The Glycoprotein Ib-IX-V Complex Is a Platelet Counterreceptor for P-Selectin

By Gabriel M. Romo,* Jing-Fei Dong,* Alicia J. Schade,§ Elizabeth E. Gardiner,† Geoffrey S. Kansas,§ Chester Q. Li,* Larry V. McIntire,* Michael C. Berndt,* and José A. López*‡

From the *Department of Medicine and the ‡Department of Molecular and Human Genetics, Baylor College of Medicine and Veterans Affairs Medical Center, Houston, Texas 77030; the †Cox Laboratory for Bioengineering, Rice University, Houston, Texas 77251; the Baker Medical Research Institute, Melbourne, Australia 3181; and the §Department of Microbiology/Immunology, Northwestern University Medical School, Chicago, Illinois 60611

Summary

We have identified platelet glycoprotein (GP) Ibα as a counterreceptor for P-selectin. GP Ibα is a component of the GP Ib-IX-V complex, which mediates platelet adhesion to subendothelium at sites of injury. Cells expressing P-selectin adhered to immobilized GP Ibα, and GP Ibα-expressing cells adhered to and rolled on P-selectin and on histamine-stimulated endothelium in a P-selectin-dependent manner. In like manner, platelets rolled on activated endothelium, a phenomenon inhibited by antibodies to both P-selectin and GP Ibα. Unlike the P-selectin interaction with its leukocyte ligand, PSGL-1 (P-selectin glycoprotein ligand 1), the interaction with GP Ibα required neither calcium nor carbohydrate core-2 branching or α(1,3)-fucosylation. The interaction was inhibited by sulfated proteoglycans and by antibodies against GP Ibα, including one directed at a tyrosine-sulfated region of the polypeptide. Thus, the GP Ib-IX-V complex mediates platelet attachment to both subendothelium and activated endothelium.

Key words: platelet adhesion • endothelium • platelet glycoproteins • selectins • PSGL-1

During inflammatory processes, endothelial selectins mediate the initial interaction of leukocytes with endothelium as the leukocytes make their way from the bloodstream into the tissues. The interaction of the selectins with leukocyte mucins, in particular a protein called P-selectin glycoprotein ligand 1 (PSGL-1), is characterized by having rapid on and off rates, allowing the leukocytes in the flowing blood to roll along the endothelium. These leukocytes then undergo a tighter interaction with the vessel wall mediated by β2 integrins on the leukocytes and receptors of the Ig superfamily on the endothelial cells (1). P-selectin is the first selectin to be expressed on the endothelial surface in response to inflammatory stimuli such as thrombin and histamine, translocating from the membranes of storage granules (the Weibel-Palade bodies) to the plasma membrane within seconds. P-selectin and the other selectins, E- and L-selectin, are type I transmembrane proteins, each having an NH₂-terminal C-type lectin domain followed by an epidermal growth factor domain and a series of short complement repeats that separate the lectin domain from the plasma membrane (2). The P-selectin interaction with PSGL-1 requires a number of posttranslational modifications in the latter molecule, including a branched, fucosylated structure on its O-linked carbohydrate side chains (3) and sulfated tyrosine residues within a cluster of acidic residues at its NH₂ terminus (4–7).

Like leukocytes, unactivated platelets can roll on the surface of inflamed endothelium, in a process dependent on endothelial selectins (8). The process is independent of platelet selectins, as platelets from mice lacking P- and E-selectin roll as efficiently as platelets from normal mice (9). Unlike the interaction of the selectins with leukocytes, their interaction with platelets does not require fucosylation of the ligand, as no difference was observed between platelets from mice lacking both α(1,3)-fucosyl transferases (FucTs), FucT IV and FucT VII, and those of normal mice. These studies were the first to demonstrate that unactivated platelets interact with endothelial cells, which are generally considered nonthrombogenic. In marked contrast, it is well...
known that when the endothelium is removed, platelets interact rapidly with the exposed matrix, initially through the platelet glycoprotein (GP) Ib-IX-V complex and subsequently von Willebrand factor (10). The GP Ib-IX-V complex comprises four polypeptides, GP Ibα, GP Ibβ, GP IX, and GP V, and can support the rolling of platelets (11) and transfect cells (12, 13) on immobilized von Willebrand factor under flow, a process similar to the rolling interaction of platelets on inflamed endothelium, albeit at much higher shear rates. GP Ibα contains the von Willebrand factor-binding site and, like PSGL-1, it is a sialomucin with a cluster of O-linked carbohydrates separating the N-H2-terminal ligand-binding region from the plasma membrane. In addition, it contains a region rich in anionic amino acids with sulfated tyrosines necessary for optimal ligand binding (14–16). A further parallel between GP Ibα and PSGL-1 is the site of synthesis of their ligands von Willebrand factor and P-selectin are synthesized and stored only in the Weibel-Palade bodies of endothelial cells and the α-granules of megakaryocytes and platelets. Given these similarities between the two molecules and the high surface density of GP Ibα on the platelet surface (25,000–50,000 copies per platelet), we sought to determine if the GP Ibα-IX-V complex might constitute a P-selectin counter-receptor.

**Materials and Methods**

Antibodies. Antibodies against P-selectin included affinity-purified polyclonal antisera produced in rabbits immunized with P-selectin purified from platelet lysates (17) and the mAB WAPS 12.2 (Zymed Labs., Inc.). Anti-GP Ibα mAbs used were: W M23 and AK3, both of which bind within the mucin-like macroglycopeptide (18); SZ2, which recognizes an epitope within the anionic/sulfated tyrosine region of GP Ibα bounded by residues Tyr276 and Glu282 (16); and AK2 (R D1 Research Diagnostics), which binds within the GP Ibα N-H2 terminus and inhibits ristocetin- and botrocetin-induced von Willebrand factor binding (16). Two von Willebrand factor mAbs were used in these studies: 6G1, which inhibits the von Willebrand factor-GP Ibα interaction (unpublished data), and 2C9, which does not inhibit the binding of von Willebrand factor to GP Ibα and was used as a control mAb (18). HECA-452 is a rat mAb (IgM) that recognizes a FucT VII–dependent carbohydrate (19, 20).

Glycocalicin Purification. Glycocalicin was isolated from outdated human platelets by a method modified from that of Hess et al. (21). Erythrocyte-free platelets were isolated from 10 liters of outdated platelet-rich plasma (obtained from the Blood Transfusion Centre, Cambridge, England) centrifuged and washed with buffer A (13 mM NaCl, 120 mM Na citrate, and 30 mM glucose, pH 7.0). After another centrifugation, the platelet pellet was suspended in 500 ml of buffer B (10 mM TRIS/HCl, 150 mM NaCl, and 2 mM CaCl₂, pH 7.4) and sonicated. The resultant suspension was then incubated at 37°C for 30 min to allow the calpain released from the platelets during sonication to cleave membrane-bound GP Ibα, releasing glycocalicin. After ultracentrifugation to remove cell components, the glycocalicin-containing supernatant was applied to a wheat germ Sepharose 4B column. Bound crude glycocalicin was eluted with 2.5% N-acetyl-d-glucosamine and 20 mM TRIS/HCl, pH 7.4. Further purification by ion exchange chromatography (on Q-Sepharose Fast-flow column; Pharmacia) was needed to remove residual contaminants. Glycocalicin was eluted with a linear salt gradient of 0–0.7 M NaCl in 20 mM TRIS/HCl, pH 7.4.

Cell Lines. Chinese hamster ovary (CHO) cells expressing a phosphatidylinositol glycan-linked form of P-selectin were a gift from Dr. C. Wayne Smith (Baylor College of Medicine, Houston, TX) (22). These cells were grown in medium comprising equal parts DMEM and F12 medium (GIBCO BR L) supplemented with 5% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. CHO and L cells expressing the GP Ibα-IX-V complex and its components have been described previously (23). In brief, CHO αβIX cells express GP Ibα, GP Ibβ, and GP IX, the three polypeptides that are minimally required for efficient cell surface expression of GP Ibα. L αβIXV cells additionally express GP V, and CHO and L αβIXV cells express GP Ibβ and GP IX but lack GP Ibα and GP V. The conditions for their growth have been described (23, 24).

Flow Cytometry. Before each experiment involving cells that express GP Ibα, the surface level of this polypeptide was first determined by flow cytometry using AK2 as previously described (25). In brief, cells grown in a monolayer were detached with 0.54 mM EDTA, washed with PBS, and resuspended in PBS containing 1% BSA. The cells were then incubated with AK2 (1 μg/ml) for 60 min at room temperature. After being washed to remove unbound antibody, the cells were incubated with an FITC-conjugated rabbit anti–mouse secondary antibody (Zymed Labs, Inc.) for 30 min at room temperature. The cells were then analyzed on a FACScan™ flow cytometer (Becton Dickinson) stimulating with laser light at 488 nm and collecting light emitted at >520 nm.

Transfections. To induce core-2 branching and α(1,3)-fucosylation of the GP Ibα carbohydrates in CHO cells, the cells were cotransfected with plasmids containing cDNAs encoding C2GnT (core 2 β(1,6)-N-acetylglucosaminyltransferase), (pCDNA1/C2GnT, a gift from Dr. Minoru Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA) and FucT VII (provided for these studies by Dr. John Lowe, Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI) using a modified SR vector containing a hygromycin resistance marker (19). Transfections were by liposome-mediated DNA delivery (LipofectAMINE; GIBCO BR L). A total of 1 μg of plasmid was used in each transfection, with the FucT VII plasmid transfected either alone or as an equal mixture with the plasmid containing the C2GnT cDNA. The cells were selected by growth in hygromycin-containing medium, for which a resistance marker is contained in the FucT VII plasmid. FucT VII expression was evaluated by flow cytometry with the antibody HECA-452, which recognizes a FucT VII–dependent epitope. Labeling and analysis were as described above for GP Ibα, except that an FITC-conjugated rabbit anti–rat antibody (Zymed Labs, Inc.) was used to label cell-bound HECA-452.

CHO-P Adhesion to Glycocalicin. The wells of 96-well microtiter plates (Nunc Immulon, Inc.) were coated with 50 μl of purified glycocalicin (20 μg/ml in PBS, pH 7.4) for 2 h at room temperature. The wells were rinsed four times over 20 min with DMEM containing 1% BSA. CHO cells and CHO cells expressing P-selectin (CHO-P) were detached from culture dishes using 0.54 mM EDTA in PBS, washed once in PBS and once in DMEM, and then incubated at 37°C for 30 min in DMEM containing 0.5 mM of 51Cr per 10⁶ cells. The cells were then washed three times by centrifugation and resuspension and then resuspended to a density of 2 × 10⁵ cells/ml in DMEM containing 1.0% BSA and
preincubated at room temperature for 15 min with either 10 mM T ris/HCl, pH 7.4, in 0.15 M NaCl (TS buffer) alone or with TS buffer containing appropriate amounts of sulfated glycans or other blocking reagents. Aliquots of 0.1 ml of the cell suspension were incubated at room temperature in four replicate microtiter wells for 15 min. The media and nonadherent cells were then removed, and the wells were rinsed three times with DMEM containing 1% BSA. S Cr-labeled cells that remained adherent to the wells were lysed in a solution containing 1% Triton X-100, and their radioactivity was assayed in a gamma counter. For studies that included anti-GP Ibα blocking antibodies, the antibodies were incubated with the immobilized glyocalcicin for 15 min at room temperature (each antibody at 40 μg/ml) before the addition of the radiolabeled cells. Each experiment was performed in triplicate; average counts from one representative experiment are reported.

Glyocalcicin Inhibition of P-Selectin Antibody Binding to CHO-P. CHO-P cells detached with EDTA were resuspended in α minimal essential medium (MEM; Gibco BRL) containing 1% BSA and aliquoted to a concentration of 2 × 106 cells/ml. Purified glyocalcicin or soybean trypsin inhibitor were then added to the cell suspensions at various concentrations and shaken gently for 15 min. Polyclonal anti-P-selectin antibody was then added to each tube (final concentration, 2 μg/ml), and the cells were incubated for an additional 45 min. The cells were then washed twice in PBS, and FITC-conjugated goat anti-rabbit antibody was added to a concentration of 8 μg/ml and incubated for 45 min. Antibody binding was then assessed by flow cytometry.

CHO Cell Aggregation. CHO cells were labeled with membrane dyes CellTracker™ Orange (5-(and -6)-(4-chloromethyl)benzoyl)amino)-tetramethylrhodamine) for CHO-P cells and CellTracker™ Green (5-chloromethylfluorescin diacetate) for CHO αβIX cells and CHO βIX (both dyes from Molecular Probes, Inc.). For this, the cells were detached from the culture dishes with 0.54 mM EDTA, centrifuged, and resuspended in culture medium. The dyes were each added to the cell suspension at 12 μM and incubated at 25°C for 60 min in the dark, with gentle shaking. After labeling, the cells were washed twice in PBS and resuspended in α-MEM, 1% BSA to a concentration of 6 × 106 cells/ml. 500-μl aliquots of CHO-P cells were then mixed with equal volumes of the other cell lines, and the mixture was shaken on a table-top shaker at six cycles per second for 10 min. Heterotypic aggregates of green and orange cells were quantitated by fluorescence microscopy. These experiments were also done with CHO αβIX cells pretreated with mocaargin, a metalloprotease from the venom of the South African spitting cobra, Naja mocambique. This protease selectively cleaves the GPI Ibα N terminus, including a region containing three sulfated tyrosine residues (16). Before the aggregation experiment, the CHO αβIX cells were incubated for 30 min with mocaargin (20 μg/ml) and then washed twice with PBS.

Preparation of Purified P-Selectin and P-Selectin Matrix. Human P-selectin was purified from outdated platelets as described previously (26). Detergent, Triton X-100, was removed from the P-selectin sample immediately before use by passage through an Extracti-Gel D column (Pierce Chemical Co.). To prepare the P-selectin matrix, glass coverslips (No. 1, 24 × 50 mm; Corning Glass Works) were coated with a solution containing 50 μg/ml P-selectin for 4 h at 37°C followed by an additional 1-h incubation with PBS containing 1% BSA to block nonspecific binding sites. The coated coverslips were then washed with 0.9% NaCl to remove unbound P-selectin.

Preparation of Primary Cultures of Human Umbilical Vein Endothelial Cells. Human umbilical cords were collected and processed for primary endothelial cell culture as described previously (27). In brief, human umbilical cords were rinsed with PBS and then treated with collagenase type II (10 μg/50 ml PBS; Worthington Biochemical Corp.) for 40 min at room temperature. Enzymatically dissociated human umbilical vein endothelial cells (HUVECS) were collected by centrifugation (10 min, 200 g) and then resuspended in M 199 medium (Gibco BRL) supplemented with 10% FBS and 1% glutamine. The cells were then plated onto glass coverslips that had previously been coated with a 1% gelatin solution for 30 min at 37°C. Cells were maintained at 37°C with 5% CO2 and 95% humidity and reached confluence in ~4-5 d.

To induce surface expression of P-selectin, monolayers of confluent HUVECS were treated with 25 μM histamine (Sigma Chemical Co.) for 12 min at room temperature (27). Stimulated cells were used immediately.

Parallel Plate Flow Chamber. The flow chamber system included a parallel plate flow chamber, an inverted stage phase contrast microscope (Diaphot-TMD; Nikon Inc.), and an image recording system. The parallel plate flow chamber was composed of a polycarbonate slab, a silicon gasket, and a glass coverslip held together by vacuum in such a way that the coverslip forms the bottom of the chamber. The coverslips were first coated with either soluble P-selectin or a monolayer of HUVECS. The chamber was maintained at 37°C by an air curtain incubator attached to the microscope. The wall shear stress was created by drawing PBS through the chamber with a Harvard syringe pump and was proportional to the fluid viscosity and flow rate and inversely proportional to the width of the chamber and height of the gap created by the gasket (28).

In studies of GPIb, IX-V-mediated cell rolling on P-selectin, 0.6 ml of a suspension of CHO αβIX, CHO βIX, L βIX, or L αβIXV cells (500,000 cells/ml) was injected into the chamber, and the cells were incubated for 1 min with immobilized P-selectin or 2 min with stimulated endothelial cells. At the end of the incubation, PBS was perfused through the chamber at a flow rate of 1.6 ml/min, generating a wall shear stress of 2 dynes/cm2 (shear rate ~182/s). Cell rolling through a single view field was recorded in real time for 4-6 min on videotape. The video data were then analyzed off line using Inovision imaging software (IC-300, M odular Image Processing Workstation; Inovision Corp.) to quantify the number and velocities of the rolling cells (27, 29). Cells considered to be rolling were those that translocated over the matrix or HUVECs while maintaining constant contact. The rolling velocity was defined as the distance a cell traveled during a defined period (μm/s).

To examine platelet rolling on activated HUVECS, platelets prepared as described below were resuspended to 2 × 108 cells/ml in PBS with 1 mM CaCl2, 0.5 mM MgCl2, and 20% erythrocytes by volume. The HUVECS were stimulated as above, and the platelet suspension was allowed to incubate for 2 min at 37°C before perfusing the chamber with the platelet suspension at a flow rate of 1.6 μl/min, generating a wall shear stress of 2 dynes/cm2 (shear rate ~120/s).

Platelet Preparation. Blood was drawn from healthy human donors into 1/6 volume of acid citrate dextrose (85 mM Na3 citrate, 111 mM dextrose, and 71 mM citric acid). The blood was centrifuged at 170 g for 15 min at 25°C to separate platelet-rich plasma from erythrocytes and leukocytes. The platelets were then pelleted from platelet-rich plasma by centrifugation at 800 g for 10 min at 25°C. The platelets were then washed by suspension in CGS buffer (13 mM sodium citrate, 30 mM glucose, and 120 mM sodium chloride, pH 6.5), centrifuged again, and resuspended to 2 × 108 platelets/ml in PBS containing calcium and magnesium.
Results

CHO Cells Expressing P-Selectin Are able to Immobilize Soluble GP Ibα (glycocalcin) in a Calcium-independent Manner. As a first step in determining if the GP Ib-IX-V complex interacts with P-selectin, we examined the binding of CHO-P cells to the soluble extracellular portion of human GP Ibα (called glycocalcin) immobilized on plastic. Glycocalcin was purified from lysates of human platelets and therefore contained all of the posttranslational modifications potentially required to constitute a platelet P-selectin receptor. The binding of CHO-P cells to glycocalcin was approximately fivefold greater than the binding of untransfected cells (n = 4, P < 0.001, Student’s t test), suggesting a specific interaction between P-selectin and GP Ibα (Fig. 1A). We then tested the ability of different reagents to block this interaction. The P-selectin interaction with PSGL-1 is dependent on two structural modifications of PSGL-1: tyrosine sulfation within a negatively charged sequence at its mature NH2 terminus and fucosylation, sialylation, and core-2 branching of its O-linked carbohydrate. P-selectin interacts with the carbohydrate motif through its C-type lectin domain in a calcium-dependent fashion. We therefore tested whether EDTA would inhibit the interaction of P-selectin with GP Ibα. Surprisingly, and in contrast to the interaction with PSGL-1, it did not (n = 3, P = 0.20, Student’s t test; Fig. 1B). However, antibodies against both GP Ibα and P-selectin did inhibit the interaction. Of the GP Ibα antibodies, the greatest inhibition of binding was observed with SZ2 (~55% inhibition; n = 3, P < 0.03, one-way ANOVA, Dunnett’s method). This antibody has been shown to recognize an epitope within the tyrosine-sulfated anionic region of GP Ibα (16), suggesting that this region plays an important role in the recognition of P-selectin. The other two GP Ibα mAbs, WM23 and AK3, directed against the GP Ibα mucin core (18), inhibited binding to a lesser extent. Binding was also almost completely inhibited by an affinity-purified rabbit polyclonal antibody against P-selectin (n = 3, P < 0.002, Student’s t test).

P-Selectin’s Interaction with GP Ibα Mimics Its Interaction with Heparin. Two findings indicated a potential similarity between P-selectin’s interaction with GP Ibα and its interaction with glycosaminoglycans. First, as shown in Fig. 1B, antibodies against P-selectin inhibited the interaction of CHO-P cells with immobilized glycocalcin. Second, as shown in Fig. 1C, several proteoglycans, including fucoidin and heparin, inhibited the adhesion of CHO-P cells to glycocalcin.

Statistical Analysis. The data were analyzed either by the unpaired Student’s t test or by one-way analysis of variance (ANOVA), depending on the nature of the data.
action with heparin: its calcium independence (17, 30) and the involvement of the GP Ibα anionic/tyrosine sulfated region. This region of GP Ibα contains three sulfated tyrosine residues (14, 16) and interacts with another of GP Ibα’s ligands, thrombin, through thrombin’s heparin-binding exosite (31, 32). We therefore studied the effect of heparin and other proteoglycans on CHO-P adhesion to glyocalcicin. Heparin and the related proteoglycan fucoidin both partially inhibited binding (n = 3, P < 0.001, one-way ANOVA, Tukey’s test; Fig. 1 C). In contrast, chondroitin sulfate, a control proteoglycan that does not block the P-selectin–PSGL-1 interaction, similarly did not affect the P-selectin–GP Ibα interaction (P > 0.1).

Glyocalcicin Blocks Antibody Binding to CHO-P Cells. We next tested the ability of glyocalcicin to interfere with the binding to CHO-P cells of a polyclonal antibody against human P-selectin. This antibody blocks the P-selectin–PSGL-1 interaction (17) and, as shown in Fig. 1 B, also blocks the adhesion of CHO-P to glyocalcicin. Glyocalcicin blocked antibody binding in a dose-dependent manner, with a 50% inhibiting concentration (IC50) of ~25 μg/ml (n = 3, P < 0.001, one-way ANOVA, Tukey’s test; Fig. 2). The results suggest that most of the binding determinants for the polyclonal antibody lie within a restricted region of P-selectin close to the GP Ibα binding site.

Posttranslational Core-2 Branching and α(1,3)-Fucosylation Are Not Required for GP Ibα Binding to P-Selectin. CHO cells synthesize simple core-1 O-linked glycans (33) and are incapable of synthesizing PSGL-1 in a form competent to bind P-selectin unless the appropriate carbohydrate-modifying enzymes are also expressed (34). Thus, CHO cells provide a good cell system to test whether carbohydrate modification of GP Ibα is required for its interaction with P-selectin. The major carbohydrate structure of platelet GP Ibα (35–37) is very similar to that reported for PSGL-1 (3), the only difference being the lack of an α(1,3)-fucosyl linkage in GP Ibα. We expressed the GP Ibα-IX-V complex in

![Figure 2](https://example.com/figure2.png)  
**Figure 2.** Inhibition of antibody binding to P-selectin. CHO-P cells were incubated in increasing concentrations of glyocalcicin or soybean trypsin inhibitor before the addition of anti-P-selectin polyclonal antibody. The binding of the antibody was evaluated by flow cytometry after incubation of the cells with an FITC-conjugated goat anti-rabbit secondary antibody. 100% binding is the binding in the absence of added protein. Glyocalcicin inhibited antibody binding, with an IC50 of ~25 μg/ml. Soybean trypsin inhibitor had no effect. Values reported are averaged from three experiments.

![Figure 3A](https://example.com/figure3A.png)  
**Figure 3A.** Assessment of the role of carbohydrate modification in the GP Ibα–P-selectin interaction. (A) Expression of the HECA-452 epitope in transfected cells. Cells transfected with cDNAs for FucT VII and C2GnT express the HECA-452 epitope. CHO βIX and CHO αpix cells were transfected with either a FucT VII expression plasmid alone (F-VII) or together with the expression plasmid for C2GnT (C2). Sham-transfected CHO βIX and CHO αpix cells served as negative controls. The appropriate carbohydrate modification was detected by cell surface binding of the antibody HECA-452, which recognizes a FucT VII–dependent epitope. Binding was determined by flow cytometry after labeling the cells with an FITC-conjugated secondary antibody. The surface level is expressed as the geometric mean fluorescence of the cell population. HL60 cells served as positive controls for the presence of the carbohydrate modification. (B) Heterotypic aggregation of CHO αpix and CHO P cells. Aggregation of green-labeled CHO αpix cells with orange-labeled CHO-P cells was induced by coincubating the cells and shaking them on a table-top shaker. The ordinate represents the percentage of the green cells found in coaggregates with orange cells. (C) Inhibition of aggregation by mocarhagin. Before commencement of the aggregation assay, CHO αpix cells were pretreated with mocarhagin, a cobra venom metalloprotease that selectively removes the GP Ibα NH₂ terminus by cleavage between Glu282 and Asp283 (16). Adherence of the cells to CHO-P was then determined as in B. Values are depicted as percentages of control binding after subtracting the background adhesion of CHO βIX cells.
CHO cells alone, with FucT VII, or with both FucT VII and the core-2 branching enzyme (C2GnT). We first assessed whether the cells carry the necessary modification by immunostaining with the antibody HECA-452, which recognizes FucT VII-dependent carbohydrate epitopes (19). Whereas the untransfected cells demonstrated only a low background fluorescence, all of the cell lines transfected with both C2GnT and FucT VII demonstrated high levels of the HECA-452 epitope (Fig. 3 A).

We then evaluated the ability of these cells to form heterotypic aggregates with cells expressing P-selectin. Cells expressing the GP Ib-IX-V complex and its components were fluorescently labeled with the membrane-permeable dye CellTracker™ Green, and the CHO-P cells were labeled with CellTracker™ Orange. Equal quantities of the two cell types were mixed and incubated with gentle shaking, and the heterotypic aggregates containing both green and orange cells were quantitated. Cells expressing GP Ibα demonstrated much greater heterotypic aggregation than the control untransfected CHO cells or CHO cells expressing a partial complex lacking GP Ibα (CHO βIX cells) (n = 3, P < 0.01, one-way ANOVA, Tukey’s test; Fig. 3 B). Expression of the carbohydrate-modifying enzymes led to only a small further increment in the formation of heterotypic aggregates, indicating that carbohydrate modification of GP Ibα is less important to the interaction with P-selectin than is the modification of PSGL-1. This finding is consistent with the lack of calcium dependence of the interaction. Calcium is required for the interaction of P-selectin with certain carbohydrate ligands but not with sulfated glycosaminoglycans such as heparin sulfate and fucoidin, which apparently involves sulfated moieties (17, 26, 30).

The involvement of the GP Ibα NH2 terminus in binding P-selectin was further implicated by studies with the cobra venom metalloprotease mocarhagin (Fig. 3 C). This protease removes the GP Ibα NH2 terminus to Glu282, including much of the anionic sulfated region (16). Mocarhagin pretreatment of CHO αβIX cells reduced heterotypic aggregation by ∼50% (n = 3, P < 0.01, one-way ANOVA, Tukey’s test).

Cells Expressing the GP Ib-IX-V Complex Roll on Immobilized P-Selectin. The P-selectin-mediated rolling of platelets on inflamed endothelium requires rapid on and off rates. Thus, for the GP Ib-IX-V complex to constitute a physiological counterreceptor for P-selectin, it should be able to support a similar rolling interaction. To test this possibility, we perfused cells expressing full or partial complexes over coverslips coated with P-selectin in a parallel plate flow chamber at a shear stress of 2 dynes/cm². Cells were observed to adhere to and roll on P-selectin (Fig. 4 A). The rolling interaction was independent of cell type, as both CHO and L cells expressing the complex rolled at similar velocities, whereas control cells lacking GP Ibα did not adhere or roll (Fig. 4 B).

Cells Expressing the GP Ib-IX Complex and Platelets Roll on Histamine-activated Endothelium. We next tested whether the GP Ib-IX-V complex could also support cell rolling on stimulated endothelial cells. Endothelial cells were collected from human umbilical veins and grown directly on gelatin-coated coverslips. When confluent, they were activated with 25 μM histamine and the coverslip was immediately placed in the parallel plate flow chamber. CHO αβIX cells were then perfused over the monolayer. Negative controls were CHO αβ IX cells perfused over untreated endothelium and CHO βIX cells perfused over activated endothelium. CHO αβIX cells adhered to and rolled on the acti-

**Figure 4.** Rolling of GP Ibα-expressing cells on immobilized P-selectin. (A) CHO cells expressing either the GP Ib-IX complex or only GP Ibβ and GP IX were allowed to settle onto coverslips coated with P-selectin in parallel plate flow chambers. After 1 min, the chambers were perfused with PBS at a constant flow rate to generate a shear stress of 2 dynes/cm². The top panels show cells before application of shear stress, and the bottom panels show the same region during perfusion. The images in the bottom panels represent a compilation of a series of frames taken over 2 s. The linear blurs represent those cells that have adhered to the matrix and are rolling on it. (B) Velocity and numbers of cells rolling on immobilized P-selectin. Both L and CHO cells were studied, with no significant differences noted between the two in either velocity or number of rolling cells. The presence of GP V also did not influence rolling. No velocity is given for cells lacking GP Ibα because no rolling cells were observed. The number of cells rolling per field were counted during a 3-min interval from the start of the perfusion.
vated endothelial monolayer but not on the unstimulated endothelium (Fig. 5). A low level of CHO βIX adhesion to activated endothelium was noted, but this did not exceed the level of adhesion of these cells to the unstimulated endothelium. Adhesion of the CHO αβIX cells was blocked by the polyclonal antibody against P-selectin (Fig. 5 and Table I).

As a final, and more physiological, test of the GP Ib-IX-V complex–P-selectin interaction, we studied the effects of antibodies to GP Ibα and P-selectin on platelet rolling on stimulated endothelium (Fig. 6). Platelet suspensions \((2 \times 10^8\) platelets/ml and 20% washed erythrocytes in PBS containing calcium and magnesium) were perfused over histamine-activated endothelium either without treatment or in the presence of antibodies against GP Ibα (SZ2 and AK2, alone and in combination), P-selectin (polyclonal anti-P-selectin), or von Willebrand factor (6G1). The number of platelets rolling was much greater on activated than on unactivated endothelium. Treatment with the two GP Ibα mAbs markedly inhibited adhesion and rolling of the platelets, with AK2 being a more potent inhibitor, reducing the number of rolling cells to the level seen on unstimulated endothelium. (We did not use AK2 in the experiments examining CHO-P adhesion to glycolocalcin because it does not bind to immobilized glycolocalcin.) Anti-P-selectin decreased platelet rolling to levels even below those of the unstimulated endothelial cells, suggesting a background level of endothelial cell activation. The anti-von Willebrand factor mAb, 6G1, did not significantly affect platelet rolling.

**Discussion**

Here we demonstrate a specific interaction between a platelet adhesion receptor, the GP Ib-IX-V complex, and P-selectin. This interaction represents a potential new paradigm for platelet adhesion, where the same receptor (GP Ib-IX-V) can support platelet adhesion to endothelial cells under the appropriate conditions of endothelial activation and to the subendothelial matrix when the endothelial cells have been removed by injury. Several interesting parallels exist between GP Ibα and PSGL-1 as cell adhesion receptors. Both are membrane mucins with elongated structures formed by the presence of a heavily O-glycosylated region rich in threonine, serine, and proline residues (38, 39). In both receptors, this mucin-like region separates the ligand-binding region from the plasma membrane (40, 41), and in both it contains tandemly repeated sequences that account for polymorphism in the human population because of variable numbers of the tandem repeats (42, 43). The major carbohydrate structures of the two receptors are very similar, with only a fucose in α(1,3) linkage in the major PSGL-1 carbohydrate chain differentiating it from the major carbohydrate species of GP Ibα. Furthermore, both receptors contain a highly acidic region with sulfated tyrosines that is important for ligand binding and lies immediately NH2-terminal to the mucin-like region (4–7,

**Table I.** Number of CHO Cells Rolling on Human Umbilical Vein Endothelium

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Unstimulated</th>
<th>No antibody</th>
<th>Anti-P-selectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO αβIX</td>
<td>7.60 ± 1.33</td>
<td>33.30 ± 3.62*</td>
<td>8.00 ± 2.07</td>
</tr>
<tr>
<td>CHO βIX</td>
<td>N/A</td>
<td>4.66 ± 2.03</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Student’s t test, *P* < 0.01.
N/A, not applicable.
The major ligands for both receptors (von Willebrand factor and P-selectin) are synthesized only in megakaryocytes and endothelial cells and are stored in the same subcellular compartments, the α-granules of platelets and megakaryocytes and the Weibel-Palade bodies of endothelial cells.

Despite all of these similarities, the interactions of the two mucins with P-selectin are quite different. Whereas PSGL-1 requires carbohydrate modification with core-2 branching and α(1,3)-fucosylation and the presence of calcium for its interaction with P-selectin, GP Ibα requires neither. This lack of a requirement for carbohydrate modification is consistent with the findings of Frenette et al. (8) that the platelet P-selectin counterreceptor does not require modification by FucTs for function.

The different carbohydrate requirements of the two P-selectin counterreceptors may not be absolute but rather may represent a difference in the relative importance of the two P-selectin binding determinants (carbohydrates and sulfated tyrosines). GP Ibα does not contain the fucosylated moiety that is so vital for PSGL-1; on the other hand, its anionic region has a much higher concentration of negative charge than does the analogous region of PSGL-1, as well as three tyrosines that are fully sulfated (14, 16). This region of GP Ibα is known to bind tightly to thrombin and does so by interacting with the same region of thrombin that binds heparin (31, 32). Thus, the anionic sulfated region of GP Ibα, with its high negative charge and sulfate groups, appears to resemble heparin. Several lines of evidence point to the importance of this heparin-like region of GP Ibα in the interaction with P-selectin. First, the calcium independence of the interaction resembles the interaction of P-selectin with heparin, as does the pattern of blockade with heparin and fucoindin, but not with chondroitin sulfate (17). Second, the interaction is inhibited by SZ2, an mAb that binds within the region and also partially blocks botrocetin-induced von Willebrand factor binding, a phenomenon known to be sensitive to the removal of tyrosine sulfates (15, 16). Finally, mocarhagin, the cobra venom metalloprotease that moves most of the heparin-like region, also inhibits binding. Thus, the GP Ibα–P-selectin interaction is almost completely independent of carbohydrate, although the failure of SZ2 or mocarhagin to fully inhibit the interaction suggests that carbohydrate may have a minor role.

The possible involvement of other regions of GP Ibα in the interaction is also suggested by the inhibition by the mAb AK2 of platelet rolling on stimulated endothelial cells. This antibody binds within the leucine-rich repeats of GP Ibα (16). However, because the three-dimensional relationship of the leucine-rich repeats with the sulfated region in three dimensions is not known, we cannot definitively make a case for the involvement of the leucine-rich repeats in the interaction. The potential involvement of all of the regions of GP Ibα will have to await more definitive studies with mutant polypeptides.

The adhesion of platelets to regions of vessel wall injury mediated by von Willebrand factor can occur at very high shear stresses, approaching 100 dynes/cm². The interaction between platelets and endothelium, on the other hand, seems to occur at much lower shear stresses, although evidence exists that platelets can interact with endothelium at shear stresses above those able to support the interaction of leukocytes with the endothelium (44). The studies reported here were carried out at venular shear stresses and do not address the possibility that the GP Ibα–P-selectin interaction may be able to support platelet rolling at shear stresses much higher than those that support leukocyte rolling.

The demonstration of an abundant receptor on the platelet surface for P-selectin brings into sharp focus the potential roles of this receptor-counterreceptor interaction in both physiological and pathological conditions. During hemostasis, the interface between the GP Ib-IX-V complex and P-selectin may be important in the interaction between activated and unactivated platelets. Activated platelets with surface-exposed P-selectin could associate with and activate previously unactivated platelets, thus providing a mechanism for propagation of platelet thrombi. Failure of
this mechanism could explain the mild bleeding diathesis of P-selectin-deficient mice (45) and could contribute to the bleeding tendency in patients with Bernard-Soulier syndrome, the genetic disorder resulting from deficiency of the GP Ib-IX-V complex (46). Nevertheless, this mechanism is likely to be only of secondary importance, as no defect in aggregation has been found in the platelets of patients with Bernard-Soulier syndrome (although a subtle defect might not be detected by conventional methods of performing aggregation).

Under certain circumstances, platelets may help neutrophils and other white cells to exit the vasculature by providing an additional ligand for newly activated endothelial cells. Such a role has been demonstrated for platelets in lymphocyte trafficking, where activated platelets bound to the lymphocytes can provide a ligand (P-selectin) for the endothelial adhesion of lymph nodes (47). Platelets interacting with activated endothelium may themselves become activated, releasing factors that may influence the behaviors of both the endothelial cells and underlying smooth muscle cells. When such a process is unchecked, such as during sepsis, the platelets may contribute to the deleterious effects of the underlying condition. In this regard, recent findings suggest that activated platelets also form stable attachments with endothelial cells mediated in part by other platelet GP receptors, such as the integrin GP Ib-IIIa (48). At this point, however, the potential biological consequences of platelet interaction with endothelium are speculative. Nevertheless, with the demonstration of such a specific interaction involving endothelial selectins (9) and the current identification of a platelet receptor for these selectins, the endothelium can no longer be considered refractory to the overtures of the platelet.

The authors thank Drs. John Lowe, Minoru Fukuda, and C. Wayne Smith for generously providing reagents and Dr. R. Cortes for helpful discussions.

We also gratefully acknowledge grant support from the National Institutes of Health (grants HL54218 to J.A. López and HL18672 to L.V. McIntire) and the National Heart Foundation of Australia (to M.C. Berndt). J.A. López and G.S. Kansas are Established Investigators of the American Heart Association.

Address correspondence to José A. López, Veterans Affairs Medical Center, Hematology/Oncology (111H), 2002 Holcombe Blvd., Houston, TX 77030. Phone: 713-794-7088; Fax: 713-794-7578; E-mail: josel@bcm.tmc.edu

Submitted: 17 May 1999 Revised: 12 July 1999 Accepted: 20 July 1999

References

15. Marchese, P., M. Murata, M. Mazzucato, P. Pradella, L. De...


