Mucin-type fusion proteins with blood group A or B determinants on defined O-glycan core chains produced in glycoengineered Chinese hamster ovary cells and their use as immunoaffinity matrices

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Assays for quantification, and methods for removal, of anti-A and anti-B antibodies are the key for the success of ABO incompatible organ transplantation programs. In order to produce tools that can be used as substrates in tests for anti-A/anti-B quantification and specificity determination or as affinity matrices in extracorporeal immunoadsorption (IA) columns, we engineered Chinese hamster ovary (CHO) cells secreting mucin-type fusion proteins carrying blood group A or B determinants on defined O-glycan core saccharide chains. Besides the P-selectin glycoprotein ligand-1/mouse immunoglobulin G2b (PSGL-1/mIgG2b) cDNA, CHO cells were transfected with plasmids encoding core 2 (β1,6GlcNAc-T1) or core 3 (β1,3GlcNAc-T6 and β1,3Gal-T5) enzymes together with α1,2Fuc-T1 or α1,2Fuc-T2 and the A or B gene-encoded α1,3GalNAcT or α1,3Gal-T, respectively. Selected clones with the correct glycoconstituent were expanded and cultured in shaker flasks and Wave biorreactors. Western blotting was used to characterize purified fusion protein and liquid chromatography–mass spectrometry was used to characterize the released O-glycans. Clones producing PSGL-1/mIgG2b carrying O-glycans with A and B determinants on type 1 (Galβ3GlcNAc) or type 2 (Galβ4GlcNAc) and type 3 (Galβ3GalNAcα) outer core saccharide chains were established. The conversion of CHO cells from exclusive inner core 1 (Galβ3GalNAc) to core 3 (GalNAcβ3GalNAc) O-glycan producers was almost complete, whereas conversion to inner core 2 (GalNAcβ6GalNAc) O-glycans was incomplete as was the α2-fucosylation of the core 1 chain. Sialylation may prevent these biosynthetic steps. The clinical utility of the blood group A and B substituted mucin-type fusion proteins as substrates in enzyme-linked immunosorbent assay or as affinity matrices in IA columns is explored.

Introduction

The blood group ABO system consists of four blood groups, A, B, AB and O, and was the first blood group system to be described. Quantitative and qualitative differences in A and B antigen expression between individuals have lead to the definition of blood group A and B subgroups, the most common of the former being A1 and A2 (Storry and Olsson 2009). The frequencies of the ABO blood groups vary in different populations. The clinical importance of the ABO system pertains to its role in transfusion medicine, where accidental transfusion across the ABO barrier is one of the major causes for transfusion-related morbidity and mortality (Storry and Olsson 2009). Traditionally, organ transplantation across the ABO blood group barrier has been avoided. However, ABO incompatible kidney transplantation from living donors is increasingly used with graft survival times similar to those of living donor ABO compatible kidneys (Genberg et al. 2008). With the use of antigen-specific immunoadsorption (IA) as an important part of some treatment protocols in ABO incompatible transplantations (Tyden et al. 2005; Kumlien et al. 2006), finding out the fine antigen specificity of the anti-A and/or the anti-B repertoire pre- and post-transplantation has become increasingly relevant (Holgersson et al. 2005).

The blood group A and B determinants are represented by the trisaccharides GalNACα3(Fucα2)Galβ-R and Galα3(Fucα2)Galβ-R, respectively. A and B determinants have been identified on at least five different outer core saccharide chains in humans, referred to as type 1 (Galβ3GlcNAc), type 2 (Galβ4GlcNAc), type 3 (Galβ3GalNAcα), type 4
(Galβ3GalNAcβ) and type 6 (Galβ4Glc) (Clausen and Hakomori 1989; Holgersson et al. 1992). A, and most likely also B, epitopes based on these different chain types can be distinguished by the immune system as suggested by the fact that mouse monoclonal antibodies have been generated that can specifically bind one or the other (Clausen and Hakomori 1989). Likewise, recipients of ABO incompatible kidney transplants that have responded to the graft by generating chain type-specific anti-A antibodies have been reported (Rydeberg et al. 1992, 1994). Recently, we have described the presence of chain type-specific A and B antibodies also in healthy blood donors (Lindberg et al. 2011). A and B antigens on the different chain types have been shown to be expressed in a tissue-specific manner (Clausen and Hakomori 1989; Holgersson et al. 1992). In short, epithelial tissues are rich in type 1, mesodermal tissues such as erythrocytes are rich in type 2 and the pancreas and kidney are rich in type 4 ABH antigens (Holgersson et al. 1992). It should be noted though that most of the studies on which these conclusions were based dealt solely with glycosphingolipid-based ABH antigens (Holgersson et al. 1992). The knowledge on the tissue-specific expression of protein-carried ABH antigens with the different chain types is much more limited.

The biosynthesis of ABH determinants is initiated by α1,2 fucosylation of the precursor chain; an enzymatic step catalyzed by the H (FUT1) or Se (FUT2) gene-encoded α1,2-fucosyltransferases (FT; Larsen et al. 1990; Kelly et al. 1995; Rouquier et al. 1995). Fucosylation is followed by the addition of an α-linked N-acetylgalactosamine (GalNAc) by the A gene-encoded α1,3-N-acetylgalactosaminyltransferase (α1,3GalNAcT) or an α-linked galactose by the B gene-encoded α1,3-galactosyltransferase (α1,3-GalT; Yamamoto et al. 1990).

Members of the big GalT family are responsible for the biosynthesis of the type 1–4 outer core saccharide chains (reviewed in Amado et al. 1999; Hennet 2002; Togayachi et al. 2006). βGal-T1, β3Gal-T2 and β3Gal-T5 can all make type 1 chain on N-linked glycans, whereas only β3Gal-T5 works on O-glycans when Chinese hamster ovary (CHO) cells are transfected with protein probes carrying N- and O-linked glycans, respectively (Holgersson and Lofving 2006). The latter enzyme has also been shown to be involved in the type 4 chain biosynthesis, since it can catalyze the formation ofGbS Cer fromGb4Cer and, hence, the Galβ3GalNAcβ outer core chain (Zhou et al. 2000). This sequence has not been found protein-linked in human blood group P erythrocytes (Yang et al. 1994) and may in humans only be present in glycolipids. The β3Gal-T7 is responsible for the biosynthesis of the core 1 O-glycan, i.e. the Galβ3GalNAcα (type 3 chain), sequence (Ju et al. 2002). Of the β3Gal-Ts described in this paragraph, only the homolog of β3Gal-T7 appears to be present in CHO cells (Liu et al. 1997, 2005). Seven β4Gal-Ts have been described (Hennet 2002). β4Gal-T1–6 can all add a galactose in a β1,4-linkage to N-acetylgalcosamine (GlNAc) residues of N-glycans, whereas β4Gal-T1 and β4Gal-T2 can also make lactose (Galβ4Glc) in the presence of α-lactalbumin (Hennet 2002). β4Gal-T7 catalyzes the addition of a galactose residue to xylose in the glycosaminoglycan core (Ly et al. 2010). The fine specificity on O-glycans of the different β4Gal-Ts is to our knowledge not known. CHO cells appear to have β4Gal-T enzymatic activity that can add a β1,4-linked galactose to core 2 (Lofving and Holgersson 2009), core 3 (Lofving and Holgersson 2009) and extended core 1 (GlCNACβ 3Galβ3GalNAc; Yeh et al. 2001) structures.

O-Glycosylation is initiated by an UDP-GalNAc-polypeptide GalNAcT, of which there are 14 isoforms in mammals (Tian and Ten Hagen 2009), adding a GalNAc to the amino acid serine or threonine. The GalNAc is further extended into eight different inner core saccharide structures, with cores 1–4 being the most common ones in humans (Tian and Ten Hagen 2009; Jensen et al. 2010). The endogenous glycosylation machinery of CHO cells only supports the biosynthesis of the inner core 1 O-glycan structure, but can be engineered to also make core 2, core 3 and extended core 1 structures by the expression of a core 2 βGalNAcT (of which there are three isoforms), the core 3 β3GalNAc-T6 and the core 1 extension β3GalNAc-T, respectively (Yeh et al. 2001; Holgersson and Lofving 2006; Lofving et al. 2008; Lofving and Holgersson 2009).

Even though ABH histo-blood group antigens based on different outer core chain types can be chemically synthesized (Meloncelli and Lowary 2009, 2010), we believe that in order to prove biological function also the type of the inner core saccharide chain, whether the glycan is O- or N-linked, and the type of carrier protein will be important (Gustafsson and Holgersson 2006; Coombs et al. 2010). Further, the strength of biological interactions involving protein receptors and carbohydrate ligands is to a large extent dependent on multivalent binding (Mammen et al. 1998; Gustafsson and Holgersson 2006; Coombs et al. 2010). Therefore, we have engineered stable CHO cells secreting a mucin-type immunoglobulin fusion protein with multivalent substitution of blood group A and B determinants on defined type 1, type 2 and type 3 outer core saccharide chains.

Results

Expression of blood group A and B substituted P-selectin glycoprotein ligand-1/mouse immunoglobulin G2b in CHO cells

P-selectin glycoprotein ligand-1/mouse immunoglobulin G2b (PSGL-1/mlgG2b) produced in CHO cells stably transfected to generate PSGL-1/mlgG2b substituted with blood group A or B determinants on the type 1 (Galβ3GlcNAc), type 2 (Galβ4GlcNAc) or type 3 (Galβ3GalNAcα) outer core saccharide chains were purified by protein A affinity chromatography and gel filtration. The transfection scheme including the enzymes used to generate stable CHO cell lines secreting the mucin-type fusion protein, PSGL-1/mlgG2b, carrying A type 1 (C-PA1-4), type 2 (C-PA2-29) or type 3 (C-PA3-46) or B type 1 (C-PB1-1H10), type 2 (C-PB2-3H10) or type 3 (L-PB3-5E8) O-glycans is shown in Figure 1.

PSGL-1/mlgG2b substituted with A determinants on type 1 (C-PA1-4), type 2 (C-PA2-29) and type 3 (C-PA3-46) chains were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Figure 2A and B, lanes 1–3) and western blot using antibodies against the mlG Fc part...
(Figure 2C, lanes 1–3), the N-terminal of PSGL-1 (Figure 2D, lanes 1–3) and the blood group A determinant (Figure 2E, lanes 1–3). In all cases, a protein of ≈300 kDa under non-reducing conditions was seen.

Similarly, SDS–PAGE (Figure 3A and B, lanes 2–4) and western blot analysis of the different blood group B substituted mucin-type fusion proteins using antibodies against the mIgG Fc part (Figure 3C, lanes 2–4), the N-terminal of PSGL-1 (Figure 3D, lanes 2–4) and the blood group B determinant (Figure 3E, lanes 2–4) also revealed a protein of ≈300 kDa under non-reducing conditions. In lane 1 (Figure 3A–E), a non-B substituted PSGL-1/mIgG2b expressed in non-glycoengineered CHO cells was applied as a reference.

A glycoprotein staining kit (Pro Q Emerald) was used in combination with Ruby to detect glycosylated as well as non-glycosylated proteins. The purified PSGL-1/mIgG2b fractions did not contain any significant amounts of contaminating proteins (Figure 2B, lanes 1–3, and Figure 3B, lanes 2–4). PSGL-1/mIgG2b produced in cells stably expressing the core 2 β1,6-N-acetylglucosaminyltransferase-1 enzyme (clone C-PA2-29 and C-PB2-3H10) or the core 3 β1,3-N-acetylglucosaminyltransferase-6 enzyme (clone C-PA1-4 and C-PB1-1H10) contained more complex glycans as indicated by their apparent higher Mr (for the A mucins compare lanes 1 and 2 with lane 3 in Figure 2C–E and for the B mucins compare lanes 2 and 3 with lanes 1 and 4 in Figure 3A–E).

The blood group A determinants on PSGL-1/mIgG2b produced in the different CHO cell lines are carried by defined type 1, type 2 and type 3 outer core saccharide chains

To confirm that the blood group A epitopes were expressed on defined outer core saccharide chains, purified PSGL-1/mIgG2b produced in clones C-P-55, C-PA3-46, C-PA2-29 and C-PA1-4 were analyzed by western blotting using mouse monoclonal antibodies (Table I with references) binding the blood group A determinant in a chain type-specific manner (Figure 4).

Blood group A type 1 determinants were detected on PSGL-1/mIgG2b produced in the CHO clone C-PA1-4 (Figure 4A, lane 2). This fusion protein was generated from the C-PA3-46 clone by stably expressing the core 3 β1,3-galactosyltransferase-6 and β1,3-galactosyltransferase-5 genes (Figure 1). A type 2 determinant was found on the mucin-type fusion protein expressed in C-PA1-4 and C-PA2-29 (Figure 4B, lanes 2 and 3, respectively). C-PA2-29 was generated by expressing the fusion protein gene, the core 2 β6GlcNAc-T1, FUT1 and the A gene in CHO cells (Figure 1). A type 3 determinant was only found on PSGL-1/mIgG2b produced in C-PA3-46 cells (Figure 4C, lane 4), which were generated by expressing FUT2 and the A gene in addition to the fusion protein gene (Figure 1). The absence of the reactivity of C-PA1-4 with the antibody specific for type 3 chain A indicates close to the complete conversion of core 1 (type 3) to core 3 (type 1).
All fusion proteins carrying A determinants reacted with a broad anti-A (Figure 4D) and bound as expected to anti-PSGL-1 (Figure 4E) and anti-mIgG (Figure 4F) antibodies.

The blood group A determinants are mainly carried by O-linked glycans on PSGL-1/mIgG2b

To confirm that the different blood group A determinants were located on O-linked, purified PSGL-1/mIgG2b produced in clone C-PA1-4, C-PA2-29 and C-PA1-4 were treated with N-glycosidase F (PNGase F). No significant reduction in anti-blood group A antibody staining intensity was observed following this treatment, suggesting that the A determinants were mainly carried by O-glycans (Figure 5A and B). Efficient cleavage of the N-glycans was confirmed by a complete mobility shift of PSGL-1/mIgG2b following PNGase F treatment (Figure 5C).

The relative blood group A epitope density on PSGL-1/mIgG2b varies with the core structure

The relative density of A epitopes on PSGL-1/mIgG2b produced in clones C-PA1-4, C-PA2-29 and C-PA3-46 was determined by enzyme-linked immunosorbent assay (ELISA; Figure 6A). The optical density (OD) ratio \([\text{Helix pomatia agglutinin (HPA) reactivity/anti-mIgG reactivity}]\) was 5.0, 2.5 and 1.8 for PSGL-1/mIgG2b produced in C-PA1-4, C-PA2-29 and C-PA3-46, respectively. The ELISA results were in agreement with the relative HPA lectin and anti-mIgG staining observed in western blot analysis (Figure 6B and C).

The blood group A type 3 epitope density is higher on PSGL-1/mIgG2b produced in Lec2 cells

The lower A epitope density on PSGL-1/mIgG2b produced in C-PA3-46 may be explained by a competition between CHO endogenous sialyltransferases and FUT2 for the core 1 precursor. To test this hypothesis, the PSGL-1/mIgG2b cDNA, FUT2 and the A gene were stably expressed in the sialylation-defective Lec2 cells (Stanley and Siminovitch 1977). The relative density of A epitopes on PSGL-1/mIgG2b produced in clone C-PA1-4 and C-PA3-46 and in Lec2 clone L-PA3-10 was determined by ELISA (Figure 6D). The optical density ratio (HPA reactivity/anti-mIgG reactivity) for PSGL-1/mIgG2b produced in L-PA3-10 was two times higher \((P<0.05, \text{Mann–Whitney } U\text{-test})\) compared with PSGL-1/mIgG2b produced in C-PA3-46. The ELISA results were in

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**Fig. 2.** SDS-PAGE and western blot analysis of purified blood group A substituted PSGL-1/mIgG2b produced in CHO cells. Purified PSGL-1/mIgG2b produced in C-PA1-4 (lane 1), C-PA2-29 (lane 2) and C-PA3-46 (lane 3). Proteins were analyzed under non-reducing conditions. Two micrograms of protein was loaded per well and glycosylated proteins were detected by Pro Q Emerald (A) followed by the detection of all proteins by Ruby (B). Lanes 1 and 2 and lane 3 of (A) and (B) were run on two different gels. For western blot analysis, 500 ng of protein was loaded per well and membranes were probed with anti-mIgG (Fc) (C), anti-PSGL-1 (D) and anti-A (E) antibodies.
agreement with the relative HPA lectin/anti-mIgG staining observed in western blot analysis (Figure 6E and F).

**Table I. Monoclonal anti-A and anti-B antibodies used**

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<th>Brand</th>
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<td>Clausen et al. (1985)</td>
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<td>Z2B-1</td>
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<td>Mouse monoclonal, IgM</td>
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<tr>
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<td>Blood group B</td>
<td>Mouse monoclonal, IgM</td>
<td>Abcam</td>
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<tr>
<td>CD-162 (PSGL-1)</td>
<td>KPL-1</td>
<td>PSGL-1</td>
<td>Mouse monoclonal, IgG1</td>
<td>BD Pharmingen</td>
<td></td>
</tr>
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</table>

**Fig. 3.** SDS–PAGE and western blot analysis of purified blood group B substituted PSGL-1/mIgG2b produced in CHO cells. PSGL-1/mIgG2b purified from CHO cells (lane 1, C-P-55; lane 2, C-PB1-1H10; lane 3, C-PB2-3H10 and lane 4, L-PB3-5E8). Proteins were analyzed under non-reducing conditions. Two micrograms of protein was loaded per well and glycosylated proteins were detected by Pro Q Emerald (A) followed by the detection of all proteins by Ruby (B). For western blot analysis, 250 ng of protein was loaded per well and membranes were probed with anti-mIgG (Fc) (C), anti-PSGL-1 (D) and anti-B (E) antibodies.

Liquid chromatography–mass spectrometric analysis of native oligosaccharides released from purified blood group A and B substituted PSGL-1/mIgG2b produced in CHO cells

The negative-mode liquid chromatography–mass spectrometry (LC-MS) base peak chromatogram and the composite spectra of native reduced O-glycans released from blood group A and B substituted PSGL-1/mIgG2b produced in different CHO clones are shown in Figure 7A–F. All major peaks in the LC-MS composite spectrum of O-glycans released from the recombinant mucin-type fusion proteins carrying blood group A and B determinants and produced in the different CHO cell lines were interpreted and tentatively assigned using the strategy described in the Materials and methods section. The mass spectrometric characterization (Figures 7 and 8) of the blood group A O-glycans identified is described in detail below. The mass spectrometric characterization of B O-glycans was done in the same manner, but identified structures only summarized below. Their tentative structures (Table II) were deduced from the results of MS and antibody binding in western blots.
Tentative blood group antigen determinants were detected on PSGL-1/mIgG2b produced in all cell clones analyzed. Ions derived from O-glycans carrying A and B type 1 determinants were predominant in the LC-MS chromatograms and spectra derived from the mucin-type fusion proteins produced in C-PA1-4 and C-PB1-1H10 (Figure 7A and D). Among O-glycans derived from PSGL-1/mIgG2b produced in the other cell clones, those carrying blood group determinants were minor constituents. Based on the LC-MS analysis and western blot, we suggest that the different CHO clones indeed produce fusion proteins carrying A and B O-glycans based on the type 1, type 2 and type 3 chain, respectively.

**Clone C-PA1-4.** The LC-MS base peak chromatogram and the composite spectrum of native O-glycans released from PSGL-1/mIgG2b produced in clone C-PA1-4 are shown in Figure 7A. The dominant [M−H] ion of m/z 936 represents a tentative GalNAcα3(Fucα2)Galβ3GlcNAcβ3GalNAcol (A type 1) structure. Two minor components with [M−H] ions of m/z 733 represent the A type 3 [GalNAcα3(Fucα2)Galβ3GalNAcol; RT 13.4, minor peak] and H type 1 (Fucα2Galβ3GlcNAcβ3GalNAcol; RT 14.8 major peak) structures, respectively. The MS² spectrum (Figure 9A) of m/z 936 shows abundant fragment ions of m/z 407 ([M−HexNAc-(dHex-)Hex-O]⁻), m/z 713 ([HexNAc-(dHex-)Hex-Nac-O⁻]), [O-(dHex-HexNAc-O)]⁻ and m/z 715 ([M−HexNAc-O⁻]). Minor peaks are seen at m/z 425 ([M−HexNAc-(dHex-)Hex-O]⁻), m/z 510 ([HexNAc-(dHex-)Hex-O]⁻, [dHex-Hex-HexNAc-O]⁻ or [O-(dHex-HexNAc-O)]⁻) and m/z 772 (M−dHex-O⁻). Based on the known specificity of the glycosyltransferases expressed in C-PA1-4 cells, western blot results (Figure 4A) and the MS² spectrum of the [M−H] ion at m/z 936, we suggest that this ion represents an O-glycan based on core 3 and with an A type 1 determinant.

In addition, the western blot indicated the presence of A type 2 determinants on the fusion protein derived from the C-PA1-4 clone (Figure 4B). The low intensity of m/z 612 in the MS² spectrum of m/z 936 suggests that it is an isotopic peak of the more intense (m/z 611), rather than the diagnostic 0.2A2α–H2O fragment of type 2 (Galβ4GlcNAc) chains (Karlsson et al. 2004). Further, no m/z 612 fragment ion was detected in the corresponding MS² of the doubly charged parent ion at m/z 4682¹. Thus, the detection of A type 2 determinants on this fusion protein by western blot may be due to antibody cross-reactivity or the presence of only small amounts of A type 2 escaping detection by MS.

**Clone C-PA2-29.** The major peaks of the composite spectrum (Figure 7B) are derived from the tentative elongated core 2 structures NeuAcα3Galβ4GlcNAcβ6(NeuAcα3Galβ3)GalNAcol ([M−H] ion of m/z 1331) and NeuAcα3Galβ4GlcNAcβ6(Galβ3)GalNAcol/Galβ4GlcNAcβ6(NeuAcα3Galβ3)GalNAcol ([M−H] ion of m/z 1040) as suggested by MS² data (not shown).
Only less abundant parent ions containing blood group A (\([\text{M-H}]\) ion of \[m/z\] 918) shows that one of the antennas consists of a NeuAc-Hex-epitope, and the minor fragment ion at \[m/z\] 569 shows that the other antenna has a composition corresponding to a blood group A epitope. A diagnostic ion for a 1,4-glycosidic linkage was observed at \[m/z\] 612 (\([0.2\text{Ar}_2\text{H}_2\text{O}])\), indicating that Gal is 1,4-linked to GlcNAc (Figure 8B). These results are in agreement with the western blot, which show a strong binding with A type 2-specific, but not with A type 1- or type 3-specific, antibodies (Figure 4).

Clone C-PA3-46. The base peak chromatogram and the composite spectrum are relatively simple with only a few peaks. The most abundant peaks at \[m/z\] 675 and \[m/z\] 966, correspond to the [M-H] ions of mono- and disialylated core 1 (Figure 7C). The only significant peak in the composite spectrum that can represent a blood group antigen is the minor \[m/z\] 733, which we suggest represents the [M-H] ion of a blood group A determinant on a type 3 chain [GalNac\(_3\) (Fuc\(_2\))Gal\(_3\)GalNac\(_4\)]. The blood group A epitope is suggested by the MS\(^2\) spectrum of \[m/z\] 733 that is dominated by the daughter ion \[m/z\] 247 (Figure 8C). This unusual fragment has been reported previously in the MS\(^2\) spectrum of a native reduced blood group A type 3 determinant and was then described as the product of a B\(_2\)-C\(_2\)H\(_4\)O\(_2\)/Y\(_{2a}\) fragmentation, i.e. an internal [dHex-Hex + O-C\(_2\)H\(_4\)O\(_2\)] fragment (Karlsson et al. 2004). Further confirmation of the blood group A epitope comes from the loss of terminal fucose (\[m/z\] 569), loss of terminal N-acetylgalactosamine (HexNAc; \[m/z\] 512) and a [-Hex-(dHex)-O/O-Hex-(dHex)-] fragment (\[m/z\] 307). This LC-MS result is in agreement with the western blot analysis, which indicates the presence of A type 3, but no A type 1 or type 2, determinants on the fusion protein produced in C-PA3-46 (Figure 4).

Figure 5. Western blot analysis of purified PSGL-1/mIgG\(_{2b}\) substituted with blood group A epitopes on different core structures prior to (-) and after (+) PNGase F treatment to remove N-linked oligosaccharides. PSGL-1/mIgG\(_{2b}\), purified from CHO cells (lanes 1 and 2, C-P-55; lanes 3 and 4, C-PA1-4; lanes 5 and 6, C-PA2-29 and lanes 7 and 8, C-PA3-46). Proteins were analyzed under reducing conditions. 250 ng of protein was loaded per well and membranes were probed with anti-A (A), AH-21 (B) and anti-mlgG (Fc) (C).

and the enzymatic specificity of expressed glycosyltransferases. Only less abundant parent ions containing blood group A determinants were detected. Glycans carrying A type 2 determinant sequences were identified in the tentative structures GalNac\(_3\) (Fuc\(_2\))Gal\(_4\)GlcNac\(_6\) (NeuAc\(_3\)Gal\(_3\)GalNac\(_4\)) ([M-H] ion of \[m/z\] 1389) and GalNac\(_3\) (Fuc\(_2\))Gal\(_4\)GlcNac\(_6\) (Gal\(_3\))GalNac\(_4\) ([M-H] ion of \[m/z\] 1098). The MS\(^2\) spectrum of the [M-H] ion of \[m/z\] 1389 is dominated by the \[m/z\] 1098, which is explained by the loss of sialic acid (Figure 8B). Further fragmentation after the loss of sialic acid (\[m/z\] 936 and \[m/z\] 918) shows that one of the antennas consists of a NeuAc-Hex-epitope, and the minor fragment ion at \[m/z\] 569 shows that the other antenna has a composition corresponding to a blood group A epitope. A diagnostic ion for a 1,4-glycosidic linkage was observed at \[m/z\] 612 (\([0.2\text{Ar}_2\text{H}_2\text{O}])\), indicating that Gal is 1,4-linked to GlcNAc (Figure 8B). These results are in agreement with the western blot, which show a strong binding with A type 2-specific, but not with A type 1- or type 3-specific, antibodies (Figure 4).

Clone C-PA3-46. The base peak chromatogram and the composite spectrum are relatively simple with only a few peaks. The most abundant peaks at \[m/z\] 675 and \[m/z\] 966, correspond to the [M-H] ions of mono- and disialylated core 1 (Figure 7C). The only significant peak in the composite spectrum that can represent a blood group antigen is the minor \[m/z\] 733, which we suggest represents the [M-H] ion of a blood group A determinant on a type 3 chain [GalNac\(_3\) (Fuc\(_2\))Gal\(_3\)GalNac\(_4\)]. The blood group A epitope is suggested by the MS\(^2\) spectrum of \[m/z\] 733 that is dominated by the daughter ion \[m/z\] 247 (Figure 8C). This unusual fragment has been reported previously in the MS\(^2\) spectrum of a native reduced blood group A type 3 determinant and was then described as the product of a B\(_2\)-C\(_2\)H\(_4\)O\(_2\)/Y\(_{2a}\) fragmentation, i.e. an internal [dHex-Hex + O-C\(_2\)H\(_4\)O\(_2\)] fragment (Karlsson et al. 2004). Further confirmation of the blood group A epitope comes from the loss of terminal fucose (\[m/z\] 569), loss of terminal N-acetylgalactosamine (HexNAc; \[m/z\] 512) and a [-Hex-(dHex)-O/O-Hex-(dHex)-] fragment (\[m/z\] 307). This LC-MS result is in agreement with the western blot analysis, which indicates the presence of A type 3, but no A type 1 or type 2, determinants on the fusion protein produced in C-PA3-46 (Figure 4).

LC-MS analysis of O-glycans released from PSGL-1/mIgG\(_{2b}\) carrying blood group B O-glycans

The LC-MS composite and the MS\(^2\) (not shown) spectra of the native O-glycans released from PSGL-1/mIgG\(_{2b}\) produced in the respective blood group B active clones (C-PB1-1H10 and C-PB2-3H10, Figure 7D and E) suggest the presence of blood group B O-glycans corresponding to the blood group A glycans described in the LC-MS results sections C-PA1-4, C-PA2-29 and C-PA3-46. One exception was the spectra of the O-glycans released from the mucin-type fusion protein produced in the Lec2 cell clone, L-PB3-5E8. Both the LC-MS composite spectrum and the MS\(^2\) spectra suggest that at least two structures of the O-glycans derived from PSGL-1/mIgG\(_{2b}\) produced in the latter cell line carried sulfate substitutions (Figure 7F). Based on the MS\(^2\) fragment ions and the available glycosyltransferases in this clone, we suggest that \[m/z\] 464 and \[m/z\] 626 represent sulfated Gal\(_3\)GalNac\(_4\) and HexGal\(_3\)GalNac\(_4\), respectively. These glycans elute from the column in several peaks in the chromatogram which correlates with different sulfate positions on the eluted structures. The MS\(^2\) spectra of the two peaks in the chromatogram with [M-H] ions of \[m/z\] 464 ions are shown in Figure 8D (RT 8.1) and E (RT 11.4). The MS\(^2\) spectrum of RT 8.1 is dominated by the fragment ions of \[m/z\] 302 ([M-H]\(^+\)) and \[m/z\] 284 ([M-H-O]\(^+\)), which both stem from a Hex-(S-)HexNacol...
parent ion (Figure 8D). This shows that CHO cells can sulfate reducing end GalNAc residues. The peak eluted at RT 11.4 has an MS2 spectrum, which suggests an S-Hex-HexNAc parent ion. The spectrum is dominated by the fragment ion of m/z 241 which represents a [S-Hex-] fragment ion (Figure 8E). Our data suggest that instead of sialylation, the Lec2 cells sulfate the corresponding sites on expressed O-glycans, e.g. the core 1 structure.

PSGL-1/ mIgG2b produced in clone C-PA1-4 can adsorb both A type 1 and type 2 chain-specific immunoglobulin G antibodies from a pool of blood group O serum

In order to assess the ability of PSGL-1/ mIgG2b carrying A type 1 and, to some degree, A type 2 determinants to adsorb anti-A antibodies, pooled blood group O serum was passed over a column with immobilized C-PA1-4 fusion protein. Remaining anti-A antibodies following adsorption was characterized in an ELISA on neoglycoproteins (NGPs) carrying A type 1–4 tetrasaccharides (Figure 9). Adsorption on the C-PA1-4 fusion protein removed A type 1 and type 2 chain-specific antibodies better than it removed A type 3 and type 4 chain-specific antibodies. In contrast, adsorption on a mixture of A type 1–4 tetrasaccharides removed antibodies specific for all A type 1–4 structures. Adsorption on A type 1 and A type 2 tetrasaccharides, respectively, removed both A type 1- and A type 2-specific antibodies, suggesting that the majority of anti-A type 1- and type 2-specific antibodies recognize an epitope shared between the A type 1 and type 2 tetrasaccharides.

Fig. 6. The relative density of blood group A epitopes expressed on different core structures on purified PSGL-1/ mIgG2b produced in CHO-K1 and Lec2 cells. The relative A epitope density on PSGL-1/ mIgG2b expressed in CHO-K1 and Lec2 cells (C-PA1-4 and C-PA2-29) was determined in H. pomatia lectin and anti-mIgG (Fc) ELISAs as described in Materials and methods. The values were normalized to PSGL-1/ mIgG2b without A epitopes (C-PA55) (A). For western blot analysis, 250 ng of protein was loaded per well (lane 1, C-PA55; lane 2, C-PA1-4; lane 3, C-PA2-29 and lane 4, C-PA3-46). Proteins were analyzed under non-reducing conditions and the membranes were probed with H. pomatia lectin (B) and anti-mIgG (Fc) (C). Graph A: the mean and standard deviation (SD) (n = 8) of one ELISA in which four different concentrations of the each of the PSGL-1/ mIgG2b fusion proteins were analyzed in duplicate are shown. In another experiment performed in a similar manner, the relative A epitope density on PSGL-1/ mIgG2b produced in CHO-K1 cells (C-PA1-4 and C-PA3-46) and Lec2 cells (L-PA3-10) was determined in H. pomatia lectin and anti-mIgG (Fc) ELISAs. The values were normalized to PSGL-1/ mIgG2b without A epitopes (C-PA55) (D). For western blot analysis, purified PSGL-1/ mIgG2b produced in CHO-K1 (lane 1, C-PA1-4 and lane 3, C-PA3-46) and Lec2 cells (lane 4, L-PA3-10) were analyzed under reducing conditions and the membranes were probed with H. pomatia lectin (E) and anti-mIgG (Fc) (F). Graph D: the mean and standard deviation (SD) (n = 12) of three ELISAs in which cell culture supernatants, harvested at three different time points, were analyzed at two different concentrations in duplicate are shown. The difference in the relative A epitope density on PSGL-1/ mIgG2b produced in clone L-PA3-10 and clone C-PA3-46 was tested for significance (P < 0.05).
Previously, we have reported on the transient expression in different host cells of the PSGL-1/mIgG2b mucin-type fusion protein modified to carry blood group A determinants (Lofting et al. 2002). The O-glycan acceptor chain specificity of the \( H \) (\( FUT1 \)) and \( Se \) (\( FUT2 \)) gene-encoded \( \alpha1,2\)-FT and

![Fig. 7. LC-MS base peak chromatograms and composite spectra of O-glycans released from blood group A and B substituted PSGL-1/mlgG2b produced in CHO cells. O-Glycans were released from PSGL-1/mlgG2b by reductive \( \beta \)-elimination. LC-MS was performed in a negative mode on a graphitized carbon column coupled to an LC/MS\(^\text{a} \) ion trap instrument. The figure is composed of chromatograms and composite spectra of O-glycans derived from the fusion protein produced in six different cell lines which have been genetically modified to produce the human blood group antigens A type 1 (A), A type 2 (B), A type 3 (C), B type 1 (D), B type 2 (E) and B type 3 (F). Carbohydrate compositions are assigned as (Hex, HexNAc, dHex, NeuAc) in the chromatograms with reducing end GalNAc included as HexNAc. Ions representing tentative blood group antigens are assigned in the composite spectra as described below. Abbreviations: A1, A type 1; A2, A type 2; A3, A type 3; B1, B type 1; B2, B type 2; B3, B type 3; H1, H type 1; H3, H type 3; S, sulfate.](https://academic.oup.com/glycob/article-abstract/23/6/720/1989431)
Fig. 8. MS² spectra of O-glycan oligosaccharide alditols released from PSGL-1/mIgG₂b produced in C-PA1-4, C-PA2-29, C-PA3-46 and L-PB3-5E8 clones representing structures carrying A type 1, A type 2, A type 3 and sulfated core 1. O-Glycans were released from PSGL-1/mIgG₂b by reductive β-elimination.
the ability of blood group A substituted PSGL-1/mIgG2b to adsorb anti-A antibodies were examined (Lofling et al. 2002). We showed that the blood group A mucin-type fusion protein was more effective in adsorbing anti-A than an equimolar amount of A trisaccharides linked to macroporous glass beads (Lofling et al. 2002). We hypothesized that this could be explained by the multivalency, spacing of O-glycans and core chain versatility provided by the mucin-type backbone (Lofling et al. 2002). Since then we have shown that, in fact, anti-A and anti-B can be core saccharide chain-specific (Lindberg et al. 2011, 2012) and that an affinity matrix containing a mixture of A or B tetrasaccharides based on the type 1–4 outer core saccharides is a better adsorber of anti-A or -B than the corresponding A or B trisaccharides (Lindberg et al. 2012). In order to assess whether having the different A and B tetrasaccharides presented on a mucin-type backbone would make an even better affinity matrix for anti-A and anti-B, we set out to generate stable CHO cells producing PSGL-1/mIgG2b having O-glycans with A or B determinants carried by the type 1 (Galβ3GlcNAc), type 2 (Galβ4GlcNAc) and type 3 (Galβ3GalNAcα) outer core saccharide chains.

As outlined in Figure 1 and described in Supplementary data and Lindberg et al. (2013), two major strategies were used to obtain the various CHO cell lines. The starting point was CHO-K1 cells adapted to growth in serum-free medium and stably transfected with the PSGL-1/mIgG2b expression plasmid to generate the C-P-55 cell line. Although the C-P-53-46 and C-PA1-4 cell lines were engineered by a sequential transfection of the fusion protein-secreting C-P-55 cells with glycosyltransferase-encoding expression plasmids, the C-PA2-29 cells were obtained following the simultaneous transfection of the C-P-55 cells with plasmids encoding the core 2 β1,6GlcNAcT, the H gene-encoded α1,2FucT and the A gene-encoded α1,3-GalNAcT. Likewise, the cell lines producing fusion proteins with blood group B determinants were all made by the simultaneous transfection of all glycosyltransferase-encoding plasmids. Sequential transfection requires careful analysis of gene expression stability after each transfection and is therefore time-consuming. Because the expression of all glycosyltransferases is required to generate the desired blood group A or B O-glycan substitution, lost expression of any of the transfected genes will disrupt the expression of the desired determinant. In contrast, co-transfection of all glycosyltransferase-encoding plasmids may increase the likelihood that they integrate in the same break point in the host cell’s genome and thereby acquire similar stability and expression profiles. Even though we have transfected cells with up to five distinct plasmids resulting in what appeared to be stable the expression of all glycosyltransferase genes and the secretion of the mucin-type fusion protein carrying the desired O-glycan substitution, we have not evaluated a high enough number of selected clones for a time long enough to be able to draw any conclusions with regard to the long-term gene expression stability.

In order to make downstream processing easier, it is a great advantage if the cell clones can be cultured under serum-free conditions. This is also absolutely necessary for any future commercial production of the fusion protein. When generating stable transfectants in the presence of serum, we have encountered problems such as lost or reduced gene expression upon the subsequent adaptation of clones to serum-free conditions. On the other hand, transfecting and selecting cells under complete serum-free conditions is hard. From our gained experience and continuous method improvements, a protocol has evolved in which CHO cells adapted to serum-free conditions are exposed to low levels of serum at the time of transfection and during initial selection. Subsequent selection and clone expansion are done under serum-free conditions (Lindberg et al. 2013). It appears as if the engineering of CHO cells to produce core 3-based A determinants is a much more efficient process than making the cells produce core 1- and core 2-based A determinants (Figure 6A–C). So while peaks explained by fragments derived from core 3-based, type 1 (or 2) chain-extended A determinants are predominant in the composite mass spectrum of O-glycans released from PSGL-1/mIgG2b produced in C-PA1-4 (Figure 7A), peaks derived from blood group A structures based on core 2 (Figure 7B) or core 1 (Figure 7C) chains are of less abundance. A likely explanation for this lies in the fact that, in these cell clones, the sialylation of the core 1 (Galβ3GalNAcα-Ser/Thr) chain is favored over GlcNAcβ1,6-extension of the GalNAc residue as in the case of core 2 or α1,2-fucosylation of the terminal Gal residue as in the case of core 1 chain-based A. That sialylation precedes, or competes out, α1,2-fucosylation is also supported by data obtained from stably transfecting the CMP-NeuAc transporter-deficient CHO cell line, Lec2 (Figure 6D–F). When comparing the A epitope density on the mucin-type fusion protein produced in Lec2 and wild-type CHO cells engineered to express A type 3 determinants on the core 1 O-glycan, the density of blood group A determinants on the type 3 chain was clearly increased when produced in Lec2 (L-PA3-10) when compared with CHO (C-PA3-46) (Figure 6D–F). However, this observation may be cell clone-dependent, because in stable CHO cells we previously have engineered, core 2 branching has been predominant over sialylation (Liu et al. 2005). Thus, high expression levels of the β1,6 GlcNAc-T1 as a result of integration of its plasmid DNA in a transcriptionally active site may override α2,6-sialylation. The fact that the HH4 antibody specific for A type 2 determinants reacted with A structures carried by the mucin-type fusion protein expressed in clone C-PA1-4 suggests that A type 2 as well as A type 1 epitopes are carried by core 3 chain.

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**LC-MS was performed in a negative mode on a graphitized carbon column coupled to an LC/MS" ion trap instrument. The MS^2 spectra are annotated with CFG black and white nomenclature with a symbol key included. The daughter ions of the [M−H] ion of m/z 936 (A) is consistent with an A type 1 O-glycan, GalαNac3(Fucα2)Galβ3GlcNacβ3GalNAcO, carried by PSGL-1/mIgG2b expressed in the C-PA1-4 cell line (Figure 7A). The MS^2 spectrum of the [M−H] ion of m/z 1389 (B) is consistent with an A type 2 structure O-glycan, GalαNac3(Fucα2)Galβ4GlcNacβ6NeuAcα3Galβ3GalNAcO, released from the fusion protein expressed in C-PA2-29 (Figure 7B). The [M−H] ion of m/z 733 generates an MS^2 spectrum (C) that is consistent with an A type 3 O-glycan, GalαNac3(Fucα2)Galβ3GalNAcO, carried by C-PA3-46-produced PSGL-1/mIgG2b (Figure 7C). The MS^2 spectra of the [M−H] ion at m/z 464 of the two isomers released from PSGL-1/mIgG2b produced in L-PB3-5E8 are consistent with Galβ3(S-)GalNAcO (D) and S-Galβ3GalNAcO (E).**

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Recombinant blood group A and B mucins

Table II. O-Glycans with blood group determinants released from PSGL-1/mIgG2b, produced in engineered CHO cells

<table>
<thead>
<tr>
<th>Cell clone</th>
<th>Composition (Hex, HexNAc, dHex, NeuAc)</th>
<th>Tentative structures carrying blood group determinants</th>
<th>[M–H]– ion (observed)</th>
<th>O-Glycan core</th>
<th>Tentative chain type</th>
<th>Tentative blood group determinant</th>
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<tr>
<td>C-PA1-4</td>
<td>1, 2, 1, 0</td>
<td>GalNAc2Galβ3GlcNAcβ3GalNAcol</td>
<td>733</td>
<td>3</td>
<td>1</td>
<td>H type 1</td>
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<tr>
<td></td>
<td>1, 2, 1, 0</td>
<td>GalNAc2Galβ3GlcNAcβ3GalNAcol</td>
<td>733</td>
<td>1</td>
<td>3</td>
<td>A type 3</td>
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<td></td>
<td>1, 3, 1, 0</td>
<td>GalNAc2Galβ3GlcNAcβ3GalNAcol</td>
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<td>3</td>
<td>1 or 2</td>
<td>A type 1 or 2</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
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<td>2</td>
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<td>3</td>
<td>1</td>
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</tr>
<tr>
<td>C-PB1-1H10</td>
<td>1, 2, 1, 0</td>
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<td>Galα3(Fuuc2)Galβ3GalNAcol</td>
<td>692</td>
<td>1</td>
<td>3</td>
<td>B type 3</td>
</tr>
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</table>

*aCarbohydrate compositions are assigned as (Hex, HexNAc, dHex, NeuAc) with reducing end GalNAcol included as HexNac.

The tentative structures are suggested based on western blotting using chain type-specific anti-A antibodies, LC-MS and the known enzymatic specificity of expressed glycosyltransferases in each clone.

O-glycans on PSGL-1/mIgG2b in this cell line. The type 1 chain is generated by stable incorporation of the β1,3GlcNAcT (core 3 enzyme) and β1,3-galactosyltransferase-5 cDNAs, whereas an endogenous β1,4-GalT most likely generates the type 2 chain on the core 3 structure. Because the LC-MS spectrum of m/z 936 of the C-PA1-4 O-glycans lack significant diagnostic ions for potential A type 2 structures, this structure must be low in abundance or explained by a cross-reacting HH4 antibody.

Even though the O-glycans of PSGL-1/mIgG2b, produced in L-PB3-10 were not analyzed by MS, the O-glycans of the fusion protein produced in the corresponding blood group B Lec2 stable cell line, L-PB3-5EH, were. Several of the O-glycans released from the fusion protein produced in this cell line carried sialyl substituitions. One potential explanation for this observation could be that the Lec2 cells strive to keep their overall negative charge constant within certain limits and that their inability to sialylate glycans is compensated for by an increased sulfation. We have made a similar observation with regard to the insect cells, Sf9 and Hi-5, which are very low in their degree of sialylation (Gautzmann et al., 2006; Loof and Holgersson 2009; Gustafsson et al. 2011). The final goal of the work presented here was, as stated above, to produce recombinant mucin-like proteins that through their multivalent substitution with A or B determinants carried by different outer and inner core saccharide chains would become efficient adsorbers of anti-A and anti-B antibodies (Lofling et al. 2002). Here, we show that the A type 1 chain-carrying mucin, C-PA1-4, removed A type 1 and type 2 chain-specific antibodies (Figure 9). Whether a combination of the different A or B mucins will be better adsorbers than the A and B type 1–4 tetrasaccharides directly linked to sepharose remains to be shown (Lindberg et al. 2012).
Also, the cost of producing the fusion proteins will be a critical determinant for their use in any clinical setting. We have recently used the blood group A and B mucins as substrates in ELISA for the semi-quantification of anti-A and anti-B antibodies (Lindberg et al. 2012). It also turned out that the mucins, in contrast to A or B neoglycolipids (Lindberg et al. 2011) or albumin-based NGPs (Lindberg et al. 2012), exhibited very low background binding of IgM (unpublished observation). Further investigations are needed to validate this finding. Yet, other applications in which the A and B mucins can be used are currently being exploited.

In conclusion, we have engineered a number of stable CHO cell lines that into the medium secrete a mucin-type fusion protein, PSGL-1/mIgG2b, carrying blood group A or B determinants on type 1, type 2 and type 3 outer and type 3, type 2 and type 1, respectively, inner core saccharide chains. Their practical use as substrates in an ELISA and in extracorporeal IA columns is currently being exploited.

Materials and methods

Transfection and clonal selection of glycoengineered CHO cells

CHO-K1 and Lec2 cells were transfected to stably express PSGL-1/mIgG2b substituted with blood group A and B determinants on defined core structures (described in Supplementary data and in Lindberg et al. 2013). The designations of CHO clones selected for large-scale cultivation are shown in the transfection scheme as outlined in Figure 1.

Large-scale cultivation of CHO cells

Selected CHO clones (Figure 1) were cultured in serum-free Ex-cell 302 (JRH Biosciences, Lenexa, KS) or ProCHO-4 (Lonza) medium in a 20L Wave bioreactor (Wave System 20/50 EH, GE Healthcare) at 37°C, pH 6.5–7.5 and 15–27 rpm (clones secreting PSGL-1/mIgG2b and blood group A substituted PSGL-1/mIgG2b) or in 3 L shaker flasks (Corning Inc., NY) at 100 rpm and 37°C with 5% CO2 (clones secreting blood group B substituted PSGL-1/mIgG2b). At regular intervals, fresh medium with 2 mM l-glutamine was added until the final volume was reached; 10 L in the wave bioreactor and 1.5 L in shaker flasks. The culture was harvested when the final cell density had reached 4–5 × 10^6 total cells/mL and the viability had dropped to 70–80%. The glucose, glutamine and pH levels were monitored daily and adjusted to optimal levels.

The cell culture supernatant was clarified by microfiltration using a 0.054 m² Millistak+ POD COCH filter (Millipore, Billerica, MA). The clarified supernatants from CHO cells secreting PSGL-1/mIgG2b and blood group A substituted PSGL-1/mIgG2b were concentrated ×20 using a 0.11-m² Pellicon 3 cassette (Millipore) connected to a Cogent M TFF system (Millipore), then further diafiltered against six volumes of phosphate-buffered saline (PBS). The clarified supernatants from CHO cells secreting blood group B substituted PSGL-1/mIgG2b, cultured to lower total volumes, were not concentrated prior to purification. All product solutions were treated with 1 mL/L protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and 0.02% NaN3 (Sigma-Aldrich) and stored at 4°C until purification.

Purification of PSGL-1/mIgG2b fusion protein

All chromatographic procedures were carried out on an ÄKTA Explorer 100 controlled by the Unicorn software (v. 5.11; GE Healthcare). The clarified supernatants from CHO cells were sterile filtered with 0.22 μm polyethyrene sulfone filter (Nalgene, Thermo Fisher Scientific, San Jose, CA) before loading onto a MabSelect SuRe column (GE Healthcare) pre-equilibrated with PBS. The column was washed with 10 column volumes (CV) of PBS, and the elution of recombinant fusion protein was achieved using 5 CV of 0.1 M sodium citrate, pH 3.0. After elution, selected fractions were pooled, neutralized with 300 µL/mL of 1 M Tris–HCl, pH 9.0, and dialyzed extensively (12–14 kDa cutoff) against MilliQ water at 4°C or desalted on a HiPrep 26/10 desalting column (GE Healthcare). After dialysis, the samples were frozen, lyophilized and stored at −80°C until further purification.

Lyophilized samples were dissolved to ~5 mg/mL in gel filtration buffer (0.1 M sodium phosphate, pH 7.2, 0.5 M sodium chloride). Gel filtration of the PSGL-1/mIgG2b was carried out on pre-equilibrated HiPrep 26/60 Sephacryl S-300 HR (blood group A substituted PSGL-1/mIgG2b) or HiPrep 26/60 Sephacryl S-400 HR (blood group B substituted PSGL-1/mIgG2b) columns (GE Healthcare). Typically, 5 mL of sample were applied on the gel filtration column and eluted with a flow rate of 1 mL/min. Eluted fractions were kept at 4°C until pooling were done on the basis of Western blot analysis. Only fractions containing mainly the dimer of PSGL-1/mIgG2b were chosen for further studies. Pooled fractions were then dialyzed as above or desalted on a HiPrep 26/10 desalting column (GE Healthcare), frozen, lyophilized and stored at −80°C.

Purification of PSGL-1/mIgG2b, expressed in Lec2 cells for SDS–PAGE and western blot analysis

Clone L-PA3-10 was cultured in serum-free ProCHO-4 medium (Lonza) in 250 mL shaker flasks (Corning) at 100 rpm and 37°C with 5% CO2. The cell culture supernatant was clarified by centrifugation at 1400 × g for 10 min. PSGL-1/mIgG2b was purified from clarified supernatant on goat anti-mlgG agarose beads (Sigma-Aldrich, 50 μL slurry per 10 mL supernatant) by rolling head over tail at 4°C overnight. The beads with fusion proteins were washed three times in PBS, dissolved in 50 μL of 4× Lithium dodecyl sulfate (LDS) sample buffer with reducing agent (Invitrogen, Carlsbad, USA) and incubated at 70°C for 10 min.

Blood group A or B epitope density on, and quantification of, PSGL-1/mIgG2b using ELISA

The concentration of PSGL-1/mIgG2b, fusion protein in supernatants and in purified fractions, and its relative blood group A epitope density, was determined by a sandwich ELISA method. The 96-well ELISA plates (Costar 3590; Corning Inc.) were coated with an affinity-purified polyclonal goat anti-mlgG (Fc) antibody (Sigma-Aldrich) at a concentration of 10 μg/mL in 50 mM carbonate buffer, pH 9.6, at 4°C overnight. The plates were blocked with 1% bovine serum albumin (BSA) in PBS, which
was also used as a dilution buffer and incubated with supernatants or purified fractions of PSGL-1/mlG2b. The plates were subsequently incubated with a peroxidase-conjugated anti-mIgG (Fc) antibody (Sigma-Aldrich) diluted 1:1000, peroxidase-conjugated lectin from HPA (Sigma-Aldrich) diluted 1:10,000 or a monoclonal anti-B (HEB-29; Abcam, UK) diluted 1:500 followed by a peroxidase-conjugated anti-mIgM antibody (Sigma-Aldrich) diluted 1:1000. All incubations were performed at room temperature for 2 h. Between and after incubations, the plates were washed with PBS containing 0.05% (v/v) Tween-20 (Sigma-Aldrich). A bound peroxidase-conjugated antibody was visualized with 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma-Aldrich). The reaction was stopped by the addition of 2 M H₂SO₄ and the absorbance read at 450 nm in a microplate reader (Tecan Sunrise™, Tecan, Männedorf, Switzerland). The PSGL-1/mlG2b concentration was estimated using for calibration a dilution series of purified mlG2bs (AbD Serotec, Oxford, UK) in blocking buffer as an internal standard. The blood group A or B epitope density on the fusion protein was determined by comparing the relative optical density from two ELISAs; HPA A or B epitope density on the fusion protein was determined by using the Pro Q Emerald 300 Glycoprotein detection kit in combination with Ruby (Molecular Probes, Leiden, the Netherlands). These gels were visualized in a Flour-S Max Multilramer carrying a CCD camera (Bio-Rad, Hercules, CA).

Chemical release of O-linked glycans from purified PSGL-1/mlG2b

Oligosaccharides to be analyzed by LC-MS analysis were released by β-elimination and analyzed as native structures. Briefly, lyophilized PSGL-1/mlG2b proteins were dissolved in water to 1 mg/mL concentration. The glycans were released in a solution of 0.5 M [or in some (AT2-29 and AT3-46) cases 1.5 M] NaBH₄ in 50 mM KOH and incubated overnight at 60°C. The reaction was stopped by adding acetic acid. Released glycans were desalted on AG50Wx8 cation exchange beads (Bio-Rad) packed on the top of a C18 Zip-tip (Millipore, Bedford, MA). 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 (five times). Dried glycans were dissolved in water for analysis by LC-MS.

MS analyses

LC-MS was performed with a graphitized carbon column coupled to an LTQ LC/MS² ion trap instrument (Thermo Fischer Scientific, San Jose, CA) as described previously (Karlsson et al. 2004), but with slight modifications. The column was packed with 5 µm Hypercarb particles (Thermo, Hypersil-Keystone, Runcorn, UK). Glycans were released with a gradient of 0–40% acetonitrile in 8 mM ammonium bicarbonate buffer and detected in a negative mode by a full scan (m/z 383–2000) followed by MS² scans of the most intense ions. The needle voltage was ~3.2 kV.

Interpretation and annotations of MS² spectra were performed with the GlycoWorkbench software (Ceroni et al. 2007, 2008). The original spectra were exported from the spectrum software viewer QualBrowser 1.2 and imported into GlycoWorkbench as peak list text files. Carbohydrate structures were drawn manually and fragment ion (B, C, Z, Y and cross-ring fragments) masses were calculated using the fragment tool of the program. Fragments were matched with the peak lists. The corresponding MS² fragment spectra for the structures listed in Table II have been submitted to Unicarb-DB (www.unicarb-db.com last accessed March 3, 2013; Hayes et al. 2011).

Sepharose-linked blood group A antigens

Blood group A substituted PSGL-1/mlG2b (C-PA1-4) was coupled to NHS-activated Sepharose 4 Fast Flow (4FF) beads (17-0906-01, GE Healthcare) according to the manufacturer’s protocol. To determine the amount of PSGL-1/mlG2b linked to the sepharose beads, a goat anti-mIgG (Fc) sandwich ELISA (see the previous procedure) was run on the coupling
solution before and after coupling. Sepharose 4FF-linked blood group A tetrasaccharides were obtained from Dextra Laboratories (Reading, UK; Lindberg et al. 2012). The following sepharose-linked oligosaccharides were used: A type 1 [GalNAcα3(Fucα2)Galβ3GlcNAcβ1-R], A type 2 [GalNAcα3(Fucα2)Galβ4GlcNAcβ1-R], A type 3 [GalNAcα3(Fucα2)Galβ3GlcNAcα1-R] and A type 4 [GalNAcα3(Fucα2)Galβ3 GlcNAcβ1-R].

Adsorption of anti-A from human blood group O serum
Pooled serum from ~100 healthy, group O blood donors were purchased from Blodcentrals Skanstull (Stockholm, Sweden). The serum was heat-inactivated at 56°C for 30 min and stored at ~8°C until being used. Heat-inactivated serum from seven non-transfused males of blood group AB was included as a negative control in ELISAs.

Sepharose beads with linked blood group A substituted PSGL-1/µgG2b (C-PA1-4) or blood group A tetrasaccharides (types 1–4) were incubated with pooled blood group O serum in propylene tubes by rolling head over tail at room temperature for 2 h. Following adsorption, the beads were removed by centrifugation at 450 × g for 10 min, and the serum was collected and stored in aliquots at ~8°C until further analyzed.

The following serum-to-bead ratios were used: 8 mL of serum to 100 µL of beads carrying PSGL-1/µgG2b (C-PA1-4) at a density of 0.63 mg/mL; 8 mL of serum to 100 µL of beads with A tetrasaccharides (types 1 and 2 at densities of 0.6 µmol/mL) and 5 mL of serum to 100 µL of beads with mixed A tetrasaccharide (types 1–4) at densities of 0.6 µmol/mL (i.e. 0.15 µmol/mL of each tetrasaccharide).

Neoglycoprotein ELISA
Human serum albumin (HSA) linked blood group A oligosaccharides were obtained from Dextra Laboratories (Lindberg et al. 2012). The following HSA NGP conjugates were used: A type 1 [GalNAcα3(Fucα2)Galβ3GlcNAcβ1-R], A type 2 [GalNAcα3(Fucα2)Galβ4GlcNAcβ1-R], A type 3 [GalNAcα3(Fucα2)Galβ3GlcNAcβ1-R] and A type 4 [GalNAcα3(Fucα2)Galβ3 GlcNAcβ1-R]. The ELISA was done as described before using alkaline phosphatase-conjugated goat polyclonal anti-human IgG (109-056-098, Jackson ImmunoResearch, West Grove, PA) diluted 1:5000 (Lindberg et al. 2012).

Statistical analysis
The difference in the relative A type 3 epitope density on PSGL-1/µgG2b, produced in CHO-K1 and Lec2 cells was tested for significance using the non-parametric Mann–Whitney U-test (Minitab8 Statistical Software, Minitab Inc., PA). A P-value of <0.05 was considered to be significant.

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Abbreviations
BSA, bovine serum albumin; CHO, Chinese hamster ovary; CV, column volumes; dHex, deoxyhexose; ELISA, enzyme-linked immunosorbent assay; FT, fucosyltransferase; Fuc, fucose; FUT1, α1,2-fucosyltransferase-1; FUT2, α1,2-fucosyltransferase-2; Gal, galactose; GalNAc, N-acetylgalactosamine; GalNACT, α1,3-N-acetylgalactosyltransferase; GalT, galactosyltransferase; GlcNAc, N-acetylgalcosamine; Hex, hexose; HexNac, N-acetyllhexosamine; HPA, Helix pomatia agglutinin; IA, immunoadsorption; LC-MS, liquid chromatography–mass spectrometry; mlg, mouse immunoglobulin; NeuAc, N-acetyllneuraminic acid; PBS, phosphate-buffered saline; PBS-T, PBS Tween-20; PNGase F, N-glycosidase F; PSGL-1, P-selectin glycoprotein ligand-1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

References


