PG-M/versican binds to P-selectin glycoprotein ligand-1 and mediates leukocyte aggregation

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Summary

P-selectin glycoprotein ligand-1 (PSGL-1), a glycoprotein expressed on the cell surface of leukocytes, binds to selectins and mediates leukocyte rolling on the vascular endothelium. Here we report that PSGL-1 binds to the C-terminal (G3 domain) of the extracellular proteoglycan PG-M/versican. Cells transfected with PSGL-1 or a shorter form containing the binding site, or cells expressing endogenous PSGL-1 aggregate in the presence of versican or G3 product. The aggregation appears to be induced by G3 multimers that bind to PSGL-1 and form a network. Endogenous versican and/or G3-containing fragments also bind to PSGL-1 in human plasma. Removal of the endogenous G3-containing fragments reduces the effect of plasma on leukocyte aggregation. Finally, the roles of G3-containing fragments in leukocyte aggregation were confirmed in a mouse model. Taken together, our results strongly support a physiologically relevant role for PSGL-1/versican binding and may have implications in the immunoresponse.

Key words: PSGL-1, Selectin, G3 domain, Interaction, Aggregation

Introduction

PG-M/versican is a chondroitin sulfate proteoglycan located in the extracellular matrix (ECM). It contains an N-terminal globular domain (G1) and a C-terminal globular domain (G3), also known as the selectin-like domain, with a large central domain containing sites for glycosaminoglycan modification (Ito et al., 1995; Kiani et al., 2002). The G3 domain contains two epidermal growth factor (EGF)-like sequences, a carbohydrate recognition domain (CRD) and a complement binding protein (CBP)-like domain. This proteoglycan is expressed by a large variety of tissues (Wight, 2002). It is important in heart development (Henderson and Copp, 1998): disruption of the versican gene results in the abnormal development of the heart, and this is lethal (Mjaatvedt et al., 1998). Versican is also highly expressed by aortic endothelial cells and vascular smooth muscle cells (Yao et al., 1994). In vitro studies have shown that versican modulates arterial and vascular smooth muscle cell adhesion, migration and proliferation (Lemire et al., 2002). As a matrix molecule, versican is known to associate with a number of molecules in the ECM and on the cell surface (Wight, 2002; Wu et al., 2004). We have demonstrated that versican enhances cell proliferation and differentiation (Zhang et al., 1998a; Zhang et al., 1998b). The EGF-like motifs in the G3 domain of versican are involved in this effect (Wu et al., 2001), and we hypothesized that versican plays this role through binding to cell surface proteins. We used a yeast two-hybrid approach with the G3 domain as bait to screen a human cDNA library, and thus identified P-selectin glycoprotein ligand-1 (or PSGL-1) as the versican binding protein. PSGL-1 is a homodimeric glycoprotein held together by disulfide-bonds expressed on the cell surface of leukocytes, which mediates leukocyte rolling on the vascular endothelium (Pouyani and Seed, 1995; Sako et al., 1993).

Leukocyte locomotion from the blood stream into tissues in response to inflammatory stimuli is a critical component of the immune response, which involves the activities of a range of adhesion and signaling molecules. The first events in this process are leukocyte adhesion and leukocyte rolling on the endothelial surface under vascular shear flow, mediated by selectins. PSGL-1 is expressed by essentially all blood leukocytes including lymphocytes, monocytes, neutrophils and platelets, and has been shown to mediate the rolling of human neutrophils on selectins (Moore et al., 1995). The N-terminal fragment of PSGL-1 is extensively glycosylated, which is critical for binding to the selectins (Leppanen et al., 2002). Here we report that PSGL-1 also binds to versican G3 domain via a motif different from the selectin-binding domain. The binding induces human leukocyte aggregation, and this finding suggests a novel role for PSGL-1 in this activity.

Materials and Methods

Materials

Lipofectin, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin/EDTA, restriction endonucleases and T4 DNA ligase were purchased from Invitrogen. ECL western blot detection kit was from Amersham. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was from Sigma. Anti-His-tag monoclonal antibody and DNA Midi-prep kit were purchased from

Qiagen. Tissue culture plates were from Nunc. Protease inhibitors and monoclonal antibody 2B1, which recognizes the C-terminal G3 domain of human versican, were from Calbiochem (San Diego, CA). All other chemicals were from Sigma. All cell lines were from American Type Culture Collection. Human cDNA library and supplies for yeast two-hybrid assays were from Clontech (Palo Alto, CA). Anti-PSGL-1 monoclonal antibody (clone KPL-1) was from BD Pharmingen. Strain CD-1 mice were purchased from Charles River.

Yeast two-hybrid assay

The versican G3 domain was inserted into the plasmid pGBDC1 to generate a construct pGBDC1-G3 for use as bait in yeast two-hybrid screening. Briefly, the G3 fragment was removed from a construct used in previous studies with *Bam*HI and *Sal*I (Zhang et al., 1998a) and inserted into *Bam*HI- and *Sal*I-digested pGBDC1 plasmid. Yeast strain JP-69-4A was co-transformed with pGBDC1-G3 and the human brain cDNA library generated in the plasmid pACT2. Library screening, colony identification, membrane binding assays and liquid binding assays were performed according to the manufacturer's instructions (Clontech, Palo Alto, CA). Sequencing of positive clones (performed by the Core Facilities of York University, Toronto) revealed a number of putative candidate genes, which may interact with pGBDC1-G3.

Strategy for generation of recombinant constructs

To identify the PSGL-1 motif binding to versican G3 domain, six PSGL-1 recombinants (P42-78, P42-278, P42-318, P42-402, P279-318, and P324-402) were generated in the yeast activation vector pGADT7. Each fragment was amplified by PCR, digested with *Eco*RI and *Xho*I, and inserted into *Eco*RI- and *Xho*I-digested pGADT7. Two primers, PSGLN42EcoRI (Table 1) and PSGLC78XhoI, were used to produce the construct P42-78; primers PSGLN42EcoRI and PSGLC278XhoI produced P42-278; primers PSGLN42EcoRI and PSGLC318XhoI produced P42-318; primers PSGLN42EcoRI and PSGLC402XhoI produced P42-402; primers PSGLN279EcoRI and PSGLC318XhoI produced P279-318; and primers PSGLN324EcoRI and PSGLC402XhoI produced P324-402.

To identify the motifs in the G3 domain that bind PSGL-1, seven constructs (G3, EGF, CRD, CBP, EGF-CRD, CRD-CBP and EGF-CBP) were generated in the binding domain vector pGBDC1. Generation of the G3 construct (pGBDC1-G3) was described above. The remaining six constructs were generated by PCR. Primers pEGFN/BamHI and pEGFC/SalIXbaI were used to synthesize EGF; primers pEGFN/BamHI and pCRDC/SalI were used to synthesize EGF-CRD; primers pCRDN/BamHI and pCRDC/SalI were used to make CRD; primers pCBPN/BamHIXbaI and pCBPC/SalI were used to produce CBP; and primers pCRDN/BamHI and pCBPC/SalI were used to synthesize CRD-CBP. Generation of EGF-CBP required two sets of primers: primers pEGFN/BamHI and pEGFC/SalIXbaI were used for EGF and pCBPN/BamHIXbaI and pCBPC/SalI were used for CBP. The PCR products were digested with either *Bam*HI and *Xba*I (for EGF) or *Xba*I and *Sal*I (for CBP), and both fragments were inserted into *Bam*HI- and *Sal*I-digested pGBDC1.

To study the effect of versican on PSGL-1-mediated cell aggregation, eight constructs were used, including mini-versican, G3, CRDCBP, CBP, PSGL, PSGL(279-402), glutathione S-transferase (GST)-PSGL and GST-PSGL(279-402). Construction of the mini-versican, G3, CRDCBP (also named G3ΔEGF) and CBP was described previously (Wu et al., 2002; Wu et al., 2001; Zhang et al., 1998a). In all of these mammalian expression constructs, a small fragment of cDNA encoding the chicken link protein signal peptide plus an epitope recognized by the monoclonal antibody 4B6 (Binette et al., 1994) was engineered to the 5′ of each construct. To isolate full-length PSGL-1, total cDNA was synthesized by reverse transcription using purified mRNA from HL60 cells. The cDNAs were subjected to PCR using two primers, PSGLN1EcoRI and PSGLC402XhoI. The PCR product was doubly digested with restriction endonucleases, *Eco*RI and *Xho*I, and inserted into *Eco*RI- and *Xho*I-digested pcDNA3. To generate GST-PSGL, the cDNA encoding the mature peptide (amino acids 42-402) was amplified using two primers, PSGLN42EcoRI and PSGLC402SalI, using PCR. The PCR product was digested with *Eco*RI and *Sal*I, and inserted into *Eco*RI- and *Sal*I-digested pGEX4T1. To generate GST-PSGL(279-402), the cDNA encoding peptide(279-402) was synthesized using two primers, PSGLN279EcoRI and PSGLC402SalI, and inserted into *Eco*RI- and *Sal*I-digested pGEX4T1 vector. Because there is a *Bam*HI site immediately upstream of the *Eco*RI site in the same reading frame, the fragment encoding peptide279-402 was obtained by digesting with *Bam*HI and *Sal*I and ligation into pcDNA3 containing a fragment encoding link protein leading peptide (Zhang et al., 1998b), producing the PSGL(279-402) construct. This leading peptide also harbors an epitope recognized by a monoclonal antibody 4B6 (Binette et al., 1994).

Construct expression and immunostaining

Astrocytoma cell line U87 and melanoma cell line A2058 were transfected with recombinant constructs using Lipofectin. Expression of the recombinant constructs was carried out by western blotting, flow cytometry and immunofluorescence staining as described previously (Chen et al., 2002; Lee et al., 2002). Immunoprecipitation was also performed as previously described (Wu et al., 2002).

For GST pull-down experiments, GST fusion peptides were immobilized on glutathione-sepharose beads and washed extensively

Table 1. Sequence and restriction endonuclease sites for oligonucleotides

Primer	Sequence
PSGLN42*Eco*RI	5′-AAA GAA TTC CAG GCC ACC GAA TAT GAG TC-3′
PSGLC78*Xho*I	5′-AAA CTC GAG AGG GGT TCC AGG CCC AGT CAG-3′
PSGLC278*Xho*I	5′-AAA CTC GAG CAC AGA AAA GGG TAT GAA CAG-3′
PSGLC278*Xho*I	5′-AAA CTC GAG CAC AGA AAA GGG TAT GAA CAG-3′
PSGLC318*Xho*I	5′-AAA CTC GAG CGC CAA GAT TAG GAT GGC CAG-3′
PSGLC402*Xho*I	5′-AAA CTC GAG CTA AGG GAG GCT GTG CAG-3′
PSGLN279*Eco*RI	5′-AAA GAA TTC TCC TCT GTT ACT CAC AAG GG-3′
PSGLN324*Eco*RI	5′-CCC GAA TTC TTC GTG TGC ACT GTG GTG-3′
PSGLN1*Eco*RI	5′-AAA GAA TTC ATG CCT CTG CAA CTC CTC CT-3′
PSGLC402*Sal*I	5′-AAA GTC GAC CTA AGG GAG GCT GTG CAG-3′
pEGFN/*Bam*HI	5′-AAA GGA TCC TGC AAA AGT AAT CCC TGC-3′
pEGFC/*Sal*I*Xba*I	5′-AAA GTC GAC TCT AGA GTC TTG CTC ACA GAG AGC-3′
pCRDN/*Bam*HI	5′-AAA GGA TCC GAG CAA GAC ACA GAG ACT-3′
pCRDC/*Sal*I	5′-AAA GTC GAC TGT TCC TTT CTT GCA GGT-3′
pCBPN/*Bam*HI*Xba*I	5′-AAA GGA TCC TCT AGA GTT GCC TGT GGT CAA CCT-3′
pCBPC/*Sal*I	5′-AAA GTC GAC GCG CCT TGA GTC CTG CCA-3′

with a buffer containing 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA and 100 mM NaCl. Culture medium collected from cells transfected with different construct or the control vector pcDNA3 was loaded to the glutathione-sepharose columns, followed by incubation at 4°C for 1 hour. The columns were washed extensively with the same buffer as above. The bound proteins were eluted by boiling the beads in 1× protein loading dye and they were then subjected to western blot analysis. To purify GST fusion proteins, the same buffer containing 1% triton X-100 was used to wash the columns extensively. The bound proteins were eluted using the same buffer containing 20 mM glutathione according to the manufacturer's instructions.

Aggregation assays

5×10^4 cells were seeded on six-well tissue culture plates, followed by the addition of 1 ml of conditioned medium (DMEM) from cells transfected with different constructs. In our expression system, we have demonstrated that products are secreted at a concentration of 1 ng/μl (Zhang et al., 1998b). The medium was mixed with the cells by gentle shaking. Cell aggregation was monitored under an inverted microscope and photographed. Cells (2×10^4 cells in 500 μl) were also seeded on 12-well tissue culture plates, followed by addition of purified G3 product (~20 ng, 0.5 ng/μl in PBS) to each well. As negative controls, total protein from vector-transfected cells was precipitated and resuspended in the same buffer and added to each well. Cell aggregation was monitored under an inverted microscope and photographed. The numbers of non-aggregated cells and cell complexes were counted to estimate cell aggregation index [=number of individual cells/(number of complexes+1)]. Three fields were selected for cell counting. In some cases, the percentage of cell aggregation was determined for comparison, where cell aggregation (%)=individual cell number of treatment/individual cell number of control.

An aggregation assay was also performed in strain CD-1 mice. In brief, 400 μl human plasma with or without addition of 200 μl polyclonal antiserum raised against chicken versican G3 domain were injected into the peritoneal cavity of the mice. Two or five days after the injection, the mice were sacrificed with CO_2, followed by an injection of 3 ml PBS in the peritoneal cavity of each mouse to allow release of leukocytes into the ascites and to dilute the leukocyte cell density. The ascites was withdrawn carefully to avoid blood contamination, and examined under a light microscope and photographed immediately.

Effect of DTT on cell aggregation

PSGL-1-expressing cells (5×10^4 cells) were treated with 10 mM DTT at room temperature for 30 minutes. The cells were pelleted and resuspended in 1 ml normal culture medium followed by addition of 1 ml conditioned medium from G3-transfected cells. Cell aggregation was monitored under an inverted microscope and photographed. Conditioned medium containing expressed G3 was also treated with 10 mM DTT for 10 minutes, and then 5×10^4 cells in 1 ml culture medium were added to examine the effect of DTT-treatment on cell aggregation. As positive controls, 5×10^4 cells in 1 ml medium were mixed with 1 ml medium containing G3 to observe cell aggregation.

Results

Interaction of versican G3 domain with PSGL-1 in yeast two-hybrid assays

We screened a human cDNA library using versican G3 domain as bait and demonstrated that the G3 domain binds to a clone encoding a fragment of PSGL-1. The full-length PSGL-1 cDNA (Fig. 1A) and six yeast two-hybrid constructs corresponding to different domains of PSGL-1 (Fig. 1B) were generated. Three constructs, namely P279-318, P42-318 and P42-402, when co-expressed with plasmid pGBDC1 containing versican G3 domain, produced positive results in a filter membrane binding assay (Fig. 1C). The smallest construct interacting with G3 was P279-318. This fragment is immediately N-terminal of the transmembrane domain and is located extracellularly. A liquid binding assay also confirmed this result (Fig. 1D).

To dissect the G3 motif(s) that interact with PSGL-1, seven constructs, containing G3, EGF, CRD, CBP, EGF-CRD, CRD-CBP and EGF-CBP were generated for yeast two-hybrid binding assays (Fig. 2A). Four of them, G3, EGF, CRD and EGF-CRD, bound to PSGL-1 (Fig. 2B). The results suggested that the EGF-like and CRD motifs interact with PSGL-1. Surprisingly, however, the presence of the CBP motif suppressed this binding. Only the combined EGF-like and CRD motifs overcame the negative effect of the CBP motif, but the mechanism is not clear. A yeast liquid culture assay showed similar results (data not shown).

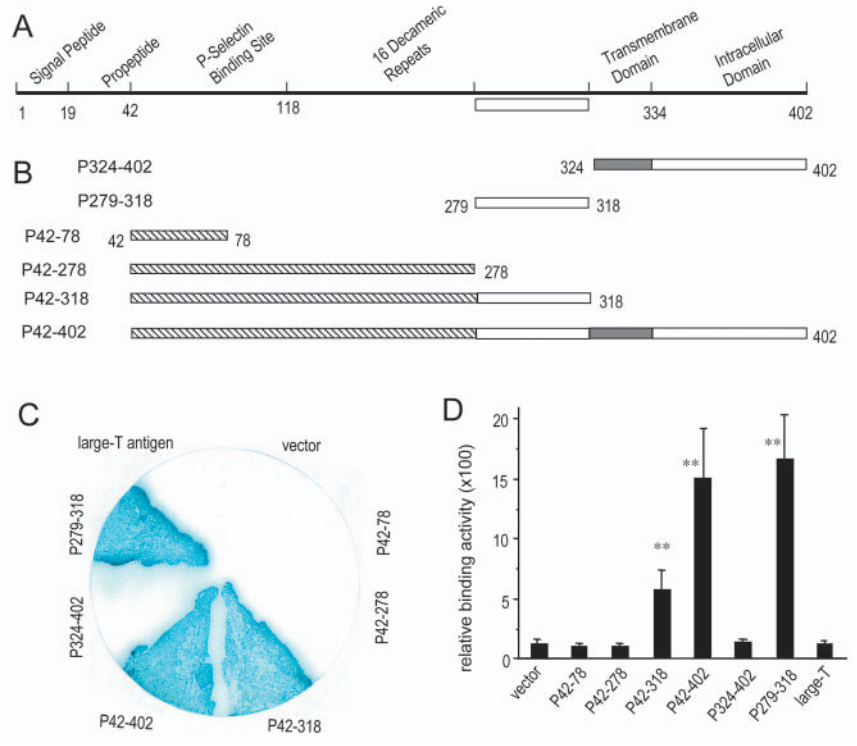

Fig. 1. Identification of the PSGL-1 fragment that interacts with versican G3 domain. (A) Amino acid structure of PSGL-1 showing motifs of the glycoprotein. (B) Recombinant constructs containing fragments of PSGL-1. (C) A filter membrane binding assay of recombinant PSGL-1 constructs with versican G3 domain was performed. Constructs containing amino acids 279-318 of PSGL bound to the G3 domain. The large-T antigen was used as a negative control. (D) Liquid binding activity assay using the same constructs as in C. Values shown are the mean±s.d. for the relative binding activity (n=3). $^{**}P<0.01$.

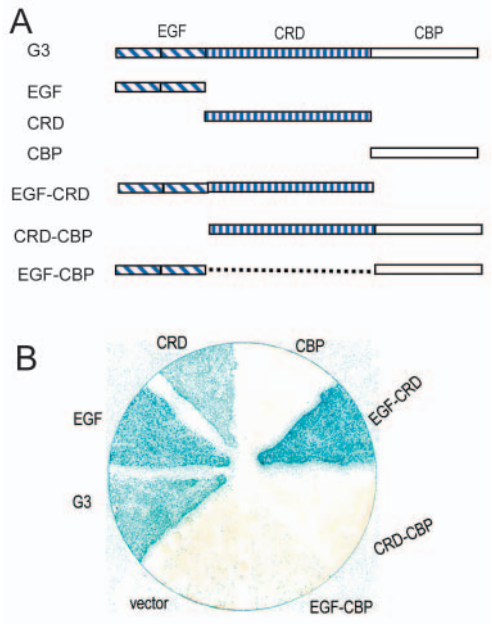

Fig. 2. Identification of motifs in the G3 domain interacting with PSGL-1. (A) Constructs containing different combinations of versican G3 domain motifs used for yeast two-hybrid assays. EGF, epidermal growth factor domain; CRD, carbohydrate recognition domain; CBP, complement binding protein domain. (B) Filter membrane binding assay of recombinant G3 constructs with PSGL-1. EGF-containing constructs interacted with PSGL-1.

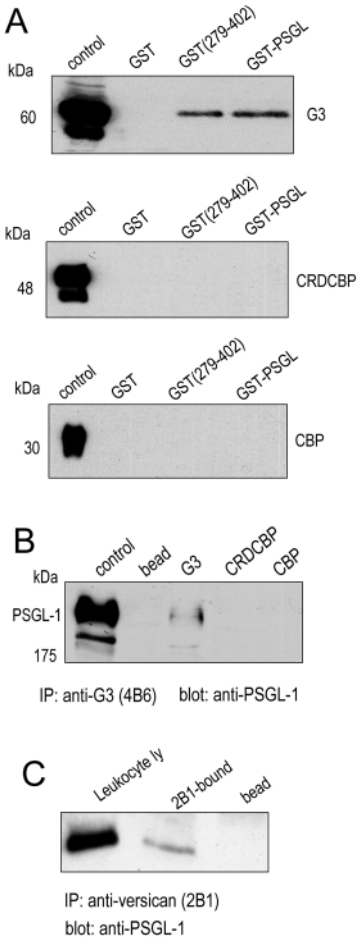

Fig. 3. Confirmation of the interaction of PSGL-1 with versican. (A) G3, CRDCBP and CBP were incubated with glutathione S-transferase (GST), GST fused with PSGL-1 or fused with amino acids 279-402 of PSGL, GST(279-402). The mixture was purified using GST affinity columns, and purified products were analyzed on a western blot probed with 4B6 that recognizes an epitope engineered in each construct. Only G3 product interacted with both fusion proteins, GST-PSGL and GST(279-402). (B) HL60 cells that express endogenous PSGL-1 were incubated with medium containing G3, CRDCBP or CBP. HL60 cell lysate prepared by sonication was incubated with protein G beads pre-incubated with bovine serum to avoid non-specific binding or protein G saturated with 4B6. After washing, proteins binding to the beads were subjected to western blotting with anti-PSGL-1 antibody. Untreated lysate served as a control. Only G3 interacted with endogenous PSGL-1. (C) Human leukocytes were lysed and centrifuged. The supernatant was subjected to immunoprecipitation using 2B1. After washing, the bound proteins were analyzed on a western blot probed with anti-PSGL-1 antibody to detect the interaction of endogenous versican and PSGL-1.

Interaction of G3 with PSGL-1 in physiological conditions

GST fusion proteins containing PSGL-1 (without the 19-amino acid signal peptide and the 22-amino acid pro-peptide), or the amino acids 279-402 of PSGL-1, GST-PSGL(279-402), were produced. The interactions of GST-PSGL and GST-PSGL(279-402) with G3, CRDCBP and CBP were tested in pull-down assays through GST affinity columns in native conditions. Only G3 product bound to the GST-PSGL and GST-PSGL(279-402) fusion proteins (Fig. 3A). To demonstrate the interaction in physiological conditions, HL60 cells, which express PSGL-1 (Fig. 3B, control), were incubated with G3-containing medium. Cell lysate was prepared and subjected to co-immunoprecipitation using 4B6. Analysis of the precipitated product on western blots probed with anti-PSGL-1 antibody indicated that PSGL-1 (240 kDa) was co-precipitated by G3 (Fig. 3B). Further confirmation was performed on primary human leukocytes using a monoclonal antibody 2B1 that recognizes the human G3 domain: PSGL-1 co-precipitated with the versican G3-containing fragments (Fig. 3C).

G3 induces aggregation of PSGL-1-transfected cells, PSGL-1-expressing cell line and primary leukocytes

The full-length PSGL-1 and PSGL(279-402), containing a link protein leading peptide (LP), the G3-binding domain, the transmembrane domain and the intracellular sequence were generated in pcDNA3. These two constructs were stably expressed in A2058 and U87 cells, respectively. Expression of PSGL-1 was analyzed by western blotting and probing with anti-PSGL-1 antibody KPL-1, whereas expression of PSGL(279-402) was confirmed with 4B6 (Fig. 4A). Cell surface localization of the products was confirmed by flow cytometry (Fig. 4B). Transfection with PSGL(279-402) obtained similar results (data not shown). When conditioned medium containing versican G3 product was added to A2058 or U87 cells stably transfected with PSGL-1 or PSGL(279-402), cell aggregation was detected almost immediately

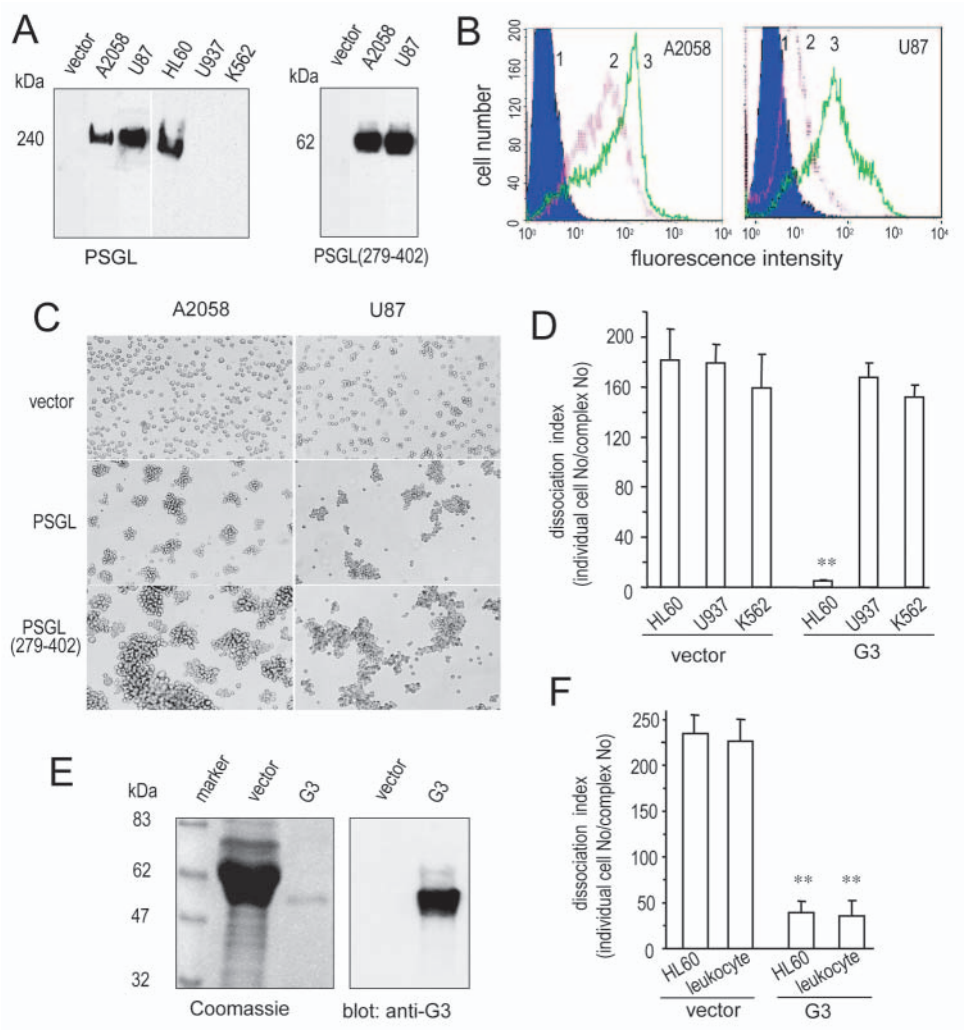

Fig. 4. PSGL-1-mediated cell aggregation in the presence of versican G3. (A) Two recombinant PSGL-1 constructs, PSGL-1 and PSGL (279-402), were stably expressed in A2058 and U87 cells. Expression of PSGL(1-402) and PSGL(279-402) was assayed by western blotting. Controls were lysates from HL60, U937 and K562 cells. (B) Flow cytometry assays confirmed cell surface localization of these proteins. Blue, negative control; pink, PSGL-1 expression; green, PSGL(279-402) expression. (C) Versican G3-containing medium was incubated with U87 and A2058 cells stably transfected with PSGL(42-402), PSGL(279-402) and a control vector respectively. Ten minutes after cell inoculation, cells expressing PSGL(42-402) or PSGL(279-402) started to aggregate. (D) The G3-containing medium was added to HL60, U937 and K562 cells. Only G3 induced aggregation of HL60 cells (n=3; $^{**}P$<0.01). (E) G3 was purified through a Ni-NTA affinity column and was analyzed by Coomassie Blue staining and western blotting with 4B6. The medium from vector-transfected cells was used as a control without purification. The strong band in the vector-transfected culture medium stained with Coomassie Blue was BSA. (F) Purified G3 and total proteins from vector-transfected cells were added to HL60 cells and human leukocytes. The purified G3 induced cell aggregation (n=3; $^{**}P$<0.01).

following product addition. The aggregation became very evident after 10 minutes of incubation (Fig. 4C).

To test the effect of G3 on cells expressing endogenous PSGL-1, G3-containing medium and medium from vector-transfected cells was added to HL60 cells, which express endogenous PSGL-1, and to U937 and K562, which do not express PSGL-1 (Fig. 4A). Although expression of PSGL-1 in U937 cells has been reported using northern blotting and flow cytometry (Vachino et al., 1995), we could not detect the PSGL-1 protein on western blots. As such, G3-containing medium only induced aggregation in HL60 cells (Fig. 4D). To confirm the role of G3 in cell aggregation, G3 was purified through a Ni-NTA affinity column. Purification of G3 (55 kDa) was verified by Coomassie Blue staining and western blotting (Fig. 4E). The purified G3 product induced aggregation of HL60 cells and human leukocytes (Fig. 4F).

We further tested if G3 could induce cell aggregation when expressed as a mini-versican product, and examined motif requirement for the induction of aggregation. Versican recombinant constructs (mini-versican, G3, CRDCBP and CBP) were generated (Fig. 5A). Expression of these constructs in U87 cells was confirmed by western blotting (Fig. 5B). Addition of products from the mini-versican or the G3 construct induced cell aggregation (Fig. 5C). Another cell line, A2058 stably transfected with PSGL-1, was incubated with G3-containing medium collected from G3-transfected U87 cells at different dilutions for different time periods. G3-induced cell aggregation was concentration dependent (Fig. 5D). Aggregation occurred after 1 minute of incubation, and increased to almost 100% aggregation after 30 minutes.

G3 induces cell aggregation by forming multimers

As G3 product expressed by bacteria did not induce cell aggregation (data not shown), we hypothesized that the three-dimensional structure of G3 is required for the induction of cell aggregation. We proposed that the G3 product expressed by mammalian cells might exist as multimeric forms through the formation of intermolecular disulfide bonds, as there are many cysteine residues in the G3 domain. A model of the putative G3 multimers formed through the linking of intermolecular disulfide bonds, and cell aggregation mediated by these multimers, and cell-surface PSGL-1 is presented in Fig. 6A. The presence of G3 multimers was confirmed by western blotting (Fig. 6B). Under non-reducing conditions, G3 migrated as multiple bands on SDS-PAGE, whereas in reducing conditions, a single major band was detected. To determine whether G3 multimers were required for cell aggregation, reducing agent dithiothreitol (DTT) was used to eliminate disulfide bonds. When U87 cells stably transfected

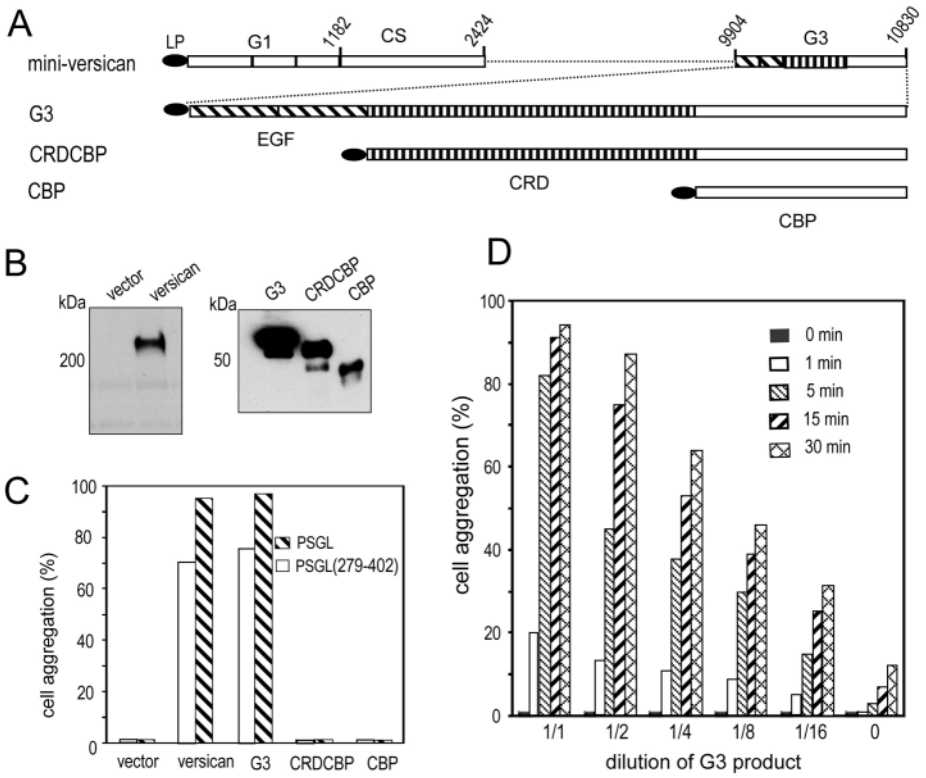

Fig. 5. The effects of mini-versican and the G3 motifs on cell aggregation. (A) Mammalian expression constructs of mini-versican, G3, CRDCBP and CBP were generated as shown. (B) Expression of these constructs in U87 cells was confirmed by western blotting with 4B6 using culture medium from transfected cells. (C) Culture medium containing mini-versican or G3-induced aggregation of cells transfected with PSGL-1 or PSGL(279-402) construct (n=3; $^{**}P$<0.01). (D) The A2058 cells stably transfected with PSGL-1 were incubated with culture medium collected from U87 cells transfected with the G3 construct at different dilutions for different time periods. The aggregation effects were reduced as G3 was diluted.

with PSGL or PSGL(279-402) were treated with DTT, addition of G3 product only partially induced aggregation (Fig. 6C). However, treatment of G3 product with DTT before incubation with the cells completely inhibited cell aggregation. This is strong evidence for the role of G3 disulfide bonds in mediating cell aggregation.

Endogenous G3-containing fragments induce leukocyte aggregation

We then investigated the effect of versican on leukocyte activity. Western blot analysis indicated that versican and versican G3-containing fragments were present in large quantities in human blood (Fig. 7A). Immunoprecipitation experiments indicated that these versican G3-containing fragments interacted with endogenous PSGL-1 expressed by human leukocytes (Fig. 7B). When the 2B1-treated and untreated plasma was added back to human leukocytes, the 2B1-treated plasma (immunodepleted) exhibited a greatly reduced ability to induce leukocyte aggregation (Fig. 7C). Human plasma was also added to human leukocytes in the presence or absence of purified GST, GST-PSGL or GST-PSGL(279-402). The addition of PSGL-1 fusion peptides blocked the effect of plasma on leukocyte aggregation (Fig. 7D). The PSGL fusion proteins also blocked the aggregation of A2058 and U87 cells stably transfected with PSGL-1 in the presence of G3 product (data not shown). The versican G3 fragment-containing human plasma and plasma mixed with polyclonal anti-G3 antiserum was injected into the peritoneal cavity of mice. It was expected that the antibody would reduce the effect of G3-containing fragments on leukocyte aggregation. Examination of ascites showed that injection of human plasma, which contained a large quantity of versican and G3-containing fragments, induced leukocyte aggregation (Fig. 7E). This effect was neutralized by the addition of anti-G3 antibody. Taken together, our results demonstrated that versican and versican G3-containing fragments are present in human blood in large quantities, and they interact with PSGL-1 under physiological conditions and induce leukocyte-leukocyte interactions. Blocking with PSGL fusion proteins or removal of endogenous G3-containing fragments inhibited this process.

Discussion

PSGL-1 is expressed by essentially all blood leukocytes including lymphocytes, monocytes, neutrophils and platelets (Xia et al., 2002). The only PSGL-1 binding partners identified to date are the selectins whose interactions mediate leukocyte rolling (Moore et al., 1995; Somers et al., 2000). Our study reveals a novel binding protein for PSGL-1, namely the versican G3 domain. In common with selectins, versican G3 domain also contains a CRD motif; for this reason, the G3 domain is also called the selectin-like domain. When PSGL-1 was identified as a G3-binding protein in our yeast two-hybrid screening, we first hypothesized that the G3 domain might bind to the same motif in PSGL-1 as the selectins. Further studies indicated that the G3 domain interacted with a novel site in the extracellular sequence. The interaction of PSGL-1 with versican G3 domain was further dissected and verified using smaller fragments of PSGL-1 in yeast two-hybrid binding assays, GST-fusion protein pull-down assays and co-immunoprecipitation of G3 incubated with cells expressing endogenous PSGL-1. The binding site, comprising amino acids 279-318 of PSGL-1, is extracellular and directly N-terminal of the transmembrane domain.

Versican is the first identified extracellular molecule that binds PSGL-1. Consistent with this, our studies reveal a novel function for PSGL-1: it mediates cell aggregation induced by the versican G3 domain. This function was very dramatic, as aggregation began within one minute of G3 addition, and the process was completed within 30 minutes. Interestingly, cells expressing a truncated form of PSGL-1 were more efficient at mediating cell aggregation than those expressing the full-length PSGL-1. This may be due to a better exposure of the binding site to the extracellular G3 product. This further confirmed that the N-terminal fragment of PSGL-1 is not required for binding to the versican G3 domain.

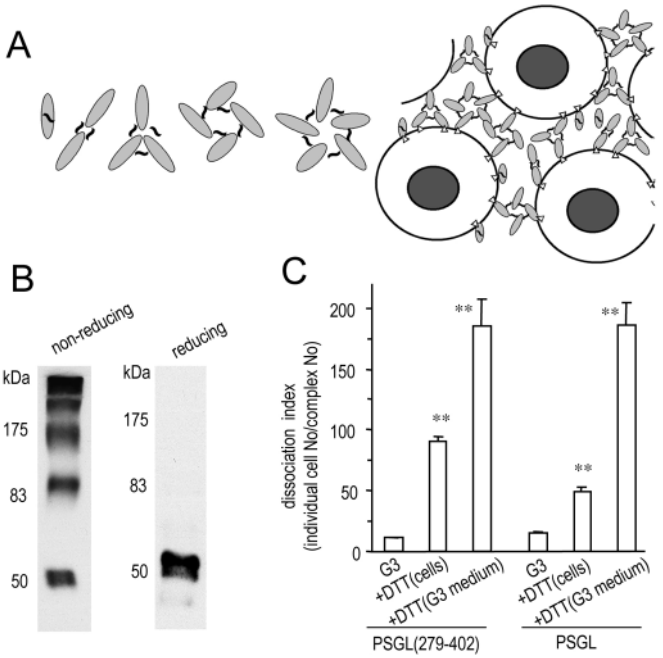

Fig. 6. G3 induces cell aggregation by forming multimeric forms. (A) A model of G3 multimers produced by the formation of intermolecular disulfide bonds (~) and cell aggregation mediated by G3 binding to PSGL-1 (Δ). (B) Culture medium was analyzed on western blots in reducing and non-reducing conditions probed with 4B6. Without DTT treatment, G3 migrated as multiple bands. In the presence of DTT, G3 migrated as a major single band. (C) U87 cells stably transfected with PSGL-1 or PSGL(279-402) were subjected to different treatments as follows: incubation with G3-containing medium; treatment with DTT before addition of G3-containing medium [+DTT (cells)] or incubation with G3-containing medium that had been treated with DTT [+DTT (G3 medium)]. When the cells were treated with DTT, a partial reduction in cell aggregation was observed. When the medium was treated with DTT, cell aggregation was completely inhibited (n=3; $^{**}P$<0.01).

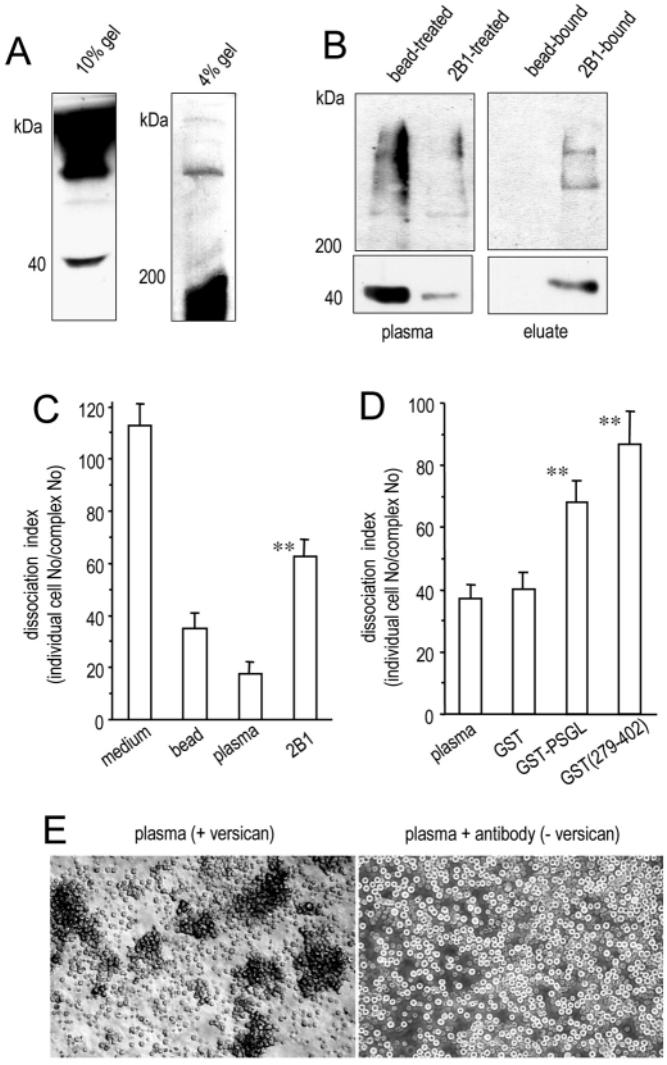

Fig. 7. Removal of G3-containing fragments from human plasma or blocking with PSGL fusion proteins reduces leukocyte aggregation. (A) Human plasma was subjected to western blot analysis probed with 2B1 to detect endogenous G3-containing fragments on 4% and 10% SDS gels. (B) Human plasma was incubated with protein G-bound 2B1 or serum-treated protein G alone. After centrifugation, treated plasma and the protein G beads were analyzed on a western blot probed with 2B1. Treatment with 2B1 reduced the amount of versican in the plasma (left panel, immunodeprivation). The binding of versican to the protein G bead-2B1 complex was confirmed (right panel). (C) The effects of anti-G3 antibody treated (immunodepleted) and untreated human plasma on human leukocyte aggregation were examined. Freshly isolated human leukocytes did not aggregate when maintained in RPMI medium containing 10% FBS. The addition of human plasma or protein G bead-treated plasma induced leukocyte aggregation. This effect diminished greatly when the plasma was immunodeprived by 2B1 treatment (n=3; $^{**}P$<0.01). (D) Human plasma was added to human leukocytes in the presence or absence of purified GST, GST-PSGL, GST(279-402). Both PSGL fusion proteins blocked human plasma-induced leukocyte aggregation (n=3; $^{**}P$<0.01). (E) Human plasma and plasma mixed with polyclonal antiserum against versican G3 domain were injected into the mouse peritoneal cavity. Two days after the injection, ascites was recovered and monocyte aggregation was examined immediately. Plasma-induced monocyte aggregation was neutralized in the presence of anti-G3 antibody.

Extensive attention has been paid to the post-translational modification of PSGL-1, as glycosylation of the N-terminal fragment is critical for PSGL-1 binding to P-, E- and L-selectins (Moore et al., 1995; Pouyani and Seed, 1995). Our study with GST-fusion protein suggests that glycosylation is not required for PSGL-1 binding to the G3 domain. The pull-down assays and GST-fusion protein blocking experiments indicated that a fragment of unmodified PSGL-1 polypeptide is sufficient for binding to the versican G3 domain. However, G3 expressed by bacteria did not induce cell aggregation (data not shown). It seems that the interaction of PSGL-1 with versican requires a simple polypeptide of PSGL-1 and a properly folded G3 domain. As an extracellular molecule, only properly folded versican will be secreted, and thus properly folded versican is always available to PSGL-1. On the other hand, as a cell surface glycoprotein, the extracellular structure of PSGL-1 is much simpler. There is only one cysteine residue on the extracellular fragment of PSGL-1 and one potential site for glycosylation in the motif involved in G3-binding. Obviously, this potential site for glycosylation is not involved in versican-binding.

Versican G3 product not only induced aggregation of cells

over-expressing PSGL-1, but also induced aggregation of cells such as HL60 cells and leukocytes, both of which express endogenous PSGL-1. This novel functional role of leukocyte aggregation mediated by PSGL-1 may be physiologically relevant. We have demonstrated that human blood contains a large amount of versican and G3-containing fragments which induced leukocyte aggregation in vitro and in vivo. Removal of endogenous versican G3-containing fragments reversed this process. Versican and versican G3-containing fragments may be important in modeling leukocyte function, as cell-cell interactions play critical roles in mediating cell activities such as cell signaling, immunoresponse, cell proliferation and survival; these all await further investigation.

The molecular mechanism underlying the induction of cell aggregation by versican G3 domain appears to be the formation of multimeric forms of the G3 domain as we reported previously (Chen et al., 2003). Here we confirmed the presence of G3 multimers through the formation of intermolecular disulfide bonds, and these multimers were disrupted by treatment of the G3 product with the reducing agent DTT. It should be noted that although DTT reduced the intermolecular disulfide bonds, there is a strong probability that DTT also reduced intramolecular disulfide bonds, thereby affecting cell structure and physiology. Evidence of this was seen when the cells were treated with DTT before the addition of G3 product: the levels of cell aggregation were much lower. Nevertheless, our results indicated that G3 formed multimers in non-reducing conditions and suggested that formation of these multimers was important in cell aggregation.

As a matrix molecule, versican is highly expressed by aortic endothelial cells (Morita et al., 1990) and vascular smooth muscle cells (Lemire et al., 1996; Yao et al., 1994). It is the major chondroitin sulfate proteoglycan in the vessel wall, where it contributes to the formation of blood vessels and angiogenesis (Zheng et al., 2004). In disease conditions, versican expression and accumulation increase after human cardiac allograft and coronary angioplasty (Lin et al., 1996; Matsuura et al., 1996). The increased expression is more evident in the thickened myxoid intimas, the atherosclerotic plaque, and in the loose ECM of restenotic lesions (Merrilees et al., 2001; Wight et al., 1997). Versican has also been found in the neointima formed in rat carotid artery after balloon-catheter denudation (Wolf et al., 1994). Versican expression is enhanced in the intimal layer of normal arteries and in the intima formed after arterial injury (Wight, 1989). The interaction of PSGL-1-expressing cells with versican is of great potential significance with respect to these disease conditions, because leukocytes are recruited to the inflammatory sites. Increased versican expression may allow leukocytes to remain in the inflammatory areas for healing purposes. A potential negative effect is that leukocyte aggregation may accelerate thrombus formation.

Furthermore, the PSGL-1-expressing leukocytes produce matrix metalloproteinases (MMPs), which cleave versican and other matrix molecules. Of all the MMPs, matrilysin (MMP-7) is expressed by macrophages in atherosclerotic plaques. Matrilysin is the most efficient at versican degradation (Halpert et al., 1996). It cleaves versican in plaques and releases versican fragments from the matrix of the vascular wall. It is conceivable that G3-containing fragments are also released in this process. As expected, these fragments would induce leukocyte aggregation and thrombus formation. Thus, versican degradation and release to plasma may contribute to intimal disorganization and thickening of the arterial wall, eventually causing fissure, hematoma, thrombosis and occlusion of the artery.

Versican degradation also occurs in mature human aorta cleaved by ADAMTS and other members of the metalloproteinases (Sandy et al., 2001). It is shed from the matrix and becomes localized in the adjacent vascular bundles around which a substantial leukocyte infiltration is concomitantly observed (Kawashima et al., 1999), or released to the blood circulation. Indeed, we have demonstrated that human plasma contains high levels of versican and G3-containing fragments. The physiological significance may be that these circulating versican and G3-containing fragments hold leukocytes in the blood stream and prevent excess extravasation of leukocytes, which could be promoted by PSGL-1-mediated leukocyte rolling on selectin-expressing endothelial cells. This would maintain the balance of leukocytes in the circulation.

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