

Platelet Glycoprotein Ib α Is a Counterreceptor for the Leukocyte Integrin Mac-1 (CD11b/CD18)

By Daniel I. Simon,* Zhiping Chen,* Hui Xu,* Chester Q. Li,^{‡§} Jing-fei Dong,^{‡§} Larry V. McIntire,[¶] Christie M. Ballantyne,[§] Li Zhang,** Mark I. Furman,^{‡‡} Michael C. Berndt,^{§§} and José A. López^{‡§||}

From the *Cardiovascular Division, Brigham and Women's Hospital, Boston, Massachusetts 02115; the [‡]Thrombosis Research Section, the [§]Department of Medicine, and the ^{||}Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030; the [¶]Cox Laboratory for Bioengineering, Rice University, Houston, Texas 77251; the ^{**}American Red Cross, Holland Laboratory, Rockville, Maryland 20855; the ^{‡‡}Center for Platelet Function Studies, Division of Cardiovascular Medicine, University of Massachusetts, Worcester, Massachusetts 01655; and the ^{§§}Baker Medical Research Institute, Prahran, Victoria 3181, Australia

Abstract

The firm adhesion and transplatelet migration of leukocytes on vascular thrombus are both dependent on the interaction of the leukocyte integrin, Mac-1, and a heretofore unknown platelet counterreceptor. Here, we identify the platelet counterreceptor as glycoprotein (GP) Ib α , a component of the GP Ib-IX-V complex, the platelet von Willebrand factor (vWf) receptor. THP-1 monocytic cells and transfected cells that express Mac-1 adhered to GP Ib α -coated wells. Inhibition studies with monoclonal antibodies or receptor ligands showed that the interaction involves the Mac-1 I domain (homologous to the vWf A1 domain), and the GP Ib α leucine-rich repeat and COOH-terminal flanking regions. The specificity of the interaction was confirmed by the finding that neutrophils from wild-type mice, but not from Mac-1-deficient mice, bound to purified GP Ib α and to adherent platelets, the latter adhesion being inhibited by pretreatment of the platelets with mocarhagin, a protease that specifically cleaves GP Ib α . Finally, immobilized GP Ib α supported the rolling and firm adhesion of THP-1 cells under conditions of flow. These observations provide a molecular target for disrupting leukocyte-platelet complexes that promote vascular inflammation in thrombosis, atherosclerosis, and angioplasty-related restenosis.

Key words: inflammation • leukocytes • platelets • adhesion • receptors

Introduction

Adhesive interactions between vascular cells play important roles in orchestrating the inflammatory response. Recruitment of circulating leukocytes to vascular endothelium requires multistep adhesive and signaling events including selectin-mediated attachment and rolling, leukocyte activation, and integrin-mediated firm adhesion and diapedesis that result in the infiltration of inflammatory cells into the blood vessel wall (1). Firm attachment is mediated by members of the β 2 integrin family, LFA-1 (α L β 2, CD11a/CD18), Mac-1 (α M β 2, CD11b/CD18), and p150,95 (α X β 2, CD11c/CD18), which bind to endothelial counterligands (e.g., intercellular adhesion molecule 1

[ICAM-1]¹) (2), to endothelial-associated extracellular matrix proteins (e.g., fibrinogen) (3), or to glycosaminoglycans (4).

Leukocyte recruitment and infiltration also occur at sites of vascular injury where the lining endothelial cells have been denuded and platelets and fibrin have been deposited. In vivo studies show that leukocytes and platelets colocalize at sites of hemorrhage, within atherosclerotic and postangioplasty restenotic lesions, and in areas of ischemia-reperfusion injury (5–8). This heterotypic interaction between platelets and leukocytes links the hemostatic/thrombotic

Address correspondence to Daniel I. Simon, Brigham and Women's Hospital, Cardiovascular Division, 75 Francis St. Tower 3, Boston, MA 02115. Phone: 617-732-7820; Fax: 617-732-5132; E-mail: dsimon@rics.bwh.harvard.edu

¹Abbreviations used in this paper: BSS, Bernard-Soulier syndrome; GP, glycoprotein; IC₅₀, 50% inhibitory concentration; ICAM-1, intercellular adhesion molecule 1; RGDS, arginine-glycine-aspartate-serine; TRAP, thrombin receptor activating peptide; vWf, von Willebrand factor.

and inflammatory responses (5). Although less well characterized, a similar sequential adhesion model of leukocyte attachment to and transmigration across surface-adherent platelets has been proposed (9–14). The initial tethering and rolling of leukocytes on platelet P-selectin (9, 10, 15) are followed by their firm adhesion and transplatelet migration, processes that are dependent on the leukocyte integrin, Mac-1 (12–14). In addition to promoting the accumulation of leukocytes at sites of platelet coverage within the vasculature, the binding of platelets to neutrophils influences key cellular effector responses by inducing neutrophil activation, upregulating expression of cell adhesion molecules (16), and generating signals that promote integrin activation (13), chemokine synthesis (17, 18), and the respiratory burst (16). Interestingly, both neutrophil–platelet and monocyte–platelet aggregates have been identified in the peripheral blood of patients with coronary artery disease (16, 19) and may be markers of disease activity (16).

Leukocyte Mac-1 binds endothelial cell adhesion molecules of the immunoglobulin superfamily, most prominently ICAM-1. This receptor is not found on platelets, although platelets express a related receptor, ICAM-2 (20). Nevertheless, Diacovo et al. (12) have shown that ICAM-2 blockade has no effect on the firm adhesion of neutrophils on monolayers of activated platelets under flow. Because activated Mac-1 binds fibrinogen, one possibility is that firm adhesion is mediated by Mac-1 binding to fibrinogen that has been immobilized on platelet glycoprotein (GP) IIb-IIIa ($\alpha_{IIb}\beta_3$). Indeed, Weber and Springer (21) found that neutrophil adhesion to activated platelets under flow was partially blocked by an antibody against GP IIb-IIIa and that platelets from a patient with Glanzmann thrombasthenia (which lack GP IIb-IIIa) did not support neutrophil accumulation nearly as well as did wild-type platelets. Nevertheless, neither of these manipulations was successful in completely blocking neutrophil accumulation, even in conjunction with ICAM-2–blocking antibodies. Against a role for the Mac-1–fibrinogen–GP IIb-IIIa axis in neutrophil arrest, Ostrovsky et al. (22) found that neither arginine-glycine-aspartate-serine (RGDS) peptides nor the replacement of normal platelets with thrombasthenic platelets affected the accumulation of the leukocytes on platelets. Despite the differences in the two studies, both groups proposed the existence of another Mac-1 receptor on the platelet surface.

Evaluation of the structural features of integrins has provided us with insights into a candidate platelet counterreceptor for Mac-1. Integrins are heterodimeric proteins composed of one α and one β subunit (23). A subset of integrin α subunits, including CD11b of Mac-1, contains an inserted domain (I domain) of \sim 200 amino acids that is implicated in ligand binding (24–28) and is strikingly similar to the A domains of von Willebrand factor (vWf) (29–31), one of which, A1, mediates the interaction of vWf with its platelet receptor, the GP Ib-IX-V complex. Through this interaction, platelets are able to adhere to regions of vascular injury in a process entirely analogous to the interaction between leukocytes and activated endothelium. Platelets recognize vWf in the subendothelium, and

roll along the region until they become activated and adhere firmly through GP IIb-IIIa (32).

One interesting aspect of the GP Ib-IX-V–vWf interaction is that both platelets and vWf circulate freely in blood, but do not interact unless vWf is immobilized on the subendothelium or in the presence of very high shear stresses (such as might be found at sites of arterial stenosis). Under static conditions in vitro, the interaction requires the presence of modulators: botrocetin, a snake venom protein, or ristocetin, a peptide antibiotic from the soil bacterium *Nocardia lurida* (33). The modulators induce conformational changes in vWf (and possibly also in GP Ib α , in the case of ristocetin) that enable the interaction.

The GP Ib-IX-V complex comprises four phylogenetically related polypeptides, GP Ib α , GP Ib β , GP IX, and GP V, of which only GP Ib α has been shown to bind ligands (34). The ligand-binding domain of this polypeptide resides within the NH₂-terminal 300 amino acids, a region containing seven 24–amino acid leucine-rich repeats, which assign this polypeptide to a large protein superfamily with similar motifs. This region is held above the platelet membrane by a heavily O-glycosylated mucin-like region called the macroglycopeptide. Following a single transmembrane domain, a cytoplasmic domain of \sim 100 amino acids mediates association of the entire complex with the cytoskeleton and with signaling proteins (33).

Because of the similarity of the vWf A1 domain and the α M I domain, we hypothesized that GP Ib α might also be able to bind Mac-1. Here, we report that GP Ib α is a constitutively expressed counterreceptor for Mac-1.

Materials and Methods

Materials. Human fibrinogen depleted of plasminogen, vWf, and fibronectin were purchased from Enzyme Research Laboratories. Porcine heparin (10,000 U/ml) was obtained from Elkins-Sinn, Inc. TGF- β 1 was from Collaborative Research, Inc., and 1,25-(OH)₂ vitamin D₃ was from Calbiochem. The snake venom metalloprotease, mocarhagin, was purified as described previously (35). Glycocalicin was purified by a minor modification of the method of Canfield et al. (36) by successive chromatography on wheat germ lectin Sepharose 6MB (Amersham Pharmacia Biotech) and jacalin agarose (Pierce Chemical Co.) (Fig. 1). A 39/34-kD disperse fragment of vWf encompassing the A1 domain (Leu480–Gly718) was purified as described previously (37). Peptide P2, corresponding to amino acid residues 377–395 in the γ chain of fibrinogen, which binds to the I domain of Mac-1 and blocks fibrinogen binding (38), was obtained from the W.M. Keck Biotechnology Resource Center (Yale University, New Haven, CT). 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF AM) was purchased from Molecular Probes.

CD11/CD18 mAbs used included the following: LPM19c, directed to the α M subunit of human Mac-1 (CD11b) and capable of blocking fibrinogen, ICAM-1, and C3bi binding (a gift of Dr. Karen Pulford, John Radcliffe Hospital, Oxford, UK) (24); M1/70, directed to the α M subunit of mouse Mac-1 (CD11b), with broad ligand blocking properties (39; American Type Culture Collection); TS1/22, directed to the α L subunit of LFA-1 (CD11a) and capable of blocking ICAM binding (provided by Dr.

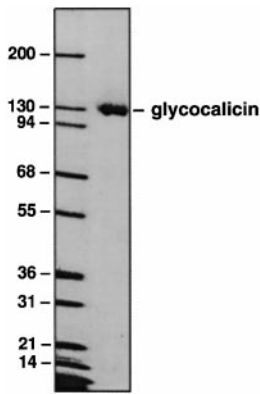


Figure 1. Purified glycofocalicin. Purified glycofocalicin from human platelets was subjected to SDS and 7.5% PAGE and stained with Coomassie brilliant blue.

Lloyd Klickstein, Brigham and Women's Hospital); and IB4, directed to the common $\beta 2$ subunit (CD18; provided by Dr. Lloyd Klickstein). The stimulating CD18 mAb KIM 127 was a gift of Dr. Martyn Robinson (Celltech Ltd., Slough, England) (40).

The GP Ib α mAbs used were: AK2, AP1, VM16d, SZ2, and WM23. All but WM23 bind within the GP Ib α ligand-binding region, within the first 282 amino acids at the GP Ib α NH $_2$ terminus. AK2 blocks vWf binding induced by both ristocetin and botrocetin and binds within the first leucine-rich repeat (amino acid residues 36–59). AP1 (provided by Dr. Dermot Kenny, Beaumont Hospital, The Royal College of Surgeons, Dublin, Ireland) also blocks both ristocetin- and botrocetin-induced vWf binding (epitope 201–268); VM16d (gift of Dr. Alexey Mazurov, Russian Ministry of Health, Moscow, Russia) only blocks botrocetin-induced vWf binding (epitope 201–268), as does SZ2 (epitope 269–282) (41). WM23 binds within the GP Ib α macroglycopeptide and does not interfere with the binding of any GP Ib α ligands (42). The polyclonal GP Ib α antibody was prepared in rabbits immunized with glycofocalicin (the soluble extracellular region of GP Ib α) and affinity-purified over a glycofocalicin Affigel 10/15 column (43).

7E3 and 10E5 (44), murine mAbs to GP IIb and/or IIIa that block platelet fibrinogen binding, were provided by Dr. Barry S. Coller (Mt. Sinai Medical Center, New York, NY). Y2/51 (Dako) is directed against CD61 (GP IIIa) and was purchased conjugated to FITC. TUK4 (Dako) is directed against CD14 on the monocyte surface and was purchased conjugated to PE. Y2/51 and TUK4 are IgG murine antibodies. Murine IgG-FITC (Dako) was used as a nonspecific isotype control. All mAbs were purified unless otherwise indicated.

Cell Lines and Culture Conditions. THP-1 monocytic cells (American Type Culture Collection) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 25 mM Hepes, penicillin, and streptomycin. Differentiation of THP-1 cells (10^6 /ml), which is accompanied by increased expression of Mac-1, was induced by treatment with 1 ng/ml TGF- β 1 and 50 nM 1,25-(OH) $_2$ vitamin D $_3$ (TGF- β /vit D $_3$) for 24 h (45). Nontransfected 293 cells (American Type Culture Collection) were maintained in DMEM/F12 supplemented with 10% fetal bovine serum, 25 mM Hepes, penicillin, and streptomycin; 293 cells expressing human LFA-1, Mac-1, or a chimeric LFA-1 receptor that contained the I domain of Mac-1 (i.e., LFA-1 [I α M]-transfected 293 cells), established as described previously (46–48), were maintained in similar media containing G418.

Preparation of Neutrophils and Platelets. Mac-1-deficient (Mac-1 $^{-/-}$) mice, generated as described previously (49), were backcrossed for >12 generations onto the C57BL/J6 strain. Murine

neutrophils were harvested from the peritoneal cavity of wild-type C57BL/J6 (Mac-1 $^{+/+}$) or Mac-1 $^{-/-}$ mice by lavage with 10 ml RPMI 4 h after the intraperitoneal injection of 1 ml sterile 3% thioglycollate broth. Isolated neutrophils were resuspended in RPMI containing low-endotoxin BSA (0.5%). Cytospin preparations stained with Giemsa revealed that >90% of the cells were neutrophils. Cell viability (>95%) was assessed in all cases using trypan blue exclusion.

Venous blood was obtained from volunteers who had not consumed aspirin or other nonsteroidal antiinflammatory drugs for at least 10 d, and was anticoagulated with 13 mM trisodium citrate. Platelet-rich plasma was prepared by centrifugation at 150 *g* for 10 min. Gel-filtered platelets were obtained by passage of platelet-rich plasma over a Sepharose 2B column in calcium-free Tyrode's Hepes buffer, as described previously (50). Platelet counts were measured using a Coulter counter (model ZM) and adjusted to 150,000/ μ l by the addition of buffer.

Adhesion Assays. Adherent cells were assayed by colorimetry (45, 51) or by loading THP-1 or 293 cells and thioglycollate-elicited murine neutrophils with BCECF AM (1 μ M) according to the manufacturer's protocol. Cells (10^5 /well) were placed in 96-well microtiter plates coated with purified GP Ib α (10 μ g/ml) or fibrinogen (10 μ g/ml) and blocked with gelatin (0.2%). Adhesion was stimulated with PMA (17 ng/ml) or the $\beta 2$ -stimulating mAb KIM 127 (5 μ g/ml). Plates were washed with 0.9% NaCl (three to five times), adherent cells were fixed in methanol for 15 min and stained with Giemsa, and adhesion was quantified by measuring absorbance at 540 nm. Alternatively, adhesion was quantified by measuring the fluorescence of BCECF AM-loaded cells using a Cytofluor II fluorescence multiwell microplate reader (PerSeptive Biosystems). The effect of anti-CD11/CD18 mAbs or soluble Mac-1 ligands (i.e., fibrinogen, heparin) on adhesion was assessed by preincubating cells with the indicated mAb (10 μ g/ml) or ligand for 15 min at 37°C; the effect of anti-GP Ib α mAbs on adhesion was investigated by incubating the indicated mAb (10 μ g/ml) with GP Ib α -coated wells for 30 min at 37°C before the addition of cells. Data are expressed as percent inhibition of maximum adherent responses of respective sets of treatment.

In the case of 293 cell adhesion experiments, low passage (1 to 3) human saphenous vein endothelial cells (provided by Dr. Peter Libby, Brigham and Women's Hospital) were grown to confluence in 96-well microtiter wells and stimulated with TNF- α (10 ng/ml) for 4 h to upregulate ICAM-1 expression (52). 293 cells were loaded with BCECF AM for 45 min at 37°C, washed, and stimulated with KIM 127 (5 μ g/ml) before adding to endothelial cell monolayers.

Purified I Domain Binding Experiments. High-binding microtiter plates (MaxiSorp; Nunc) were coated with purified I domain (10 μ g/ml), obtained as described previously (38), in Tris-buffered saline (TBS), pH 7.4, and then blocked with buffer containing 0.5% gelatin. Biotinylated glycofocalicin (0–50 μ g/ml) was added to each well in TBS containing 1 mM CaCl $_2$ and MgCl $_2$ and 0.5% gelatin, and plates were incubated for 60 min at 25°C. After washing, bound glycofocalicin was quantified with avidin peroxidase. Specific binding was determined by subtracting binding to wells coated with gelatin alone and accounted for up to 40% of the total binding.

Neutrophil Adhesion to Surface-adherent Platelets. Neutrophil adhesion to surface-adherent platelets was investigated as described previously (12). Gel-filtered human platelets ($\sim 1.5 \times 10^7$) were added to 96-well microtiter plates coated overnight with 0.2% gelatin. After 45 min at 37°C, unbound platelets were removed by washing. Neutrophils (1.5×10^5) were loaded with

1 μM BCECF AM, washed twice, and then added to each well for 60 min at 37°C in 5% CO₂. After washing, neutrophil adhesion was quantified as the percentage of total cells adherent by measuring the fluorescence of BCECF AM-loaded cells using a Cytofluor II fluorescence multiwell microplate reader (PerSeptive Biosystems). Fluorescence of input neutrophils before washing served as a measure of total cell number. The effect of mAbs on neutrophil adhesion to platelets was assessed as described above for purified GP Ib α ; the effect of the snake venom metalloprotease, mocarhagin, which cleaves GP Ib α at peptide bond 282–283 (35), on leukocyte adhesion to platelets was examined by preincubating surface-adherent platelets with mocarhagin for 30 min at 37°C. Data are expressed as percent inhibition of maximum adherent responses of respective sets of treatment.

Whole Blood Detection of Platelet–Leukocyte Aggregates. Leukocyte–platelet aggregates were measured by two-color flow cytometry in a FACSCalibur™ flow cytometer (Becton Dickinson) by slight modifications of methods described previously (19). Peripheral blood was drawn from a healthy volunteer or, as indicated, from a patient with Bernard-Soulier syndrome (BSS) (53) who had not ingested aspirin or other antiplatelet drugs during the previous 10 d. The first 2 ml of drawn blood was discarded. Blood was then drawn into a 3.2% sodium citrate Vacutainer (Becton Dickinson). The sample was diluted 1:1 with modified Tyrode's Hepes buffer, pH 7.4, and then immediately incubated at 22°C for 10 min with either (a) buffer alone; (b) 0.5 μM ADP (Biodata); or (c) 5 μM thrombin receptor activating peptide (TRAP; 14-mer; Calbiochem) and saturating concentrations of Y2/51-FITC and TUK4-PE. The samples were then fixed at 22°C for 10 min to a final concentration of 1% formalin (Polysciences, Inc.) and 1.5 \times HBSS concentrate (GIBCO BRL). The samples were then diluted with 500 μl of distilled H₂O, vortexed, and incubated at 22°C for 10 min to lyse the red blood cells. Monocytes and neutrophils were identified by CD14-PE positivity and their characteristic light scatter.

THP-1 Adhesion to Glycocalicin under Flow. The interaction of TGF- β 1/vit D₃-stimulated THP-1 cells with immobilized glycocalicin was examined using a parallel-plate flow chamber system, which has been described previously (54). Glass coverslips, which form the bottom of the chamber, were coated with purified glycocalicin (100 $\mu\text{g}/\text{ml}$) by immersing them in the glycocalicin solution for 3 h at 37°C. Residual nonspecific binding sites were then blocked with 3% BSA for 30 min. The chamber was then assembled and mounted onto an inverted-stage microscope (DIAPHOT-TMD; Nikon) equipped with a silicon-intensified target video camera (model C2400; Hamamatsu) connected to a video cassette recorder. Cells were suspended in culture medium and the suspension was brought to room temperature. 1 ml of the cell suspension (10⁶ cells/ml) was then injected into the chamber and incubated for 1 min. The cells were allowed to settle onto the glycocalicin substrate for 1 min; the chamber was then perfused with the PBS at a flow rate calculated to generate fluid shear stress of 2 dyn/cm². The attachment and rolling of cells in a single-view field were recorded in real time for 3 min, and the video data were then analyzed using imaging software (IC-300 Modular Image Processing Workstation; Inovision Corp.) to calculate the rolling velocities. The rolling events scored were ligand specific as confirmed in parallel determinations on control substrates coated with heat-stable antigen (HSA). In the study to inhibit glycocalicin, the coverslip was incubated with the GP Ib α mAb VM16d (25 $\mu\text{g}/\text{ml}$) for 30 min at room temperature and washed before assembly of the chamber. To test the effect of Mac-1 inhibition, the THP-1 cells were incubated

with LMP19c (25 $\mu\text{g}/\text{ml}$) for 30 min at room temperature before injection into the chamber. Rolling cells were those observed to be moving in the direction of flow while maintaining constant contact with the glycocalicin substrate.

Statistics. All data are presented as the mean \pm SD. Groups were compared using the nonpaired *t* test. *P* values < 0.05 were considered significant.

Results

Mac-1–expressing Cells Bind to GP Ib α . Given the homology between the receptor-binding vWf A1 domain and the Mac-1 I domain, we hypothesized that Mac-1 would bind the platelet GP Ib-IX-V complex. To assess this potential interaction, we assayed the adhesion of Mac-1-bearing cells to purified glycocalicin, the soluble extracellular domain of GP Ib α . We have previously shown that stimulation of THP-1 monocytic cells (which constitutively ex-

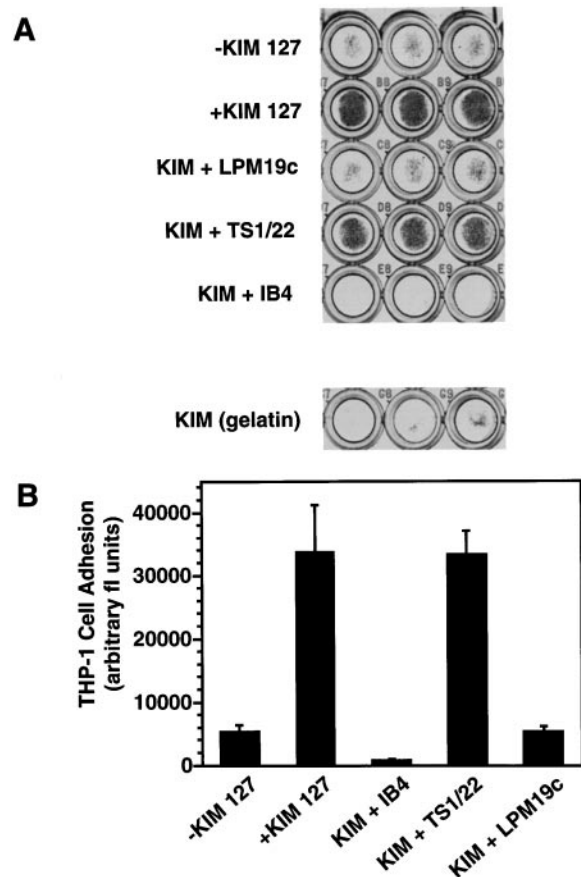


Figure 2. THP-1 cell adhesion to GP Ib α is Mac-1 dependent. Cytokine-treated THP-1 cells were added to glycocalicin (GP Ib α)-coated and gelatin-blocked wells. Adhesion was promoted by the addition of the stimulating mAb KIM 127 (5 $\mu\text{g}/\text{ml}$). The effect of anti-CD18 (IB4), anti-CD11a (TS1/22), and anti-CD11b (LPM19c) mAbs on adhesion was assessed as described in Materials and Methods. After washing, (A) adherent cells were stained with Giemsa and (B) adhesion was quantified by measuring the fluorescence of BCECF AM-loaded THP-1 cells. Triplicate determination (mean \pm SD) representative of three to five separate experiments.

press Mac-1) with TGF- β 1 and 1,25-(OH) $_2$ vitamin D $_3$ increases Mac-1 surface expression \sim 2.0-fold (45). When cytokine-treated THP-1 cells were stimulated with either phorbol ester (PMA) or KIM 127, an mAb to CD18 that induces a change in the conformation of CD18 and promotes both LFA-1- and Mac-1-dependent adhesion (40), they adhered robustly to wells coated with GP Ib α and blocked with gelatin, but not to wells coated with gelatin alone (Fig. 2). Adhesion of the cells to GP Ib α was inhibited by IB4, an anti-CD18 mAb that blocks both LFA-1- and Mac-1-dependent functions, indicating that adhesion is CD18 dependent (Fig. 2, and Table I). THP-1 cell adhesion to GP Ib α was also completely blocked by LPM19c, an mAb that binds to the I domain of the α M subunit of Mac-1 (CD11b) and blocks the binding of fibrinogen,

Table I. Summary of Inhibition of KIM 127-stimulated THP-1 Adhesion to GP Ib α by Antibodies or Soluble Ligands

Antibody or ligand	Receptor (epitope)	Percent inhibition
IB4	Anti-CD18	99 \pm 1*
TS1/22	Anti-CD11a (I domain)	10 \pm 13
LPM19c	Anti-CD11b (I domain)	92 \pm 12*
Polyclonal	Anti-GP Ib α	80 \pm 17*
Polyclonal	Rabbit IgG control	16 \pm 20
VM16d	Anti-GP Ib α (aa residues 201-268)	83 \pm 16*
AP1	Anti-GP Ib α (aa residues 201-268)	86 \pm 10*
AK2	Anti-GP Ib α (aa residues 36-59)	16 \pm 6
SZ2	Anti-GP Ib α (sulfated tyrosine residues 269-282)	8 \pm 6
WM23	Anti-GP Ib α (macroglycopeptide)	11 \pm 5
7E3	Anti-GP IIb-IIIa, - α v β 3, -Mac-1	16 \pm 8
10E5	Anti-GP IIb-IIIa	18 \pm 6
Fibrinogen (2 μ M)	Mac-1	99 \pm 1*
P2 γ 377-395 (10 μ M)	Mac-1	0
Heparin (200 U/ml)	Mac-1	93 \pm 9*
vWF A1 (20 μ g/ml)	GP Ib α	83 \pm 13*

The adhesion of cytokine-treated THP-1 cells to glyocalicin (GP Ib α)-coated microtiter wells was stimulated by the addition of KIM 127 (5 μ g/ml) in the presence and absence of antibodies directed to or soluble ligands of Mac-1 and GP Ib α . Polyclonal antibodies were added at 20 μ g/ml and purified mAbs at 10 μ g/ml as described in Materials and Methods. Soluble ligand concentrations are indicated. CD11/CD18 mAbs included IB4, TS1/22, and LPM19c; GP Ib α mAbs used were AK2, AP1, VM16d, SZ2, and WM23. Data are expressed as percent inhibition of maximal KIM-stimulated adhesion by the antibodies or soluble ligands (mean \pm SD, n = 3-5). aa, amino acids.

* P < 0.01.

C3bi, and ICAM (24). In contrast, the anti-LFA-1 mAb TS1/22 had no effect on adhesion. Similar results were obtained with integrin mAbs whether THP-1 cell adhesion was stimulated with KIM 127 or PMA, ruling out the possibility that the mAbs inhibited adhesion by interfering with the binding of KIM 127 to CD18.

To confirm that Mac-1 mediates the adhesion of THP-1 cells to GP Ib α and to eliminate the possibility that mAbs to Mac-1 inhibit THP-1 cell adhesion indirectly, we assessed the adhesion of 293 cells transfected with either LFA-1 or Mac-1 to GP Ib α . Flow cytometry confirmed similar expression levels of LFA-1 and Mac-1 (Fig. 3 A). Both LFA-1-transfected and Mac-1-transfected 293 cells adhered robustly to human endothelial cells expressing ICAM-1 (Fig. 3 B), indicating that these transfected 293 cells are functional. Mac-1-expressing 293 cells adhered to GP Ib α , and this adhesion was enhanced by KIM 127 (Fig. 3 C). KIM 127-stimulated adhesion was blocked by mAbs directed to both CD11b and CD18, and to GP Ib α (VM16d). Neither untransfected 293 cells nor LFA-1-transfected 293 cells bound to GP Ib α .

Neutrophil Binding to GP Ib α Requires Mac-1. To investigate further the role of Mac-1 in leukocyte adhesion to GP Ib α , we examined the binding of neutrophils from wild-type (Mac-1 $^{+/+}$) and Mac-1-deficient (Mac-1 $^{-/-}$) mice to GP Ib α . Mac-1 $^{+/+}$ neutrophils bound to GP Ib α , adhesion that was enhanced by PMA pretreatment (Fig. 4). In contrast, Mac-1 $^{-/-}$ neutrophils showed significantly reduced adhesion to GP Ib α , whether in the absence or presence of PMA. Taken together, these observations indicate that Mac-1 mediates adhesion to purified GP Ib α .

The Mac-1 I Domain Serves as a Recognition Site for GP Ib α . The adhesion of Mac-1-bearing THP-1 cells to GP Ib α was inhibited by LPM19c (Fig. 2), an mAb that binds to the I domain of the α M subunit of Mac-1 (CD11b). To establish definitively that the Mac-1 I domain serves as a recognition site for GP Ib α , we transfected 293 cells with a chimeric LFA-1 receptor that contained the I domain of Mac-1 (i.e., LFA-1 [I α M]-transfected 293 cells). Flow cytometry showed comparable expression of LFA-1 (I α M), LFA-1, and Mac-1 in transfected 293 cells (Fig. 3 A). LFA-1-transfected 293 cells did not adhere to GP Ib α (Fig. 3 C); in contrast, LFA-1 (I α M)-transfected 293 cells adhered robustly to GP Ib α (Fig. 5 A), indicating that the I domain of Mac-1 is required for adhesion to GP Ib α . We next investigated whether GP Ib α would directly bind to purified I domain of Mac-1. Biotinylated glyocalicin bound to immobilized I domain in a concentration-dependent manner over the range 0-200 nM (Fig. 5 B).

To evaluate further the I domain as a recognition site on Mac-1 for GP Ib α , we examined the ability of Mac-1 I domain ligands (e.g., fibrinogen [24] and heparin [4]) to inhibit THP-1 adhesion to GP Ib α . Soluble fibrinogen (50% inhibitory concentration [IC $_{50}$] \sim 1 μ M) and heparin (IC $_{50}$ \sim 75 U/ml) both inhibited adhesion in a dose-dependent manner (Fig. 6, A-C). Inhibition by fibrinogen suggested to us the possibility that the GP Ib α and fibrinogen binding sites in Mac-1 may be partially overlapping. To exclude

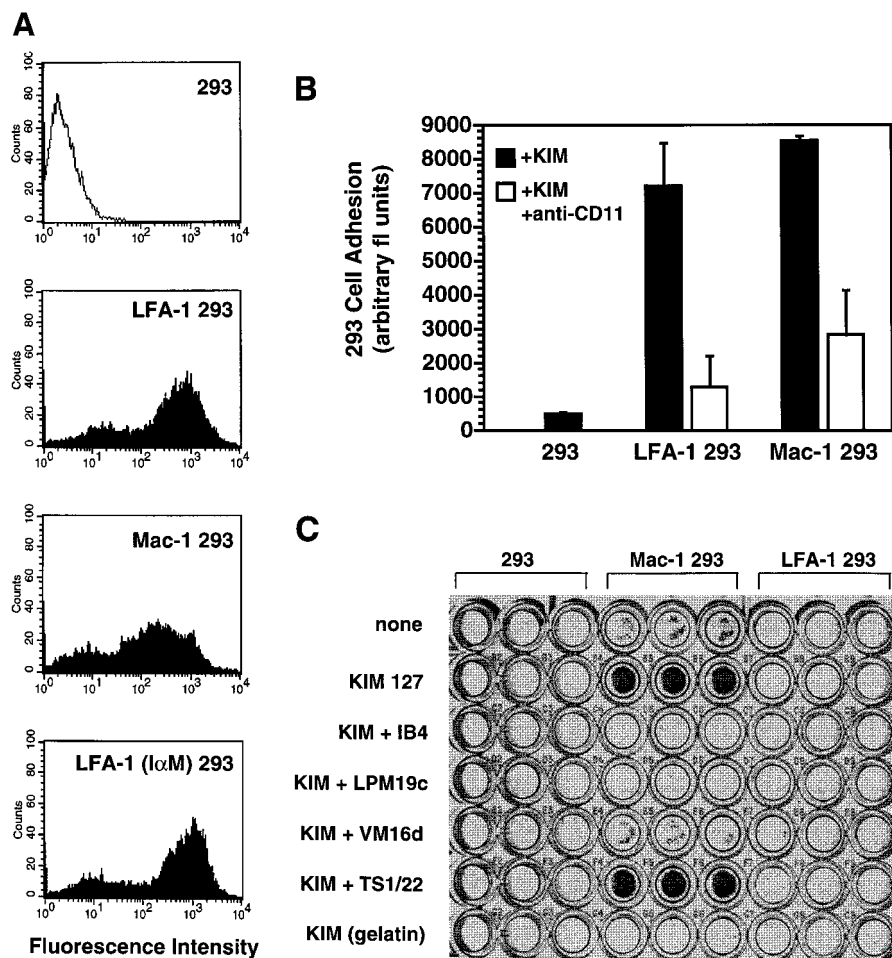


Figure 3. Mac-1 expression is required for adhesion to GP I β α . (A) Expression of CD11/CD18 integrin in 293 cells was confirmed by flow cytometry using the anti-CD18 mAb IB4. Equivalent expression of CD18 is observed in 293 cells transfected with human LFA-1, Mac-1, or a chimeric LFA-1 containing the I domain of Mac-1, designated LFA-1 (α M). (B) Adhesion of untransfected 293 cells and 293 cells transfected with human Mac-1 or LFA-1 to human endothelial cells stimulated with TNF- α to express ICAM-1. Adhesion was promoted by the addition of the stimulating mAb KIM 127 (5 μ g/ml) and quantified by measuring the fluorescence of BCECF AM-loaded 293 cells; triplicate determination (mean \pm SD) representative of two separate experiments. (C) 293 cells were added to glycolocalicin-coated and gelatin-blocked wells. Adhesion was promoted by the addition of KIM 127 (5 μ g/ml). After washing, adherent cells were stained with Giemsa. Triplicate determination representative of three separate experiments.

potential nonspecific steric hindrance of fibrinogen, we examined the effect of peptide P2, corresponding to amino acid residues 377–395 in the γ chain of fibrinogen, that binds to the I domain of Mac-1 and blocks fibrinogen

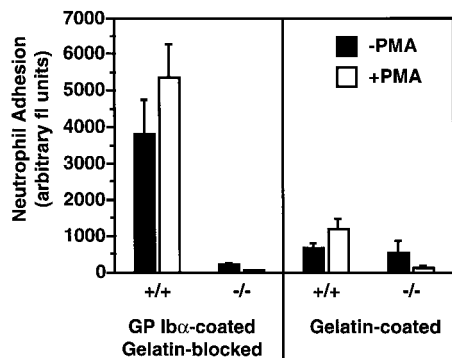


Figure 4. Neutrophil binding to GP I β α requires Mac-1. Thioglycolate-elicited neutrophils from wild-type (Mac-1^{+/+}) or Mac-1-deficient (Mac-1^{-/-}) mice were added to glycolocalicin-coated and gelatin-blocked wells in the absence (black bars) and presence (white bars) of PMA (17 ng/ml). Adhesion was promoted by the addition of PMA (17 ng/ml). Adhesion was quantified by measuring the fluorescence of BCECF AM-loaded neutrophils. Triplicate determination (mean \pm SD) representative of three separate experiments.

binding ($IC_{50} \sim 1 \mu$ M) (38). P2 had no effect on THP-1 adhesion to GP I β α , suggesting that the GP I β α and fibrinogen binding sites are probably distinct.

To evaluate the interaction between Mac-1 and GP I β α more quantitatively and to confirm that Mac-1 did not recognize a binding site present solely on immobilized GP I β α , we tested soluble GP I β α for its ability to inhibit Mac-1-dependent THP-1 cell adhesion to immobilized GP I β α (Fig. 6 B) or fibrinogen (Fig. 6 C). Soluble GP I β α inhibited THP-1 adhesion to wells coated with either GP I β α ($IC_{50} = 0.5 \mu$ M) or fibrinogen ($IC_{50} = 0.25 \mu$ M). Conversely, the vWf A1 domain (20 μ g/ml), a ligand for GP I β α , blocked binding of THP-1 cells to immobilized GP I β α (percent inhibition = 83 ± 13) (Table I).

Identifying the Mac-1 Binding Site within GP I β α . We next turned our attention to identifying the Mac-1 interaction site within GP I β α by assessing the effect of monoclonal and polyclonal antibodies to GP I β α on Mac-1-dependent THP-1 cell adhesion to glycolocalicin (Fig. 7, and Table I). Polyclonal anti-GP I β α , but not control rabbit IgG, significantly reduced THP-1 cell adhesion. VM16d and AP1, which map to the leucine-rich COOH-terminal flanking region of GP I β α (amino acids 201–268), inhibited KIM 127-stimulated adhesion of THP-1 cells to GP

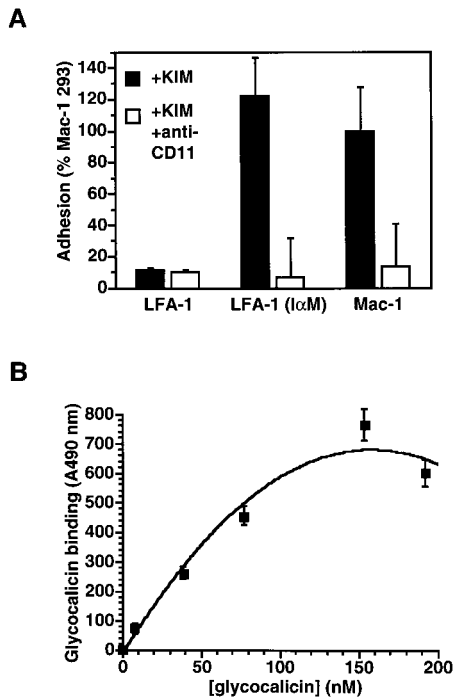


Figure 5. Mac-1 I domain serves as a recognition site for GP Ib α . (A) 293 cells transfected with human Mac-1, LFA-1, or a chimeric LFA-1 containing the I domain of Mac-1 (LFA-1 [I α M]) were added to glyco-calcin-coated and gelatin-blocked wells. Adhesion was promoted by the addition of the stimulating mAb KIM 127 (5 μ g/ml) and quantified by measuring the fluorescence of BCECF AM-loaded 293 cells. Triplicate determination (mean \pm SD) representative of three separate experiments. (B) Interaction of purified glyco-calcin and Mac-1 I domain. Specific binding of biotinylated glyco-calcin (0–200 nM) to purified I domain-coated and gelatin-blocked wells as described in Materials and Methods (B). Triplicate determination (mean \pm SD), $n = 2$.

Ib α (percent inhibition: VM16d = 83 ± 16 ; AP1 = 86 ± 10). A similar inhibitory effect of these mAbs was noted when THP-1 cell adhesion to GP Ib α was stimulated by PMA (percent inhibition: VM16d = 90 ± 4 ; AP1 = 84 ± 2). In contrast, neither AK2, directed to the first leucine-rich repeat (amino acids 36–59), nor WM23, directed to the macroglycopeptide mucin core region of GP Ib α , had any significant effect on THP-1 cell adhesion. An irrelevant mAb against GP IIB-IIIa (10E5) also had no significant effect on THP-1 cell adhesion.

Our observation that heparin inhibited Mac-1-dependent adhesion to GP Ib α led us to consider the possibility that the region within GP Ib α containing sulfated tyrosine residues (an anionic stretch between residues Asp269 and Asp289 which contains three sulfated tyrosines, Tyr276, Tyr278, and Tyr279) might be involved in the interaction with Mac-1. We tested this possibility by assessing the effect of SZ2, an anti-GP Ib α mAb that maps within the region containing the sulfated tyrosines and requires sulfation for its epitope. SZ2 did not affect THP-1 cell adhesion to GP Ib α , suggesting that heparin likely inhibits adhesion by binding the Mac-1 I domain and interfering with GP Ib α binding rather than by directly mimicking a binding site on GP Ib α (4).

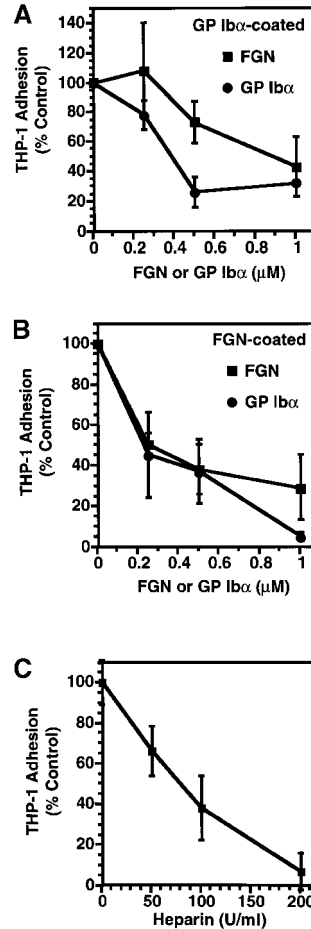


Figure 6. Mac-1–GP Ib α binding and Mac-1 I domain ligands. Cytokine-treated THP-1 cells were added to (A) glyco-calcin-coated or (B) fibrinogen (FGN)-coated wells. Adhesion was promoted by the addition of KIM 127 (5 μ g/ml) in the presence of increasing concentrations (0–2 μ M) of soluble GP Ib α (○) or the soluble Mac-1 I domain ligand fibrinogen (■). (C) The adhesion of THP-1 cells to glyco-calcin (GP Ib α)-coated wells in the presence of increasing concentrations of a second Mac-1 I domain ligand, heparin (0–100 U/ml), was also examined. Adhesion was quantified by measuring the fluorescence of BCECF AM-loaded cells and expressed relative to maximal adhesion stimulated with KIM 127. Mean \pm SD, $n = 3$.

Glyco-calcin Supports the Rolling and Firm Adhesion of THP-1 Cells under Flow. To evaluate the potential for the GP Ib α –Mac-1 interaction to support the adhesion of blood cells under flow, we perfused THP-1 cells over coverslips coated with a glyco-calcin matrix using a parallel-plate flow chamber system. The cells were either kept in their native state or treated with TGF- β 1 and 1,25-(OH) $_2$ vitamin D $_3$ to increase Mac-1 expression. THP-1 cells were able to adhere to the glyco-calcin, whether or not they were induced to differentiate, but did not adhere to control, BSA-coated coverslips (Fig. 8). However, undifferentiated cells that adhered to the glyco-calcin exhibited mainly rolling behavior, whereas differentiated cells adhered more firmly. The antibodies LMP19c and VM16d both greatly decreased the number of attached cells, confirming the involvement of both Mac-1 and GP Ib α , respectively, in the adhesion of THP-1 cells under flow.

Mac-1 and GP Ib α Facilitate the Interaction between Leukocytes and Platelets. Finally, to establish that Mac-1 and GP Ib α facilitate the heterotypic interaction between leukocytes and platelets, we assayed the adhesion of wild-type and Mac-1-deficient neutrophils to platelets. Thioglycolate-elicited Mac-1 $^{+/+}$ neutrophils bound to adherent

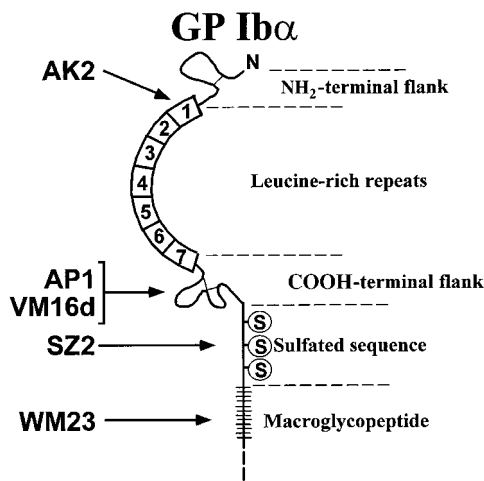


Figure 7. Epitope map of mAbs to GP Ib α . Schematic representation of the extracellular domain of GP Ib α . The GP Ib α mAbs employed were: AK2, AP1, VM16d, SZ2, and WM23. AK2 binds within the first leucine-rich repeat (amino acid residues 36–58). AP1 and VM16d bind to the COOH-terminal flanking and leucine-rich repeat region (201–268). SZ2 maps to the sulfated tyrosine residues encompassing amino acids 268–282 (reference 41). WM23 binds within the macroglycopeptide region of GP Ib α (reference 42).

platelets, and this adhesion was promoted by PMA (Fig. 9 A). In contrast, Mac-1^{-/-} neutrophils demonstrated markedly reduced adhesion to platelets. Adhesion of Mac-1^{+/+} neutrophils was also blocked by the rat anti-mouse Mac-1 mAb M1/70 (percent inhibition = 95 \pm 5) and the anti-GP Ib α mAb VM16d (percent inhibition = 55 \pm 10). Furthermore, Mac-1^{+/+} leukocyte adhesion to platelets was

inhibited dose-dependently by soluble glycolalgin (percent inhibition = 72 \pm 18) and by pretreatment of adherent platelets with the snake venom metalloprotease, mocarhagin (percent inhibition = 89 \pm 8), which cleaves GP Ib α at peptide bond 282–283 as the only detectable proteolytic event on the platelet surface (35) (Fig. 9 B). Taken together, these observations indicate that neutrophil adhesion to platelets is primarily mediated by Mac-1 and GP Ib α .

Finally, to provide additional evidence supporting a role for GP Ib α in platelet binding to leukocytes, we assessed the presence of leukocyte–platelet aggregates in whole blood obtained from a normal volunteer or a patient with BSS. We have previously shown that spontaneous or agonist-induced leukocyte–platelet aggregate formation require an interaction between P-selectin glycoprotein ligand 1 (PSGL-1) and P-selectin that is strengthened by integrins (19). Leukocyte–platelet aggregates were decreased in the circulation of a patient with BSS compared with a normal control (2 vs. 7% platelet-positive neutrophils; Fig. 10). Moreover, these leukocyte–platelet aggregates were less likely to form in the BSS patient after agonist stimulation with either 0.5 μ M ADP (4 vs. 21% platelet-positive neutrophils) or 5 μ M TRAP (13 vs. 86% platelet-positive neutrophils). Mac-1 dependence in leukocyte–platelet aggregate formation in this assay was confirmed by the fact that the anti-Mac-1 mAb LPM19c completely inhibited agonist-induced platelet–neutrophil complex formation.

Discussion

In this study, we have identified a direct interaction between the leukocyte integrin Mac-1 and platelet GP Ib α . The following evidence was obtained for this interaction: (a) mAbs to both Mac-1 and GP Ib α inhibited THP-1 cell

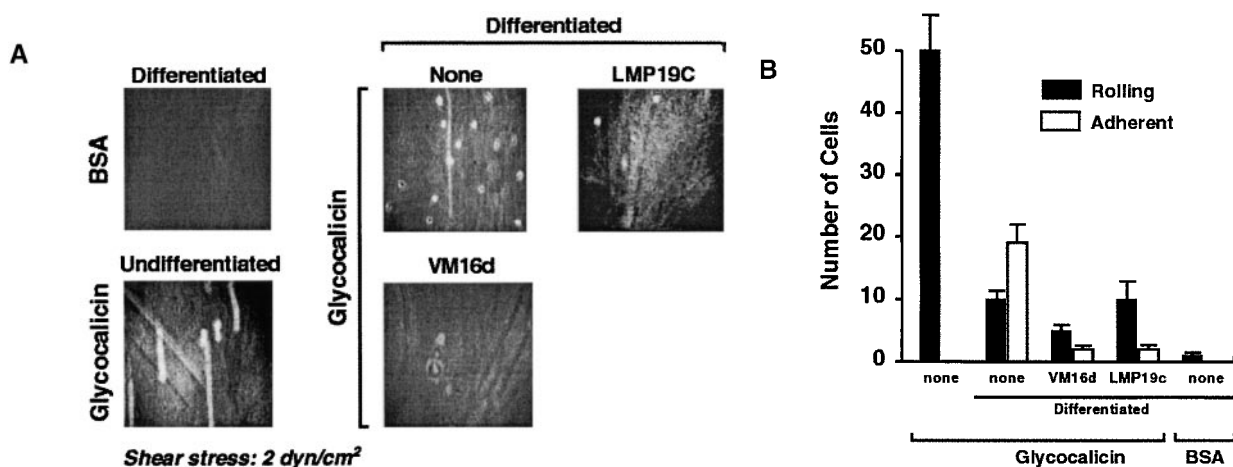


Figure 8. Adhesion of THP-1 cells to glycolalgin under flow. (A) Video images of THP-1 cells rolling on or firmly adherent to immobilized glycolalgin at a wall shear stress of 2 dyn/cm² in a parallel-plate flow chamber. Images were created using a digital image processing system to snap frames of previously recorded experiments. The cells were either untreated (Undifferentiated) or induced to differentiate with TGF- β 1 and 1,25-(OH)₂ vitamin D₃ then injected into the chamber and allowed to settle on the matrix for 1 min. The chamber was then perfused with buffer at a velocity calculated to generate the desired shear stress. Images were created by overlapping 30 frames taken over 1 s. The effect of mAbs VM16d (anti-GP Ib α) and LMP19c (anti-CD11b) were assessed by preincubating, respectively, either the coverslip or the cells with saturating concentrations of antibody. BSA-coated coverslips were used as a control matrix. (B) Quantitation of rolling and firmly adherent cells from experiments such as those represented in A (mean \pm SEM, *n* = 4).

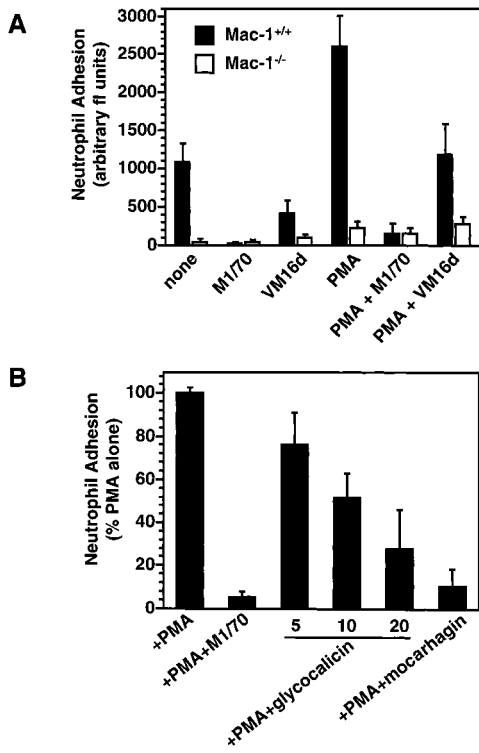


Figure 9. Neutrophil binding to platelets requires Mac-1 and GP I β α . (A) Thioglycollate-elicited neutrophils from wild-type (Mac-1^{+/+}, black bars) or Mac-1-deficient (Mac-1^{-/-}, white bars) mice were added to surface-adherent platelets. Adhesion was promoted by the addition of PMA (17 ng/ml). The contribution of Mac-1 or GP I β α to wild-type neutrophil adhesion to platelets was assayed by the addition of the rat anti-mouse CD11b mAb, M1/70 (10 μ g/ml), or the anti-GP I β α mAb, VM16d (10 μ g/ml), respectively. (B) The effect of soluble glycocalicin (0–20 μ g/ml) and the snake venom metalloprotease, mocarhagin, which cleaves GP I β α at peptide bond 282–283, on neutrophil adhesion was also examined. Adhesion was quantified by measuring the fluorescence of BCECF AM-loaded neutrophils. Triplicate determination (mean \pm SD) representative of three separate experiments.

adhesion to purified GP I β α ; (b) 293 cells that express Mac-1, but not LFA-1, bound strongly to GP I β α , and this adhesion was inhibited specifically by mAbs; (c) wild-type, but not Mac-1-deficient, neutrophils adhered to platelets and to purified GP I β α ; (d) neutrophil adhesion to platelets was inhibited by mAbs to Mac-1 and GP I β α and by pre-treatment of the platelets with the snake venom metalloprotease, mocarhagin, whose major platelet substrate is GP

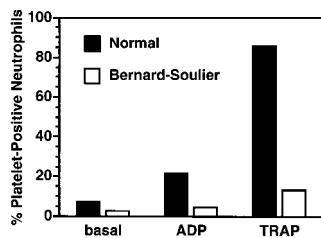


Figure 10. Absence of GP I β α reduces neutrophil-platelet aggregates in whole blood. Leukocyte-platelet aggregates in peripheral blood obtained from a normal volunteer (black bars) or a patient with BSS (white bars) were measured by two-color flow cytometry as described in Materials and Methods. Formation of leukocyte-platelet aggregates was

stimulated by incubating blood with ADP (0.5 μ M) or TRAP (5 μ M). Data shown are mean values of duplicate determinations.

I β α (35); and (e) basal and agonist-stimulated leukocyte-platelet aggregates were decreased in whole blood of a patient with BSS compared with a normal control.

By virtue of binding diverse ligands including, among others, fibrin(ogen) (55, 56), ICAM-1 (57), factor X (58), C3bi (55), high molecular weight kininogen (59), and heparin (4), Mac-1 regulates important leukocyte functions including adhesion, migration, coagulation, proteolysis, phagocytosis, oxidative burst, and signaling (49, 60–62). However, these ligands do not account for all of Mac-1's adhesive interactions. Although previous studies have shown that Mac-1, the primary fibrin(ogen) receptor on leukocytes, directly facilitates the recruitment of leukocytes at sites of platelet and fibrin deposition (12–14), the precise platelet counterreceptor was unidentified.

I or A domains are regions of \sim 200 amino acids that are present in 1 or more copies in many proteins involved in cell-cell, cell-matrix, and matrix-matrix interactions (25, 31). This superfamily motif is present in integrin α subunits, including CD11a, CD11b, CD11c, CD11d, CD49a, CD49b, and α E (25), the complement proteins Factors B and C2 (63, 64), collagens (65, 66), and vWf (29). For CD11b, experimental evidence supports the notion that the I domain is responsible for the binding of all Mac-1 ligands except for factor X (24, 67). vWf has three similar domains, in this case termed A domains. The first and third of these, A1 and A3, mediate binding to GP I β α and collagen, respectively (68). High-resolution crystal structures of the CD11b I domain and the vWf A1 domain show that both of these domains adopt a classic α/β "Rossman" fold (25, 69). The Mac-1 I domain also contains a metal ion-dependent adhesion site (MIDAS) for binding protein ligands, a motif, however, not present in the vWf A1 domain due to the presence of an arginine and an alanine instead of a serine and an aspartate, respectively, at two of the critical amino acids forming the MIDAS motif. The observation that mutations of the α M I domain that correspond to gain-of-function mutations of the vWf A1 domain also alter the binding activity of Mac-1 (48) supports the notion that Mac-1 and vWf may be functionally similar with respect to GP I β -IX-V binding.

The binding of GP I β α to vWf and Mac-1 has several similarities and some interesting differences. First, as expected, the binding involves the homologous I or A domains. In addition, in both cases binding requires a conformational change of the A or I domain, in the case of vWf requiring ristocetin, botrocetin, or shear stress, and in the case of Mac-1 requiring activation of the integrin to its ligand-competent form. Distinguishing the two interactions is the distinct pattern of inhibition by GP I β α antibodies. AK2, for example, is a potent inhibitor of vWf binding to GP I β α , whether induced by ristocetin, botrocetin, or shear stress. This antibody failed to inhibit the interaction of Mac-1 with GP I β α . VM16d, on the other hand, does not inhibit vWf binding to GP I β α , except as induced by botrocetin, but is a potent inhibitor of Mac-1 binding. However, the sites are not completely distinct, as indicated by the observation that the isolated vWf A1 do-

main blocks the interaction of Mac-1-expressing cells with GP Iba and by the ability of the mAb AP1, which also blocks ristocetin- and botrocetin-induced vWf binding, to inhibit the interaction.

The identification of the interaction between GP Iba and Mac-1 provides a tantalizing lead into the nature of leukocyte-platelet adhesion, helping to clarify the sequential adhesion model of neutrophil attachment to surface-adherent platelets proposed by Diacovo et al. (12). Nevertheless, our data do not rule out the possibility of additional platelet surface receptors for Mac-1. Other potential Mac-1 ligands present on the platelet membrane include fibrinogen (bound to GP IIb-IIIa) (55, 56), ICAM-2 (70), high molecular weight kininogen (59), and glycosaminoglycans (4). A leukocyte-platelet interaction mediated by fibrinogen bridging between Mac-1 and GP IIb-IIIa has been discounted by Ostrovsky et al. (22), who found that neither RGDS peptides nor the replacement of normal platelets with thrombasthenic platelets (i.e., lacking GP IIb-IIIa) affected the accumulation of the leukocytes on platelets, and recently by Furman et al. (71), who found that GP IIb-IIIa antagonists and RGDS peptides did not reduce leukocyte-platelet aggregate formation in whole blood.

Other interactions contributing to Mac-1-independent leukocyte-platelet complex formation include thrombospondin bridging between GP IV receptors on platelets and monocytes (72), and P-selectin on activated platelets binding with leukocyte PSGL-1 (73, 74). Nevertheless, under the experimental conditions employed in the present study, which assayed the adhesion of activated neutrophils (i.e., thioglycollate-elicited peritoneal neutrophils) to surface-adherent platelets after vigorous washing, the predominant interaction between neutrophils and platelets appeared to be between Mac-1 and GP Iba.

These present observations also suggest a possible target for therapeutic intervention. In particular, the distinct difference in the inhibitory patterns of GP Iba antibodies suggests that it might be possible to prevent leukocyte attachment to platelets by targeting GP Iba without inhibiting platelet adhesion to the vessel wall. Our recent observations have identified Mac-1 as a molecular determinant of neointimal thickening after experimental arterial injury that produces endothelial denudation and platelet and/or fibrin deposition. We found that antibody-mediated blockade (7) or selective absence (75) of Mac-1 impaired transplatelet leukocyte migration into the vessel wall, diminishing medial leukocyte accumulation and neointimal thickening after experimental angioplasty or endovascular stent implantation. Therefore, future studies aimed at identifying the precise binding site(s) responsible for Mac-1-GP Iba binding might provide a molecular strategy for disrupting leukocyte-platelet complexes that promote vascular inflammation in thrombosis, atherosclerosis, and angioplasty-related restenosis.

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