Targeting Platelet-Leukocyte Interactions: Identification of the Integrin Mac-1 Binding Site for the Platelet Counter Receptor Glycoprotein Iba

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Abstract

The firm adhesion and transplatelet migration of leukocytes on vascular thrombus are dependent on the interaction of the leukocyte integrin Mac-1 (αMβ2, CD11b/CD18) and the platelet counter receptor glycoprotein (GP) Iba. Previous studies have established a central role for the I domain, a stretch of ~200 amino acids within the αM subunit, in the binding of GP Iba. This study was undertaken to establish the molecular basis of GP Iba recognition by αMβ2. The P201-K217 sequence, which spans an exposed loop and amphipathic α4 helix in the three-dimensional structure of the αM domain, was identified as the binding site for GP Iba. Mutant cell lines in which the αM I domain segments P201-G207 and R208-K217 were switched to the homologous, but non-GP Iba binding, αL domain segments failed to support adhesion to GP Iba. Mutation of amino acid residues within P201-K217, H210-A212, T213-I215, and R216-K217 resulted in the loss of the binding function of the recombinant αM I domains to GP Iba. Synthetic peptides duplicating the P201-K217 sequence, but not scrambled versions, directly bound GP Iba and inhibited αMβ2-dependent adhesion to GP Iba and adherent platelets. Finally, grafting critical amino acids within the P201-K217 sequence onto αL, converted αLβ2 into a GP Iba binding integrin. Thus, the P201-K217 sequence within the αM I domain is necessary and sufficient for GP Iba binding. 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 counter ligands (e.g., intercellular adhesion molecule [ICAM]-1; 2), endothelial-associated extracellular matrix proteins (e.g., fibrinogen; 3), or 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.
A similar sequential adhesion model of leukocyte attachment to and transmigration across surface-adherent platelets has been proposed (5). The initial tethering and rolling of leukocytes on platelet P-selectin (6) are followed by their firm adhesion and transplatelet migration, processes that are dependent on αMβ2 (5).

Our laboratory has focused on identifying the platelet counter receptor for αMβ2. Evaluation of the structural features of integrins provides insight into candidate platelet counter receptors for αMβ2. Integrins are heterodimeric proteins composed of one α and one β subunit. A subset of integrin α subunits, including αM, contains an inserted domain (I domain) of ~200 amino acids that is implicated in ligand binding (7–9) and strikingly similar to the A domains of von Willebrand factor (vWF; 10), one of which, A1, mediates the interaction of vWF with its platelet receptor, the glycoprotein (GP) Ib–IX–V complex. 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 αMβ2 and reported that GP Ibα is indeed a constitutively expressed counter receptor for αMβ2 (11). Furthermore, under the conditions used in these studies, the predominant interaction between neutrophils and platelets appeared to be between αMβ2 and GP Ibα (11).

The αM I domain contributes broadly to the recognition of ligands by αMβ2 (12) and specifically to the binding of GP Ibα (11). This region has been implicated in the binding of ICAM-1 (13), iC3b (14), fibrinogen (12, 13), and neutrophil inhibitory factor (NIF; 15), as well as GP Ibα. Previous studies suggested that overlapping, but not identical, sites are involved in the recognition of iC3b, fibrinogen, and NIF (16, 17). Although the binding sites for iC3b, NIF, and fibrinogen in the αM I domain have been mapped extensively (18–23), the recognition site for GP Ibα is unknown.

In this study, we have localized the binding site for GP Ibα within the αM I domain. The strategy developed was based on the differences in the binding of GP Ibα to the αM I and αM domains and involved several independent approaches, including screening of mutant cells, synthetic peptides, site-directed mutagenesis, and gain in function analyses. The binding site for GP Ibα was localized within the segment αM I (P201–K217). The grafting of two amino acids within this segment into the αM I domain converted it to a GP Ibα–binding protein. Thus, a small segment that has a defined structure within the αM I domain is necessary and sufficient for GP Ibα binding.

Materials and Methods

Materials. The soluble extracellular region of GP Ibα (sGP Ibα; i.e., glycosylcalcin) was purified as we previously reported (11). Human fibrinogen depleted of plasminogen, vWF, and fibronecrtin was purchased from Enzyme Research Laboratories.

The CD11/CD18 mAbs used included the following: LPM19c, directed to the αM I domain (provided by K. Pulford, Radcliffe, Oxford, United Kingdom; 12); OKM1, directed to the αM subunit of human Mac-1 (American Type Culture Collection [ATCC]); M1/70, directed to the αM subunit mouse αMβ2 (ATCC; 24); CBRM1/5, an activation-specific αM reporter antibody (provided by T. Springer, Harvard Medical School, Boston, MA; 25); TSI/22, directed to the αI subunit of αMβ2 and capable of blocking ICAM-1 binding (ATCC); MEM-83, an activation-specific αI reporter antibody (Caltag); and IB4, a blocking mAb directed to the β2 subunit (ATCC). The stimulating CD18 mAb KIM127 (26) was provided by M. Robinson (Celltech Ltd., Slough, United Kingdom). mAb24, a β2 activation reporter antibody (27), was provided by N. Hogg (Imperial Cancer Research Fund, Lincoln’s Inn Fields, London, United Kingdom).

Peptides were obtained from the W.M. Keck Biotechnology Resource Center at Yale University. The peptides were diluted in DMSO and stored at −80°C.

Cell Lines and Culture Conditions. THP-1 monocytic cells (ATCC) were maintained and differentiated with 1 ng/ml TGF-β1 and 50 nM 1,25-(OH)2 vitamin D3, as previously described (28). 293 cells expressing human αIβ2, αMβ2, or mutant αMβ2 receptors were established and maintained as previously described (16, 20, 23, 29).

Segment Switches by Site-directed Mutagenesis. To systematically define the GP Ibα–binding site in αM I, a homologue-scanning mutagenesis strategy was implemented (20). Accordingly, guided by the crystal structure (8, 9), the hydrated surface of the αM I domain was replaced with sequences of the αM I domain in segments of 7–11 amino acids. To apply this approach to the αM I domain (~200 amino acids), 16 segments were switched (20, 23). Site-directed mutagenesis of the αM I domain was performed using QuickChange Site-Directed Mutagenesis Kit (Stratagene). The mutations introduced and the mutagenic primers used have been reported (23). The appropriate DNA sequence of the entire I domain (from T130 to A135) was confirmed for each mutant before transferring back into the αM I subunit cDNA.

Transient Transfection. The expression vector pcDNA3.1 (Invitrogen) was used for cloning αM, αI, and β2 from human leukocyte cDNA library. 293 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) with 24 μg DNA/vessel for 4 h, according to the manufacturer’s instructions. After transfection, the medium was replaced with full growth medium. The functional assays were prepared 48 h after transfection.

Flow Cytometry. FACStar® analyses were performed to assess the expression of wild-type and mutated forms of αMβ2 and αIβ2 on the surface of transfected 293 cells, as previously described (11). Platelet P-selectin expression was assessed using FITC-conjugated AK-4 or isotype control (BD Biosciences).

Preparation of Neutrophils and Platelets. Neutrophils from wild-type (Mac-1+/+) and Mac-1-deficient (Mac-1−/−; 30) C57Bl/J6 mice were harvested and purified from the peritoneal cavity after the intraperitoneal injection of 1 ml sterile 3% thioglycollate broth, as previously described (11).

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, which also contained 100 nM prostaglandin E1. 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 containing 100 nM prostaglandin E1, as previously described (11).

Adhesion Assays. Adherent cells were assayed by loading 293 cells and thioglycollate-elicated murine neutrophils with 1 μM 2’,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxyethyl ester (BCECF AM), according to the manufacturer’s instructions (Molecular Probes). 106 cells/well were placed in 96-well
well microtiter plates coated with 10 μg/ml sGP Ibα or 10 μg/ml fibrinogen and blocked with 0.2% gelatin. Adhesion was stimulated with 20 ng/ml PMA or 5 μg/ml of the B2-stimulating mAb KIM 127. Plates were washed and adhesion was quantified by measuring the fluorescence of BCECF AM–loaded cells using a Cytofluor II fluorescence microplate reader (PerSeptive Biosystems). The effect of anti-αM mAb on adhesion was assessed by preincubating cells with 10 μg/ml LPM19c. The effect of peptides M1–M8 on adhesion was investigated by incubating the indicated peptide with sGP Ibα–coated wells for 30 min at 37°C before the addition of cells. Data are expressed as percent adhesion of control treatment.

Neutrophil Adhesion to Surface-adherent Platelets. Neutrophil adhesion to surface-adherent platelets was investigated as previously described (11). The effect of αM peptides on leukocyte adhesion to platelets was examined by preincubating surface-adherent platelets with peptide (1–1,000 nM) or vehicle for 30 min at 37°C. Data are expressed as percent adhesion of control treatment.

Site-directed Mutagenesis, Expression, and Purification of I Domain Fusion Proteins. The cDNAs of αM domain (675 nucleotides, R137–S489), αL domain (630 nucleotides, P113L–S380), and mouse αM domain (675 nucleotides, L135–S480) were cloned and inserted into the pGEX-5X-3 expression vector (22). All wild-type and mutant I domains were expressed as glutathione S-transferase (GST) fusion proteins. Mutations were created in these I domains and intact αM by oligonucleotide-directed mutagenesis. The selective introduction of the desired mutations into the I domains was confirmed by DNA sequence analyses. The GST-I domain fusion proteins were purified by adsorption onto glutathione-Sepharose 4B (Amersham Biosciences) and eluted with buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM glutathione, 2.5 mM CaCl2. Such preparations of the fusion proteins were >90% pure as assessed by SDS-PAGE.

GP Ibα Binding to the I Domains. To test the interaction of the wild-type αM, αL, and chimeric I domains with biotinylated GP Ibα, 96-well plates (Immulon 4BX; Dynex Technologies Inc.) were coated with the I domains at 50 μg/ml and blocked with 2% BSA. sGP Ibα in 20 mM Tris⋅HCl, pH 7.6, containing 100 mM NaCl and 2 mM CaCl2, was added to the wells and incubated for 1 h at 37°C. After washing, bound GP Ibα was detected using avidin–alkaline phosphatase and p-nitrophenyl phosphate. Background reaction on BSA-coated wells was subtracted.

BIAcore Surface Plasmon Resonance Analysis. Real-time protein–protein interactions were examined using surface plasmon resonance on a BIAcore 1000 (BIAcore AB). Immobilization of peptides was performed via thiol coupling onto a sensor chip C1 using 20 mM Tris, 150 mM NaCl, pH 7.4, as running buffer at a flow rate of 5 μl/min⁻¹. Modified versions of peptides containing an amino-terminal cysteine residue were synthesized and then diluted in 10 mM acetic buffer, pH 5.0 (coupling buffer). The peptide was loaded on the chip for 16 min and immobilization occurred via thiol disulfide exchange. Identical treatment was applied to the reference flow cell without peptide (control surface). For analysis, sGP Ibα diluted into running buffer was injected at a flow rate of 10 μl/min. Binding (resonance unit [RU]) was measured as a function of time(s). Binding data (after subtracting nonspecific background binding to the control surface) are presented as sensorgrams, binding curves, and Scatchard plots (31).

In Vitro Analysis of Cellular Adhesion under Laminar Flow Conditions. The laminar flow chamber used in this assay has been described (32). 25 mm² diameter glass coverslips (Asistent; Carolina Biological Supply Company) were coated with a solution of 20 μg/ml soluble P-selectin (provided by R. Camphuizen, Genetics Institute, Cambridge, MA) and 40 μg/ml sGP Ibα for 2 h at room temperature. Nonspecific interactions were blocked with PBS containing 1% human serum albumin for 1 h at room temperature and with 0.1% Tween 20 immediately before use. The effect of M2 and scM2 peptides on THP-1 cell rolling and firm arrest was examined by incubating 50 μM of the peptide with the ligand-coated glass coverslip for 1 h at room temperature. To block PSGL-1 and αMβ2 interactions, THP-1 cells were incubated with 20 μg/ml KPL-1 (BD Biosciences) or 20 μg/ml LPM19c mAb, respectively, for 20 min before perfusion. αMβ2 was activated by treating cells with 10 μg/ml KIM127 for 5 min before perfusion. The rolling and firm adhesive events scored were ligand specific as confirmed in parallel determinations on control substrates coated with human serum albumin. Coverslips were inserted in the flow chamber and 0.6 × 10⁶/ml THP-1 cells were drawn across at an estimated shear stress of 0.75 dynes/cm² using a syringe pump (Harvard Apparatus). After 3 min of perfusion, the number of rolling and arrested cells was quantified in each of five random 10x fields (area ~0.3 mm²) by an investigator blinded to treatment.

Statistics. Data are presented as the mean ± SD or SEM. Groups were compared using the nonpaired t test. Rolling and arrest data were analyzed using repeated measure ANOVA with a Bonferroni corrective for multiple comparisons. P values <0.05 were considered significant.

Results

Binding of GP Ibα to Mutant Cell Lines. Our previous report indicated that the αM domain serves as a recognition site for GP Ibα (11). The adhesion of αMβ2-bearing cells to sGP Ibα was inhibited by LPM19c, an mAb that binds to the αM domain. To establish definitively that the αM domain serves as a recognition site for GP Ibα, we transfected 293 cells with wild-type αMβ2, αLβ2, or a chimeric αLβ2 receptor that contained the I domain of αMβ2 (i.e., αL(1αMβ2)β2, transfected 293 cells). αLβ2-transfected 293 cells did not adhere to sGP Ibα. In contrast, αL(1αMβ2)β2-transfected 293 cells adhered robustly to sGP Ibα in a manner similar to αMβ2–transfected 293 cells, indicating that the αM domain is required for adhesion to sGP Ibα. These data suggest that although αMβ2 and αLβ2 are highly homologous integrins, the lack of binding of sGP Ibα to αLβ2 might be the result of sequence and/or structural differences.

As the first step to define the binding site for GP Ibα within the αM domain, mutant cell lines, each expressing a mutant αMβ2 in which a short αM domain sequence, corresponding to a structural unit in the crystal structure (8, 9), was replaced for the corresponding region of the αL domain, were tested for their adhesion to immobilized sGP Ibα. 16 segments were switched from their original αM domain to their counterparts in αL (16, 20, 23). These segment swaps are placed throughout the P147–Q134 region of the αM domain, D132–A138 and are intended to cover its entire hydrated surface (8, 9). For such experiments to be readily interpretable, cell lines expressing high and similar levels of wild-type and mutant receptors were selected by cell sorting using mAbs OKM1 and IB4 followed by cloning by limiting dilution. The expression of the receptors as assessed by the mean fluorescence intensity (MFI) in
FACS® analyses differed by less than twofold from the internal wild-type control (not depicted).

The results of adhesion of 16 mutants to sGP Iβx are summarized in Fig. 1. The data are expressed as the percent adhesion of the wild-type αβ2-expressing cells to sGP Iβx. Substitutions for the following regions of the αM domain abrogated adhesion: αM(P147–R152), αM(M153–T159), αM(E162–L170), αM(P201–G207), αM(R208–K217), αM(K245–R261), αM(E262–L267), αM(D273–K279), αM(S281–P286), and αM(F297–T307). These cell lines form a group of negative mutants. As the essential control, the αβ2-expressing cells adhered poorly to sGP Iβx, consistent with lack of interaction of αβ2 with sGP Iβx.

The lack of adhesion of these mutants was not the result of decreased surface expression of the receptor because there was no correlation between the level of expression and adhesion. Specifically, surface expression of the αM(Q200–G207) mutant was 1.5-fold higher than that of the cells expressing the wild-type αMβ2 cells, but adhesion was abrogated completely. Surface expression of negative mutants, αM(P147–R152), αM(M153–T159), αM(E162–L170), αM(P201–G207), αM(R208–K217), αM(K245–R261), αM(E262–L267), αM(D273–K279), αM(S281–P286), and αM(F297–T307). These cell lines form a group of negative mutants. As the essential control, the αβ2-expressing cells adhered poorly to sGP Iβx, consistent with lack of interaction of αβ2 with sGP Iβx.

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**Table I. Effect of α₃MI Domain Peptides on α₃β₂ 293 Cell Adhesion to sGP Ibα and Fibrinogen**

| Peptide or mAb | α₃MI domain sequence | Peptide sequence | Percent α₃β₂ 293 adhesion to sGP Ibα | Percent α₃β₂ 293 adhesion to fibrinogen |
|----------------|----------------------|------------------|-------------------------------------|----------------------------------------|
| M1             | P147–T159            | PHDFRNMKEFVST    | 76 ± 18                             | 118 ± 27                               |
| M2             | P201–K217            | PITQLLGRKTHATGIRK| 26 ± 13a                            | 102 ± 46                               |
| M3             | K245–R261            | KFGDPLGVEDYPEADR | 94 ± 20                             | 136 ± 32                               |
| M4             | D275–I287            | DAFRSEKSQELNTI   | 110 ± 36                            | 115 ± 8                                |
| M5             | F297–T307            | FQVNENEALKT      | 96 ± 16                             | 105 ± 12                               |
| M6             | Q190–S197            | QNNPNNPRS        | 93 ± 17                             | 107 ± 3                                |
| M7             | E162–L170            | EQLKKSKTIR      | 79 ± 31                             | 103 ± 3                                |
| M8             | E178–T185            | EEFRIHFT        | 66 ± 27                             | 104 ± 11                               |
| C-M2           | P201–K217            | CPTQGLGRTHTATGIRK| 26 ± 1b                             | ND                                     |
| scM2           |                     | LGTRITHQRTGPTIKL| 80 ± 15                             | ND                                     |
| LPM19c         | Anti-α₃MI domain     |                  | 6.3 ± 5.7b                          | 6.1 ± 6.6b                             |

The adhesion of α₃β₂-expressing 293 cells to sGP Ibα- or fibrinogen-coated microtiter wells was stimulated by the addition of 5 μg/ml KIM 127 in the presence and absence of 50 μM α₃MI domain peptides or 10 μg/ml antibody (LPM19c). Data are expressed as percent adhesion with vehicle alone. Mean ± SD, n = 3–5.

†P < 0.01.

**Figure 2.** Effect of M2 peptide on α₃β₂-dependent adhesion to GP Ibα. 293 cells expressing α₃β₂ were added to GP Ibα-coated wells preincubated with 0–50 μM M2 or scM2 (C) peptides. Adhesion was promoted by the addition of 5 μg/ml of the stimulating mAb KIM 127 and quantified by measuring the fluorescence of BCECF AM-loaded cells. Data represent mean ± SD, n = 3 independent experiments.

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†P < 0.01.

Thus, BIAcore analysis revealed real-time, direct binding for MI domain peptides or 10 μg/ml antibody (LPM19c). Similar results were obtained when purified sGP Ibα in running buffer containing 2 mM MgCl₂ were injected over the chip surface and the sensorgrams were recorded. As shown in Fig. 3, the sensorgrams detected little interaction between sGP Ibα and control chips or chips containing immobilized scM2. Significant interaction responses were detected for sGP Ibα with M2. Apparent equilibrium dissociation constant was estimated from the equilibrium resonance signal as a function of analyte (sGP Ibα) concentration (kD ~20 μM) and calculated by Scatchard analysis (kD = 17 μM). Similar results were obtained when purified sGP Ibα in running buffer containing 2 mM CaCl₂ were injected over the chip surface (not depicted). Thus, BIAcore analysis revealed real-time, direct binding for bimolecular interactions between GP Ibα and M2.

**GP Ibα Binding to “Triple Mutants” of the α₃MI Domain.** To begin localization of specific amino acid residues within the α₃MI domain involved in GP Ibα recognition, recombinant fragments containing wild-type α₃MI domain, R115–S340, wild-type α₃I domain, P120–S340, and a mutant α₃MI domain were expressed as GST fusion proteins in *Escherichia coli*. The mutant α₃MI domain contained a swap of the P201–K217 segment, which we implicated in sGP Ibα binding to the corresponding residues of the α₃I domain. The recombinant proteins were purified from the bacterial lysates on glutathione-Sepharose. A facile binding assay for quantifying sGP Ibα binding to the recombinant I domains was developed by measuring the binding of biotinylated sGP Ibα to the recombinant I domains immobilized onto 96-well plastic plates. As shown in Fig. 4, GP Ibα exhibited minimal reaction with α₃I domain. Binding was observed and was similar with both human and mouse α₃I domains. With the α₃MI domains, binding was dependent on the sGP Ibα con-
were subtracted from those obtained with immobilized peptides. (B) Plot of sGP Ibα in running buffer injected over control chips (dashed line) or chips immobilized with Cys-M2 (solid line) or Cys-scM2 (dotted line) for 4 min at a flow rate of 10 μl/min followed by running buffer alone. In all cases, sensograms obtained with control flow cells were subtracted from those obtained with immobilized peptides. (B) Plot of RU as a function of sGP Ibα concentration. The inset shows Scatchard analysis to obtain apparent equilibrium dissociation constant (kD (dotted line) or 4 min at a flow rate of 10 μl/min followed by running buffer alone. In all cases, sensograms obtained with control flow cells were subtracted from those obtained with immobilized peptides. (B) Plot of RU as a function of sGP Ibα concentration. The inset shows Scatchard analysis to obtain apparent equilibrium dissociation constant (kD = 17 μM). RU, resonance unit.

Figure 3. BIAcore analysis of the interaction between GP Ibα and M2. (A) 0–100 μM sGP Ibα in running buffer injected over control chips (dashed line) or chips immobilized with Cys-M2 (solid line) or Cys-scM2 (dotted line) for 4 min at a flow rate of 10 μl/min followed by running buffer alone. In all cases, sensograms obtained with control flow cells were subtracted from those obtained with immobilized peptides. (B) Plot of RU as a function of sGP Ibα concentration. The inset shows Scatchard analysis to obtain apparent equilibrium dissociation constant (kD = 17 μM). RU, resonance unit.

Figure 4. GP Ibα binding to recombinant I domains. Biotinylated sGP Ibα was added at the indicated concentrations to αM1 domain GST fusion proteins immobilized on microtiter wells. The various I domains are: human αM1 domain (○), mouse αM1 domain (●), and the αL domain (■). Incubations were performed in the presence of 2 mM Ca2+, and samples were processed as indicated in Materials and Methods. Each dataset is the mean ± SEM of at least three independent experiments.

To begin to identify the individual residues within the αM1 domain “swap” mutant with biotinylated sGP Ibα was greatly diminished compared with wild-type αM1 domain and similar to that of the αL domain. Essentially, no specific binding of sGP Ibα to this mutant I domain was detected. This result is consistent with our BIAcore binding experiments showing a direct interaction between sGP Ibα and immobilized M2 peptide corresponding to this P201–K217 segment.

To begin to identify the individual residues within the P201–K217 segment that mediated sGP Ibα recognition, a series of six triple mutants were created. In each of these triple mutants, a set of three consecutive amino acids within the αM1 domain was changed to the corresponding αL residues. If the αM and αL residues were the same, the amino acid was mutated to alanine. After the DNA sequence of each mutant I domain was confirmed, it was expressed in E. coli and purified on glutathione-Sepharose. When analyzed by SDS-PAGE, each mutant migrated as a single band of ~52 kDa (not depicted). sGP Ibα binding to each triple mutant was then assessed. The results in Fig. 5 A show the binding of each of the six triple mutants to 50 nM biotinylated sGP Ibα. Of the six triple mutants, three, H210–A212, T213–F215, and R216–K217, showed a significant reduction in sGP Ibα binding. Three mutants, P201–T203, Q204–L206, and G207–T209, did not impair binding to GP Ibα. These results, taken in light of the observation that mutating the entire region spanning P201–G207 abolished cell adhesion to sGP Ibα (Fig. 1), suggest that the inactivation of sGP Ibα binding requires alteration of more than one residue within the P201–G207 (i.e., no single residue within the triple mutants, P201–T203, Q204–L206, and G207–T209, decreases affinity detectably), or the conformation of the P201–G207 segment, which corresponds to a portion of a loop within the αM1 domain, is necessary for binding, and, again, no single substitution alters the conformation of this region sufficiently to prevent binding.

GP Ibα Binding to “Single Point Mutants” of the αM1 Domain. Next, within the three triple mutants with reduced sGP Ibα binding, each of the three amino acids was mutated individually to the corresponding residue in αL domain or in case of identical residues in two I domains, the amino acid was replaced with an alanine. After confirming the DNA sequences of these single mutants, each of the GST fusion proteins was purified. The mutant fusion protein carrying the K217Y substitution was insoluble. Therefore, K217 was mutated to Ala, and this αM1 domain with a K217A substitution was readily purified, yielding a total of eight single mutants. The capacity of the eight single mutants to bind sGP Ibα was summarized in Fig. 5 B. Within each of the three triple mutants with reduced sGP Ibα recognition, only one of the three single mutants exhibited reduced sGP Ibα binding. These three single mutants showing reduced binding were T213A, T213G, and R216N.

Development of a Chimeric αL Domain with GP Ibα Binding Activity. Loss of GP Ibα binding function in these single mutants could reflect direct involvement of the specific residues in GP Ibα binding or conformational perturbation of the resulting αM1 domain due to substitutions at these positions. A gain in function approach was used to distinguish between these possibilities by introducing the identi-
fied point mutations into the αI domain. T211 is conserved in both αM and αI domains. Therefore, chimeric I domains containing either a single G213T substitution or two substitutions, G213T and N216R, in the αI domain backbone were created. These mutant αI domains were expressed as GST fusion proteins, purified, and their sGP Ibα binding properties were evaluated. The chimeric I domain harboring both the G213T and N216R mutations bound sGP Ibα with an affinity substantially greater than wild-type αI domain and the I domain containing the single G213T mutation (Fig. 6). Indeed, the binding capability of double substituted chimeric I domain was comparable to the wild-type αM I domain. To confirm that these two amino acid residues are sufficient to impart sGP Ibα recognition to the mutated αI domain, we performed additional BIAcore binding assays with immobilized αI domain peptide (C201HVKMLLTNGAINY217, termed C-L2) corresponding to the P201–K217 sequence within αM I domain and the double substituted mutant C201HVKMLLTNTFTAIRY217 (C-mul2). Binding assays were also performed with a mutant M2 peptide containing T211A, T213G, and R216N substitutions (C201PITQLLGRTHAAGGIN217, termed C-muM2) corresponding to the three single mutants showing reduced binding in the purified mutant I domain binding assays (Fig. 5 B). The sensorsgrams detected little interaction between 50 μM sGP Ibα and chips containing immobilized C-L2 (RU = 10) or C-muM2 (RU = 25). Significant interaction responses were detected for sGP Ibα with C-mul2 (RU = 224).

Role of the Identified Amino Acids in the Context of Intact αMβ2. The role of T213 and R216 in the GP Ibα binding function of the intact receptor was investigated. The T213G and R216N substitutions were introduced into the cDNA for the αL subunit using site-directed mutagenesis and coexpressed with the cDNA for the β3 subunit in 293 cells. Wild-type αLβ2 and αMβ2 were also transiently expressed in these cells as controls. 48 h after transfection, the cells were detached from tissue culture plates, and receptor expression levels were evaluated by FACS®. αM expression was evaluated with OKM1, β3 expression with IB4, and αL expression with TS1/22. The expression levels of both the α and β subunits of the integrins were comparable (not depicted). Next, the function of the receptors was assessed by evaluating their adhesion to immobilized sGP Ibα. PMA, Mn2+, or their combination were used to activate the integrins on the cells. As shown in Fig. 7, mock-transfected cells or cells expressing wild-type αLβ2 showed little adhesion to sGP Ibα under all conditions. When αMβ2-expressing cells were stimulated with PMA, Mn2+, or their combination, their adhesion to sGP Ibα increased markedly compared with the nonstimulated cells. The chimeric αMβ2 cells adhered to sGP Ibα considerably better than the αLβ2 cells or mock-transfected cells. The adhesion of the chimeric αLβ2 cells approached that of the wild-type αMβ2 cells. We considered whether inserting these amino acid residues into αL might affect the activation state of chimeric αLβ2, such that the increase in binding might reflect allosteric changes in the inte-

Figure 5. GP Ibα binding to αM domain triple mutants (A) and αI domain single mutants (B). The GST fusion proteins were adsorbed onto wells of microtiter plates, and sGP Ib binding was measured using a 50-nM concentration of ligand. Each dataset is the mean ± SEM of at least three independent experiments.

Figure 6. GP Ibα binding to the chimeric αI domain. Biotinylated sGP Ibα binding was measured as in Fig. 1 to wild-type αM domain (●), the αI domain (□), the chimeric αI domain (○) containing the single G(213)T mutation, and the chimeric αI domain containing the double G213T and N216R substitutions (▲). Values are presented as the mean ± SEM of at least three separate experiments.
to platelets is mediated primarily by the
under these experimental conditions, neutrophil adhesion
platelets. Taken together, these observations indicate that

that such PMA treatment up-regulated P-selectin expres-
type neutrophils was blocked by the rat anti–mouse
wild-type adhesion

mAb M1/70 (percent wild-type adhesion

mAb M1/70 (percent wild-type adhesion

activated

Figure 7. GP Ibα binding to the chimeric αβ₂. Adhesion of mock-

transfected 293 cells (open bars), αβ₂-transfected cells (solid bars), αβ₂-

transfected cells (gray bars), and cells expressing the chimeric αβ₂ receptor

striped bars) to sGP Ibα-coated wells. The percentage of input cells

adherent after washing was calculated. Values are the mean ± SEM of

three individual experiments.



30 nM) wild-type neutrophil adhesion to

Figure 8. Neutrophil binding to platelets is inhibited by M2 peptide.

Neutrophils from wild-type (Mac-1+/+) or Mac-1–deficient (Mac-1−/−, ◆)
mice were added to surface-adherent platelets. Adhesion was promoted
by the addition of 17 ng/ml PMA. The contribution of αβ₂ to wild-
type neutrophil adhesion to platelets was also assayed by the addition of 10
µg/ml rat anti–mouse αβ₂ mAb M1/70 (□). The effect of 0–1,000 nM
M2 (●) and scM2 (▲) on neutrophil adhesion was also examined. Adhe-
sion was quantified by measuring the fluorescence of BCECF AM–
loaded neutrophils. Data are expressed as percent wild-type neutrophil
adhesion with vehicle alone (mean ± SD, n = 3 independent experiments).

M2 Peptide Abrogates the Firm Adhesion of THP-1 Cells un-
der Flow. To evaluate the potential for M2 to modulate
the adhesion of blood cells under flow, we perfused THP-1

cells that express Mac-1 over coverslips coinmunized

with soluble P-selectin and GP Ibα using a parallel plate

flow chamber system (0.75 dynes/cm²). The number of

rolling and arrested cells was quantified on five random

fields after 3 min of perfusion. THP-1 cells rolled and ar-

rested on coverslips cocoated with P-selectin and sGP Ibα

and the number of rolling (control vs. scM2, P > 0.05) or

arrested (control vs. scM2, P > 0.05) cells was unaffected

by scM2 (Fig. 9 A). In contrast, M2 peptide inhibited

THP-1 cell arrest, thereby increasing the number of rolling
cells visualized. The effect of M2 on cell adhesion was simi-
lar to treatment with LPM19c, an anti-CD11b mAb that
blocks αβ₂-dependent binding to sGP Ibα, thereby con-
fiming the involvement of αβ₂ in cell adhesion. Both cell

rolling and arrest were abolished by treating cells with

KPL-1, an anti–PSGL-1 mAb that blocks P-selectin bind-
ing. The effect of M2 on cell arrest was also quantified.

Thus, on coverslips incubated with vehicle or scM2, 64
and 66% of THP-1 cells arrested, respectively (Fig. 9 B).

After treatment with M2 peptide only 34% of cells arrested
(P < 0.05). Similarly, after preincubation of THP-1 cells

with LPM19C, only 33% of cells arrested (P < 0.05). KPL-1
antibody treatment abrogated almost all rolling and subse-
quient arrest (<10%; P < 0.01).

Discussion

In this study, we have identified the P²⁰¹–K²¹⁷ segment, which spans an exposed loop and amphipathic α4 helix in
the three-dimensional structure of the αM domain, as the
binding site for platelet GP Ibα. This conclusion is sup-
ported by the following data: (a) mutant cell lines in which the
αM domain segments P²⁰¹–G²⁰⁷ and R²⁰⁸–K²¹⁷ were

Figure 9. Neutrophil rolling and arrest on coverslips.

Peptide Abrogates the Firm Adhesion of THP-1 Cells un-
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the adhesion of blood cells under flow, we perfused THP-1

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αM domain segments P²⁰¹–G²⁰⁷ and R²⁰⁸–K²¹⁷ were

Figure 7. GP Ibα binding to the chimeric αβ₂. Adhesion of mock-

transfected 293 cells (open bars), αβ₂-transfected cells (solid bars), αβ₂-

transfected cells (gray bars), and cells expressing the chimeric αβ₂ receptor

striped bars) to sGP Ibα-coated wells. The percentage of input cells

adherent after washing was calculated. Values are the mean ± SEM of

three individual experiments.
switched to the homologous αI domain segments failed to support adhesion to sGP Ibα, (b) mutation of amino acid residues within P\textsuperscript{P201–K217}, H\textsuperscript{H210–A212}, T\textsuperscript{T213–I215}, and R\textsuperscript{R216–K217} resulted in the loss of the binding function of the recombinant αM\textsubscript{I} domains to biotinylated sGP Ibα, (c) synthetic peptides duplicating the P\textsuperscript{P201–K217}, but not scrambled versions, directly bound GP Ibα and inhibited αM\textsubscript{β2}-dependent adhesion to sGP Ibα and adherent platelets, and (d) grafting key amino acids within the P\textsuperscript{P201–K217} sequence onto αI converted αIβ2 into a GP Ibα binding integrin.

By virtue of binding diverse ligands including, among others, fibrinogen (34, 35), ICAM-1 (36), factor X (37), C3bi (34), high molecular weight kininogen (38), and heparin (4), αM\textsubscript{I} regulates important leukocyte functions including adhesion, migration, coagulation, proteolysis, phagocytosis, oxidative burst, and signaling (30, 39–42). However, these ligands do not account for all of αM\textsubscript{β2}’s adhesive interactions. Although previous studies have shown that αM\textsubscript{β2} directly facilitates the recruitment of leukocytes at sites of platelet and fibrin deposition (5), the precise platelet counter receptors, including GP Ibα (11) and JAM-3 (43), have been elucidated only recently.

In this study, we have identified key elements of the binding site for GP Ibα within the αI domain. The strategy to define the ligand binding site was based on the difference in the sGP Ibα binding properties of the αM\textsubscript{I} and αI\textsubscript{I} domains and entailed four complimentary approaches. In the first approach, a series of homologue-scanning mutants, used previously to map the binding regions for NIF, iC3b, and fibrinogen (16, 20, 23), were screened for adhesion to sGP Ibα. In this case, 16 mutants at the hydrated surface of the αI\textsubscript{I} domain were replaced with the corresponding segments from the homologous αI domain, which does not bind GP Ibα. 11 mutants lacked the ability to support adhesion sGP Ibα, and alteration of two other regions, αM\textsubscript{I}(E\textsuperscript{178–T185}) and αM\textsubscript{I}(ΔE\textsuperscript{262G}263), resulted in the partial loss of adhesive function. Thus, the initial insight provided by these mutant receptors indicated that the sGP Ibα binding interface within the αI domain was composed of several nonlinear sequences.

The second approach entailed the use of synthetic peptides duplicating the sequences of the critical segments in the αM\textsubscript{I} domain. These analyses showed that two critical segments, αM\textsubscript{I}(P\textsuperscript{201–G207}) and αM\textsubscript{I}(R\textsuperscript{208–K217}), may contain amino acid residues that participate directly in binding GP Ibα because the peptide M2 that spanned P\textsuperscript{201–K217} bound sGP Ibα and inhibited αM\textsubscript{β2}-dependent adhesion to sGP Ibα and adherent platelets. The negative results for other peptides (M1, M3–M5, and M7) synthesized to correspond to other negative mutants (Fig. 1 and Table I) do not exclude a role for other αI domains in binding function. These segments may play an accessory role in ligand binding or the short peptides may simply not assume the appropriate conformation for recognition by the ligand.

Finally, segments implicated in the binding of sGP Ibα by the negative mutants, other than P\textsuperscript{P201–K217}, may reflect interference by αI domain residues rather than residues that actually participate in binding. It is also possible that the activation of αM\textsubscript{β2}, rather than ligand binding per se, was affected adversely by the introduction of these αI segments within αI domain. Because these same mutants have been used in previous studies (17, 20, 23) to analyze interaction of other activation-dependent ligands with αM\textsubscript{β2}, such effects on activation would need to specifically perturb GP Ibα recognition.

To obtain direct evidence that the αM\textsubscript{I}(P\textsuperscript{P201–K217}) sequence constitutes the functional binding site for the GP Ibα, we turned to a third approach using site-directed mutagenesis of the αI domain. Binding experiments with purified sGP Ibα and GST–I domain mutants provided independent confirmation that the P\textsuperscript{P201–K217} segment is important for sGP Ibα binding because mutations of these residues resulted in significant loss of sGP Ibα binding. These experiments also served to narrow further the binding region to H\textsuperscript{H210–K217} and subsequently identified three single mutants showing reduced binding to sGP Ibα (T\textsuperscript{T211A}, T\textsuperscript{T213G}, and R\textsuperscript{R216N}). Of these, T\textsuperscript{T211} in αI domain is conserved in the αI domain. Thus, it was the conversion of the residue to A that perturbed GP Ibα binding, suggesting that this substitution exerts a negative influence on the conformation of the αM\textsubscript{I} domain that is required for GP Ibα recognition, rather than participating directly in ligand contact. The positioning of this residue on the interior of the α-helix and facing the central core of the αM\textsubscript{I} domain, according to the crystal structures (8, 9), supports this interpretation. In contrast, T\textsuperscript{T213} and R\textsuperscript{R216} are appropriately positioned, including when the recent crystal structure of the GP Ibα/vWF A domain (44) is used as a template.

In the fourth approach, the two amino acids (T\textsuperscript{T213}G and R\textsuperscript{R216N substitutions}) within the αM\textsubscript{I}(P\textsuperscript{P201–K217}) were grafted...
into the corresponding positions of the αI domain in the context of intact αβ. As demonstrated in Figs. 6 and 7, this manipulation imparted GP Ibα binding capacity to the chimeric molecule. Thus, the role of amino acids within the αm(P201-K217) sequence in GP Ibα binding, which initially was inferred from the loss in function experiments, was verified by the gain in function approach. Taken together, the four approaches substantiated independently the role of αm(P201-K217) in GP Ibα binding and provided evidence that two to three critical residues participate in ligand docking.

To date, several lines of evidence have emphasized that multiple ligands share overlapping binding sites within αβ2, including the fact that one ligand (e.g., NIF) is capable of blocking the interaction of multiple ligands (C3bi, ICAM-1, fibrinogen) with the receptor (15, 45). However, although the binding sites for these ligands might be overlapping, they need not be identical (16, 17). Ustinov and Plow (22) have proposed a mosaic model in which many of the same loops and helices of the αm domain, which has been implicated in both NIF and fibrinogen recognition (23). Key contact amino acids in this loop for fibrinogen binding are E248, D254, and P257, whereas Y252 and E258 are involved in NIF binding. This same αm domain segment is also involved in C3bi recognition by αβ2 (17).

Under the experimental conditions used in this study, which assayed the adhesion of activated neutrophils to surface-adherent platelets after vigorous washing, the predominant interaction between neutrophils and platelets appeared to be mediated by αβ2 binding to GP Ibα, based on the ability of M2 to inhibit >80% of neutrophil adhesion. The enhanced potency of M2 with respect to inhibiting neutrophil adhesion to platelets (IC50 = 30 nM) compared with BIAcore (kD = 17 μM) is possibly secondary to the fact that leukocytes express αβ2 receptors and platelets express GP Ibα in their native conformations. In contrast, BIAcore experiments used sGPIbα with immobilized M2 peptide.

Our data do not rule out the possibility of additional platelet surface receptors for αβ2. Other potential αβ2 ligands present on the platelet membrane include fibrinogen (bound to GP IIb-IIIa; 34, 35), ICAM-2 (46), high molecular weight kininogen (38), and JAM-3 (43). However, a leukocyte–platelet interaction mediated by fibrinogen bridging between αβ2 and GP IIb/IIIa has been discounted by Ostrovsky et al. (47), 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. Although αβ2 binds ICAM-1, this receptor is not found on platelets. Platelets express a related receptor, ICAM-2 (48), but Diacovo et al. (5) have shown that ICAM-2 blockade has no effect on the firm adhesion of neutrophils on monolayers of activated platelets under flow. Santoso et al. (43) have reported recently that αmβ2 may also bind to platelet JAM-3, cooperating with GP Ibα to mediate neutrophil–platelet adhesive contacts (43).

The present observations also suggest a possible target for therapeutic intervention. In particular, the specificity of M2 inhibitory action toward GP Ibα (i.e., noninhibitory toward fibrinogen) suggests that it might be possible to prevent leukocyte attachment to platelets by targeting GP Ibα without inhibiting other αβ2 functions. Our recent observations have identified αmβ2 as a molecular determinant of neointimal thickening after experimental arterial injury that produces endothelial denudation and platelet deposition. We found that antibody-mediated blockade (49) or selective absence (50) of αmβ2 impaired transplatelet leukocyte migration into the vessel wall, diminishing medial leukocyte accumulation and neointimal thickening after experimental angioplasty or endovascular stent implantation. Therefore, this study identifying the precise binding site responsible for αmβ2–GP Ibα interaction 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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Note added in proof. Hoffmeister et al. (Hoffmeister, K.M., E.C. Josophson, N.A. Isaac, H. Clausen, J.H. Hartwig, and T.P. Stossel. 2003. Glycosylation restores survival of chilled blood platelets. Science. 301: 1531–1534.) have recently reported that chilled platelets are cleared by hepatic macrophage αβ2 receptors via an interaction between platelet GP Ibα and a non–1-domain, lectin-binding site within αβ2.

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