

Glycan Recognition

Glycan characterization of pregnancy-specific glycoprotein 1 and its identification as a novel Galectin-1 ligand

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Abstract

Pregnancy-specific beta 1 glycoprotein (PSG1) is secreted from trophoblast cells of the human placenta in increasing concentrations as pregnancy progresses, becoming one of the most abundant proteins in maternal serum in the third trimester. PSG1 has seven potential N-linked glycosylation sites across its four domains. We carried out glycomic and glycoproteomic studies to characterize the glycan composition of PSG1 purified from serum of pregnant women and identified the presence of complex N-glycans containing poly LacNAc epitopes with α 2,3 sialylation at four sites. Using different techniques, we explored whether PSG1 can bind to galectin-1 (Gal-1) as these two proteins were previously shown to participate in processes required for a successful pregnancy. We confirmed that PSG1 binds to Gal-1 in a carbohydrate-dependent manner with an affinity of the interaction of 0.13 μ M. In addition, we determined that out of the three N-glycosylation-carrying domains, only the N and A2 domains of recombinant PSG1 interact with Gal-1. Lastly, we observed that the interaction between PSG1 and Gal-1 protects this lectin from oxidative inactivation and that PSG1 competes the ability of Gal-1 to bind to some but not all of its glycoprotein ligands.

Key words: galectin, glycosylation, pregnancy specific glycoproteins

Introduction

The Pregnancy-specific beta-1 glycoprotein (PSG) and the closely related Carcinoembryonic antigen cell adhesion molecule gene families are members of the immunoglobulin (Ig) superfamily (Kammerer and Zimmermann 2010). The serum concentration of PSGs rapidly increases as pregnancy progresses, reaching approximately 200 µg/mL at term (Towler et al. 1976). Only species with hemochorial placentation express these glycoproteins. All have several PSG-encoding genes that arose by gene duplication (Chang et al. 2013; McLellan et al. 2005; Sudmant et al. 2015; Zhou and Hammarstrom 2001). PSG1 is believed to be the highest expressed of the 10 human PSG genes, and most studies have centered on the elucidation of its functions. PSG1 binds to heparan sulfate proteoglycans, the latency-associated peptide of TGF- β 1 and to the platelet integrin α IIb β 3 (Blois et al. 2014; Lisboa et al. 2011; Shanley et al. 2013). The interaction of PSG1 with the ligands listed above has been shown to mediate the reported proangiogenic and immuno-regulatory functions of PSG1 and its ability to inhibit the interaction of platelets with fibrinogen (Martinez et al. 2013; Martinez et al. 2012; Moore and Dveksler 2014; Shanley et al. 2013).

PSG1 is composed of four domains; one Ig variable-like domain at its N-terminus (N-domain) followed by three Ig constant-like domains (A1, A2 and B2) and a short hydrophilic carboxy-terminal tail. Messenger RNAs potentially coding for splice variants lacking particular domains or with differences in the length of the C-terminal tail have been described (Zhou and Hammarstrom 2001). There are seven potential N-glycosylation sites in the PSG1 sequence that map to the N- (Asn61, 104 and 111), A1- (Asn199) and A2- (Asn259, 268 and 303) domains. In addition, several serine and threonine residues, which could potentially be candidates for O-glycosylation, are present in the primary sequence. However, only limited information is available regarding the biochemical composition and site-occupation of PSG1 glycans. An early study of native PSG1 measured the content of its monosaccharides as 6.2% Neu5Ac, 5.8% Gal, 13%GlcNAc, 6.5% Man and 1.1% Fuc and the authors suggested that PSG1 may not be decorated by O-linked glycans (Osborne et al. 1982). Consistent with this report, studies performed with the lectin concanavalin A suggested that a large proportion of native PSG1 from healthy pregnant women likely contains N-glycan structures (Halmesmaki et al. 1987; Koistinen et al. 1981). A more recent histochemical study of gestational diabetes mellitus detected enhanced fluorescent staining of patient serum PSG1 with *A. aurantia* lectin, which has a binding preference for the α 1,6 linked core-fucose of N-glycans (Jones et al. 2007; Matsumura et al. 2007). This enhanced imaging in gestational diabetes mellitus indicated that normal PSG1 N-glycans are likely to be partially fucosylated on their cores.

We have carried out a comprehensive glycomics and glycoproteomics investigation of native PSG1. The results of this study indicate the presence of mutiantennary and poly-N-acetyl-lactosamine (LacNAc) elongated moieties with mainly α 2,3-linked sialic acid terminals, which suggests that PSG1 may interact with members of the galectin family. Because galectin-1 (Gal-1) has been shown to regulate several important biological processes during pregnancy including angiogenesis, immune homeostasis, cell adhesion, trophoblast invasion and cell cycle progression through intracellular or extracellular mechanisms, we explored whether PSG1 is a Gal-1 ligand (Barrientos et al. 2014; Blois et al. 2019; Than et al. 2015). PSG1 and Gal-1 are secreted by the placenta and increase in the maternal circulation as pregnancy progresses (Ngo et al. 2018; Tirado-Gonzalez et al. 2013). Interestingly, PSG1 and Gal-1 both participate in the regulation of

some of the same processes required for pregnancy success described above but whether these proteins interact has not been explored previously (Blois et al. 2014; Lisboa et al. 2011; Moore and Dveksler 2014; Warren et al. 2018). In addition, several reports indicate that pregnancy pathologies including spontaneous miscarriage, intrauterine growth restriction and preeclampsia are linked to glycosylation alterations (Lee et al. 2011; Roberts et al. 2007; Vukasovic et al. 2015; Zhang et al. 2015). Here we report that PSG1 binds to Gal-1 and that this interaction is mediated by glycans in more than one domain of the protein. Recombinant glycoforms of PSG1 were generated and compared for their ability to bind to Gal-1 and protect this lectin from oxidative inactivation and to compete the interaction of Gal-1 with a soluble ligand and with cell surface receptors. We propose that PSG1 contributes to the stabilization of Gal-1 in the placental extracellular space and also hypothesize that PSG1 could modulate the interaction of Gal-1 with its previously identified ligands in the placenta.

Results

N-glycomics analysis of pooled native PSG1

The N-glycan profile of native PSG1 from pooled serum of pregnant women was characterized initially by MALDI-TOF mass spectrometry (Figure 1A). Multiantennary complex N-glycans are the major species in the N-glycan pool of native PSG1, consisting from mono- to tetra-antennary structures (e.g. m/z 1981, 2792, 3602 and 4413). The highest mass m/z signal for an N-glycan released from this protein was detected at m/z 4587, corresponding to a tetra-sialylated tetra-antennary glycan with core-fucosylation. Some glycans have a potential bisecting GlcNAc (e.g. m/z 2850 and 3211). Sialylation with NeuAc is seen as virtually a global modification to cap the antennary LacNAc on most of these complex glycans. Biantennary structures fully capped by NeuAc, m/z 2792 and 2966 are the most abundant glycans. Similarly, fully sialylated structures are the most abundant tri- and tetra-antennary glycans observed (e.g. m/z 3602 and 4413). Mono-fucosylation is observed on about 50% of complex glycans and this can be attributed to core-fucosylation, which was confirmed by MS/MS analysis (see below). Furthermore, the hybrid type structures detected (e.g. m/z 2186 and 2360) share the features of fucosylation and sialylation with the complex structures as described above. High mannose structures were also detected in this glycan pool albeit at relative low abundance (e.g. m/z 1579 and 1783).

MS/MS fragmentation analysis was carried out for the majority of assigned molecular ions by MALDI-TOF/TOF analysis in order to validate tentative assignments and to gain additional structural information. Three exemplifying spectra are shown (Supplementary Figure S1) for parental molecular ions m/z 1981, 2156 and 3211. A diagnostic b-ion, m/z 847, appears in the MS/MS spectra of all the parental molecular ions revealing a general presence of a trisaccharide sialyl-LacNAc moiety. A series of corresponding y-ions (e.g. 1157, 1402 and 1606) for this moiety in the spectrum of parental ion m/z 1981 validates its assignment and sequential arrangement of residual monosaccharides. Core-fucosylation can be validated on parental molecular ions, such as m/z 2156, by a diagnostic y-ion, m/z 474 and its corresponding b-ion, m/z 1704. Additionally, a fragment y-ion, m/z 2952, was observed in the MS/MS spectrum of parental ion m/z 3211 corresponding to loss of a GlcNAc from a non-reducing end. This indicates the presence of either a truncated antenna or a bisecting GlcNAc.

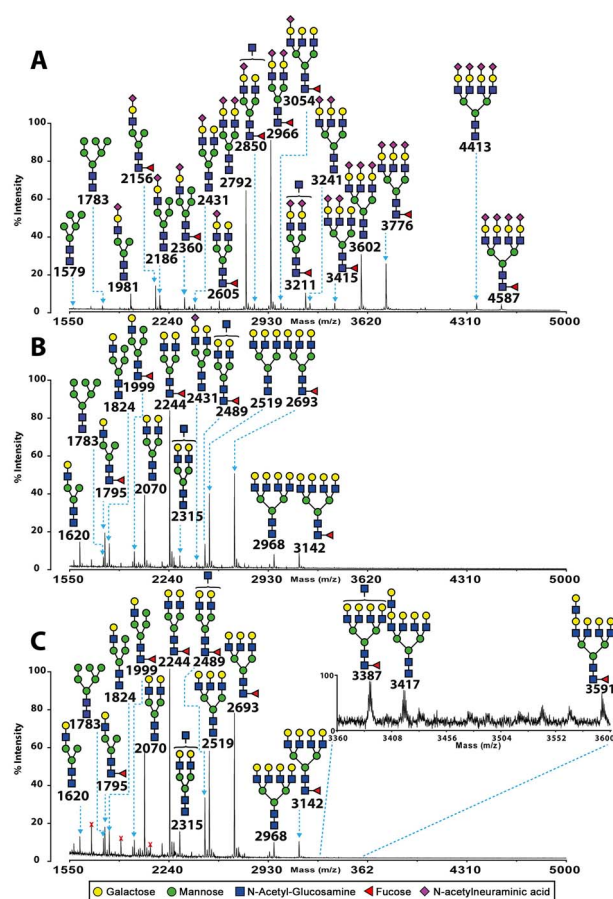


Fig. 1. N-glycan profile of human native PSG1. MALDI-TOF MS spectrum of permethylated N-glycans from native PSG1 (A), native PSG1 after α 2,3 linkage-specific desialylation by sialidase S (B) and native PSG1 after broad specificity desialylation by sialidase A (C). Data were acquired from the 50% acetonitrile/water fraction after purification by a C18 reversed phase chromatography. All molecular ions are observed as $[M + Na]^+$ in the positive mode. The assignments of structures are based on compositional information, MS/MS fragmentations and knowledge of biosynthetic pathways. Monosaccharides assigned above brackets indicate the composition with undetermined glycosidic linkages. Poly-hexoses contaminations are indicated by red crosses above corresponding signals.

There are two different expected structures for a sialyl-LacNAc moiety, which can be distinguished by the linkage of sialic acid to the LacNAc unit as either α 2,3 or α 2,6. The latter configuration of the sialic acid can block the binding of the LacNAc unit to Gal-1 (Stowell et al. 2008). Therefore, we conducted sialidase treatments to assess the linkage specificity of NeuAc residues on the sialyl-LacNAc antenna of pooled PSG1 N-glycans. Native PSG1 was treated with Sialidase S, which can specifically cleave off sialic acid residues with an α 2,3 linkage. After the sialidase S treatment (Figure 1B), a nearly complete cleavage of NeuAc residues on the N-glycans was observed as a predominant mass shift from sialylated N-glycans (e.g. m/z 1981, 2156, 2966, 3602, 3776, 4413 and 4587; Figure 1A) in the untreated group to their fully desialylated products (e.g. m/z 1620, 1795, 2070, 2244, 2519, 2693, 2968 and 3142; Figure 1B). We observed a small number of low abundance NeuAc-bearing N-glycans, such as m/z 2431, after treatment and this incomplete cleavage may be correlated to the linkage-related resistance of NeuAc residues (α 2,6) to the enzyme activity. Therefore, we conclude that the vast majority of NeuAc residues on the native PSG1 N-glycans have an α 2,3 linkage

while a small minority of residues have an α 2,6 linkage. We also conducted sialidase A treatment on another aliquot of the PSG1 N-glycans to cleave off NeuAc residues with all possible linkages (α 2,3/6/8/9) (Figure 1C). A complete removal of NeuAc residues from the native PSG1 N-glycans is evident as there was no detection of molecular ions corresponding to NeuAc-bearing N-glycans after treatment and a complete mass shift of molecular ions to fully desialylated products from sialylated N-glycans in the untreated group was observed (Figure 1A). Desialylated tetraantennary N-glycans with either an additional LacNAc unit or GlcNAc residue were identified as m/z 3387, 3417 and 3591, respectively, due to reduced heterogeneity after complete removal of sialic acids. The presence of an additional LacNAc unit on m/z 3417 and 3591 indicates the potential of native PSG1 N-glycans to have poly-LacNAc extension forming elongated antennary chains. Furthermore, the presence of m/z 3387 reveals that native PSG1 N-glycans may have bisecting GlcNAc on their tri- and tetra-antennary structures.

In summary, the N-glycomic characterization of native PSG1 pooled from the sera of pregnant women reveals that the N-glycosylation of this protein is dominated by multiantennary complex glycans. Sialylation is nearly universal on complex and hybrid glycans forming sialyl-LacNAc antennae with a majority of α 2,3 type linkage. Core-fucosylation is also identified on the majority of complex and hybrid glycans, and there is a minor presence of glycans with GlcNAc which is most likely bisecting and poly-LacNAc extension.

Glycoproteomics of pooled native PSG1

Secondly, we conducted a glycoproteomic analysis for glycopeptides derived from native PSG1 following tryptic digestion, in order to determine the site-occupancy and define site-specific glycoforms to the extent possible using our previously described LC-ES-MS methods (Panico et al. 2016). The majority of the native PSG1 material was used for the above glycomics studies. That restricted both the data quality but also the strategy for overall molecular coverage which would normally demand the use of multiple digests. Nevertheless, the data found on four of the sites were of interest and are reported here. There are seven potential N-glycosylation sites in the PSG1 sequence, and we found evidence for the presence of glycosylation for four of these sites, Asn61, Asn199, Asn268 and Asn303 (Table I) in this tryptic data set. Although three of the sites were not observed (for experimental reasons including predicted peptide molecular size and shortage of material) that does not infer the absence of glycosylation at those sites since the free peptides were not found. Full conclusions cannot be drawn without the availability of further PSG1 material for study. Two glycopeptides, (one found to correspond to incomplete tryptic digestion), are from the N-domain and contain the N-glycosylation site (Asn61). One glycopeptide carries the single N-glycosylation site, Asn199, in the A1 domain, and two glycopeptides were identified with N-glycans at positions Asn268 and Asn303 found in the A2 domain (Supplementary Figure S2). A double miss-cleavage was observed for the glycopeptide containing the Asn303 site. The identity of the glycopeptides detected were each validated by MS/MS analysis as shown in Table I and the exemplars in Supplementary Figure S2 and Supplementary Figure S3.

The N-glycosylation site Asn61 of the N-domain of native PSG1 was found in two tryptic digestion peptides with the amino acid sequences, ⁴⁵VSEKDVLLVHNLQNLTYGIWYK⁶⁹ (miscleavage) and ⁵⁰DVLLVHNLQNLTYGIWYK⁶⁹, respectively. Both glycopeptides were seen to carry a mixture of glycoforms, with main components being fully sialylated bi-, tri- and tetra-antennary glycans

Table I. Summary of the LC-MS glycoproteomics data showing identified glycopeptides from tryptically digested native PSG1. The site of glycosylation is in bold

Monoisotopic Mass (m/z)	Observed Mass (m/z)	Residue number	Glycopeptide assignments
1605.09 ⁴⁺	1605.03 ⁴⁺	45–69	VSEKDVLLLVHNLPQNLTGYIWYK + 4NeuAc 4LacNAc 3Man 2GlcNAc
1641.47 ⁴⁺	1641.40 ⁴⁺	45–69	VSEKDVLLLVHNLPQNLTGYIWYK + 4NeuAc 4LacNAc 3Man Fuc 2GlcNAc
1313.36 ⁴⁺	1313.29 ⁴⁺	45–69	VSEKDVLLLVHNLPQNLTGYIWYK + 2NeuAc 2LacNAc 3Man Fuc 2GlcNAc
1440.90 ⁴⁺	1440.80 ⁴⁺	45–69	VSEKDVLLLVHNLPQNLTGYIWYK + 3NeuAc 3LacNAc 3Man 2GlcNAc
1477.41 ⁴⁺	1477.36 ⁴⁺	45–69	VSEKDVLLLVHNLPQNLTGYIWYK + 3NeuAc 3LacNAc 3Man Fuc 2GlcNAc
1604.96 ⁴⁺	1605.10 ⁴⁺	45–69	VSEKDVLLLVHNLPQNLTGYIWYK + 4NeuAc 4LacNAc 3Man 2GlcNAc
1284.17 ⁵⁺	1284.09 ⁵⁺	45–69	VSEKDVLLLVHNLPQNLTGYIWYK + 4NeuAc 4LacNAc 3Man 2GlcNAc
1316.62 ³⁺	1316.90 ³⁺	50–69	DVLLLVHNLPQNLTGYIWYK + NeuAc LacNAc 3Man 2GlcNAc
1365.31 ³⁺	1365.90 ³⁺	50–69	DVLLLVHNLPQNLTGYIWYK + NeuAc LacNAc 3Man Fuc 2GlcNAc
975.393 ³⁺	975.32 ³⁺	195–200	LSETNR + 2NeuAc 2LacNAc 3Man 2GlcNAc
1558.34 ³⁺	1558.90 ³⁺	266–285	SENYTYIWWLNGQSLPVSPR + GlcNAc NeuAc 2LacNAc 3Man Fuc 2GlcNAc
1587.68 ³⁺	1587.90 ³⁺	266–285	SENYTYIWWLNGQSLPVSPR + 2NeuAc 2LacNAc 3Man Fuc 2GlcNAc
1191.01 ⁴⁺	1191.20 ⁴⁺	266–285	SENYTYIWWLNGQSLPVSPR + 2NeuAc 2LacNAc 3Man Fuc 2GlcNAc
1241.78 ⁴⁺	1241.96 ⁴⁺	266–285	SENYTYIWWLNGQSLPVSPR + GlcNAc 2NeuAc 2LacNAc 3Man Fuc 2GlcNAc
1318.55 ⁴⁺	1318.50 ⁴⁺	266–285	SENYTYIWWLNGQSLPVSPR + 3NeuAc 3LacNAc 3Man 2GlcNAc
1055.04 ⁵⁺	1055.06 ⁵⁺	266–285	SENYTYIWWLNGQSLPVSPR + 3NeuAc 3LacNAc 3Man 2GlcNAc
1355.07 ⁴⁺	1354.97 ⁴⁺	266–285	SENYTYIWWLNGQSLPVSPR + 3NeuAc 3LacNAc 3Man Fuc 2GlcNAc
1084.25 ⁵⁺	1084.40 ⁵⁺	266–285	SENYTYIWWLNGQSLPVSPR + 3NeuAc 3LacNAc 3Man Fuc 2GlcNAc
1246.30 ⁴⁺	1246.22 ⁴⁺	294–315	ILILPSVTRNETGPYQCEIRDR + 2NeuAc 2LacNAc 3Man Fuc 2GlcNAc

with and without core fucosylation, see for example m/z 1440.9⁴⁺ and m/z 1477.4⁴⁺ in Table I.

The only N-glycosylation site in the A1 domain, Asn199, was found in the glycopeptide, ¹⁹⁵LSETNR²⁰⁰, in the LCMS trace, modified principally with a fully sialylated biantennary complex glycan with and without core fucosylation at m/z 975.4³⁺ and 1024.0³⁺.

There are two N-glycosylation sites on the A2 domain of native PSG1, Asn268 and Asn303. The tryptic glycopeptides found for site Asn268 carry sialylated biantennary and triantennary glycans with and without core fucosylation and some evidence of partial modification by a potential bisecting GlcNAc, for example via the signal at m/z 1558.3³⁺ in Table I. At site Asn303 of this domain, the glycopeptide ²⁹⁴ILILPSVTRNETGPYQCEIRDR³¹⁵ was found to carry fully sialylated biantennary complex glycans, again with and without core fucosylation.

Native PSG1 binds to Gal-1

The glycomic and glycoproteomic studies of native PSG1 described above strongly suggested that PSG1 could be a galectin ligand. We

first determined whether native PSG1 can bind to Gal-1 by ELISA using a dimeric form of murine Gal-1 (Gal-1Fc). As a control, we utilized a Gal-1 double-mutant (dmGal-1Fc) in which important amino acids required for carbohydrate binding were substituted (W69G, H45L) (Cedeno-Laurent et al. 2010). Our data shows that native PSG1 binds to Gal-1 while it does not bind to dmGal-1 (Figure 2A). To further confirm that the interaction between native PSG1 and Gal-1 is carbohydrate-dependent we used lactose, a competitive inhibitor of Gal-1 binding. We observed a significant decrease in binding between PSG1 and Gal-1 when lactose was added (Figure 2B), whereas sucrose had no effect. Using surface plasmon resonance (SPR), we confirmed the interaction between human Gal-1 and native PSG1 (Figure 2C). Next, we defined the kinetics of the interaction between Gal-1 and native PSG1 (Figure 2D). To determine whether the interaction between Gal-1 and PSG1 was also observed using a recombinant form of the protein, we utilized PSG1 made in CHO-K1 cells. We selected this cell line because it has been previously shown to decorate proteins with complex glycans with LacNAc groups (North et al. 2010). We observed that PSG1 made in our stably transfected

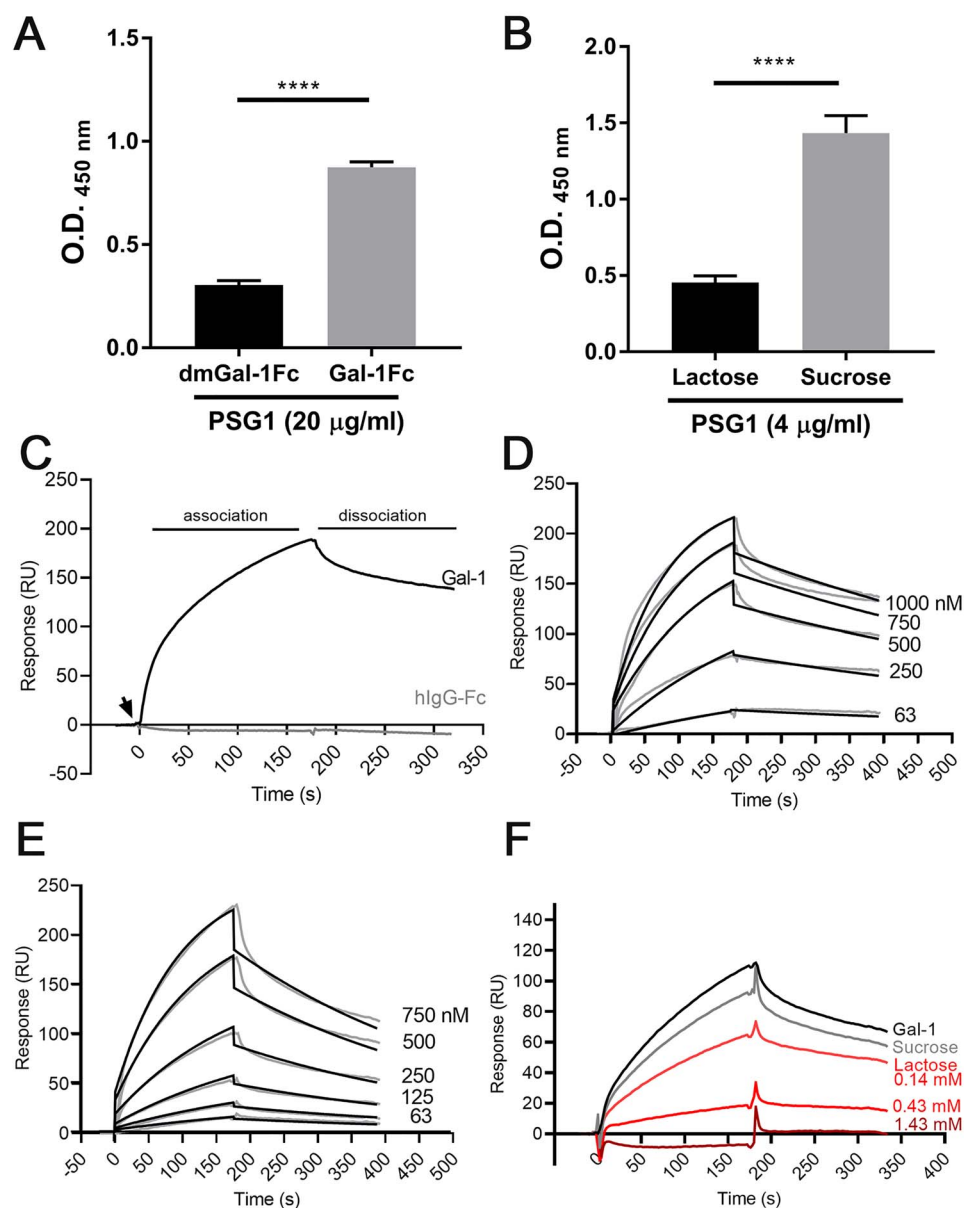


Fig. 2. Native PSG1 and recombinant PSG1 made in CHO-K1 cells bind to Gal-1 with similar affinity in a carbohydrate-dependent manner. **(A)** Native PSG1 was coated on wells and after blocking, 2 µg/mL of Fc-tagged mutated Gal-1 (dmGal-1), which cannot bind to LacNAc structures due to two amino acid substitutions, or wild type Gal-1 were added. After washing, binding was detected with HRP-conjugated anti-Fc Ab. **(B)** rGal1 was coated on wells and after blocking, native PSG1 (4 µg/mL) was added in the presence of 100 mM lactose or 100 mM sucrose, as indicated. PSG1 binding was detected with an anti-PSG1 biotin-labeled MAb. **(C)** SPR sensorgram of the interaction of Gal-1 (black line) and hlgG-Fc used as negative control (grey line) at 1 µM with immobilized native PSG1. The beginning of the Gal-1 and hlgG-Fc injections are indicated with an arrow head, and the association and dissociation phases are specified. A control surface without immobilized protein was used as a control for potential unspecific binding to the biosensor surface and subtracted from the binding of Gal-1 to the PSG1 surface. **(D)** Sensorgrams of the interaction of Gal-1 with native PSG1. Serial dilutions of Gal-1 ranging from 1 to 0.01 µM were injected during 3 min over a CM5 biosensor chip with immobilized native PSG1. SPR sensorgrams for each protein concentration are shown as grey lines while the fitted data are shown as black lines. **(E)** Sensorgrams of the interaction of Gal-1 with recombinant PSG1 represented as in **(B)**. **(F)** Sensorgrams of the interaction of Gal-1 at 0.25 µM with immobilized recombinant PSG1 in the absence (black) or presence of 1.43 mM of sucrose (grey) or increasing concentrations of lactose (from light to dark red).

CHO-K1 cell line also bound to Gal-1 (Figure 2E). The calculated affinity of the interaction of native and recombinant PSG1 for Gal-1 were quite similar, with an estimated K_D of 0.13 and 0.25 µM, respectively (Table II). In agreement with the previous results, we observed that the interaction between recombinant PSG1 and Gal-1 was inhibited by the addition of lactose and not sucrose (Figure 2F). Overall, these results indicate that the recombinant PSG1 generated

under these conditions closely reflects the interaction between native PSG1 and Gal-1.

The N and A2 domains of PSG1 mediate the interaction with Gal-1

As described above, while native PSG1 has seven potential N-linked glycosylation sites only four sites mapping to the N, A1 and A2

Table II. Kinetics constants and affinity of the interaction of Gal-1 with native and recombinant PSG1

Gal-1	K_a M ⁻¹ s ⁻¹ (mean ± SE)	k_d s ⁻¹ (mean ± SE)	KD μM	Rmax (mean ± SE)	chi ²
Recombinant PSG1	$(1.0 \pm 0.08) \times 10^4$	$(2.7 \pm 0.02) \times 10^{-3}$	0.25	294 ± 2.2	6.51
Native PSG1	$(1.1 \pm 0.09) \times 1$	$(1.4 \pm 0.09) \times 10^{-3}$	0.13	230 ± 1.9	4.99

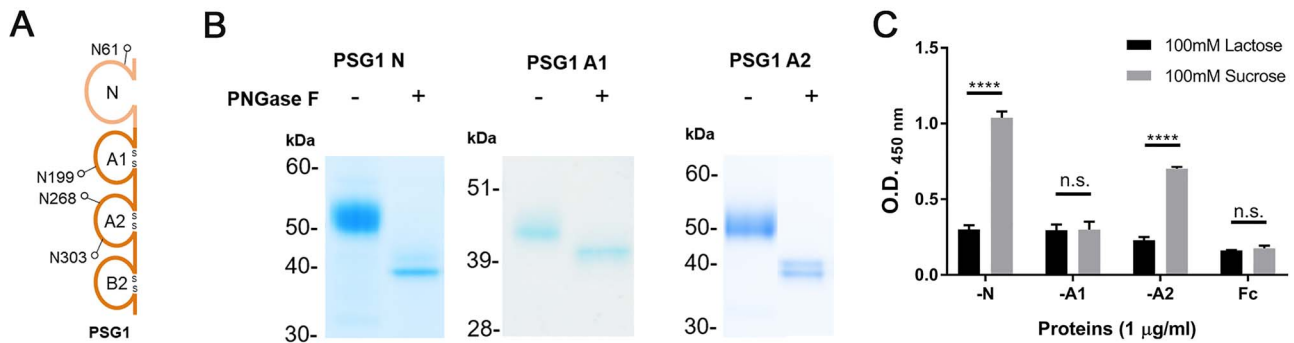


Fig. 3. The N- and the A2-domains of PSG1 bind to Gal-1. **(A)** Model of PSG1 representing the sites in the N, A1 and A2 domains confirmed to be occupied by N-glycans indicated by lollipops. **(B)** Recombinant N, A1 and A2 proteins were treated with PNGase F (+) and after incubation, the products and their corresponding untreated controls (–) were separated on SDS-PAGE and stained with GelCode. **(C)** Recombinant Gal-1 (10 μg/mL) was coated on wells and after blocking, PSG1N-Fc, PSG1A1-Fc, PSG1A2-Fc, and the control protein Fc were added in the presence of 100 mM lactose or 100 mM sucrose, as indicated. After washing, binding was detected with HRP-conjugated anti-Fc Ab.

domains of the protein were confirmed to be occupied by N-glycans (Figure 3A). We generated recombinant single PSG1 domains with an Fc tag as described in the materials and methods section. The recombinant N, A1 and A2 domains were incubated with PNGase F and after treatment we observed that they migrated faster upon separation on SDS-PAGE than the untreated proteins indicating a decrease in molecular weight (Figure 3B). Because the Fc tag is glycosylated, we also incubated the Fc control protein with PNGase F and as expected observed a small decrease of roughly 2.2 kDa in its molecular weight (data not shown). Since the observed reduction in molecular weight of the domains was larger than that of the protein consisting of just the Fc portion following PNGase F treatment, our results indicate that these three domains are decorated by glycans. We then investigated which of these three domains interact with Gal-1. Our results show that the N and A2 domains of PSG1 interact with Gal-1. This interaction was inhibited by lactose but not by sucrose (Figure 3C). Therefore, the major contributors of the interaction of recombinant PSG1 with Gal-1 are the glycans in the N and A2 domains.

The cell line utilized to generate recombinant PSG1 affects its ability to bind Gal-1

We generated PSG1 in CHO-K1 cells from our stable single cell clone grown in a FiberCell bioreactor, by transient transfection of ExpiCHO cells using the standard protocol and in Expi293 cells (Figure 4A and data not shown). In addition, PSG1 was generated in Hi5 insect cells and in HEK293 cells deficient in N-acetylglucosaminyltransferase I (GnTI) (data not shown and Figure 4A). We observed that recombinant PSG1 made in insect cells and in GnTI-deficient cells did not bind to Gal1-Fc over the Fc control (data not shown and Figure 4B), while PSG1 made in CHO-K1, ExpiCHO using the standard protocol and Expi293 cells significantly bound to Gal-1Fc (Figure 4B and data not shown).

The differential binding of recombinant PSG1 made in CHO-K1 cells and GnTI-null cells to Gal-1 was further confirmed by SPR analysis and immunoblotting with biotin-labeled Gal-1 (Figure 4C and Supplementary Figure S5).

PSG1 protects Gal-1 from oxidative inactivation

Gal-1 has six cysteine residues, some of which are susceptible to oxidation resulting in Gal-1 inactivation (Bianchet et al. 2000; Liao et al. 1994). Binding to specific glycan ligands enhances Gal-1 dimerization and reduces sensitivity to oxidative inactivation of the protein (Cho and Cummings 1995, Di Lella et al. 2010, Stowell et al. 2009). We investigated whether the interaction of PSG1 with Gal-1 can protect this lectin from inactivation by oxidation, measured by a decrease in binding to its well-characterized ligand asialofetuin (ASF), after exposure to hydrogen peroxide (H₂O₂).

Gal-1 without lactose or β-mercaptoethanol (2ME), which are routinely employed to preserve its activity, was incubated with 20 mM H₂O₂ or phosphate buffered saline (PBS) as indicated in the materials and methods section. We observed that Gal-1 incubated with H₂O₂ had in average a decrease of 75% in its ability to bind to ASF when compared to Gal-1 incubated with PBS whether the lectin was free in solution or mixed with Sepharose beads coupled to bovine serum albumin (BSA) (data not shown). We then tested whether PSG1 generated in CHO-K1 and in GnTI-deficient cells were able to protect Gal-1 from the observed reduction in its ability to bind to ASF after oxidation. Gal-1 mixed with the beads prepared as described in the materials and methods section was exposed to 20 mM H₂O₂ and the oxidation reaction was stopped by the addition of catalase. As expected, Gal-1 eluted from the beads coupled with the different proteins bound minimally and with no significant difference to the control wells that were coated with 1% BSA (data not shown). On the other hand, Gal-1 eluted from the beads coupled to PSG1 generated in CHO-K1 cells showed significantly more binding to ASF, represented

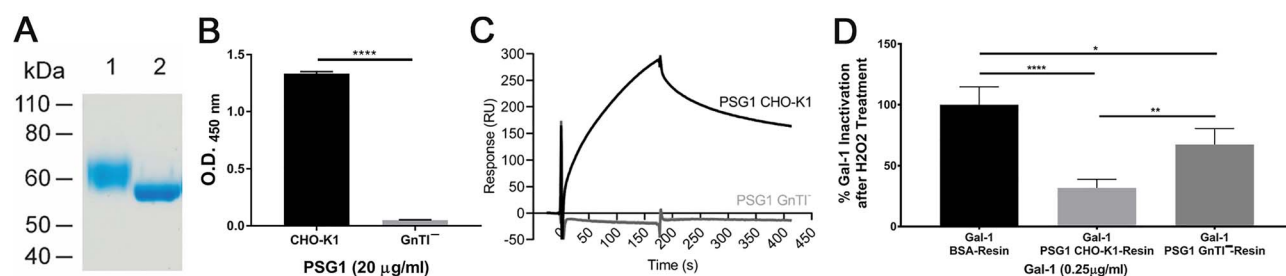


Fig. 4. Differences in binding of two glycoforms of PSG1 to Gal-1 and ability to mediate protection from oxidative inactivation. **(A)** Recombinant PSG1 produced in CHO cells (lane 1) or N-acetylglucosaminyltransferase I (GnTI)-deficient cells (lane 2) were purified using a HisTrap column followed by gel filtration chromatography. Purified proteins were separated on SDS-PAGE and stained with GelCode Blue. **(B)** Recombinant PSG1 generated in CHO-K1 cells or GnTI-deficient cells was coated on wells and after blocking, 2 µg/mL of Fc control protein or Fc-tagged recombinant human Gal1 (hGal1-Fc) were added. Binding was detected with HRP-conjugated anti-Fc Ab. Fc control values were subtracted from hGal1-Fc values. **(C)** SPR sensorgram showing the interaction of Gal-1 at 1 µM with PSG1 produced in CHO-K1 or GnTI-deficient cells. **(D)** BSA, PSG1 made in CHO-K1 cells or PSG1 made in GnTI-deficient cells were coupled to CnBr-Sepharose beads. Gal-1 was applied to each resin, followed by the addition of 20 mM H₂O₂. The oxidation reaction was stopped with catalase and Gal-1 was eluted from each resin with 100 mM Lactose, dialyzed against PBS and its concentration determined by ELISA. ASF or BSA were coated on wells and after blocking, oxidized Gal-1 recovered from each resin was added to the ASF or BSA-coated wells at 0.25 µg/mL. Gal-1 binding was detected with anti-Gal-1 biotin-labeled Ab. Results are expressed as percentage of Gal-1 inactivation after H₂O₂ treatment and values are derived from absorbance values of each sample treatment by the formula listed in the materials and method section.

as reduced % inactivation after exposure to H₂O₂, than Gal-1 recovered from the beads coupled to BSA or PSG1 made in GnTI-deficient cells (Figure 4D). Altogether, these results demonstrate that the interaction between PSG1 and Gal-1 can protect Gal-1 from oxidative inactivation.

Ability of PSG1 to compete the binding of Gal-1 to cell surface receptors and to soluble fibronectin

We examined whether binding of PSG1 to Gal-1 could compete the binding of Gal-1 to its previously identified soluble ligand fibronectin (FN) (Ozeki et al. 1995) or to glycoprotein receptors on the cell surface. We performed these experiments with different concentrations of PSG1 as its concentration varies at different stages of gestation (Towler et al. 1976). We observed that PSG1 made in CHO-K1 cells at 2 and 20 µg/mL competed the binding of Gal-1 to 2 µg/mL FN, while as expected, PSG1 made in GnTI⁻ cells did not compete the interaction between FN and Gal-1 (Figure 5A). Next, we assessed the ability of PSG1 to compete the binding of biotin-labeled Gal-1 to the membrane of the human Jurkat T cell line. Our flow cytometric analysis shows that PSG1 generated in CHO-K1 cells dose-dependently inhibited the interaction of Gal-1 with Jurkat cells at 2 and 20 µg/mL but did not compete Gal-1 binding when added at 0.2 µg/mL (Figure 5B). On the other hand, PSG1 made in GnTI-null cells did not inhibit the interaction of Gal-1 with Jurkats at any of the concentrations tested. Finally, we explored the ability of PSG1 to compete the binding of Gal-1 secreted by the Swan71 extravillous trophoblast (EVT) cell line to its own cell membrane. Interestingly, neither 2 µg/mL nor 20 µg/mL of PSG1 generated in CHO-K1 cells competed the binding of Gal-1 to these cells (Figure 5C). These results suggest that while PSG1 may compete the binding of Gal-1 to soluble or membrane bound glycans, factors such as affinity, concentration and whether Gal-1 is already bound to its ligand prior to the addition of PSG1, affects its ability to function as a competitor.

Discussion

We characterized the N-glycosylation profile of native PSG1 revealing a glycan repertoire dominated by multiantennary complex glycans

with high levels of sialylation and partial core-fucosylation. Potential bisecting and poly-LacNAc extensions were also observed. These antennary modifications are generally consistent with previous findings of N-glycans from mammalian placentas studied in five different species and glycomic studies of human trophoblast cells indicating a possibly similar specific glycosylation pattern for placental glycoproteins (Chen et al. 2016; Jones et al. 2007). The trophoblast cells N-glycans contained α2,3 linked NeuAc which would allow binding to galectins, again consistent with the linkage of NeuAc found on native PSG1 and its interaction with Gal-1.

Based on the results of the mass spectrometric glycosylation analysis and the well-recognized importance of Gal-1 for a successful pregnancy, we investigated the potential interaction of these proteins (Blois et al. 2007; Freitag et al. 2013; Ramhorst et al. 2012; Tirado-Gonzalez et al. 2013). We observed that native and recombinant forms of PSG1 interact with wild type murine and human Gal-1 but not with a mutated form of Gal-1 or the control protein consisting of the Fc tag. The affinity of the interaction of PSG1 with Gal-1 is very similar to that reported previously for other ligands such as recombinant CD146, CD60 and neuropilin-1 (de la Fuente et al. 2014; Hsieh et al. 2008; Jouve et al. 2013). Both ELISA and SPR analysis showed that the addition of lactose significantly inhibited Gal-1 from binding to PSG1 and therefore we can conclude that the interaction of these proteins is mediated by glycans in PSG1.

PSG1 isolated from pregnant serum and PSG1 generated in CHO-K1 cells in a bioreactor bound to Gal-1 with similar affinity. The similar binding kinetics and affinity indicates that this recombinant protein is a reliable tool to perform functional studies. Site-occupancy and site-specific glycoforms were revealed by the glycoproteomics study of native PSG1. We identified glycans on four out of the seven potential N-glycosylation sites. The relative distribution of the characterized N-glycans was not uniform across the protein domains, more highly processed tri- and tetra-antennary glycans were found on N-glycosylation sites Asn61 and Asn268 on the N- and A2-domains. However, less branched biantennary glycans are observed on site Asn199 of the A1 domain. The differences of glycosylation on these sites are most likely caused by steric constraints resulting in differential accessibilities of processing enzymes.

The results of the ELISA show that out of the three N-glycosylation-carrying domains, only the N and A2 domains of

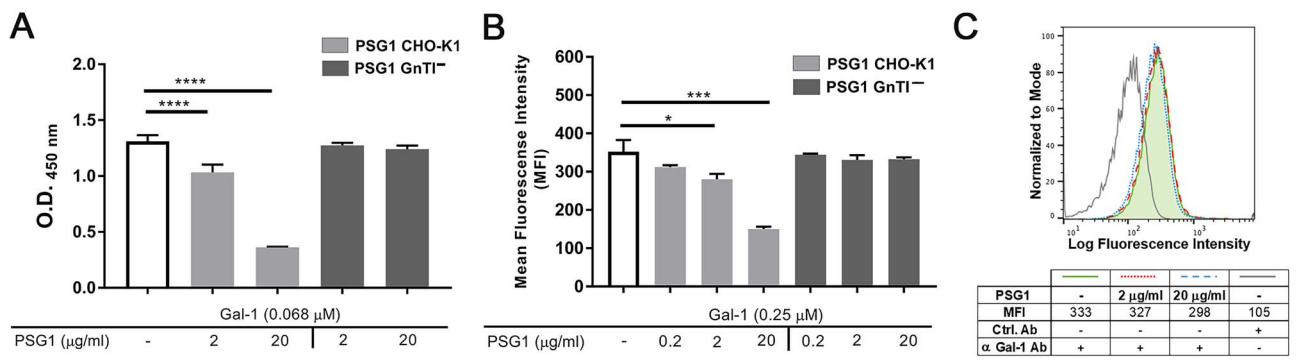


Fig. 5. Ability of PSG1 to compete the binding of Gal-1 to cell surface receptors and to soluble fibronectin. **(A)** FN and 3% BSA were coated on wells and after blocking, Gal-1 pre-incubated with PBS (–), PSG1 generated in CHO-K1 cells or in GnTI-deficient cells at the indicated concentrations was added to the wells. Binding of Gal-1 was detected with a biotin-labeled anti-Gal-1 Ab followed by streptavidin-HRP. The numbers shown in the figure represent the O.D. values obtained following subtraction of the O.D. values of the BSA-coated wells. **(B)** Jurkat cells were incubated with biotin-labeled Gal-1 in the presence or absence of PSG1 generated in CHO-K1 cells or in GnTI-deficient cells at the indicated concentrations. Binding of biotinylated Gal-1 to the cells was detected with streptavidin-APC. **(C)** Swan 71 cells were incubated in FACS buffer or in FACS buffer containing 2 or 20 μ g/mL PSG1 as indicated. After washing, Gal-1 bound to the cells was detected with biotin-labeled anti-Gal-1 Ab followed by streptavidin-APC. Cells incubated with biotin-labeled isotype control Ab followed by streptavidin-APC were used as control.

recombinant PSG1 interact with Gal-1. While these studies were not performed with native PSG1 due to the limited amount of material, this is likely the case for native PSG1 although this awaits confirmation. The other members of the human PSG family share a similar domain organization with PSG1 although some lack an A2 domain (Moore and Dveksler 2014). Thus, the N-domain of other PSGs may also bind to Gal-1. While we have not tested all members of the family, our preliminary data indicates that PSG4, 7 and 8 can also bind to Gal-1. Interestingly, PSG4 has only two potential N-linked glycosylation sites in the N-domain lacking the one at position Asn61. Whether the interaction of PSG4 with Gal-1 is mediated only through the A2 domain or if the other sites present in the N-domain are decorated by glycans and contribute to this interaction remains to be determined (Zhou and Hammarstrom 2001). Galectins have been shown to have preference for more branched N-complex ligands (Hirabayashi et al. 2002). The presence of less processed N-complex glycans in the A1 domain of PSG1 may explain the absence of a detectable interaction between this domain and Gal-1 since the affinity of Gal-1 for complex N-glycans increases with an increase in the branching number, whereas the affinity for monovalent N-glycans is relatively low (Hirabayashi et al. 2002).

PSGs are synthesized in the placenta of other mammalian species including mice, rats and non-human primates and recently genes encoding for potential PSGs have been described in bats (Chemnitz et al. 1982; Kammerer et al. 2017; Rebstock et al. 1993; Stevens et al. 1976). The importance of glycans in murine PSGs has been previously studied; glycosylation of murine PSG17 and PSG19 is required for their interaction with the receptor CD9 (Ha et al. 2008). Whether the PSGs of other species besides humans can bind to Gal-1 remains to be investigated. Gal-1 functions in the regulation of different processes during the establishment, development and maintenance of pregnancy including regulation of trophoblast migration and modulation of maternal immune response and placental angiogenesis, which is supported by observations in Gal-1 null mice (Freitag et al. 2013; Than et al. 2015). Intriguingly, a gradual and parallel increase in both Gal-1 and PSG1 concentrations in plasma occurs throughout pregnancy that drops to background levels after delivery (Pluta et al. 1979; Tirado-Gonzalez et al. 2013). The circulating levels of Gal-1 in maternal serum are dysregulated in pregnancy complications and sev-

eral studies indicate its potential use as marker for preeclampsia, miscarriage and recurrent fetal loss (Hirashima et al. 2017; Jeschke et al. 2007; Schnabel et al. 2016; Than et al. 2008; Tirado-Gonzalez et al. 2013). As previously stated, Gal-1 is inactivated in the extracellular environment due to oxidation resulting in the formation of intramolecular disulfide bridges that precludes Gal-1 dimer formation and ligand binding (Lopez-Lucendo et al. 2004; Tracey et al. 1992; Yu et al. 2015). However, how circulating Gal-1 is able to maintain its binding activity within the oxidative extracellular environment is currently unknown. We assessed whether the interaction with PSG1 could protect Gal-1's ability to interact with a known ligand following exposure to H₂O₂-mediated oxidation (Dam et al. 2005). We observed that the interaction with PSG1 reduced the sensitivity of Gal-1 to oxidative inactivation as demonstrated by the preservation of its carbohydrate binding activity. Thus, we hypothesize that high concentrations of Gal-1 ligands such as PSG1 may enable the maintenance of a reservoir of active Gal-1 and provide a control mechanism to regulate the extracellular availability of this lectin. Importantly, whether PSG1 and Gal-1 are found complexed in the maternal-fetal interface and/or in maternal serum has not been determined in our studies.

We also tested the ability of PSG1 to compete with Gal-1 for binding to its previously characterized soluble ligand, fibronectin (FN) and to cell surface glycoprotein receptors. We observed that PSG1 inhibited the binding of Gal-1 to FN-coated on the well. It is important to consider, however, that the post-translational modifications of FN vary in different tissues and between the soluble and the extracellular matrix forms of this protein (Carsons et al. 1987; Hsiao et al. 2017). In addition, besides the differences in concentration of PSG1 and Gal-1 during gestation, the glycosylation of FN in the placenta is not defined, it could vary at different developmental stages and possibly differs from that of the FN utilized in our studies. Therefore, the biological relevance of this observation should be taken with caution. Based on the mean concentration of PSG1 in the third trimester of pregnancy, the highest concentration of PSG1 utilized in the competition experiments was 20 μ g/mL (Rattila et al. 2019). In addition and as discussed above, other members of the PSG family besides PSG1, likely bind to Gal-1. Therefore at present we cannot evaluate the actual concentration of PSGs in vivo that could

potentially act as competitors of Gal-1. We also observed that glycans in PSG1 competed Gal-1's binding to Jurkat cells in a concentration-dependent manner while, as expected, PSG1 decorated with high mannose glycans did not compete Gal-1 binding to these cells. Gal-1 has been shown to induce apoptosis of Th1 cells contributing to its anti-inflammatory activity (Toscano et al. 2007). The glycoprotein receptors implicated in this Gal-1 activity include CD43, CD45 and CD7 (Hernandez et al. 2006, Nguyen et al. 2001, Pace et al. 2000). In vivo, the concentration of PSGs, Gal-1 and the affinity of Gal-1 for its receptors in Th1 cells will ultimately determine whether PSGs can inhibit the pro-apoptotic effects of this lectin.

PSG1 tested up to a concentration of 20 µg/mL could not displace the Gal-1 already bound to the membrane of an EVT cell line. Gal-1 is highly expressed in invasive trophoblasts and membrane bound Gal-1 has been proposed to regulate their migration by interacting with the $\beta 1$ chain of $\alpha 5 \beta 1$ integrin (Kolundzic et al. 2011; Tirado-Gonzalez et al. 2013; Vicovac et al. 1998). The interaction of Gal-1 with $\alpha 5 \beta 1$ was shown to be dependent on the glycosylation of the integrin (Lagana et al. 2006; Moiseeva et al. 2003; Sanchez-Ruderisch et al. 2011). Differences in $\alpha 5 \beta 1$ glycosylation were observed between invasive and non-invasive trophoblasts, and reports indicate an association of altered glycosylation of $\beta 1$ integrin with pregnancy complications (Liao et al. 2015; Moss et al. 1994; Zhang et al. 2015). Interestingly, PSG1 binds to $\alpha 5 \beta 1$ and regulates EVT migration although this interaction is not mediated by glycans in PSG1 (Rattila et al. 2019) and unpublished data. In addition, we have recently shown that some EVTs express PSG1 (Rattila et al. 2019). Therefore, Gal-1 acting in an autocrine manner, the concentration of PSGs defined by the gestational stage, the glycan composition of integrin $\alpha 5 \beta 1$, and the expression of other Gal-1 ligands in EVTs likely modulate the migration and invasive capacity of these cells.

Besides, Gal-1 other galectins including Gal-3, -9 and -13 have been shown to play important roles during pregnancy (Bozic et al. 2004; He et al. 2018; Huppertz et al. 2008; Lajko et al. 2018; Li et al. 2019; Maquoi et al. 1997; Sammar et al. 2018; Yang et al. 2012). While both Gal-3 and Gal-9 are known to bind to poly LacNAcs, there is no consensus on whether Gal-13 binds to carbohydrates (Kamili et al. 2016; Su et al. 2018; Than et al. 2009). Also, both Gal-3 and Gal-9 can interact with glycans displaying terminal $\alpha 2,3$ sialylation (Hirabayashi et al. 2002), but only Gal-3 is known to interact with some glycans terminating in $\alpha 2,6$ sialylation (Stowell et al. 2008). Future experiments should assess whether additionally to Gal-1, PSGs can also interact with other members of the galectin family.

Materials and methods

Protein production and purification

In total, 140 micrograms of PSG1 were obtained from 25 mls of serum of pregnant women. Briefly, 1 mL serum samples, which tested positive for hCG consistent with pregnancy, were obtained from the Clinical Pathology Laboratory at Holy Cross Hospital, Silver Spring, MD, USA, prior to their disposal. No identifiers were associated with the serum samples and the protocol was considered exempt from review by the Institutional Review Board.

PSG1-His, PSG1-Fc and the PSG1N-Fc were harvested from the supernatant of stably transfected CHO-K1 cell lines grown in a C2003 hollow fiber cell culture cartridge (FiberCell Systems, Frederick, MD). The protein consisting of only the Fc tag, the A1 domain and the A2 domain fused to the Fc tag were generated by

transfection of ExpiCHO cells (Thermo Fisher Scientific) following the standard protocol and the supernatants were harvested 4–5 days posttransfection. All proteins with an Fc tag consisting of the hinge region and CH2 and CH3 domains of the IgG1 heavy chain were generated by cloning the specific cDNA in-frame into the pFUSE-IgG1-e3-Fc1 vector (InvioGen, San Diego, CA) and were purified from the supernatant on protein A columns as previously described (Ballesteros et al. 2015). GST-PSG1 was generated in Hi5 insect cells as previously reported (Snyder et al. 2001). The human Gal-1 cDNA was synthesized by GenScript USA Inc. (Piscataway, NJ) cloned into pFUSE-IgG1-e3-Fc1 and the protein was generated by transient transfection of Expi293 cells (Thermo Fisher Scientific) as per manufacturer's instructions and purified on a protein A column. The production and purification of murine dmGal-1 and wild-type Gal-1 has been previously described (Cedeno-Laurent et al. 2010). PSG1 V5His was made by transient transfection of GnTI-deficient HEK293 cells (Thermo Fisher Scientific). The 6xHis-tagged proteins were purified with a HisTrap column (GE Healthcare) followed by gel filtration chromatography as previously reported (Warren et al. 2018). All proteins were checked for purity by separation on 4–20% NuPAGE Bis-Tris gels followed by staining of the gel with GelCode Blue (Thermo Fisher Scientific). Recombinant Gal-1 was purchased from PeproTech (Rocky Hill, NJ). For the experiment shown in Figure 4, Gal-1 was generated as previously described and dialyzed with PBS to remove lactose and DTT (Feng et al. 2013).

Reduction and carboxymethylation of native PSG1

Fifty micrograms of pooled native PSG1 was dissolved into 50 µL 10 mM dithiothreitol (DTT) solution in degassed 0.6 M Tris pH 8.5 and incubated at 37°C for 1 h. Carboxymethylation was performed by addition of 50 µL 60 mM iodoacetic acid in 0.6 M degassed Tris pH 8.5 and incubation of the sample at RT in the dark for 90 min. The reaction was terminated by dialysing the sample in a dialysis cassette for 48 h against 50 mM ammonium bicarbonate (Ambic) buffer, pH 8.4, with frequent changes.

Tryptic digestion of native PSG1, release of N-glycans and derivatisation

One µg/µL trypsin (Sigma) solution was prepared in 50 mM Ambic buffer (pH 8.4). Trypsin was added to the protein in a 1:50 (w/w) ration in 200 µL 50 mM Ambic buffer (pH 8.4). The sample was then incubated at 37°C for 14–16 h and the proteolytic digestion was terminated by heating the solution to 100°C for 3 min with subsequent addition of a drop of acetic acid. Digested products were purified by reversed-phase chromatography (Classic C18 cartridge, Waters) against propan-1-ol/5% (v/v) acetic acid.

N-glycans were released from tryptic digested glycopeptides of the sample by addition of 8 units of PNGaseF (Roche) to the sample in 200 µL of 50 mM Ambic buffer (pH 8.4) following a 24 h incubation at 37°C. The released N-glycans were purified from the aqueous mixture by reserved phase chromatography (Classic C18 cartridge, Waters) against propan-1-ol/5% (v/v) acetic acid. The glycan containing fraction (5%AA) was lyophilised and permethylated using sodium hydroxide as previously described (Dell et al. 1993).

Sialidase S/A digestions

An aliquot of lyophilised and purified N-glycans of native PSG1 was dissolved in 250 µL 100 mM sodium acetate buffer (pH 5.5) to which 170 mU Sialidase S/A was added for a 12 h incubation at 37°C.

Another 170 mU sialidase S/A was then added following a further 12 h incubation under the same condition. The reaction was terminated by lyophilisation and digested N-glycans were then purified from the mixture by reversed phase chromatography (Classic C18, Waters). The digested and purified N-glycans were permethylated by the same sodium hydroxide procedure as above.

MALDI-TOF and TOF/TOF for N-glycan determination

Permethylated N-glycans from previous steps were resolubilised in 10 μ L methanol. One microliter of this methanol solution was mixed with 1 μ L of 20 mg/mL 3,4-diaminobenzophenone assisting matrix solution against 75%(v/v) acetonitrile in a 1:1 ratio. One microliter of this mixture was spotted onto a metal plate and the N-glycans were subjected to MALDI-TOF and TOF/TOF analysis in the 4800 MALDI-TOF/TOFTM Analyzer (Applied Biosciences). The data for N-glycan determination was acquired in instrument's positive mode and collision energy was set to 1 kV for TOF/TOF with argon as collisional gas. We estimate that a total of approximately 50 μ g of native PSG1 was utilized for the various MALDI-MS experiments.

In solution digestion of native PSG1 for glycoproteomics

Approximately 10 μ g of native PSG1 was solubilized in 50 mM ammonium bicarbonate buffer pH 8.4 (20 μ L). The protein was reduced at 56°C for 30 min with dithiothreitol prepared in the 50 mM ammonium bicarbonate buffer (10 μ L of a 10 mM solution). This was followed by alkylation for 30 min at RT in the dark with iodoacetic acid, also prepared in the ammonium bicarbonate buffer (10 μ L of a 55 mM solution). Digestion was performed by using Promega sequencing grade modified trypsin in 1:50 enzyme to protein ratio at 37°C overnight. Digestion was halted by acidification using formic acid to a final concentration of 0.2%. The sample was snap frozen in dry ice and concentrated to a few microliters on a speed vac.

Glycoproteomic analysis

Glycoproteomic analysis of the native PSG1 was conducted as previously described (Panico et al. 2016). Briefly, the tryptic digest was solubilized in 0.1% (v/v) formic acid before being analyzed on a Q-Star Pulsar Hybrid mass spectrometer (Applied Biosystems) coupled to a Dionex nano LC system. Separation was conducted using a C18 reverse phased column (Acclaim PepMap 100: 75 μ m ID, 15 cm length) with mobile phase A (5% acetonitrile/0.05% formic acid) and mobile phase B (95% acetonitrile/0.04% formic acid). Separation occurred using a 90 min gradient with separation occurring between 15–70% B over 40 min. Data was acquired in DDA mode and analyzed with the aid of Analyst QS 1.1 software. Raw data files were converted to mgf format before submission to Mascot (www.matrixscience.com) for searching against the Swissprot database. Search criteria included: mass values set at monoisotopic, peptide and fragment mass tolerance set at 150 ppm, one missed cleavage, carboxymethylation of cysteine residues set as fixed modification and methionine oxidation set as variable modification. Probability based scores generated protein hits based on how well the precursor masses and MS/MS fit the data. The percentage coverage of PSG1 was 44%. After confirmation of PSG1 in the Mascot search, the raw data was inspected manually for glycosylation.

ELISAs

The direct interaction of PSG1 with human Gal-1 was determined by ELISA. Wells of a 96-well plate were coated with PSG1 in PBS overnight at 4°C. The coated wells were washed with PBS, 0.05% Tween-20 and the residual binding sites blocked with 3% BSA in PBS. After washing, 2 μ g/mL of human Gal-1Fc, murine Gal-1Fc, murine dmGal-1Fc or the Fc control protein were added to the wells and incubated overnight at 4°C. The following day, the wells were washed and bound proteins were detected with HRP-conjugated goat antihuman IgG Fc (Thermo Fisher Scientific) followed by the addition of TMB substrate (BD Biosciences). The reaction was stopped with sulfuric acid, and the plate read at 450 nm in an ELISA plate reader. Alternatively, wells were coated overnight at 4°C with 10 μ g/mL of human Gal-1 (PeproTech). After washing and blocking with 3% BSA in PBS, rPSG1 or native PSG1 were added to the wells at 20 or 4 μ g/mL for an overnight incubation at 4°C. Bound PSG1 was detected with biotin-labeled anti-PSG MAb#4, followed by streptavidin-HRP (BD Biosciences). For some experiments, wells were coated overnight with 10 μ g/mL of PSG1 in PBS. After washing and blocking with 3% BSA in PBS, human Gal-1 (PeproTech) was added to the wells at 0.25 μ g/mL in PBS for 2 h at RT. Bound Gal-1 was detected with biotin-labeled polyclonal anti-Gal-1 Ab BAF1152 (R&D systems), followed by streptavidin-HRP. To determine whether binding of PSG1 to Gal-1 is glycan-mediated, 100 mM of β -lactose (Sigma) was used as a competitive inhibitor of Gal-1. Sucrose (Sigma) at the same concentration was used as a negative control due to its very low affinity for this lectin. Briefly, wells of a 96-well plate were coated with 10 μ g/mL of human Gal-1. The wells were washed and blocked with 3% BSA in PBS. Lactose or sucrose were added to the wells overnight at 4°C. After washing, full length, the individual PSG1 domains or native PSG1 were added at 1 μ g/mL or 4 μ g/mL, respectively, in PBS with sucrose or lactose. Bound full length or native PSG1 was detected with biotin-labeled anti-PSG MAb#4 followed by streptavidin-HRP, as described above. Bound PSG1 domains were detected with HRP-conjugated goat anti-human IgG Fc, followed by addition of TMB substrate. The ability of recombinant PSG1 to compete the binding of Gal-1 to fibronectin (FN) was tested by ELISA. Briefly, wells of a 96-well plate were coated with 2 μ g/mL of FN (R&D systems; catalog # 1030-FN) in PBS overnight at 4°C and after washing wells were blocked with 3% BSA/PBS. Gal-1 (1 μ g/mL) was incubated with PSG1 generated in CHO-K1 cells or in GnTI-deficient cells at 2 and 20 μ g/mL in 0.5% BSA/PBS for 1 hr at RT in siliconized tubes. After the incubation with PSG1, Gal-1 was added to the wells for 1 hr at RT and following washes, Gal-1 binding was detected with biotin-labeled anti-Gal-1 Ab followed by streptavidin-HRP as described above.

PNGase F treatment

Between 10 and 20 μ g of the PSG1N, PSG1A1 and PSG1A2 domains followed by an Fc tag or the Fc only protein were incubated with 40 mM DTT for 10 min at 55°C and PNGase F (225 units/reaction) for 2 hr at 37°C as recommended by the manufacturer (New England Biolabs). The proteins before and after PNGase F treatment were separated side by side on 4–20% NuPAGE Bis-Tris gels followed by staining with GelCode Blue for protein visualization.

Expression, purification, carbamidomethylation and biotinylation of recombinant human Gal-1

Expression of rGal-1 was performed using the pET30 Ek/Lic vectors in the *Escherichia coli* BL21 (DE3) (Novagen) cells and induced

by 0.1 mM isopropyl D-thiogalactoside (Sigma–Aldrich) at 23°C for 16 h in 3 L of LB medium containing 100 µg/mL ampicillin and 30 µg/mL kanamycin. The soluble proteins extracted with Bugbuster (Novagen) containing 1 mM PMSF and 0.07% beta-mercaptoethanol (2-ME) were loaded onto a lactose–sepharose column. After washing the column with 0.07% 2-ME in 1:10 PBS [PBS (1:10)/2-ME], the rGal1 was eluted with 0.1 M lactose in PBS (1:10)/2-ME. Carbamidomethylation of rhGal1 was performed as reported earlier (Feng et al. 2013). Purified rGal-1 was absorbed on a 1 mL of DEAE–sepharose pre-equilibrated with PBS (1:10)/2-ME, and incubated for 1 h at 4°C with slow agitation. The resin was poured into a column and after extensive washing with PBS (1:10), the column was overlaid with 3 mL of 0.1 M iododacetamide/0.1 M lactose and incubated for 1 h at 4°C in the dark. After washing the column with 50 mM lactose in PBS (1:10), the bound protein (crhGal-1) was eluted with PBS (1:10)/0.5 M NaCl/0.1 M lactose. Biotinylation of rhGal-1 was performed with 1 mg/mL EZ-Link Sulfo-NHS-Biotin (Pierce) in accordance with the manufacturer's recommendations (Nita-Lazar et al. 2016).

Detection of Gal-1 binding in cells by flow cytometric analysis

Jurkat cells (clone E6–1) (American Type Culture Collection) were grown in RPMI 1640 (Corning) supplemented with 10% FBS (Innovative Research Inc.), 100 U/mL and 100 µg/mL of penicillin and streptomycin (Corning), respectively, at 37°C with 5% CO₂. One million cells per tube were resuspended in a final volume of 100 µL of FACS buffer (PBS with 2% BSA and 0.02N Na₃) containing biotin-labeled carbamidomethylated Gal-1 in the presence or absence of PSG1 at the concentrations indicated. Cells were incubated on ice for 30 min and then washed with FACS buffer. Cells were then stained with streptavidin-APC at 0.5 µg/1 × 10⁶ cells for 30 min on ice. The human first trimester trophoblast cell line Swan 71, provided by Dr. Gil Mor (Yale University School of Medicine) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) supplemented with 10% FBS and penicillin/streptomycin in a 37°C humidified incubator with 5% CO₂, was detached with Accutase (Innovative Cell Technologies) (Straszewski-Chavez et al. 2009). One million cells per tube were resuspended in a final volume of 100 µL of FACS buffer (PBS with 2% BSA) containing PSG1 at 2 or 20 µg/mL. The cells were incubated for 1 h on ice, after which they were washed with FACS buffer. Biotin-labeled anti-Gal-1 Ab (1 µg) was added for 1 h on ice, the cells were washed, and streptavidin-APC was added at a concentration of 0.5 µg/1 × 10⁶ cells and incubated for 30 mins on ice. Cells were stained with Sytox blue (Life Technologies) and analyzed using the BD LSR II (BD Biosciences). Fifty thousand total events were collected for each condition using the FACS Diva software (BD Biosciences), and the FlowJo software V10.0.8 (BD Biosciences, San Jose, CA, USA) was utilized for post-acquisition analysis.

SPR experiments

SPR experiments were performed in a BIAcore 3000 instrument (GE Healthcare) at a flow rate of 10 µL/min and 25°C using HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% v/v Surfactant P20) as running buffer. Purified recombinant PSG1 or native PSG1 were immobilized onto a CM5 sensor chip (GE Healthcare) in 10 mM sodium acetate pH 4.0 using standard amine coupling chemistry. A control surface where no protein was

immobilized was used as control to correct for background and potential nonspecific binding. Gal-1 (PeproTech) was injected over a range of concentrations (3 µM to 100 nM) for 180 s followed by a 240 s dissociation period. To regenerate the surface after each Gal-1 injection, a solution containing 1 M NaCl and 20 mM NaOH in HBS-EP buffer was injected for 30 s. Real-time data were analyzed using the BIAevaluation 4.1 software (GE Healthcare), and kinetic profiles were fitted using a global 1:1 binding algorithm to estimate the association (*k_a*) and dissociation (*k_d*) constant, and the affinity (*K_D*) of the interaction. In addition, we calculated the affinity (*K_D*) of the interaction of native PSG1 with Gal-1 in equilibrium using a steady-state calculation (Supplementary Figure S4). For competition experiments, sucrose at a final concentration of 1.43 mM or lactose (0.14, 0.43 or 1.43 mM) were incubated with Gal-1 (0.25 µM) in HBS-EP buffer for 30 min at RT. As a control, Gal-1 at the same concentration and buffer without added disaccharide was incubated for 30 min at RT. These Gal-1 preparations were injected over a SPR biosurface with immobilized recombinant PSG1 or native PSG1 using the same flow, running buffer, temperature and regeneration conditions described above. PSG1 produced in CHOK1, ExpiCHO High Titer or GnTI-deficient cells were immobilized in independent biosurfaces of a CM5 sensor chip as indicated before for recombinant or native PSG1. Gal-1 at 1 µM was injected simultaneously over the three different surfaces and a control surface with no protein immobilized. The same experimental conditions as the ones used to study the interaction of native and recombinant PSG with Gal-1 were used to test these interactions. Real-time sensorgrams were aligned using the BIAevaluation 4.1 software (GE Healthcare).

Gal-1 immunoblotting analysis

PSG1 preparations, between 0.75 µg and 1.29 µg, were heated at 95°C followed by the addition of 2X Laemmli buffer. Samples were loaded onto a SDS-PAGE 4–15% gradient gel. The gels were transferred onto PVDF membranes and blocked with 5% BSA overnight. Biotin-labeled human Gal-1 was added at a concentration of 1 µg/mL and was incubated for 1.5 h at RT. Membranes were then washed five times with PBST (1 × PBS, 0.01% Tween 20). Blots were then incubated with streptavidin-HRP for 45 min at RT, and then washed five times with PBST. The blots were developed using chemiluminescence substrate.

Protection of Gal-1 following treatment with hydrogen peroxide

To assess whether the interaction between PSG1 and Gal-1 protects Gal-1 from oxidation, we first established the conditions in which we could observe a significant reduction of Gal-1 binding to its well-characterized glycoprotein ligand ASF by exposure to hydrogen peroxide (H₂O₂) (Salomonsson et al. 2010). Gal-1 was dialyzed in 1 × PBS to remove β-mercaptoethanol (2ME) after which it was incubated with 20 mM H₂O₂ or PBS for 2 h at RT. The reaction was stopped by the addition of catalase (Sigma) and the Gal-1 samples were concentrated using Amicon Ultra-4 3 K MW cut off centrifugal filter units (Millipore) and their concentration determined. To test the activity of the Gal-1 preparations subjected to the treatment conditions described above, we assessed their ability to bind to ASF. Briefly, wells of a 96-well plate were coated with 5 µg/mL ASF or with 1% BSA used as control. The wells were then blocked with 3% BSA and following washes, 0.25 µg/mL Gal-1 was added for 3 h at RT. The wells were washed and bound Gal-1 was detected with

biotin-labeled goat anti-human Gal-1 antibody (R&D Systems) followed by Streptavidin-HRP and TMB substrate and the plate was read at 450 nm in an ELISA plate reader.

After the reduction in Gal-1 activity was established as described above, three different resins were prepared as follows: rPSG1 made in CHO-K1 cells, rPSG1 made in HEK293 GnTI-deficient cells or BSA were coupled to CnBr-activated-Sepharose beads at 10 mg of protein per ml of resin. Following the coupling reaction, the resins were thoroughly washed with PBS and poured into a Pierce™ column (Catalog # 89896, Thermo Fisher Scientific). Recombinant human Gal-1 was dialyzed against PBS to remove 2ME used to preserve its activity. Following dialysis, 50 µg of Gal-1 were incubated with the PSG1-Sepharose or the BSA-Sepharose beads for 2 h at RT followed by a 6 h incubation with 20 mM H₂O₂. The oxidation reaction was stopped by the addition of 300 U/mL of catalase (Sigma) and Gal-1 was eluted from the protein-coupled Sepharose beads with 100 mM Lactose and dialyzed against PBS to remove the lactose. Gal-1 was concentrated using Amicon Ultra-4 3 K MW cut off centrifugal filter units (Millipore) and the concentration of the recovered Gal-1 following dialysis was determined using a commercially available ELISA (R&D Systems). To assess the activity of Gal-1 eluted from the beads, we determined their ability to bind to ASF by ELISA as described above. Background levels of Gal-1 binding to control wells coated with 1% BSA were subtracted from the absorbance values of Gal-1 binding to ASF. Results are expressed as percentage of Gal-1 inactivation after H₂O₂ treatment and are derived from absorbance values of each sample using the following formula: % of Gal-1 inactivation_i = (absorbance value of Gal-1 from sample_i/mean absorbance value of Gal-1 from BSA-resin) × 100%.

Statistical analysis

Results are expressed as mean ± SD. The statistical analyses were performed using a two-tailed Student's *t*-test or two-way ANOVA test. Post-hoc comparisons were performed using Sidak's test. All data were analyzed using Prism computer software (GraphPad). Differences were considered significant when *P* < 0.05.

Supplementary data

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

Conflict of interest statement

None declared.

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