Pichia pastoris-produced mucin-type fusion proteins with multivalent O-glycan substitution as targeting molecules for mannose-specific receptors of the immune system

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Mannose-binding proteins like the macrophage mannose receptor (MR), the dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) and mannose-binding lectin (MBL) play crucial roles in both innate and adaptive immune responses. Immunoglobulin fusion proteins of the P-selectin glycoprotein ligand-1 (PSGL-1/mlIgG2b) carrying mainly O-glycans and, as a control, the α1-acid glycoprotein (AGP/mlIgG2b) carrying mainly N-linked glycans were stably expressed in the yeast Pichia pastoris. Pichia pastoris-produced PSGL-1/mlIgG2b was shown to carry O-glycans that mediated strong binding to mannose-specific lectins in a lectin array and were susceptible to cleavage by α-mannosidases including an α1,2- but not an α1,6-mannosidase. Electrospray ionization ion-trap mass spectrometry confirmed the presence of O-glycans containing up to nine hexoses with the penta- and hexasaccharides being the predominant ones. α1,2- and α1,3-linked, but not α1,6-linked, mannose residues were detected by 1H-nuclear magnetic resonance spectroscopy confirming the results of the mannosidase cleavage. The apparent equilibrium dissociation constants for binding of PNGase F-treated mannosylated PSGL-1/mlIgG2b to MR, DC-SIGN and MBL were shown by surface plasmon resonance to be 126, 56 and 16 nM, respectively. In conclusion, PSGL-1/mlIgG2b expressed in P. pastoris carried O-glycans mainly comprised of α-linked mannoses and with up to nine residues. It bound mannose-specific receptors with high apparent affinity and may become a potent targeting molecule for these receptors in vivo.

Keywords: DC-SIGN / mannosose-binding lectin / mannose receptor / mucin / Pichia pastoris

Introduction

Mannose-binding proteins play crucial roles in both innate and adaptive immune responses. Attempts to elucidate their detailed ligand specificity have intensified in recent years because of their potential use as targeting receptors for vaccines (Keler et al. 2004) and other therapeutics (Irache et al. 2008).

The mannose receptor (MR or CD206, previously known as the macrophage MR, MMR), a C-type lectin abundantly expressed on macrophages and dendritic cells (DCs), binds glycoconjugates with high affinity and promotes endocytosis through several carbohydrate recognition domains (CRDs; Taylor et al. 1990, 1992). Different CRDs in conjunction with an extended conformation enable it to distinguish between endogenous and exogenous glycosylated molecules (Taylor and Drickamer 1993; Napper et al. 2001), thus participating in as diverse functions as immune responses to pathogens such as Mycobacterium tuberculosis (Schildesinger 1993) and the regulation of endogenous protein levels (e.g. tissue plasminogen activator; Otter et al. 1991). The MR is a type 1 transmembrane protein. The extracellular part consists of a cysteine-rich domain, a fibronectin type I repeat and eight CRDs (Taylor et al. 1990). Binding studies have shown that CRD4 is the only one of the eight CRDs capable of binding mannoside on its own, albeit with low affinity. The affinity is considerably improved upon the involvement of CRD5 in the binding, while high-affinity binding of natural ligands requires the involvement of CRD4–8 (Taylor et al. 1992; Taylor and Drickamer 1993).

Another mannose-binding receptor is DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN...
or CD209; Geijtenbeek, Kwon, et al. 2000; Geijtenbeek, Torensma, et al. 2000). This receptor has a more restricted expression pattern than the MR as it is mainly expressed on immature and mature DCs with crucial functions in DC trafficking, T-cell interactions and pathogen recognition (Geijtenbeek et al. 2002). It is a type II membrane protein consisting of an extracellular part including a CRD and a neck domain, a transmembrane and a cytoplasmic region. The latter region contains several internalization motifs, allowing DC-SIGN to recycle between the cell surface and endosomes. DC-SIGN has been shown to preferentially bind to high-mannose structures (Feinberg et al. 2001; Mitchell et al. 2001). Binding of DC-SIGN to the internal pentasaccharide GlcNAcβ1,2Manα1,3[β1,2Manα1,6]Man revealed that two monomers, cross-linked by the oligosaccharide, are involved in binding. The trimannose part of this pentasaccharide is present in both high-mannose and complex N-glycan structures but binding to the trimannose core in the latter is prevented by steric interference caused by different anomeric linkages. On the cell surface, it appears that high-affinity binding of high-mannose structures is achieved by heterotramer formation via the neck region (Mitchell et al. 2001; Bernhard et al. 2004). Despite the seemingly high affinity for high-mannose structures, it has been shown that in the tetramer formation also multivalent monomeric mannose, as in, for example, Man$_{30}$-bovine serum albumin (BSA), is bound with high affinity by DC-SIGN (Mitchell et al. 2001).

The mannose-binding lectin (MBL) is yet another C-type lectin which selectively binds mannose-containing oligosaccharides. MBL belongs to the collectins, a group of proteins comprised of a collagenous region, a neck region and a CRD (Dommet et al. 2006). MBL is made up of three identical polypeptide chains twisted around each other via the collagenous region. It is produced by the liver and secreted into the serum where it functions in activation of complement, in opsonophagocytic processes, in modulation of inflammation as well as in promotion of apoptosis (Dommet et al. 2006). As for DC-SIGN, the oligomerization of MBL seems important for its carbohydrate-binding properties. Most common are tri- and tetrameric homo-oligomers, referred to as MBL-I and -II, where it has been shown that the tri- and tetramer structures have similar affinities for both mannose- and N-acetylglucosamine-based glycoconjugates. The tetramer shows a considerably higher binding capacity and a lower dissociation rate when compared with the trimeric structure. On the other hand, no differences were seen between the two oligomers with regard to the complex formation with the MBL-associated serine protease-1, -2 and -3 (Teillet et al. 2005). Conformational studies have shown that the CRDs exhibit substantial flexibility in their orientation (Jensenius et al. 2009), which is likely to play a significant role for recognition of different ligands in a multivalent fashion, for example, on a bacterial or viral surface.

Even though the mannose-specific receptors to some extent have evolved to protect us against the invasion of pathogens like fungi that carry mannlosylated glycans on their surfaces, their fine specificity toward more complex mannosylated structures is incompletely characterized. It is also not clear whether the binding of these receptors is restricted to high-mannose type N-glycans or whether O-glycans carrying oligomannose structures can also be bound. To address this question, a P-selectin glycoprotein ligand-1 immunoglobulin fusion protein (PSGL-1/mIgG$_{2b}$) carrying mainly O-glycans was expressed in _P. pastoris_ and characterized with regard to its binding to the MR, DC-SIGN and MBL. An α1-acid glycoprotein immunoglobulin fusion protein (AGP/mIgG$_{2b}$) carrying mainly N-glycans was used for comparison in the binding studies. Further, O-glycans carried by the mucin-type fusion protein (PSGL-1/mIgG$_{2b}$) were characterized by exoglycosidase cleavage, Western blotting, mass spectrometry (MS) and 1H-nuclear magnetic resonance (1H-NMR) spectroscopy.

**Results**

**PSGL-1/mIgG$_{2b}$ and AGP/mIgG$_{2b}$ expression in _Pichia pastoris_**

The biomass concentration (expressed as g dry cell weight per liter cultivation volume) was after glycerol batch and fed-batch phases 29.1 ± 0.8 g/L and 44.2 ± 1.0 g/L and grew to ~55 g/L for both strains after 40–60 h of induction (Figure 1). Although there was no significant difference (P = 0.05) in biomass generation between the PSGL-1/mIgG$_{2b}$ and AGP/mIgG$_{2b}$ expressing strains during the glycerol batch and glycerol fed-batch phases, there appeared to be a slight difference during the methanol fed-batch phases. According to the regression models fitted to the data with a 95% confidence interval, fusion protein concentration in the culture supernatant reached 92 ± 12 mg/L for PSGL-1/mIgG$_{2b}$ and 13.4 ± 2.5 mg/L for AGP/mIgG$_{2b}$ after 48 h induction (Figure 1). Fusion protein accumulation in the culture supernatant stopped after ~50 h of induction for both strains.

![Fig. 1](https://academic.oup.com/glycob/article-abstract/21/8/1071/1988319) Bioreactor cultivation of _P. pastoris_ clones. Variations in fusion protein concentration in culture supernatant and dry cellular mass during the methanol fed-batch (induction) phase. PSGL-1/mIgG$_{2b}$ concentration (crosses); AGP/mIgG$_{2b}$ concentration (filled squares); dry cellular mass for PSGL-1/mIgG$_{2b}$ expression (filled triangles); dry cellular mass for AGP/mIgG$_{2b}$ expression (filled diamonds). Quadratic regression models were fitted to the data using a significance level of 0.05. Statistical analysis was based on at least duplicate cultivations.
PSGL-1/mIgG₂b and AGP/mIgG₂b produced in P. pastoris are both expressed as dimers and are heavily mannosylated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analyses of purified PSGL-1/mIgG₂b produced in Chinese hamster ovary (CHO)-K1 (lane 1) and P. pastoris (lane 2) cells revealed a protein of 200–350 kDa under non-reducing conditions (Figure 2A–D). The apparent MW of AGP/mIgG₂b was ~120 kDa under non-reducing conditions, which is likely to represent the dimeric form of AGP/mIgG₂b (Figure 2A–C and E, lane 3). All three proteins (lanes 1–3) bound anti-mouse IgG (Figure 2C), while as expected only the PSGL-1 fusion proteins bound anti-PSGL-1 (Figure 2D). The anti-PSGL-1 antibody bound considerably weaker to P. pastoris- (lane 2) when compared with CHO-produced (lane 1) PSGL-1/mIgG₂b (Figure 2D). The AGP fusion protein was reactive with the anti-AGP antibody (Figure 2E, lane 3). Pichia pastoris-produced PSGL-1/mIgG₂b (lane 2), AGP/mIgG₂b (lane 3) and the bovine thyroglobulin control (lane 4), known to contain high-mannose type N-glycans (Rawitch et al. 1993), bound strongly to the concanavalin A (Con A) lectin (Figure 2F). Also PSGL-1/mIgG₂b expressed in CHO-K1 cells reacted with the Con A lectin (Figure 2F, lane 1), albeit considerably weaker than the P. pastoris-produced mucin-type fusion protein. This binding is due to the presence of internal mannoses in the N-glycan pentasaccharide core as verified by abolished binding upon PNGase F cleavage (see section “PSGL-1/mIgG₂b produced in P. pastoris carries mannose-containing O-glycans”). In addition to bands corresponding to the major glycoforms of the PSGL-1 and AGP Ig fusion proteins, bands of lower and higher molecular weight were found.

**PSGL-1/mIgG₂b produced in P. pastoris carries mannose-containing O-glycans**

The binding of Con A to CHO-produced PSGL-1/mIgG₂b was abolished by PNGase F-treatment (Figure 3A; compare lane 1 with lane 2), showing that CHO-produced PSGL-1/mIgG₂b carries mannose residues only as part of its N-glycans. In contrast, Con A binding to P. pastoris-produced PSGL-1/mIgG₂b resisted PNGase F-treatment (Figure 3B; compare lane 3 with lane 4) indicating that the carbohydrate determinants recognized by the Con A lectin were carried also on O-glycans. The complete shift in molecular size without any appearance of intermediary sized forms of PSGL-1/mIgG₂b upon PNGase F-treatment suggests that the enzymatic release was complete (Figure 3C and D, compare lane 1 with lane 2, and lane 3 with lane 4). As noted above, anti-PSGL-1 binds very weakly to P. pastoris-produced PSGL-1/mIgG₂b (Figure 3D, lane 3). However, binding is increased upon PNGase F-treatment (Figure 3D, lane 4).

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**Fig. 2.** SDS–PAGE and Western blot analysis of PSGL-1/mIgG₂b and AGP/mIgG₂b produced in P. pastoris cells. PSGL-1/mIgG₂b purified from CHO cells (lane 1) or P. pastoris cells (lane 2), AGP/mIgG₂b purified from P. pastoris (lane 3) and bovine thyroglobulin (lane 4; positive control for Con A). Five micrograms of protein were loaded per well and glycosylated proteins detected by Pro Q Emerald (A) followed by a detection of all proteins by Ruby (B). For Western blot analyses, 500 ng of protein were loaded per well and membranes probed with anti-mIgG(Fc) (C), anti-PSGL-1 (D), anti-AGP (E) and Con A (F).
of *P. pastoris*-produced PSGL-1/mIgG2b do not contain α1,6-linked mannoses either terminally or subterminally. Enzymatic cleavage with a non-linkage-specific α-mannosidase resulted in a considerably larger molecular weight shift of the fusion protein compared with that when only an α1,2-mannosidase was used (Figure 4A, lane 6). This implies that a fraction of *O*-glycans on PSGL-1/mIgG2b produced in *P. pastoris* contains α-linked mannoses that are not α1,2- or α1,6-linked. PNGase F-treated PSGL-1/mIgG2b was also incubated with a β-mannosidase for different time periods and at different temperatures. Overnight digestion at 37°C resulted in total degradation of the fusion protein demonstrated by the lack of anti-PSGL-1 binding (not shown). Incubation at 50°C for 2 h resulted in some remaining anti-PSGL-1 binding (not shown), but most of the fusion protein was still degraded suggesting the presence of proteases in the β-mannosidase solution provided by the manufacturer. Protein degradation was avoided by addition of a protease inhibitor cocktail to the β-mannosidase incubation at 50°C for 2 h (Figure 4A, lane 7). No molecular weight shift was seen, indicating that terminal β-linked mannose is not present in the *O*-glycans of *P. pastoris*-produced PSGL-1/mIgG2b provided that the β-mannosidase was not also inhibited by the protease inhibitor cocktail (cf. lanes 2 and 7). Mannosidase-treated PSGL-1/mIgG2b was analyzed with regard to its binding to Con A (Figure 4B); a binding that verified the results seen in Figure 4A. However, additional bands of lower molecular weight and not detected by anti-PSGL-1 were seen (lanes 2–7). They most likely represent fusion protein break-down products devoid of the anti-PSGL-1 epitope.

**PSGL-1/mIgG2b and AGP/mIgG2b produced in *P. pastoris* are highly mannosylated and show completely different lectin-binding patterns compared with CHO-produced PSGL-1/mIgG2b**

A lectin array was used in order to establish a glycan profile for PSGL-1/mIgG2b produced in *P. pastoris* and CHO as well as for AGP/mIgG2b produced in *P. pastoris* (Figure 5). In summary, *P. pastoris*-produced fusion proteins reacted strongly with MBLs such as Con A, GNA, HHL, LCA, NPA, PSA and UDA (for lectin specificity, Table 1). However, the signal of Calsepa, which also recognizes mannose, was weak indicating that this lectin recognizes a mannosylated structure that is not present on either AGP/mIgG2b or PSGL-1/mIgG2b. *Pichia pastoris*-produced fusion proteins were further characterized by weak expression of galactose (BPL, ECA, RCA120) and fucose (AAL, AOL, LTL, TJA-II and UEA-I). CHO-produced PSGL-1/mIgG2b on the other hand showed binding specific for complex-type *N*-glycans (ACG, DSA, ECA, PHA-E and RCA120), fucose (AAL, AOL, TJA-II and TxlCI) and the determinants carried by *O*-glycans, Tn, sialyl-T and disialyl-T (ABA, ACA, BPL, Jacalin, MAH and MPA).

The total binding intensity of AGP/mIgG2b was much weaker than that of PSGL-1/mIgG2b, indicating that the overall glycan expression level is considerably lower for AGP/mIgG2b. This is consistent with the fact that AGP is a globular protein with *N*-linked glycans only, while PSGL-1 is a heavily *O*-glycosylated mucin-type protein with 53 potential *O*-glycosylation sites.
MS of permethylated oligosaccharides released from purified, recombinant PSGL-1/mIgG2b produced in P. pastoris

O-glycans released by reductive β-elimination from P. pastoris-produced PSGL-1/mIgG2b were permethylated and characterized using electrospray ionization MS (ESI-MS). Five peaks corresponding to fragments with masses explained by the sodiated molecular ions of permethylated Hex2-6 structures were seen (Figure 6A). These findings correspond well with previous analyses of O-glycans from P. pastoris-derived
glycoproteins (Trimble et al. 2004). In addition, three peaks corresponding to \(\text{Hex}_{3,9}\) structures were seen. The \(\text{Hex}_{3,8}\) structures were confirmed by MS\(_x\) analyses (see representative daughter ion spectra; Figure 6B–E), while the peak most likely corresponding to \(\text{Hex}_9\) was too small for MS\(_x\) analysis.

NMR analyses confirm the presence of \(\alpha\)- but not \(\beta\)-linked mannoside residues in \(O\)-glycans from recombinant PSGL-1/mIgG\(_{2b}\). Figure 7 shows the anomeric region of the one-dimensional and two-dimensional double quantum-filtered correlated spectroscopy (DQF COSY) spectra of reduced \(O\)-glycans released from PSGL-1/mIgG\(_{2b}\), revealing a number of both \(\alpha\)- and \(\beta\)-signals as well as several broader resonances of most likely protein origin. The \(\beta\)-signals appearing at 4.725 ppm (GlcNAc\(\beta\)) and 4.461 ppm (GlcNAc\(\beta,4\)) and an \(\alpha\)-signal at 5.245 ppm (GlcNAc\(\alpha\)) probably represent the first two residues of \(N\)-linked glycocalcic core structures (GlcNAc\(\beta,4\), GlcNAc\(\alpha/\beta\); van Halbeek et al. 1980, 1981; Jars et al. 1995). A third \(\beta\)-signal can be seen at 4.655 ppm whose origin cannot be determined at present. The remaining \(\alpha\)-signals are all due to mannosides. Literature values for \(O\)-linked mannoose structures from \(P.\) pastoris are lacking but closely related structures from \(S\). cerevisiae have been characterized by MS and/or NMR. In the case of \(S\). pombe, linear structures up to three mannosides (Man\(1\),2Man\(1\),2Man–ol) have been found (Gemmill and Trimble 1999). From the COSY spectrum in Figure 7, the H1/H2 connectivities corresponding to Man\(1\),2Man–ol (5.01/4.01 ppm) and Man\(1\),2Man\(1\),2Man–ol (5.07/4.09 ppm and 5.26/4.03 ppm) are found. These values are precisely in accord with those found for the \(S\). pombe structures (Gemmill and Trimble 1999). It is to be noted that no NMR evidence was found in \(P\). pastoris for the presence of the Gal\(1\),2Gal\(1\),3Man\(1\),2 hybrid structures up to the pentasaccharide level as in \(S\). pombe. It is clear, however, that structures longer than three sugar residues are present since the H1/H2 connectivities at 5.42/4.08 and 5.22/3.89 ppm remain to be assigned. Considering that the MS data above indicated linear oligosaccharide sequences (Figure 6A–C) and that only the Man\(1\),2 glycosidase trimmed the non-reducing end(s) and that the Man\(1\),6 glycosidase was ineffective also at the penultimate level suggests that Man\(1\),3 residues are blocking further trimming. In fact, literature values for internal Man\(3\) residues found in \(N\)-glycans vary from 5.33/4.10 to 5.41/4.09 ppm when followed by one or two Man\(1\),2 residues (Dorland et al. 1981). Thus, the H1/H2 connectivity at 5.42/4.08 ppm in Figure 7 is consistent with the presence of a penultimate Man\(1\),3 residue which minimally would result in a Man\(1\),2Man\(1\),3Man\(1\),2Man–ol sequence. Furthermore, the H1/H2 connectivity at 5.22/3.89 ppm may then be rationalized as stemming from the Man\(1\),2 residue preceding Man\(1\),3. Inspection of both the one-dimensional and COSY spectra reveals, moreover, that most peaks consist of overlapping components as well as minor cross peaks at 5.25/4.06, 5.19/3.94 and 5.01/4.05 ppm (not seen at the gain used in Figure 7) in close proximity of the major ones suggesting the presence of even longer sequences.

**Multivalent expression of oligomannose on**

**P. pastoris-produced PSGL-1/mIgG\(_{2b}\) and AGP/mIgG\(_{2b}\)**

**confers high-affinity binding to recombinant MR, DC-SIGN and MBL as revealed by surface plasmon resonance**

The apparent equilibrium dissociation constants, \(K_D\), for \(P.\) pastoris-produced AGP/mIgG\(_{2b}\) and PSGL-1/mIgG\(_{2b}\) were in the nanomolar range for all recombinant receptors, indicating specific, high-affinity binding in all cases. The dissociation constants for each combination are listed in Table II. In contrast, no binding was observed for \(\alpha\)-mannose, and poor

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Table I. Specificities of lectins in the lectin array

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<th>Lectin no.</th>
<th>Lectin</th>
<th>Reported specificity</th>
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<tbody>
<tr>
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<tr>
<td>3</td>
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<td>Fuc(1)-6GlcNAc, (\alpha)-D-Glc, (\alpha)-D-Man</td>
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<tr>
<td>4</td>
<td>UEA-I</td>
<td>Fuc(1)-2Gal(1)-4GlcNAc</td>
</tr>
<tr>
<td>5</td>
<td>AOL</td>
<td>Fuc(1)-6GlcNAc (core fucose)</td>
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<td>AAL</td>
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</table>

These data were collected from lectin vendors and reports found by internet searches. Copied from (http://www.gpbio.jp) with permission from GP Biosciences.
binding was observed for oligomannose-9 (data not shown) and CHO-produced PSGL-1/mIgG2b. Therefore, no affinity constants could be calculated for these compounds.

**MR, DC-SIGN and MBL bind with high affinity to both N- and O-glycan oligomannose structures**

The apparent equilibrium dissociation constants ($K_D$) for untreated *P. pastoris*-produced PSGL-1/mIgG2b ranged from 16 to 307 nM for all recombinant receptors. PNGase F-cleavage of the $N$-glycans did not significantly affect the binding to MR or DC-SIGN, suggesting that the $O$-glycans are very important for binding of the mannosylated mucin-type fusion protein to these receptors (Table II). Representative curves for the binding of PNGase F-treated PSGL-1/mIgG2b to the MR, DC-SIGN and MBL are shown in Figure 8.

**Discussion**

*Pichia pastoris*-produced PSGL-1/mIgG2b and AGP/mIgG2b displayed specific, high-affinity binding to the MR, DC-SIGN and MBL, while CHO-produced PSGL-1/mIgG2b, which carries complex-type $N$-glycans and $O$-glycans without terminal mannoses, displayed poor binding. These findings are generally consistent with previous studies which have indicated that the CRDs of the MR and MBL specifically interact with single terminal mannose residues and that the CRD of DC-SIGN binds selectively to high-mannose type $N$-glycans (Taylor et al. 1992; Weis et al. 1992; Feinberg et al. 2001). Upon the removal of the high-mannose type $N$-glycans from *P. pastoris*-produced PSGL-1/mIgG2b by PNGase F-treatment, no major differences in binding affinities to the receptors were observed. The results show that mannosylated $O$-glycan structures are capable of mediating high-affinity binding to the MR, MBL and DC-SIGN similar to what has been reported before for high-mannose type $N$-glycans. Although it may be expected that the terminal mannoses of $O$-glycans on the *P. pastoris*-produced PSGL-1/mIgG2b can mediate binding to the MR and MBL, previous studies on the selective binding of DC-SIGN to high-mannose type $N$-glycans have not indicated that it can bind mannosylated $O$-glycan structures (Feinberg et al. 2001).

The results suggest that the $O$-glycans of *P. pastoris*-derived PSGL-1/mIgG2b were mainly composed of Man$_{2,6}$ oligosaccharides in which the individual mannose residues were $\alpha$1,2- and $\alpha$1,3-linked, but not $\alpha$1,6-linked. These findings are generally consistent with previous studies. The Man$_{2,5}$ $O$-glycans of both endogenous cell wall proteins and the kringle 1-4 domain of human plasminogen expressed in *P. pastoris* had the mannose residues $\alpha$1,2-linked (Duman et al. 1998). The $O$-glycans of human bile salt-stimulated lipase secreted by *P. pastoris* were mainly comprised of $\alpha$1,2-linked Man$_{2,4}$ oligosaccharides (Trimble et al. 2004). Some Man$_{3,6}$ structures were also identified, in which the terminal mannose of...
Man₆ and the terminal and subterminal mannoses of Man₆ were β₁,₂-linked (Trimble et al. 2004). This is in contrast to the O-glycans found on PSGL-1/mIgG₂b which appeared not to contain any β-linkages. When Toxoplasma gondii surface antigen I (SAGI) and gelatinase B were expressed and secreted by P. pastoris, O-glycans were also shown to include α₁,₃- and α₁,₆-linkages besides α₁,₂ (Van den Steen et al. 1998; Letourneur et al. 2001). In contrast to O-glycans in

Fig. 6 Continued
Our results are consistent with this, as α1,3-linkages were only identified as penultimate mannose residues.

Reports have suggested that synthetic oligolysine-based dimannoside clusters with at least a trilysine core are good...
ligands for the MR (Frison et al. 2003). The (Man\(_{\alpha1,6}\)Man)\(_5\)Lys\(_4\) cluster was shown to bind better than (Man\(_{\alpha1,3}\)Man)\(_5\)Lys\(_4\), which in turn bound better than (Man\(_{\alpha1,2}\)Man)\(_5\)Lys\(_4\) clusters (Frison et al. 2003). Hence, the type of linkage between the terminal and subterminal mannose residues may influence the ability to interact with the CRDs of the MR and maybe also the CRDs of MBL and DC-SIGN. Despite the lack of \(\alpha1,6\)-linked mannoses on its \(O\)-glycans, \(P\). \textit{pastoris}-produced PSGL-1/mIgG\(_2\) bound with higher affinity to the MR than any of the oligolysine-based dimannoside clusters.

The larger Man\(_{7,8}\) \(O\)-glycans observed on PSGL-1/mIgG\(_2\) have not been confirmed in previous studies, although hyper-O-glycosylation has been suggested for \(P\). \textit{pastoris}-produced SAGI (Letourneur et al. 2001).

Dimeric AGP/mIgG\(_2\) has the potential to carry 12 \(N\)-linked glycans, which upon expression in \(P\). \textit{pastoris} are likely to be of the high-mannose type. The presence of different AGP/mIgG\(_2\) species of different molecular weight indicates variation in \(N\)-glycan size and site occupancy. Based on other studies, these \(N\)-glycans are likely to be Man\(_{8,9}\)GlcNA\(_2\) oligosaccharides, although the majority of them might be Man\(_{8,9}\)GlcNA\(_2\) oligosaccharides (Trimble et al. 1991; Montesino et al. 1998; Blanchard et al. 2008).

The poor binding of \(D\)-mannose and oligomannose-9 to the MR, MBL and DC-SIGN suggests that interactions between individual CRDs of the receptors and a single, \(P\). \textit{pastoris}-produced \(O\)-glycan would be weak and that other complementary interactions are important to generate the high binding affinity observed between PNGase F-treated PSGL-1/mIgG\(_2\) and the different receptors. Such complementary interactions could be multivalent binding and non-specific interactions between the protein part of PSGL-1/mIgG\(_2\) and the CRDs of the receptors (Lee et al. 1992; Coombs et al. 2010). The distinguishing feature of \(P\). \textit{pastoris}-produced PSGL-1/mIgG\(_2\) is its potential to present a large number, potentially 106, of \(O\)-glycans on an extended polypeptide core, as opposed to the

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**Table II.** Apparent equilibrium dissociation constants (\(K_D\)) for binding of PSGL-1/mIgG\(_2\) and AGP/mIgG\(_2\) to MMR, DC-SIGN and MBL

<table>
<thead>
<tr>
<th>Analyte/receptor</th>
<th>MMR (nM)</th>
<th>DC-SIGN (nM)</th>
<th>MBL (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(P). \textit{pastoris}-produced PSGL-1/mIgG(_2)</td>
<td>307</td>
<td>40.3</td>
<td>16</td>
</tr>
<tr>
<td>PNGase F-treated (P). \textit{pastoris}-produced PSGL-1/mIgG(_2)</td>
<td>126</td>
<td>56.2</td>
<td>67.7</td>
</tr>
<tr>
<td>(P). \textit{Pastoris}-produced AGP-1/mIgG(_2)</td>
<td>84.1</td>
<td>22.4</td>
<td>40.8</td>
</tr>
<tr>
<td>CHO-produced PSGL-1/mIgG(_2)</td>
<td>—</td>
<td>—</td>
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**Fig. 7.** NMR analyses of \(O\)-glycans released from PSGL-1/mIgG\(_2\) produced in \(P\). \textit{pastoris} cells. The anomeric region of the one-dimensional and two-dimensional DQF COSY spectra of reduced \(O\)-glycans obtained at 600 MHz and 25°C. The residual water signal (HDO) was used as internal standard and set to 4.80 ppm. The numbers above the anomeric glycan resonances represent approximate intensities (the resonances appearing at 5.22 ppm were arbitrarily set to a two-proton intensity). Di-s, tri-s and penta-s stand for di-, tri- and pentasaccharide, respectively. Cross peaks marked with an asterisk represent artifacts.

**Fig. 8.** Surface plasmon resonance analysis of the concentration-dependent binding of PNGase F-treated PSGL-1/mIgG\(_2\) to the MR, DC-SIGN and MBL. The graphs show response units vs time for binding of PNGase F-treated PSGL-1/mIgG\(_2\) to the MR (A), DC-SIGN (B) and MBL (C) at concentrations of 10, 2, 0.4, 0.08, 0.016, 0.0032 and 0.00064 \(\mu\)M.
more scarcely placed N-glycans on a globular protein such as AGP/mlgG2b. It has been suggested that MBL is particularly well suited to bind repeating sugar structures of microbial origin (Weis and Drickamer 1994). The distinct conformation of PSGL-1/mlgG2b with the PSGL-1 part covered with O-glycans and being at least 200 Å long, resembles in a way a microbial surface and should therefore be well suited to present terminal mannos residues to the three CRDs of MBL.

Similar to MBL, the MR seems to rely on several active CRDs to generate strong interactions with an oligosaccharide ligand. However, in contrast to MBL which present multiple CRDs by oligomerization, the MR carries its eight CRDs within a single polypeptide (Taylor et al. 1992; Mullin et al. 1997). It has been suggested that the MR adopts an extended conformation possibly reaching ~380 Å from the cell surface where CRD4 and 5 may be close enough to interact with terminal mannos in a single high-mannose type N-glycan (Mullin et al. 1997). Simultaneous interaction with other CRDs of the MR may require more widely spaced terminal mannos. PSGL-1/mlgG2b, with its extended conformation might be expected to provide terminal mannos which could potentially interact with MR CRDs other than 4 and 5. In fact, it has been shown that CRD4–8 are required to achieve high-affinity binding to multivalent glycoconjugates which would imply that PSGL-1/mlgG2b O-glycans interact with at least these CRDs of the MR (Taylor et al. 1992; Taylor and Drickamer 1993).

One would expect affinity enhancement through multivalency not to be as pronounced for DC-SIGN as for MBL or the MR, because it carries only a single CRD which has been shown to bind with high affinity to high-mannose type N-glycans (Mitchell et al. 2001). This has been suggested to reflect extensive interactions between the CRD and the oligosaccharide outside the primary mannos-binding site (Mitchell et al. 2001). However, PNGase F-treated P. pastoris-produced PSGL-1/mlgG2b bound with the same high affinity to DC-SIGN as to the MR and MBL. This suggests that the N-glycans played a minor part in the high-affinity interactions observed and that multivalent binding occurred to the O-glycans of PSGL-1/mlgG2b. This would be consistent with the finding that Man340-BSA has been shown to bind to the tetrameric extracellular domains of DC-SIGN with affinities in the nM range, although the mannos were monomeric and not of the high-mannose type N-glycans. The authors suggested that the large number of mannos residues scattered over a larger surface could generate a substantial affinity enhancement (Mitchell et al. 2001).

The high-affinity interactions between mannosylated PSGL-1/mlgG2b and MR, MBL and DC-SIGN suggest that this glycoprotein can be used as a targeting molecule for these receptors also in vivo. The MR and DC-SIGN are endocytic receptors which have the potential to improve antigen internalization and subsequent T-cell presentation (East and Isacke 2002; Engering et al. 2002), whereas MBL is a soluble plasma protein which participates in the activation of the complement system (Turner 1996). Conjugation of antigens to mannosylated PSGL-1/mlgG2b may provide an efficient way to target antigens to these receptors and thereby enhance antigen-specific immune responses. It has been shown that the type of mannosylation carried by the antigens is critical for the kind of response they elicit (Luong et al. 2007). For example, extensive O-linked mannosylation generated increased lymphoproliferative responses, while the presence of N-linked mannosylation was associated with decreased proliferative responses (Luong et al. 2007). The different conformations and carbohydrate substitutions of PSGL-1/mlgG2b may thus provide the means to modulate the immune responses toward conjugated antigens.

Materials and methods

Chemicals

Ammonium hydroxide, BSA, dithiothreitol (DTT), ethylene-diaminetetraacetic acid (EDTA), glucose, hydrochloric acid, sodium bicarbonate, sodium chloride, sodium citrate, sodium hydroxide, sodium phosphate, sulfuric acid, tris-base and Triton X-100 were purchased from Sigma-Aldrich (St Louis, MO). Calcium chloride, magnesium chloride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxy succinimide (EDC/NHS), ethanolamine, glycine and sodium acetate were purchased from Biacore, GE Healthcare (Uppsala, Sweden). Glutamine was from Invitrogen (Carlsbad, CA).

Construction of expression plasmids for subsequent P. pastoris transfection

The cDNA-encoding PSGL-1/mlgG2b was polymerase chain reaction (PCR)-amplified from the PSGL-1/mlgG2b expression plasmid (Liu et al. 1997) using 5′-CGC GGG AAT TTC AGC TGT GGG ACA CCT GGG-3′ and 5′-CGG GGA TCG GGG TAC A TT TAC CCG GAG ACC GGG AGA TG-3′ as forward and reverse primers, respectively, and was ligated into the multiple cloning site of the pPICZα vector (Invitrogen) following EcoRI digestion. The cDNA-encoding AGP/mlgG2b was PCR amplified from the AGP/mlgG2b expression plasmid (Holgersson and Lofling 2006) using 5′-CGA GAT CCC TGG AAA CCC CCT ACC TGG-3′ and 5′-CGG GGA TCG TCT ATT TAC CCG GAG ACC GGG AGA TG-3′ as forward and reverse primers, respectively. The AGP/mlgG2b fragment was digested by EcoRI and KpnI (New England BioLabs, Ipswich, MA) and, likewise, subcloned into the multiple cloning site of the pPICZα vector (Invitrogen). The sequences were confirmed by DNA sequencing.

Plasmid integration and selection of high-producing P. pastoris clones

The vectors pPICZαA:PSGL-1/mlgG2b and pPICZαA:AGP/mlgG2b were amplified in Escherichia coli XL-1 Blue using 25 µg/mL Zeocin™ (Invitrogen) as selective drug. Following purification, the vectors were linearized by PmeI (New England BioLabs) and transformed into P. pastoris GS115 cells according to the standard procedures (Easy Comp™, Invitrogen). Transformants of the Mut+ phenotype were subsequently identified by growing nine clones from each transformation on MDH (minimal dextrose medium + histidine) agar (1.34% w/v yeast nitrogen base, 4 × 10^{-3}% w/v histidine,
4 × 10⁻⁵% w/v biotin, 2% w/v glucose, 1.5% w/v agar) and on MMH (minimal methanol + histidine) agar (MDH but with 0.5% methanol instead of glucose) using P. pastoris GS115/Mut² and P. pastoris GS115/pPICZ/lacZ/Mut² as negative and positive controls, respectively. To screen for high-expressing clones, seven transformants of each transformation exhibiting the Mut² phenotype were selected and inoculated in buffered glycerol complex medium (BMGY; 1% w/v yeast extract, 2% w/v peptone, 1.5% w/v glycerol, 1.34% w/v yeast nitrogen base, 100 mM potassium phosphate, pH 6.0, 4 × 10⁻⁷% w/v biotin) and grown for 24 h at 29°C, at 180 rpm (Shake Incubator model 481, Thermo Fisher Scientific, Waltham, MA). This was followed by a 72-h induction period in buffered methanol-complex medium (0.5% v/v methanol, 1% w/v yeast extract, 2% w/v peptone, 1.34% w/v yeast nitrogen base, 100 mM potassium phosphate, pH 6.0, 4 × 10⁻⁷% w/v biotin) at 29°C, 180 rpm. MDH agar, MMH agar and BMGY components were purchased from Sigma-Aldrich with the exception of peptone and yeast extract which were from Merck (Darmstadt, Germany). Cell culture supernatants were then harvested by centrifuging at 10,000 × g for 10 min at 4°C, and the concentration of PSGL-1/mIgG2b and AGP/mIgG2b in the supernatants was assessed by enzyme-linked immunosorbent assay (ELISA) using a goat anti-mouse IgG (Fc) antibody (see section “Quantification of fusion protein using ELISA”).

**Construction of an expression plasmid for subsequent CHO transfection**

The PSGL-1/mIgG2b expression plasmid (Liu et al. 1997) was modified to contain an enterokinase (EK) cleavage site (Asp Asp Asp Asp Lys) by PCR amplification using 5'-CGC GGG CAT CCC CCT TAT CGT CAT CGT CCT GCT TCA CAG AGA TGT GTT CTC GGA-3' and 5'-CGC GGG AAG CTT ACC ATG GGG TCT CTC CAA CCG CTG G-3' as forward and reverse primers, respectively, and subsequent ligation following Hind3 and BamH1 digestion (New England BioLabs). The EK cleavage site can be used for down-stream release of the mIgG2b part of PSGL-1/Fc. The sequence was confirmed by DNA sequencing.

**Transfection and clonal selection of CHO cells**

CHO-K1 (ATCC®, Manassas; cat. no. CCL-61) cells, gradually adapted to serum-free medium, were seeded in Ex-cell 302 (JRH Bioscience, Lenexa, KS) medium in a Corning® (Corning Inc., NY) CellBind 75 cm² flask at a density of 1 × 10⁶ cells/mL. The cells were transfected with the Avr2 (New England Biolabs) linearized PSGL-1/Fc/mIgG2b expression plasmid, using Lipofectamine 2000 CD (Invitrogen), according to the manufacturer’s instructions. Forty-eight hours after transfection, the cells were incubated in selection medium containing 6 µg/mL puromycin (Sigma-Aldrich). The selection medium was changed every second to third day. After ~2 weeks, dead cells were removed using Dead Cell Removal MicroBeads (Miltenyi Biotech, Auburn, CA), according to the manufacturer’s instructions. Viable cells were single-cell cloned in 96-well plates and expanded in selection medium for ~2 weeks. To screen for high expressing clones, the concentration of PSGL-1/Fc/mIgG2b in the supernatants was assessed by ELISA using a goat anti-mouse IgG(Fc) antibody (see section “Quantification of fusion protein using ELISA”). The clone C-P55 was selected for further expansion and a second single-cell cloning step was performed in ProCHO-4 (Lonza, Basel, Switzerland) medium to generate the clone C-P55.2.

**Bioreactor cultivation of P. pastoris clones**

An inoculum was prepared by inoculating 50 mL BMGY media in a 500 mL shake flask with P. pastoris GS115 Mut² encoding either PSGL-1/mIgG2b or AGP/mIgG2b. The culture was incubated at 180 rpm (Shake Incubator model 481, Thermo Electron Cooperation) at 29°C until OD₆₀₀ was ~2. The bioreactor cultivations were conducted according to a methanol-limited fed-batch strategy (Invitrogen Pichia Fermentation Process Guidelines Version B 053002) in 1 L bioreactors (Biobundle, Applikon, The Netherlands) with an initial volume of 750 mL BMGY supplemented with 4% w/v glycerol and 1 g/L histidine. The glycerol batch phase was conducted at 29°C, pH 6.0. To reduce pH to 3.5 for the methanol fed-batch phases (induction phase), the pH controller was set to 3.5 during the glycerol fed-batch phase and allowed to be lowered by the metabolic activity of the cells. The pH was maintained by automatic addition of 15% NH₄OH. During the glycerol batch phase, the dissolved oxygen (DO) concentration, measured by a pO₂ electrode, was kept at 30% of oxygen saturation by keeping the agitation fixed at 700 rpm and varying the aeration and supply of pure oxygen as needed. The pO₂ electrode was calibrated before inoculation with oxygen saturation at 29°C, pH 6.0, one atmosphere and aeration of 0.75 L/min and an agitation of 700 rpm. After the initial glycerol was consumed, indicated by a DO value of 100%, the cells were fed with 50 mL of a 50% w/v glycerol including 12 mL PTM₁ (0.6% CuSO₄ × 5H₂O, 8 × 10⁻³% NaHCO₃, 0.3% MnSO₄ × H₂O, 0.02% NaMoO₄ × 2H₂O, 2 × 10⁻³% boric acid, 0.05% CoCl₂, 2% ZnCl₂, 6.5% FeSO₄ × 7H₂O, 0.02% biotin, 0.5% v/v H₂SO₄) salts per liter of glycerol at a rate of 12.5 mL/h. The DO was maintained at 30%. Following a 10 min starvation period, a 1.6 mL/h/L feed of pure methanol with 12 mL PTM₁ salts per liter of methanol was initiated. The feed was linearly increased to 5 mL/h/L over a 20 h period after which the feed was kept constant for the remainder of the cultivation. Aeration and pure oxygen feed were adjusted to maintain DO at 30%. Preliminary experiments indicated that histidine was never limiting when 5 mL of a 3.5% histidine solution was injected to the bioreactor prior to induction. Cell culture supernatants were harvested by centrifuging at 10,000 × g for 10 min at 4°C and filtered through sterile, 0.2 µm pore size, polyether sulfone (PES) vacuum filters (TPP, Trasadingen, Switzerland). The supernatants were finally treated with 0.5 mL of protease inhibitor cocktail (Sigma-Aldrich) per liter supernatant and stored at 4°C.

**Large-scale cultivation of CHO cells**

The C-P55.2 cell line was cultured in serum-free ProCHO4 medium (Lonza) in repeated batch mode in a 20 L wave bioreactor (Wave System 20/50 EH, GE Healthcare). The bioreactor was inoculated at 0.79 × 10⁶ viable cells/mL in a
volume of 5.2 L. At regular intervals, fresh cultivation medium with 2 mM glutamine was added as a bolus until the final volume in the bioreactor reached 10.3 L. The culture was harvested when the final cell density had reached $4.6 \times 10^6$ total cells/mL and the viability had dropped to 88%. The glucose, glutamine and pH levels were monitored daily and adjusted at optimal levels using sodium bicarbonate. Total cultivation time was 11 days. Cell culture supernatant was clarified by microfiltration using a $0.054 \text{m}^2$ Millistak+ POD C0HC filter (Millipore, Billerica, MA). The clarified supernatant was concentrated 20-fold 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). Finally, the product solution was treated with 1 mL/L protease inhibitor cocktail (Sigma-Aldrich) and 0.02% NaN₃ (Sigma-Aldrich) and stored at 4°C until purification.

Purification of PSGL-1/mIgG₂b and AGP/mlG₂b fusion proteins

All chromatographic procedures were carried out on an ÄKTAExplorer 100 controlled by the Unicorn software (v. 5.11; GE Healthcare). The clarified supernatants were sterile filtered with 0.22 μm PES filter (TPP) before loading onto a MabSelect SuRe column (GE Healthcare) pre-equilibrated with PBS. The column was washed with 10 column volumes (CVs) of PBS, and elution of recombinant fusion protein was achieved using 5 CVs 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 then dialyzed extensively (12–14 kDa cutoff) against MilliQ water at 4°C. After dialysis, the samples were frozen, lyophilized and stored at −80°C before 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 a pre-equilibrated HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare). Typically, 5 mL sample was applied to 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. Pooled fractions were then dialyzed as above, frozen, lyophilized and stored at −80°C.

Quantification of fusion protein using ELISA

The concentrations of recombinant fusion protein in supernatants and in purified fractions were determined by a two-antibody sandwich ELISA method. The 96-well ELISA plates (Corning Inc.) were coated with an affinity-purified, polyclonal goat anti-mouse IgG(Fc) antibody (Sigma-Aldrich) at a concentration of 10 μg/mL at 4°C overnight. The plate was blocked with 1% BSA in PBS which was also used for dilution of fusion protein as well as the second, detecting antibody (peroxidase-conjugated, anti-mouse IgG(Fc) antibody; Sigma-Aldrich, 1:1000). All incubations lasted for 2 h. Between and after incubations, the plates were washed with PBS containing 0.5% (v/v) Tween 20. 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. The fusion protein concentration was estimated using for calibration a dilution series of purified mouse IgG₂b (AbD Serotec, Oxford, UK) in blocking buffer as an internal standard.

PNGase F-treatment

After dialysis and lyophilization, purified PSGL-1/mlG₂b was dissolved in sodium phosphate buffer (0.1 M, pH 7.6) with 25 mM EDTA and 2% Triton X-100 and incubated at 100°C for 10 min. Samples were allowed to cool to room temperature, after which 60 U of PNGase F (Roche, Basel, Switzerland) per milligram protein were added and samples incubated at 37°C overnight. For control samples, an equal volume of buffer was added instead of PNGase F. PNGase F-treated PSGL-1/mlG₂b, was re-purified on a HiPrep 26/60 Sephacryl S-300 HR column as described (see section “Purification of PSGL-1/mlG₂b and AGP/mlG₂b fusion proteins”).

Mannosidase treatment

PNGase F-treated and re-purified P. pastoris-produced PSGL-1/mlG₂b was treated with α1,2- and α1,6-mannosidases (Prozyme, San Leandro, CA) and non-linkage-specific α- and β-mannosidases (Sigma-Aldrich), α1,2- and α1,6-mannosidases were used at 15 and 40 U/g fusion protein, respectively. Incubations were performed in the reaction buffers provided by the manufacturer at 37°C for 20 h. α-mannosidase was used at 0.25 g/L and the incubation performed in 0.05 M citrate buffer, pH 4.6, at 37°C for 20 h. The β-mannosidase had a specific activity of 5–30 U/mL of which 25 μL was used per 100 μg of fusion protein. Incubations were performed in 0.01 M NaOAc, pH 4.5, at 37°C for 20 h or at 50°C for 2 h with or without protease inhibitor cocktail (Sigma-Aldrich).

SDS–PAGE and western blotting

Recombinant proteins were analyzed by SDS–PAGE under reducing (5 mM DTT) and non-reducing conditions using 3–8% tris-acetate gradient gels and tris-acetate SDS running buffer (Invitrogen). Precision protein standards (Candycane, Molecular Probes, Leiden, the Netherlands, or Hi-Mark, Invitrogen) were applied as reference for protein molecular weight determination. Protein gels were stained using the Pro Q Emerald 300 Glycoprotein Detection Kit in combination with Ruby (Molecular Probes). These gels were visualized in a Fluor-S Max Multimager carrying a CCD camera (Bio-Rad, Hercules, CA). Separated proteins were also electrophotographically blotted using iBlot (Invitrogen) in combination with nitrocellulose membranes (Invitrogen). Western blot membranes were probed with biotinylated Con A (Vector, Burlingame, CA) 10 μg/mL, a mouse anti-PSGL-1 antibody (clone KPL-1, BD PharMingen, San Diego, CA) diluted 1:1000, and an anti-CD159 (AGP) antibody (DakoCyntomation, Denmark) diluted 1:500. Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG F(ab)₂ (Sigma-Aldrich) diluted 1:50,000 and goat anti-rabbit IgG(H+L) (Sigma-Aldrich) diluted 1:10,000. Peroxidase-conjugated
NeutrAvidin (Pierce) 10 ng/mL was used to detect Con A binding. Bound lectins and antibodies were visualized by chemiluminescence using the ECL kit according to the manufacturer’s instructions (GE Healthcare). Bovine thyroglobulin (Sigma-Aldrich) was used as a Con A positive control.

Glycan profiling of PSGL-1/mIgG2b and AGP/mIgG2b using lectin arrays

Purified CHO- and P. pastoris-produced PSGL-1/mIgG2b and purified P. pastoris-produced AGP/mIgG2b were sent to GP Biosciences Ltd (Yokohama 225-0012, Japan; www.gpbio.jp) for glycan profiling analyses using their lectin arrays. Briefly, samples were labeled by Cy3 fluorescent dye and applied to a LecChip™. Bound fusion proteins were detected using the GlycoStation™ Reader 1200 and the results analyzed using the GlycoStation™ Tools Pro. For a more detailed description of the method, please see www.gpbio.jp/pdf/GlycoStation_ProductGuide_ver.1.0_E-L.pdf and Kondoh et al. (2009).

Chemical release and permethylation of O-linked glycans from purified PSGL-1/mIgG2b

Oligosaccharides were released by β-elimination as described (Carlstedt et al. 1993). The solution containing released oligosaccharides was evaporated under a stream of nitrogen at 45°C, and the oligosaccharides permethylated according to Ciucanu and Kerek (1984), with slight modifications as described (Hansson and Karlsson 1993).

MS analyses

ESI-MS in the positive-ion mode was performed using an LCQ ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Samples were dissolved in methanol:water (1:1) and introduced into the mass spectrometer at a flow rate of 5–10 μL/min. Nitrogen was used as sheath gas and the needle voltage set to 4.0 kV. The temperature of the heated capillary was set to 200°C.

Proton NMR spectroscopy

O-glycans were prepared as described (see section “Chemical release and permethylation of O-linked glycans from purified PSGL-1/mIgG2b”) yielding alditols at the reducing end of the O-glycans. The sample was subsequently lyophilized twice after having been dissolved in 100% D2O and was thereafter dissolved in 0.5 mL of the same solvent. 1H-NMR spectra were acquired on a Varian 600 MHz spectrometer at 25°C. Two-dimensional DQF COSY spectra were recorded by the standard pulse sequence (Marion and Wuthrich 1983).

Real-time surface plasmon resonance spectroscopy and data evaluation

Analyses were performed using a Biacore 2000 instrument (Biacore, GE Healthcare). Recombinant human MMR (cat. no. 2534-MR/CF), DC-SIGN/Fc chimera (cat. no. 161-DC) and MBL (cat. no. 2307-MB/CF) were purchased from R&D Systems (Minneapolis, MN) and immobilized on a CM5 sensor chip using amine coupling chemistry according to the manufacturer’s instructions. Briefly, activation of the surface was made with EDC/NHS 1:1 at a flow rate of 10 μL/min for 7 min. The receptors were dissolved in sodium acetate buffer, pH 4.5, at concentrations of 20 μg/mL for MR, 20 μg/mL for DC-SIGN and 10 μg/mL for MBL, and were immobilized at 10 μL/min for 7 min. Deactivation of excess reactive groups was made with ethanolamine, 20 μL/min for 7 min. The immobilization levels of the receptors were 11445 RU for MR, 8946 RU for DC-SIGN and 5040 RU for MBL. The analytes—PNGase F-treated and untreated PSGL-1/mIgG2b with mannose structures (P-PM) or with mono- and disialylated core 1 (C-P55.2) and AGP/mIgG2b with mannose structures (P-AM)—were dissolved in HBS-P buffer with 1 mM CaCl2 and 1 mM MgCl2 and injected on the CM5 sensor chip with a rate of 20 μL/min for 4 min with a 2.5 min waiting time. The analyte concentrations ranged from 0.64 nM to 10 μM. Regeneration of the surfaces was achieved by injection of glycine, pH 2.2, at 30 μL/min for 40 s. One channel on the CM5 sensor chip was immobilized only with buffer and was used as blank sensograms for subtraction of the bulk refractive index background. Data were calculated using BIAevaluation 4.1 software (Biacore, GE Healthcare) and the apparent equilibrium dissociation constants (K_D) were calculated by plotting steady-state binding levels against the analyte concentrations for several concentrations simultaneously.

The immobilized surface of the sensor chip was tested with mannan (Dextra Laboratories, Reading, UK) or mannosylated PSGL1/mIgG2b, to verify that the surface was still active after the regeneration procedure. In addition, D-mannose (Dextra Laboratories) and oligomannose-9 (Dextra Laboratories) were also analyzed for possible binding to the receptors.

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Conflict of interest

J.H. is acting CSO, shareholder and board member of Recopharma AB.

Abbreviations

AGP, α1-acid glycoprotein; BMGY, buffered glycerol complex medium; BSA, bovine serum albumin; CHO, Chinese hamster ovary; Con A, concanavalin A; CRD, carbohydrate recognition domain; CV, column volume; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin; DO, dissolved oxygen; DQF COSY, double quantum-filtered
correlated spectroscopy; DTT, dithiothreitol; EDC/NHS, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxy-succinimide; EDTA, ethylenediaminetetraacetic acid; EK, enterokinase; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; MBL, mannose-binding lectin; MDH, minimal dextrose medium + histidine; MMH, minimal methanol + histidine; MR, mannose receptor; MS, mass spectrometry; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PES, polyether sulfone; PSGL-1, P-selectin glycoprotein ligand-1; SAGI, Toxoplasma gondii surface antigen I; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

References


