptoms have been conjunctivitis, exemplified in the Dutch avian influenza H7N7 outbreak 2003 and H5N1 outbreaks in Hong Kong 1997 and 2003 [20]. Some of the infected cases also developed traditional influenza symptoms [20]. This suggests that avian influenza may initiate infection in the ocular epithelium of humans from where the virus can spread to the respiratory tract. Replication of avian influenza virus in human ocular cells *in vitro* and the ability of multiple strains of avian and human influenza to cause severe respiratory tract infections in ferrets by inoculation *via* the ocular route seem to confirm this hypothesis [21, 22]. This route of infection is facilitated by Siaα2,3 expression on ocular epithelium of humans, while Siaα2,6 is found on mucins in the tear fluid [11].

In vitro studies on the recombinant H5 derived from the highly pathogenic avian H5N1 A/Vietnam/1203/04 strain suggest that it binds almost exclusively to Siaα2-3, and that the inner carbohydrate chain is of less importance [23, 24].

Vaccines against the latest pandemic influenza (porcine H1N1, 2009) proved to be efficacious in approximately two thirds of the cases, but with big variations within different groups [25]. Traditional vaccine production with chicken eggs is both expensive and time consuming. Although novel techniques utilizing reverse genetics in order to produce viral proteins in cell lines can speed up the process considerably [25], there is still a need for treatment of influenza when no vaccine is yet available, such as when a novel influenza virus emerges.

The surface antigens HA and NA have been suggested as targets for inhibitory molecules, in order to prevent infection and ameliorate disease. This has been demonstrated with Tamiflu (Oseltamivir) and Relanza (Zanamivir) [26], which irreversibly bind to influenza NA. Speaking against these drugs is their relatively low efficacy and that escape-mutants have started to appear [27–29].

Efficient inhibition of HA seems to require multivalent display of the sialic acid moiety and is further improved by optimizing its density and spacing, as well as the size of the carrier molecule [14, 17, 30–32]. Monovalent sialic acid derivatives have shown low affinity to influenza with $K_i = 10^{-2}$–10^{-5} M [33, 34], while multimeric Sia conjugates may inhibit at levels below 10^{-5} M [17] and references within).

Selectins and their glycoprotein ligands are important in leukocyte trafficking, including migration across the vascular endothelial cells during inflammation and lymphocyte migration to lymphoid organs [35]. The P-selectin glycoprotein ligand-1 (PSGL-1, CD162) is a heavily *O*-glycosylated mucin-like protein. The specific binding of PSGL-1 to P-selectin (CD62P) is dependent on sialyl-Lewis x glycosylation and tyrosine sulfation at specific residues [36].

Previously, we have published a number of articles describing the concept of using glyco-engineered cell lines in which we produced a mucin-like fusion protein with tailored glycosylation by expressing a genetically fused extracellular part of P-selectin glycoprotein ligand-1 with the Fc part of mouse IgG_{2b} (PSGL-1/mIgG_{2b}) in cells transfected with specific glycosyltransferases. [31, 37–41]. The frequent *O*-glycosylation of the mucin part of PSGL-1/mIgG_{2b} provides the scaffold for multivalent

display of bioactive carbohydrate determinants, which makes it suitable as an inhibitor of carbohydrate-binding bacterial adhesins [42], toxins [43], antibodies [31, 38, 44], and viral surface proteins; the topic of this paper. The aim of this investigation was to evaluate if recombinant mucins with tailored glycosylation may serve as efficient binding partners of the H5N1 A/Vietnam/1203/04 hemagglutinin.

Results

P-selectin glycoprotein ligand-1/mouse immunoglobulin G_{2b} is mainly expressed as a dimer in C-P55, 293-P and C-PSLex cells

PSGL-1/mIgG_{2b} produced in C-P55, 293-P and C-PSLex was affinity purified, gel filtered and analyzed by SDS-PAGE and Western blot (Fig. 1, lane 1–3). Staining of the SDS-PAGE gel with Pro Q Emerald (stains glycosylated proteins) followed by Ruby (stains all proteins) revealed strong glycosylated bands above the 180 kDa marker (Fig. 1a and b). Minor glycosylated bands were also seen below the 180 kDa MW marker and may represent degradation products of PSGL-1/mIgG_{2b}. Western blots probed with anti-PSGL-1 and anti-IgG (Fc-specific) antibodies stained strongly with bands migrating between the 268 and 460 kDa MW markers (Fig. 1c and d). Running the gel under reducing conditions roughly halves the apparent MW of the protein on the gel, suggesting that the PSGL-1/mIgG_{2b} molecule is dimeric (data not shown). The theoretical molecular weight of dimeric PSGL-1/mIgG_{2b} is only 117 kDa, indicating that the degree of glycosylation is high. The anti-IgG (Fc-specific) antibody staining of PSGL-1/mIgG_{2b} produced in C-P55 (Fig. 1d, lane 1) showed weak binding to additional bands between 117 and 268 kDa, most likely representing degradation products of PSGL-1/mIgG_{2b} carrying the IgG Fc-moiety.

Characterization of O-glycans released from PSGL-1/mIgG_{2b} produced in C-P55, 293-P and C-PSLex cell lines with liquid chromatography mass spectrometry

Purified PSGL-1/mIgG_{2b} protein produced in the C-P55, 293-P, and C-PSLex cell lines was subjected to β-elimination in order to release *O*-linked glycans. After wash-up, the reduced non-derivatized *O*-glycans were analyzed with liquid chromatography - mass spectrometry in negative mode (Fig. 2). The *O*-glycans were separated on the column with an increasing acetonitrile gradient and in each scan (*m/z* 383–2000) the top three most abundant ions were fragmented to give MS^2 spectra. This allows the collection of a comprehensive data-set with MS^2 spectra of most separated glycans. The MS^2 spectra of all annotated spectra in this report have been submitted to the UniCarb-DB (http://www.unicarb-db.org/) for public access.

C-P55. The chromatogram and spectrum of *O*-glycans derived from PSGL-1/mIgG_{2b} produced in the C-P55 cell line are shown in Fig. 2a, with the major ions annotated with CFG cartoons. We suggest that the dominant ions in the spectrum represent mono- and disialylated core 1 ([M—H] ions of *m/z* 675.3 and 966.3), which are also found with acetylated sialic acid ([M—H] ions of *m/z* 717 and 1008). Non-sialylated core 1 is also observed ([M—H] ion of *m/z* 384). A minor *m/z* 1040.3 ion may represent a tentative core 2 structure carrying a type 2 chain with terminal sialic acid, *i.e.* Siaα3Galβ4GlcNAcβ6(Galβ3)GalNAcol or Galβ4GlcNAcβ6(Siaα3Galβ3)GalNAcol as suggested by the MS^2 spectrum (not shown) and knowledge of available glycosyltransferases in CHO cells. In conclusion, the LC-MS analysis shows that the major *O*-glycan structures in the C-P55 cell line are mono- and disialylated core 1.

HEK-293-P. The chromatogram and spectrum of *O*-glycans derived from PSGL-1/mIgG_{2b} produced in the 293-P cell line are shown in Fig. 2b. Similar to the C-P55 derived *O*-glycan

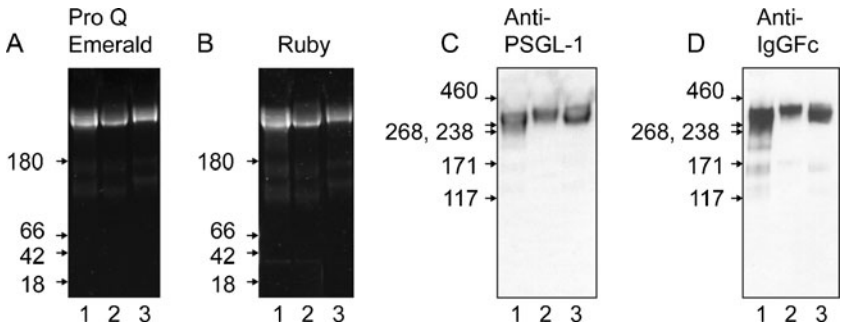

Fig. 1 Western blot and SDS-PAGE analysis of the P-selectin glycoprotein ligand-1/mouse immunoglobulin G_{2b} fusion protein produced in C-P55, C-PSLex and 293-P cells. PSGL-1/mIgG_{2b} produced in CP55 (lane 1), C-PSLex (lane 2) and 293-P (lane 3) was affinity purified, gel filtered and analyzed with SDS-PAGE under non-reducing conditions on Tris-Acetate 3-8 % gels. Gel staining of SDS-PAGE with Pro Q Emerald (A), which stains glycosylated proteins, and Ruby (B), which stains all proteins, revealed strong glycosylated bands above the 180 kDa marker (5 μg protein/lane). Western blotting with anti-PSGL-1 (C) and anti-IgGFc (D) strongly stained proteins migrating between the 268 and 460 kDa MW marker (0.5 μg/lane)

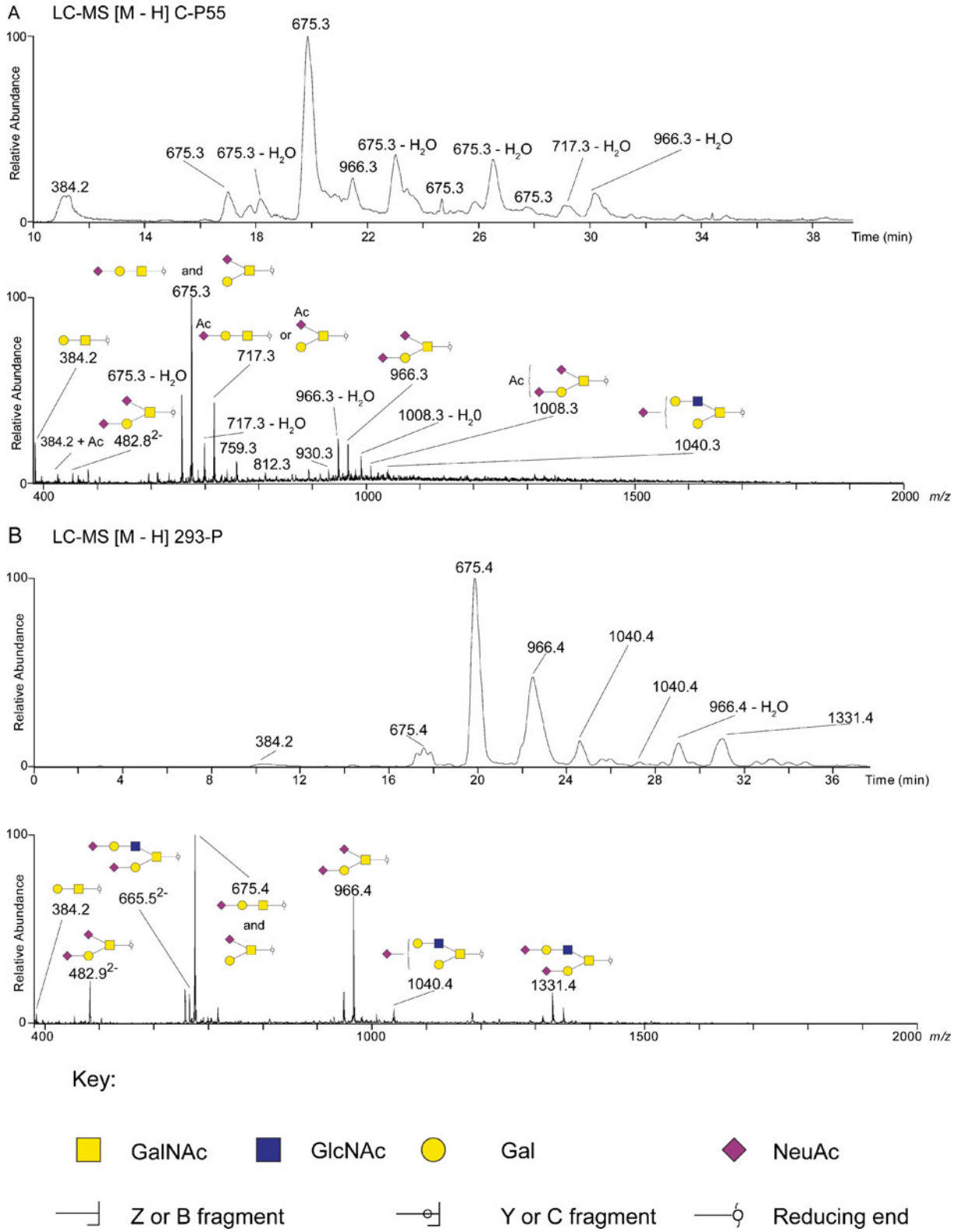

Fig. 2 LC-MS chromatogram and spectra in negative mode of *O*-glycan oligosaccharide alditols released from PSGL-1/mIgG$_{2b}$ produced in C-P55, 293-P and C-PSLex cells. Major compositions are assigned in the base peak chromatogram and spectra of alditols derived from C-P55 (A), 293-P (B) and C-PSLex (C and D). Tentative carbohydrate structures are assigned with cartoons according to the nomenclature suggested by Varki *et al.* [68]. The fragment ions of the C-PSLex LC-MS^2 spectrum of [M—H] ion of *m/z* 749 (D) are annotated according to nomenclature suggested by Domon and Costello [69]

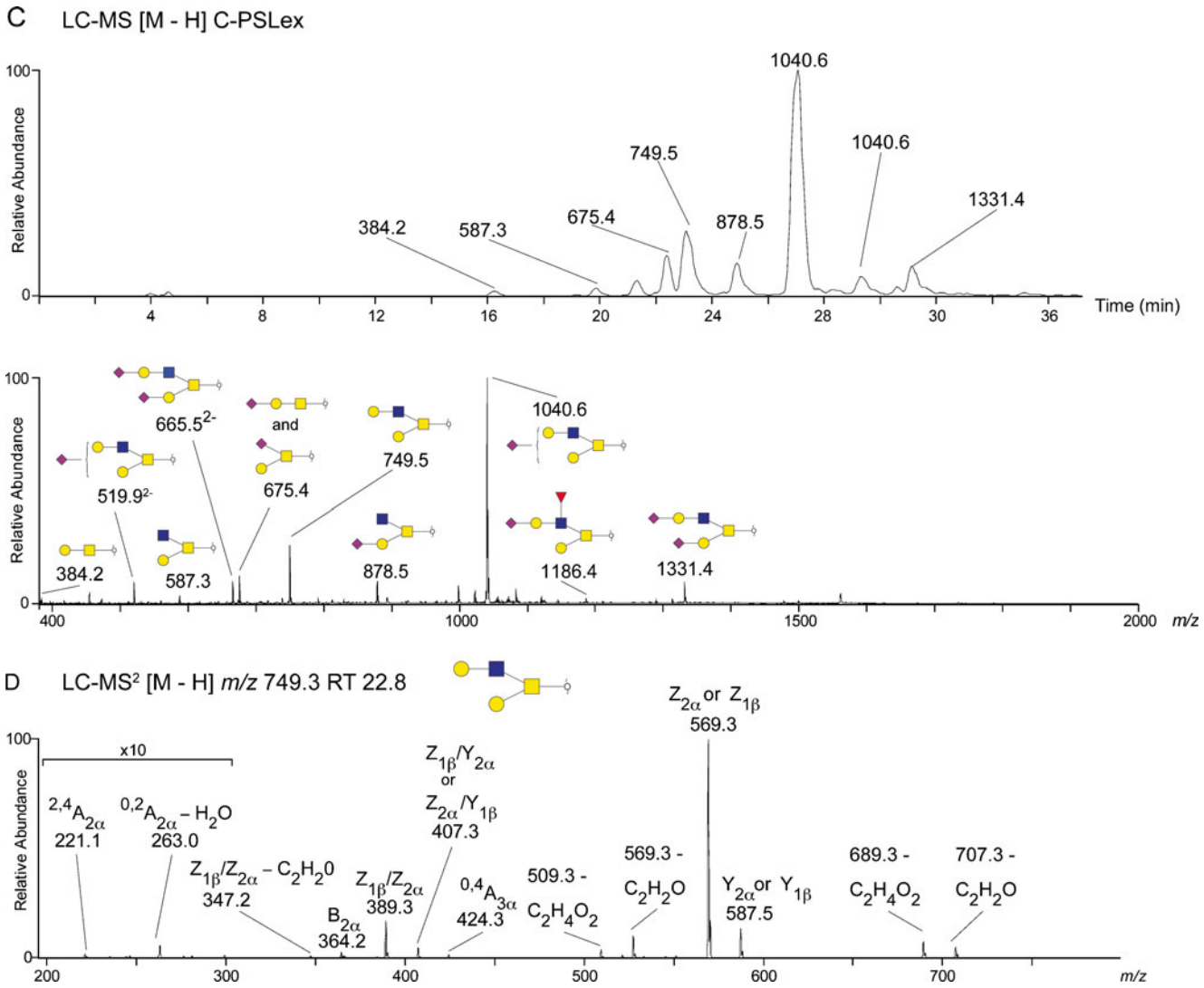

Fig. 2 (continued)

spectrum, we suggest that the major ions represent mono- and disalylated core 1, but significant ions representing tentative lactosamine on core 2 with one or both chains terminated with sialic acid are also observed (*m/z* 1040.4 and 1331.4).

C-PSLex The chromatogram and composite spectrum of *O*-glycans derived from PSGL-1/mIgG$_{2b}$ produced in the C-PSLex cell line are shown in Fig. 2c. In contrast to the spectra of C-P55 and 293-P, the major ion in the C-PSLex derived spectrum represents monosialylated lactosamine on core 2 (*m/z* 1040.6). The position of the sialic acid could not be determined. A range of smaller but significant ions representing core 2 (*m/z* 587.3) and extended structures thereof were also observed, including sialylated core 2 (*m/z* 878.5), tentative lactosamine on core 2 (*m/z* 749.5) and tentative lactosamine on core 2 with both chains terminated with sialic acid (*m/z* 1331.4). Core 1 with and without sialic acid was also observed (*m/z* 384.3 and *m/z* 675.4). The minor ion *m/z* 1186.4 represents a tentative SLe^x structure on core 2, *i.e.* Siaα3Galβ4(Fucα3)GlcNAcβ6(Galβ3)GalNAcol.

An LC-MS based method by which a 1-4 linkage of terminal Hex or HexNAc residues can be confirmed has previously been developed (Schulz *et al.* 2002). The presence of the diagnostic $^{0,2}A_{2\alpha}$ [M – H] fragment ion of *m/z* 263.0 in the MS^2 spectrum of the *m/z* 749.3 mother ion suggest that the terminal Hex is 1-4 linked (Fig. 2d). We therefore suggest that the *m/z* 1040.6 ion represents sialylated lactosamine on core 2, Siaα3Galβ4GlcNAcβ6(Galβ3)GalNAcol.

Lectin Western blot indicates the presence of terminal Siaα2-3 on lactosamine on PSGL-1/mIgG$_{2b}$ produced in 293-P and C-PSLex cell lines

PSGL-1/mIgG$_{2b}$ produced in C-P55, 293-P and C-PSLex cells was affinity purified and gel filtered prior to being

analyzed by Western blot using antibodies and lectins. PSGL-1/mIgG$_{2b}$ purified from C-P55 (lane 1), 293-P (lane 2) and C-PSLex (lane 3) stained strongly with both goat anti-mouse IgG Fc-HRP (Fig. 3a) and anti-PSGL-1 (Fig. 3b), while PSGL-1/mIgG$_{2b}$ from P-PM (control, lane 4) stained strongly only with goat anti-mouse IgG Fc-HRP, which has been noted before [39].

Western blot using MAL-I recognizing sialylated lactosamine (Siaα2-3Galβ1-4GlcNAc), binds strongly to PSGL-1/mIgG$_{2b}$ produced in 293-P and C-PSLex cells (Fig. 3c, lane 2 and 3), but only weakly to PSGL-1/mIgG$_{2b}$ produced in C-P55 (Fig. 3c, lane 1). Binding was also seen to bovine fetuin which has been shown to carry sialylated lactosamine structures (Fig. 3c, lane 6) [45].

MAL-II recognizes monosialylated core 1 (Siaα2-3Galβ1-3GalNAc) and showed strong binding to PSGL-1/mIgG$_{2b}$ produced in C-P55 and 293-P (Fig. 3d, lane 1 and 2) and weaker binding to C-PSLex (Fig. 3D lane 3), while no binding was seen to the fusion protein produced in *Pichia pastoris* (P-PM, Fig. 3d, lane 4). The lack of MAL-I binding to the positive controls AGP, which reportedly carries terminal Siaα2-3 and Siaα2-6 [46], and Siaα2-3-LacNAc-APE-HSA indicates that the controls may present Siaα2-3 in a different manner than the fusion proteins.

SNA (recognizing sialic acid α2-6-linked to terminal galactose) Western blots revealed staining of bovine fetuin and human transferrin which both carry sialic acid in an α2-6-linkage to lactosamine [45, 47]. No staining was observed to PSGL-1/mIgG$_{2b}$ produced in C-P55, 293-P and C-PSLex. The results of the lectin experiments are in good agreement with the LC-MS structural characterization of the *O*-linked glycans derived from the fusion proteins (Fig. 2a–d).

The avian influenza H5 hemagglutinin binds strongly to sialylated PSGL-1/mIgG$_{2b}$ and Siaα2-3-LAcNAc-APE-HSA in a Biacore assay

The H5N1 A/Vietnam/1203/04 hemagglutinin (ligand) was immobilized on the dextran surface with amine coupling chemistry and the fusion proteins or controls (analytes) flowed over the surface and binding was measured in real-time. Representative sensorgrams of the fusion protein derived from C-P55 (Fig. 4a), 293-P (Fig. 4c), C-PSLex (Fig. 4e), positive control Siaα2-3-LAcNAc-APE-HSA (Fig. 5a), negative controls LacNAc-APE-HSA (Fig. 5c) and P-PM (Fig. 5d) are shown. The binding curves obtained for the different fusion proteins are similar with a rapid buildup of analyte-ligand complex and most of the curves stabilizing near steady-state at the end of the injections (Fig. 4a, 4c and 4e). In contrast, the positive control Siaα2-3-LacNAc-APE-HSA curves have a slower association and do not reach steady-state (Fig. 5a). The dissociation phase of the fusion proteins and Siaα2-3-LacNAc-APE-HSA derived curves display an initial sharp drop in response signal, while the subsequent dissociation was markedly slow, which indicates stable binding of the analyte to the ligand. The negative controls lacking the sialic acid that is needed for specific binding to hemagglutinin did not bind well. *Pichia pastoris* derived PSGL-1/mIgG$_{2b}$ (P-PM), which is heavily mannosylated, and LacNAc-APE-HSA bound weakly to H5 and generated curves below zero after reference subtraction. These results show that no specific binding occurred with these analytes (Fig. 5c and 5d).

Estimations of affinity

None of the sensorgrams could be fitted to a simple 1:1 binding model in order to calculate K_a and K_d. This indicates that the analyte-ligand interactions are more complex. The steady-state section of the curves near the end of the injections was thus used in order to calculate the dissociation equilibrium constant, K_D. The response (RU) *vs.* concentration was plotted and fitted to a simple 1:1 model fit available in the Scrubber 2 software. The fitted concentration plots and the calculated kinetic constants are shown in Fig. 4b, 4d and 4f and Table 1. The C-P55, 293-P and C-PSLex derived sensorgrams showed similar binding curves. Consequently, the equilibrium dissociation constant, K_D, was comparable between the different PSGL-1/mIgG$_{2b}$ proteins analyzed: C-P55 0.8±0.1 nM; 293-P 0.8±0.1 nM and C-PSLex 3.1±1 nM

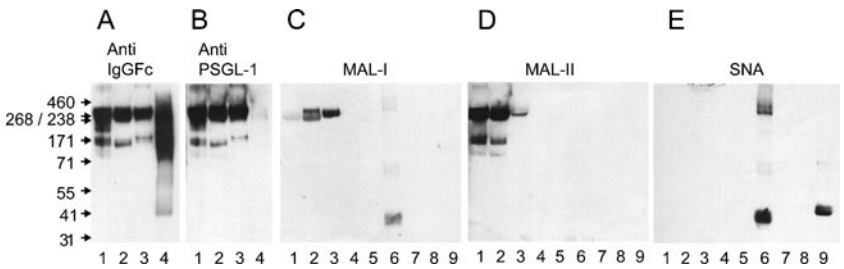

Fig. 3 Lectin Western blot analyses of PSGL-1/mIgG$_{2b}$ produced in C-P55, 293-P and C-PSLex. The following samples were applied: lane 1, C-P55; 2, 293-P; 3, C-PSLex; 4, P-PM (control); 5, Galα1-3Gal-BSA; 6, bovine fetuin; 7, human AGP-1; 8, 3′-Neu5Ac-*N*-Acetyllactosamine-APE-HSA; 9, human transferrin. Five μg of fusion protein, fetuin, AGP-1 and transferrin, and 500 ng of BSA-neoglycoconjugates were loaded per lane

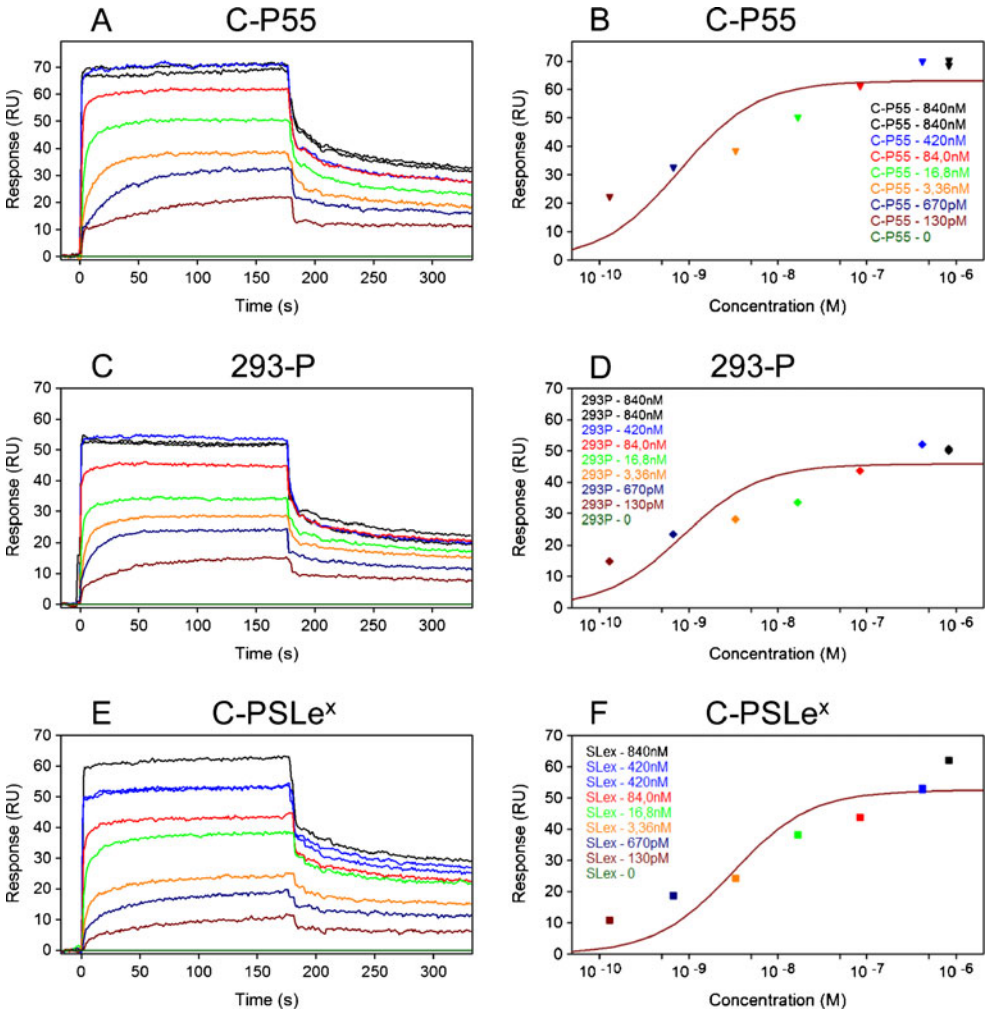

Fig. 4 SPR sensorgrams and steady-state concentration plots of PSGL-1/mIgG$_{2b}$ binding to immobilized H5. Recombinant H5 (A/H5N1/Vietnam/1203/2004) ligand was immobilized on CM5 dextran surfaces with amine coupling chemistry. The binding curves and concentrations plots of steady-state curves are shown for injected analytes: PSGL-1/mIgG$_{2b}$ produced in C-P55 (A and B), 293-P (C and D) and C-PSLex (E and F), respectively

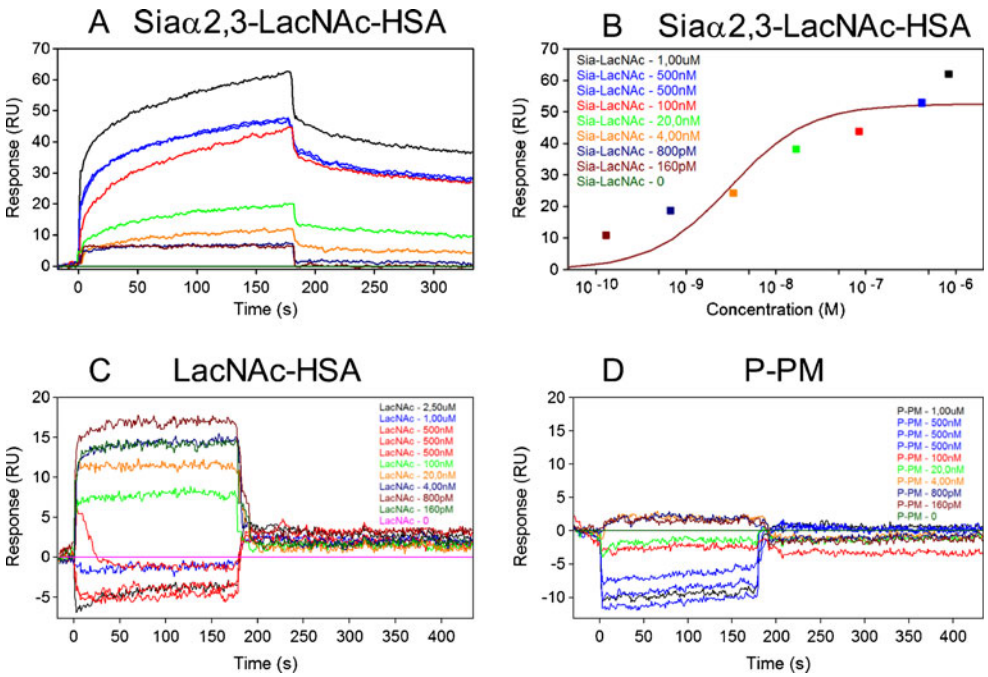

Fig. 5 SPR sensorgrams and steady-state concentration plots of positive and negative controls. Recombinant H5 (A/H5N1/Vietnam/1203/2004) ligand was immobilized on CM5 dextran surfaces with amine coupling chemistry. The binding curves and concentrations plots of steady-state curves are shown for the positive control Siaα2-3-LacNAc-APE-HSA (A and B). The binding curves of the negative controls LacNAc-APE-HSA and PSGL-1/mIgG$_{2b}$ produced in *Pichia pastoris* (P-PM) are shown in (C) and (D), respectively

Table 1 Equilibrium dissociation constants (K_D) of PSGL-1/mIgG$_{2b}$ and neoglycoconjugates for binding to H5 (A/Vietnam/1203/04), determined with 1:1 binding model of steady-state curves (160-180 s)

Analyte	Equilibrium dissociation constant (K_D)±standard deviation of fit (nM)
C-P55 produced PSGL-1/mIgG$_{2b}$	0.8±0.1*
293-P produced PSGL-1/mIgG$_{2b}$	0.8±0.1
C-PSLex produced PSGL-1/mIgG$_{2b}$	3.1±0.3
Siaα2,3LacNAc-HSA	29±2
LacNAc-HSA	n.d[a]
P-PM produced PSGL-1/mIgG$_{2b}$	n.d

[a] not determined

*standard deviation of fit

(Table 1). The K_D value for Siaα2-3-LacNAc-APE-HSA obtained in the same manner was 29±2 nM. The true K_D values for the fusion proteins and Siaα2-3-LacNAc-APE-HSA may be greater than or equal to the values obtained because the response *vs.* concentration plots did not reach a clear plateau at high concentrations, which indicates that the surface was not saturated (Fig. 4b, 4d and 4f). The obtained affinity values are the sum of multiple binding sites and will thus reflect the avidity, rather than the affinity.

Discussion

The aim of this study was to evaluate if recombinant mucins with tailored glycosylation may serve as efficient binders of the highly pathogenic H5N1 A/Vietnam/1203/04 hemagglutinin. We chose to monitor the avian H5N1 A/Vietnam/1203/04 strain since it is considered to be one of the most threatening of the emerging avian influenza strains, with very high death rates in reported outbreaks [19].

PSGL-1/mIgG$_{2b}$ produced in stable cell lines carry Siaα2-3 on different core chains

In order to find a suitable binding partner of the avian H5 hemagglutin, we have produced PSGL-1/mIgG$_{2b}$ carrying the Siaα2,3Gal terminal on different carbohydrate core chains. For this purpose the cell lines C-P55, 293-P and C-PSLex were used. The C-P55 cell line is only transfected with the cDNA encoding PSGL-1/mIgG$_{2b}$ and represents the mother cell line that we further engineered in order to produce the fusion protein carrying the ABH blood group antigens on type 1, 2 and 3 chains [38]. CHO cells mainly express core 1 based *O*-glycans and have limited capacity for terminal Siaα2-6 sialylation [48, 49]. In addition, we have shown that there is an active endogenous β1,4-galactosyltransferase which will elongate core 2 structures with a type 2 chain, if a core 2 substrate is provided [31]. The LC-MS spectrum of *O*-glycans from PSGL-1/mIgG$_{2b}$ derived from the C-P55 cell line was dominated by ions representing a tentative Siaα3Galβ3GalNAcol *O*-glycan (Fig. 2a). The isomeric Galβ3(Siaα6)GalNAcol glycan that eluted earlier from the column is also observed but is a less abundant ion. An unexpected finding was the minor ion representing a tentative core 2 structure carrying a type type 2 chain with terminal sialic acid, *i.e.* Siaα3Galβ4GlcNAcβ6(Galβ3)GalNAcol or Galβ4GlcNAcβ6(Siaα3Galβ3)GalNAcol. Contamination with culture medium-derived glycans is less likely since the cells were grown in serum-free medium. However, it is possible that silent genes have somehow been activated. The CHO genome contains homologues to 99 % of all human glycosylation-associated transcripts, but only half of them are expressed [48].

The 293-P cell line (HEK-293 origin) has the capacity to produce the fusion protein with simple elongated core 2 structures, including sialylated lactosamine. We have previously utilized 293-T cells to probe the activity of human Se and H α1,2 glycosyltransferases and showed that the blood group A epitope can be produced in 293-T cells that are transfected with the genes encoding these enzymes and the blood group A gene [41]. The HEK-293 cells are of human origin but it is not clear if these cells have the capacity to make glycans with terminal Siaα2,6Gal. The 293-P derived LC-MS spectrum contained a mixture of tentative Siaα3Galβ3GalNAcol and Siaα3Galβ3(Siaα6)GalNAcol, with small amounts of tentative mono- (Siaα3Galβ4GlcNAc(Galβ3)GalNAcol) and disialylated (Siaα3Galβ4GlcNAc(Siaα3Galβ3)GalNAcol) type 2 on core 2 chain structures (Fig. 2b). In a variant of the HEK-293 cells, 293-T, we previously detected tentative *O*-linked Galα1-3Gal epitopes and NeuGc glycans [42]. In this study, none of these structures were detected among the released *O*-glycans from PSGL-1/mIgG$_{2b}$ produced in 293-P cells (Fig. 2c). A possible explanation to the discrepancy is that the 293-T cells were grown with serum-containing medium, while the 293-P cells were grown in serum-free medium.

The C-PSLex cell line was originally developed together with C-PLeb in order to produce mucins with the binding epitopes of *H. pylori*, SLe^x and Le^b [42]. The cell line was established by transfecting CHO-K1 cells with cDNA encoding the core 2 β1,6*N*-acetylglucosaminyl transferase I (C2 GnT-I) and FUT-VII [50]. Even though the levels of *O*-linked SLe^x was limited, the activity of the core 2 enzyme was enough to generate high levels of sialylated lactosamine [42]. The C-PSLex derived LC-MS spectrum was dominated by Siaα3Galβ4GlcNAc(Galβ3)GalNAcol (Fig. 2c).

The lectin Western blot staining indicated the presence of Siaα2-3-terminated core 1 on PSGL-1/mIgG$_{2b}$ produced in

C-P55 and 293-P (Fig. 2d, lane 1 and 2) and Siaα2-3-terminated lactosamine on PSGL-1/mIgG$_{2b}$ derived from 293-P and C-PSLex (Fig. 2c, lane 2 and 3). The lack of SNA binding to all fusion proteins (Fig. 2e, lane 6 and 9) indicates that terminal Siaα2-6Gal is not abundant on the fusion proteins.

H5 binds strongly to multivalent Siaα2-3 on PSGL-1/mIgG$_{2b}$ and Siaα2-3-LacNAc-APE-HSA

A Biacore biosensor analysis was set up where the recombinant H5 A/Vietnam/1203/04 hemagglutinin was immobilized on the chip surface and the fusion proteins or controls (analytes) flowed over the surface at different concentrations with binding monitored in real-time. Strong binding to the immobilized H5 was observed for the fusion proteins produced in C-P55, 293-P and C-PSLex (Fig. 4). The neoglycoconjugate control Siaα2,3-LacNAc-APE-HSA, also bound strongly to the H5 surface (Fig. 5), while LacNAc-APE-HSA and P-PM lacking sialic acid did not appear to bind specifically to hemagglutinin since reference subtraction generated negative response curves (Fig. 5). High levels of background binding were noted for all analytes, possibly through interaction with the dextran surface. Using a chip surface with lower degree of carboxymethylation (CM4) with reduced negative charge might have circumvented this problem. The surfaces with immobilized hemagglutinin were not stable enough for consecutive experiments with different analytes. Therefore new surfaces were coated for each analysis. Since the surfaces differed in the immobilized levels of hemagglutinin, direct comparison of the relative responses of the different fusion proteins may not be possible. The fusion proteins and Siaα2-3-LacNAc-APE-HSA did not saturate the surface as demonstrated by the almost linear concentration *vs.* log response plots (Fig. 4b, 4d, 4f and 5b). Injections of fusion proteins at higher concentrations sometimes resulted in erratic behaviour of the curves, but we believe that all fusion proteins have a similar binding capacity. The multivalency of the fusion proteins and H5 may explain the slow decrease in response signal during dissociation (buffer injection starting at 180 s, Fig. 4a, 4c, 4e and 5a). Initially, weakly bound proteins come off, but subsequent dissociation is slow due to the strong binding of PSGL-1/mIgG$_{2b}$. The multiple binding sites may lead to a Velcro-effect where many Siaα2-3 molecules contribute to the binding to H5.

PSGL-1/mIgG$_{2b}$ - H5 binding avidity

The K_D values of PSGL-1/mIgG$_{2b}$ produced in C-P55, 293-P and C-PSLex were calculated from the sensorgrams of steady-state curves using a simple 1:1 model. The calculated values for all fusion proteins were in the low nanomolar range, with the K_D value of Siaα2-3-LacNAc-APE-HSA being slightly worse than the different PSGL-1/mIgG$_{2b}$ variants (Table 1). The calculated affinities are representing the combined strength of all binding sites involved in the binding complex and reflect the avidity, rather than the affinity. The calculated K_D values for the fusion proteins and Siaα2-3-LacNAc-APE-HSA may be lower than the real K_D values because the concentration plots did not appear to reach saturation (Fig. 4b, 4d, 4f and 5b). Both the mucins and the influenza H5 have multiple binding sites, which are not accounted for in the simple 1:1 binding model. Even if such a model existed, it would not be feasible to use it because the exact valency of the mucins and the immobilized HA is not known in this system. The experimental set-ups of previous SPR studies that have measured the affinity of influenza virus and recombinant hemagglutinin to various carbohydrate receptors vary greatly and the methods used to determine the kinetic constants were not always the same or not always shown. This should be taken into account when comparing the determined K_D values of these studies. However, it is interesting to note that the SPR-obtained K_D values of recombinant H5 binding to glycoconjugates [24], influenza virus binding to gangliosides (H1N1 and H3N2 [51]) and influenza virus binding to the glycoprotein fetuin (H1N1 [52]) have all been reported to be in the low nanomolar range. This is in the same range as the K_D values we report in this study.

H5 receptor specificity

The PSGL-1/mIgG$_{2b}$ proteins used in this assay do not carry a single homogenous well-defined carbohydrate structure (LC-MS spectra of Fig. 2a–d) as would be preferred if the goal is to determine the fine receptor specificity of the H5N1 (A/Vietnam/1203/04) hemagglutinin. Based on the data obtained by LC-MS (Fig. 2a–d), lectin Western blot (Fig. 3a–e) and the Biacore sensorgrams (Figs. 4 and 5), we suggest that the H5N1 (A/Vietnam/1203/04) hemagglutinin requires Siaα2-3Gal for binding. However, it does not discriminate between the different core saccharide chains carrying this disaccharide, *i.e.* Siaα3Galβ3GalNAc, mostly abundant in C-P55 (Fig. 2a), or Siaα3Galβ4GlcNAcβ6(Galβ3)GalNAc which is the dominant structure on PSGL-1/mIgG$_{2b}$ produced in C-PSLex (Fig. 2c).

Our reported binding specificity of H5 (A/Vietnam/1203/04) is in close agreement with a recent Biacore biosensor study determining the fine receptor specificity of H5 (A/Vietnam/1203/04) using multivalent defined glycoconjugates. H5 binds equally well to poly(acrylic acid)amide (PAA) conjugates carrying sialylated core 1 and sialylated lactosamine in a Biacore single cycle kinetics assay, while notably other strains were more discriminating in their binding epitope requirements [24]. The calculated K_D values were in the low nanomolar range for Siaα2,3 on core 1 (2.9 nM), type 1 (2.0 nM) and type 2 (1.6 nM) chains, while no binding was observed to α2-6-linked sialic acid on type 2 chain. In another study,

Stevens *et al.* set up a glycan microarray assay with an immobilized library of glycans and glycoproteins. The highest binding signal of recombinant H5N1 A/Vietnam/1203/04 hemagglutinin was observed to α_1-acid glycoprotein (orosomucoid) [23]. α_1-acid glycoprotein is heavily glycosylated and is known to carry Siaα2-3 and Siaα2-6 in a multivalent fashion (5*N*-glycosylation sites) [46]. Among the glycoconjugates screened, structures carrying terminal Siaα2-3- bound well, while almost no binding was observed to glycans carrying Siaα2-6-. The best glycoconjugate ligands were substituted with sulfate, such as sulfated sialylated core 1 (Siaα3Galβ3(S6-)GalNAcα−), sulfated sialylated lactosamine (Siaα3Galβ4(S6-)GlcNAcβ-) and sulfated sialyl Lewis x (Siaα3Galβ4(S6-)(Fucα3) GlcNAcβ). Taken together, these studies demonstrate that H5 bind to several receptors carrying terminal Siaα2-3, while Siaα2-6 binding is absent, and that multivalent presentation of the receptor yields a strong binding.

PSGL-1/mIgG$_{2b}$ as a potential inhibitor of influenza virus

There is a general understanding that multivalency is important in biological systems, including ligand receptor interactions [53]. In the case of virus, the multiple copies of binding molecules in the virus envelope result in a near irreversible association to the host membrane. In order to inhibit virus attachment, a suitable inhibitor must thus bind with higher affinity than the natural HA receptor, be present in higher concentration than the natural receptor or possess an even higher avidity than the natural HA receptor. The first strategy includes using synthetic mono- or multivalent compounds, while the inhibitory effect of PSGL-1/mIgG$_{2b}$ would be to competitively inhibit the binding of virus hemagglutinin by presenting the natural Siaα2-3Gal receptor in multiple copies. Studies on deformable and non-deformable spherical multivalent nanoparticles, suggest that good binders of virus particles should be large and not too rigid [53]. A large flexible binding partner can interact with several hemagglutinin molecules on the surface of virus particles, which a small and solid inhibitor cannot [53]. Although the strong binding between PSGL-1/mIgG$_{2b}$ and H5 observed in the Biacore assay indicate that the binding is multivalent, it remains to be shown if PSGL-1/mIgG$_{2b}$ can also bind multiple H5 trimers in the envelope of avian influenza A virus. While some of the synthetic multivalent binding partners used to study the fine receptor specificity of influenza may not be appropriate for *in vivo* use due to toxicity problems [17, 54], we believe the toxicity of PSGL-/mIgG$_{2b}$ is low. In this study we have demonstrated that PSGL-1/mIgG$_{2b}$ with tailored glycosylation bind specifically to avian influenza H5 *in vitro*. However, introducing foreign glycoproteins into the human body (in circulation or topical treatment) always warrants great caution since unintended immune responses and rapid clearance should be avoided. One concern may be that recombinant PSGL-1/mIgG$_{2b}$ may somehow interfere with the natural binding between human PSGL-1 and P- and E-selectins if introduced in high concentrations. Conceivably, this risk can be avoided if recombinant PSGL-1/mIgG$_{2b}$ does not carry the sialyl-Lewis x epitope and sulfation that is required for specific binding [36]. Our analysis of PSGL-1/mIgG$_{2b}$ suggest that C-P55 and 293-P may thus be more suitable than C-PSLex in this regard (Fig. 2a–c).

The mouse IgG Fc portion of PSGL-1/mIgG$_{2b}$ may be immunogenic if introduced in humans, and the fusion protein may also be targeted to Fc gamma receptors with unwanted immune reaction as a result. In order to largely avoid these problems a fusion protein carrying a human IgG Fc portion should thus be designed, preferably non-glycosylated to avoid Fc gamma receptor targeting [55].

We have previously shown that mannosylated PSGL-1/mIgG$_{2b}$ can improve immune responses in mice (possibly by targeting immune lectins such as DC-SIGN, MR and MBL) [37, 39] and that PSGL-1/mIgG$_{2b}$ carrying different blood group determinants bind specifically to chain type specific human antibodies [38, 44]. However, for a multivalent glycan inhibitor, both these types of immune reactions are undesirable.

Siglecs are a group of sialic-acid binding membrane bound lectins that belong to the immunoglobulin supergene family. Cell bound Siglecs may bind to glycan determinants terminated with sialic acid within the same cell membrane (*cis* binding) or on other cells (*trans* binding). The role of the many Siglecs is still being explored but they appear to be involved in immune cell-cell interactions and may have a regulatory role [56]. Some pathogens bind to Siglecs and thereby improve uptake and possibly modulate the immune response [56]. It seems that the function of Siglecs is closely related to the balance between *cis* and *trans* binding. It is thus possible that high concentrations of sialylated glycoproteins such as PSGL-1/mIgG$_{2b}$ may interfere with this balance with unknown immune-modulatory effects.

The effect of glycosylation on pharmacokinetic stability of therapeutic proteins has been studied for a long time [57, 58]. It has been realized that many lectins are involved in clearance of (glyco-)proteins from the circulation and that insufficient glycosylation lead to rapid clearance from the blood stream [59]. We believe that the large size (avoid excretion *via* kidneys) and terminal sialylation should render an acceptable half-life of PSGL-1/mIgG$_{2b}$.

A family of C-type lectins in innate immunity has been suggested to be pattern recognition receptors (PRRs) similar to Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs) [60, 61]. PRR binding may trigger many immunological events such as phagocytosis by macrophages, activation of natural killer cells or induction of genes encoding costimulatory molecules, cytokines or chemokines. Several PRR lectins recognize Lewis blood

group antigens, why these glycans should be avoided on a multivalent glycan inhibitor [60].

The use of a sialic acid receptor that is identical to the natural binding target of the virus lowers the risk of escape mutations, which have started to appear against the neuraminidase inhibitors [30] [29]. An inhibitor that closely resembles the natural receptor of HA may also become a substrate of influenza neuraminidase. Hydrolysis of sialic acid by neuraminidase has been suggested to contribute to release of budding virus particles from infected cells [62]. However, a greater understanding of what role the neuraminidase plays during penetration of the mucus layer and subsequent binding to the host cell surface is needed. Inhibiting an enzyme such as neuraminidase has been more efficient compared to blocking an attachment site such as hemagglutinin. This may be explained by the notion that an enzyme has a high turnover number, while the hemagglutinin molecule may be used only once [63]. Because there is a fine balance between the activities of NA and HA it should be possible to inhibit host cell attachment with a multivalent inhibitor carrying the natural influenza receptors without further modifications of the binding epitope. A possible future application in man of an avian influenza inhibitor could be as a topical treatment of the eyes based on the notion that avian influenza may gain access to the respiratory tract *via* the ocular route.

Material and methods

Lectins, antibodies and neoglycoconjugates

Goat anti-mouse IgG Fc (cat no M4280), goat anti-mouse IgG Fc-HRP (cat no A2554), goat anti-mouse IgG(Fab)-HRP (cat no A2304) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The mouse anti-PSGL-1 antibody, CD162 (clone KPL-1, cat no 556052), was bought from BD PharMingen (San Diego, CA). Biotinylated *Maackia amurensis* lectins MAL-I, MAL-II and *Sambucus nigra* agglutinin (SNA) were acquired from Vector laboratories (Burlingame, CA, USA). *N*-Acetyllactosamine-aminophenylethyl (APE)-human serum albumin (HSA) (MW 72340) and 3′-Neu5Ac-*N*-Acetyllactosamine-APE-HSA (MW 70590) was purchased from IsoSep (Botkyrka, Sweden). Both have a substitution grade of 10 mol carbohydrate/mol protein. Galα1-3Gal-BSA was bought from Dextra Laboratories (Reading, UK). Bovine fetuin (F3385), human alpha 1-acid glycoprotein, AGP-1, (G9885) and human transferrin (T2036) were purchased from Sigma-Aldrich.

Engineered cell lines

HEK-293 cells were transfected with plasmids encoding the PSGL-1/mIgG_{2b} fusion protein resulting in the stable cell line 293-P, as described previously [31], with the exception of using HEK-293 cells (ATCC CRL-1573) instead of 293-T cells [42]. The C-P55 cell line was established by transfection with cDNA encoding PSGL-1/mIgG_{2b} as described before [38]. The stable cell line, C-PSLex, expressing the fusion protein, the C2 β1,6GnTI and FUT-VII was generated as previously described [50].

Large scale cultivation of 293 and CHO cells

Large scale culture of 293-P was performed on a Wave bioreactor (Wave System 20/50 EH, GE Healthcare, Uppsala, Sweden; 20 L bag) in 293 s serum free media (293 s CDM serum free media, Lonza) supplemented with penicillin 50 U/ml, 50 μg/ml streptomycin (PEST, Invitrogen, Carlsbad, USA), L-glutamine 4 mM/ml (Invitrogen), Puromycin 2 μg/ml (Invitrogen) and dextransulfate 50 μg/ml (Mr 5000, Fluka, Sigma-Aldrich). The culture was started at a cell density of 0.74×10^6 cells/ml in a volume of 5.7 L. A continuous cell medium feed set to 39 ml/h facilitated steady growth and pH was maintained above 7.0 with sodium carbonate until a maximum volume of 10.8 L was reached. Cell viability and pH was monitored daily and the culture was stopped when viability dropped to 3.2×10^6 cells/ml (77 % viability). Culture of C-PSLex and C-P55 were performed as described previously [31, 38].

Purification of PSGL-1/mIgG_{2b}

The 293-P cell culture supernatant was prepared for subsequent chromatographic purification of the secreted fusion proteins by removing the cells using microfiltration and concentrating the cleared supernatant 20x followed by buffer exchange to PBS using diafiltration, as described previously [42]. A protease inhibitor cocktail (1 mL/L, Sigma-Aldrich) and 0.02 % NaN_3 (Sigma-Aldrich) were added to the product solution, which was stored at 4 °C until purification. Affinity purification and size exclusion chromatography was performed on an ÄKTAExplorer 100 instrument with MabSelect SuRe (protein A) and Sephacryl S-300 columns as described previously [42]. Purification of PSGL-1/mIgG_{2b} derived from cell culture supernatants of C-PSLex and C-P55 was performed as described previously with the exception that the fusion protein derived from C-PSLex was eluted from the size exclusion column Sephacryl S-200 column with 3 M sodium thiocyanate [31, 38].

Purified fractions were pooled based on WB analysis, dialyzed against water (12–14 kDa cutoff, Spectrapor, Houston, TX, USA), lyophilized and stored in −80 °C until further use.

Quantification of PSGL-1/mIgG_{2b} with ELISA

The concentrations of recombinant PSGL-1/mIgG_{2b} protein in supernatants and in purified fractions were

determined by a two-antibody sandwich ELISA method as described previously [42].

SDS-PAGE and Western blot

The fusion protein was analyzed with WB on blotted nitrocellulose membranes and directly on SDS-PAGE gels stained with the ProQ Emerald 300 Glycoprotein Detection kit and Ruby stain (Molecular Probes, Leiden, the Netherlands) as described previously [42]. Lectins and antibodies that were used are listed in a separate section above.

Chemical release of O-linked glycans from purified PSGL-1/mIgG$_{2b}$

Glycans were released by reductive β-elimination and analyzed as native reduced structures. Briefly, lyophilized PSGL-1/mIgG$_{2b}$ proteins were dissolved in water to 1 mg/ml concentration. The glycans were released in a solution of 1.5 M $NaBH_4$ in 50 mM KOH and incubated over night at 60 °C. The reaction was stopped with addition of concentrated acetic acid. The released glycans were desalted on a homemade column composed of AG50WX8 cation exchange beads (Bio-Rad, Hercules, CA, USA) packed on top of a micro-tip with glass wool as stopper. Samples were eluted with water and dried in a SpeedVac concentrator. Borate complexes were removed with 1 % acetic acid in methanol and subsequent vacuum centrifugation (5x). The dried glycans were dissolved in water for analysis on LC-MS.

Mass spectrometry

Liquid chromatography electrospray MS/MS was performed on a graphitized carbon column coupled to an LTQ LC/MS^n ion trap instrument. (ThermoFinnigan, San Jose, CA). The method was previously developed by Karlsson *et al.* [64]. The column was packed with 5 μm Hypercarb particles (Thermo, Hypersil-Keystone, Runcorn, UK). The glycans were eluted with a gradient from 0–40 % acetonitrile in 8 mM NH_4HCO_3 buffer and detected in negative mode by full scans (*m/z* 383–2000) followed by MS^2 scans of the most intense ions. The needle voltage was −3.2 kV. Interpretation and annotation of MS^n spectra was facilitated by the GlycoWorkBench software which allows *in-silico* fragmentation of glycans [65, 66]. Identification of structures was also based on diagnostic fragments ions and fragment ion pattern matching to structures found in the glycan MS database Unicarb-DB (http://www.unicarb-db.org/), last accessed March 15 2013 [67].

Establishing a SPR method with immobilized hemagglutinin

A Biacore biosensor analysis was set up in which the H5 hemagglutin (ligand) was immobilized on the dextran surface with amine coupling chemistry and the fusion proteins or controls (analytes) were flowed over the surface. The advantage with this approach is that only a limited amount of hemagglutinin is needed, while the disadvantage is that the relative instability of the hemagglutin protein makes regeneration of the surface difficult. HA is meta-stable and will undergo a conformational change between pH 5.0–5.5 which means that low pH is not suitable during immobilization and regeneration of the chip surface [5]. After testing a range of buffers (acidic-, basic-, hydrophobic- and ionic- conditions) 30 s spikes of 1 M NaCl, 0.05 % P20 was selected as regeneration regimen. However, the activity of the surface still decreased slightly for each cycle so each surface with immobilized H5 hemagglutinin was only used for two consecutive experiments.

Realtime SPR analysis

The analyses were performed using a Biacore 2000 instrument with a research-grade CM5 sensor chip (Biacore, GE Healthcare). The ligand, recombinant full length H5N1 A/Vietnam/1203/04 (iha-010, ProSpec, Ness Ziona, Israel) (70 kDa, >90.0 % pure as determined by SDS-PAGE) was immobilized using amine coupling chemistry. Briefly, the surfaces were activated for 7 min with a 1:1 mixture of 0.1 M NHS (*N*-hydroxysuccinimide) and 0.1 M EDC (3-(*N*, *N*-dimethylamino) propyl-*N*-ethylcarbodiimide) at a flow rate of 5 μl/min. The ligand was injected at a concentration of 16 μg/ml in 10 mM sodium maleate buffer pH 6.7 (Sigma-Aldrich), 0.05 % surfactant P20 (GE Healthcare), for 30 min at 5 μl/ml flow rate resulting in a density of approximately 3000–5000 response units (RU). The following levels of hemagglutinin ligand were immobilized for the presented sensorgrams: C-PSLex 2500 RU; C-P55 5000 RU; 293-P 3300 RU; P-PM 3600 RU; Siaα2,3-LacNAc-APE-HSA 4200 RU and LacNAc-APE-HSA 4100 RU. Channel one on the CM5 sensor chip was only activated/deactivated and was used as reference sensorgram for subtraction of buffer effects. All the surfaces were blocked with a 7 min injection of 1 M ethanolamine, pH 8.0. The analytes, (C-P55 (~300 kDa), C-PSLex (~300 kDa), 293-P (~300 kDa) and P-PM (~250 kDa)) were dissolved in HBS-EP (10 mM HEPES, 150 mM NaCl, 0, 005 % P20, pH 7.4), GE Healthcare) supplemented with 0.05 % P20 and injected over the flow cells in a concentration series of 0.13, 0.67, 3.36, 16.8, 84, 420 and 840 nM at a flow rate of 20 μl/min and a temperature of 25 °C. Positive control Siaα2-3-LacNAc-HSA was injected with the dilution series 0.16, 0.8, 4.0, 20, 100 500 and 1,000 nM. The association complex was built up during 180 s and then dissociated for 600 s. Surfaces were regenerated with 30 s injection of 1 M NaCl 0.05 % P20 followed by 10 min stabilization time. Samples were injected in decreasing concentration order with at least one duplicate and buffer blanks. All experiments

except the negative control (LAcNAc-APE-HSA) were performed at least twice. Data were collected at 1 Hz and fitted using a 1:1 interaction model available in the Scrubber 2 software (BioLogic software, Campbell, Australia). The equilibrium dissociation constant (K_D) was calculated from steady-state sections of the curves between 160 and 180 s.

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Conflicts of interest J.H. is a part time CEO/CSO, board member and shareholder of Recopharma AB.

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