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ORIGINAL ARTICLE

Activation of latent transforming growth factor-β1, a conserved function for pregnancy-specific beta 1-glycoproteins

James Warren [1], Michelle Im[2], Angela Ballesteros[3], Cam Ha[1], Tom Moore[4], Fanny Lambert[5], Sophie Lucas[5], Boris Hinz[2], and Gabriela Dveksler [1,*]

[1]Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20817, USA [2]Laboratory of Tissue Repair and Regeneration, Matrix Dynamics Group, Faculty of Dentistry, University of Toronto, Toronto, 150 College St., FG234, ON M5S3E2, Canada [3]Molecular Physiology and Biophysics Section, National Institute on Neurological Disorders and Stroke (NINDS-NIH), Bethesda, MD 20892, USA [4]School of Biochemistry and Cell Biology, University College Cork, College Road, Cork T12 K8AF, Ireland [5]Institut de Duve, Université catholique de Louvain, Avenue Hippocrate 75 - B1.74.04, B-1200 Brussels, Belgium

*Correspondence address. Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA. Tel: +1(301) 295-3332; Fax: +1(301) 295-1640; E-mail: gabriela.dveksler@usuhs.edu orcid.org/0000-0003-3037-1155

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STUDY QUESTION: Do all 10 human pregnancy-specific beta 1-glycoproteins (PSGs) and murine PSG23 activate latent transforming growth factor-β1 (TGF-β1)?

SUMMARY ANSWER: All human PSGs and murine PSG23 activated latent TGF-β1.

WHAT IS KNOWN ALREADY: Two of the 10 members of the PSG1 family, PSG1 and PSG9, were previously shown to activate the soluble small latent complex of TGF-β1, a cytokine with potent immune suppressive functions.

STUDY DESIGN, SIZE, DURATION: Recombinant PSGs were generated and tested for their ability to activate the small latent complex of TGF-β1 in a cell-free ELISA-based assay and in a bioassay. In addition, we tested the ability of PSG1 and PSG4 to activate latent TGF-β bound to the extracellular matrix (ECM) or on the membranes of the Jurkat human T-cell line.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Recombinant PSGs were generated by transient transfection and purified with a His-Trap column followed by gel filtration chromatography. The purified PSGs were compared to vehicle (PBS) used as control for their ability to activate the small latent complex of TGF-β1. The concentration of active TGF-β was measured in an ELISA using the TGF-β receptor II as capture and a bioassay using transformed mink epithelial cells that express luciferase in response to active TGF-β. The specificity of the signal was confirmed using a TGF-β receptor inhibitor. We also measured the binding kinetics of some human PSGs for the latent-associated peptide (LAP) of TGF-β using surface plasmon resonance and determined whether PSG1 and PSG4 could activate the large latent complex of TGF-β1 bound to the ECM and latent TGF-β1 bound to the cell membrane. All experiments were performed in triplicate wells and repeated three times.

MAIN RESULTS AND THE ROLE OF CHANCE: All human PSGs activated the small latent complex of TGF-β1 ($P < 0.05$ vs. control) and showed similar affinities (KD) for LAP. Despite the lack of sequence conservation with its human counterparts, the ability to activate latent TGF-β1 was shared by a member of the murine PSG family. We found that PSG1 and PSG4 activated the latent TGF-β stored in the ECM ($P < 0.01$) but did not activate latent TGF-β1 bound to glycoprotein A repetitions predominant (GARP) on the surface of Jurkat T cells.

LIMITATIONS, REASONS FOR CAUTION: The affinity of the interaction of LAP and PSGs was calculated using recombinant proteins, which may differ from the native proteins in their post-translational modifications. We also utilized a truncated form of murine PSG23 rather than the full-length protein. For the studies testing the ability of PSGs to activate membrane-bound TGF-β1, we utilized the T-cell line Jurkat and Jurkat cells expressing GARP rather than primary T regulatory cells. All the studies were performed *in vitro*.

WIDER IMPLICATIONS OF THE FINDINGS: Here, we show that all human PSGs activate TGF-β1 and that this function is conserved in at least one member of the rodent PSG family. *In vivo* PSGs could potentially increase the availability of active TGF-β1 from the soluble and matrix-bound latent forms of the cytokine contributing to the establishment of a tolerogenic environment during pregnancy.

LARGE-SCALE DATA: None.

STUDY FUNDING/COMPETING INTEREST(S): The research was supported by a grant from the Collaborative Health Initiative Research Program (CHIRP). No conflicts of interests are declared by the authors.

Key words: pregnancy-specific beta 1-glycoproteins / latent TGF-β1 / extracellular matrix / latency-associated peptide of TGF-β / GARP protein/heparan-sulfate

Introduction

Pregnancy-specific beta 1-glycoproteins (PSGs) are expressed throughout human pregnancy by the syncytiotrophoblast and are secreted to the maternal circulation (Zhou *et al.*, 1997). PSG1 is expressed at the highest level when compared to the other PSGs and we have shown that it activates the latent form of transforming growth factor (TGF)-β1 (Shanley *et al.*, 2013; Blois *et al.*, 2014). There are 10 human protein coding genes on the long arm of chromosome 19, named *Psg1*—*Psg9*, and *Psg11* (*Psg10* is believed to be a non-coding pseudogene in most individuals) that expanded into a multigene family by duplication and subsequent divergence (Teglund *et al.*, 1994). PSGs belong to the carcinoembryonic antigen (CEA) family, which also includes the CEA-related adhesion molecules (CEACAMs) and are believed to have evolved from the ancestral membrane-bound CEACAM1 (Kammerer and Zimmermann, 2010). PSG-related genes are present in human, chimpanzee, orangutan, rhesus macaque, African green monkey and baboon. In addition to higher primates, PSGs are found in mice and rats (Kammerer and Zimmermann, 2010). Rodent PSGs are expressed in spongiotrophoblasts and in trophoblast giant cells (Rebstock *et al.*, 1993; Kromer *et al.*, 1996; Wynne *et al.*, 2006). Interestingly, in all species that express PSG-like proteins multiple *Psg* genes are found. For example, there are 11 human *Psg* genes, 15 *Psg* genes in baboons, 5 *Psg* genes in rats and 17 in mice (Zhou and Hammarstrom, 2001; McLellan *et al.*, 2005a). As stated above, PSGs have expanded from a single gene by duplication and it remains unknown whether, after expansion, different genes have acquired different functions.

An independent expansion of rodent and primate PSG families has been suggested and there is a lack of orthologous relationship between primate and rodent PSGs (McLellan *et al.*, 2005b). A major difference between rodent and primate PSGs is observed in the domain organization of these glycoproteins with rodent PSGs expressing several Ig-variable like-domains and primate PSGs having only one at the N-terminus (McLellan *et al.*, 2005a; Moore and Dveksler, 2014). Therefore, it is possible that different PSGs have different functions within the same species and that unique functions have evolved as PSGs diversified in sequence and structure between primates and rodents. In this study, we analyzed all 10 human PSGs and PSG23, one of the two murine PSGs that constitutes the bulk of *Psg* gene expression in the placenta, for their ability to activate latent TGF-β1 (Ball *et al.*, 2004; McLellan *et al.*, 2005a).

The importance of TGF-β1 in human and murine pregnancy has been recognized by many investigators as this cytokine is involved in many processes essential for pregnancy success including decidualization, trophoblast invasion, angiogenesis and immune regulation (Graham *et al.*, 1992; Jones *et al.*, 2006; Zhao *et al.*, 2006; Stoikos *et al.*, 2008; Ingman and Robertson, 2009). A major reservoir of latent TGF-β1 is in the extracellular matrix (ECM) of many tissues, including the placenta (Graham *et al.*, 1992; Isogai *et al.*, 2003; Jones *et al.*, 2006; Horiguchi *et al.*, 2012). Therefore, we tested whether human PSG1 was able to activate this cytokine deposited in the ECM. Another reservoir of latent TGF-β1 is located on the surface of stimulated CD4 +CD25+ Foxp3+ regulatory T cells (Tregs). Tregs suppress other immune cells in a contact-dependent manner and they are indispensable for the maintenance of peripheral tolerance including maternal immune tolerance to the semi-allogeneic fetus (Aluvihare *et al.*, 2004; Josefowicz *et al.*, 2012; Hosseini *et al.*, 2018). Immune suppression by Tregs implies release of active TGF-β1 from protein complexes located on their surface, in which latent TGF-β1 is bound and presented by transmembrane protein glycoprotein A repetitions predominant (GARP) for activation by integrin $\alpha_V\beta_8$ (Nakamura *et al.*, 2001; Tran *et al.*, 2009; Stockis *et al.*, 2009b, 2017; Worthington *et al.*, 2011; Cuende *et al.*, 2015). We thus also tested if two members of the human PSG family, PSG1 and PSG4, were able to activate latent TGF-β1 presented by GARP on the surface of T cells.

Results

All human PSGs activate latent TGF-β1

Previous studies from our group showed that PSG1 and PSG9 can activate the small latent form of TGF-β1 (Blois *et al.*, 2014; Jones *et al.*, 2016). In addition, we observed that while more than one domain of PSG1 can activate the latent cytokine, the B2 domain was by far the most efficient (Ballesteros *et al.*, 2015). As shown in Fig. 1A, some human PSGs (PSG2, 5 and 11) lack either the A1 or A2 domains but all human PSGs have a B2 domain. The amino acid identity of the B2 domain of the different PSGs ranges from 72% to 95%. We generated recombinant human PSG1, 2, 3, 4, 5, 6, 7, 8 and 11 with a 6xHis and a V5 tag at the C-terminus in Expi293 cells (Shanley *et al.*, 2013). All recombinant PSGs were detected with the anti-PSG MAb6799 (Fig. 1B). Using PSG1 single domain constructs, we determined that this Ab binds to a conserved epitope in the A1 and A2 domains (Supplementary Fig. S1) and does not react with the N- or the B2 domain (data not shown). The yield of recombinant PSGs varied greatly, with PSG3 resulting in the lowest amount even when we attempted its production in a different cell line (ExpiCHO) or with a different tag (Fc).

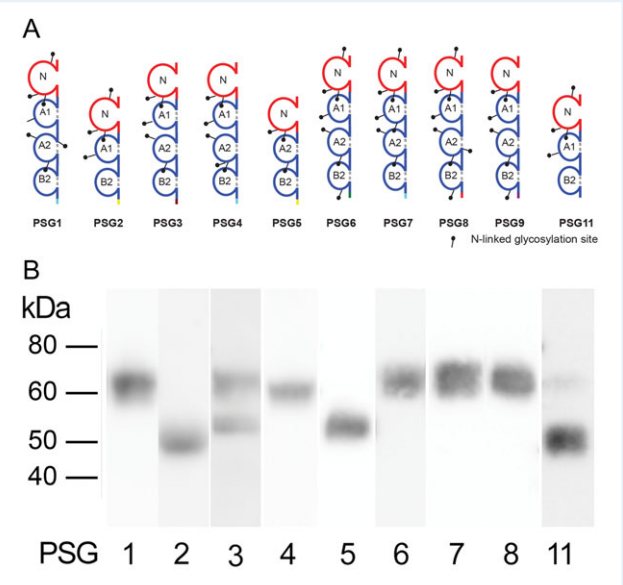

Figure 1 Western blot analysis of recombinant pregnancy-specific beta 1-glycoprotein. (**A**') Schematic representation of human pregnancy-specific beta 1-glycoprotein (PSG) family members. Red color-coded 'N' domains are Ig variable-like domains, and blue 'A' and 'B' domains are Ig constant-like domains. Black 'lollipops' indicate potential N-linked glycosylation sites. Note that atypical amino and carboxy terminal sequences are also color-coded. (**B**) Recombinant PSGs were generated and purified as described under the materials and methods section and loaded onto a 4%–20% NuPAGE gel. The proteins (1 μg/lane) were transferred to a polyvinylidene difluoride membrane and probed with anti-PSG MAB6799.

We tested all human PSGs for their ability to activate the SLC of TGF-β1 using a bioassay in which stably transfected transformed mink lung epithelial cells (TMLECs) express luciferase in response to biologically active TGF-β (Abe *et al.*, 1994). We found that all PSGs tested activated TGF-β at 15 μg/ml or higher concentration and the specificity of the response was further confirmed by the lack of luciferase signal when the cells were incubated with a specific TGF-β receptor I inhibitor (Fig. 2A and data not shown). In addition, we determined that these PSGs activate the SLC utilizing an ELISA-based protocol with the TGF-β receptor II as capture as previously described (Blois *et al.*, 2014) (Fig. 2B). The concentration that consistently showed activation by the different protein preparations in both assays was 15 μg/ml, the equivalent to ~233 nM for the full-length PSGs containing the four domains (N, A1, A2 and B2). For some PSGs (PSG1, 2, 4, 5, 6, 7, 8 and 11), we also tested their ability to activate latent TGF-β1 at 30 μg/ml and we observed that the concentration of active TGF-β1 obtained was higher when PSGs were used at 30 μg/ml rather than 15ug/ml (data not shown). PSG1 and PSG4 were also tested at 140 nM and showed significant TGF-β activation activity albeit the fold activation observed over control was lower than the one observed at the higher PSG concentration (data not shown).

We also compared the kinetics of the interaction of the different human PSGs with the LAP of TGF-β1, the polypeptide that confers latency to the cytokine. Fig. 3 shows representative sensograms of recombinant PSG1, PSG2, PSG4, PSG5 and PSG8 interaction with a LAP biosurface obtained by surface plasmon resonance (SPR). All the PSGs tested showed an affinity (KD) for LAP in the micromolar range, ranging from 4.36 to 11.1 μM for PSG1 and PSG5, respectively (Table I). PSG5 presented a slightly lower affinity (2.3 times) due to a reduced association constant when compared to the other PSGs. On the other hand, PSG1, PSG2, PSG4, and PSG8 presented identical kinetics for the interaction with LAP, with an association constant (k_a) of around $3 \times 10^3\ M^{-1}\ s^{-1}$ and a dissociation constant (k_d) of $15 \times 10^{-3}\ s^{-1}$ approximately (Table I). Unexpectedly, we did not detect binding of PSG7 to immobilized LAP even after testing different preparations of the protein made in Expi293 or ExpiCHO cells or after increasing the PSG7 concentration. We were not able to calculate the kinetics of the PSG3, PSG6 and PSG11 interaction with LAP as these experiments require substantial amount of protein and we obtained lower quantity of these recombinant proteins compared to that of the other PSGs.

PSG1 activates latent TGF-β deposited on the extracellular matrix (ECM)

We have previously shown that PSG1 binds to heparan-sulfate (HS; Lisboa *et al.*, 2011). The ECM is rich in HS proteoglycans and therefore we tested whether PSG1 binds to the ECM (Esko *et al.*, 2009; Neill *et al.*, 2015). We observed that PSG1 binds to the ECM and that this interaction is competed by heparin, a highly sulfated form of HS (Fig. 4). In addition, we observed that PSG1 activates TGF-β in the presence of heparin (2 μg/ml) in the TMLEC reporter bioassay; when heparin was added to the reporter cells together with 280 nM of PSG1, we obtained a luciferase signal corresponding to 381 ± 7.4 pg/ml of TGF-β compared to 372 ± 4.4 pg/ml in the absence of heparin. Treatment of the cells with vehicle rather than PSG1 resulted in a concentration of TGF-β in the supernatant of the reporter cells of 96.6 ± 7.3 pg/ml in the presence of heparin and of 97.2 ± 3.1 pg/ml in the absence of heparin. These results suggested that the interaction of PSG1 with HS and LAP of TGF-β1 occurs through different epitopes in the protein. Therefore, we wondered whether PSGs might activate latent TGF-β deposited by cells in the ECM. To answer this question, we took advantage of MEF derived from TGF-β deficient mice, which do not deposit latent TGF-β in the matrix. MEF from wild-type and TGF-β deficient mice were seeded on wells for 7–10 days to allow for ECM accumulation (Tesseur *et al.*, 2006). After removal of the cells, TMLEC TGF-β reporter cells were added to the ECM-containing wells in the presence or absence of PSG1. As shown in Fig. 5, PSG1 was able to activate latent TGF-β secreted and stored in the ECM deposited by MEF from wild-type cells as determined by the increase in relative luminescence. As expected, the ECM deposited by MEF from TGF-β1-deficient mice showed comparable levels of active TGF-β1 to those from wild-type ECM in the absence of PSG1 (Fig. 5).

PSG1 and 4 do not activate latent TGF-β1 presented by GARP on the surface of transfected human T cells

We tested if PSGs were also able to activate latent TGF-β1 from GARP/latent TGF-β1 complexes on the surface of human T cells. Jurkat + GARP cells, but not parental non-infected Jurkat cells, express very high levels of surface GARP/latent TGF-β1 complexes (Stockis *et al.*, 2009a). Because a very low amount of TGF-β that could be below the levels of detection by available ELISAs could result in phosphorylation of the TGF-

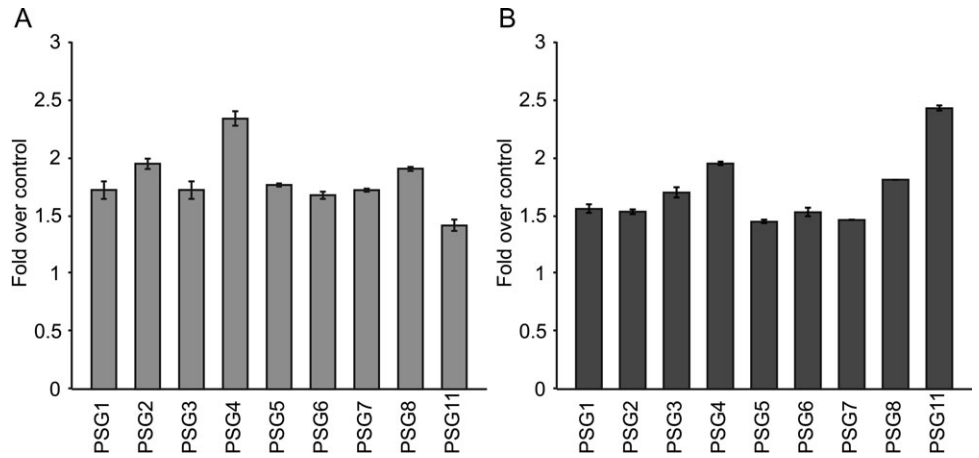

Figure 2 All human PSGs activate the small latent complex of transforming growth factor-β1. (**A**) PSGs (15 μg/ml) or an equal volume of PBS (control) were incubated with 50 ng/ml of small latent complex (SLC) for 1 h at 37°C in DMEM-0.1% ITS and then added to plasminogen activator inhibitor-1 reporter mink lung epithelial cells (TMLEC). The treated TMLECs were incubated for 16–18 h and then lysed. The luciferase activity in the lysate was analyzed with a GloMax luminometer. (**B**) PSGs (15 μg/ml) or an equal volume of PBS (control) were incubated with 50 ng/ml of SLC for 1 h at 37°C in PBS. The samples were then transferred to wells of a 96-well coated human transforming growth factor-β1 (TGF-β) receptor II-Fc. After 2 h, the plates were washed, and active TGF-β1 was detected with a biotinylated-antibody specific for mature TGF-β1. All experiments were performed in triplicate wells in three independent experiments. Results are expressed as fold difference over control and all values are significant ($P < 0.05$) over control. Significance was calculated using the Student's *t*-test.

β signal transducer Suppressor of Mothers Against Decapentaplegic 2 (SMAD2) in this assay, PSG1 and PSG4 were processed with an additional purification step as indicated in the methods section. PSG1 was selected as it is the highest expressed in pregnancy and we also included PSG4 as we had higher amounts of this protein compared to the other PSGs and we predicted that an additional purification step would result in substantial protein loss. Cells were incubated for 4 hours with PSG1 or PSG4 and assessed for phosphorylation of SMAD2 (pSMAD2) by Western Blot as a read out for TGF-β1 activation. As shown in Fig. 6, PSG1 and PSG4 induced low levels of pSMAD2, equivalent to 10 pg/ml of active rhTGF-β1, whether surface GARP/latent TGF-β1 complexes were present or absent on Jurkat cells. This indicated that PSG1 and PSG4 can activate soluble latent TGF-β1 produced by Jurkat cells, whether they express GARP or not. Expression of high levels of GARP/latent TGF-β1 on the cell surface did not increase active TGF-β1 production in the cells. In addition, we could not detect any interaction between soluble PSG1 and surface GARP/latent TGF-β1 complexes by immunoprecipitation (data not shown). Altogether, we concluded that although PSGs can activate soluble latent TGF-β1 or latent TGF-β1 present in the ECM, it does not appear to interact or activate latent TGF-β1 in complex with GARP on the surface of T cells.

Murine PSG23 activates latent TGF-β

Rodent PSGs have a relatively low sequence conservation compared to their human counterparts and assignment to the PSG subgroup has only been possible by comparing expression patterns (Rebstock *et al.*, 1993). Of the 17 murine PSGs (PSG16-32) fourteen of them, including PSG23, are composed of three N-domains and a single A-domain (N1-N2-N3-A). Past efforts in our laboratory to generate full-length murine PSG17, 22 and 23 in CHO-K1 cells resulted in very low protein yields while we were able to generate murine PSGs composed of the first domain and the A-domain (Ha *et al.*, 2008; Wu *et al.*, 2008; Sulkowski *et al.*, 2011). We transfected ExpiCHO and Expi293 cells in an attempt to generate full-length PSG23 but the amount of protein recovered was very low and insufficient to test its activity. Therefore, we generated recombinant proteins composed of the N_1 and A-domain (PSG23 N_1A myc His) and a protein composed of just the N1-domain of PSG23 (PSG23N_1-Fc). These proteins were tested for their ability to activate TGF-β1, as described for human PSGs. We found that both PSG23 N_1A myc His (Fig. 7A and B) and PSG23N_1-Fc (Fig. 7C and D) were able to activate TGF-β1 in a dose-dependent manner.

Materials and Methods

Production of recombinant PSGs

Recombinant PSGs were generated as previously described with minor modifications (Houston *et al.*, 2016). Plasmids were transiently transfected into Expi293 cells and the supernatants were harvested 6 days post-transfection (Thermo Fisher Scientific). Clarified supernatants were bound to a His-trap column (GE Healthcare, Chicago, IL, USA) in the presence of 10 mM imidazole and were eluted with 200 mM imidazole. The proteins eluted at 200 mM imidazole were buffer-exchanged into PBS and concentrated using Amicon Ultra-15 10 K MW cut off centrifugal filter units (Millipore, Burlington, MA, USA) and further purified by gel filtration chromatography on a HiLoad 16/200 Superdex 200 pg column (GE Healthcare). The proteins which corresponded to the expected molecular weight were then further concentrated prior to running on a gel. For experiments using Jurkat cells, PSG1 and PSG4 were further purified to remove any latent TGF-β bound to the protein using anti-latent-associated peptide (LAP) of TGF-β1 antibody (Ab) (Blois *et al.*, 2014). Briefly, 200 μg of anti-LAP Ab (R&D Systems, Minneapolis, MN, USA) were coupled to 10 mg of activated magnetic beads (Dynabeads® M-280 Tosylactivated) following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Two miligrams of PSG1 or PSG4 were incubated with 10 mg of anti-LAP Ab-coupled beads, prepared as described above, at 4°C overnight and the

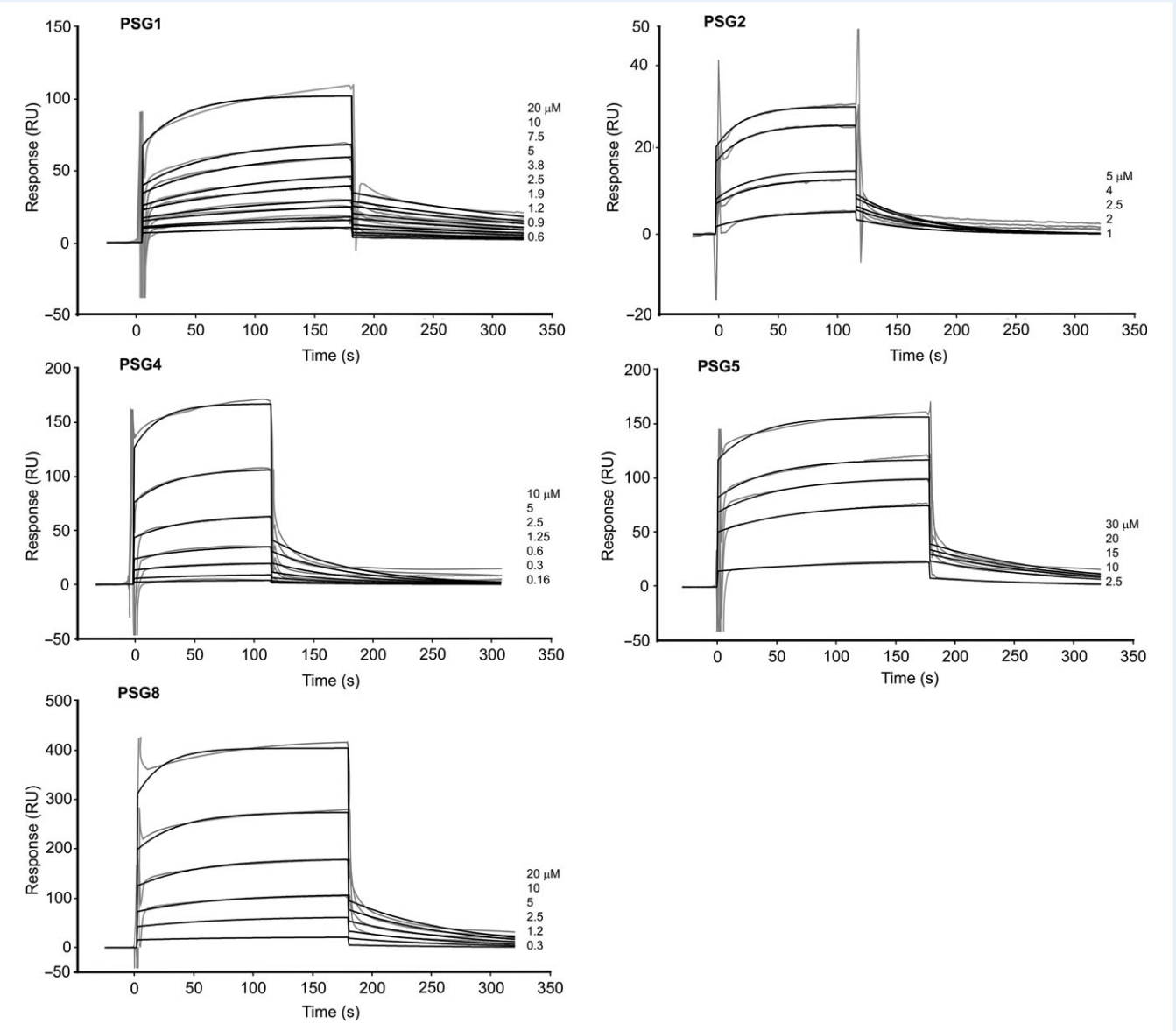

Figure 3 Human PSGs bind to latent-associated peptide of TGF-β1. Purified PSG1, PSG2, PSG4, PSG5 and PSG8 proteins were injected a several concentrations ranging from 30 to 0.16 μM over a CM5 biosensor chip with immobilized latent-associated peptide (LAP). The data were analyzed using a simultaneous fit algorithm to calculate the kinetic parameters of the interaction that are presented in Table 1. The KD values for each interaction are shown. Surface plasmon resonance sensograms for each response are shown as gray lines and fitted curves are shown as black lines.

protein that did not bind to the beads (flow through) was concentrated with an Amicon Ultra-15 centrifugal filter and utilized for the experiments. For quantitation purposes, all proteins employed in our studies were separated on 4–20% NuPAGE Bis-Tris gels at different dilutions alongside known concentrations of bovine serum albumin (BSA) (Thermo Fisher Scientific) used as standards. After staining with GelCode Blue (Thermo Fisher Scientific), the proteins were quantitated by densitometry. The identity of the proteins was confirmed by Western blotting with the anti-human PSG pan specific monoclonal antibody 6799 (MAB6799) (clone# 684 701, R&D systems). To map the MAB6799 binding site, we generated Fc-fusion protein of the PSG1 A1 and A2 domain. The cDNA coding these domains was synthesized by GenScript (Piscataway, NJ, USA) and cloned into the Eco-RI-Bgl II sites of the pFuse-IgG1 e3-Fc1 vector (InvivoGen, San Diego, CA, USA). The plasmids were transfected into ExpiCHO cells under the manufacturer's instructions (Thermo Fisher Scientific) and the recombinant proteins in the collected medium were purified using a 1-ml HiTrap protein A column (GE Healthcare). Bound proteins were eluted with 0.1 M glycine [pH 2.8] and neutralized with 1 M Tris-HCL [pH 8.0] and were quantitated following separation on 4–20% NuPAGE Bis-Tris gels as indicated above. The N-domain of PSG1 with the Fc tag (PSG1N-Fc), the Fc control protein, PSG1-Fc, PSG23N_1-Fc and PSG23N_1A myc His were generated as previously described (Wu *et al.*, 2008; Sulkowski *et al.*, 2011; Blois *et al.*, 2014; Ballesteros *et al.*, 2015).

Surface plasmon resonance

The interaction of PSGs with the human LAP of TGF-β1 was analyzed using a Biacore 3000 instrument (GE Healthcare), as previously described

Table I Binding kinetics rate constants of the interaction of pregnancy-specific beta 1-glycoproteins with the human latent-associated peptide of transforming growth factor-β1.

	ka M^{-1} s^{-1} (mean ± SE)	kd s^{-1} (mean ± SE)	KD μM	Rmax (mean ± SE)	chi^2
PSG1	$(1.4 \pm 0.3) \times 10^3$	$(6.1 \pm 0.4) \times 10^{-3}$	4.36	44.2 ± 0.5	3.0
PSG2	$(3.2 \pm 0.3) \times 10^3$	$(15.8 \pm 0.5) \times 10^{-3}$	4.88	18.3 ± 0.7	0.2
PSG4	$(3.2 \pm 0.1) \times 10^3$	$(15.1 \pm 0.3) \times 10^{-3}$	4.72	61.4 ± 1.1	2.8
PSG5	$(0.9 \pm 0.3) \times 10^3$	$(9.9 \pm 0.2) \times 10^{-3}$	11.1	56.5 ± 1.1	3.0
PSG8	$(3.2 \pm 0.1) \times 10^3$	$(15.6 \pm 0.2) \times 10^{-3}$	4.79	126.0 ± 1.3	1.6

The kinetic data shown in Fig. 3 for the interaction of the different human pregnancy-specific beta 1-glycoproteins (PSGs) with latent-associated peptide were fitted using a 1:1 Langmuir binding model for the estimation of the association and dissociation rates (ka and kd, respectively), and dissociation constant (KD = ka/kd). The maximum response (R_{max}) and chi^2 values for the fitted data are also included. The '*n*' of the SE for the ka, kd and R_{max} shown for each protein corresponds to the number of sensograms or protein dilutions used in each calculation and represented in Fig. 3 ($n = 10$ for PSG1, $n = 5$ for PSG2, $n = 7$ for PSG4, $n = 5$ for PSG5 and $n = 6$ for PSG8).
The SE reported for each parameter represents an estimate of how sensitive the fitting performed is to the observed changes in that parameter after testing several dilutions of each protein. Chi^2 is a measure of the average deviation of the experimental data from the fitted curve.

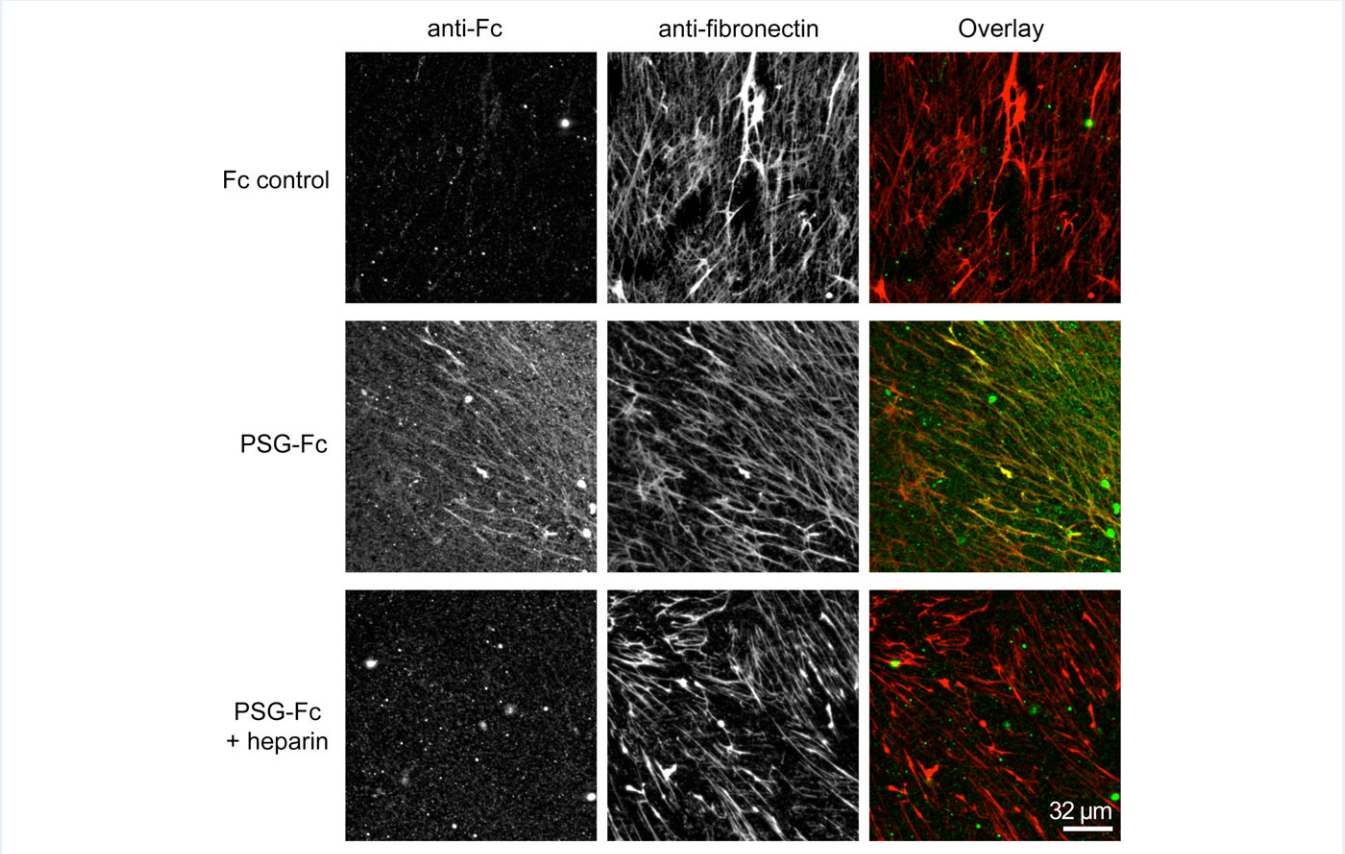

Figure 4 PSG1 binds to the extracellular matrix in a heparan-sulfate dependent manner. PSG1-Fc (20 μg/ml) in the presence or absence of heparin or the Fc protein control were added to decellularized fibroblast matrix for 1 h at room temperature, washed extensively and fixed with 3% paraformaldehyde. Slides were then incubated with anti-fibronectin antibody followed by tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG or FITC-conjugated anti-human Fc. Fluorescence microscopy images were acquired using an Axio Imager upright microscope equipped with an AxioCam HRm camera and ZEN software with 40× magnification. The right most panels show an overlay image of the PSG1 detection anti-Fc (green) and anti-fibronectin (red) images. Scale bar indicates 32 μm.

(Ballesteros *et al.*, 2015). Briefly, carrier-free recombinant human LAP (R&D systems) was coupled via amine-coupling to a flow cell of a CM5 sensor chip unit until a level of 2000 resonance units (RUs) was reached. A control cell was prepared following the same protocol without the addition of LAP. PSGs were injected separately into the flow cells at a rate of 10 μl/min at 25°C. At least to protein preparations were tested for each

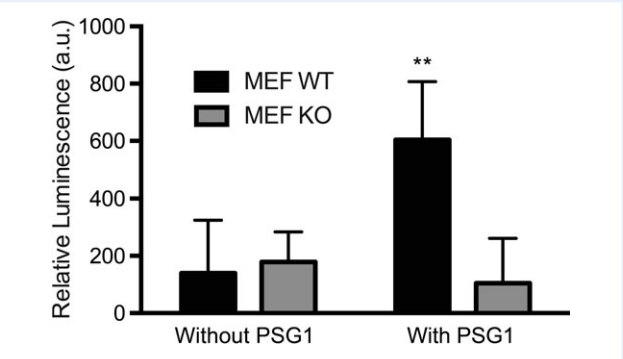

Figure 5 PSG1 activates latent TGF-β bound to the extracellular matrix. Mouse embryonic fibroblasts (MEF) from wild-type or TGF-β1-deficient mice were cultured for 7–10 days to lay matrix. After decellularization, TMLEC TGF-β reporter cells were seeded on the MEF matrix with or without 20 μg/ml of PSG1 in TMLEC medium and incubated overnight at 37°C. Cells were washed, lysed and luciferase activity in the lysed TMLECs was determined 16–20 h later. All experiments were performed in triplicate wells in three independent experiments. Significance was calculated using the Student's *t*-test. $^{**}P \leq 0.01$.

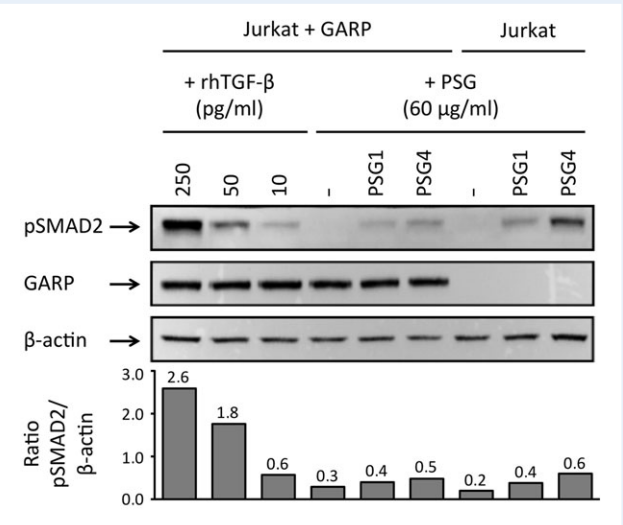

Figure 6 PSGs do not activate latent TGF-β1 presented by GARP on transfected human T cells. Jurkat cells, untransfected or stably transfected with glycoprotein A repetitions predominant (GARP) (Jurkat + GARP), were incubated in serum-free medium in the presence or absence of 60 μg/ml of PSG1 or PSG4, or with the indicated concentration of recombinant, active human TGF-β1 (rhTGF-β1) as controls. Cell lysates were collected after 4 h and analyzed by Western blot with antibodies against phosphorylated SMAD2 (pSMAD2) as a readout for active TGF-β1 production, anti-GARP or β-actin as loading controls. Bar graphs show quantification of enhanced chemiluminescence signals represented as ratios of pSMAD2 to β-actin.

PSG. Several concentrations of the same PSG were injected during the association phase for 2–3 min and the dissociation phase was carried out over a 2-min period. Kinetic data were analyzed using BIAevaluation software (GE Healthcare) and the association and dissociation constants were calculated assuming a 1:1 Langmuir binding model. All binding curves were corrected for background and bulk refractive index contribution by subtraction of the reference flow cell.

Activation of the small latent complex of TGF-β1 by PSGs

To detect active TGF-β1 we performed two assays using triplicate wells for each condition tested (Ballesteros *et al.*, 2015). In the cell-free system assay, proteins were incubated with 50 ng/ml of small latent complex (SLC) (R&D Systems) at 37°C for 1 h in a final volume of 0.1 ml of PBS in siliconized tubes. The samples were then transferred to wells of a 96-well Nunc Maxisorb plate that had been coated overnight with recombinant human TGF-β receptor II-Fc (R&D systems) and blocked with PBS-1%BSA. After 2 h, the plates were washed, and active TGF-β1 was detected with a biotinylated-antibody specific for mature TGF-β1 (BAF240, R&D systems). In the second assay, bioactive TGF-β was determined using the TGF-β responsive plasminogen activator inhibitor-1 reporter mink lung epithelial cells line (TMLEC) provided by Dr Rifkin (New York University, NY, USA) following incubation of the proteins with 50 ng/ml of SLC at 37°C for 1 h in a final volume of 0.1 ml of serum-free DMEM supplemented with 0.1% insulin–transferrin–sodium selenite (Sigma-Aldrich, St. Louis. MO, USA) in siliconized tubes, which was then added to the cells for 16–18 h prior to lysis of the cells to measure relative luciferase units (Abe *et al.*, 1994). To verify that the observed increase in luciferase was the result of TGF-β binding to its receptor in the TMLEC cells, the experiments were carried out in the presence of 5uM of the TGF-β receptor I kinase inhibitor SB431542, as previously described (Blois *et al.*, 2014). Two-fold dilutions of active TGF-β1 (R&D Systems) starting at 2000–62.5 pg/ml were used as standards to determine the linearity of the signal in these assays. Controls included SLC incubated with the same volume of PBS (vehicle) or a protein consisting of the Fc tag. We observed no differences between these two controls in any of our assays.

Immunofluorescence detection of PSG1 in the ECM

Cell culture dishes (60 mm) were pre-coated with 20 μg/ml gelatin (dissolved in PBS) and incubated overnight at 4°C. MRC-5 fibroblasts (ATCC, Manassas, VA, USA) were then seeded and cultured for 7–10 days to lay down matrix. Cells were gently removed with 20 mM NH_4OH with 0.5% Triton X-100, followed by washes with PBS. PSG1-Fc (20 μg/ml dissolved in PBS) or the Fc protein control was then added to the decellularized matrix for 1 h at room temperature, washed extensively and fixed with 3% paraformaldehyde. Antibodies used were directed against total fibronectin (rbAb, Sigma-Aldrich, F3648), secondary goat anti-rabbit-IgG conjugated to tetramethylrhodamine isothiocyanate (TRITC: Sigma-Aldrich, F9887), and FITC-conjugated anti-human Fc antibody (Sigma-Aldrich, F9512). Fluorescence microscopy images were acquired using an Axio Imager upright microscope equipped with an AxioCam HRm camera and ZEN software with 40× magnification (Zeiss, Oberkochen, Germany). To determine if binding of PSG1-Fc was mediated by HS, PSG1-Fc or Fc control was added to the matrix in the presence of heparin (50 μg/ml).

PSG1 activation of latent TGF-β in the ECM

Mouse embryonic fibroblasts (MEF) from wild-type or TGF-β1-deficient mice provided by Dr Wyss-Coray (Stanford University, CA, USA) were seeded into cell culture dishes (35 mm) for 7–10 days to lay matrix (Tesseur *et al.*, 2006). After decellularization performed as described above, TMLEC TGF-β reporter cells were seeded on the MEF laid matrix (60 000 cells/cm^2) with or without 20 μg/ml of PSG1 in TMLEC medium

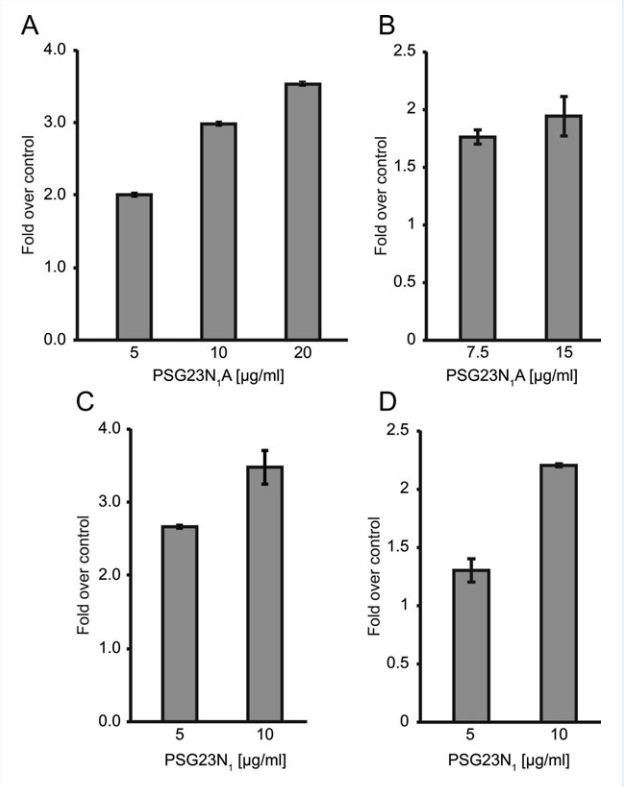

Figure 7 Murine PSG23 activates the SLC of TGF-β1. Recombinant PSG23 consisting of the N_1 and A domains (Part **A** and **B**) or just the N1-domain (Part **C** and **D**) at the indicated concentrations were tested for their ability to activate SLC in an ELISA-based assay (Parts A and C) or with the TMLEC reporter cells (Parts B and D) as described in Material and methods section. A protein consisting of the Fc tag at the same concentration was used as control. All experiments were performed in triplicate wells in three independent experiments. Results are expressed as fold difference over control and all values are significant ($P < 0.05$) over control. Significance was calculated using the Student's *t*-test.

(DMEM containing heat-inactivated 1% FBS) and incubated overnight at 37°C. Cells were then washed, lysed using 1 × Cell Culture Lysis Reagent (Promega, Madison, MI, USA), and 20 µl of lysate was transferred to each well of a white, opaque 96-well plate. Luciferase activity in the lysed TMLECs, used as a read out for the presence of active TGF-β, was determined 16–20 h later.

Activation of latent TGF-β1 from GARP/latent TGF-β1 complexes on the surface of T cells

Jurkat cells (clone E6-1) were obtained from the American Type Culture Collection (Manassas, VA, USA). Clone E6-1 was transduced with a lentivirus encoding GARP to generate Jurkat + GARP, as previously described (Stockis *et al.*, 2009a). Jurkat and Jurkat + GARP cells were incubated in X-vivo 10 serum-free medium (#04-380Q, Lonza, Walkersville, MD, USA) for 4 hours at 37°C in the presence or absence of 60 µg/ml of recombinant PSG1 or PSG4, or with the indicated concentrations of recombinant, active TGF-β1 as controls. Cells were lysed and submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Blots were incubated with primary Abs against phopshorylated SMAD2 (pSMAD2) (#3108; Cell Signaling Technology, Danvers, MA, USA), GARP (Plato-1; Enzo Life Sciences, Farmingdale, NY, USA), or β-actin (A5441; Sigma-Aldrich), then secondary horse-radish peroxidase-coupled Abs, and revealed with an ECL substrate (Thermo Fisher Scientific) on a Fusion Solo 4 S system (Vilber, Collégien, France). Quantifications were performed with Bio1D (Vilber).

Discussion

Many placental hormones, including PSGs, are encoded by multigene families (Hughes *et al.*, 2000). As previously suggested, the increased gene dosage for some placental proteins that share the same function could be the outcome of the maternal-fetal conflict (Haig, 1993). Human PSG genes exhibit rapid coding sequence divergence and a relatively high frequency of gene conversion between different PSG family members compared to the genome average (Chang *et al.*, 2013). Microarray studies have suggested that a high level of ethnic-specific PSG gene copy number variations (12–30 copies) exists in the population. This implies that some individuals could lack one or more PSG genes (natural knockouts) while other individuals may have two or more copies of a particular PSG gene (Chang *et al.*, 2013). Two recent studies have reported a deletion of PSG11 in some individuals, making PSG11 one of the few genes in which a complete human knockout has been identified (Zhao *et al.*, 2012). Owing to a lack of information regarding the function of the different PSGs, the significance of missing a PSG gene or having multiple copies of others cannot be evaluated.

In both human and rodents, there is some evidence that indicates that a small number of PSGs produces the bulk of the mRNA, likely due to differences in promoter sequences, and also that the level of expression increases for most PSGs as pregnancy progresses (Chamberlin *et al.*, 1994; Camolotto *et al.*, 2010; Shanley *et al.*, 2013). Our preliminary results obtained after the analysis of 40 serum samples with the PSG1 Quantikine ELISA kit (R&D systems) indicates that the mean PSG1 concentration is ~20 µg/ml. While the concentration of the other PSGs in serum remains unknown, it is important to note that the local concentration of PSGs at the maternal-fetal interface (that is the site of PSG secretion) is likely much higher than that measured in circulation.

As shown in Fig. 1, PSG potential N-linked glycosylation sites range from eight to three. The effect of glycosylation on the activity of these proteins has not been evaluated but based on the study of other glycoproteins, glycosylation likely differs based on culture conditions and the cell line utilized for recombinant protein production (Nam *et al.*, 2008; Croset *et al.*, 2012; Jain *et al.*, 2017). We previously expressed PSG1 in CHO-K1 and MEFs and found that it activated TGF-β1 (Blois *et al.*, 2014). For the experiments described here, all full-length PSGs were expressed in Expi293 cells but we have also tested PSG4 made in ExpiCHO cells, PSG6 in Hi5 insect cells and PSG1 made in ExpiCHO and the NSO (human B cell) cell line (Snyder *et al.*, 2001). While all these recombinant proteins activated latent TGF-β1 at 280 nM (data not shown), we did not perform a thorough study to determine the possible effects of differences in glycostructures in these PSGs and potential subtle differences in their ability to activate TGF-β1. In addition, the activity of the different glycoforms may differ from the native protein due to potential differences in half-life in circulation and immunogenicity (Higel *et al.*, 2016).

Previously we reported that PSG1 and PSG9 activate the SLC of TGF-β1, the results presented here indicate that all 10 members of the human PSG family share this biological activity (Blois *et al.*, 2014; Jones *et al.*, 2016). We proposed that PSG1 could have therapeutic potential in diseases in which an increase in Tregs could be beneficial (Blois *et al.*, 2014). Therefore, to determine whether a human PSG other than PSG1 could be more efficient at activating latent TGF-β1 and could potentially perform better as a therapeutic, we compared their binding kinetics for LAP *in vitro*. All the recombinant human PSGs tested (PSG1, PSG2, PSG4, PSG5 and PSG8) presented similar kinetics and affinity for LAP as assayed by SPR. These data suggest a common and similar ability to directly interact with LAP in all human PSGs.

The SLC of TGF-β1 can be secreted from cells in complex with latent TGF-β binding protein-1 (LTBP-1) forming the large latent complex (LLC) (Robertson *et al.*, 2015). Fibronectin immobilizes LTBP-1 and thus stores latent TGF-β1 in the ECM (Klingberg *et al.*, 2018). Our results indicate that PSGs could bind to HS proteoglycans in the ECM and activate the LLC deposited in it. The same results were obtained when using PSG4 (data not shown). In addition to being secreted as soluble SLC or deposited in the ECM in association with LTBP-1, latent TGF- β1 can also be retained and presented on the surface of Tregs via its interaction with GARP, a transmembrane protein that is required for TGF-β1 activation by these cells (Cuende *et al.*, 2015). Our results suggest that PSG1 and PSG4 do not interact or activate latent TGF-β1 from GARP/latent TGF-β1 complexes.

Previous studies on some members of the murine PSG family have shown that there is conservation of functions between the human and murine PSGs studied. For example, like human PSG1, murine PSG22 and 23 induce endothelial tube formation and PSG23 inhibits the interaction of fibrinogen with platelets (Sulkowski *et al.*, 2011; Blois *et al.*, 2012; Shanley *et al.*, 2013). Gene expression data from RT-PCR of placental RNA and Expressed Sequence Tag (EST) database analysis revealed that there are considerable differences in the expression levels between murine PSGs, and that *Psg23* is one of the highest expressed (Ball *et al.*, 2004; McLellan *et al.*, 2005a). Despite the lack of sequence conservation, shared functions including the ability to induce endothelial tubulogenesis and binding to αIIbβ3 integrin have been described for some members of the human and murine PSG families (Blois *et al.*, 2012; Shanley *et al.*, 2013). Our study indicates that members of the murine PSG family, represented here by one of the highest expressed members, may also contribute to immune tolerance in pregnancy although the concentration of murine PSG23 in serum remains to be determined (Wynne *et al.*, 2006). We performed our studies with two different recombinant PSG23 proteins. The first is composed of the N1-domain of PSG23 with an Fc tag and the second has the N1 and A domains followed by the myc and 6xHis tags at the C-terminus. Our results revealed the ability of the truncated forms of murine PSG23 to activate TGF-β1 as observed for the human PSG counterparts. Interestingly while in human PSG1, the B2 domain is the most efficient as a TGF-β activator, the N1-domain of murine PSG23 is far better as an activator than the N-domain of PSG1 when comparing their activities at same molar concentration (Ballesteros *et al.*, 2015). In addition, preliminary data from our laboratory indicate that the B2 domain of PSG1 and not the N-domain mediates its ability to interact with HS while, as we previously reported, the N1-domain of PSG23 binds to HS (Sulkowski *et al.*, 2011). Because the other two variable like-domains of PSG23 (N_2 and N_3) may contribute to its interaction with latent TGF-β, we hope to be able to calculate the affinity of PSG23 for latent TGF-β in the future as we continue to test different expression conditions to increase the yield of full-length PSG23. Our preliminary studies with recombinant murine PSG17 consisting of only of the N1-domain showed that PSG17 can also activate TGF-β1.

The studies presented here indicate that all human PSGs activate the soluble SLC of TGF-β1. Therefore, as shown for PSG1, all PSGs may potentially activate the ECM-bound LLC of TGF-β1. Our initial characterization indicates that the ability to activate TGF-β1 by human PSGs is conserved in murine PSGs, despite their low amino acid sequence conservation. These observations strengthen the hypothesis that PSGs, present in species with hemochorial placentation, contribute to the tolerogenic environment during pregnancy by virtue of their ability to increase the availability of the active form of soluble and matrix-bound TGF-β1.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

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Authors' roles

S.L., B.H. and G.D. conceived and designed the research; J.W., C.H., M.I., A.B. and F.L. performed the research; T.M. contributed essential reagents; all authors gave input on writing the manuscript; G.D. wrote the manuscript.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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