Platelet Factor 4 Binds to Vascular Proteoglycans and Controls Both Growth Factor Activities and Platelet Activation*

Received for publication, September 30, 2016, and in revised form, January 18, 2017 Published, JBC Papers in Press, January 23, 2017, DOI 10.1074/jbc.M116.760660

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Platelet factor 4 (PF4), or chemokine (CXC motif) ligand 4 (CXCL4), is a 7.8-kDa protein synthesized by megakaryocytes and packaged in the α-granules of platelets as a tetrameric complex bound to serglycin GAG chains (1). Upon platelet activation, PF4 is released into the circulation where it has roles in both inflammation and wound healing, and these activities are thought to be modulated through interactions with the GAG chains of proteoglycans.

PF4 has been found in the blood vessel wall within minutes following removal of the endothelium and platelet attachment to the denuded basement membrane (2). It binds to endothelial cells through integrins αvβ3, αvβ5, and α5β1 (3) and also to thrombomodulin on the endothelial cell surface through its chain (4). PF4 inhibits heparin binding growth factors, including FGF2 and VEGF165, binding to their receptors through its protein core of ~18 kDa. The type and sulfation of GAG chains that decorate serglycin are cell type-dependent mechanisms in which connective tissue mast cells decorate serglycin with heparin (14), whereas mucosal tissue mast cells decorate serglycin with CS (15), neutrophils and platelets decorate serglycin with CS, and macrophages decorate serglycin with CS and HS (16).

Serglycin is an intracellular proteoglycan produced by hematopoietic cells including neutrophils, mast cells, macrophages, and platelets (11), as well as cells of non-hematopoietic origin including smooth muscle and endothelial cells (12, 13). The name serglycin was derived from the serine-glycine repeat structure where up to eight GAG chains can decorate the protein core of ~18 kDa. The type and sulfation of GAG chains that decorate serglycin are cell type-dependent mechanisms in which connective tissue mast cells decorate serglycin with heparin, whereas mucosal tissue mast cells decorate serglycin with CS (15), neutrophils and platelets decorate serglycin with CS, and macrophages decorate serglycin with CS and HS (16).

Serglycin knock-out mice exhibit a reduced platelet aggregation response and reduced levels of platelet α-granule proteins including PF4, β-thromboglobulin, and platelet-derived growth factor that are thought to be related to defects in packaging and secretion of α-granule proteins, including proteases, that are required for platelet activation (17).

Perlecan is an HS proteoglycan present in the basement membrane that binds and supports the signaling of heparin binding growth factors including FGFs and VEGFs (18, 19). In addition, perlecan supports platelet adhesion via the integrin α2β1 (20, 21) but does not activate platelets (22).

This study demonstrated that platelet serglycin is decorated with CS and DS and that PF4 binds to the CS/DS chains. Additionally, PF4 has a higher affinity for perlecan HS chains than preventing the formation of a stable heparin-anti-thrombin III-thrombin ternary complex (9).

The presence of heparin-PF4 immune complexes suggests that PF4 has a high affinity for heparin and that it is not a normally occurring complex in the blood stream. Heparin-induced thrombocytopenia can occur via both non-immune and immune mediated complications of heparin administration in some patients (10). Immune-mediated heparin-induced thrombocytopenia is caused by the formation of heparin-PF4 complexes that are recognized as foreign, resulting in the formation of antibodies against the complex. These antibodies bind to the heparin-PF4 complex and platelets themselves, increasing coagulation and reducing levels of circulating platelets.

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Serglycin is located in the α-granules of platelets where it is bound to many cytokines and proteases, including PF4, until they become activated (15). Serglycin knock-out mice exhibit a reduced platelet aggregation response and reduced levels of platelet α-granule proteins including PF4, β-thromboglobulin, and platelet-derived growth factor that are thought to be related to defects in packaging and secretion of α-granule proteins, including proteases, that are required for platelet activation (17).

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This study demonstrated that platelet serglycin is decorated with CS and DS and that PF4 binds to the CS/DS chains. Additionally, PF4 has a higher affinity for perlecan HS chains than
serglycin GAG chains and can be competed from complexes with serglycin in the presence of perlecan. The binding of PF4 to perlecan inhibits both FGF signaling and platelet activation.

Results

Characterization of Platelet-derived Serglycin—Peptide identification of proteins present in the proteoglycan-enriched platelet extract by mass spectrometry identified two cell surface proteoglycans, betaglycan and syndecan-1, as well as the intracellular proteoglycan serglycin (Table 1). This fraction also contained PF4, indicating that it may be closely associated with the proteoglycans in this fraction.

Platelet-derived serglycin was analyzed by Western blotting and detected as a smear between apparent relative molecular mass ($M_r$) of 75,000 and more than 250,000, which corresponded to the expected size of serglycin decorated with GAGs (Fig. 1A, first lane). It should be noted that the protein standards migrate faster than glycosylated proteins; therefore the $M_r$ reported are estimates. Treatment of the serglycin with chondroitinase (C’ase) ABC to digest GAG chains containing both CS and DS disaccharides resulted in a reduction of the $M_r$ of the smear to ~25,000, corresponding to the protein core indicating that platelet-derived serglycin contained ~45,000–225,000 CS and/or DS (Fig. 1A, second lane). Treatment of the serglycin with C’ase ACII that digests only terminal CS disaccharides resulted in a shift in $M_r$ of the platelet-derived serglycin in the range of 70,000 to more than 250,000, but not the generation of the protein core (Fig. 1A, third lane). Similarly C’ase B only removes terminal DS disaccharides and resulted in a shift in $M_r$ of the platelet derived serglycin to 65,000 to more than 250,000, but not the generation of the protein core (Fig. 1B, fourth lane). Because C’ases ACII and B are exoglycosidases, it indicated that the GAG chains that decorated this form of serglycin contained both CS and DS disaccharides (Fig. 1B). This would account for the small shift in $M_r$ when treated with either C’ase ABC or B, because once a terminal disaccharide was encountered that the enzyme could not digest, then digestion would cease. Treatment of the sample with heparinase III (HepIII) indicated that the platelet-derived serglycin was not decorated with HS because there was no change in $M_r$ compared with the undigested sample (Fig. 1A, first and fifth lanes).

Proteoglycan-enriched platelet extract was also analyzed by ELISA for the presence of serglycin (Fig. 1C). Low levels of serglycin were detected in the undigested sample; however, upon digestion with either C’ase ABC or C’ase B, significantly higher ($p < 0.05$) levels of serglycin were detected, indicating that CS and DS disaccharides decorated the protein core of serglycin and that these GAG chains hindered the access of the antibody to the epitopes present on the protein core. Treatment of the proteoglycan-enriched platelet extract with HepIII did not alter the level of serglycin detected compared with the undigested sample, confirming that platelet-derived serglycin was not decorated with HS (Fig. 1C).

Serglycin was found to contain the 4-sulfated stub structure after C’ase ABC digestion, but not C’ase B digestion, indicating that only CS disaccharides were present close to the protein core and were decorated with 4-sulfated CS stubs (Fig. 1D). Un- and 6-sulfated CS stubs were not detected after digestion with either C’ase ABC or B (data not shown). The CS chain structures present in the proteoglycan-enriched platelet extract were reactive with the CS antibody clone CS-56 (Fig. 1E), indicating the presence of CS disaccharides with the sequence GlcUA-GalNAc(4S)-GlcUA(2S)-GalNAc(6S) (23). There was a background level of reactivity with the CS chain antibody clones LY-111 and MO-225 (data not shown).

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a reduction in level of complexes with $M_r$ of 100,000 to greater than 250,000 and the presence of PF4 monomers at $M_r$ 8,000 (Fig. 3B, lanes 2 and 4). Incubation of recombinant serglycin with PF4 followed by digestion with either C’ase ACII or HepIII resulted in a broader range of complexes with $M_r$ of 50,000 to greater than 250,000 and the presence of PF4 monomers at $M_r$ 8,000 (Fig. 3B, lanes 3 and 5). Incubation of recombinant serglycin with PF4 followed by digestion with both C’ase ABC and HepIII resulted in the presence of PF4 monomers at $M_r$ 8,000 (Fig. 3B, lane 6). These data indicated that PF4 bound to the recombinant serglycin predominantly via by CS, DS, and HS/heparin. These data also indicated differences in the binding of PF4 to recombinant serglycin compared with platelet serglycin because of the presence of the HS/heparin.

A sandwich ELISA was also performed following incubation of recombinant serglycin with PF4 and GAG digestion. This assay revealed that PF4 bound to the recombinant serglycin via both CS and DS, with DS involved to a significantly greater ($p < 0.05$) extent than recombinant serglycin when coated at the same concentration (Fig. 4A). PF4 did not bind to endothelial perlecan that had been treated with HepIII to remove its HS chains (Fig. 4A), indicating that PF4 only bound to the HS that decorated the protein core. PF4 bound to recombinantly expressed perlecan domain V via both its CS and HS chains (Fig. 4B).

Surface plasmon resonance studies indicated that PF4 bound to immobilized heparin, perlecan, and serglycin in a dose-dependent fashion (Fig. 5). The association rate was similar for each condition with equilibrium reached rapidly followed by a slow dissociation (Fig. 5, A–C). The equilibrium constants were 25, 100, and 50 nM for heparin, serglycin, and perlecan, respectively, indicating that PF4 had the highest affinity for heparin followed by perlecan and serglycin (Fig. 5D). Control experiments performed with perlecan predigested with Hep III to remove HS and serglycin predigested with both C’ase ABC and HepIII to remove CS/DS and HS gave baseline sensorgrams indicating that growth factor binding was only to the GAG chains (data not shown). Analysis of the level of PF4 binding to heparin (Fig. 5A) indicated that one PF4 tetramer bound per heparin chain. This analysis is not possible for the proteoglycans because the relative contribution of the protein and GAG chains to the response units measured by the instrument cannot be determined.
The relative affinity of PF4 for perlecan and serglycin was analyzed in an ELISA assay where serglycin was immobilized on the ELISA plate and incubated with PF4 followed by incubation with perlecan. Perlecan was able to release PF4 from serglycin as detected by a significant ($p < 0.05$) reduction in the level of PF4 bound to serglycin after incubation with perlecan compared with without soluble perlecan treatment (Fig. 6). However, when the PF4-serglycin complex was incubated with perlecan that had been digested with HepIII to remove its HS chains, there was no change in the level of PF4 bound to serglycin, indicating that the protein core of perlecan did not release PF4 from serglycin. Incubation of the PF4-serglycin complex with perlecan domain V was also able to release PF4 from serglycin, and this interaction was found to be dependent on the presence of the HS chains. Treatment of the PF4-serglycin complex with GAG digestion enzymes alone under the same conditions had no effect on the level of PF4 bound to serglycin because of the short incubation times used in this assay. This indicated that PF4 had a higher affinity for perlecan HS than serglycin GAG chains.

PF4 Inhibits the Activity of FGF2 Bound to Heparin and Perlecan—The ability of ternary complexes to form between heparin, FGF2, and FGF receptor 1c (FGFR1c) and signal in the presence of PF4 was analyzed in BaF-32 cells transfected with FGFR1c (Fig. 7). Heparin and FGF2 were used as a positive control for the assay, whereas cells in the presence of medium or growth factor alone were used as negative controls. BaF-32 cells expressing FGFR1c responded to FGF2 in the presence of heparin to a level significantly above ($p < 0.05$) the medium only control; however, in the presence of PF4 at concentrations of 0.64 – 2.56 µM, the level of signaling was significantly reduced ($p < 0.05$) compared with the positive control (Fig. 7A). Perlecan in the presence of FGF2 was able to signal to a similar extent.
as the positive control; however, in the presence of PF4, the level of signaling was significantly reduced ($p < 0.05$) and at a level comparable to the negative control as well as perlecan in the absence of growth factor (Fig. 7B). These data indicated that PF4 could inhibit the binding and signaling of FGF2 through both heparin and perlecan HS.

**PF4 Activates Platelets, Whereas GAG Bound PF4 Does Not Activate Platelets**—Platelet activation was determined by measuring P-selectin expression. Type I collagen was used as a positive control for the assay, whereas freshly isolated platelets were used as the negative control (Fig. 8A). Exposure of platelets to PF4 was found to increase P-selectin expression, but not to the same extent as exposure to collagen type I. Exposure of platelets to heparin, endothelial perlecan, or recombinant serglycin did not activate platelets (Fig. 8B). Preincubation of PF4 with perlecan, serglycin, or heparin before exposure to the platelets inhibited the PF4 mediated activation of platelets (Fig. 8C), suggesting that the GAG chains modulated the activity of PF4.

**Discussion**

This study demonstrated that platelets contain serglycin decorated with GAG chains that consist of both CS and DS disaccharides with 4-sulfated CS closer to the protein core and part of the linkage tetrasaccharide structure. This finding extends previous reports that platelet serglycin is decorated with CS containing 4-sulfated disaccharides (17).
PF4 was found to bind to platelet serglycin through both CS and DS as well as perlecan through HS chains. Additionally, PF4 bound to recombinant serglycin via CS, DS, and HS/heparin chains further supporting the affinity of PF4 for a variety of GAG chains. Previous analyses of the affinity of PF4 for various GAG chains revealed that PF4 had the highest affinity for heparin followed by CSD or E, HS, and CSA, B, or C (25, 26) and that this binding involved lysine residues in PF4 binding to the sulfate groups on the GAG chains (1). Heparin had the highest affinity for PF4 in this study with a $K_D$ of 30 nM that was similar to previous reports in the range of 16–60 nM (1, 27, 28). The affinity of PF4 for proteoglycans was explored for the first time in this study and indicated that PF4 had a higher affinity for perlecan decorated with HS than serglycin predominantly decorated with CS/DS. PF4 has been established to be a tetrameric complex at physiological pH and ionic strength via NMR and neutron scattering (29, 30). Models of these studies support the hypothesis that the heparin chain wraps around the PF4 tetramer (30). Although this arrangement is likely with exposure of PF4 tetramers to single GAG chains, physiologically it is likely that the PF4 will be exposed to serglycin with eight GAG chains and perlecan with three GAG chains. Given the close proximity of the GAG chains on both serglycin and perlecan, there is limited flexibility for the chains to wrap around the PF4 tetramers as is possible with an isolated GAG chain. Thus there is the possibility of multiple GAG chains binding the PF4 tetramers as depicted in Fig. 9; however, this model remains to be experimentally verified.

Following vascular injury, platelets are known to adhere to the denuded vascular basement membrane, particularly to collagen, inducing platelet aggregation, activation, and the release of PF4. PF4 is released from platelets in complex with serglycin (31). Thus the release of PF4 from serglycin GAG chains by perlecan HS, as demonstrated in this study, is a likely mechanism by which PF4 is transferred to the vascular basement.
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![Diagram of Proteoglycan GAG Chains](Image)

**FIGURE 9.** Schematic of proposed mechanism of PF4 binding to proteoglycan GAG chains. The proposed model involves multiple GAG chains on proteoglycans, including serglycin and perlecan, binding PF4.

membrane as observed in vivo (2). Additionally, under physiological conditions, endothelial cell surface GAG chains bind basal levels of platelet-derived PF4 (32).

PF4 binds to the surface of resting and activated platelets and supports platelet aggregation (31). This study also demonstrated that PF4 promotes platelet activation; however, platelets did not become activated when exposed to PF4 bound to either serglycin or perlecan GAG chains, suggesting that the binding of PF4 to proteoglycans can modulate platelet activation events.

PF4 is reported to be angiostatic as demonstrated in a chicken chorioallantoic membrane assay (8). The angiostatic effect of PF4 has been ascribed to its binding to the same regions of HS as growth factors involved in angiogenesis including FGF2 and VEGF (7). Additionally, PF4 can inhibit the binding of FGF2 to both fibroblast cell ECM and cell surface receptors (5) through binding to HS. The present study supported these findings using the BaF32 cell assay where the addition of PF4 could dose-dependently inhibit the binding and signaling of FGF2 that require the formation of ternary complexes with FGF2, the FGFRIc cell surface receptor, and either heparin or perlecan HS. FGF2 is produced by vascular endothelial cells and acts as both a mitogenic and migratory signal for these cells. Indeed the addition of PF4 to cultures of endothelial cells inhibited their migration (5). Together these data suggest that both PF4 and FGF2 bind to similar regions of HS to inhibit the HS-dependent signaling of FGF2 via FGFR receptors.

PF4 also exerts its angiostatic activity via HS-independent mechanisms as it was able to inhibit VEGF_{121} induced cell proliferation but did not interfere with its binding to the VEGF receptor, flk-1 (6). HS-independent mechanisms include binding integrins on the endothelial cell surface (3), inhibiting growth factor binding to receptors and binding directly to FGF2 and preventing dimerization (7).

PF4 preferentially binds to newly formed blood vessels providing a mechanism to control their growth (33). Thus one of the physiological roles of PF4 may be to control normal and neoplastic vascularization through controlling the activities of growth factors. Thus it is hypothesized that the binding of PF4 to various GAG chains that decorate proteoglycans in the vasculature controls angiogenesis in wound healing through modulating the activity of growth factors and platelet activation.

**Experimental Procedures**

**Materials**—Chondroitinase ABC ((EC 4.2.2.4) purified from *Proteus vulgaris* proteinase free lyase I), B (EC 4.2.2) purified from *Flavobacterium heparinum*, ACII purified from *Arthro-
mm Bicine, 5 mm BisTris, 0.2 mm EDTA, 50 μg/ml SDS, 10% (v/v) methanol, pH 7.2) in a semi-dry blotter at 300 mA and 20 V for 60 min. The membrane was blocked with 1% (w/v) BSA in TBS (20 mm Tris base, 136 mm NaCl, pH 7.6) with 0.1% (v/v) Tween 20 (TBST) for 2 h at 25 °C followed by incubation with primary antibody diluted in 1% (w/v) BSA/TBST for 2 h at 25 °C. Membranes were subsequently rinsed with TBST, incubated with secondary HRP conjugated antibodies (1:50,000) for 45 min at 25 °C, and rinsed with TBST and TBS before being imaged using chemiluminescence reagent (Femto reagent kit; Pierce) and X-ray film.

ELISA—Proteoglycan-enriched samples (10 μg/ml based on Coomassie Blue protein assay), with and without glycosaminoglycan digestion, were coated onto high binding 96-well ELISA plates (Greiner) for 2 h at 25 °C. Wells were rinsed twice with Dulbecco's PBS, pH 7.4 (DPBS), followed by blocking with 0.1% (w/v) casein in DPBS for 1 h at 25 °C. Wells were rinsed twice with DPBS with 1% (v/v) Tween 20 (PBST) followed by incubation with primary antibodies diluted in 0.1% (w/v) casein in DPBS for 2 h at 25 °C. Wells were rinsed twice with PBST followed by incubation with biotinylated secondary antibodies (1:1000) diluted in 0.1% (w/v) casein in DPBS for 1 h at 25 °C, rinsed again twice with PBST, and then incubated with streptavidin-HRP (1:500) for 30 min at 25 °C. Binding of the antibodies to the samples was detected using the colorimetric substrate, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid), and absorbance was measured at 405 nm.

A sandwich ELISA was performed by coating ELISA plates with a rabbit polyclonal anti-serglycin capture antibody in 0.1 M sodium carbonate buffer, pH 9.6, for 16 h at 4 °C. Wells were rinsed twice with DPBS followed by blocking with 0.1% (w/v) casein in DPBS for 2 h at 25 °C. Wells were rinsed with PBST followed by incubation with the proteoglycan-enriched samples (10 μg/ml based on Coomassie Blue protein assay) for 2 h at 25 °C and subsequent detection with primary and secondary antibodies as for the standard ELISA. Data for both the ELISA and sandwich ELISA were corrected for background absorbance.

PF4 Binding Assays—The binding of PF4 to recombinant serglycin prepared as described previously (24), immunopurified endothelial derived perlecan prepared as described previously (35, 36), and recombinant perlecan domain V prepared as described previously (37) was assessed by Western blotting, ELISA, and surface plasmon resonance. For Western blotting, PF4 (5 μg/ml) was incubated with serglycin (2 μg/ml) in solution for 16 h at 37 °C and either analyzed without further modification or treated with glycosaminoglycan digestion enzymes prior to analysis.

For ELISA, plates were coated with either serglycin, endothelial perlecan, or perlecan domain V at a concentration of 10 μg/ml for 16 h at 4 °C prior to blocking with 1% (w/v) casein in PBS for 2 h at 25 °C. This was followed by incubation with 1 μg/ml PF4 for 2 h at 37 °C and then detection with the mouse monoclonal anti-PF4 antibody. The binding of PF4 to the proteoglycans was also analyzed after glycosaminoglycan digestion prior to immobilizing on the ELISA plates. The background level of PF4 binding to casein was also measured, and absorbance values were subtracted from sample absorbance values.

For the displacement assay, ELISA plates were coated with 10 μg/ml serglycin that was either untreated or treated with Case ABC, HepIII, or both Case ABC and HepIII for 16 h at 4 °C. The plates were then blocked with 1% (w/v) casein in PBS for 2 h at 25 °C followed by incubation with 8 μg/ml PF4 for 30 min at 37 °C. Plates were then incubated with either 10 μg/ml perlecan or 10 μg/ml perlecan domain V that was untreated or after glycosaminoglycan digestion for 1 h at 37 °C. As a control, selected wells were incubated with 0.05 unit/ml glycosaminoglycan digestion enzymes alone for 1 h at 37 °C. The plates were then analyzed for bound PF4 using the mouse monoclonal anti-PF4 antibody by the ELISA technique.

Surface Plasmon Resonance—Heparin (Sigma H3393; 17–19 kDa), recombinant serglycin, and endothelial perlecan were biotinylated as described previously (38, 39). The interaction between PF4 and either serglycin or endothelial perlecan or heparin was analyzed with a Biacore 2000 (GE Healthcare). Streptavidin-coated sensor surfaces (GE Healthcare) were coated by injection of 100 μl of 10 μg/ml biotinylated heparin, endothelial perlecan, or recombinant serglycin at 5 μl/min (diluted in DPBS, pH 7.4). The surfaces were then blocked by injection of 150 μl of 1% (v/v) BSA in DPBS at 5 μl/min. The binding of PF4 (0.1, 0.5, and 1 μM) was detected in DPBS, pH 7.4, at a flow rate of 20 μl/min at 25 °C using an injection volume of 50 μl. Sensorgrams were analyzed using BiACore 2000 evaluation software 3.0. Sensorgrams were fitted with differential rate equations for the association and dissociation rates. Control experiments were performed with samples that had been predigested with either Case ABC or HepIII and PF4 binding to blocked streptavidin chips.

Baf32 Cell Proliferation Assays—Baf32 cells are from an IL-3-dependent and HSPG-deficient myeloid B cell line that has been stably transfected with FGFR1c (40, 41). Baf32 cells are a model system developed to identify heparan sulfate and heparin structures that interact with FGFs and their receptors. The readout of this assay is cell proliferation, which indicates the formation of ternary complexes in situ. Baf32 cells were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 10% (v/v) WEHI-3BD conditioned medium, 100 units/ml penicillin, and 100 μg/ml streptomycin. WEHI-3BD cells were maintained in RPMI 1640 medium supplemented with 2 g/liter sodium bicarbonate, 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin, and the conditioned medium was collected three times per week and stored at −20 °C until required. For the mitogenic assays, the Baf32 cells were transferred into IL-3 depleted medium for 24 h prior to experimentation and seeded into 96-well plates at a density of 2 × 10⁴ cells/well in the presence of FGF2 (0.03 nM), heparin (30 nM), or immunopurified endothelial perlecan (4 pM) and PF4 (0.125–2.5 μM). The cells were incubated for 96 h in 5% CO₂ at 37 °C, and the amount of cells present was assessed using the MTS reagent (Promega, Madison, WI) by adding to the cell cultures for 6 h prior to measuring the absorbance at 490 nm.

Platelet Activation—Platelets were harvested from human donors under ethics approval from the University of New South Wales. Blood was collected in acid citrate dextrose anticoagulant-treated vacutainers. Platelet-rich plasma was prepared by centrifugation of the blood at 350 × g for 20 min at 25 °C fol-
lowed by careful removal of the upper platelet-rich layer. Platelet-rich plasma was then centrifuged at 1200 × g for 10 min to yield a platelet pellet with platelet poor plasma as the supernatant. The platelet pellet was resuspended in Tyrode’s buffer (1.8 mM CaCl₂, 1 mM MgCl₂, 2.7 mM KCl, 136.9 mM NaCl, 0.4 mM NaH₂PO₄, 11.9 mM NaHCO₃, 5.6 mM d-glucose, and 0.1 unit/ml apyrase) and centrifuged again at 1200 × g for 10 min. The supernatant was discarded, and the platelets were resuspended in Tyrode’s buffer to a concentration of 2 × 10⁷ platelets/ml. Platelet suspensions (2 × 10⁷ platelets/ml) in Tyrode’s buffer were used to either PF4 (4 μg/ml), collagen type I (30 μg/ml), 10 μg/ml recombinant serglycin, 10 μg/ml endothelial perlecán, 10 μg/ml heparin, or mixtures of 10 μg/ml serglycin and 4 μg/ml PF4, 10 μg/ml perlecán and 4 μg/ml PF4, or 10 μg/ml heparin and 4 μg/ml PF4 for 5 min at 37 °C in the presence of FITC-labeled anti-CD62P (1 μg/ml, clone 9E1; BioScientific Pty. Ltd., Gymea, Australia) detects P-selectin on the surface of activated platelets. The reaction was stopped by adding paraformaldehyde to a final concentration of 1% (w/v). The fluorescence intensity of 10,000 platelets was analyzed using flow cytometry (FACSscan; Becton Dickinson).

Statistical Analysis—A one-way analysis of variance (ANOVA) was performed, and the results of p < 0.05 were considered significant. The experiments were performed in triplicate and repeated.

Author Contributions—M. S. L. and J. M. W. conceived and coordinated the study. M. S. L. designed and performed the experiments shown in Figs. 5 and 8. B. C. designed and performed the experiments shown in Figs. 1–4 and 6. B. L. F. designed and performed the experiments shown in Fig. 7. M. S. L. wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

Acknowledgments—We thank B. Caterson (Cardiff University, Cardiff, UK) for the gift of the IB5, 2B6, and 3B3 hybridoma culture supernatants and T. Achilles (University of Patras, Patras, Greece) for the supply of rabbit polyclonal anti-serglycin antibody. We also acknowledge the technical assistance of Marie Labeye.

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