An Isoleucine-based Allosteric Switch Controls Affinity and Shape Shifting in Integrin CD11b A-domain*

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In response to cell activation signals, integrins switch from a low to a high affinity state. Physiologic ligands bind to integrins through a von Willebrand Factor A-type domain. Crystallographic studies revealed two conformations of this domain, “closed” and “open.” The latter crystallizes in complex with a pseudoligand or ligand, suggesting that it represents the high affinity state; data linking structure and activity are lacking however. In this communication, we expressed stable low and high affinity forms of integrin CD11b A-domain and determined their binding isotherms and crystal structures. The low affinity form, generated by deleting an N-terminal extension intrinsic to the domain, did not bind to physiologic ligands, and crystallized in the closed conformation. The high affinity form was generated by either deleting or substituting an invariant C-terminal Ile316, wedged into a hydrophobic socket in the closed form, but displaced from it in the open structure. Both mutants crystallized in the open conformation, and the Ile316 → Gly-modified integrin displayed high affinity. Structural differences between the low and high affinity forms were detected in solution. These data establish the structure-function correlates for the CD11b A-domain, and define a ligand-independent isoleucine-based allosteric switch intrinsic to this domain that controls its conformation and affinity.

Integrins are heterodimeric receptors that mediate vital cell-cell and cell-matrix adhesive interactions (1). Integrins bind to physiologic ligands in a divalent cation-dependent manner and require a solvent-exposed acidic residue in their respective ligands for binding. Integrin interactions with physiologic ligands and crystal structures. The low affinity form, generated by deleting a N-terminal extension intrinsic to the domain, did not bind to physiologic ligands, and crystallized in the closed conformation. The high affinity form was generated by either deleting or substituting an invariant C-terminal Ile316, wedged into a hydrophobic socket in the closed form, but displaced from it in the open structure. Both mutants crystallized in the open conformation, and the Ile316 → Gly-modified integrin displayed high affinity. Structural differences between the low and high affinity forms were detected in solution. These data establish the structure-function correlates for the CD11b A-domain, and define a ligand-independent isoleucine-based allosteric switch intrinsic to this domain that controls its conformation and affinity.

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described previously (13, 20, 21). Site-directed mutagenesis was carried out in pGEX-4T-1 vector as described previously (13). The following mutagenic primers were used: 5′-AIFGA forward, 5′-TATAATGCTAGCG-GCCCTCCGGAGGAGCTCCTAGAAGATG-3′; AIF reverse, 5′-CTCAGTGGAGTGCCTCCAGGAGGCTGACGTTG-3′; and 5′-CTACGTTAACATCCCTGAGCAAAGCCCTTCT- C′-3′. Introduction of the respective mutation was confirmed by direct DNA sequencing. The recombinant DNA work used standard molecular biology protocols. The Pseu-Bspa-restricted cDNA fragment of the CD11b A-domain (11bA) containing the mutation was subcloned into the Pseu-Bspa-restricted CD11b cDNA and cloned into 11bpcDNA plasmid (which contains a full-length human CD11b). Recombinant human 11bA spanning amino acid residues Glu123–Gly321 (11bA123–321) numbering based on the full-length coding region (22), Glu123–Lys315 (11bA123–315), lacking residues Ile316–Phe-Ala and therefore the surface terminal of helix α7) or containing an Ile316 to Gly substitution (11bA123–315–Gly) were expressed as GST fusion proteins in Escherichia coli (4), thrombin-cleaved, and purified as described previously (6). The cysteine residue at position 128 was replaced by serine in all the expressed GST-A-domain fusion forms to prevent formation of disulfide-linked dimers after thrombin cleavage (data not shown). Purity was confirmed by SDS-polyacrylamide gel electrophoresis analysis. 

Crystalization, Data Collection, and Structure Determination—

Crystals were grown using 10 mg/ml protein and the hanging drop vapor diffusion method as described previously (6). 11bA123–315 and 11bAIle-316 crystallized under the above conditions, but formed crystals at room temperature. Crystals started to form within a week, grew to a typical size of 0.3 × 0.5 × 0.4 mm in 2 weeks, and belonged to the tetragonal space group P43 (Table I). 11bA123–321 did not crystallize under the above conditions, but formed crystals at room temperature using 10% polyethylene glycol 4000, 0.1 M sodium acetate, pH 4.5, 5 mM MnCl2 as precipitant; 11bA123–315 and 11bA123–315–Gly did not form crystals in this buffer. The 11bA123–321 crystals belong to space group I4122 (Table I). The two crystal forms are not related.

Single 11bA123–315 and 11bA123–315–Gly crystals were used to collect, respectively, 2.3 and 3.0 Å resolution data sets, at 100 K, on beamline X12B of the National Synchrotron Light Source at Brookhaven National Laboratory using a charge-coupled device detector. A 2.6 Å resolution data set was collected from a single 11bA123–321 crystal, using an in-house rotating anode generator/imaging plate system. Data were integrated and reduced with the HKL package (23). Structures were determined by molecular replacement. We used the refined 1.8 Å Mg2+ structure (residues Asp132 to Lys315) as a starting model for 11bA123–315 and 11bA123–315–Gly. The refined structure of F302W 11bA (residues Gly302 to Gly321), which is similar to the Mn2+ structure (Protein Data Bank accession code 1jzm) (16), was used as the starting model for 11bA123–315–Gly. The models were modified by deleting ions and water molecules. Rigid body refinement was initially used to improve each solution. Subsequent refinement rounds consisted of alternating cycles of torsion-angle dynamics and restrained individual isotropic B factor refinement protocols in XPLOR (24). In each case, 5% of the data were excluded from the refinement to monitor the free R-factor (24). After each refinement round, the models were inspected with (2Fo − Fc) and (Fo − Fc) electron density maps and modified using O (25). For 11bA123–315, NCS restraints were used in early refinement and gradually released as the resolution increased. Solvent and metal ions were added during later stages of model refinement based on peaks >3σ in (Fo − Fc) difference maps, reasonable hydrogen bond distances, and refined temperature factors of less than 50 Å2. The final models comprise all non-hydrogen atoms of residues Asp132 to Lys315 for 11bA123–315 and 11bA123–315–Gly, and residues Asp132 to Gly321 for 11bA123–315. Data collection and refinement statistics are shown in Table I.

Protein Binding to 11bA—Ligand binding was measured using surface plasmon resonance (BIAcore AB, Uppsala, Sweden). The physiologic ligands iC3b, fibrinogen, CD54, the antagonist NIF, and the mAbs 904 or 44a were covalently coupled individually via primary amines to the dextran matrix of separate CM5 sensor chips. BSA-immobilized BSA-immobilized 11bA was flowed over the chip at 5 μl/min at different times. TBS (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 2 mM MgCl2 and 0.005% P20 (BIAcore AB) was used as the running buffer unless otherwise indicated. 1 mM NaCl in 20 mM Tris-HCl, pH 8.0, was used to remove the bound proteins and to regenerate the surface. Binding was measured as a function of time, and binding isotherms were determined as described previously (26). After subtracting background binding to the BSA-immobilized 11bA, the binding was expressed as a percentage of binding to WT, after correcting for the degree of surface expression using binding of mAb 904 (13).

RESULTS AND DISCUSSION

Generation of Stable High and Low Affinity Forms of 11bA in Solution—in attempting to express stable and homogeneous forms of the low and high affinity forms of 11bA123–315, we took note of the following unexplained observations. First, integrin A-domains that bind to their respective physiologic ligands contain a N-terminal amino acid extension (16 amino acids N-terminal to Gly127 in CD11b); domains lacking this extension bind physiologic ligands poorly (4, 27–30). Second, one major difference in the crystal structure of the open and closed forms is the position of Ile316 close to the C terminus. In the closed form, Ile316 is part of the last turn of helix α7 and fits tightly into a conserved hydrophobic pocket (Fig. 1, a–d). In the open structure, Leu316 replaces Ile316 in this pocket (Fig. 1, c–f). Ile316 is strictly conserved in all integrin A-domains (Fig. 1g). Third, some crystal and NMR structures of other integrin A-domains (3, 5–7) show structural flexibility in the C-terminal helical turn of α7, suggesting a potential physiologic regulatory role. We evaluated the structural and functional consequences of modifying Ile316 in integrin 11bA that lacks the bulk of the extrinsic N-terminal extension.

The ligand binding properties of 11bA123–321 and 11bA123–315 were determined using surface plasmon resonance (6). 11bA123–321 showed no binding to the activation-dependent physiologic ligands, complement iC3b, fibrinogen, and CD54 (ICAM-1) (Fig. 2, a and e), in the presence of MgCl2. In contrast, 11bA123–315 displayed high affinity binding to all three ligands (Fig. 2, b, d, and f). The divalent cations Mn2+ and Mg2+, but not Ca2+ (each at 2 mM), supported the interaction of 11bA123–315 with iC3b (Fig. 2b); no binding of iC3b to 11bA123–315 was observed in the presence of 2 mM EDTA (Fig. 2b). Physiologic ligand binding to 11bA123–321 in the presence of Mn2+, Mg2+, Ca2+, or EDTA was minimal (Fig. 2a). The structural differences observed between the low and high affinity forms did not require the presence of a physiologic ligand, as mAb 44a, which binds in the C-terminal region of 11bA12 (12), displayed a ~500-fold difference in affinity between the low and high affinity forms (Fig. 2, k and l). Both A-domain forms bound equally well to the activation-independent antagonist NIF (Fig. 2, g and h), and to mAb 904 (Fig. 2, i and j), indicating that the differences observed are not caused by variations in A-domain concentrations. These data establish the feasibility of expressing stable and homogeneous low and high affinity forms of 11bA and show that structural differences between the two can be detected in solution in the absence of physiologic ligands. 

Crystal Structures of 11bA123–321 and 11bA123–315—To provide a structural basis for the functional differences observed in solution, we determined the crystal structures of 11bA123–321 and 11bA123–315. The crystal structure 11bA123–321 was that of the closed conformation (Fig. 3, a, c, and e), with a Mn2+ ion occupying MIDAS, as shown previously (16). On the other
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FIG. 1. Ile316 coordination pocket. a and b, surface representation of the CD11bA domain crystal structure in its closed (a) and open (b) states, with the C-terminal α7 helix outlined green, and the socket for isoeucine (SILEN) residues (Ile335, Leu336, Ile336, Tyr337), lying within a 4-Å radius from Ile316) shown in yellow. An arrow points to the MIDAS face at the top. As can be seen, MIDAS and SILEN lie on almost opposite ends of the A-domain structure. c and d, magnified face view of SILEN in the closed (c) and open (d) conformations. In the closed form opposite ends of the A-domain structure. isoleucine (SILEN) residues (Ile135, Leu164, Ile236, Tyr267, lying within a 4-Å radius from Ile 316) shown in red. Leu312 moves to cover SILEN in the open structure (d). a-d were built using GRASP (Barry Honig, Columbia University, New York). e and f, stereo views of the Ile316 coordination socket in the closed (e) and open (f) conformations (16). SILEN residues are shown in blue, and Ile316 and Leu312 in red. g, sequence alignment of the nine human integrin A domains, of which only the respective α7 helices are shown (residues 302–318 in CD11b). The invariable Ile316 is outlined in red. Sequences are aligned based on the structural superimposition of the domains of CD11b, CD11a, CD49a, and CD49b.

FIG. 2. Functional analysis of 11bA123–321 and 11bA123–315 A-domains using BLAcore™. Sensorgrams recording the interactions of 11bA123–321 (a, c, e, g, i, k) or 11bA123–315 (b, d, f, h, j, l) with the activation-dependent ligand iC3b (a, b), fibrinogen (c, d), or CD54 (e, f), and the activation-sensitive mAb 44a (k, l). All three physiologic ligands bound to 11bA123–321, but not to 11bA123–315, in the presence of 2 mM Mg2+ (a–l, bold lines). iC3b binding to 11bA123–315 was supported by 2 mM Mn2+ (b, dashed line) or 2 mM Mg2+ (b, bold line). Background binding to BSA-coated chip was subtracted (this amounted to 12–17% of total binding when Mg2+ or Mn2+ are used in the buffer but was ~85% when calcium was included in the buffer). Minimal binding occurred in the presence of 5 or 2 mM Ca2+ (b, dotted line and —), respectively, and no binding was observed in the presence of 2 mM EDTA (b, —). Mn2+ (b, dashed line), Mg2+ (b, solid line) or 2 mM EDTA (b, —) did not support binding of 11bA123–321 to iC3b. mAb 44a bound with ~500-fold higher affinity to 11bA123–321 versus 11bA123–315 (Kd of 2 mM versus 870 nM, respectively) or 2 mM EDTA (a, —) did not support binding of 11bA123–321 to 11bA123–315 versus 11bA123–315 (Kd of 2 mM versus 870 nM, respectively). Binding of 11bA123–321 and 11bA123–315 to the activation-independent natural antagonist, NIF (g, h), and to the activation-independent mAb 904 (i, j) were comparable. To quantitatively determine the affinity, a range of different concentrations of A-domains were flowed over the sensor chips (data not shown). The binding data (in the presence of Mg2+) were analyzed by the linear transformation method (37), giving Kd values (mean ± S.D., n = 2) of 0.46 ± 0.15 μM (for iC3b), 0.25 ± 0.07 μM (for Fg); and 0.22 ± 0.04 μM (for CD54).
octahedral coordination distinguishes it from solvent (31). Third, the average unrestrained distance from this metal ion to coordinating ligands (2.28 Å) is more close to Ca\(^{2+}\) in the octahedral coordination (2.35 Å) than Mg\(^{2+}\) (2.07 Å) or Mn\(^{2+}\) (2.17 Å) (32). The refined B-factor for calcium is slightly lower than that of the surrounding side chains but is still within the margin of error for a 2.3-Å resolution. These data suggest that Ca\(^{2+}\) may be coordinated in MIDAS in the open conformation, at least under the nonphysiologic crystallization conditions used here. An alternative interpretation of the above structural and functional data is that Ca\(^{2+}\) does bind under physiologic conditions to the open form but exerts a suppressive effect on physiologic ligand binding. Measurements of Ca\(^{2+}\) affinity to the high versus low affinity A-domain states may help in differentiating between these two possibilities. The above data also support previous findings that the nature of the metal ion per se is not sufficient to induce the open state (6, 7).

An Ile\(^{316}\) to Gly Substitution in 11bA Crystallizes in the Open Form and Generates High Affinity 11bA and Holoreceptor—

Ile\(^{316}\) is invariable in all integrin a\(^\alpha\)A-domains cloned to date (Fig. 1g). We determined the effect of removing the hydrophobic side chain “finger” of this isoleucine on affinity and structure of the A-domain. The 11bAIle-316\(^{3}\)Gly form exhibited “high affinity” (Fig. 4a). The same substitution created in the holoreceptor dramatically increased its ligand binding activity (Fig. 4b). The 11bAIle-316\(^{3}\)Gly crystal structure (Table I) was essentially identical to that of the open 11bA 123–315 form, including the predicted presence of calcium in MIDAS; the two structures can be superimposed onto each other with an root mean square deviation of 0.17 Å for main chain atoms. Taken together, these data indicate that an Ile\(^{316}\)-based switch, intrinsic to this domain, acts allosterically to regulate its ligand binding affinity and topology. A conserved hydrophobic intramolecular socket (SILEN, socket for isoleucine), fastens the Ile\(^{316}\) finger in the closed conformation; Ile\(^{316}\) is replaced by Leu\(^{312}\) at this site in the open structure (Fig. 1, a–f). SILEN is formed by the hydrophobic side chains of Ile\(^{135}\), Leu\(^{164}\), Ile\(^{236}\), and Tyr\(^{267}\) both in the closed and open conformations (Fig. 1, c–f) and are either identical or conserved in all the other A-domains. In the
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CD11b/CD18 integrin, certain mutations that lie outside MIDAS produce gain-of-function effects in the holoreceptor and are believed to act allosterically (12, 18, 19, 33). Similar data were recently reported in the CD11a/CD18 heterodimer, while this manuscript was in preparation (33). All these studies were carried out in the holoreceptors and therefore did not determine whether allosteric regulation is intrinsic to the A-domain, since potential interdomain interactions and/or other quaternary effects in the holoreceptor may also be operative. While the transition from the closed to the open conformation undoubtedly involves multiple intradomain realignments, the data presented here suggest that interference with SILEN may be a key component in allosteric regulation. We note that mutations that up-regulate integrin function occur in or around SILEN. For example, substitution of the α1–β loop of CD11b with that of CD11a generates a constitutively active integrin (19). This region includes Leu1-44, one of the SILEN residues. Integrin activation also occurs in an Leu1-44 to Phe substitution (18), which predictably makes SILEN smaller and therefore less accommodating to Ile316. Other activating mutations involving Glu313, Asp319, Lys321, and Phe324 lie at the bottom of the structure, in close proximity to SILEN (18), and may thus exert their effects through interference with the proper coordination of the Ile316 finger in SILEN. The inhibitory effect of certain mAbs with epitopes on the opposite side of MIDAS (e.g., mAb 44a, the epitope of which spans residues on the top of SILEN (12)) may similarly be explained through stabilization of the SILEN pocket. The binding site for the CD11a/CD18 inhibitor lovastatin (34) includes two of the four SILEN residues (Leu133 and Tyr287) in CD11a, which correspond to Ile315 and Tyr297, respectively, in CD11b, and may therefore act by stabilizing the low affinity state. Interestingly, an Ile316 to Ala substitution in CD11a (equivalent to Ile316 in CD11b) was found to increase ligand binding affinity of the CD11a/CD18 heterodimer (33), suggesting that the present observations may extend to other A-domains.

Implications for Mechanisms of Activation by Inside-out Signaling—Integrins exist in low and high affinity states in the absence of ligand (2). However, the high affinity state of the ligand binding A-domain has so far been seen crystallographically only in complex with a pseudoligand (3, 6) or ligand (15), leading to the suggestion that the ligand triggers or stabilizes the active state. The low and high affinity forms of 11bA reported here assume different structures even in solution and in the absence of ligand, as revealed by the differential binding of mAb 44a. Although not strictly proven, it is likely that the differences detected in solution correspond to the open and closed conformers. Generation of the high affinity 11bA in a stable form should now permit an examination of this question.

Previous studies in all integrin A-domains expressed to date have shown that the presence of a short N-terminal amino acid extension extrinsic to the A-domain itself allows the respective domain to bind to its physiologic ligands. Removal of this N-terminal extension or reducing it to four residues, as shown here, generates a low affinity state that assumes the closed form. These data suggest that the N-terminal extension plays a role in affinity regulation within the domain. The underlying structural basis for this effect is unknown, since none of the residues in the N-terminal extension are included in the three-dimensional structures of this domain. It has been observed, however, that residues within this extension regulate ligand binding in A-domains. First, naturally occurring point mutations in this segment of the vWF A1 domain cause gain-of-function phenotypes in patients with type IIb vWf disease (35). This region also contains an activating mutation in CD11b (18). Second, structural data from the CD49b A-domain also show that three residues that extend beyond the α7 helix can pack into a crevice formed in part by N-terminal residues, bringing the N and C termini into close spatial proximity (5). Flexibility of the C-terminal residues in α7 has also been observed in the crystal (36) and NMR (8) structures of the CD11a A-domain. Based on these data, we speculate that the N-terminal extension in the isolated A-domain may loosen Ile316 coordination into SILEN, thus allowing some molecules to exist in the high affinity open form. A similar mechanism may be operative in the holoreceptor; inside-out signals may through intramolecular repacking, free the N-terminal extension to access and therefore modify SILEN. Validation of such a mechanism will require the three-dimensional structure determination of a whole integrin.

The ability to express a stable form of the high affinity A-domain and to identify the structural basis of A-domain affinity switching should have broad biologic and pharmaceutical applications. Elucidation of the structures of other A-domains in their open conformation in the absence or presence of physiologic ligands may now be feasible. Also, integrin affinity is increased in many common diseases. High affinity forms of integrin A-domains could be of value therapeutically or utilized in high throughput screens to develop effective small molecule antagonists.

Acknowledgments—We thank Dr. Malcolm Capel and his staff at the Brookhaven National Laboratory for assistance in data collection. We acknowledge the Massachusetts General Hospital crystallography facility for providing in-house data collection equipment.

| Data collection and summary of refinement results |
|---------------------------------|
| Space group | 11bA123–321 | 11bA123–315 | 11bA1le316–Gly |
| Unit cell constants (Å) | P2₁,2,2 | a = 48.1, b = 121.5, c = 74.6 | P4₁ | a = b = 45.7, c = 94.8 | a = b = 45.2, c = 95.0 |
| No. of unique reflections | 13,990 | 7,955 | 3,749 |
| Resolution (Å) | 8.0–2.6 | 8.0–2.3 | 8.0–3.0 |
| Rmerge (%) | 7.7 (23) | 8.8 (28) | 11.2 (30.4) |
| Completeness (%) | 99.3 (98.4) | 91.1 (80.5) | 95.3 (73.6) |
| Redundancy | 5.9 | 2.3 | 2.7 |
| R-factor (%) | 21.9 | 18.8 | 20.8 |
| R-free (%) | 30.0 | 24.8 | 28.8 |
| Solvent molecules | 82 | 62 | 20 |
| Metal ions | 2 (Mn²⁺) | 1 (Ca²⁺) | 1 (Ca²⁺) |
| Root mean square deviations from ideal values | | |
| Bond lengths (Å) | 0.007 | 0.007 | 0.006 |
| Bond angle (°) | 1.3 | 1.3 | 1.25 |

*a Rmerge = ∑|I| − ⟨I⟩/2I, where I is the observed intensity and ⟨I⟩ is the average intensity from multiple observations of symmetry-related reflections.
*b Numbers in parentheses shows values for highest 0.1 Å resolution bin.
*c R-factor = ∑|Fo − Fc|/∑|Fc|, where T is a test set containing a randomly selected 5% of the reflections omitted from the refinement.
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J. Biol. Chem. 2000, 275:38762-38767.
doi: 10.1074/jbc.C000563200 originally published online October 16, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C000563200

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