Structural basis of the leukocyte integrin Mac-1 I-domain interactions with the platelet glycoprotein Ib

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Key Points

- The integrin Mac-1 I-domain interaction with platelet receptor GPIba has been mapped by NMR.
- The GPIba LRR capping α-helix coordinates directly to the Mac-1 MIDAS Mg2+ ion via an acidic residue.

Cell-surface receptor interactions between leukocyte integrin macrophage-1 antigen (Mac-1, also known as CR3, αMβ2, CD11b/CD18) and platelet glycoprotein Ibα (GPIba) are critical to vascular inflammation. To define the key residues at the binding interface, we used nuclear magnetic resonance (NMR) to assign the spectra of the mouse Mac-1 I-domain and mapped the residues contacting the mouse GPIba N-terminal domain (GPIbaN) to the locality of the integrin metal ion-dependant adhesion site (MIDAS) surface. We next determined the crystal structures of the mouse GPIbaN and Mac-1 I-domain to 2 Å and 2.5 Å resolution, respectively. The mouse Mac-1 I-domain crystal structure reveals an active conformation that is stabilized by a crystal contact from the α7-helix with a glutamate side chain completing the octahedral coordination sphere of the MIDAS Mg2+ ion. The amino acid sequence of the α7-helix and disposition of the glutamic acid matches the C-terminal capping region α-helix of GPIba effectively acting as a ligand mimetic. Using these crystal structures in combination with NMR measurements and docking analysis, we developed a model whereby an acidic residue from the GPIba leucine-rich repeat (LRR) capping α-helix coordinates directly to the Mac-1 MIDAS Mg2+ ion. The Mac-1:GPIbaN complex involves additional interactions consolidated by an elongated pocket flanking the GPIbaN LRR capping α-helix. The GPIbaN α-helix has an HxxxE motif, which is equivalent by homology to RxxxD from the human GPIbaN. Subsequent mutagenesis of residues at this interface, coupled with surface plasmon resonance studies, confirmed the importance of GPIbaN residues H218, E222, and the Mac-1 MIDAS residue T209 to formation of the complex.

Introduction

The integrin receptor macrophage-1 antigen (Mac-1, also known as CR3, integrin αMβ2, or CD11b/CD18) is expressed on the surface of myeloid leukocytes and mediates numerous responses of these cells critical to innate immunity.1 The Mac-1 receptor contributes to the recruitment, firm adhesion, and transendothelial migration of leukocytes at sites of vascular injury and facilitates tissue inflammation.2 Biochemical and cell-based studies have characterized the interactions of the Mac-1 integrin with diverse ligands, including plasma protein fibrinogen,9 complement protein fragment iC3b,4 high-molecular-weight kininogen,5 and the cell-surface receptors platelet glycoprotein Ibα (GPIba)2 and intercellular adhesion molecule 1 (ICAM-1).6 The Mac-1 integrin exists in an inactive conformation and, when activated by a variety of stimuli, undergoes a structural change to an active form capable of binding to ligands with high affinity.7,8 Concurrently, allosteric changes occur on ligand binding that result in “outside-in” cell signaling.9
Mac-1-deficient mice show delayed thrombosis, but largely unimpaired hemostasis. Blocking the Mac-1-GP1bα interaction prevents neutrophil extracellular trap formation, and an antibody targeting Mac-1 was shown to block inflammation. Inhibiting the Mac-1 I-domain with the small molecule allosteric regulator leukaderrin-1 has demonstrated efficacy in animal models of inflammatory disease.

For the majority of Mac-1 interactions described, the inserted αM-subunit, or “I-domain,” is the principal ligand-binding domain. The Mac-1 I-domain contains a Mg$_2^+$ iC$_3$b,4 but the complex between Mac-1 and GPIb has been extensively studied. X-ray structural analysis of both the mouse (aspartate or glutamate) from coordinating ligands. A crystal structure has defined the interaction between Mac-1 and iC$_3$b,4 but the complex between Mac-1 and GPIb and the principal ligand fibrinogen are poorly understood at the molecular level. Here, nuclear magnetic resonance (NMR) assignment of the mouse Mac-1 I-domain, together with X-ray structural analysis of both the mouse GP1bα and Mac-1 I-domain, has enabled us to map the binding surface and model the interaction site for the GP1bα leucine-rich repeat (LRR) N-terminal domain (GP1bαN) with the MIDAS face of Mac-1.

Materials and methods

Protein expression, purification, and characterization

A complementary DNA fragment encoding mouse GP1bαN residue E1 to T266 (mature protein sequence numbering) was cloned into the pMT-puro vector for expression using the DES system (Invitrogen). At the N terminus, the signal sequence corresponds to a Drosophila homolog of the immunoglobulin binding chaperone protein signal sequence, and a polyhistidine tag sequence (TGTHHHHHH) was added at the C terminus. Site-specific mutations H218A and E222A were introduced using the QuikChange method (Stratagene) and confirmed by DNA sequencing. The mouse amino acid sequence numbering is defined by alignment with the mature human GP1bα sequence, such that H218 corresponds to H234 in the UniProt entry GP1BA_MOUSE, which includes the signal sequence. Drosophila S2 cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum at 28°C and transfection was performed in each case using calcium phosphate. Cells were grown for an additional 48 h before selection with puromycin to establish stable cell lines. Serum-free Express Five (Invitrogen) insect culture media was collected containing secreted proteins. Initial capture of the crude product was performed using Ni-sepharose affinity chromatography, followed by gel filtration and ion exchange chromatography (supplemental Figure 1). Protein identity and successful removal of the signal peptide was confirmed by trypsin digest mass spectrometry, analyzed using the Mascot Database (ProteinID, University of York) shown in supplemental Figures 1 and 2.

The mouse Mac-1 I-domain gene fragment (P129 to A318; mature protein sequence numbering) was cloned into the pGEX-4T-1 vector for expression as an N-terminal GST-fusion using Escherichia coli. All mutants were produced using Q5 site-directed mutagenesis. XL1-Blue and BL21 cells were transformed using calcium chloride for storage and expression, respectively. BL21 cells were grown in LB media (for surface plasmon resonance [SPR] and mass spectrometry) or M9 minimal media (for NMR spectroscopy) supplemented with $^{15}$N ammonium chloride and/or $^{13}$C glucose as required for isotope labeling. GST-affinity chromatography and incubation with thrombin resulted in the separation of the tag (residual Gly-Ser remains at the N terminus) and further purification using cationic exchange chromatography. Mass spectrometry, gel filtration studies, NMR line width analysis, and diffusion measurements indicate that the recombinant Mac-1 I-domain is monomeric in solution (supplemental Figures 3 and 4).

NMR spectroscopy

NMR data were recorded at 25°C on a Bruker 800 MHz Avance III spectrometer with a QCI cryoprobe using Topspin 3.1 software. Backbone resonances ($^{15}$C/$^{13}$N) were assigned using HNCO, HN(C$_n$)CO, Cβ(C$_n$)NH, and Cβ(C$_n$)(CO)NH experiments. Backbone assignment (>146/182 residues) was facilitated by a number of locally perturbing single-point mutations (to Ala/Gly) and through selective unlabeling experiments (Glu and Lys, in particular). $^{15}$N-TROSY 2D spectra were collected in titration experiments at a Mac-1 concentration of 120 μM in 25 mM potassium phosphate buffer (pH 7.0), 25 mM NaCl, 5% D$_2$O, and 0.02% NaN$_3$ using 1.5 equivalents of MgCl$_2$ to ensure metal complexation. $^{15}$N-TROSY spectra were collected at 0.2 molar ratio intervals up to a 2:1 excess of GP1bαN. Chemical shift perturbations (CSPs) were calculated using a weighting of $^{1}$H and $^{15}$N shifts according to: CSP = $\sqrt{(1/2 \times (\delta_{1H}^2 + 0.14 \times \delta_{15N}^2))}$. For structural modeling, the standard deviation (SD) from the mean CSP was calculated and mean + SD and mean + 2 × SD used for surface mapping.

Quantitation of Mac-1 I-domain ligand binding

SPR was performed using a BIAcore 3000 (GE Healthcare). Ligands of recombinant mouse GP1bαN, GP1bαN variants H218A and E222A, and fibrinogen (rF) were amine-coupled to a CMS sensor chip (GE Healthcare); a reference cell was prepared by blank amine-coupling. Reombinant Mac-1 I-domain wild-type and T209A variants samples at protein concentrations ranging from 3.125 μM to 200 μM, in running buffer (10 mM N$_2$H$_2$O$_2$, 50 mM NaCl, 150 mM MgCl$_2$) were injected at a flow rate of 50 μL/min. Kinetic studies of the Mac-1 I-domain wild-type and T209A constructs were conducted as a dilution series, in the presence and absence of Mg$^{2+}$ against the immobilized GP1bαN. Each of the series contains a minimum of 10 concentrations (11.25-200 μM) and was repeated a minimum of 2 times. The Mg$^{2+}$-ion dependence of the GP1bαN interaction was tested against a wild-type Mac-1 I-domain concentration series (6-100 μM) in which the running buffer contained 5 mM EDTA to sequester metal ions. Binding to the immobilized GP1bαN was also evaluated using a dilution series of the antibody Xia2B2 (emfret ANALYTICS) with protein concentration of 3 to 50 nM. The CM5 chip was regenerated with 2 M NaCl after each binding experiment. Binding curves were generated by subtracting the appropriate controls and the resulting curves were analyzed using BIAevaluation software or a steady-state equilibrium approach using a Hill plot generated with Prim 6 (GraphPad Software Inc.).

Protein crystallization and crystal structure determination

Recombinant mouse GP1bαN was concentrated to 12 mg/mL and subjected to sparse matrix screens of Morpheus, Proplex, Index, PACT, JCSG+, and MIDAS using the sitting drop vapor diffusion method. Crystals of GP1bαN grew in Morpheus condition D2 containing 0.2 M mixture of 1,6-hexanediol; 1-butanol; 1,2-propanediol;
2-propanol; 1,4-butanediol; 1,3-propanediol, 0.1 M imidazole; MES monohydrate (acid) pH 6.5, 20% v/v ethylene glycol; and 10% w/v PEG 8000. Crystals of the mouse Mac-1 I-domain (6 mg/mL) grew in the JCSG+ screen condition D7. 0.2 M lithium sulfate, 0.1 M tris(hydroxymethyl)aminomethane, pH 8.5, 40% PEG 400. Crystals were cryocooled in liquid nitrogen and X-ray diffraction data were collected using the ID30B beamline at the ESRF (GP1bxN) and Diamond beamline I24 (Mac-1 I-domain). Data were processed with XDS and molecular replacement was performed with PHASER using a human GP1bxN\textsuperscript{15} and Mac-1 I-domain structure\textsuperscript{7} (Table 1). Partial model building was performed using BUCCANEER and completed manually using COOT and refined with REFMAC5 (deposited PDB code: 6EJX).

**Molecular docking calculations**

Three-dimensional models of the Mac-1 I:GP1bxN docked complex structures were calculated using HADDOCK\textsuperscript{16} with template Mac-1 I-domain (PDB code: 1IDO) and GP1bxN (PDB code: 1MOZ) crystal structures used. Active residues were defined based on the Mac-1 I-domain NMR titration experiments: E244, G143, and N147 plus the MIDAS Mg\textsuperscript{2+} ion. Active residues for GP1bxN were D222 and R218.

**Results**

**NMR assignment and structural analysis of the Mac-1 I-domain**

A bacterially expressed mouse Mac-1 I-domain construct (residues 129-318) was isotopically \(^{1}H/^{15}N\) labeled and the NMR spectra assigned using heteronuclear 3-dimensional NMR experiments. The \(^{1}H/^{15}N\) heteronuclear single quantum coherence is highly dispersed with sharp resonances indicative of well-ordered secondary structure in both the Mg\textsuperscript{2+} free and bound state (supplemental Figure 5). We also prepared and characterized 2 Mac-1 mutants, T209A and F302W, which have previously been reported as "pseudo-open" (T209A) and "open" (F302W) variants of the I-domain.\textsuperscript{17,18} The side chain of T209 coordinates directly with the Mg\textsuperscript{2+} ion only in the "open" conformation of the Mac-1 I-domain,\textsuperscript{4,7} and previous crystallographic studies have defined a large allosteric conformational shift between "open" and "closed" states, which links the switching of the coordination of the metal ion from D140 to T209 to a shift in the position of helix \(\alpha_7\). T209 has also been shown to play a direct and critical role in Mac-1 ligand binding to ICAM-1 and iC3b.\textsuperscript{6} The CD spectra of the mutants and wild-type Mac-1 are superimposable, showing no significant changes in protein secondary structure and folding (supplemental Figure 1B). NMR of the \(^{15}N\)-labeled mutant proteins confirms retention of the core structure; however, extensive perturbations across the MIDAS face are evident in both mutants (supplemental Figure 5).

**NMR analysis of the Mac-1 I-domain interaction with GP1bxN**

Recombinant mouse GP1bxN was expressed and purified from insect cells (supplemental Figures 1 and 2). NMR chemical shifts (\(^{1}H/^{15}N\)) are particularly sensitive to changes in chemical environment and the conformation associated with ligand binding and were used to characterize the GP1bxN interaction with the Mac-1 I-domain. A buffered solution of GP1bxN was titrated into a 120 \(\mu\)M solution of \(^{15}N\)-Mac-1 I-domain in the presence of 1.5 molar equivalents of Mg\textsuperscript{2+}, and \(^{1}H/^{15}N\) heteronuclear single quantum coherence spectra were collected over a range of ligand concentrations up to a Mac-1 I:GP1bxN molar ratio of 1:2.

A significant number of fast-exchange CSPs are observed in response to ligand binding. However, a number of residues within the Mac-1 I-domain C-terminal region (helix \(\alpha_7\)) showed some evidence of signal broadening and peak distortion in the bound state. Statistically significant CSP effects are identified for 16 residues of the Mac-1 I-domain (Figure 1) and enable us to identifying 2 well-defined binding patches. The first group of residues, which are in close proximity or intimately involved in MIDAS Mg\textsuperscript{2+} coordination, includes D140, S142, G143, I145, and D242 together with the adjacent solvent-exposed residues N147, K210, and E244. Residues D242, E244, and D273 cluster on the surface of the I-domain to form a negatively charged patch that is adjacent to the MIDAS. The second group of Mac-1 residues I265, R266, F275, G293, E294, H295, and V296 (Figure 1D-E) are distal to the MIDAS and appear to be linked to it through conformational changes propagated away from the metal center.

**Crystal structures of the mouse GP1bxN and Mac-1 I-domain**

We next determined the crystal structure of mouse Mac-1 I-domain to 2.5 \(\AA\) resolution. This revealed the mouse Mac-1 I-domain in an open conformation with the MIDAS metal ion coordinated by T209, S142, S144, and a glutamic acid residue (E314) ligand mimetic supplied by a crystal contact from the C-terminal helix (\(\alpha_7\)) of the

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**Table 1. Crystallographic data collection and refinement statistics**

| Sample                  | Mouse GP1bxN | Mouse Mac-1 I |
|-------------------------|--------------|---------------|
| **Data collection**     |              |               |
| Space group             | P2_12_12_1   | P4_1_2_2      |
| **Cell dimensions**     |              |               |
| a, b, c (\(\AA\))      | 61.5, 72.8, 164.0 | 62.9, 62.9, 336.2 |
| \(\alpha, \beta, \gamma\) (\(^{\circ}\)) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Resolution, \(\AA\)     | 29.9, 2.0    | 45.9, 2.5     |
| \(R_{work} \)          | 11.1         | 12.5          |
| \(I_0; C(C(1/2)) \)‡   | 7.0 (1.9); 0.997 (0.78) | 8.2 (1.5); 0.903 (0.68) |
| Completeness (%)\(^\dagger\) | 99.0 (90.0) | 86.7 (67.2) |
| Redundancy\(^\dagger\)  | 4.3 (1.3)    | 6.2 (2.5)     |
| **Refinement**          |              |               |
| Number of reflections   | 48095        | 21236         |
| \(R_{work}/R_{free} \)  | 0.192/0.237  | 0.222/0.271   |
| B factors, \(\AA^2\)    | 29.8         | 48.2          |
| RMS deviations          |              |               |
| Bond lengths, \(\AA\)   | 0.018        | 0.021         |
| Bond angles, \(^{\circ}\) | 1.96         | 1.85          |

\(R_{work}, R_{free} \) calculated with SCALA.

\(I_0; C(C(1/2)) \) and \(R_{work} \) collected using the ID30B beamline at the ESRF (GP1bxN).

Values in parentheses are for the highest resolution shell.

\(R_{work} = \sum(h)|S|/\sum(h)|S|/|S|, where \(I\) is the observed intensity and \(<I>_o\) is the average intensity of multiple observations from symmetry-related reflections calculated with SCALA.

\(R_{free} = \sum(h)|S|/\sum(h)|S|/|S|, where \(F_o\) and \(F_c\) are the observed and calculated structure factors, respectively. \(R_{work} \) computed as in \(R_{work} \), but only for 5% randomly selected reflections, which were omitted in refinement, calculated using REFMAC.

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Figure 1. Mapping the Mac-1 I-domain binding site with GP1bαN by NMR. (A) Selected peaks showing CSP effects between bound and unbound (the first panel for G228 is shown as a control, in which no perturbation is observed). (B) Representation of CSP effects, with dashed lines showing statistically significant perturbation (lower line, mean CSP +1 SD; upper line, mean CSP + 2 SD) residues above the latter. (C) CSP effects mapped to the Mac-1 surface with residues in darker pink corresponding to...
The C-terminal Mac-1 I-domain α-helix consists of the amino acid sequence 310-NQLQE-314, which resembles the sequence from the α-helix in the GPIbα N capping region spanning residues 218-HWLQE-222, and is equivalent structurally in terms of the α-helical disposition of the glutamic acid residue. Figure 2B shows that the α-helix sits in a groove on the surface of the Mac-1 I-domain packing against residues F246 and R208, forming multiple hydrogen bonded contacts between the sidechains of N310 and N306 and the sidechains of E244 and K277 on the MIDAS face. For comparison, crystal structures are available for the human Mac-1 I-domain as an open conformation in complex with ligand iC3b, simvastatin, and stabilized by a crystal contact. The mouse and the human Mac-1 I-domain crystal structures are superposed in Figure 2D, revealing the MIDAS metal ion coordinating acidic residue consistently occupies an identical position simultaneously bonding to both the metal ion and the hydroxyl of T209. Subtle differences occur across the MIDAS face with the introduction of K277 in the mouse Mac-1 forming a salt bridge to E244, replacing the K279-E244 salt bridge observed in the human structure. A comparison of structures shows that mouse GP1bαN H218 and E222 occur with the same disposition as human GP1bαN R218 and D222, respectively, and a sequence alignment of this region reveals that charged residues at these positions are conserved across other mammalian species.

Docking of the GPIbαN and Mac-1 I-domain 3-dimensional structures

Previous cell-based and biochemical studies identified residues 218 through 228 from GP1bα as the principal determinants of the Mac-1 interaction encompassing the human GP1bαN LRR C-terminal capping α-helix, which is important for the interaction with Mac-1. This structure reveals overall features of the LRR solenoid flanked by N- and C-terminal disulphide-bonded capping regions and R-loop, as observed previously for the human GP1bαN structure. The junction between the C-terminal capping region α-helix and the LRR solenoid forms an elongated pocket (Figure 3A, dotted elliptical line). In a notable departure from the human sequence, Figure 3B-D reveals that the mouse GP1bαN has the substitutions H218 and E222 that, by analogy with the human GP1bαN structure, are also surface exposed (Figure 3B). A comparison of structures shows that mouse GP1bαN H218 and E222 occur with the same disposition as human GP1bαN R218 and D222, respectively, and a sequence alignment of this region reveals that charged residues at these positions are conserved across other mammalian species (Figure 3C).
N223 of GP1b as R208 from Mac-1 forms hydrogen bonds to the side chains of respectively. Flanking interactions also occur on the right of Figure 4B hydrogen bond to the side chains of N221 and R217 of GP1b orange in Figure 4B, residues G143 and S144 of Mac-1 (lower left) and they were used as templates for docking.

Docking was performed using NMR distance restraints between the Mac-1 MIDAS Mg\(^{2+}\) ion and residues F192, S194, Y215, and W219 (Figure 4C). As viewed down the central projection of the GP1bxN capping \(\alpha\)-helix, colored orange in Figure 4B, residues G143 and S144 of Mac-1 (lower left) hydrogen bond to the side chains of N221 and R217 of GP1bx, respectively. Flanking interactions also occur on the right of Figure 4B as R208 from Mac-1 forms hydrogen bonds to the side chains of N223 of GP1bx, and its own H195 main chain carbonyl. The Mac-1 I-domain F246 side chain inserts into an elongated hydrophobic pocket in the GP1bxN LRR capping region defined by GP1bx residues F192, S194, Y215, and W219 (Figure 4C).

**SPR analysis of the Mac-1 I:GP1bxN interaction**

To further evaluate the contribution of amino acid side chains at the center of the proposed mouse Mac-1 I:GP1bxN interface, we used site-directed mutagenesis coupled with SPR. To establish the assay, the SPR response was first observed with Mac-1 I-domain analyte and immobilized full-length fibrinogen, which has been characterized in detail from previous studies. SPR analysis (Figure 5A) shows a high-affinity interaction with evidence of multiple binding modes; an estimated \(K_d\) of 30 \(\mu\)M was derived from the equilibrium values of response units was consistent with previous reports.

A ligand surface of GP1bxN was generated through amine-coupling to a CM5 chip. To determine the integrity of the GP1bxN surface, a dilution series with the antibody Xia.B2 (emfret ANALYTICS) was conducted and analysis of sensorgrams resulted in a \(K_d\) of 34 nM, consistent with the anticipated high affinity for an antibody-antigen interaction, confirming the immobilized GP1bxN is forming an active surface. Sensorgrams were recorded using wild-type Mac-1 I-domain as the analyte and high-quality SPR curves were obtained (Figure 5B, top), such that association and dissociation profiles fitted well to a 1:1 Langmuir simultaneous binding model. A calculated \(K_d\) of 64 \(\mu\)M was in good agreement with the steady-state equilibrium \(K_d\) value of 121 \(\mu\)M.

Our model of the Mac-1 I:GP1bxN complex shows that the Mac-1 I-domain T209 is a pivotal residue at the binding interface not only
by coordinating the MIDAS metal ion, but also in forming a hydrogen bond from the β-OH to the carboxylate of GPIbaN D222. The binding to GPIbaN of the Mac-1 T209 alanine variant (T209A) elicited neither a significant signal response nor a clear association binding curve (Figure 5B, bottom), which was similarly the case with wild-type Mac-1 with EDTA in excess (5 mM) to remove the metal ion from the MIDAS (data not shown). These experiments demonstrate that the bound Mg2+ ion is critical to the interaction and confirm that the observed distal CSP effects in the NMR titration data arise from allosteric effects propagated away from the MIDAS site, rather than to a second ligand-binding site. In contrast with GPIbaN, at least 1 of these binding interactions is still evident from the SPR analysis with full-length fibrinogen and Mac-1 T209A, although the overall binding affinity is greatly reduced (Figure 5A, bottom). This is consistent with a primary binding site around the MIDAS surface, along with a second, low-affinity Mac-1 I-domain site.3

We further tested the model of the Mac-1 I:GPIbaN complex by generating recombinant mouse GP1baN variants H218A and E222A, which were purified with a similar protocol and produced a similar yield to the wild-type protein (supplemental Figure 1A). SPR studies using the immobilized GPIbaN mutants with the wild-type Mac-1 I-domain as the analyte revealed that GP1baN H218A and E222A substitutions led to a loss of SPR response, preventing the sensorgrams from being fitted using either kinetic or equilibrium binding models, indicating significant impairment of the interaction with the Mac-1 I-domain (Figure 5C).

Discussion

Interactions among innate immune cells, platelets, and plasma proteins have been implicated in pathways leading to thrombosis,10,27-29 and yet very few of these protein interactions have been fully described. To the best of our knowledge, we provide the first detailed description of the complex formed between the leukocyte integrin Mac-1 I-domain and the platelet receptor GPIbaN using a combination of NMR, crystallography, site-directed mutagenesis, and SPR binding studies. We establish the principle that the Mac-1 I-domain in the open conformation has a groove on the surface formed by the extended sidechains of F246 and R208 and other residues that can accommodate an α-helix appropriately positioned by an acidic side chain coordinating the MIDAS metal ion and the side chain hydroxyl of T209. We show that the GPIbaN receptor LRR C-terminal capping α-helix uses a key acidic residue (D222 in the human; E222 in the mouse ortholog) to coordinate to the vacant site in the metal ion coordination shell of the Mac-1 MIDAS Mg2+ ion.

The presence of the integrin-binding acidic residue at the end of an α-helix in GPIbaN is reminiscent of the crystal structure of the Mac-1 I-domain complex with iC3b, whereby the integrin MIDAS metal ion binding residue D1247 of iC3b is also placed at the end of an
α-helix. A comparison of the Mac-1 Ii:C3b and Mac-1 I:GPibαN complexes reveals the MIDAS-coordinating α-helix is oriented differently (90° rotation) in each ligand relative to the Mac-1 I-domain surface. In addition, unlike GPibαN, iC3b does not use the negatively charged patch (E244) on the surface of the Mac-1 I-domain for binding, but in common with GPibαN it does use Mac-1 I-domain residue R208. These interactions suggest that the ability of the Mac-1 I-domain to bind a host of specific ligands involves a common binding surface and fits with the negatively charged patch (E244) on the surface of the Mac-1 I-domain. In addition, unlike GPibαN, the D248 side chain in both the open and closed conformation is not present on the surface to interact with a ligand. Mac-1 residues T213 and R216 are very close to key MIDAS face residues R208 and F246 and thus may affect binding indirectly, or, alternatively, R216 could form part of a second binding site for GPibαN.

Overall, we believe this is the first detailed description of the interface formed between an integrin receptor and an LRR protein revealing an elongated pocket in the GPibαN LRR C-terminal capping region, which is key to the interaction. The Mac-1 integrin is not the only integrin to engage multiple ligands and, interestingly, the more distantly related integrin α2β1 binds to collagen as the principal ligand and has a well-characterized additional interaction with the LRR C-terminal capping region of the matrix protein chondroadherin. The binding site for the α2β1 integrin is localized to the chondroadherin sequence of residues LRRWLEAK, which resembles the FRRWLQDN sequence we report for GPibαN. Previous authors have speculated that the groove adjacent to the α-helix could be used by the integrin as described here. This structural scaffold describing an integrin-LRR protein interaction will facilitate the development of agents targeting Mac-1 or GPibαN for the modulation of thrombotic and inflammatory disorders.
Acknowledgments

The authors acknowledge the Diamond Light Source for provision of synchrotron radiation in using the beamline I24 (Mac-1) and the European Synchrotron Radiation Facility (GIPIbn) and thank Phillip Williams for analysis of SPR data and Bernhard Neiswander (emfret ANALYTICS) for providing the Xia.B2 antibody used in the SPR studies and Keith McCrae for the kininogen complementary DNA.

The work was supported in part by a British Heart Foundation grant RG/12/9/29775 and IG/16/1/32140 (J.E.), and by the School of Chemistry at the University of Nottingham and the EPSRC for studentship support (J.M.). The nuclear magnetic resonance facilities are supported by the School of Chemistry and were funded from the Nottingham HEFCE Capital Investment Fund allocation (2009).

Authorship

Contribution: J.A.L., M.S.S., and J.E. designed the research project; J.M. collected and assigned the nuclear magnetic resonance data with technical assistance from H.E.L.W., with nuclear magnetic resonance analysis of additional mutants by C.A.; cloning, protein expression, and isotopic labeling was carried out by J.M. with additional mutants produced by A.F.; X-ray analysis of mouse GP1balphaN was carried out by M.S. and Mac-1 I-domain by R.N. and S.G.C.; surface plasmon resonance experiments were conducted by J.M., S.S.W., and A.D.M.; all authors discussed the results; and J.E., M.S.S., J.A.L., and J.M. contributed to the writing of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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