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CONFORMATIONAL REGULATION OF INTEGRIN STRUCTURE AND FUNCTION

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■ **Abstract** Integrins are a structurally elaborate family of heterodimers that mediate divalent cation-dependent cell adhesion in a wide range of biological contexts. The inserted (I) domain binds ligand in the subset of integrins in which it is present. Its structure has been determined in two alternative conformations, termed open and closed. In striking similarity to signaling G proteins, rearrangement of a Mg^{2+}-binding site is linked to large conformational movements in distant backbone regions. Mutations have been used to stabilize either the closed or open structures. These show that the snapshots of the open conformation seen only in the presence of a ligand or a ligand mimetic represent a high-affinity, ligand-binding conformation, whereas those of the closed conformation correspond to a low-affinity conformation. The C-terminal α-helix moves 10 Å down the side of the domain in the open conformation. Locking in the conformation of the preceding loop is sufficient to increase affinity for ligand 9000-fold. This C-terminal "bell-rope" provides a mechanism for linkage to conformational movements in other domains. The transition from the closed to open conformation has been implicated in fast (<1 s) regulation of integrin affinity in response to activation signals from inside the cell. Recent integrin structures and functional studies reveal interactions between β-propeller, I, and I-like domains in the headpiece, and a critical role for integrin EGF domains in the stalk region. These studies suggest that the headpiece of the integrin faces down toward the membrane in the inactive conformation and extends upward in a "switchblade"-like opening motion upon activation. These long-range structural rearrangements of the entire integrin molecule involving multiple interdomain contacts appear closely linked to conformational changes in the I domain, which result in increased affinity and competence for ligand binding.

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AN OVERVIEW OF INTEGRIN STRUCTURE AND FUNCTION

Integrins are adhesion molecules with noncovalently associated α- and β-subunits that mediate cell-cell, cell–extracellular matrix, and cell-pathogen interactions. As their name implies, integrins integrate the cytoskeleton with points of attachment in the extracellular environment to mediate force-resistant adhesion, polarization in response to extracellular cues, and cell migration. Integrin-dependent physiological processes include tissue morphogenesis, inflammation, wound healing, and regulation of cell growth and differentiation (38, 82).

Nineteen different integrin α-subunits and 8 different β-subunits have been reported in vertebrates (36, 38), forming at least 25 $\alpha\beta$ heterodimers and perhaps

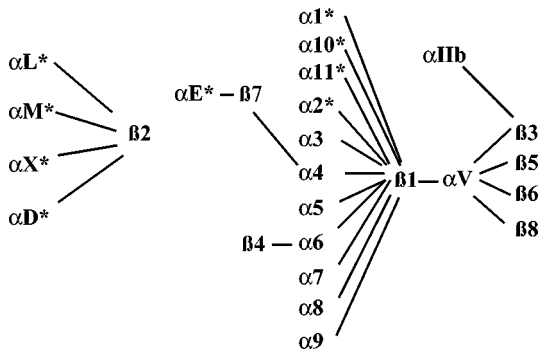

Figure 1 Integrin α- and β-subunits form 24 heterodimers that differ in ligand recognition and inside-out and outside-in signaling. Half of the α-subunits contain I domains (*asterisks*).

making the integrins the most structurally and functionally diverse family of cell-adhesion molecules (Figure 1). These integrins differ with respect to which cell surface, extracellular matrix, or inflammatory ligands they bind, the mechanisms by which their binding activity for ligands is activated, the types of cytoskeletal components to which they bind, and the types of signaling pathways they activate within cells.

The most unusual feature of integrins compared to other adhesion molecules is that the ability of their extracellular domains to bind ligands can be activated on a timescale of <1 s by signals within the cell (inside-out signaling). This is particularly evident with integrins on platelets and leukocytes in the bloodstream. Activation of integrins on these cells enables platelets to bind to injured vessel walls and fibrin clots, and enables leukocytes to bind to vessel walls and subsequently to migrate across endothelium to participate in immune and inflammatory processes. Multiple mechanisms, including conformational change in integrins (affinity regulation) and clustering and association with the cytoskeleton (avidity regulation), have been proposed to explain these events (2, 6, 19, 22, 27, 55, 87, 94). There is abundant evidence with antibodies for conformational change in many of the different extracellular integrin domains. However, it has been questioned whether conformational change is a result of ligand binding or a cause of ligand binding (affinity regulation), and it has been suggested that avidity regulation is the most important process for regulating ligand binding (2). Recently, multiple structures have been determined for the I domain that is a key ligand-binding domain in many integrins. I domains became embroiled in similar controversies as to whether conformational differences seen in crystal structures were physiologically relevant, and whether conformational change could regulate ligand binding or was merely a consequence of ligand binding. Now, through mutational and further structural studies, it is clear that conformational change in integrin I domains is of key physiologic importance for regulating the affinity for ligand. We are also beginning to

understand how signals are transmitted from one domain to another in the complex multidomain architecture of integrins, and thus to appreciate the molecular basis for both the inside-out and outside-in signaling mechanisms. Furthermore, under development are drugs that bind to integrin I domains and inhibit ligand binding not by binding to the active site, but by binding to an allosteric site and stabilizing the inactive conformation. This review focuses on these exciting recent advances on the conformational regulation of ligand binding by integrin I domains and places them within the broader context of integrin structure and function.

INTEGRIN DOMAINS

Integrins contain two noncovalently associated, type I transmembrane glycoprotein α and β-subunits with extracellular domains of >940 and >640 residues, respectively (Figure 2). The intracellular domains are short, except for the cytoplasmic domain of integrin $\beta 4$, which is specialized to connect to the keratin cytoskeleton and contains fibronectin type III domains (16). The overall shape

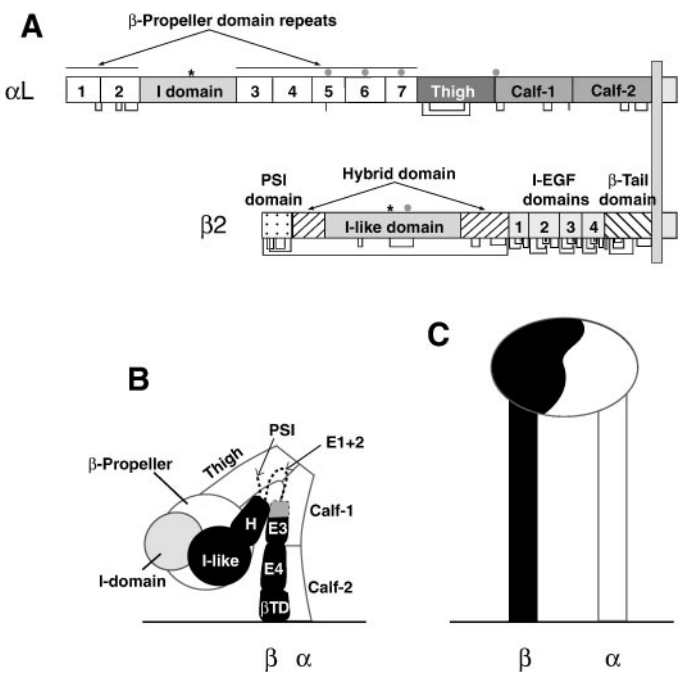

Figure 2 Integrin architecture. (*A*) Organization of domains within the primary structure of $\alpha L\beta 2$. (*B*) Arrangement of domains within the three-dimensional crystal structure of $\alpha V\beta 3$ (99), with an I domain added. (*C*) Commonly seen appearance of integrins in the electron microscope (20).

of the extracellular domain is known from electron microscopy (20, 91, 95). A globular headpiece binds ligand, and two long stalk regions containing C-terminal segments from the α- and β-subunits connect the ligand-binding headpiece to the transmembrane and C-terminal cytoplasmic domains. Recently, the structure of the extracellular fragment of integrin $\alpha V\beta 3$, which lacks an I domain, was reported at 3.1 Å resolution (99). Of the 12 domains predicted to be present, 8 domains, and a portion of a ninth, were resolved (Figure 2*B*). A complementary NMR structure of a $\beta 2$ integrin fragment (4) reveals the structure of some of the missing domains and defines the disposition of residues important in integrin activation. This information leads to a novel interpretation of the $\alpha V\beta 3$ structure and a model of integrin activation (4), which is discussed near the end of this review. This is that the bent conformation seen in the crystal structure (Figure 2*B*) is inactive and that the extended conformation commonly seen in electron micrographs represents the active conformation (Figure 2*C*). The overall picture is that activation results in a switchblade-like upward movement of the headpiece, which is coupled to conformational movements within ligand-binding domains that increase affinity for ligand.

The α-Subunit

THE β-PROPELLER DOMAIN The N-terminal region of the integrin α-subunit contains seven segments of about 60 amino acids each with weak homology to one another, which have been predicted to fold into a seven-bladed β-propeller domain (83) (Figure 2*A*). The trimeric G protein β-subunit contains a β-propeller domain with the same topology. The β-propeller model has received strong support from mapping of epitopes that are far apart in sequence but close in the predicted structure (69) and from the finding that Ca^{2+}-binding motifs in propeller β-sheets 4–7 are more similar to motifs found in turns between β-strands than to EF-hand motifs in turns between α-helices (84). Mutagenesis studies show that ligand-binding residues cluster to one portion of the top and side of the β-propeller (40). The $\alpha V\beta 3$ crystal structure is in agreement with these conclusions (99).

About half of the integrin α-subunits contain no I domain (Figure 1). In these integrins, the β-propeller domain appears to directly participate in ligand binding (36). In integrins that contain I domains, the β-propeller domain can cooperate in binding to some but not other ligands, as in αM (100); or play no direct role, as in αL (59, 79).

THE I DOMAIN Half of the integrin α-subunits contain a domain of about 200 amino acids, known as an I domain or a von Willebrand factor A domain (Figures 1, 2, and Figure 3). I domains are the major ligand-binding sites in integrins that contain I domains (17, 62). The I domain is inserted between β-sheets 2 and 3 of the β-propeller domain (83). The three-dimensional structure of the I domain (48) shows that it adopts the dinucleotide-binding or Rossmann fold, and within this class of folds it shows the greatest similarity to small G proteins and

trimeric G protein α-subunits. The structural relationship of the integrin I domain and β-propeller domains to G protein α- and β-subunits, respectively, is quite interesting and may reflect functional similarities in conformational regulation of ligand binding (47, 83). A divalent cation coordination site designated the metal ion-dependent adhesion site (MIDAS) in the I domain binds negatively charged residues in ligands much in the same way that the Mg^{2+} in G proteins coordinates to the γ-phosphate of GTP. I domain structure and function is described in detail below.

THE α-SUBUNIT STALK REGION The region C-terminal to the β-propeller domain comprises a large portion of the α-subunit extracellular domain of about 500 residues. Much of this C-terminal region appears to correspond to the stalk region visualized in electron micrographs and is predicted to consist of domains with a two-layer β-sandwich structure (58). The crystal structure reveals the presence of three β-sandwich domains in this region, designated the thigh, calf-1, and calf-2 domains (99) (Figure 2*A*, *B*).

The β-Subunit

THE PSI DOMAIN The N-terminal cysteine-rich region of residues 1–50 shares sequence homology with membrane proteins including plexins, semaphorins, and the c-met receptor; it has therefore been termed the PSI domain for plexins, semaphorins, and integrins (8). This region in integrin β-subunits has seven cysteines, six of which are shared with other PSI domains, and is predicted to have two α-helices. The first of the seven cysteines forms a long-range disulfide to the C-terminal cysteine-rich region in the β-subunit (Figure 2*A*) (10). These cysteine-rich regions cooperate to restrain the integrin in the inactive conformation (103).

THE I-LIKE DOMAIN Integrin β-subunits contain a highly evolutionarily conserved domain of about 240 residues, spanning from about residue 100 to 340. This domain contains a putative metal-binding DXSXS sequence motif similar to that of the MIDAS in the I domain, a similar secondary structure (48), and weak but detectable sequence homology to the I domain (71); therefore, it has been termed the I-like domain (Figure 3). This region is a hotspot for point mutations that result in a lack of association of the integrin β2 subunit with α-subunits, or loss of function, and cause leukocyte-adhesion deficiency (7). The I-like domain appears to directly bind ligand in integrins that lack I domains and to indirectly regulate ligand binding by integrins that contain I domains. There is a large interface between the β-propeller domain and the I-like domain (99), as originally deduced by their mutual dependence for folding (31, 33) and from antibody epitopes (72, 102).

THE C-TERMINAL STALK AND THE EGF-LIKE DOMAINS The C-terminal portion of the extracellular domain extends from about residue 340 to 700. Much of this is cysteine-rich and corresponds to the β-subunit stalk region. From about residue

435 to 600 are four cysteine-rich repeats that are EGF-like (4, 89, 92, 99, 101). These have been designated integrin-EGF (I-EGF) domains (4, 89). Many activating antibodies, or antibodies that bind only when integrins are activated, bind to the C-terminal region of the β1, β2, and β3 subunits (36). Mapping in more detail shows that these mAb map within the EGF-like modules (57). This portion of the β-subunit is important in signal transmission and is discussed in more detail below.

THE OVERALL STRUCTURE AND FUNCTION OF INTEGRIN I DOMAINS

Ligand-Binding Function

I domains have been implicated as major ligand-binding sites in those integrins in which they are present. The evidence includes mapping of mAb that inhibit ligand binding to the I domain (17, 32), the requirement of residues in the MIDAS and in the surrounding area for ligand binding (32, 41), and the ability of isolated I domains to bind ligands (62, 75). Deletion of the I domain does not affect expression of the $\alpha\beta$ heterodimer (50, 100). Deletion of the I domain of the integrin αL abolishes recognition of all known ligands (50, 100); however, deletion of the I domain of the integrin αM abolishes binding to some ligands and diminishes binding to others (101). The role of the β-propeller domain in this residual binding suggests that in some cases both the β-propeller domain and I domain can directly contribute to ligand binding. However, in the case of the integrin αL, when the I domain is locked in the proper conformation (see below) and compared to the heterodimer, the isolated I domain is sufficient to give equal adhesiveness in cell-based assays (59); and binding to the monomeric, soluble ligand ICAM-1 of equal affinity in surface plasmon resonance assays (79).

I Domain Structure

Crystal and NMR structures have been determined for I domains from the integrin αM (1, 47, 48), αL (39, 49, 73, 74), α2 (25, 26), and α1 (66, 76) subunits. The I domain adopts the dinucleotide-binding or Rossmann fold, with α-helices surrounding a central β-sheet (Figure 4). There are six major α-helices and several short α-helices that differ between I domains (Figure 3). The β-sheet contains five parallel and one anti-parallel β-strand. The top face of the I domain contains the MIDAS. β-strands and α-helices tend to alternate in the secondary structure (Figure 3), with the α-helices wrapping around the domain in counterclockwise order when viewed from the top (Figure 4). The cation in the MIDAS is ligated by five side chains located in the β1-α1, α2-α3, and β4-α4 loops on the top of the domain (Figures 3, 4). The first of these loops contains three coordinating residues in a sequence that is a signature of I domains, DXSXS. Divalent cations have long been known to be universally required for ligand binding by integrins, and in I domains the metal-coordinating residues, and the residues

surrounding the metal-binding site are important for ligand binding. Many of the proteins with dinucleotide or Rossmann folds are enzymes that have an active site and a Mg^{2+}-binding site at the top face, and the Mg^{2+} often coordinates the phosphate group of NAD, ATP, or GTP, which are substrates or cofactors for these enzymes. Of these proteins, the most closely related to integrin I domains are the small G proteins such as ras. The I domain and small G protein folds differ only in one α-helix and in reversing the order of the $\beta 2$ and $\beta 3$ strands in the β-sheet.

TWO DIFFERENT CONFORMATIONS FOR INTEGRIN I DOMAINS

The αM I Domain

Early on, the integrin αM I domain was found to crystallize in two different conformations (47). There was controversy about whether the different conformations were physiologically relevant or an artifact of the lattice contacts in crystals (1, 52). The two conformers were at first termed the Mn^{2+} and Mg^{2+} forms because they were crystallized in the presence of these metals and bound them at the MIDAS; later, they were termed the closed and open conformers, respectively. The latter terminology is much less confusing because further studies have shown that the closed conformation can be seen with Mg^{2+}, Mn^{2+}, Cd^{2+}, or no metal in the MIDAS (1, 74); the open conformation can be seen with Mg (48), Co^{2+}, Zn^{2+}, and probably Mn^{2+}, Cd^{2+}, and Ni^{2+} in the MIDAS (26). What clearly distinguishes the closed and open I domain conformations is that in the two open structures determined, an acidic residue donated either by a ligand (26) or a ligand-mimetic lattice contact (48) coordinates to the metal in the MIDAS, whereas there is no ligand-like contact in the large number of closed structures that have been determined. Instead, a water molecule is present at the equivalent coordination position (Figure 5*A*). The closed and open conformations differ not only in the coordination of residues in the I domain with the MIDAS, but in the structure of surrounding loops and in the position of the C-terminal α-helix (Figure 6*A*).

In the MIDAS, five residues in the I domain and several water molecules contribute oxygen atoms to the primary and secondary coordination sphere surrounding the metal (Figure 5*A*). Compared to most other binding sites for Mg^{2+} in protein crystal structures, the MIDAS is unusual in its content of serine and threonine residues; however, this feature is also shared with G proteins. Ca^{2+} is generally not seen to be coordinated by serine and threonine side chains. Instead, Ca^{2+} prefers more polarized oxygen atoms as found in acidic and amide amino acid side chains and in carbonyl groups of the backbone. The metal-oxygen distances are also smaller for Mg^{2+} than for Ca^{2+}. The shorter interatomic distances and the less-polar nature of the oxygens seen with Mg^{2+} are thought to reflect a greater covalent character for the Mg^{2+} ligand bond compared to a greater ionic character for the Ca^{2+} ligand bond (47). In the open conformation of the MIDAS, two serines and

one threonine are in the primary coordination sphere, whereas two aspartic acid residues contributed by the I domain are in the secondary coordination sphere and fix the positions of coordinating water molecules (Figure 5*A*). Notably, the glutamic acid contributed by the ligand or ligand mimetic donates the only negatively charged oxygen to the primary coordination sphere in the open conformation. The lack of any charged group in the primary coordination sphere donated by the I domain is hypothesized to enhance the strength of the bond between the metal and the acidic residue in the ligand. The most intensively studied ligand for an I domain is ICAM-1; domain 1 of ICAM-1 binds to the I domain of integrin $\alpha L\beta 2$. The binding sites have been mapped by mutagenesis, and the structure of IgSF domains 1 and 2 of ICAM-1 has been determined (5, 12). By far the most important binding residue in ICAM-1 is a glutamic acid residue near the center of the binding site; therefore, this residue has been hypothesized to directly coordinate to a Mg^{2+} in the I domain MIDAS (85).

In the closed conformation of the I domain, the threonine moves from the primary to the secondary coordination sphere, and one of the aspartic acid residues moves from the secondary to the primary coordination sphere (Figure 5*A*). This is consistent with the idea that an energetically favorable MIDAS requires at least one primary coordination to a negatively charged oxygen, and when this is not provided by a ligand, there is a structural rearrangement within the I domain to provide this from within the MIDAS. The backbone and side chain rearrangements in the I domain are accompanied by a 2.3 Å sideways movement of the metal ion away from the threonine and toward the aspartic acid on the opposite side of the coordination shell (Figure 5*A*). A water molecule takes the place of the ligand-mimetic glutamic acid to complete the coordination sphere.

The structural rearrangement of the MIDAS is coupled to backbone movements of the loops that bear the coordinating residues. Linked structural shifts occur in the hydrophobic core, in neighboring loops on the top of the I domain, and in α-helices on the side of the domain (Figure 6*A*). In the largest movement in the transition from the closed to open structure, the C-terminal helix, $\alpha 6$, moves 10 Å down the side of the domain. This requires a repacking of the hydrophobic face of $\alpha 6$ against the side of the domain. At the N terminus of $\alpha 6$, Phe-302, which inserts into a hydrophobic cavity in the top of the closed domain, becomes completely exposed as a consequence of the dramatic reshaping of the $\beta 6$-$\alpha 6$ loop. The $\alpha 6$ helix is distant from the ligand-binding site; however, its remarkable movement provides a mechanism to link conformational movements in I domains to movements elsewhere in integrins.

The $\alpha 2$ I Domain

Recently, the structure of the $\alpha 2$ I domain has been determined in the absence of ligand (25) and in the presence of a collagen peptide ligand (26). The triple-helical collagen peptide contains a critical Gly-Phe-hydroxyPro-Gly-Glu-Arg sequence, and the Glu of this sequence ligates the MIDAS. The differences between the

ligand-bound and nonliganded $\alpha 2$ I domains are remarkably similar to the differences between the αM I domains with and without a ligand-mimetic lattice contact (Figure 6*B*); when the differences in Cα carbon backbone positions are plotted, they are remarkably similar (26). Furthermore, exactly the same changes are seen in the residues that make primary coordinations to the metal at the MIDAS (Figure 5*B*). Thus, the liganded and nonliganded conformations of the $\alpha 2$ I domain adopt the open and closed conformations, just as seen for αM (Figure 6*B*). The I domains of $\alpha 2$ and αM are only 27% identical in sequence and are among the most distantly related of integrin I domains; thus, it is to be expected that the open and closed conformations will be a general feature of integrin I domains.

The $\beta 6$-$\alpha 6$ loop adopts conformations that are canonical for the open and closed structure. In the open conformations, the backbone conformation of the $\beta 6$-$\alpha 6$ loops are almost identical in αM and $\alpha 2$ (Figure 7). The conformation is quite different in the closed conformation, yet the conformation of this loop is almost identical for the closed conformations of αM and $\alpha 2$, as well as for αL and $\alpha 1$ (Figure 7). Remarkably, only one of the five residues in the $\beta 6$-$\alpha 6$ loop is identical in sequence between αM and $\alpha 2$ (Figure 3). The high conservation of this loop is also emphasized by the success of modeling it in the open comformation of αL (see below).

There is one unique feature of $\alpha 2$ compared to αM and αL. An additional helix called helix C extends from the top of the closed $\alpha 2$ I domain near the MIDAS. This helix appears to sterically hinder binding of collagen to the MIDAS. In the transition to the open conformation, there is a "slinking" motion in which this helix unwinds and residues are added to the following helix (Figure 6*B*); the loss of the C helix appears to open the binding site for collagen (26).

The αL I Domain

Both mutation of αL to lock in the open and closed conformations and NMR studies have provided evidence for conformational change. Multiple crystal structures have been determined for the αL I domain in the closed conformation; these structures, determined with Mg^{2+}, Mn^{2+}, or no metal at the MIDAS, are similar to one another except that the C-terminal helix differs in conformation (39, 73, 74). In these closed αL I domain structures, there is no downward movement of the helix or restructuring of the preceding loop as seen in the closed-to-open transition of $\alpha 2$ or αM I domains. Instead, the helix differs in conformation and whether it packs closely against the hydrophobic core of the domain or moves away from it as a result of lattice contacts. The NMR solution structure of the αL I domain shows that the protein in solution also adopts the closed conformation, regardless of whether Mg^{2+}, Mn^{2+}, or no divalent cation is present (49). The NMR solution structure also demonstrates that the C-terminal α-helix, although well formed, is highly flexible, probably as a result of breathing or segmental motion.

NMR spectroscopy was further used to probe conformational change of the αL I domain upon binding to ligand (37). Change in the chemical environment of

specific residues in the αL I domain was investigated in the presence or absence of a fragment of ICAM-1 containing domains 1 and 2. Domain 1 includes the αL binding site and the most critical residue for ligand binding, Glu-34 (5, 12). Two distinct clusters of affected residues were identified. One cluster of residues localized around the MIDAS on the upper face of the I domain. Another group of perturbed residues clustered at a different location in the domain, around the C-terminal α-helix and the opposing face of the central β-sheet. Although the solution structure of the I domain in complex with ICAM-1 was not determined, the regions that are chemically shifted upon binding to ICAM-1 are similar to the regions that differ most between the closed and open conformations of the αM and α2 I domains.

IS SHAPE-SHIFTING IN INTEGRIN I DOMAINS PHYSIOLOGICALLY RELEVANT FOR REGULATION OF THEIR AFFINITY FOR LIGAND?

The above studies demonstrated that conformational changes occur when ligands are bound to I domains. However, they did not establish whether these changes result from induced fit upon ligand binding, or if they are physiologically relevant for regulating the affinity for ligand of I domains and the integrins in which they are present. These have been hotly debated issues in the integrin field. For some years, it has been proposed that after cellular activation, signals are transmitted to the extracellular domains of integrins that alter the conformation of their ligand-binding site, and hence affinity for ligand (22, 56). More recently, evidence has also accumulated that lateral redistribution and clustering of integrins, or so-called avidity regulation, may alter cellular adhesion independently of a change in affinity for ligand (88). Indeed, it has been suggested that conformational change in integrins is overemphasized and is a consequence of ligand binding rather than a cause (2). The binding of many antibodies to integrins is stabilized or induced by ligands, leading to the term ligand-induced-binding sites (LIBS) for these epitopes. Although many of the same antibodies can activate ligand binding, this can be argued to be a consequence of stabilizing the ligand-bound conformation and hence the integrin-ligand complex. The keys to resolving these issues were (*a*) whether the conformational changes seen in crystal structures were physiologically relevant, i.e., altered affinity for ligand as predicted, (*b*) whether the change in affinity was substantial, and (*c*) whether conformational alterations in I domains occurred on the cell surface in physiological circumstances.

Parallels with G Proteins

Clues to the physiological relevance of conformational change in I domains are provided by the strikingly similar changes in metal coordination that are linked to backbone movements in signal-transducing G proteins (47). In the small G protein ras, a Mg^{2+} ion is present in the catalytically active GTP-binding site

(63). The GTP-bound form, which is active in binding to and stimulating effector molecules, and the inactive GDP-bound form have Mg^{2+} ion coordinations that are analogous to those of the open and closed I domains, respectively (Figure 6*C*). In the GTP-bound form, the Mg^{2+} ion binds directly to a serine, a threonine, and two water molecules; the other two primary coordinations are to β- and γ-phosphate oxygen atoms of GTP. There is a secondary coordination to an aspartate. On hydrolysis of the GTP with release of the γ-phosphate, the coordination to the γ-phosphate oxygen is lost, and other Mg^{2+} coordinations are altered. The bond to the threonine is lost, and a direct coordination to the aspartate is gained. The loss of coordination to the threonine results in a flip in the switch I loop containing the threonine, and this is linked to a large change in the adjacent switch II loop (Figure 6*C*). These changes abolish binding of effector molecules at the switch region (63). G proteins and I domains differ in the loops bearing the residues that coordinate Mg^{2+} and in the backbone regions that move. The movement of the C-terminal α-helix in I domains appears to be a specialized feature that allows conformational communication between domains because it is not seen in G proteins.

In the case of G proteins, conformational change is highly regulated by the intrinsic GTPase activity, GTPase-activating G proteins (GAPs), and guanine nucleotide exchange factors, and is central to the mechanism of regulating binding to effector molecules and hence regulating intracellular signaling pathways. Thus interactions with other proteins regulate G protein conformation. Conformational change in I domains is similarly regulated by interactions with other integrin domains.

Conformation-Sensitive mAb

Many mAbs to integrins have been reported that either bind only when the integrin is activated or induce activation themselves (2). However, few activation-dependent mAb block ligand binding and thus appear to recognize the ligand-binding site. The only such mAb reported to I domains is CBRM1/5, which recognizes the I domain of αM (18). This mAb does not bind to resting peripheral blood neutrophils, but after these cells are activated through G protein–coupled chemoattractant receptors, or with a drug that activates protein kinase C, 10% or 30% of the $\alpha M\beta 2$ on the surface of individual cells binds CBRM1/5 mAb. Although it recognizes only a subset of the $\alpha M\beta 2$ on the cell surface, CBRM1/5 mAb completely blocks ligand binding by cells, showing that it recognizes the active subset of molecules.

Evidence to support the physiological relevance of conformational change seen in the open and closed form of αM I domain crystal structure was provided with CBRM1/5 mAb (68). This mouse anti-human mAb is specific for six residues that differ between the human and mouse amino acid sequences. The residues in the epitope are present in two different amino acid segments that are structurally adjacent and near the MIDAS (68) (Figure 8). The first three residues, P147, H148, and R151, are located at the top of the $\alpha 1$ helix and are preceded

immediately by three residues that coordinate Mg^{2+} and form the DXSXS motif of the MIDAS: D140, S142, and S144. The last three residues, K200, T203, and L206, are in the loop that contains T209, which is directly coordinated to the metal in the open conformation and indirectly coordinated in the closed conformation. Both groups of residues are widely exposed regardless of activation as judged by reactivity with other antibodies. These results suggest that the selectivity of CBRM1/5 for the active state is not due to "unmasking" of the epitope by other integrin domains but to "shape-shifting" in the I domain itself. Comparison between the superimposed opened and closed structures shows that P147, H148, and R151 differ remarkably in position and in side chain orientation, and hence in relationship to the three other residues in the epitope (Figure 8). Cα atom movements that average 2.4 Å are tightly linked to the 2.0 Å movement of S144 of the MIDAS. In addition, H148 and R151 are adjacent to the loop preceding the C-terminal α-helix in the closed conformation, and both are more exposed in the open conformation, owing to the movement of this loop that accompanies the large downward shift of the C-terminal α-helix. Thus, CBRM1/5 recognizes shape-shifting in the αM I domain near the MIDAS and the C-terminal α-helix. Documentation of shape-shifting in these regions of the I domain upon activation of integrins on the cell surface provides strong evidence that conformational change seen in the open and closed I domain structures is physiologically relevant and occurs within the context of intact integrin $\alpha\beta$ heterodimers. Since CBRM1/5 mAb blocks ligand binding, it clearly does not recognize a ligand-induced-binding site (LIBS). Therefore, the induction of the CBRM1/5 epitope on cell surface $\alpha M\beta 2$ is a consequence of changes within $\alpha M\beta 2$ itself and not a consequence of ligand binding.

Mutations Designed to Stabilize the Open and Closed Conformers of I Domains

To measure how transition between the open and closed conformations of the I domain regulates affinity for ligand, mutations have been introduced to stabilize a particular conformation, and tested for effect on ligand binding.

αM In the αM I domain, Phe-302 is buried in the closed conformation and exposed in the open conformation; therefore, mutation to a hydrophilic residue should favor the open conformation (51). To this end, the mutation Phe-302 → Trp was designed and was claimed to stabilize the open conformation and to increase ligand binding (51); however, this claim is not without controversy. First, Trp is not a hydrophilic residue, and mutants with a hydrophilic Arg substitution were not expressed. Second, the study was internally inconsistent. It was reported both that the overall affinity of the mutant was unchanged and that there was a 2.3-fold increase in the proportion of molecules in the active conformation. For both to be true, there would have to be a corresponding 2.3-fold decrease in the affinity for ligand of the active species. Third, a subsequent study showed that the Phe-302 → Trp mutant crystallized in the closed conformation and that the Trp was buried

(98). Finally, another study showed at best only a slight increase in binding by the same mutant (80).

A different approach was taken to stabilize particular conformations of the αM I domain and to investigate the physiological significance of these conformations (80). The computational algorithm ORBIT, developed by Dahiyat & Mayo (15), rationally designs amino acid sequences that stabilize a particular backbone structure. Using ORBIT, sequences were selected that minimized the energy of either the open or closed conformation of the αM I domain. Back calculations showed that mutations that stabilized the open conformation also destabilized the closed conformation and vice versa. To avoid mutations that could directly alter the ligand binding face or alter contacts with other domains in intact integrins, only hydrophobic core residues were allowed to mutate. Three different designed open I domains, each containing 8 to 13 mutations, showed increased binding to ligand when expressed on the cell surface in $\alpha M\beta 2$ heterodimers, whereas designed closed or wild-type I domains did not (80). Similar results were obtained when I domains alone, in the absence of any other integrin domains, were expressed on the cell surface with an artificial C-terminal transmembrane domain. The CBRM1/5 mAb reacted with $\alpha M\beta 2$ containing the designed open but not designed closed or wild-type I domains. Furthermore, the designed closed I domain was resistant to activation. $\alpha M\beta 2$ heterodimers containing wild-type I domains, but not designed closed I domains, bound ligand in response to activating mAb. These results demonstrated that the open and closed conformations correspond to ligand binding and inactive conformations, respectively.

The closed conformation appears to be the low-energy conformation of the I domain and the default conformation adopted by the I domain in resting integrin heterodimers on the cell surface. In $\alpha M\beta 2$ heterodimers, and in isolation on the cell surface, the wild-type I domain behaved like the designed closed I domain in lack of expression of the CBRM1/5 epitope and lack of ligand binding. This suggests that the closed conformation is adopted in the inactive state by integrins on the cell surface. Calculation of the energies of αM I domains crystallized in the open and closed conformations also shows that the closed conformation is of lower energy (80).

Mutation in the αM I domain of the single residue Ile-316, located in the last half of the C-terminal α-helix, is sufficient to favor the open conformation (99). The side chain of Ile-316 packs in a hydrophobic pocket between the C-terminal α-helix and the opposing β-sheet in the closed conformation, but due to the downward movement of this helix in the open conformation, this residue cannot pack against the side of the domain in the open conformation and is not visualized in the crystal structure of the open conformer (47). To test the hypothesis that packing of Ile-316 wedged into this hydrophobic socket might constrain the αM I domain in the closed conformation, recombinant soluble αM I domains were truncated just before Ile-316 ($r11bA^{123\text{-}315}$) or Ile-316 was mutated to Gly ($r11bA^{Ile\text{-}316\text{-}Gly}$) (98). These mutants showed increased affinity for the ligands iC3b, fibrinogen, and ICAM-1 compared to the wild-type I domain ($r11bA^{123\text{-}321}$), as revealed by surface

plasmon resonance (98). Thus, the absence of the Ile-316 side chain clearly favors the open, ligand-binding conformation in solution. The r11bA$^{\text{Ile-316-Gly}}$ mutant crystallized in the open conformation when a ligand-mimetic crystal contact was present. However, because the wild-type r11bA$^{123\text{-}315}$ also crystallized in the open conformation when a ligand-mimetic crystal contact was present (98), as also observed in the first αM I domain crystal structure (48), the crystal studies do not reveal which conformation is present in solution, nor do they reveal whether the Ile-316 mutation by itself is sufficient for conversion to the open conformation in the absence of a ligand-mimetic contact.

ENGINEERED DISULFIDE BONDS WITHIN αL I DOMAINS Locking the αL I domain in the open conformation (59, 60, 79) has relied on models, since all αL structures thus far show the closed conformation (39, 49, 73, 74). The open conformation of the αL I domain was modeled by using the open αM I domain as a template in regions where the closed and open conformations differed. Positions were sought where pairs of residues could be mutated to cysteine that could form a disulfide bond, and the disulfide could form only in one conformation. The positions that were found bracket the loop between the C-terminal α-helix and preceding β-strand (Figure 9). To lock this loop in its two alternate conformations, pairs of cysteines were introduced either at residues 287 and 294 for the open conformation, or at residues 289 and 294 for the closed conformation. In surface plasmon resonance measurements of binding to ICAM-1, the soluble locked-open I domain molecule showed a 9000-fold increase in affinity compared to wild type, which was reversed by disulfide reduction. Locking the I domain open increases its on-rate, which is consistent with conformational change being rate limiting for binding of the wild-type I domain (Table 1). The affinity of the locked-closed conformer was similar to wild type (79). Furthermore, the affinity and kinetics of the soluble locked-open αL I domain for ICAM-1 are comparable to that measured independently (44) for intact, activated αLβ2 (Table 1). Thus, the αL I domain, when locked in the open conformation, is sufficient for full-affinity binding.

TABLE 1 The affinity for ICAM-1 of the locked-open αL I domain is equal to that of intact αLβ2

Immob. ligand	**Analyte**	$\mathbf{k_{on}}$ **(M^{-1} s^{-1})**	$\mathbf{k_{off}}$ **(s^{-1})**	$\mathbf{K_D}$ **(μM)**
sICAM-1	WT I domain	2950 ± 440	4.95 ± 0.85	1670 ± 100
sICAM-1	Closed I domain	2110 ± 400	2.84 ± 0.27	1760 ± 70
sICAM-1	Open I domain	$139{,}000 \pm 8000$	0.0257 ± 0.0015	0.185 ± 0.012
Open I domain	sICAM-1	$107{,}000 \pm 3000$	0.0275 ± 0.0028	0.258 ± 0.024
αLβ2*	sICAM-1	$224{,}000 \pm 69{,}000$	0.0298 ± 0.0069	0.133 ± 0.041

Binding kinetics measured by surface plasmon resonance. Data are from (79), except for measurements on αLβ2.
*Data from (44).

The locked-open and -closed I domains were also tested for adhesiveness in the context of intact $\alpha L\beta 2$ on the cell surface (59). $\alpha L\beta 2$ containing the locked-open I domain was constitutively and maximally active for adhesion to ICAM-1, whereas $\alpha L\beta 2$ heterodimers containing wild-type or locked-closed I domains failed to support adhesion. $\alpha L\beta 2$ containing the wild-type I domain was activatable for adhesion by activating mAb or Mn^{2+}, whereas $\alpha L\beta 2$ containing the locked-closed I domain was resistant to such activators. The results with soluble I domains and cell surface heterodimers clearly demonstrated that reshaping the $\beta 6$-$\alpha 6$ loop is fully sufficient for regulation of the affinity of the ligand-binding site at the MIDAS because only the conformation of the $\beta 6$-$\alpha 6$ loop is directly restrained by the disulfide bond. Therefore, inside-out signals relayed from the cytoplasm could be propagated to the ligand-binding site by pulling down the C-terminal, $\alpha 6$ helix, thereby reconfiguring the $\beta 6$-$\alpha 6$ loop. The C-terminal α-helix may act like a bell-rope in relaying conformational signals.

As discussed above, there has been controversy about the contribution to regulation of adhesiveness of lateral movements on the cell surface (clustering, avidity regulation) and conformational change in the ligand-binding site (affinity regulation). In part to address this issue, I domains were expressed on the cell surface in isolation from other integrin domains using an artificial transmembrane domain (59). In contrast to native $\alpha L\beta 2$, the cell surface I domains contained only a single transmembrane domain derived from the platelet-derived growth factor receptor and truncated five residues into the cytoplasmic domain. Isolated wild-type or locked-closed I domains did not support adhesion, whereas the isolated locked-open I domain was as strongly adhesive for ICAM-1 as fully activated intact $\alpha L\beta 2$ heterodimer at an equivalent cell surface density. These findings demonstrate that affinity regulation is fully sufficient to regulate cell adhesiveness and that interactions between integrins or other components mediated by integrin cytoplasmic, transmembrane, or any extracellular domains other than the I domain are not required. However, these findings do not rule out a role for avidity regulation or a link between conformational change in integrins and clustering.

Mutations Near the C-Terminal α-Helix in αL

Mutations around the interface between the C-terminal α-helix and the opposing β-sheet affect ligand-binding activity, underscoring the significance of conformational changes occurring around the C-terminal α-helix. Systematic mutagenesis of this region has revealed mutations that both increase and decrease ligand binding by $\alpha L\beta 2$, apparently by affecting the relative stability of the open and closed conformations, or by affecting interactions with nearby domains that regulate I domain conformation (37). One of these residues, Ile-306, corresponds to Ile-316 of αM, which stabilizes the closed conformation by fitting in a hydrophobic socket (98). Consistent with the observations with the αM I domain, in intact $\alpha L\beta 2$ substitution of Ile-306 with alanine increased adhesion to ICAM-1

(37). However, a soluble αL I domain truncated at residue 305 and hence lacking Ile-306 did not show increased affinity for ICAM-1 (M. Shimaoka & T. A. Springer, unpublished data). In isolated αL I domains, the C-terminal α-helix is mobile in NMR structures (49), shows variable conformations in crystal structures, and does not pack well against the body of the domain (73, 74). Therefore, the effect on ligand binding of mutation of residue 306 in intact αLβ2 but not in isolated I domains suggests that the C-terminal α-helix is well-packed against the body of the I domain in intact αLβ2 and that interactions with other domains are important for the conformation of the C-terminal α-helix. Mutation in αLβ2 of another hydrophobic residue in the same pocket underlying the C-terminal α-helix, I235A, activated ligand binding (37). Thus, in the context of intact αLβ2, mutations in the hydrophobic pocket appear to favor the open conformation of the I domain.

Therapeutic Antagonists Directed to Integrin I Domains

In vivo experiments using antibodies and gene disruption have shown that binding of αLβ2 to ICAMs is important in leukocyte trafficking in inflammation, lymphocyte homing, and T lymphocyte interactions with antigen-presenting cells in immune reactions (9, 82). These findings suggested that antagonists of αLβ2 could be useful for the therapy of autoimmune diseases. Indeed, a blocking mAb directed to the αL I domain was shown to be efficacious in phase 3 clinical studies of patients with psoriasis (29). High-throughput screening of large chemical libraries has led to the identification by more than three different pharmaceutical companies of small molecules that inhibit binding of αLβ2 to ICAM-1 (39, 42, 45, 53, 54, 96, 97). The compounds are highly specific for αLβ2 compared to αMβ2. Remarkably, each of the independently discovered lead compounds, which belong to different chemical classes, binds to the hydrophobic pocket between the C-terminal α-helix and the β-sheet, as documented by NMR or crystallography at three different companies (39, 45, 53) (Figure 10). This binding site is distant from the ligand-binding site at the MIDAS. Together with the finding that the drug–I domain complexes crystallize in the closed conformation, this suggests that the compounds allosterically inhibit binding to ICAM-1 by favoring the closed conformation. In agreement with this hypothesis, αLβ2 containing a mutant I domain locked open with an engineered disulfide bridge is completely resistant to inhibition by drug compound (59). In contrast, αLβ2 heterodimers containing I domains of wild-type or with single cysteine substitutions are susceptible to drug compound, as is αLβ2 containing the locked-open I domain after disulfide reduction with dithiothreitol. Thus, the drug compounds inhibit LFA-1 function by binding to the closed conformation of the I domain and blocking the conformational transition to the open form that is active in binding to ICAMs and mediates cell-cell adhesion. The ability of these compounds to inhibit cell adhesion in vitro and in vivo provides strong evidence that a change in affinity, and not a change in avidity through clustering on the cell surface, is responsible for physiologic regulation of adhesiveness.

The Mechanistic Basis of I Domain Activation

The above studies demonstrate that (*a*) I domain conformation dramatically regulates affinity for ligand, (*b*) the open conformation is sufficient to maximally activate cell adhesion independently of the transmembrane and cytoplasmic domains, (*c*) drug compounds that lock I domains in the closed conformation inhibit cell adhesion, and (*d*) antibodies detect changes on integrins in physiologically activated cells that are intrinsic to the integrin and not dependent on ligand binding. It is inescapable that regulation of I domain conformation regulates cell adhesion by integrins. Nonetheless, we do not know the details of the regulatory molecular changes that precede ligand binding. There could be a stable change in I domain conformation, or a single integrin molecule could equilibrate between the closed and open conformations, with cellular activation shifting the equilibrium. Alternatively, activation might lower the energy barrier for conformational change and make the open conformation kinetically accessible upon binding of ligand or mAbs such as CBRM1/5. Furthermore, after activation and prior to ligand binding, the I domain might exist in a conformation intermediate between the closed and open conformations.

DOMAIN-DOMAIN INTERACTIONS IN INTACT INTEGRINS IN TRANSMISSION OF SIGNALS TO AND FROM THE I DOMAIN

In the following section we address how, in the context of an intact integrin heterodimer, conformational signals are transmitted to and from the I domain. The evidence is consistent with signal transmission through the C-terminal α-helix, the linker that follows this helix, and through contacts at the bottom of the I domain.

Interactions Between the Three Domains in the Headpiece

The C-terminal linker connecting the I domain to the β-propeller domain is much longer than the N-terminal linker. In the primary structure of the α-subunit, the I domain is inserted between blades (β-sheets) 2 and 3 of the β-propeller domain, with its N terminus immediately following the last β-strand (strand 4) of blade 2. A pair of cysteines conserved only among I domain–containing α-subunits is predicted to form a disulfide that connects the loop between β-strands 2 and 3 in β-sheet 2 to the linker that follows β-strand 4 in β-sheet 2. There are only three residues from this disulfide-bridged cysteine to the first residue defined in I domain structures, indicating that the N-terminal linker closely tethers the I domain to the β-propeller domain. On the other hand, the C-terminal linker of the I domain is much longer and thus may permit much greater conformational motion. This linker is $\sim$20 amino acid residues long and connects the end of the C-terminal α-helix of the I domain to β-strand 1 in blade 3 of the β-propeller. Many of the residues are serines, which suggests flexibility.

Since the closed conformation is favored energetically in isolated I domains and appears to be the default conformation adopted in the basal, inactive state of integrins on the cell surface, conversion to the open conformation would require an external input of energy. This would have to come from movements at interdomain contacts. The most likely type of motion is a downward movement of the C-terminal α-helix, which could be induced by exertion of a bell-rope-like pull on a segment within the C-terminal linker region.

The integrin α-subunit β-propeller and I domains are in close proximity to the β-subunit I-like domain. In the crystal structure of integrin $\alpha V\beta 3$ (99), the MIDAS of the β-subunit I-like domain is positioned close to the loop in the β-propeller in which the I domain is inserted (*asterisk* in Figure 11). It is possible that the open and closed conformations of the I domain are regulated by interaction of the C-terminal linker with the β-propeller and/or the I-like domain at this site. Because this site is equivalent to the ligand-binding site in integrins that lack I domains, alterations in the interaction of the linker with the MIDAS of the I-like domain may occur that are analogous to those that regulate interactions with ligands in integrins that lack I domains. In summary, we predict that three structural units, the I, β-propeller, and I-like domains, make a ternary interaction interface where structural rearrangements of the latter two domains affect the conformation of the I domain.

The Bottom of the I Domain and the C-Terminal Linker

Consistent with a role for domain-domain interactions in regulating I domain conformation, mutation of exposed residues near the bottom of the I domain and in the linker region can regulate ligand binding. In one study, 17 sequence segments distributed over all faces of the human αM I domain were swapped with corresponding mouse segments (68). Of these, only three substitutions, all located on the bottom face, increased binding to ligand and expression of the CBRM1/5 activation epitope. In another investigation (104), two segments at the bottom of the αM I domain were swapped with corresponding segments of the αL I domain; each swap activated ligand binding. Interestingly, similar substitutions at the bottom of the von Willebrand factor A1 domain, which is highly homologous to integrin I domains, have been found to activate ligand binding and result in spontaneous binding of von Willebrand factor to platelets (24). Such mutations were identified because they are responsible for the abnormal platelet aggregation and thrombocytopenia in patients with type 2B von Willebrand disease. Conformational movements analogous to those in integrin I domains may regulate ligand binding in von Willebrand factor A domains; however, thus far only small movements have been detected in crystal studies of a type 2B A domain mutant (13).

Mutational and antibody epitope studies suggest a role for the C-terminal linker region in regulating ligand binding by the I domain and in conformational movements. The last α-helical residue defined in I domain structures is equivalent to Tyr-307 in αL; Ser-327 approximates the beginning of the β-propeller domain,

leaving a linker from residues 308–326. Some mutations in this linker sequence, K314A and L317A, activate ligand binding by $\alpha L\beta 2$, while other mutations at the linker, Y307A and E310A, inactivate $\alpha L\beta 2$ (37). This suggests that contacts between the linker and other domains modulate the conformation of the I domain. This supports the idea that signals could be propagated via the C-terminal linker sequence to the C-terminal α-helix, and then to the MIDAS. In further support of conformational movement of the linker region, CBR LFA-1/1, a conformation-sensitive αL mAb (61a) maps to residues 301 to 338, which includes all of the linker and the last two turns of the C-terminal α-helix (32, 59).

Regulation by the I-Like Domain

Recent evidence shows that the I-like domain regulates the conformation of the I domain. Previously, it was thought that in I domain–containing integrins, the I-like domain made a direct contribution to ligand binding because mutations in the MIDAS of the I-like domain, and mAb directed to the I-like domain, inhibited ligand binding. However, when locked in the open conformation the isolated αL I domain is sufficient to give a monomeric binding affinity equivalent to that of activated $\alpha L\beta 2$ and it also gives equivalent adhesiveness when present on the cell surface. Remarkably, ligand binding by $\alpha L\beta 2$ containing an I domain locked open with a disulfide was resistant to many mAb that fully inhibited ligand binding by activated wild-type $\alpha L\beta 2$ and that bound equally well to locked-open and wild-type $\alpha L\beta 2$ (60). There were two classes of mAb specific for the I domain: those that inhibited wild-type and locked-open $\alpha L\beta 2$ equally well and those that inhibited wild-type but had no effect on locked-open $\alpha L\beta 2$. The former mAb appear to directly block the ligand-binding site; the latter mAb cannot block the ligand-binding site and therefore appear to favor the closed conformation of the I domain. Most importantly, all mAbs to the I-like domain inhibited ligand binding by wild-type but had no effect on locked-open $\alpha L\beta 2$. These mAb had been mapped to multiple epitopes located in three widely separated sites on the molecular surface of the I-like domain. Furthermore, disulfide reduction with dithiothreitol restored the susceptibility of the disulfide-locked receptor to the inhibitory mAbs, showing that the mutant receptors override the blocking effect of mAbs because their conformation is fixed. This clearly demonstrates that the anti-$\beta 2$ I-like domain mAbs exert their effect in an allosteric manner rather than by directly competing with the ligand. Thus, in $\beta 2$ integrins, the I-like domain does not directly participate in ligand binding and appears to affect ligand binding indirectly by regulating the conformation of the I domain (60). Allosteric inhibition of ligand binding by other β-subunit mAbs has also been reported (64).

Regulation by Ca^{2+} and Mn^{2+}/Mg^{2+}

Observations on the effect of Ca^{2+} and Mn^{2+} ions on ligand binding by I domain–containing integrins also favor a regulatory rather than a direct role for the β-subunit I-like domain. High concentrations of Ca^{2+} are known to be inhibitory against many I domain–containing integrins. As described above, the Ser and

Thr side chains in the MIDAS strongly disfavor Ca^{2+} coordination. Furthermore, in contrast to results with intact $\alpha 2\beta 1$ and $\alpha L\beta 2$, binding of isolated $\alpha 2$ (68) and αL (M. Shimaoka & T. A. Springer, unpublished data) I domains to their ligands is not inhibited by mM concentrations of Ca^{2+}. Moreover, Mn^{2+}, a well-known strong activator of integrins, does not appear to activate by binding to the I domain's MIDAS because (*a*) Mn^{2+}-loaded αM and αL I domains crystallize in the closed conformation (47, 74); (*b*) the wild-type isolated I domain shows equivalent adhesiveness in Mg^{2+} and Mn^{2+} (43); and (*c*) the locked-open αL I domain shows identical affinities and adhesiveness to ICAM-1 in Mg^{2+} and Mn^{2+} (M. Shimaoka & T. A. Springer, unpublished data).

The I-like domain is the best candidate for mediating the effects of Mn^{2+} and Ca^{2+}. A recently described Ca^{2+}-binding site in the I-like domain of the $\alpha V\beta 3$ structure is adjacent to the MIDAS of the I-like domain and thus has been termed the ADMIDAS (99) (Figure 11). The ADMIDAS and the MIDAS of the I-like domain are likely to be the inhibitory Ca^{2+}- and stimulatory Mn^{2+}-binding sites, respectively. The existence of the conserved MIDAS sequence in the β-subunit's I-like domain, and the requirement of the MIDAS residues for ligand binding by both integrins that contain and lack I domains (88), have been the major basis for the proposed direct involvement of the β-subunit in ligand binding by integrins containing I domains. Based on the evidence that the I-like domain plays a regulatory rather than a direct role in ligand binding by I domain–containing integrins, we propose an alternative model. We propose that in the active conformation of the I-like domain, it binds to a ligand-like segment in the α-subunit, most likely in the I domain linker, and thereby exerts the downward pull on the bell-rope that remotely opens the conformation of the ligand-binding site of the I domain.

Signal Transmission Through the Stalk Region

We have discussed interactions among the three domains in the headpiece, the β-propeller domain, I domain, and I-like domain, that regulate ligand binding by the I domain. We now discuss how signals are relayed from the membrane through the stalk regions to the headpiece. The first step in inside-out signaling appears to be the disruption of interactions between the juxtamembrane regions of the α- and β-subunits, which leads to the separation or movement apart of the juxtamembrane regions. Early studies suggested complementary interactions between the αIIb and $\beta 3$ subunits near the junction between the transmembrane and cytoplasmic domains (35). Mutations near the junction between the transmembrane and cytoplasmic domains, as well as deletion of the cytoplasmic domains, have been repeatedly shown to result in integrin activation. Recently, Lu et al. found that replacement of the cytoplasmic tails of the αL and $\beta 2$ subunits with complementary α-helices that formed a heterodimeric α-helical coiled-coil kept the receptor in a low-affinity state (61). By contrast, replacement with noncomplementary α-helices resulted in constitutive activation of $\alpha L\beta 2$. Similarly, movement apart at the C-terminal region of the extracellular domain was shown to activate a soluble version of the integrin $\alpha 5\beta 1$. The low-affinity receptor was converted into a fully

active one by a pure conformational manipulation that released a covalent constraint introduced at the C-terminal end of the stalks (92). These findings suggest that conformational movements in the ligand-binding headpiece are induced by movement apart of the cytoplasmic and transmembrane domains. In physiologic settings, this would be initiated by signals from inside the cell.

Much experimental evidence suggests that in integrin activation, major structural rearrangements occur in the stalk regions of the α- and β-subunits, particularly in the β-subunit, and that separation of the α and β stalks occurs. Many mAbs that recognize "activation epitopes," which become exposed upon receptor activation, map to the stalk regions, particularly to the PSI domain (65) and I-EGF domains 2–4 of the β-subunit (3, 20, 57, 78, 86, 93, 102). Also, a subset of anti-stalk antibodies activate integrins upon binding, probably by acting as a "wedge" to break domain-domain contacts and open up the stalk region (34). Furthermore, amino acid residues have been identified that participate in restraining the integrin αXβ2 in its resting conformation. Species-specific differences at these residues cause activation when the α- and β-subunits are derived from different species. These residues are present in the PSI and I-EGF domains 2 and 3 of the β-subunit, suggesting that these domains contact the α-subunit in the resting but not active conformation (103).

A Switchblade-Like Model for Integrin Activation

Recently, we proposed a model for integrin activation that is based on both functional and structural data (4). The crystal structure of integrin αVβ3 reveals four α-subunit domains and five β-subunit domains, but it lacks the PSI domain, I-EGF domains 1 and 2, and 27% of I-EGF domain 3 (99). Complementary NMR data reveal the structure of integrin β2 I-EGF module 3; together with perturbation data, it is demonstrated that the I-EGF domain $2 + 3$ module pair has a rigid and extended structure (4). The combined data on I-EGF modules $2 + 3 + 4$ demonstrate a single continuous structural unit with rigid module-module interfaces that is suitable for conveying structural motion from the membrane to the headpiece.

The αVβ3 extracellular domain structure has an overall V-shaped organization, in which the ligand-binding headpiece bends back toward the base of the stalk region and is oriented toward the cell membrane (99) (Figure 11*A*). Although reported to represent the active conformation, this genuflected conformation appears unfavorable for ligand binding. αVβ3 was crystallized with Ca^{2+} and no Mg^{2+} (99). Ca^{2+} is known to stabilize integrins in the inactive conformation, and this has been directly demonstrated for soluble αVβ3; ligand binding requires Mg^{2+} or Mn^{2+} (81). Furthermore, at the juxtamembrane region, the C termini of the αV and β3 extracellular domains are close together (99); close association of the juxtamembrane regions maintains integrins in the inactive state (61, 91). Moreover, αVβ3 was crystallized in the absence of ligand; in crystal structures of integrin I domains, the active conformation has only been seen in the presence of a ligand or ligand mimetic (26, 48). Finally, electron microscopy of integrins shows that they adopt an extended, open conformation when bound to ligand that permits binding of mAb to activation epitopes in the β-subunit stalk (20).

Superposition of β2 I-EGF modules 2 and 3 on the partial I-EGF module 3 in $\alpha V\beta 3$ allows the orientation of functionally important residues to be visualized and provides strong support for the idea that the bent conformation represents the inactive conformation. The residues in β2 I-EGF3, which are in an $\alpha\beta$ interface that restrains integrins in the inactive state (103), are on the face pointing toward calf domain 1 of the α-subunit stalk (*yellow spheres*, Figure 11). The residues participating in the KIM127 activation epitope in β2 I-EGF2 (*red spheres*) and in the CBR LFA-1/2 and MEM48 activation epitopes in I-EGF3 (*green spheres*) are masked in the bent conformation (Figure 11*A*). However, in the extended, unbent conformation, there is no domain that could mask these epitopes (Figure 11*B*). The transition from the bent (Figure 11*A*) to the extended conformation (Figure 11*B*) provides a mechanism for unmasking of these activation epitopes. Furthermore, the β-subunit bends back on itself much more than the α-subunit, providing an explanation for the observation that the vast majority of activation epitopes are present on the β-subunit.

Therefore, we have proposed that activation triggers a switchblade-like opening motion that extends the ligand-binding headpiece of the integrin heterodimer away from the plasma membrane (Figure 11) (4). In integrin activation by biological inside-out signaling, movement apart of the juxtamembrane domains (61, 91) may lead to dislocation of the I-EGF3 contact with calf domain 1, which in turn triggers the switchblade-like opening. This drastic change in the overall orientation and interaction between domains would not only reposition the headpiece in a more favorable orientation for ligand binding, but is also hypothesized to be linked to change in conformation of the I-like domain and in turn the conformation of the I domain. The work on isolated I domains described above suggests that repositioning of the headpiece, in the absence of shape-shifting in the I domain, is insufficient to activate ligand binding. Because bending will mask many antibody epitopes in the stalk region, particularly on the β-subunit stalk, which is severely jackknifed at the bend (99) (Figure 11), binding of antibodies to these epitopes would favor the extended integrin conformation, producing a mechanism for antibody-induced integrin activation.

THE RELATIVE IMPORTANCE OF CONFORMATIONAL CHANGE AND CLUSTERING IN INSIDE-OUT AND OUTSIDE-IN SIGNALING

Inside-Out Signaling: Affinity or Avidity?

We have focused on regulation of an integrin's ligand-binding activity by conformational shape-shifting within a single receptor molecule. However, many reports suggest that upregulation of integrin-mediated adhesion by activated cells is achieved by receptor clustering on the cell surface (i.e., avidity augmentation) rather than by, or together with, an increase in affinity of individual receptors (2, 27, 55, 87, 94). Clustering of receptors on the cell surface would no doubt increase overall cell-adhesive efficiency, particularly when the ligands are

di- or multivalent and have a similar clustered distribution on the opposing cell or substrate. However, experimental evidence for the formation of integrin clusters on activated cells is rather qualitative and in most cases is demonstrated experimentally by either a large dot-like or polarized staining pattern after cell fixation. Furthermore, it is difficult to know whether clustering triggers ligand binding or is a result of ligand binding that is triggered by an increase in receptor affinity. Receptors on cells, including integrins, are known to redistribute to sites on cells where they can bind ligand as a consequence of their capture in ligand-receptor complexes. Moreover, real-time imaging has shown that the formation of visible clusters in adhering cells occurs long after the first contacts are made (46, 70), whereas the ligand-binding activity of integrins on circulating cells such as leukocytes and platelets must be upregulated in a matter of seconds in vivo.

Intermediate Affinity States?

The dismissal of affinity alteration as the mechanism of increased cell adhesion often is based on the inability to detect increased binding of soluble ligands to cells that clearly exhibit increased adhesiveness (2). Thus, avidity regulation is adopted as the alternative explanation for increased cellular adhesiveness, not because it has been directly demonstrated, but because there is a lack of evidence for affinity regulation. In some cases, certain stimuli that cause increased cellular adhesiveness do result in a measurable increase in soluble ligand binding (affinity regulation is inferred), and other stimuli that also cause increased cell adhesiveness do not augment soluble ligand binding (avidity regulation is inferred) (14, 27, 87). The observation that the affinity of the αL I domain for ligand can range all the way from a K_D of 200 nM for the locked-open I domain to 2 mM for the wild-type or locked-closed I domain suggests that the lack of binding of soluble ligand should be interpreted with great caution (79).

It is reasonable to propose that in integrin heterodimers on the cell surface, I domains could exist not just in two affinity states with K_D of 200 nM (open) and K_D of 2 mM (closed), but also in many intermediate states. This could result from equilibration between two states, with the affinity representing the time-averaged population of the two states, from the existence of true conformational intermediates along the shape-shifting pathway, or from differences in the kinetics of I domain opening. Activation of $\alpha L\beta 2$ on the cell surface to an intermediate affinity with a K_D of 20 μM for ICAM-1 would not be detectable by ligand binding to cells because the K_D of 200 nM of the locked open I domain is just barely within the range detectable by conventional assays for ligand binding to cells. However, a K_D of 20 μM should be sufficient to activate cell adhesion, based on measurements with other cell-adhesion molecules (21). Thus, conformational alterations in integrins resulting in an intermediate affinity for ligand could be the initial event in inside-out activation, which would allow cells to surmount the threshold from a nonadhesive to an adhesive phenotype. After cells make the initial contact to the ligand-bearing surface, clustering of integrins may further stabilize the adhesion machinery.

Outside-In Signaling

A similar argument is applicable to the debate over the possible involvement of conformational alterations in outside-in signaling. Because of the multivalent nature of the physiological ligands of integrins, ligation will result in the clustering of integrins on the cell surface, the enabling recruitment of signaling molecules to the cytoplasmic face of the adhesion complex, and initiating downstream signaling events. Therefore, it is generally accepted that it is the clustering of receptors that drives outside-in signal transduction (28). However, as discussed above, gross structural changes in the heterodimer including a swichblade-like opening appear to be linked to other more subtle changes, including the conformation of the I domain. The open conformation of the I domain has not yet been visualized in the absence of, and thus may be stabilized by, bound ligand. In other words, binding to ligand could stabilize not only the conformational shift of the I domain, but also the gross structural rearrangements in the whole receptor molecule. Activation of the receptor, and ligand binding, may reinforce one another in stabilizing a fully open conformation, consistent with the appearance of the antibody epitopes known as ligand-induced binding sites. Thus, ligand binding may stabilize or induce changes in the integrin transmembrane and cytoplasmic domains that mediate outside-in signaling. It is possible that overall outside-in signal transduction is achieved as a sum of intermolecular clustering and intramolecular conformational modulation. In fact, induction of integrin clustering alone is insufficient to reproduce full outside-in signaling events (30).

CODA

In summary, structures have been determined and models built for integrin I domains in two different conformations, open and closed. Mutational and functional studies demonstrate that the open conformation binds ligand with high affinity, and the closed conformation either does not bind ligand or binds with low affinity. In physiologic activation of integrins on the cell surface, studies with antibodies demonstrate that conformational change precedes ligand binding. However, it is not known whether these changes correspond precisely to transition from the closed to open conformation, or transition to an intermediate conformation, since thus far the open conformation has been visualized only in the presence of a ligand or ligand mimetic. The structural changes in I domains are similar to those in small G proteins, particularly around the metal-binding site; however, metal-binding site rearrangement is linked to large motions in different backbone segments.

In I domains, the linkage to the C-terminal α-helix segment provides a mechanism for propagating conformational change from one domain to another. Locking in alternative conformations of the loop preceding this C-terminal α-helix demonstrates that conformational movement here is linked to a dramatic 9000-fold increase in affinity of the ligand-binding site around the MIDAS. The C-terminal linker of the I domain is located in an interface between the β-propeller and I-like domains that constitutes the ligand-binding site in integrins that lack I domains.

Interactions at this site of the linker in integrins that contain I domains may mimic interactions of ligands with the I-like domain MIDAS in integrins that lack I domains, and these interactions may provide a mechanism for transmitting conformational motion to the I domain.

Structural information on $\alpha V\beta 3$ and the $\beta 2$ I-EGF3 domain reveals the location of sites that are functionally important in integrin activation and suggests a model for activation. In the inactive conformation, the headpiece faces the membrane. In activation, the headpiece extends upward in a switchblade-like motion. Interfaces between I-EGF modules 2 and 3 and the headpiece are broken, and activation epitopes hidden by the bend are exposed. These long-range rearrangements of the global interdomain architecture are coupled to conformational changes within the ligand-binding site that increase affinity for ligand. Movement apart of the juxtamembrane segments of the α- and β-subunits, which is set in motion by intracellular signaling cascades, appears to initiate the rearrangements in the extracellular domain.

Exactly how integrin heterodimers achieve signal transduction in both directions, to what extent conformational change within receptor molecules is responsible for these events, and the details of signal transmission between domains within these complex molecular machines await further biochemical, structural, and cell biological studies. The complexities of these molecules are appropriate to the sophisticated and diverse functions they mediate in connecting the intracellular and extracellular environments. Much more remains to be learned about how these molecules function in general, as well as how different integrin heterodimers are specialized for diverse tasks. There is no doubt that the understanding of these events at the molecular level will reveal exciting biological and structural principles and will also greatly advance our ability to devise therapeutics to control the pathophysiologies mediated by this important family of cell-adhesion molecules.

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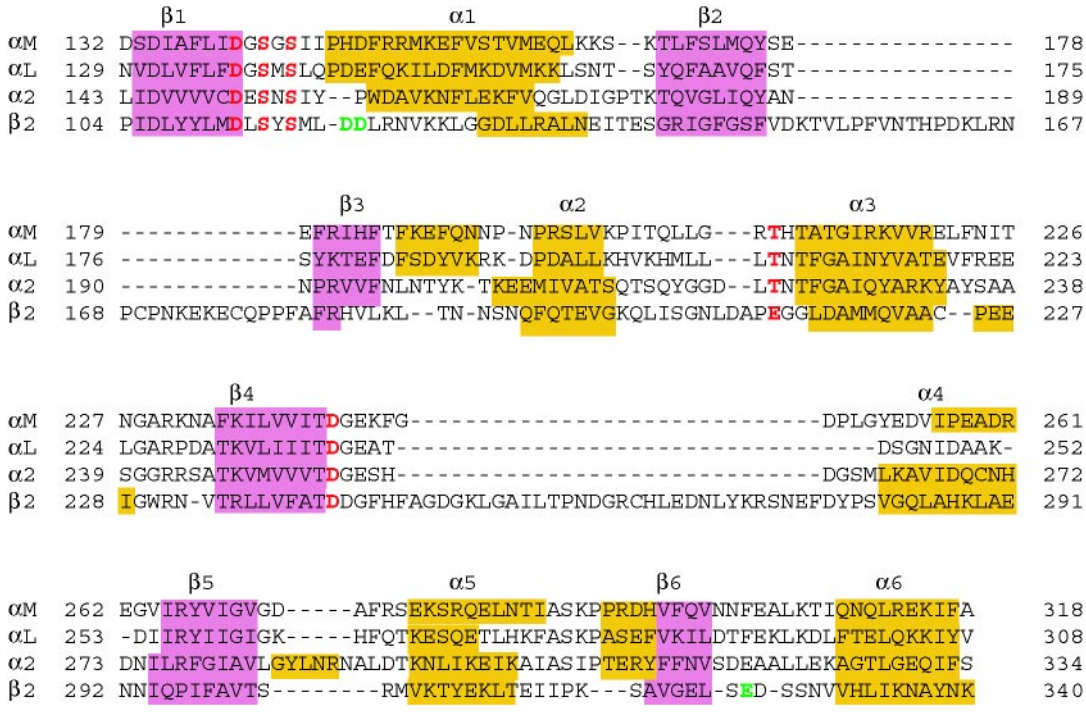

Figure 3 Structure-based sequence alignment of α-subunit I domains, and the β2 subunit I-like domain. The closed conformers of αM (1JLM) (47), αL (1ZON) (74), and α2 (1AOX) (25) I domains, and the β3 I-like domain (99) were superimposed with 3D MALIGN of MODELLER (77) using a gap penalty of 4 Å, and five iterations of alignment with superposition of Cα, Cβ, Cα, Cβ, and finally Cα atoms. Gaps in loops were closed up. The β2 I-like domain was aligned by sequence to that of β3. α-helices and β-strands in the I domains and β3 I-like domain are highlighted in *gold* and *magenta*, respectively. Residues that coordinate the metal in the MIDAS are *red*, those that coordinate the Ca^{2+} in the ADMIDAS are *green*.

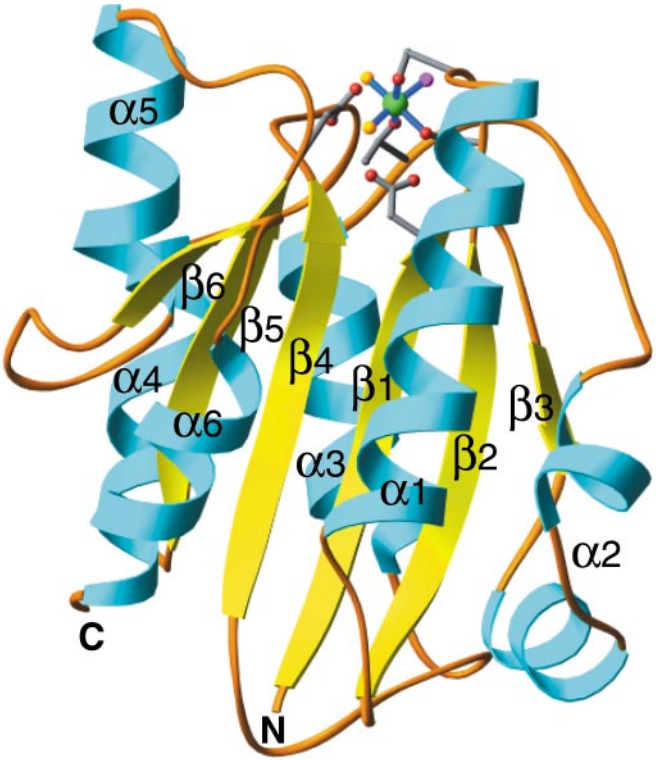

Figure 4 Ribbon diagram of the αM I domain in the open conformation. The β-strands (*yellow*), α-helices (*cyan*), and the N and C termini are labeled. The Mg ion is shown as a *green sphere*. Side chains of residues that form primary or secondary coordinations to the metal ion (D140, S142, S144, T209, and D242) are shown with *gray* bonds and carbon atoms and *red* oxygen atoms. Coordinating water molecule oxygens are *gold*, and the oxygen of the ligand-mimetic Glu from another I domain is *purple*. All ribbon diagrams in this review were prepared with Ribbons (11).

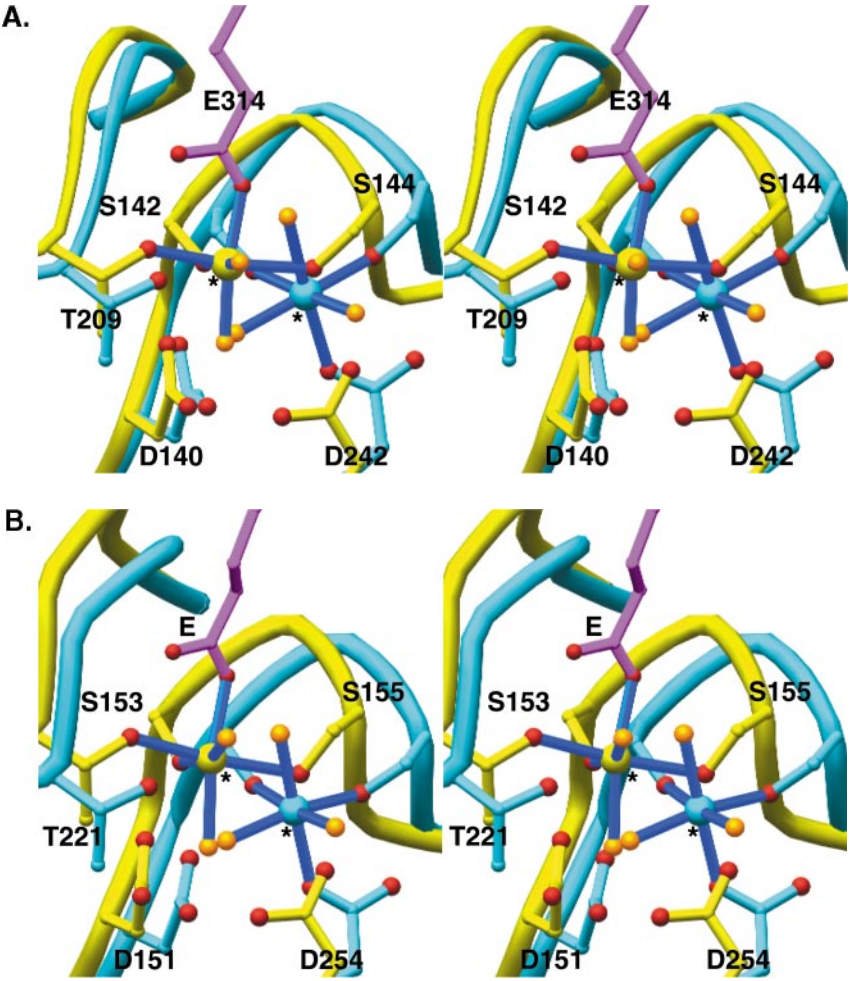

Figure 5 Stereo view of alternative conformations of the MIDAS. (*A*) αM. (*B*) α2. The backbone, coordinating side chain bonds, and metals (labeled with *asterisks*) are shown in *yellow* (open conformation) and *cyan* (closed conformation). The coordinating glutamate residue bonds from the ligand-mimetic neighboring αM I domain (E314) in αM and collagen peptide ligand (E) in α2 are in *purple*. Primary coordination bonds to the metals are in *blue*. Oxygen atoms of the coordinating side chains and water molecules are *red* and *gold*, respectively. I domains were superimposed on one another in turn, so all were in the same orientation as the closed 1JLM αM structure (48). The 1IDO open αM structure (47) was superimposed on 1JLM using residues 132–141, 166–206, 211–241, 246–270, and 287–294. The 1AOX closed α2 structure (25) was superimposed on 1JLM using residues 145–153, 180–189, 192–199, 222–240, 246–256, and 268–282. The 1DZI open α2 structure (26) was then superimposed on 1AOX using residues 143–152, 173–216, 223–253, and 259–282. The closed αL structure 1ZON (74) was superimposed on 1JLM using residues 131–141, 167–189, 201–221, 231–241, and 255–262 (see Figure 7).

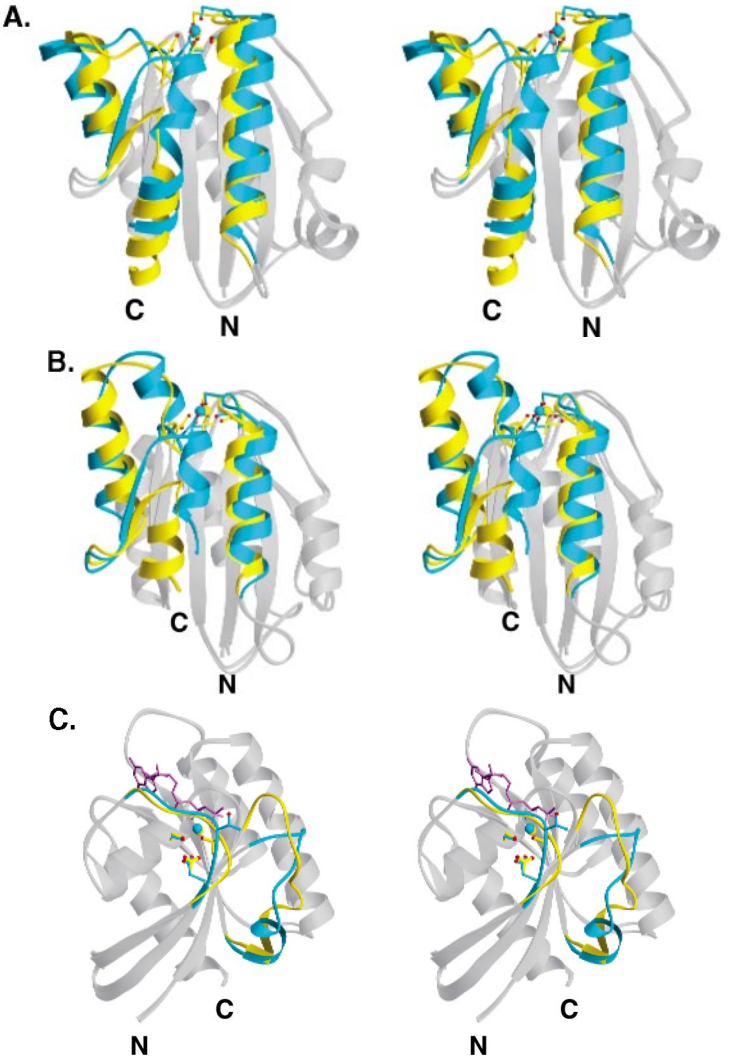

Figure 6 Stereo view of the alternative conformations of I domains and the small G protein ras. (*A*) αM I domain. (*B*) α2 I domain. (*C*) ras p21 G protein. The regions of significant difference between the superimposed conformers are shown in *yellow* (open or active) and *cyan* (closed or inactive). Similar backbone regions are in *gray*. Metal atoms and coordinating side chain bonds and carbon atoms are in *yellow* (open or active) and *blue* (closed or inactive); oxygen atoms are *red*. The coordinating residues are S142, S144, T209, and D242 in αM; S153, S155, T221, and D254 in α2; and S17, T35, and D57 in ras p21. In the active conformer of ras p21, GDP-CP, a GTP analog, is in *purple*. The I domains were superimposed as in Figure 5. The active (GTP-bound) and inactive (GDP-bound) ras structures 6Q21 and 1Q21 (63) were superimposed using residues 1–29, 39–59, and 77–167. The orientations in *A* and *B* are identical, but differs in *C* to display the conformational changes in G proteins that differ in topological location from I domains.

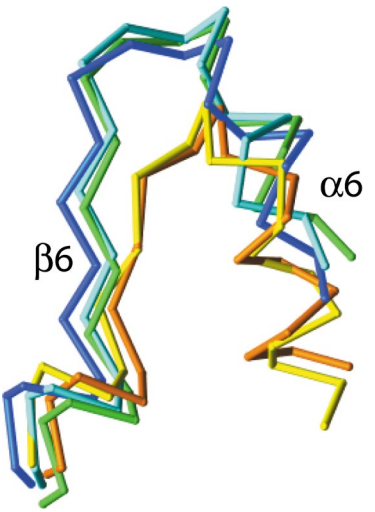

Figure 7 The loop between the most C-terminal β-strand (β6) and α-helix (α6) in I domains has a canonical conformation in open structures and a different canonical structure in closed structures. Loops are shown for open (αM, *yellow*; α2, *gold*) and closed (αM, *blue*; α2, *green*; αL, *dark blue*) conformations. For clarity, only residues 290–310 of αM, 306–326 of α2, and 280–300 of αL, which are of equal length in the closed and open structures and in all three I domains, are shown. I domains were superimposed as described in Figure 5.

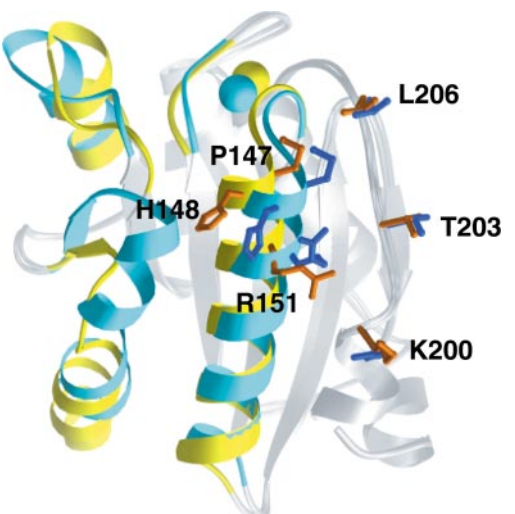

Figure 8 Alteration of the CBRM1/5 epitope in open and closed αM I domains. The metals and regions where conformational changes are significant are shown in *yellow* (open) and *blue* (closed). Other backbone regions are *gray*. Side chains of the CBRM1/5 epitope are in *gold* (open) and *dark blue* (closed). The open and closed αM I domain structures were superimposed as described in Figure 5.

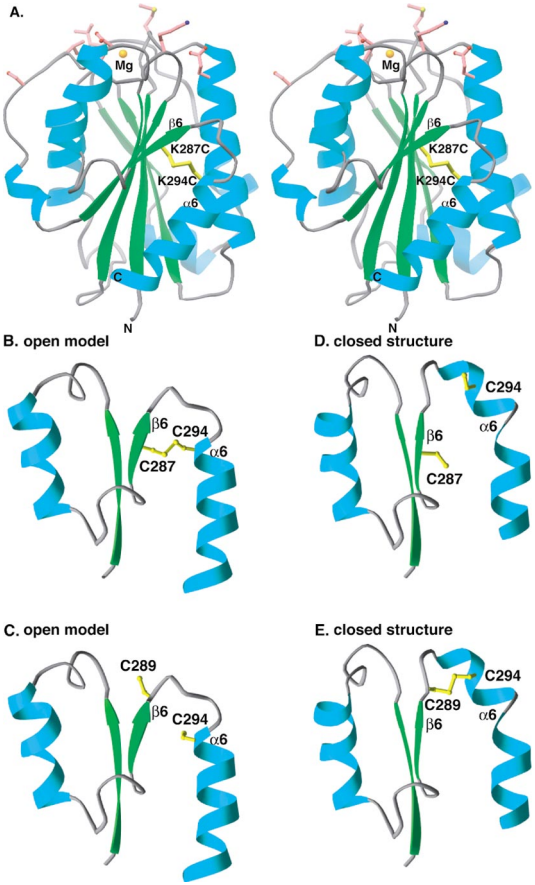

Figure 9 Locking in αL I domain conformations with engineered disulfide bridges. (*A*) Stereodiagram of the high-affinity model of the αL I domain, with mutations to introduce a disulfide bond. The side chains and disulfide bond of C287 and C294 are shown in *yellow*. The Mg^{2+} ion of the MIDAS is shown as a *gold sphere*. Side chains of residues important in binding to ICAM-1 and ICAM-2 are shown with *rose-pink* side chains and *yellow* sulfur, *red* oxygen, and *blue* nitrogen atoms. These residues, defined as important in species-specific binding to ICAM-1 (32), or by at least a twofold effect on binding to ICAM-1 or ICAM-2 upon mutation to alanine (23), are M140, E146, T175, L205, E241, T243, S245, and K263. Note that these residues surround the Mg^{2+} ion and are distant from the disulfide. (*B–E*) Predicted disulfide bonds that are selective for open or closed conformers of the αL I domain. The K287C/K294C mutation (*B*, *D*) and L289C/K294C mutation (*C*, *E*) were modeled in both open (*B*, *C*) and closed (*D*, *E*) I domain conformers. For clarity, only residues 254–305 of the models are shown. The four models were superimposed using residues not involved in conformational shifts and are shown in exactly the same orientation. The downward movement of the α6 helix in panels *B* and *C* compared to *D* and *E* is readily apparent. The remodeling of the loop connecting β6 and α6 is accompanied by a reversal in the orientation of the side chain of residue 289. Figure from (79).

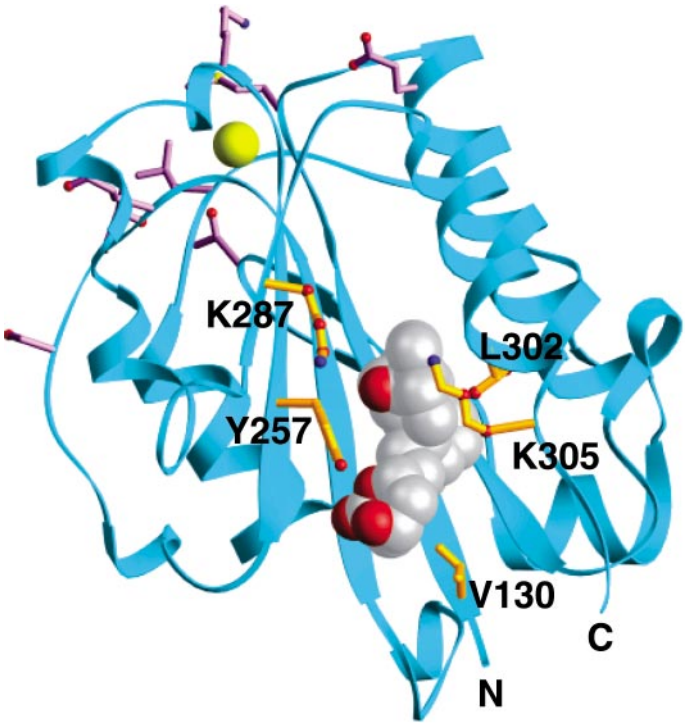

Figure 10 The drug-binding pocket of the αL I domain (39). The small drug molecule bound in the hydrophobic pocket between the β-sheet and the α-helix 6 is shown in CPK with *silver* carbon atoms and *red* oxygen atoms. Side chains within the binding pocket are labeled and shown with *gold* bonds and carbon atoms, *red* oxygen atoms, and *blue* nitrogen atoms. V233, which is also in the pocket, is hidden by the drug molecule. The residues critical for binding to ICAM-1 or ICAM-2 are shown as in Figure 9 and are clearly distal from the small molecule-binding site. The crystal structure is of the αL I domain bound to lovastatin (39); it appears to be a coincidence that lovastatin inhibits both αLβ2 and β-hydroxy methylglutaryl coenzyme A reductase.

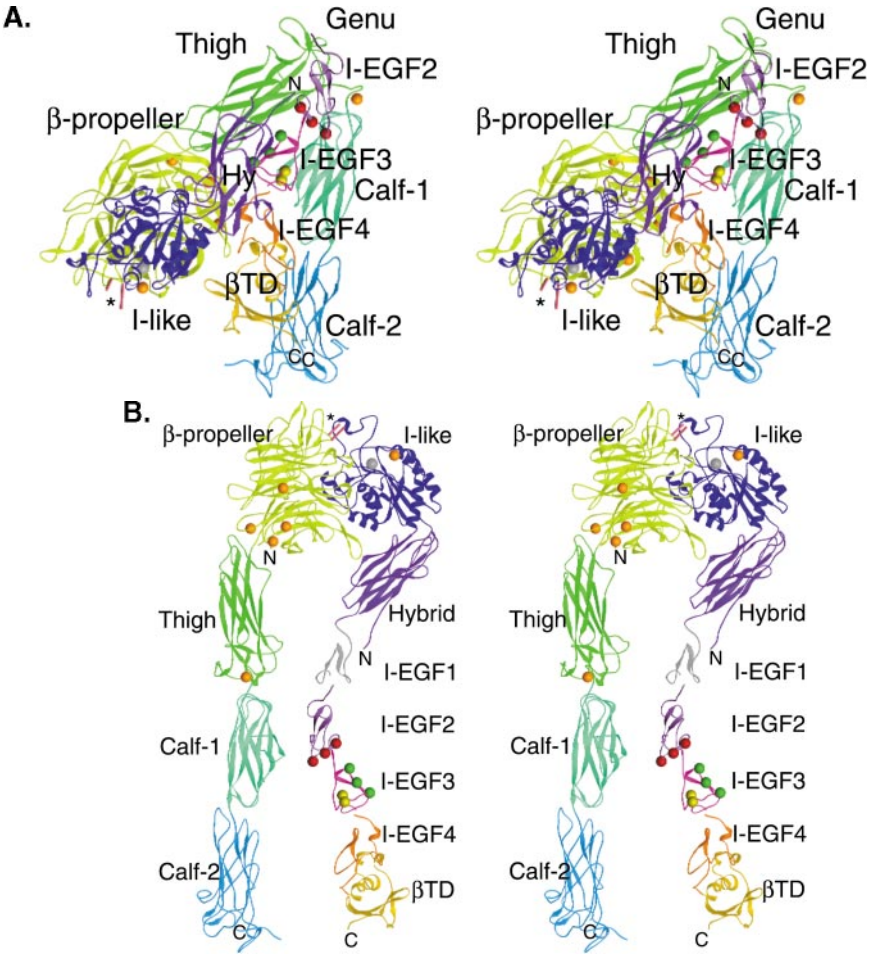

Figure 11 Stereo ribbon diagrams of alternative conformations of the extracellular segment of integrins. (*A*) Bent conformation observed in the crystal structure of $\alpha V\beta 3$ (99). (*B*) Model of an upright, active integrin, including segments from $\alpha V\beta 3$ (99) and I-EGF domains 2 and 3 of $\beta 2$ (4). Each domain is shown in a different color and labeled (Hy, hybrid; βTD, β-tail domain). Two *red cylinders* and an *asterisk* show where the I domain would be inserted. Ca^{2+} and Mg^{2+} ions are *gold* and *silver spheres*, respectively. The Mg^{2+} is not present in the structure but is added to show the position of the MIDAS. The genu (knee) is where the headpiece bends over the stalk region. In panel *B*, the integrin is straightened at the bend (99) to resemble the ligand-binding conformation observed in the electron microscope (20) (Figure 2*C*). The missing I-EGF1 domain is modeled in *gray*. I-EGF2 and a portion of I-EGF3 are also missing in the $\alpha V\beta 3$ structure; I-EGF domains 2 and 3 from $\beta 2$ (4) were added by superimposition on $\beta 3$ EGF-3. Cα atoms of functionally important residues in $\beta 2$ I-EGF2 and 3 are shown as large *spheres*: *red*, the epitope of the activating and activation-dependent KIM127 mAb; *green*, the epitope of the activating MEM48 and CBR LFA-1/2 mAbs (57); and *yellow*, residues critical for association of αX and $\beta 2$ subunits (103).