

Therapeutic Antagonists and the Conformational Regulation of the α_2 Integrins

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Abstract: The α_2 integrins are validated therapeutic targets for inflammatory disorders. Two distinct mechanistic classes of small molecule inhibitors, termed I allosteric and / I-like allosteric antagonist, have recently been developed. The

I allosteric antagonists bind underneath the C-terminal helix of the I domain and stabilize the I domain in the inactive closed conformation. By contrast, the / I-like allosteric antagonists bind to the α_2 I-like domain MIDAS and disrupt conformational signal transmission between the I and the I-like domain, leaving the I domain in a default inactive form. Furthermore, the two classes of the antagonists have opposite effects on integrin conformation; the I allosteric antagonists stabilize the bent conformation, whereas the / I-like allosteric antagonists induce the extended conformation with inactive I domain. The small molecule antagonists to the α_2 integrin highlight the importance of the structural linkages within and between integrin domains for transmission of the conformational signals and regulation of the overall conformation.

INTRODUCTION

The α_2 integrins ($\alpha_L\beta_2$ or LFA-1; $\alpha_M\beta_2$ or Mac-1; $\alpha_X\beta_2$ or p150,95; and $\alpha_D\beta_2$) are a family of integrins, which share the β_2 -subunit and are expressed exclusively on leukocytes. $\alpha_L\beta_2$ and $\alpha_M\beta_2$ play critical roles in leukocyte migration into sites of inflammation, and in the case of $\alpha_L\beta_2$, into lymphoid tissues [1, 2]. $\alpha_L\beta_2$ is required for a wide variety of cell-cell interactions including T cells with antigen-presenting cells, B cells with T cells, and NK cells with target cells, and acts as a co-stimulatory molecule in essentially all T cell responses, correlating with its participation in formation of the immunological synapse [3].

$\alpha_L\beta_2$ binds exclusively to IgSF cell surface molecules termed ICAMs, whereas $\alpha_M\beta_2$ binds to ICAM-1, fibrinogen, complement component iC3b, and many other protein ligands. A major endothelial ligand for both $\alpha_L\beta_2$ and $\alpha_M\beta_2$ is ICAM-1, which comprises five tandem Ig-like domains [4]. The binding sites for $\alpha_L\beta_2$ and $\alpha_M\beta_2$ are located in domain 1 and domain 3 of ICAM-1, respectively. The ability of the α_2 integrins to bind ligand is dynamically enhanced by intracellular signals (see below).

As shown by a rare genetic disorder leukocyte adhesion deficiency I, where mutation of the α_2 integrin subunit results in null or severely reduced expression of all α_2 integrin heterodimers, the α_2 integrins are essential for host defense against microorganisms. Furthermore, α_2 integrins are important in the pathogenesis of leukocyte-mediated tissue injuries in inflammation and autoimmunity [5]. Of the

leukocyte integrins, only $\alpha_L\beta_2$ is expressed on most lymphocytes, whereas $\alpha_M\beta_2$ predominates on neutrophils with some $\alpha_L\beta_2$ and $\alpha_X\beta_2$ also present. Diseases in which neutrophils are important, such as ischemia-reperfusion injuries in cerebral and myocardial infarction and shock, have usually been targeted with antagonists to all α_2 integrins or to $\alpha_M\beta_2$. In contrast, diseases in which lymphocytes are important, such as psoriasis, rheumatoid arthritis, and organ transplant rejection have usually been targeted with antagonists of $\alpha_L\beta_2$ [5 - 8]. Phase III clinical trials in psoriasis with a mAb to the α_L I domain have resulted in FDA approval of efalizumab [9].

The α_2 and α_4 integrins on leukocytes both play important roles for leukocyte-endothelial interaction in inflammation by binding to ICAM-1 and VCAM-1, respectively, and are validated therapeutic targets for the treatment of inflammatory diseases [9-11]. A major structural and functional difference between the α_2 and α_4 integrins is that the α_2 integrins contain, in the β_2 -subunit, an 'extra', inserted (I) domain that functions as a major ligand binding domain. However, one class of potent small-molecule antagonists of α_2 integrins, the / I-like allosteric antagonists, share key structural features with α_4 integrins. Indeed, as we describe below, / I-like allosteric α_2 integrin antagonists have recently been shown to function analogously to α_4 integrin antagonists, except that the former block binding of an intrinsic ligand whereas the latter block binding of extrinsic ligands. In this review, we focus on mechanism of action of small-molecule antagonists to the α_2 integrins, and relevance to the α_4 integrins. In a related review, we have covered a broader range of integrin therapeutic targets [12]. The reader is referred elsewhere for reviews on the small molecules and antibodies to the α_2 integrins, and the results of animal model studies and human clinical trials [8, 9, 13, 14].

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OVERVIEW OF INTEGRIN STRUCTURE

Integrins comprise two non-covalently associated, type I transmembrane glycoprotein α and β subunits. Each subunit contains a large extracellular portion with multiple domains, a single transmembrane domain, and except for integrin α_4 , a short cytoplasmic tail. The integrin α subunit ectodomain of >940 residues contains 4 domains (5 in I domain-containing

integrins) and the β subunit of ~640 residues contains 8 domains (Fig 1). The N-terminal portions of the α and β subunits fold into the globular headpiece, which is connected through α and β tailpiece domains to the membrane [15-18]. The α -subunit β -propeller domain and the β -subunit I-like domain form the integrin head, a ligand-binding structure in the headpiece (Fig 1F). About half of the β -subunits (β_1 , β_2 , β_{10} , β_{11} , β_L , β_M , β_X , β_D and β_E) contain an inserted (I)

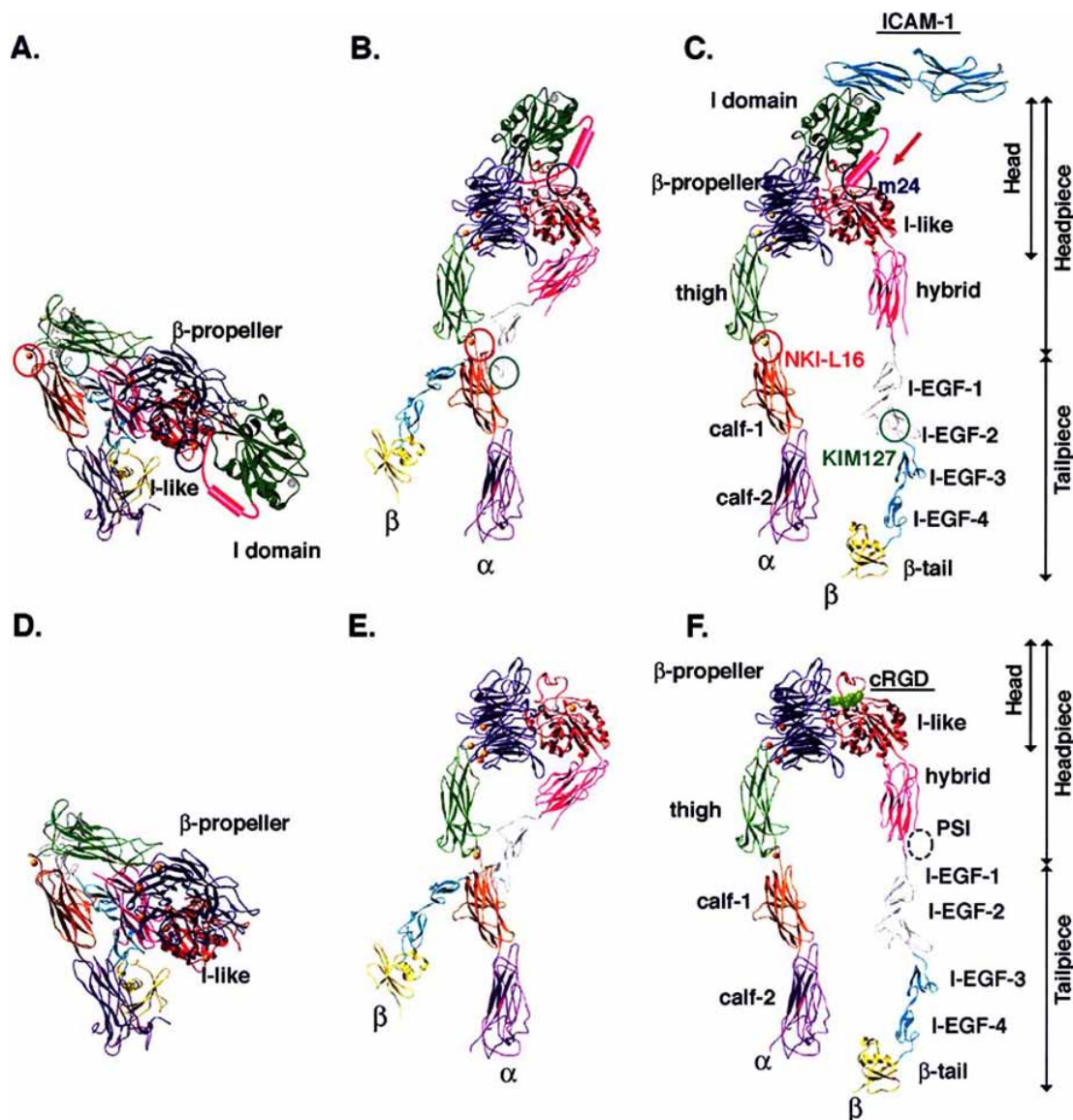


Fig. (1). Domain organization and global conformational changes of the extracellular portion of integrins. Three distinctive conformations are illustrated for a representative I domain containing integrin $\alpha_L \beta_2$ (A-C) and a representative non I domain-containing integrin $\alpha_V \beta_3$ (D-F). The conformations except for the I domain are based on crystal [18, 30], NMR [53], and electron microscopy [35] structures. The α_L I domain and its complex with the N-terminal 2 domains of ICAM-1 are cartoons based on crystal structures [27]; the C-terminal I domain β -helix is represented by a cylinder (A-C). The I domain is joined at the point of its insertion in the β -propeller domain but its orientation is arbitrary; the I domain and ICAM-1 are shown at slightly larger scale for emphasis. Ribbon representations were prepared as described [62, 71]. (A&D) Bent conformation (low-affinity) (B&E) Extended conformation with closed headpiece (intermediate-affinity) (C&F) Extended conformation with open headpiece (high-affinity) bound by the N-terminal two domains of ICAM-1 (C) or cyclic RGD peptide (cRGD) (F) (shown as a space-filling representation). Ca^{2+} and Mg^{2+} ions are spheres. Locations of epitopes of activation-dependent mAbs to $\alpha_L \beta_2$ are shown by circles and labeled. Integrin α and β subunit and domain names are shown and ligand names are shown and underlined.

domain, which is located at the top of the integrin head (Fig 1C). Integrin domains including the α -subunit thigh, β -subunit PSI and hybrid domains, and those in the tailpiece, are not directly involved in ligand-binding. However, these domains are important for conformational regulation of integrin ligand binding activity. They present a huge interface that buries more than 4,000 Å² of solvent-accessible surface area between the headpiece and tailpiece and between the α and β tailpiece in the bent, low affinity conformation (Fig 1A&D). Rearrangements of integrin domains, which are initiated by inside-out and/or outside-in signaling, result in destabilizing this interface and shifting the conformational equilibrium towards the active state (see below).

DOMAINS IN THE INTEGRIN HEAD

I Domain

α -subunit I domains and β -subunit I-like domains adopt an α/β fold with a metal ion dependent adhesion site (MIDAS) on the “top” of the domain, whereas the C and N-terminal connections are on the distal “bottom” face [18-21] (Fig 2). Metal-dependent interactions through the MIDAS of each of these domains plays a central role in ligand recognition. The I domain, which is inserted between blade 2 and 3 of the α -propeller domain of the α -subunit (Fig. 1C & Fig. 2) [22], is a major ligand-binding domain and recognizes ligand directly when it is present [23, 24]. The ability of the I domain to bind ligand is controlled by conformational changes; the affinity of the I domain for its ligand is enhanced by downward axial displacement of its C-terminal helix, which is conformationally linked to alterations of the MIDAS loops and Mg²⁺ coordination [25-27] (Fig 3). In the case of α_L2 , compared to the default, low affinity conformation, downward displacements by one and two turns of helix lead to intermediate- and high-affinity conformations with ~500 and 10,000-fold increased affinity, respectively [27].

Conversely, binding of ligand to the MIDAS of the I domain induces conformational change by stabilizing the high affinity conformation. These changes include rearrangements in metal coordination in the MIDAS and backbone movements in the loops surrounding the MIDAS, which are linked to a downward axial displacement of the C-terminal helix (Fig. 3) [19, 27, 28].

I-like Domain

The β -subunit I-like domain, which is inserted in the hybrid domain of the β -subunit, directly binds ligand in integrins that lack I domains in the α -subunit (Fig 1F). By contrast, when the I domain is present, the I-like domain functions indirectly by regulating the I domain (Fig. 1C). Compared to the I domain, the I-like domain contains two long loops, including one which is important for determining ligand specificity, and is referred to as the specificity-determining loop (SDL) [29]. On either side of the MIDAS, the I-like domain contains two adjacent metal coordination sites, the ADMIDAS (adjacent to MIDAS) and LIMBS (ligand-associated metal binding site) (Fig 1) [18, 30]. The MIDAS is directly involved in ligand-recognition, whereas the ADMIDAS and LIMBS are negative and positive

regulatory sites for ligand-binding at the MIDAS, respectively [31].

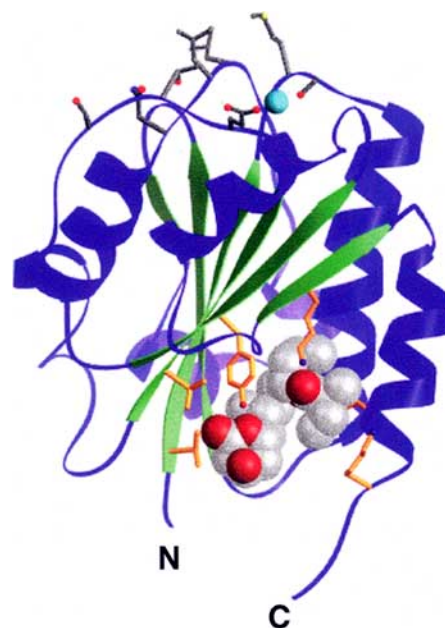


Fig. (2). Ribbon diagram of the α_L I domain in complex with an I allosteric antagonist [57]. The I domain adopts a Rossmann fold in which the central β -strands are surrounded by 7 α -helices. A Mg²⁺ ion is located at the metal ion-dependent adhesion site (MIDAS) on the “top” of the domain, whereas the C- and N-terminus, which connect to the α -propeller domain, are on the bottom. The I allosteric antagonist (shown by a space-filling representation with white carbon atoms and black oxygen atoms) binds in the hydrophobic pocket underneath the C-terminal α -helix and stabilizes the I domain in the closed conformation. The side chains within the antagonist-binding pocket are shown. The residues that interact with ICAM-1 in the crystal structure [27] are located around the MIDAS, distal from the antagonist binding site.

The function of the I-like domain appears to be regulated by conformational changes similar to those observed in the I domain, in which a downward movement of the C-terminal α -helix allosterically alters the geometry of the MIDAS and increases the affinity for ligand [20, 32]. Mutation L358A in the C-terminal 7 helix of the α_1 I-like domain induces the active conformation of the I-like domain that binds ligand with high-affinity [33]. An outward swing of the hybrid domain relative to the I-like domain has been observed in the high affinity, ligand-bound state (Fig. 1B&C, E&F), and is thought to be coupled to the downward shift of the C-terminal α -helix of the I-like domain [34-36].

α -propeller Domain

The N-terminal region of the integrin α -subunit contains seven repeats of about 60 amino acids, which fold into a seven-bladed α -propeller domain [18, 22] (Fig 1). A β -propeller domain with the same topology is also found in the trimeric G-protein β -subunit. The α -propeller domain directly participates in ligand recognition in those integrins that lack I domains [37].

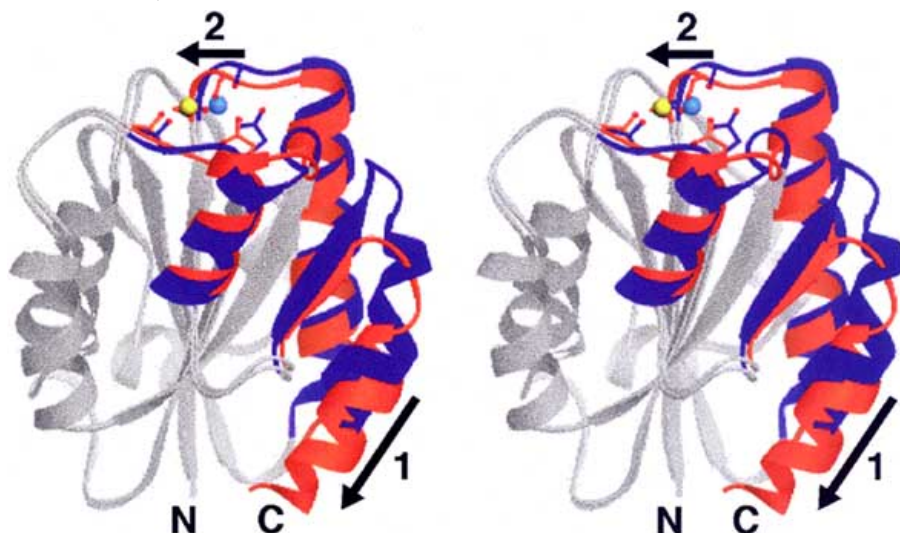


Fig. (3). Stereo view of alternative conformations of the α_M I domain. The regions with significant conformational changes are shown in blue (closed, low-affinity [72]) and orange (open, high-affinity [19]). Regions with similar backbones are in gray. Mg^{2+} ions and side chains of metal-coordinating residues at the MIDAS are in blue (closed, low-affinity) and yellow (open, high-affinity) with red oxygen atoms. In inside-out signaling, a downward pull of the C-terminal helix (shown by an arrow 1) induces a rearrangement of the MIDAS to the open configuration (arrow 2). In outside-in signaling, the rearrangement of the MIDAS by ligand-binding (arrow 2) results in a downward shift of the C-terminal helix (arrow 1). In both cases, the conformational signals are relayed in the same pathway but in the opposite direction [27]. Ribbon diagrams are prepared as described [20].

DOMAIN ORGANIZATION OF THE INTEGRIN HEAD

The structure of $\alpha_V\beta_3$ that lacks an I domain reveals that the I-like domain makes extensive contact with the β -propeller domain, with the "top," ligand-binding faces of each domain oriented at about 90° to one another (Fig. 1F) [18]. Loops in blades 2, 3, and 4 of the β -propeller domain are prominent in the ligand binding site. The structure of $\alpha_V\beta_3$ in complex with a cyclic peptide containing an Arg-Gly-Asp (RGD) sequence demonstrated binding to both the α and β subunits at the β -propeller-I-like domain interface (Fig. 1F). The Asp carboxylate side chain coordinates directly to the metal of the β subunit I-like domain MIDAS, while the Arg side chain binds to the α subunit β -propeller domain [30].

In I domain-containing integrins, the bottom of the I domain is connected at its N- and C-terminal linkers to a loop between blade 2 and 3 of the β -propeller domain. A long and flexible C-terminal linker of the I domain is predicted to locate near the β -propeller/I-like domain interface, corresponding to the ligand-binding face in integrins that lack I domains [20] (Fig 1A-C). An invariant Glu residue in the I domain C-terminal linker, Glu-310 in the α_L subunit, is important in I domain activation [26]. I domain activation is induced by a downward pull on the C-terminal α -helix or linker [27, 38, 39]. It has been proposed that this universally conserved Glu residue in the I domain linker is an "intrinsic ligand," and that binding of the activated α_2 I-like domain to this intrinsic ligand pulls the C-terminal α -helix of the I domain downward, and activates high affinity for ligand (see below, Fig. 1C)[20, 32, 40]. Direct evidence in support of this hypothesis is provided by second site reversion mutations in α_L -Glu 310 and a residue near the α_2 I-like MIDAS, α_2 -Ala 210 [41]. Individual α_L -

E310C and α_2 -A210C mutations abolish ligand binding, whereas the double α_L -E310C/ α_2 -A210C mutant constitutively binds ligands, as a result of an inter-subunit disulfide bond that pulls α_L residue 310 into a position close to the α_2 MIDAS.

STRUCTURAL BASIS OF SIGNAL TRANSMISSION

Integrins transmit bi-directional signals, termed outside-in or activating signals, and inside-out or priming signals [42]. Integrins trigger "outside-in" activating signals in response to ligand binding [43, 44]. Ligand binding induces structural rearrangements in the ligand-binding domain, which convey conformational signals, through the extracellular domains, to the cytoplasm [27, 28, 45]. In B and T cell responses, binding of $\alpha_L\beta_2$ to ICAMs augments proliferation and protects against apoptosis [46, 47]. Conversely, in inside-out signaling or priming by integrins, signals received by other receptors activate intracellular signaling pathways that impinge on integrin cytoplasmic domains, and make the extracellular domain competent for ligand binding on a time-scale of less than 1 s [20, 48-50]. This unique property enables leukocytes to rapidly respond to signals in the environment, such as foreign antigen or chemoattractants, to activate adhesion and direct cell migration.

Recently, the basis for bi-directional signal transmission across the membrane by integrins has been explained. The integrin α and cytoplasmic tails associate with each other and constrain the integrin in its inactive form. Dissociation of the α cytoplasmic tails by intracellular signals leads to the activation of the extracellular parts of the integrin [16, 35, 45, 51, 52]. In the latent, low affinity state, the integrin assumes a bent conformation (Fig. 1A&D) [35, 53]. Separation of the α and β subunit cytoplasmic domains is

linked to separation of the transmembrane domains and the membrane-proximal segments of the α and β extracellular domains, which destabilizes the interface between the headpiece and tailpiece, and induces a switchblade-like opening to an extended conformation (Fig. 1B&E), re-orienting the ligand binding face with activation epitopes in the tailpiece exposed.

In the extended conformation, two different conformations of the headpiece, termed closed (Fig. 1B&E) and open (Fig. 1C&F), are seen [35]. In the bent conformation only the closed conformation of the headpiece is present [18]. Therefore, extension facilitates adoption of the open conformation of the headpiece, which corresponds to the ligand-bound, high affinity conformation [35]. Linked changes in α -propeller loops may also occur. This is the last step in ligand binding by integrins that lack I domains, in which the active form of the I-like domain directly binds ligand in cooperation with parts of the α -propeller domain. By contrast, in the case of I domain-containing integrins, a further step is involved in activation of ligand-binding: the active form of the I-like domain interacts with the 'intrinsic ligand' in the C-terminal I domain linker and executes a downward pull of the linker, which converts the I domain into the active form that binds extrinsic ligands such as ICAM-1.

In solution and apparently on the cell surface as well, integrins are not fixed in a particular conformation, but equilibrate between them [35] (Fig. 1). Whether the equilibrium favors the bent, low affinity conformation or the extended, high affinity conformation is affected by the presence of activating intracellular factors and the concentration of extracellular ligands. Activation by signals within the cell (priming) induces straightening and stabilizes the extended form. Binding of extracellular ligands also stabilizes the extended conformation and therefore enhances the separation of integrin tails, which transmits signals to the cytoplasm (ligand-induced activation). Therefore, transition from the bent to the extended conformation is a bi-directional, allosteric mechanism for relaying conformational signals between the integrin headpiece and the cytoplasmic domains. All biological integrin ligands are multivalent, and therefore can also induce integrin clustering, which appears to be required, in addition to conformational change, for outside-in signaling.

ANTAGONISTS TO α_2 INTEGRINS AND RELEVANCE TO α_4 INTEGRINS

Direct Inhibition of Ligand Binding to the I Domain

Since the top face of the I domain around the MIDAS directly binds ligands (Fig. 2), binding of inhibitors to the same site on the I domain is a straight-forward way to block function competitively. Indeed, many inhibitory mAbs map to the I domain [23, 54] and the proximity of mAb epitopes to the MIDAS metal ion correlates with how well mAbs block function [55]. Although most inhibitory I domain mAbs directly block ligand binding, some that bind more distal from the MIDAS inhibit ligand binding indirectly, similarly to the I allosteric antagonists discussed below [56].

I Allosteric Antagonists

As discussed above, the affinity of the I domain is allosterically regulated by conformational changes. Downward axial displacement of the C-terminal α -helix of the I domain is linked to structural rearrangements at the ligand binding site, the MIDAS (Fig. 1C & Fig. 3). One class of small molecule inhibitors binds underneath the C-terminal α -helix of the α_L I domain (Fig. 2) [57-59]. The antagonists bind to and stabilize the closed conformation of the I domain, inhibit conversion to the high affinity, open conformation, and thereby allosterically inhibit ligand binding to the distal MIDAS site (Fig. 2). Therefore, this class of inhibitors is termed I allosteric antagonists (Fig. 4A). The mode of action of the antagonists is confirmed by a mutant α_L I domain that is locked in the open high-affinity conformation with an engineered disulfide bond that stabilizes the position of the C-terminal α -helix (the high-affinity I domain) [25, 38, 56]. $\alpha_L\alpha_2$ containing the locked open, high-affinity I domain is resistant to inhibition by I allosteric antagonists [56] (Table 1).

Binding of I allosteric antagonists to the pocket under the C-terminal α -helix of the I domain affects not only the conformation of the I domain regionally but also the conformation of the $\alpha_L\alpha_2$ heterodimer globally (Table 1). Some I allosteric antagonists perturb binding of I-like domain mAbs [60]. Furthermore, the antagonists reduce exposure by the activating agent Mn^{2+} of activation-dependent epitopes in the α_2 I-like domain as well as in the α and β subunit legs [61, 62]. Downward movement of the I domain C-terminal α -helix appears to be tightly linked to the binding of the intrinsic ligand Glu-310 in the α_L I domain linker to the MIDAS of the α_2 I-like domain (Fig. 5G). In the absence of this interaction, activation of the I-like domain is inhibited, as shown by suppression of exposure by Mn^{2+} of the m24 epitope in the I-like domain. Suppression of KIM127 and NKI-L16 epitope exposure in the α and β legs shows that the I allosteric antagonists shift the conformational equilibrium toward the bent conformation (Table 1). Therefore, the conformation of the I domain is linked, apparently through the I-like domain, to the conformation of the leg domains. These results highlight the extensiveness of conformational linkages within integrins.

/ I-like Allosteric Antagonists

The I-like domain of the α_2 subunit of $\alpha_L\alpha_2$ is a regulatory domain. A class of $\alpha_L\alpha_2$ and $\alpha_M\alpha_2$ small molecule antagonists patented by Roche and Genentech [63, 64] (Fig. 4B & Table 1) has recently been found to bind near the interface between the I domain and the I-like domain [60, 62]. These inhibitors are polysubstituted (*S*)-2-benzoylamino propionic acids, in common with some inhibitors of $\alpha_4\alpha_1$, that lacks an I domain (Fig. 4B). Indeed, compound #1 (Fig. 4B), which was used as a lead to develop $\alpha_L\alpha_2$ and $\alpha_M\alpha_2$ antagonists at both Genentech and Roche, was initially discovered during random screening as an $\alpha_4\alpha_1$ antagonist at Roche during a collaboration with Genentech, and inhibits $\alpha_L\alpha_2$ and $\alpha_4\alpha_1$ with similar μM potency (J. Tilley, personal communication). This novel class of $\alpha_L\alpha_2$ antagonists cannot inhibit binding of isolated intermediate- or high-affinity I

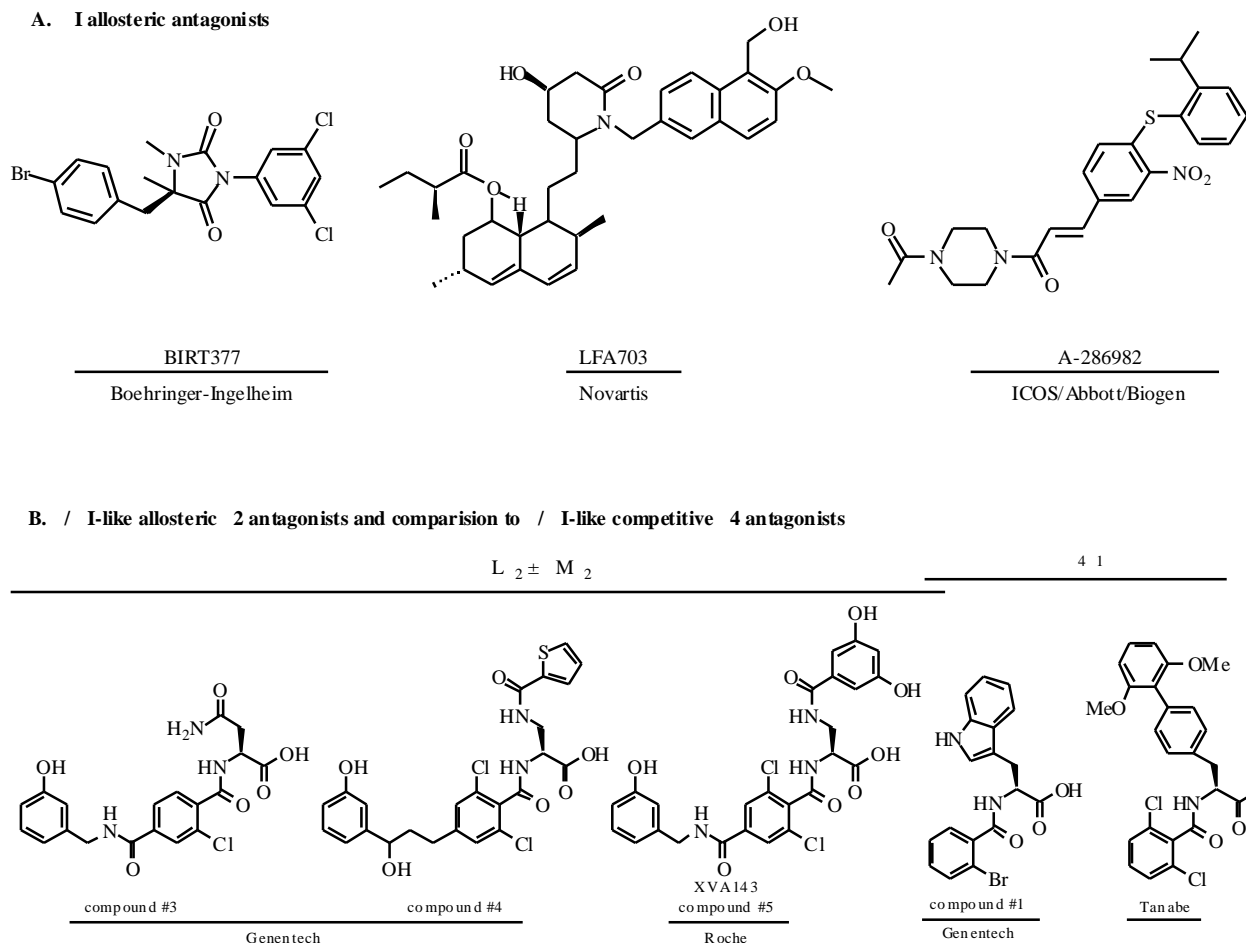


Fig. (4). Chemical structures of small molecule α_2 integrin antagonists. **A.** / I allosteric antagonists to the α_L I domain. BIRT377 [58], LFA703 [73], and A-286982 [59] are shown. **B.** / I-like allosteric antagonists to $\alpha_L \alpha_2$ and $\alpha_M \alpha_2$ are compared to / I-like competitive antagonists to $\alpha_4 \beta_1$. The antagonists are all polysubstituted (S)-2 benzoylamino propionic acids. Compounds #1, #3, #4 [64, 65], XVA143 [60, 63], and a Tanabe $\alpha_4 \beta_1$ antagonist [74] are shown. The integrins antagonized by these compounds, which both contain I domains ($\alpha_L \alpha_2$ and $\alpha_M \alpha_2$) and lack I domains ($\alpha_4 \beta_1$) are indicated.

domains to ICAM-1, whereas purified monomeric ICAM-1 in solution does inhibit. Furthermore, the compounds cannot block interaction with ICAM-1 substrates of the wild-type isolated I domain on the cell surface under shear stress, whereas / I allosteric antagonists, LFA703 and BIRT377, block this interaction (Table 1) [62]. Therefore, the Genentech compounds do not mimic ICAM-1 and do not bind to the MIDAS of the α_L I domain, as previously suggested [65].

Inhibitors of integrins that lack I domains, such as those that are based on the Arg-Gly-Asp (RGD) sequence, bind to both the integrin α and β subunits at the α -subunit-propeller/ β -subunit I-like domain interface [30] (Fig. 5C). Several of this class of antagonists to integrins that lack I domains have been found to make an association of the integrin α and β subunits resistant to SDS at room temperature [66]. / I-like allosteric antagonists (Fig. 4B), but not / I allosteric antagonists (Fig. 4A), share with antagonists of integrins that lack I domains a key structural feature, the absolute requirement for a free carboxyl group,

and often share other structural features as well. It was reasoned that if / I-like allosteric antagonists bound to a similar site, they might also stabilize the integrin complex to SDS. In the absence of added compounds, $\alpha_L \alpha_2$ was dissociated by SDS and the α_L and α_2 subunits migrated individually in SDS-PAGE, in both reducing and non-reducing conditions (Fig. 6A, lanes 1 and 6). By contrast, when treated with / I-like allosteric antagonists, but not with / I allosteric antagonists, the α_L and α_2 subunits migrated together as an $\alpha_L \alpha_2$ complex in SDS-PAGE (Fig. 6A, lanes 2-5, 7-9). Stabilization to SDS by the inhibitors was dependent on divalent cations, because EDTA abolished complex formation (Fig. 6A, lanes 11-13) [62].

The stabilization by the / I-like allosteric antagonists of $\alpha_L \alpha_2$ complexes to SDS treatment was observed not only in wild-type $\alpha_L \alpha_2$ (Fig. 6B, lane 1 and 2) but also in $\alpha_L \alpha_2$ mutants such as $\alpha_L \alpha_2$ containing the mutant locked open I domain (Fig. 6B, lanes 3 and 4) and $\alpha_L \alpha_2$ with the I domain deleted from the α_L subunit (I-less, Fig. 6B, lanes 7 and 8)

Table 1. Comparison of the α_2 Small Molecule Antagonists.

	Antagonist	
	I allosteric (e.g. BIRT377)	/ I-like allosteric (e.g. XVA143)
Inhibition ¹		
WT $\alpha_1 \alpha_2$	+	+
HA $\alpha_1 \alpha_2$	-	-
Isolated WT α_1 I domain	+	-
WT α_2	-	+

Impact on epitopes ²		
Activation-independent		
α_1 I domain (R7.1)		
α_1 -propeller domain (TS2/4)		
α_2 I-like domain (IB4)		
α_2 leg domain (CBRLFA-1/2)		
Activation-dependent		
α_1 leg domain (NKI-L16)	3	4
α_2 I-like domain (m24)	3	4
α_2 leg domain (KIM127)	3	4

Requirement of cation for antagonist binding ⁵	-	+

SDS-stable complex formation ⁶		
WT $\alpha_1 \alpha_2$	-	+
WT α_2	-	+

¹Ligand-binding of $\alpha_1 \alpha_2$ containing the wild-type (WT) or locked open high-affinity (HA) I domain was tested by binding of soluble, polyvalent ICAM-1 [62]. Binding of isolated wild-type I domain was studied under shear stress in a flow chamber, in which rolling interactions of cells expressing the wild-type I domain with ICAM-1 substrate was readily detected [39]. Cell adhesion to iC3b substrate of cells expressing wild-type α_2 was described [75].

²Expression of mAb epitopes in $\alpha_1 \alpha_2$ on K562 cells was studied by immunofluorescent flow cytometry [62] in the presence or absence of the antagonists.

³Basal exposure of the epitopes in $\text{Mg}^{2+}/\text{Ca}^{2+}$ as well as upon activation by Mn^{2+} was suppressed.

⁴Exposure of the epitopes was enhanced in the presence of $\text{Mg}^{2+}/\text{Ca}^{2+}$.

⁵Based on the requirement of divalent cation for the antagonists to impact epitope expression (α_1 allosteric and / I-like allosteric antagonists) [60, 62] and to form SDS-stable complexes (α_1 / I-like allosteric antagonists) [62].

⁶Stabilization of $\alpha_1 \alpha_2$ association to SDS treatment at room temperature was described [62].

[67], demonstrating that the compounds bind to $\alpha_1 \alpha_2$ regardless of the conformation or the presence of the I domain. However, when one of the metal-coordinating sidechains in the MIDAS of the α_2 I-like domain was mutated (α_2 -S114A), the SDS-stable complex was not formed by any of the compounds (Fig. 6B, lane 6 and not shown) in spite of intact non-covalent association of both subunits prior to SDS addition as shown by co-precipitation (Fig. 6B) [62]. These results demonstrate the crucial role of the α_2 MIDAS in the action of these compounds.

The ability to stabilize $\alpha_1 \alpha_2$ association, and the lack of requirement for the I domain, suggests that / I-like allosteric antagonists may bridge the α_2 I-like domain MIDAS either to the subunit -propeller domain or to the linker between the I domain and -propeller domain, which

is present in the I-less LFA-1. Thus it should be possible to build subunit specificity into the compounds, as is indeed illustrated by the compound series [62]. Based on the evidence that these compounds bind to the α_2 I-like MIDAS, stabilize $\alpha_1 \alpha_2$ association, and can show selectivity for the subunit, they are designated as / I-like allosteric antagonists.

In contrast to I allosteric antagonists which stabilize the bent conformation, / I-like allosteric antagonists induce the extended conformation. The / I-like allosteric antagonists increased binding of activation-dependent mAbs to the α_1 leg, α_2 I-like, and α_2 leg domains, whereas they reduced binding of some mAbs to the α_2 I-like domain (Table 1) [62]. RGD-like, ligand-mimetic antagonists to integrins that lack I domains, which are designated as / I-

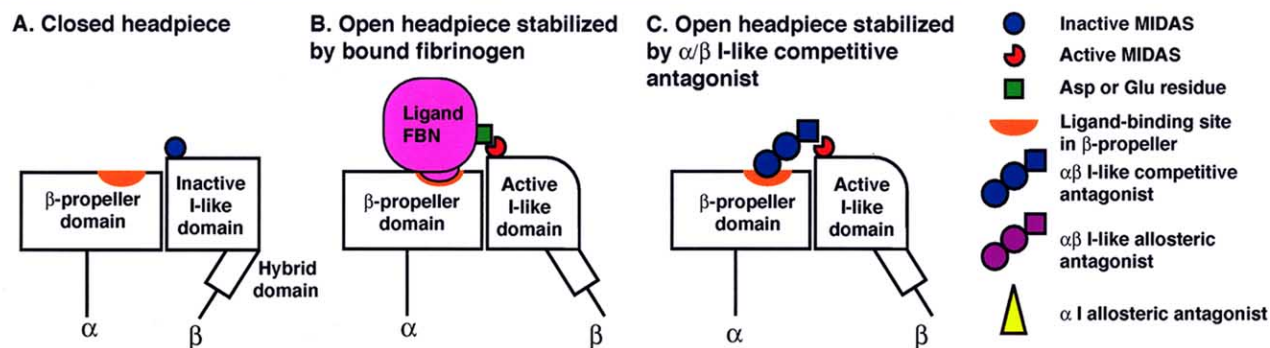
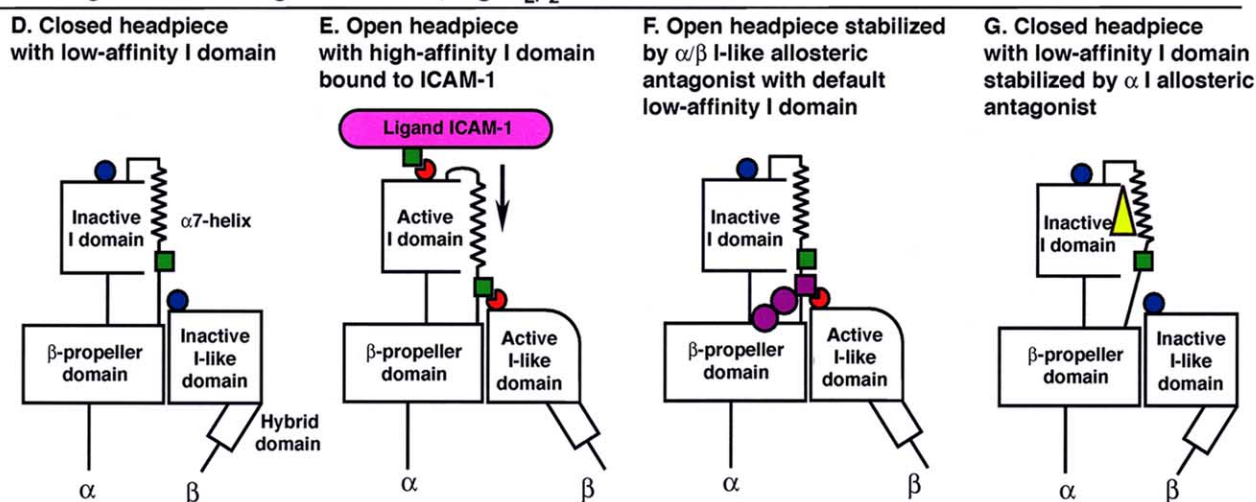
A-C. Integrins lacking an I domain, e.g. $\alpha_{IIb}\beta_3$ **D-G. Integrins containing an I domain, e.g. $\alpha_L\beta_2$** 

Fig. (5). Mechanisms of inhibition and impact on integrin conformation of small molecule antagonists. Cartoons illustrate conformational changes in the headpiece of a representative integrin that lacks an I domain, $\alpha_{IIb}\beta_3$ (A to C), and a representative integrin that contains an I domain, $\alpha_L\beta_2$ (D to G). For clarity, only headpiece domains are shown, and domains in legs are simplified as lines. The outward-oriented (obtuse angled) hybrid domain correlates with exposure of activation-dependent epitopes in the legs and adoption of the extended conformation.

like competitive antagonists, can also stabilize the active conformation of the integrin (Fig. 5A-C) [35, 68-70]. The similarities in requiring a carboxyl group, stabilizing subunit association, and inducing the extended conformation are consistent with the findings that $\alpha_L\beta_2$ I-like allosteric and $\alpha_{IIb}\beta_3$ I-like competitive antagonists bind to similar sites. The findings suggest that $\alpha_{IIb}\beta_3$ I-like allosteric antagonists bind to the MIDAS of the $\alpha_{IIb}\beta_3$ I-like domain as mimics of the intrinsic ligand in the linker between the I and β -propeller domains, competitively inhibit binding of the intrinsic ligand, and consequently leave the I domain in the low energy, inactive, closed conformation (Fig. 5D-F). At the same time, the $\alpha_{IIb}\beta_3$ I-like allosteric antagonists stabilize the I-like domain in its active configuration by mimicking intrinsic ligand binding, as shown by induction of activation epitopes in the $\alpha_{IIb}\beta_3$ I-like domain and α_L and β_2 legs (Fig. 5F) [62]. Thus, as a consequence of I-like domain activation, the $\alpha_{IIb}\beta_3$ allosteric antagonists stabilize the extended integrin conformation. It is interesting that the antagonists inhibit I domain activation, whereas they stabilize the rest of the integrin in the active conformation.

Like $\alpha_L\beta_2$ I-like allosteric antagonists, many mAbs to the $\alpha_{IIb}\beta_3$ I-like domain block ligand binding indirectly [56]. However, in contrast to the $\alpha_{IIb}\beta_3$ I-like allosteric antagonists that stabilize the I-like domain in the active conformation, inhibitory I-like domain mAbs stabilize the I-like domain in the inactive conformation as shown by suppression of activation-dependent epitopes in the I-like domain and leg domains [76]. Thus, binding of inhibitory I-like domain mAbs appears to favor, through stabilization of the I-like domain in the inactive form, the bent conformation and closed inactive I domain, supporting a pivotal role of the I-like domain in structural linkage between the I domain and leg domains.

CONCLUDING PERSPECTIVES

The two distinct classes of small molecule allosteric antagonists to $\alpha_{IIb}\beta_3$ integrins, together with the $\alpha_L\beta_2$ I-like competitive antagonists to integrins that lack I domains, enhance the understanding of multiple structural linkages between integrin domains that enable bi-directional communication between the ligand binding site and the

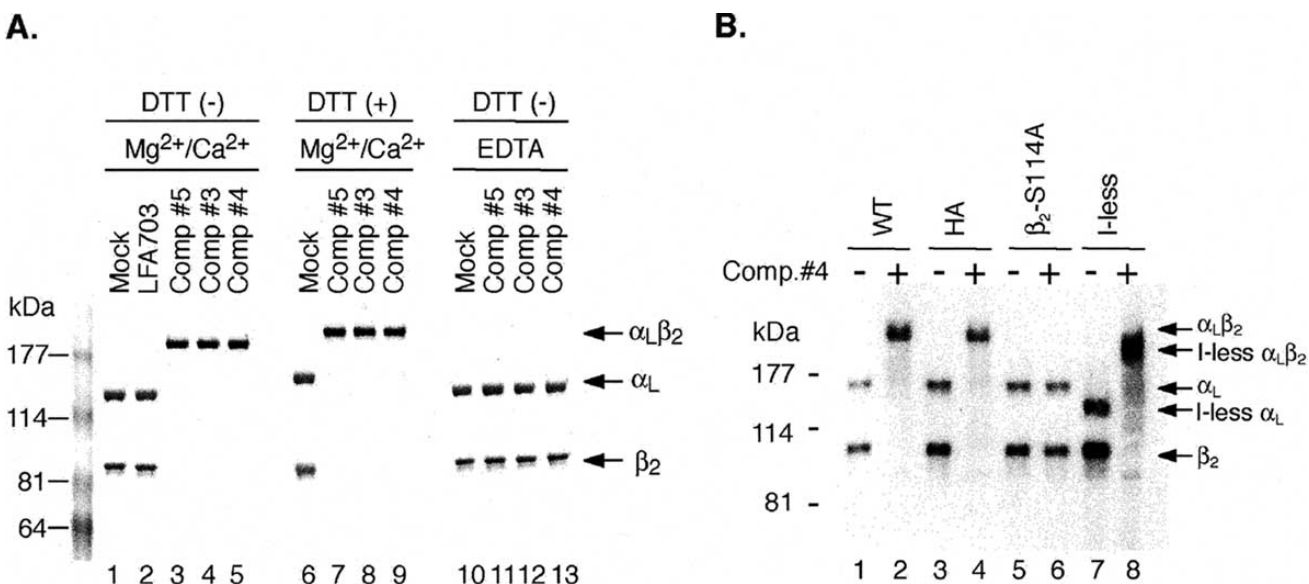


Fig. (6). Effect of the $\alpha_L\beta_2$ integrin antagonists on wild-type and mutant $\alpha_L\beta_2$ measured by stabilization of $\alpha_L\beta_2$ complexes in SDS-PAGE [62]. **A.** SDS-PAGE and Coomassie blue staining of soluble recombinant $\alpha_L\beta_2$ in the presence or absence of the inhibitors and 10 mM DTT. **B.** Immunoprecipitation of wild-type and mutant $\alpha_L\beta_2$ heterodimers. Lysates of surface-biotinylated K562 cell transfectants, which were pretreated with inhibitors (100 μ M) or DMSO (1:1000), were immunoprecipitated with mAb TS/4 to $\alpha_L\beta_2$ and detected on western blots by streptavidin and luminescence.

cytoplasmic domains, and regulate transition between the bent, low affinity conformation and the extended, high affinity conformation. Thus far, all potent small molecule antagonists to I domain-containing integrins are allosteric inhibitors, whereas those to integrins that lack I domains are ligand-mimetic, competitive inhibitors. It will be interesting to see whether potent, competitive small molecule antagonists to I domains can be discovered. Determination of the structure of I domain-containing integrin heterodimers and their complexes with ligands or small molecule antagonists should accelerate development of antagonists and understanding of conformational regulation of integrins.

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