Platelet Glycoprotein Ibα 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) Ibα, 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 Ibα–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 Ibα 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 Ibα and to adherent platelets, the latter adhesion being inhibited by pretreatment of the platelets with mocarhagin, a protease that specifically cleaves GP Ibα. Finally, immobilized GP Ibα 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 β2 integrin family, LFA-1 (αLβ2, CD11a/CD18), Mac-1 (αMβ2, CD11b/CD18), and p150,95 (αXβ2, 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

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

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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 ~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 Ibα, in the case of ristocetin) that enable the interaction.

The GP Ib-IX-V complex comprises four phylogenetically related polypeptides, GP Ibα, GP Ibβ, GP IX, and GP V, of which only GP Ibα has been shown to bind ligands (34). The ligand-binding domain of this polypeptide resides within the NH_2-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 ~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 αM I domain, we hypothesized that GP Ibα might also be able to bind Mac-1. Here, we report that GP Ibα 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-β1 was from Collaborative Research, Inc., and 1,25-$(OH)_2$ vitamin D_3 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 dispase 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 αM 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 αM subunit of mouse Mac-1 (CD11b), with broad ligand blocking properties (39; American Type Culture Collection); TS1/22, directed to the αL subunit of LFA-1 (CD11a) and capable of blocking ICAM binding (provided by Dr.

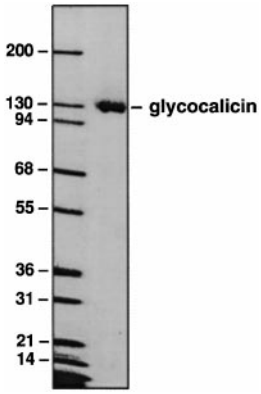

Figure 1. Purified glycocalicin. Purified glycocalicin from human platelets was subjected to SDS and 7.5% PAGE and stained with Coomassie brilliant blue.

Lloyd Klickstein, Brigham and Women's Hospital); and IB4, directed to the common β2 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 Ibα mAbs used were: AK2, AP1, VM16d, SZ2, and WM23. All but WM23 bind within the GP Ibα ligand-binding region, within the first 282 amino acids at the GP Ibα 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 Ibα macroglycopeptide and does not interfere with the binding of any GP Ibα ligands (42). The polyclonal GP Ibα antibody was prepared in rabbits immunized with glycocalicin (the soluble extracellular region of GP Ibα) and affinity-purified over a glycocalicin 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-β1 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 [IαM]-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/μl 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 μM) according to the manufacturer's protocol. Cells (10^5/well) were placed in 96-well microtiter plates coated with purified GP Ibα (10 μg/ml) or fibrinogen (10 μg/ml) and blocked with gelatin (0.2%). Adhesion was stimulated with PMA (17 ng/ml) or the β2-stimulating mAb KIM 127 (5 μg/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 μg/ml) or ligand for 15 min at 37°C; the effect of anti-GP Ibα mAbs on adhesion was investigated by incubating the indicated mAb (10 μg/ml) with GP Ibα–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 μg/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 μg/ml), obtained as described previously (38), in Tris-buffered saline (TBS), pH 7.4, and then blocked with buffer containing 0.5% gelatin. Biotinylated glycocalicin (0–50 μg/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 glycocalicin 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 (~1.5×10^7) 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% CO_2. 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 Ibα; the effect of the snake venom metalloprotease, mocarhagin, which cleaves GP Ibα 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× HBSS concentrate (GIBCO BRL). The samples were then diluted with 500 μl of distilled H_2O, 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-β1/vit D_3–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 μg/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; Hammatus) 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 (10^6 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/cm^2. 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 Ibα mAb VM16d (25 μg/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 μg/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 ± SD. Groups were compared using the nonpaired *t* test. *P* values < 0.05 were considered significant.

Results

Mac-1–expressing Cells Bind to GP Ibα. 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 Ibα. We have previously shown that stimulation of THP-1 monocytic cells (which constitutively ex-

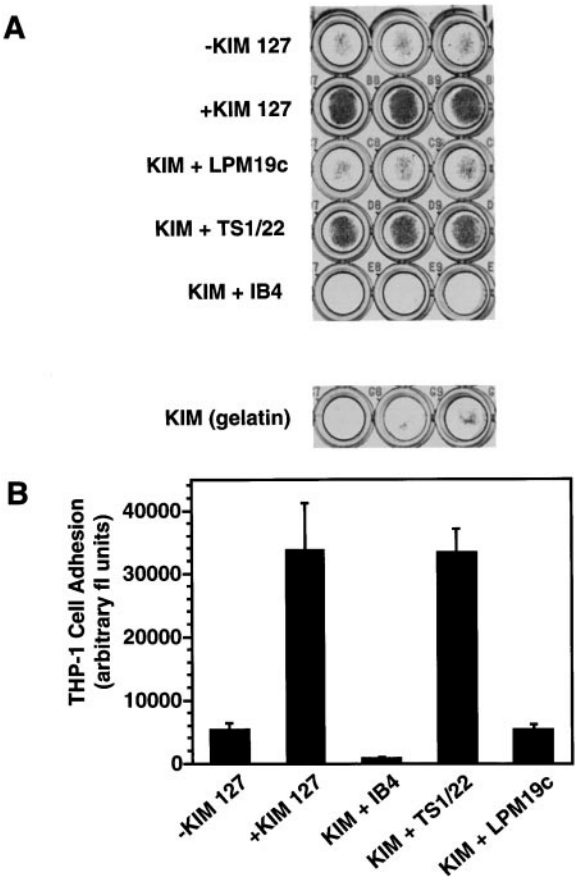

Figure 2. THP-1 cell adhesion to GP Ibα is Mac-1 dependent. Cytokine-treated THP-1 cells were added to glycocalicin (GP Ibα)–coated and gelatin-blocked wells. Adhesion was promoted by the addition of the stimulating mAb KIM 127 (5 μg/ml). The effect of anti-CD18 (IB4), anti-CD11a (TS1/22), and anti-CD11b (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 ± SD) representative of three to five separate experiments.

press Mac-1) with TGF-β1 and 1,25-$(OH)_2$ vitamin D_3 increases Mac-1 surface expression ~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 Ibα and blocked with gelatin, but not to wells coated with gelatin alone (Fig. 2). Adhesion of the cells to GP Ibα 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 Ibα was also completely blocked by LPM19c, an mAb that binds to the I domain of the αM subunit of Mac-1 (CD11b) and blocks the binding of fibrinogen, 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.

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

Antibody or ligand	Receptor (epitope)	Percent inhibition
IB4	Anti-CD18	99 ± 1*
TS1/22	Anti-CD11a (I domain)	10 ± 13
LPM19c	Anti-CD11b (I domain)	92 ± 12*
Polyclonal	Anti-GP Ibα	80 ± 17*
Polyclonal	Rabbit IgG control	16 ± 20
VM16d	Anti-GP Ibα (aa residues 201–268)	83 ± 16*
AP1	Anti-GP Ibα (aa residues 201–268)	86 ± 10*
AK2	Anti-GP Ibα (aa residues 36–59)	16 ± 6
SZ2	Anti-GP Ibα (sulfated tyrosine residues 269–282)	8 ± 6
WM23	Anti-GP Ibα (macroglycopeptide)	11 ± 5
7E3	Anti-GP IIb-IIIa, -αvβ3, –Mac-1	16 ± 8
10E5	Anti-GP IIb-IIIa	18 ± 6
Fibrinogen (2 μM)	Mac-1	99 ± 1*
P2γ377–395 (10 μM)	Mac-1	0
Heparin (200 U/ml)	Mac-1	93 ± 9*
vWF A1 (20 μg/ml)	GP Ibα	83 ± 13*

The adhesion of cytokine-treated THP-1 cells to glycocalicin (GP Ibα)–coated microtiter wells was stimulated by the addition of KIM 127 (5 μg/ml) in the presence and absence of antibodies directed to or soluble ligands of Mac-1 and GP Ibα. Polyclonal antibodies were added at 20 μg/ml and purified mAbs at 10 μg/ml as described in Materials and Methods. Soluble ligand concentrations are indicated. CD11/CD18 mAbs included IB4, TS1/22, and LPM19c; GP Ibα 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 ± SD, $n = 3$–5). aa, amino acids.
*$P < 0.01$.

To confirm that Mac-1 mediates the adhesion of THP-1 cells to GP Ibα 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 Ibα. 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 Ibα, 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 Ibα (VM16d). Neither untransfected 293 cells nor LFA-1–transfected 293 cells bound to GP Ibα.

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

The Mac-1 I Domain Serves as a Recognition Site for GP Ibα. The adhesion of Mac-1–bearing THP-1 cells to GP Ibα was inhibited by LPM19c (Fig. 2), an mAb that binds to the I domain of the αM subunit of Mac-1 (CD11b). To establish definitively that the Mac-1 I domain serves as a recognition site for GP Ibα, we transfected 293 cells with a chimeric LFA-1 receptor that contained the I domain of Mac-1 (i.e., LFA-1 [IαM]–transfected 293 cells). Flow cytometry showed comparable expression of LFA-1 (IαM), LFA-1, and Mac-1 in transfected 293 cells (Fig. 3 A). LFA-1–transfected 293 cells did not adhere to GP Ibα (Fig. 3 C); in contrast, LFA-1 (IαM)–transfected 293 cells adhered robustly to GP Ibα (Fig. 5 A), indicating that the I domain of Mac-1 is required for adhesion to GP Ibα. We next investigated whether GP Ibα would directly bind to purified I domain of Mac-1. Biotinylated glycocalicin 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 Ibα, we examined the ability of Mac-1 I domain ligands (e.g., fibrinogen [24] and heparin [4]) to inhibit THP-1 adhesion to GP Ibα. Soluble fibrinogen (50% inhibitory concentration [IC_{50}] ~ 1 μM) and heparin (IC_{50} ~ 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 Ibα and fibrinogen binding sites in Mac-1 may be partially overlapping. To exclude

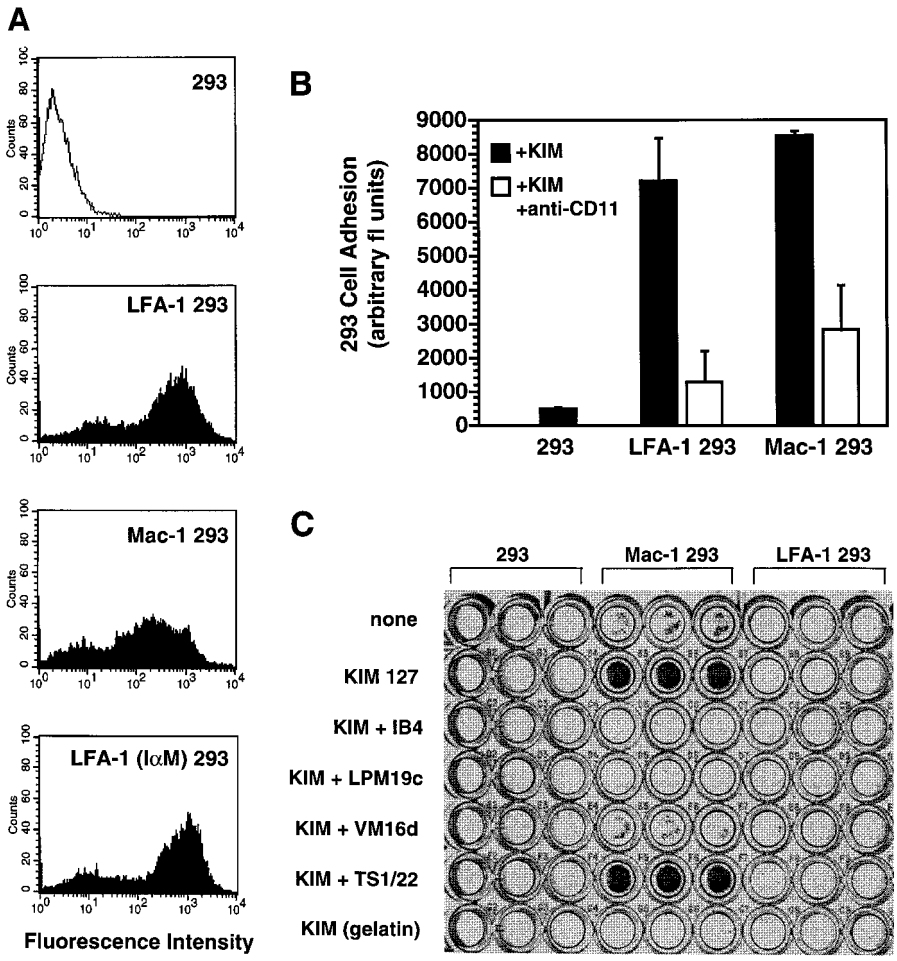

Figure 3. Mac-1 expression is required for adhesion to GP Ibα. (A) Expression of CD11/CD18 integrin in 293 cells was confirmed by flow cytometry using the anti-CD18 mAb IB4. Equivalent expression of CD18 is observed in 293 cells transfected with human LFA-1, Mac-1, or a chimeric LFA-1 containing the I domain of Mac-1, designated LFA-1 (IαM). (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 μg/ml) and quantified by measuring the fluorescence of BCECF AM–loaded 293 cells; triplicate determination (mean ± SD) representative of two separate experiments. (C) 293 cells were added to glycocalicin-coated and gelatin-blocked wells. Adhesion was promoted by the addition of KIM 127 (5 μg/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 binding ($IC_{50} \sim 1$ μM) (38). P2 had no effect on THP-1 adhesion to GP Ibα, suggesting that the GP Ibα and fibrinogen binding sites are probably distinct.

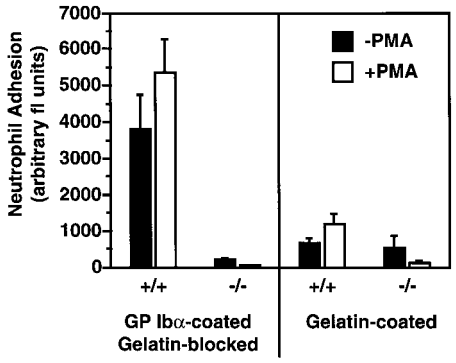

Figure 4. Neutrophil binding to GP Ibα requires Mac-1. Thioglycollate-elicited neutrophils from wild-type (Mac-$1^{+/+}$) or Mac-1–deficient (Mac-$1^{-/-}$) mice were added to glycocalicin-coated and gelatin-blocked wells in the absence (black bars) and presence (white bars) of PMA (17 ng/ml). Adhesion 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 ± SD) representative of three separate experiments.

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

Identifying the Mac-1 Binding Site within GP Ibα. We next turned our attention to identifying the Mac-1 interaction site within GP Ibα by assessing the effect of monoclonal and polyclonal antibodies to GP Ibα on Mac-1–dependent THP-1 cell adhesion to glycocalicin (Fig. 7, and Table I). Polyclonal anti–GP Ibα, 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 Ibα (amino acids 201–268), inhibited KIM 127–stimulated adhesion of THP-1 cells to GP

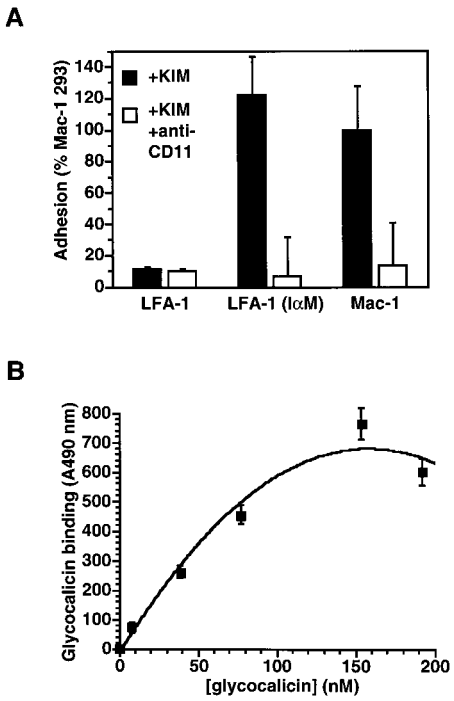

Figure 5. Mac-1 I domain serves as a recognition site for GP Ibα. (A) 293 cells transfected with human Mac-1, LFA-1, or a chimeric LFA-1 containing the I domain of Mac-1 (LFA-1 [IαM]) were added to glycocalicin-coated and gelatin-blocked wells. Adhesion was promoted by the addition of the stimulating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

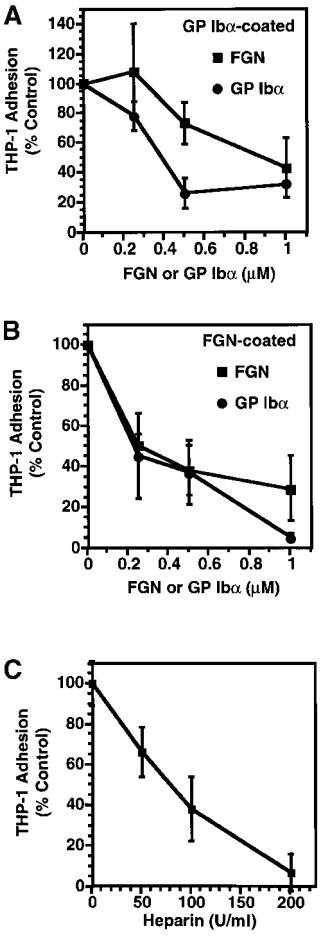

Figure 6. Mac-1–GP Ibα binding and Mac-1 I domain ligands. Cytokine-treated THP-1 cells were added to (A) glycocalicin-coated or (B) fibrinogen (FGN)-coated wells. Adhesion was promoted by the addition of KIM 127 (5 μg/ml) in the presence of increasing concentrations (0–2 μM) of soluble GP Ibα (○) or the soluble Mac-1 I domain ligand fibrinogen (■). (C) The adhesion of THP-1 cells to glycocalicin (GP Ibα)–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 ± SD, $n = 3$.

Ibα (percent inhibition: VM16d = 83 ± 16; AP1 = 86 ± 10). A similar inhibitory effect of these mAbs was noted when THP-1 cell adhesion to GP Ibα 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 Ibα, 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 Ibα led us to consider the possibility that the region within GP Ibα 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 Ibα 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 Ibα, suggesting that heparin likely inhibits adhesion by binding the Mac-1 I domain and interfering with GP Ibα binding rather than by directly mimicking a binding site on GP Ibα (4).

Glycocalicin Supports the Rolling and Firm Adhesion of THP-1 Cells under Flow. To evaluate the potential for the GP Ibα–Mac-1 interaction to support the adhesion of blood cells under flow, we perfused THP-1 cells over coverslips coated with a glycocalicin matrix using a parallel-plate flow chamber system. The cells were either kept in their native state or treated with TGF-β1 and 1,25-$(OH)_2$ vitamin D_3 to increase Mac-1 expression. THP-1 cells were able to adhere to the glycocalicin, 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 glycocalicin 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 Ibα, respectively, in the adhesion of THP-1 cells under flow.

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

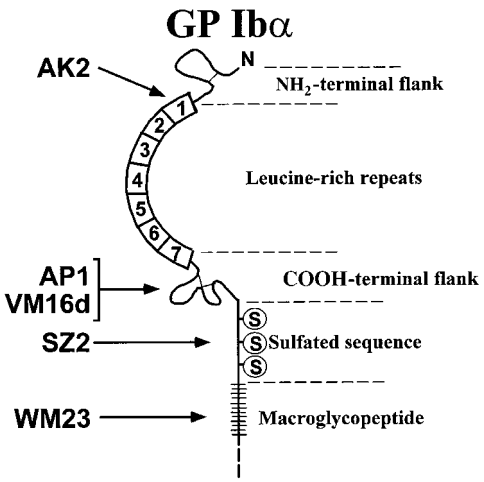

Figure 7. Epitope map of mAbs to GP Ibα. Schematic representation of the extracellular domain of GP Ibα. The GP Ibα mAbs employed were: AK2, AP1, VM16d, SZ2, and WM23. AK2 binds within the first leucine-rich repeat (amino acid residues 36–58). AP1 and VM16d bind to the COOH-terminal flanking and leucine-rich 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 Ibα (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 ± 5) and the anti-GP Ibα mAb VM16d (percent inhibition = 55 ± 10). Furthermore, Mac-$1^{+/+}$ leukocyte adhesion to platelets was inhibited dose-dependently by soluble glycocalicin (percent inhibition = 72 ± 18) and by pretreatment of adherent platelets with the snake venom metalloprotease, mocarhagin (percent inhibition = 89 ± 8), which cleaves GP Ibα 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 Ibα.

Finally, to provide additional evidence supporting a role for GP Ibα 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 μM ADP (4 vs. 21% platelet-positive neutrophils) or 5 μM 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 Ibα. The following evidence was obtained for this interaction: (a) mAbs to both Mac-1 and GP Ibα inhibited THP-1 cell

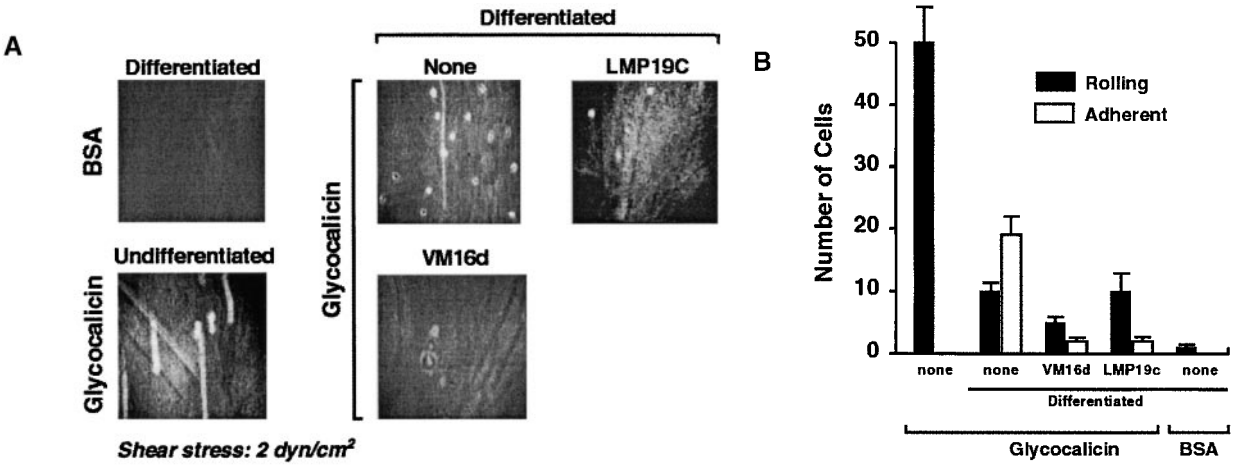

Figure 8. Adhesion of THP-1 cells to glycocalicin under flow. (A) Video images of THP-1 cells rolling on or firmly adherent to immobilized glycocalicin at a wall shear stress of 2 dyn/cm^2 in a parallel-plate flow chamber. Images were created using a digital image processing system to snap frames of previously recorded experiments. The cells were either untreated (Undifferentiated) or induced to differentiate with TGF-β1 and 1,25-$(OH)_2$ vitamin D_3 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 Ibα) 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 ± SEM, $n = 4$).

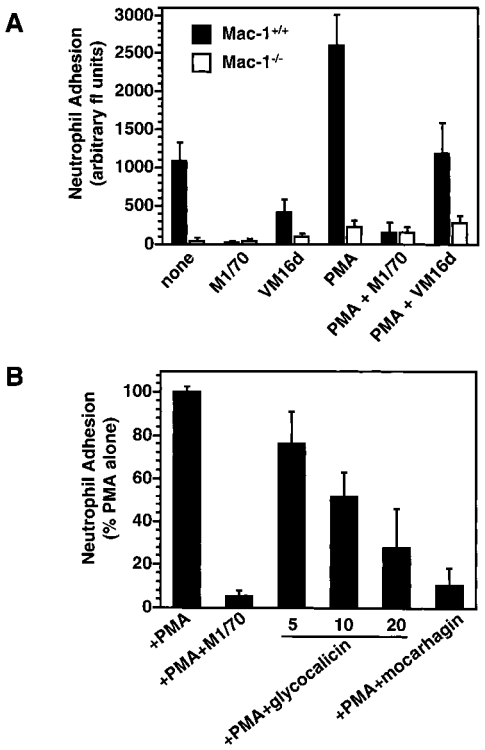

Figure 9. Neutrophil binding to platelets requires Mac-1 and GP Ibα. (A) Thioglycollate-elicited neutrophils from wild-type (Mac-$1^{+/+}$, black bars) or Mac-1–deficient (Mac-$1^{-/-}$, white bars) mice were added to surface-adherent platelets. Adhesion was promoted by the addition of PMA (17 ng/ml). The contribution of Mac-1 or GP Ibα to wild-type neutrophil adhesion to platelets was assayed by the addition of the rat anti–mouse CD11b mAb, M1/70 (10 μg/ml), or the anti-GP Ibα mAb, VM16d (10 μg/ml), respectively. (B) The effect of soluble glycocalicin (0–20 μg/ml) and the snake venom metalloprotease, mocarhagin, which cleaves GP Ibα 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 ± SD) representative of three separate experiments.

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

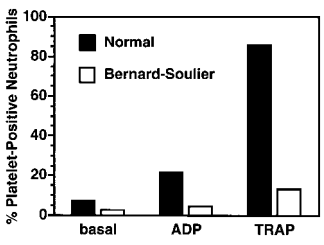

Figure 10. Absence of GP Ibα reduces neutrophil–platelet aggregates in whole blood. Leukocyte–platelet aggregates in peripheral blood obtained from a normal volunteer (black bars) or a patient with BSS (white bars) were measured by two-color flow cytometry as described in Materials and Methods. Formation of leukocyte–platelet aggregates was stimulated by incubating blood with ADP (0.5 μM) or TRAP (5 μM). Data shown are mean values of duplicate determinations.

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 ~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 αE (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 Ibα 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 αM 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 Ib-IX-V binding.

The binding of GP Ibα 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 Ibα antibodies. AK2, for example, is a potent inhibitor of vWf binding to GP Ibα, whether induced by ristocetin, botrocetin, or shear stress. This antibody failed to inhibit the interaction of Mac-1 with GP Ibα. VM16d, on the other hand, does not inhibit vWf binding to GP Ibα, 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 Ibα 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 Ibα 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 Ibα.

These present observations also suggest a possible target for therapeutic intervention. In particular, the distinct difference in the inhibitory patterns of GP Ibα antibodies suggests that it might be possible to prevent leukocyte attachment to platelets by targeting GP Ibα 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 Ibα 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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