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Integrins: Bidirectional, Allosteric Signaling Machines

Review

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In their roles as major adhesion receptors, integrins signal across the plasma membrane in both directions. Recent structural and cell biological data suggest models for how integrins transmit signals between their extracellular ligand binding adhesion sites and their cytoplasmic domains, which link to the cytoskeleton and to signal transduction pathways. Long-range conformational changes couple these functions via allosteric equilibria.

Integrins are the major metazoan receptors for cell adhesion to extracellular matrix proteins and, in vertebrates, also play important roles in certain cell-cell adhesions. In addition to mediating cell adhesion, integrins make transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways. Since the recognition of the integrin receptor family around 15 years ago (Hynes, 1987), they have become the best-understood cell adhesion receptors. Integrins and their ligands play key roles in development, immune responses, leukocyte traffic, hemostasis, and cancer and are at the heart of many human diseases—genetic, autoimmune, and others. They are the target of effective therapeutic drugs against thrombosis and inflammation, and integrins are receptors for many viruses and bacteria.

Because of these multifarious functions, integrins have been and are being studied intensively and there has been continuous, rapid progress over the past 15 or so years (averaging over a 1000 papers a year for the past decade). Over the past year there have been particularly rapid advances in understanding integrin structure and function because of the elucidation of the 3D structures of one integrin (Xiong et al., 2001, 2002) and parts of others. These structural analyses have revealed some surprises but are also beginning to make sense of an enormous body of prior data on integrins. In this review, I will first give a brief overview of the integrin family to set the context and then review the recent structural data and experiments arising from them, which give insight into some long-standing questions concerning integrin functions. It is impossible to be exhaustive in such a review of integrins, so I have resorted to summary figures and tables and cited other reviews for more details on specific aspects.

The Integrin Receptor Family: Evolution and Complexity

Integrins are restricted to the metazoa; no homologs are detected in prokaryotes, plants, or fungi (Whittaker and Hynes, 2002). The simplest metazoa, sponges and cnidaria, have integrins (Burke, 1999; Hughes, 2001) and it is clear that primitive bilateria had at least two integrin $\alpha\beta$ heterodimers, the descendents of which persist to this day in organisms as diverse as flies, nematodes, and vertebrates (Hynes and Zhao, 2000). Indeed, that is the entire set of integrins in *Caenorhabditis elegans*; one β subunit and two α subunits forming two integrins. Orthologs of these two integrins are recognized in *Drosophila melanogaster* and in vertebrates, although vertebrates have expanded each set (Figure 1). One set (blue in Figure 1) recognizes the tripeptide sequence, RGD, in molecules such as fibronectin and vitronectin in vertebrates and tiggrin in *Drosophila*, whereas the other set (purple in Figure 1) mediates adhesion to basement membrane laminins. It is plausible that evolution of integrins was necessary to allow the cell-matrix adhesion intrinsic to metazoa, and as diploblastic organisms evolved, the two cell layers may have evolved separate integrins to mediate their asymmetric interactions with the basal lamina; representatives of these two primordial integrins are detected in all higher metazoan phyla.

Expansions of the integrin subunit set have occurred in different phyla. Figure 1 shows the complete mammalian set (based on extensive searches of the human and mouse genomic sequences, C.A. Whittaker and R.O.H., unpublished data), comprising 8 β and 18 α subunits, so far known to assemble into 24 distinct integrins. Orthologs of more than half these subunits have, so far, been found only in chordates, including most of the β subunits and all the nine α subunits that have an extra inserted domain, known as an I or A domain (see later). In addition to the ancient RGD and laminin receptor subfamilies mentioned above, vertebrates have a set of collagen receptors with inserted I/A domains (α1, α2, α10, α11) and a pair of related integrins (α4β1, α9β1), which recognize both ECM proteins such as fibronectin and Ig-superfamily cell surface counterreceptors such as VCAM-1. Vertebrates also have a set of leukocyte-specific integrins (Figure 1), which also recognize Ig-superfamily counterreceptors and mediate heterotypic cell-cell adhesion. Most integrins recognize relatively short peptide motifs and, in general, a key constituent residue is an acidic amino acid (see more below). The ligand specificities rely on both subunits of a given $\alpha\beta$ heterodimer and are significantly more complex than shown in Figure 1 (see reviews cited in Figure 1 for more details about the diverse ligand specificities of integrins).

Each of the 24 integrins shown in Figure 1 appears to have a specific, nonredundant function. In part, this is evident from the details of their ligand specificities (not shown in Figure 1) but is most clearly shown by the phenotypes of knockout mice (Table 1). Genes for the β subunits and all but four of the α subunits have been knocked out and each phenotype is distinct, reflecting the different roles of the various integrins. The phenotypes range from a complete block in preimplantation development (β1), through major developmental defects (α4, α5, αv, β8), to perinatal lethality (α3, α6, α8, αv, β4,

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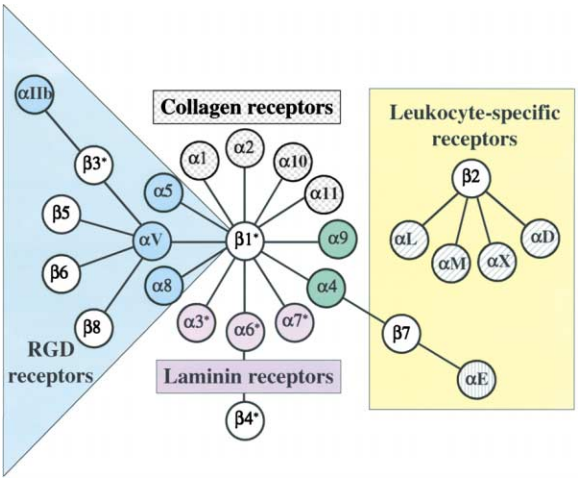

Figure 1. The Integrin Receptor Family

Integrins are αβ heterodimers; each subunit crosses the membrane once, with most of each polypeptide (>1600 amino acids in total) in the extracellular space and two short cytoplasmic domains (20–50 amino acids). The figure depicts the mammalian subunits and their αβ associations; 8 β subunits can assort with 18 α subunits to form 24 distinct integrins. These can be considered in several subfamilies based on evolutionary relationships (coloring of α subunits), ligand specificity and, in the case of β2 and β7 integrins, restricted expression on white blood cells. α subunits with gray hatching or stippling have inserted I/A domains (see text). Such α subunits are restricted to chordates, as are α4 and α9 (green) and subunits β2-β8. In contrast, α subunits with specificity for laminins (purple) or RGD (blue) are found throughout the metazoa and are clearly ancient (see text). Asterisks denote alternatively spliced cytoplasmic domains. A few extracellular domains are also alternatively spliced (not shown). Further information on integrin subunit structures and details of ligand specificity are given in several extensive reviews (Hemler, 1999; Plow et al., 2000; van der Flier and Sonnenberg, 2001).

β8) and defects in leukocyte function (αL, αM, αE, β2, β7), inflammation (β6), hemostasis (αIIb, β3, α2), bone remodeling (β3), and angiogenesis (α1, β3) as well as others (see Table 1; Hynes, 1996, 2002; DeArcangelis and Georges-Labouesse, 2000; Sheppard, 2000; Bouvard et al., 2001). There is not space here to discuss the details of all these phenotypes; the relevant point is that the integrins play diverse and important roles in most biological processes. How do they accomplish this?

Transmembrane Connections and Signaling

In addition to their roles in adhesion to ECM ligands or counterreceptors on adjacent cells, integrins serve as transmembrane mechanical links from those extracellular contacts to the cytoskeleton inside cells. For all integrins except α6β4, the linkage is to the actin-based microfilament system, which integrins also regulate and modulate. The β4 subunit differs from all the others; its cytoplasmic domain being much larger, ~1000 amino acids long instead of around 50, and making connections to intermediate filaments instead of to actin. The submembrane linker proteins connecting the cytoplasmic domains of integrins to the cytoskeleton are multiple and their interactions are complex. We will return later to a discussion of the roles of some of them in controlling integrin functions but there is not space here to review the integrin-cytoskeleton links; details can be found in recent reviews (Zamir and Geiger, 2001; van der Flier and Sonnenberg, 2001).

In part related (both in cause and in effect) to the integrin-mediated assembly of cytoskeletal linkages, ligation of integrins also triggers a large variety of signal transduction events (Figure 2) that serve to modulate many aspects of cell behavior including proliferation, survival/apoptosis, shape, polarity, motility, gene expression, and differentiation. These signal transduction pathways are complex, like those emanating from receptors for soluble factors (e.g., G protein-coupled and kinase receptors). Indeed, many integrin-stimulated pathways are very similar to those triggered by growth factor receptors and are intimately coupled with them (Figure 2). In fact, many cellular responses to soluble growth factors, such as EGF, PDGF, LPA, and thrombin, etc., are dependent on the cell's being adherent to a substrate via integrins. That is the essence of anchorage dependence of cell survival and proliferation and integrins lie at the basis of these phenomena (Assoian, 1997; Schwartz and Assoian, 2001; Frisch and Screaton, 2001). It is now very well established that integrin-mediated signals are necessary in normal cells to block apoptosis (via PI3-kinase and Akt) and to stimulate cell cycle progression (via ERK and cyclin D1, etc.). In oncogenically transformed cells, these anchorage (integrin)-dependent signals are instead provided by oncogenes or by loss of tumor suppressor genes. Again, this is too complex an area to review here in detail and the reader is referred to more specialized reviews; see Figure 2, which summarizes the main messages—integrins are full-fledged signal transduction receptors, at least as important to cells as more traditional growth factor receptors.

Regulation of Integrin Function: Activation and Inactivation

Many integrins are not constitutively active; they can be, and often are, expressed on cell surfaces in an inactive or "OFF" state, in which they do not bind ligands and do not signal. This is very important for their biological functions, as is most evident from considering integrins on circulating blood cells.

The major platelet integrin, αIIbβ3, also known as GPIIb/IIIa, is present at high density on circulating platelets where it is inactive. If it were not, platelets would bind their major ligand, fibrinogen, from the plasma and aggregate, leading to thrombosis. On platelet activation, αIIbβ3 is activated from within the cell, so that it can bind fibrinogen, von Willebrand factor, and fibronectin, leading to strong adherence to the vessel wall and, by crosslinking via fibrinogen, to aggregation with other platelets. The importance of this activation of αIIbβ3 for hemostasis is clear from the phenotypes of mice lacking either subunit (see Table 1); these mice show major defects in hemostasis and have a bleeding disorder that is an excellent model of the human genetic disease Glanzmann thrombasthenia (GT), which arises from mutations in the genes for αIIb or β3 (Kato, 1997). Antagonists of αIIbβ3/fibrinogen binding, either antibodies or low molecular reagents based on the integrin recogni-

Table 1. Integrin Gene Knockout Phenotypes

α1	V, F	No immediately obvious developmental defects, reduced tumor vascularization	Gardner et al., 1996; Pozzi et al., 2000, 2002
α2	V, F	Few immediately obvious developmental defects, delayed platelet aggregation and reduced binding to monomeric collagen, reduced mammary gland branching	Holtkotter et al., 2002; Chen et al., 2002
α3	P	Kidney tubule defects, reduced branching morphogenesis in lungs, mild skin blistering, lamination defects in neocortex	Kriedberg et al., 1996; DiPersio et al., 1997; Anton et al., 1999
α4	E11/14	Defects in placenta (chorioallantoic fusion defect) and heart (epicardium, coronary vessels). Chimeras show defects in hematopoiesis.	Yang et al., 1995; Arroyo et al., 1996, 1999
α5	E10-11	Defects in mesoderm (posterior somites) and vascular development, neural crest apoptosis. Chimeras show muscular dystrophy	Yang et al., 1993; Goh et al., 1997; Taverna et al., 1998
α6[a]	P	Severe skin blistering, other epithelial tissues also defective. Lamination defects in cortex and retina.	Georges-Labouesse et al., 1996, 1998
α7	V, F	Muscular dystrophy, defective myotendinous junctions	Mayer et al., 1997
α8	P	Small or absent kidneys, inner ear hair cell defects	Muller et al., 1997; Littlewood Evans et al., 2000
α9	V	Die within 10 days of birth, chylothorax due to lymphatic duct defect	Huang et al., 2000
α10		Not reported	
α11		Not reported	
αv	E10/P	Two classes: embryonic lethality due to placental defects, perinatal lethality with cerebral vascular defects probably due to neuroepithelial defects, cleft palate. Most blood vessels develop normally	Bader et al., 1998; McCarty et al., 2002
αIIb[b]	V, F	Hemorrhage, no platelet aggregation	Tronik-Le Roux et al., 2000
αL	V, F	Impaired leukocyte recruitment	Schmits et al., 1996
αM	V, F	Defective phagocytosis and apoptosis of neutrophils, mast cell development defects, adipose accumulation.	Coxon et al., 1996; Tang et al., 1997; Dong et al., 1997
αX		Not reported	
αD		Not reported	
αE	V, F	Greatly reduced numbers of intraepithelial lymphocytes.	Schon et al., 1999
β1	E6.5	Peri-implantation lethality, ICM deteriorates, embryos fail to gastrulate. Extensive analyses of chimeras.	Fässler and Meyer, 1995; Stephens et al., 1995; Brakebusch et al., 1997
β2[c]	V, F	Leukocytosis, impaired inflammatory responses, skin infections, T cell proliferation defects	Scharffetter-Kochanek et al., 1998
β3[b]	V, F	Hemorrhage, no platelet aggregation, osteosclerosis, hypervascularisation of tumors	Hodivala-Dilke et al., 1999; McHugh et al., 2000; Reynolds et al., 2002
β4[a]	P	Severe skin blistering, other epithelial tissues also defective	van der Neut et al., 1996; Dowling et al., 1996
β5	V, F	No immediately obvious developmental defects	Huang et al., 2000
β6	V, F	Inflammation in skin and airways, impaired lung fibrosis—all probably due to failure to activate TGFβ	Huang et al., 1996; Munger et al., 1999
β7	V	Deficits in gut-associated lymphocytes—no Peyer's patches, reduced intraepithelial lymphocytes (IEL).	Wagner et al., 1996
β8	E10/P	Two classes: embryonic lethality due to placental defects, perinatal lethality with cerebral vascular defects probably due to neuroepithelial defects. Most blood vessels develop normally.	Zhu et al., 2002

Reference citations are listed but not given in the reference list. They can be found in PubMed or in several extensive reviews, which also discuss the implications of the results as well as work with chimeric mice and recent work using conditional and tissue-specific ablation of integrins (Hynes, 1996; De Arcangelis and Georges-Labouesse, 2000; Sheppard, 2000; Bouvard et al., 2001).

Abbreviations: E, embryonic lethal (day of lethality); P, perinatal lethal; V, viable; F, fertile.

[a,b,c] Human mutations in these genes lead to disease (Hogg and Bates, 2000)

[a] α6β4 Epidermolysis bullosa (JEB-PA)—skin blistering (Pulkkinen and Uitto, 1999)

[b] αIIbβ3 Glanzmann thrombasthenia (GT)—bleeding (Kato, 1997)

[c] β2 Leukocyte adhesion deficiency (LAD)—failure in leukocyte recruitment (Etzioni et al., 1999)

tion sequence, are effective antithrombotic drugs (Coller, 1997; Scarborough and Gretler, 2000). The activation of αIIbβ3 is triggered by thrombin, ADP, or epinephrine, all of which act through G protein-coupled receptors, or by von Willebrand factor signaling through its receptor (GPIb/V/IX), or by collagen signaling through its receptors GPVI and the integrin α2β1. This last is an example of another important general principle, namely that integrins frequently intercommunicate, serving to activate (as in this case) or inhibit each other (Schwartz and Ginsberg, 2002; Hynes, 2002).

Leukocytes offer other examples of the importance

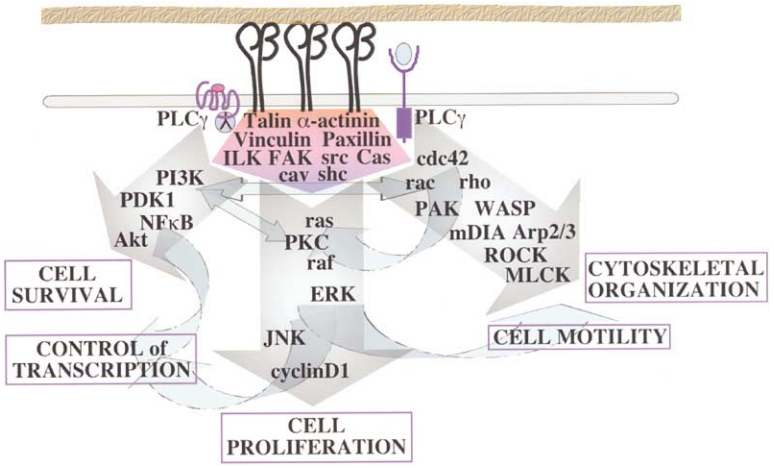

Figure 2. Integrin Signaling

A decade ago, ideas about integrin signaling were in their infancy (Hynes, 1992). It was clear that integrins synergized with other cell surface receptors including growth factor receptors to activate largely unknown signaling pathways to affect cell proliferation and differentiation, cell shape and migration, and other events. These signal transduction mechanisms could be subverted by oncogenes such as pp60^{src} to give anchorage independence of growth. Our current view remains the same in outline but many detailed signal transduction pathways have now been elucidated.

The major signal transduction pathways and many of the key players in them are shown, leading to the major effects on cell behavior mediated by integrins, often acting in concert with G protein-coupled or kinase receptors for soluble factors. The major submembranous, integrin-associated links between integrins and these signal transduction pathways are contained within the pink-purple pentagon beneath the clustered integrins. Details of the interactions of these linker/adaptor proteins and of the signal transduction pathways are omitted, as are other known players in these processes. Readers are referred to several excellent reviews for further details (Clark and Brugge, 1995; Schwartz et al., 1995; Yamada and Miyamoto, 1995; Clark and Hynes, 1997; Giancotti and Ruoslahti, 1999; Danen and Yamada, 2001; Wu and Dedhar, 2001; Schwartz and Ginsberg, 2002; Miranti and Brugge, 2002).

of inactive integrins and their regulated activation. Members of the β2 integrin subfamily (also known as CD11/18) are expressed on most white blood cells but, when these cells are "resting," these integrins become inactive. When the cells become activated, for example by cytokines, the β2 integrins are rapidly activated and the cells become adhesive for their counterreceptors, in this case, Ig superfamily molecules such as ICAMs. These are expressed on endothelial cells, allowing attachment of leukocytes to the vessel wall, or on other cells, allowing phenomena such as phagocytosis, cytotoxic killing, or lymphocyte help. As in the case of platelets, it is important that the β2 integrins are inactive on the surfaces of resting leukocytes (to avoid inflammation) and that they can be rapidly activated (to allow immune function). Defects in either have pathological consequences. Clear support for the importance of these processes comes from the phenotypes of mice lacking one or more of the β2 integrins or their ligands (Table 1; Rosenkranz and Mayadas, 1999) and from the genetic disease leukocyte adhesion deficiency (LAD), which arises from mutations in the gene for β2 integrin. LAD patients suffer from leukocytosis and the failure to recruit leukocytes to sites of infection, leading to early death (Etzioni et al., 1999). In contrast, blockade of β2 integrins, and of α4 integrins, which mediate similar functions on lymphocytes, is a very promising avenue for therapy of a variety of inflammatory and autoimmune diseases (Gottlieb et al., 2000; Jackson, 2002).

While these vascular processes offer particularly clear examples of the importance of inactivation and activation of integrin function, not all integrins have been shown to undergo such extremes of activity. However, it is believed that many, perhaps all, integrins may behave similarly, albeit in a less absolute and more localized fashion, during processes such as cell migration, neurite outgrowth, and so forth, when it is important for cells to regulate their adhesion in a temporal and spatial fashion (Lauffenburger and Horwitz, 1996). This concept of regulation of integrin function from within the cell has commonly been called "inside-out" signaling to distinguish it from "outside-in" signaling, as depicted in Figure 2 (Hynes, 1992; Ginsberg et al., 1992). Both obviously involve transmembrane signals, the nature of which has been difficult to decipher. Major insights come from recent structural information on integrins and from experiments stimulated by and/or reinterpreted in light of the structural results, and we will return later to a discussion of the nature of integrin activation.

Integrin Structure: Extracellular Domains

The first domain of integrins to be crystallized was the I/A domain inserted into half of the mammalian α subunits (Figure 1). Lee et al. (1995a) determined the structure of this domain from αMβ2 (CD11b/CD18, CR3) and showed it to be a Rossmann fold with a core of parallel β sheets surrounded by amphipathic α helices. Within the extended family of Rossmann folds, the integrin I/A domains form a subset of the larger group of VWA domains found in a wide variety of proteins (Tuckwell, 1999; Whittaker and Hynes, 2002). VWA domains are around 180 amino acids long and many appear to be involved in protein-protein interactions. The I/A domains of integrin α subunits comprise the ligand binding sites of these integrins.

Lee et al. (1995a) defined a metal ion coordination site at the "top" of the I/A domain of αM, involving residues from three separate loops of the I/A domain. Interestingly, a glutamate from an adjacent molecule in the crystal formed part of the coordination sphere. It was already well established that integrins require divalent cations for ligand binding and that an aspartate (D) or glutamate (E) residue is key to the integrin recognition site of all ligands (including ICAM-1, a ligand for αMβ2). This had led to the idea that the ligand D/E might participate together with residues from the integrin in joint coordination of a divalent cation. The structure determined by Lee et al. (1995a) fitted this idea very well and they

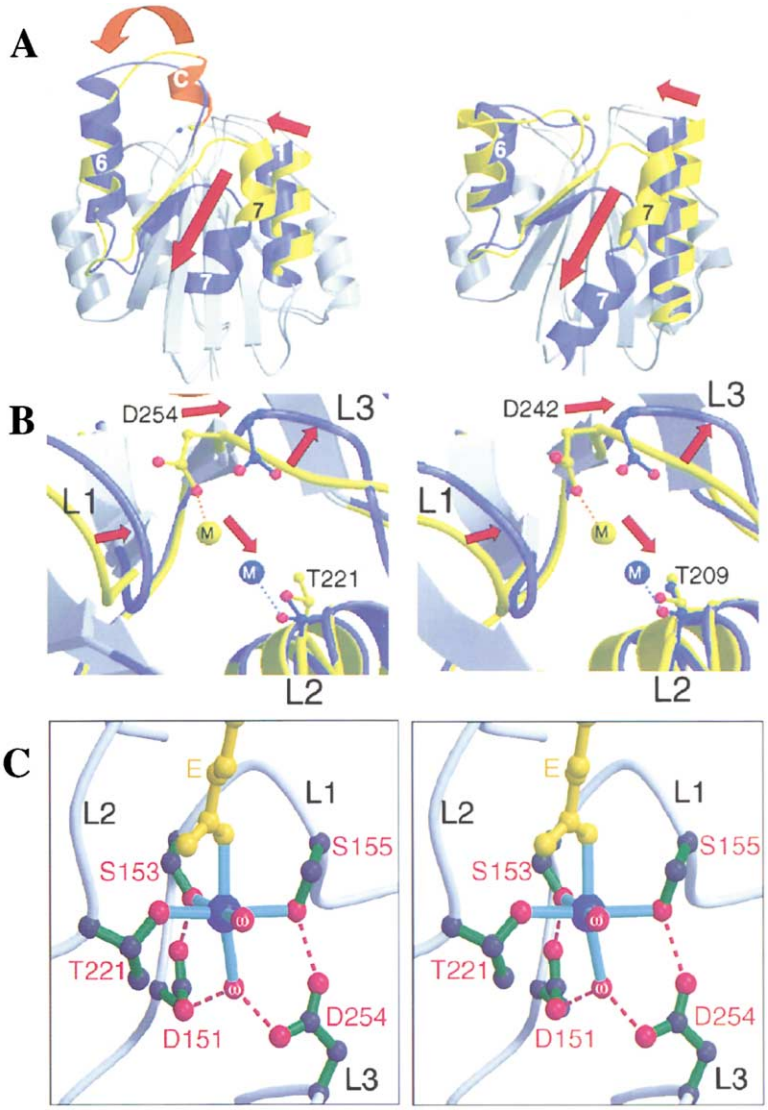

Figure 3. Integrin I/A Domain Structure and Conformational Change

(A) Comparison of I/A domain structures of α2 (left) and αM (right). In each case, regions showing large changes between the two states; open/liganded (blue) and closed/unliganded (yellow) are indicated, and the shifts on ligation are shown by red arrows. Note the shift from the C helix (red; specific to collagen binding I/A domains) into the α6 helix and the large downward shift of the C-terminal α7 helix on binding of ligand to α2. αM shows a very similar downward shift of the C-terminal helix.

(B) Close-up of the movements of the metal ion and loops around the MIDAS site in α2 (left) and αM (right) with color-coding as in (A). Again note the strong similarity in the conformational changes occurring in the two domains. The movement of the loops is coordinated with the movement of the metal ion, which switches its coordination from a D in loop L3 to a T in loop L1. Changes in L1 and L2 lead to the reorganization of αC and α7 shown in (A).

(C) Stereo diagram of the MIDAS motif of α2 with the glutamate residue (E) from the ligand (yellow) coordinating the metal ion (blue). Residues from the loops of the I/A domain coordinate the metal ion either directly or through water molecules (ω). An additional residue (E256 from L3) has been omitted for clarity.

All panels from Emsley et al. (2000).

coined the term metal ion-dependent adhesion site (MIDAS). They also pointed out a homologous segment embedded within the β subunit and sharing hydropathy and secondary structure predictions and a MIDAS motif. This segment of the β3 subunit had already been implicated in ligand binding by crosslinking, genetic, and mutagenesis data (D'Souza et al., 1988; Bajt and Loftus, 1994; Loftus et al., 1994). This prediction was followed up by more elaborate secondary structure predictions (Tozer et al., 1996; Tuckwell and Humphries, 1997; Huang et al., 2000), and refined HMM models now reliably predict a VWA domain within integrin β subunits. These conclusions have been confirmed within the last year by the determination of the structure of the entire extracellular domain of integrin $\alpha v\beta 3$ (see below).

Additional structures of I/A domains followed and it became clear that the domains could take on two conformations, "open" and "closed," differing in the coordination of the metal at the MIDAS site (Lee et al., 1995b; Qu and Leahy, 1995, 1996; Emsley et al., 1997). It was proposed that ligand binding was coupled to a conformational change within the I/A domain and this was elegantly confirmed by the determination of the structure of the I/A domain of α2 with and without a model ligand based on the recognition sequence in collagen (Figure 3; Emsley et al., 1997, 2000). Comparisons among all the I/A domain structures lead to the clear deduction that the ligand does indeed coordinate the metal ion in the MIDAS site via a carboxylate group and this is coupled to alterations in metal coordination by residues within the integrin MIDAS motif. These in turn are coupled to conformational shifts within the domain: lateral movements of the loops containing the MIDAS residues and longer-range movements in the C-terminal helix of the I/A domain, which moves around 10 Å down the side of the domain when ligand binds (Figure 3). Liddington and colleagues (Lee et al., 1995b; Loftus and Liddington, 1997) noted the strong parallels between these conformational changes in I/A domains and those occurring in GTPases such as ras and G proteins, which also contain Rossmann nucleotide binding folds. It is easy to imagine how such conformational changes

could propagate to the rest of the molecule, to which the I/A domain is coupled via its adjacent N and C termini, and we will return later to this important allosteric property of integrins.

Xiong et al. (2000) expressed I/A domains of αM that adopt each of the two forms (open or closed) and showed that only the open form binds ligands. Springer and colleagues have also exploited the structural information to produce I/A domains of αL locked in the open and closed states by disulfide bonds engineered into the C-terminal helix to lock it into the up (closed) or down (open) position and shown that these two forms differ markedly in affinity for ligand (Lu et al., 2001a; Shimaoka et al., 2001, 2002). The open form is high affinity or "active" and the closed form is low affinity or "inactive," and the conformational switch between them is coupled with ligand binding or with known activation stimuli such as activating antibodies or Mn^{2+} ions.

Half the mammalian α subunits and all known nonchordate integrins lack an inserted I/A domain (Figure 1), but it is clear that these α subunits also contribute ligand binding specificity. How do they do that? Springer (1997) predicted that the 7-fold repeat in the extracellular domain of all α subunits folds into a 7-bladed β propeller like that in the β subunit of G proteins (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996) and predicted that this might complex with the I/A domain embedded within the integrin β subunit by analogy with the Gα/Gβ complex in G proteins. This prediction has also been confirmed by the αvβ3 structure.

The solution by Arnaout and colleagues of the crystal structure of the extracellular domain of αvβ3 (Xiong et al., 2001) represents a truly major advance in the integrin field. In addition to confirming the predictions of an I/A domain within the β subunit and of a β-propeller domain within the α subunit in an association very like that of Gα and Gβγ, it revealed the structure of much of the rest of the extracellular domains of both subunits (Figure 4; Xiong et al., 2001, 2002). The propeller domain and the β-I/A domain are complexed to form the ligand binding head of the integrin, which is attached to two legs, one from each subunit, as predicted from a large body of electron microscopic, biophysical, and other data. The N-terminal propeller domain of the α subunit is attached to an elongated leg formed of three β sandwich domains termed thigh, calf1, and calf2. The β subunit domain organization is a bit more complex; although the β-I/A domain is at the distal end of the molecule (furthest from the C-terminal membrane insertion site), it is not at the N terminus of the primary sequence. Instead, it is inserted into a loop in a so-called hybrid domain, another β sandwich domain with some homology with I-set Ig domains. The hybrid-I/A domain unit is preceded in the sequence by an N-terminal 54-residue PSI domain, which in the 3D structure lies below the hybrid-I/A domain "head" and is disulfide bonded to the distal end of the β subunit leg. This leg is made up of four tandem cystine-rich repeats highly characteristic of integrin β subunits. The first and second are poorly resolved in the crystal, but the third and fourth are clearly folded into EGF-like folds. An NMR structure of the second and third cystine-rich repeats of β2 (Beglova et al., 2002) confirms their EGF-like pattern including an extra fourth cystine pair characteristic of these repeats, which I will refer to as I-EGF repeats. The four I-EGF repeats are followed by a C-terminal disulfide-bonded β sheet domain termed the β-tail domain.

As mentioned, this structure confirmed many predictions and conformed with much preexisting data concerning integrin structure (see Humphries, 2000, 2002; Shimaoka et al., 2002, for relevant reviews relating earlier data to the structure). The big surprise was that, instead of being extended as depicted in Figure 4B and as expected from published EM images of integrins, the αvβ3 integrin in the crystal structure was bent over at a 135° angle with a "genu" between the thigh and calf domains of αv and a similar bend in the I-EGF 2/3 region of the β3 leg (Figure 4A). This surprising structure raises very interesting questions and has already stimulated experiments to which I will return below.

The structure determined by Xiong et al. (2001) was obtained in a Ca^{2+} buffer and lacked bound ligand, conditions usually yielding inactive integrins. The MIDAS motif did not have a clear cation engaged, although an adjacent site (ADMIDAS) did and other cations bind at other sites within both subunits. Subsequent structures obtained after diffusing cycloRGDF and Mn^{2+} into the crystal showed cycloRGDF bound at the αβ interface with the arginine residue binding the propeller domain of the α subunit and the aspartate joining the coordination sphere of a Mn^{2+} ion bound at the MIDAS site (Figure 4C; Xiong et al., 2002). Changes occurred in the loops at the top of the I/A domains, similar to those seen in α-I/A domains, but the 10 Å shift in the C-terminal helix characteristic of ligand bound I/A domains from α subunits was not observed in the β3 I/A domain. Several possibilities have been suggested: (1) the β3 I/A domain is constitutively active, even in the absence of ligand (Xiong et al., 2001, 2002), (2) the lattice contacts in the crystal prevent the full conformational change and activation (Liddington, 2002), or (3) activation of the I/A domain in β subunits occurs somewhat differently (Mould et al., 2002; Liddington, 2002). Mould et al. (2002) report an activation-dependent antibody that binds the α1 helix at the base of the β-I/A domain near the contact with the hybrid domain. Many function-blocking and -activating antibodies bind the α1 and α2 helices in this part of the β-I/A domain (Takada and Puzon, 1993), also suggesting a propagated conformational change in this region not seen in the cycloRGDF-αvβ3 crystal.

Much of the top surface of the propeller is occluded by the apposed β-I/A domain in the crystal structure (Xiong et al., 2001), including residues known to be involved in interactions with ligands and to contain epitopes for blocking antibodies against several integrins (Humphries, 2000, 2002). It has been known for a long time that RGD peptides and small ligands can bind integrins that are not fully activated, whereas larger ligands such as fibrinogen and fibronectin cannot (Coller, 1986; Beer et al., 1992). Mould et al. (1997) showed that the RGD of fibronectin interacts with the β-I/A domain, whereas the synergy site in the adjacent Fn3 repeat interacts with the propeller domain. Dual interaction of these two sites appears to be necessary for strong binding of α5β1 integrin to fibronectin (Garcia et al., 2002). These data suggest that a fully active ligand-engaged integrin must undergo some opening up at the interface between the β-I/A domain and the propeller domain.

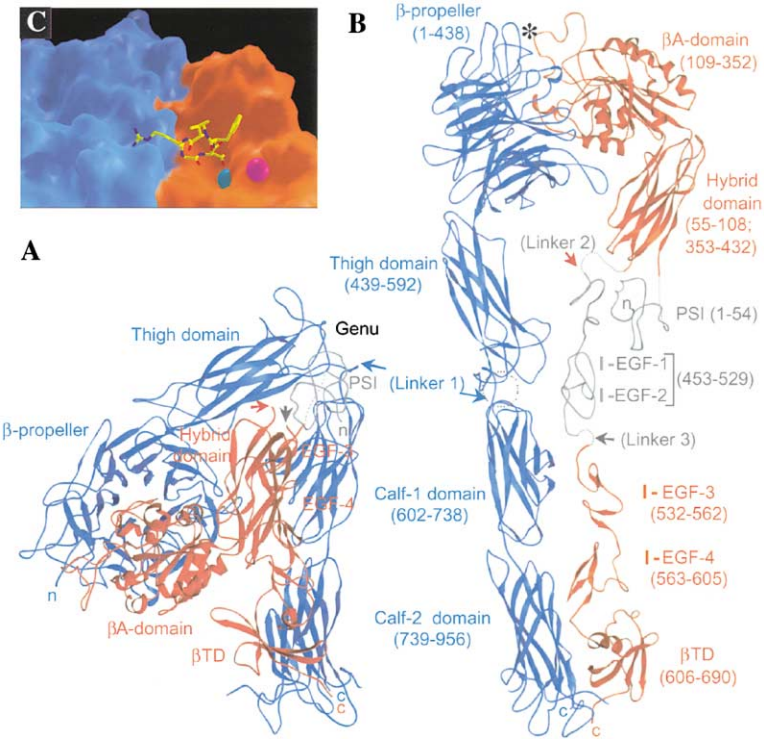

Figure 4. Three-Dimensional Structure of the Integrin αvβ3

(A) The structure of the unliganded αvβ3 is shown as a ribbon diagram with the αv subunit in blue and the β3 subunit in red. In the crystal the integrin is folded over at a bend or "genu," with the head (propeller, β-I/A, and hybrid domains) bent over toward the C termini of the legs which would be inserted into the membrane in an intact integrin. The domains are hard to see in this view and are more readily visualized in (B).

(B) The structure in (A) has been unfolded by straightening it out at the "genu" of the αv subunit by 135° and rotating the thigh 120° around its axis, with similar adjustments to the β3 structure. The structures of the linker segments (1 in the αv, 2 and 3 in the β3) and of the PSI domain and I-EGF repeats 1 and 2 are not well resolved and are approximate estimates only. The structure reveals two legs (~160 Å × ~20 Å) extending from the membrane insertion site at the C termini to the head at the top. The head is ~90 Å × 60 Å × 45 Å and comprises three domains: a β propeller domain at the N terminus of the αv subunit and an I/A domain inserted into a loop on the top of the hybrid domain in the β subunit. The N-terminal PSI domain is curled in below the hybrid domain and is known to be linked by a disulfide bond to the I-EGF-1 repeat, although this connection is not resolved in the crystal structure. The apposition of the propeller and I/A domains is highly similar to that of G proteins. A 3_{10} helix from the I/A domain reaches out to the propeller and inserts an arginine residue into the central channel of the propeller. This arrangement is very similar to the arrangement of a lysine in the α2 helix of the switch II region of Gα inserted into the propeller domain of Gβ. The asterisk marks the loop into which I/A domains are inserted in some integrin α subunits, although not αv.

(C) Surface representation of the cyclo RGDF peptide bound to the interface between the α subunit propeller (blue) and the β subunit I/A domain (red). The aspartate (D) of the ligand coordinates a Mn^{2+} ion (cyan) and the arginine (R) binds to aspartate residues in loops on top of the propeller. The second Mn^{2+} ion (violet) is in the ADMIDAS site.

(A) and (B) are from Xiong et al. (2001); (C) is from Xiong et al. (2002).

This would resemble the separation of the homologous Gα and Gβ domains in activated G proteins and seems a very reasonable working hypothesis for integrins (Liddington, 2002; Liddington and Ginsberg, 2002). Such a model receives some support from EM images of integrins in the presence of ligand peptides. Hantgan et al. (1999) report some separation of the α and β heads of αIIbβ3 in the presence of RGD peptides and Takagi et al. (2002) detect changes in the relationship between the head and the hybrid domain of αvβ3 as a consequence of RGD binding. Since the C-terminal helix of the β-I/A domain connects to the hybrid domain, if it were to undergo a downward shift like that shown by the corresponding helix in α-I/A domains, that would necessarily be coupled to changes in β-I/A-hybrid domain organization that could well include rotation away from contact with the propeller domain, opening it up for further interactions with ligands (Figure 5).

The αvβ3 integrin lacks an α-I/A domain, but the site of insertion of I/A domains in those α subunits that have one falls between blades 2 and 3 of the propeller domain, and this position is marked in Figures 4 and 5. Since α-I/A domains contain the ligand binding sites of the corresponding integrins, we need to consider how ligand binding may differ between the two classes of integrin, those with and without α-I/A domains. We will return to consider further models for ligand binding and activation of the extracellular domains after we have reviewed recent data on the cytoplasmic domains of integrins to which events at the ligand binding sites must be coupled.

Cytoplasmic Domains: Structures and Interactions

Despite the fact that integrins' cytoplasmic domains are much smaller than their extracellular domains (generally less than 50 amino acids) they play a vital role in integrin functions and have been the subject of intensive analysis. Paradoxically we have a less clear picture of their 3D structure than we do for the large extracellular domains, although recent work has produced some major insights.

The cytoplasmic domains are the sites of interaction with, and linkage to, the cytoskeletal and signaling partners of integrins (see Figure 2). There is an extensive literature on the many proteins that have been reported to interact with α or β cytoplasmic domains but I will not attempt to review most of that work (see Burridge and Chrzanowska-Wodnicka, 1996; Critchley et al., 1999; Calderwood et al., 2000; Zamir and Geiger, 2001, for reviews). For our present considerations, it is most relevant to consider data that indicate that integrin cytoplasmic domains can regulate the activation state of integrins; that is, affect the structure and function of the

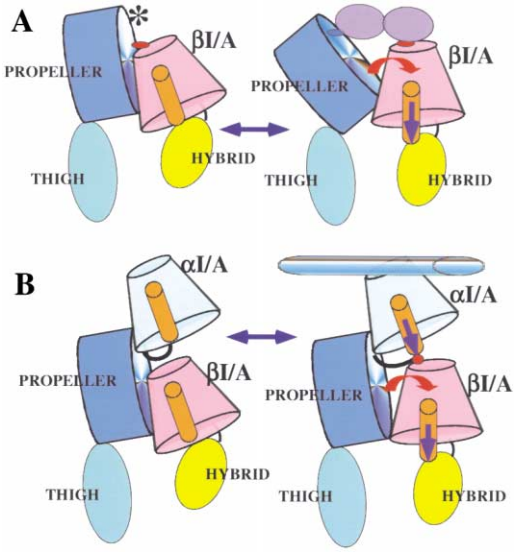

Figure 5. Hypothetical Models for Ligand binding to Integrin Heads

(A) An integrin without an I/A domain in the α subunit, such as αvβ3 (note; only the head region is shown). A small ligand such as cyclo RGDF binds at the interface between the propeller and the β-I/A domain (see Figure 4). The model proposes that the C-terminal helix (orange) moves down, causing the β-I/A domain (pink) to rotate away from the propeller domain opening up the top of the propeller to engage larger ligands such as fibronectin (lilac). It is known that the RGD motif in fibronectin engages the β-I/A domain while the synergy site in the adjacent Fn3 domain engages the propeller (Mould et al., 1997), consistent with this model, although the degree of opening shown is hypothetical and could easily vary among integrins.

(B) An integrin with an α-I/A domain such as α2β1. The ligand, collagen, binds to the top of the α-I/A domain (pale blue) causing a 10 Å downward shift of the C-terminal helix (Figure 3), which is attached to an extended loop containing a conserved glutamate (red dot). It is proposed that this could bind to the MIDAS site in the β-I/A domain (Alonso et al., 2002) and act upon it as a ligand relay. The β-I/A domain is proposed to transmit conformational change to the hybrid domain as in (A). Springer and colleagues (Shimaoka et al., 2002) have concentrated on inside-out activation of β2 integrins and thus have focused on how the α-I/A domains become activated. They have suggested that the C-terminal helix acts like a bell rope to pull open the I/A domain. This is the reciprocal of the ligand-relay model. The change is an allosteric one and the equilibrium can be driven from either end.

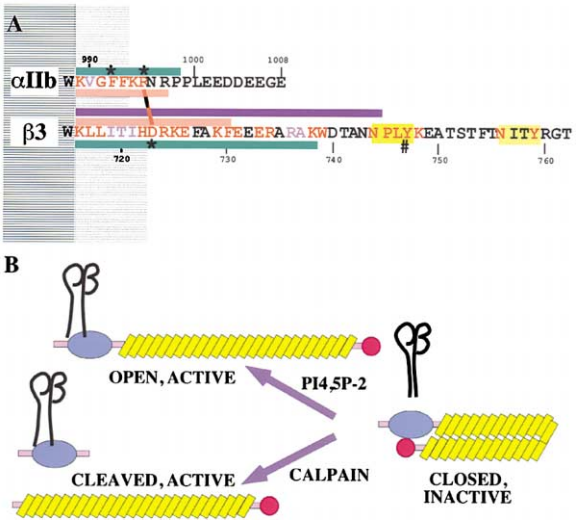

Figure 6. Interactions between and with Integrin Cytoplasmic Domains

(A) Sequences of the cytoplasmic tails of αIIb and β3. The membrane-spanning segment is usually considered to end at the W within the darker gray shaded area (lipid bilayer). The immediately membrane-proximal segments are highly conserved (red denotes conservation in the vast majority of subunits, lilac denotes conservation in more than half). Conserved NxxY motifs are highlighted in yellow. Deletion of the conserved membrane-proximal segment from either subunit leads to activation, as do point mutations marked by asterisks (see text). The proposed salt bridge between R995 and D723 is marked by a red bar (Hughes et al., 1996). The pink bars denote regions showing interaction between subunits and the green bars denote α-helical segments, both deduced from NMR data (Vinogradova et al., 2002). The purple bar denotes segment of β3 showing interaction with talin head by NMR (Vinogradova et al., 2002) consistent with cell biological results (Calderwood et al., 1999, 2002; Patil et al., 1999). Talin binding also requires Y747 (hatch mark). Since the affinity of talin head for β3 tail is much higher than that between the two tails, binding of talin undoes the clasp between the cytoplasmic domains in the same way as mutations in the membrane-proximal region (asterisks). Armulik et al. (1999) report that the conserved membrane-proximal segments can be buried in the lipid bilayer (lighter gray shading). If so, then the transmembrane segments are atypically long (28–30 residues) and Armulik et al. suggest that interactions with cytoplasmic proteins could pull the conserved segments out of the bilayer, offering an alternative or additional way in which binding of proteins such as talin could alter integrin conformation leading to activation (see also Figure 7B).

(B) Talin can be activated for binding to β tails by cleavage (Yan et al., 2001) to release the FERM domain-containing head (blue) or by interaction with PIP2 (Martel et al., 2001). In each case, the talin head binds the β cytoplasmic domain leading to separation of the tails (see [A] and text). Intact talin does not interact with integrin tails and is depicted as folded upon itself with the head domain occluded by the tail of talin, by analogy with ERM proteins (Pearson et al., 2000), although the tertiary structure of talin is unknown. The talin tail comprises a series of short α-helical segments (yellow) and an actin binding domain (red).

extracellular domains. There is a considerable body of data indicating that the cytoplasmic domains of the α and β subunits can interact to control the activation states of integrins. These analyses have proceeded furthest for the platelet integrin, αIIbβ3, which as discussed earlier is tightly regulated so that it is inactive on resting platelets but rapidly activated by thrombogenic stimuli. Ginsberg and colleagues have investigated the roles of the αIIb and β3 cytoplasmic domains in this regulation. They have shown that the short αIIb cytoplasmic domain acts as a negative regulator of activation. Deletion of the entire domain (see Figure 6A) or of just the highly conserved GFFKR sequence produces a constitutively active integrin (O'Toole et al., 1991, 1994). Similarly, the conserved membrane-proximal segment of β3 is also necessary (Hughes et al., 1995). They proposed that R995 of αIIb forms a salt bridge with D723 of β3; mutation of either one to alanine yields a constitutively active integrin, whereas a charge reversal, αIIbR995D/β3D723R, restored the inactive state (Hughes et al., 1996). Based on these and other results, Ginsberg and colleagues suggested several models, all relying on interaction between the membrane-proximal segments of αIIb and β3 to restrain the integrin in an inactive state (Williams et al., 1994; Woodside et al., 2001). Separation, twisting,

pistoning, and hinging of the tails were all considered as mechanisms to allow activation. More recent data favor models involving separation of the cytoplasmic domains as a key step in integrin activation. Evidence comes from recent NMR analyses and from cell biological studies.

Binding between the cytoplasmic domains of αIIb and β3 could be detected by surface plasmon resonance and was ablated by deletion of KVGFFKR or by an R995A mutation (Vallar et al., 1999). The affinity was low (K_d = 7–50 μM depending on divalent cation concentration), which may explain why initial efforts to determine structures of the interacting domains were largely unsuccessful (Ulmer et al., 2001; Li et al., 2001). However, Weljie et al. (2002) detected α-helical structure and intersubunit interactions using synthetic peptides representing the membrane-proximal segments. Vinogradova et al. (2002) demonstrated interactions between membrane-proximal helices in both subunits, using the entire cytoplasmic domains, and also demonstrated that they were disrupted by point mutations (F992A or R995D) already known to interfere with inactivation by αIIb cytoplasmic domain in the intact integrin (see earlier discussion). These data are summarized in Figure 6A, which also collects together information from a different, complementary set of experiments.

Calderwood et al. (1999, 2002) showed that the head domain of talin binds to the cytoplasmic domains of β3 and other β subunits via a PTB domain within the conserved FERM domain of talin; Y747 of β3 is necessary for this interaction. The NPLY motif is believed to form a β turn, and NMR data on β3 cytoplasmic domain support this idea (Ulmer et al., 2001). Vinogradova et al. (2002) therefore analyzed the effects of talin head on the NMR signals of the αIIb and β3 cytoplasmic domains; talin head bound to β3 but not to αIIb. The interactions extended from K716 to N744, completely overlapping the region of β3 interaction with αIIb (see Figure 6A). Furthermore, talin head ablated the interaction between the αIIb and β3 tails (Vinogradova et al., 2002), consistent with its much higher affinity for β3 tail (K_d ~100 nM; Calderwood et al., 1999, 2002). Thus, the head of talin binds to the β3 tail and separates it from the αIIb tail.

Talin head was also shown to bind to and activate integrins (Calderwood et al., 1999, 2002), entirely consistent with the model that interactions between αIIb tail and β3 tail keep the integrin in an inactive state and separation is necessary for activation (see Figure 6B). In order for talin's head domain to trigger this activation, it must be exposed. This can be accomplished by expressing recombinant fragments of talin (Calderwood et al., 1999, 2002; Patil et al., 1999), by calpain cleavage, which separates the head from the tail (Yan et al., 2001), or by phosphatidyl inositols (Martel et al., 2001) as depicted in Figure 6B. The mapping of the interaction to a PTB domain within the talin head (Calderwood et al., 2002), which binds to the NPxY motif conserved in β3 and in most other integrin β subunits, raises the very interesting possibility that other PTB-containing proteins may also interact with β tails leading to activation of integrins (Liddington and Ginsberg, 2002). Among candidates for such a role is FAK, which like talin has a FERM domain containing a PTB domain. As mentioned earlier, multiple proteins have been reported to bind to integrin tails, most often those of β subunits. Others of these could act similarly to talin head or, alternatively, could bind elsewhere in the tail, such as the distal portion of β tail, which does not appear to interact with the α tail (Figure 6A).

Integrin Activation: Transmembrane Connections and Long-Range Conformational Changes

If activation of integrins by inside-out signaling involves separation of the α and β cytoplasmic tails, how is that signal transmitted to the ligand binding site(s) 10–20 nm away at the far end of the extracellular domain? Recent results are beginning to reveal possible mechanisms, despite the fact that there is not a structure for an intact integrin, only for the separate intracellular and extracellular domains.

β2 integrins, like αIIbβ3, are dependent on their membrane-proximal cytoplasmic domains to maintain an inactive state; deletion of either α or β segments yields active integrins (Lu et al., 2001b). Furthermore, replacement of the αL and β2 tails by, respectively, acidic and basic coiled-coil domains restored the inactive state. This is analogous to the charge-reversal experiment with αIIbβ3 and confirms that αβ tail associations also restrain β2 integrins in an inactive state. To take the analysis further, Takagi et al. (2001) eliminated both the tails and the transmembrane domains from α5β1 and replaced them with acidic and basic coiled coils joined by a disulfide bond. This generated a soluble α5β1 dimer. As predicted, this clamped, soluble α5β1 did not bind its ligand, fibronectin, but it could be activated by cleaving the C-terminal clamp; that is, by allowing the α and β stalks (legs) to separate, which was confirmed by EM. This experiment shows that the C-terminal cytoplasmic domain clasp or the engineered C-terminal clamp, whether inside or outside the membrane, constrain integrins in an inactive state but release of these constraints, allowing separation of the stalks/legs of the extracellular domains, leads to activation of the ligand binding site in the head.

The idea that conformational changes in the extracellular domain near the membrane can be linked to changes in the ligand binding domain in the head of integrins is far from a new one. A decade ago Weisel et al. (1992) demonstrated that αIIbβ3 bound to fibrinogen tends to show widely separated tails. This is effectively the reciprocal result of the experiment of Takagi et al. (2001) with α5β1 and fibronectin. In another early experiment, Du et al. (1993) showed cooperative activation between the binding of fibrinogen to the head of the αIIbβ3 integrin and binding of a monoclonal antibody to the first 90 amino acids of the β3 stalk adjacent to the membrane. The distance between these two sites as revealed by EM was 16 nm. The antibody had originally been isolated as recognizing a ligand-induced binding site (anti-LIBS) and its binding was enhanced by fibrinogen binding to the head of the integrin. Importantly, binding of the antibody also enhanced the affinity of the integrin for fibrinogen, i.e., the activation was *reciprocal*.

So we now have a picture of long-range conformational changes linking the C-terminal ends of an integrin's legs, i.e., the membrane-proximal regions both outside and inside the membrane, to ligand binding at the

head. There is, in fact, a great deal of evidence in support of this concept, including many activating and activation-sensitive monoclonal antibodies that frequently map in the β stalk regions (reviewed in Humphries, 2000, 2002; Shimaoka et al., 2002), as well as biophysical data (e.g., Hantgan et al., 1999, and earlier work) and the electron microscopy already mentioned. The challenge is to understand how conformational changes in the head domains associated with ligand binding are coupled reciprocally with alterations, probably separation, at the base of the legs and in the cytoplasm. How can we fit these results with the newly available structural data? The structure offers some potential solutions but also the complication represented by the bent structure observed in the crystal (Figure 4A).

Xiong et al. (2001, 2002) suggested that the bent form is the active form of the integrin. However, others have argued that it is more likely to be the inactive state, based on details of the conformation of the β-I/A domain and the fact that it was crystallized in the absence of ligand (Liddington, 2002; Liddington and Ginsberg, 2002; Shimaoka et al., 2002). The latter interpretation would fit much better with EM images of ligand bound integrins (Weisel et al., 1992; Du et al., 1993), which show an extended structure like that shown in Figure 4B. Beglova et al. (2002) mapped epitopes for activation-specific monoclonal antibodies to specific residues in I-EGF repeats 2 and 3 and noted that these residues would be buried in the bent form of the integrin. They proposed, therefore, that the bent form represents the inactive state and that activation occurs by a "switch-blade" opening of the integrin into an extended shape and a separation of the legs. Such a conformational change could expose the epitopes for activation-specific antibodies, many of which are known to bind to the I-EGF repeats or to the PSI domain, which is also buried in the genu (the structure is not well resolved there). Takagi et al. (2002) went on to show that integrins clamped in the inactive state predominantly adopt a bent shape as seen by EM, whereas integrins activated by Mn^{2+} or by cyclo RGDfV were predominantly in an extended form. They showed that the clamped, bent form did not bind ligand, whereas the activated, extended form did. Finally they presented evidence that integrins on cell surfaces can be trapped in a bent and inactive state by an engineered disulfide bond that, when released, allows their activation. These results conform well with the idea that the bent form seen in the crystal represents the inactive state of the integrin and that activation comprises straightening and separation of the legs. This is, of course, also in good agreement with the data on cytoplasmic domain separation (Figure 6, see prior discussion). These concepts are schematized in Figure 7, which shows two ways in which the bent form might be related to the membrane. These differ in the orientation of the membrane-proximal "ankles" of the legs relative to the membrane; this is of course unknown at present. In the switch-blade (Beglova et al. 2002; Shimaoka et al., 2002) or "flick-knife" (Liddington, 2002) model, the "calves" of each leg are perpendicular to the membrane and the head domain is very close to the cell surface. In a variant "angle-poise" model, the legs are bent over closer to the membrane and extend like an angle-poise lamp during activation. The latter model would place the head domain in a better position to interact with macromolecular ligands. There are currently no data available to distinguish between these two possibilities.

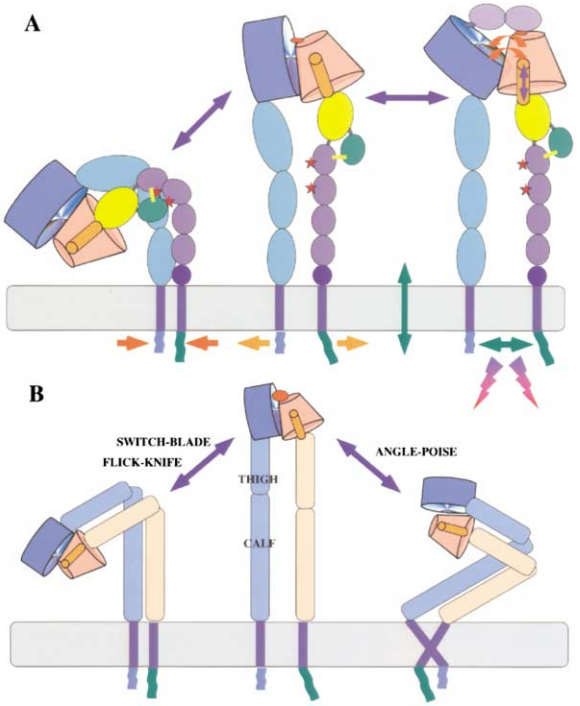

Figure 7. Models for Long-Range Allosteric Changes Giving Bidirectional Signaling by Integrins

(A) Integrin in its bent form is presumed to be inactive. Activation can occur either by ligand binding or by effects on the cytoplasmic domains, leading to straightening and separation of the legs. Alterations in the orientation of the propeller and I/A domains are coupled to changes in the hybrid domain (yellow) by movement of the C-terminal helix of the I/A domain (orange). The hybrid domain, in turn, is linked to the I-EGF domains (purple) via the PSI domain (green), which is disulfide bonded (yellow line) to the first I-EGF domain. Straightening and separation of the legs exposes activation epitopes in the I-EGF domains (red stars) and in the PSI domain (not shown). Separation of the cytoplasmic domains is accompanied by conformational changes in them, allowing binding of cytoplasmic proteins (see Figure 2) and signaling (lightning). All changes are reversible equilibria and can operate in either direction, allowing both outside-in and inside-out signaling. See text for discussion and references.

(B) Two models for the proposed straightening up of integrins during activation. The switch-blade or flick-knife model (Beglova et al., 2002) and an alternative angle-poise model differ in the way in which the C termini of the legs relate to the transmembrane segments (which is unknown). The angle-poise model incorporates the possibility that the transmembrane helices may be especially long and could change orientation and/or move in and out of the membrane during activation (Armulik et al., 1999; see Figure 6A). The angle-poise model would place the ligand binding site in a more accessible position for macromolecular ligands.

Thus, the preponderance of the evidence strongly favors models in which activation of the ligand binding domain in the head and binding of ligand are coupled, via long-range conformational changes in the legs (probably including straightening and separation), to separation of the bases of the legs and the attached transmembrane and cytoplasmic domains. This cou-

pling is bidirectional and reciprocal and is best viewed in terms of an allosteric equilibrium, or series of equilibria (Figure 7). Binding of extracellular ligand would therefore enhance separation of the cytoplasmic domains, allowing their interaction with cytoskeletal and signal transduction molecules, that is, outside-in signaling (Figure 2). Reciprocally, separation of the cytoplasmic domains by talin and perhaps other activators would activate the head for ligand binding, that is, inside-out signaling (Figure 6). The distinction between these two forms of integrin signaling has been conceptually useful over the past decade, but they should actually be viewed as two reflections of the same allosteric equilibrium. Either cytoplasmic or extracellular interactions can trap the equilibrium in the active state, enhancing thereby the function at the opposite end of the integrin. One could also imagine cytoplasmic interactors that could trap the equilibrium in the inactive state, stabilizing the integrin in the "off" state as on resting platelets or leukocytes. Similarly, antibodies that activate integrins or recognize only the active state presumably trap the equilibrium in the active state, and some function-blocking antibodies are also known to act allosterically rather than at the actual ligand binding site and presumably act by trapping the equilibrium in the inactive state; I will discuss an example of this below.

Figure 7 depicts an integrin lacking an α-I/A domain. We need to consider how the situation might differ for those integrins with an inserted α-I/A domain. In these integrins, ligand is wholly or largely bound by the α-I/A domain. As discussed earlier, recombinant α-I/A domains can bind ligand with the same affinity and specificity as intact integrins, especially when locked in the active conformation. Many inactivating mutations and epitopes for function-blocking antibodies lie in the α-I/A domains and deletion of these domains inactivates the integrins. As discussed above, the active conformation of α-I/A domains shows a downward shift of the C-terminal helix (Figure 3). This could clearly propagate a conformational change to other domains of the integrin. Alonso et al. (2002) have suggested that a highly conserved glutamate just C-terminal to the C-terminal helix could act as a pseudoligand for the β-I/A MIDAS site, acting as a ligand relay (Figure 5B). They present mutagenesis data in support of this model, although it, like all the other models discussed here, will need further confirmation. Consistent with the model is the fact that there exist both mutations in, and function-blocking antibodies against, the β-I/A domain that preclude binding of ligand at the α-I/A domain unless the α-I/A domain is locked in the open position when it becomes immune to such inhibition (Lu et al., 2001c). Based on these and other results, Shimaoka et al. (2002) proposed that the α-I/A domain is activated by allosteric interactions with the β-I/A domain and proposed that the C-terminal helix acts like a bell rope to open the α-I/A domain. This is entirely compatible with the ligand-relay model (Figure 5). Thus, it appears likely that integrins containing α-I/A domains function in essentially the same way as those which lack that extra domain, differing only in that there is an extra step in the chain of linked conformational changes connecting the ligand binding site with the cytoplasmic domains.

Open Questions and Future Directions

Although the models presented (Figures 5–7) are consistent with a broad range of data, including the 3D structures, they remain working hypotheses and require experimental tests. Foremost among these is the pressing need for integrin/ligand cocrystals to investigate the conformation of ligand bound integrins. Does an activated integrin actually stand up as implied by Figures 4B and 7? Do the legs separate? What exactly are the conformational changes in the β-I/A domain and the domains in the legs? Much immunological evidence demonstrates the existence of conformational changes in these domains, but what exactly are they? In fact, we lack any definitive structures for the PSI domain and several of the I-EGF domains. There are inconsistencies between the disulfide bonding patterns for I-EGF-3 deduced from the X-ray crystallography (Xiong et al., 2001) and by NMR (Beglova et al., 2002). Could these perhaps reflect possible disulfide interchanges within the integrin, as has been suggested (Yan and Smith, 2000, 2001; O'Neill et al., 2000)? What is the significance of the other divalent cations bound to integrins? Does the β-I/A domain change conformation in the same way as the α-I/A domain? Does its C-terminal helix move down, altering the relationship between the β-I/A and hybrid domains? Does the β-I/A domain rotate away from the propeller, opening up the top of the integrin for more extensive interactions with macromolecular ligands? Are there intermediate, stable conformers and different activation states, as suggested by some data? Do all integrins undergo extreme changes in conformation or are some more subtle in their approach? Is there linkage between α-I/A and β-I/A domains as suggested in Figure 5B? Why do some integrins have α-I/A domains, anyway?

Although the current data favor the model that the inactive state of integrins is a bent form as seen in the X-ray structure (or something very like it), this requires further investigation and its generality needs testing. The results of Takagi et al. (2002) clearly show that soluble β3 integrins and those on the cell surface do adopt a bent, inactive form, which can be induced to extend by appropriate manipulations, However, it would be helpful to be able to monitor this process in living cells, perhaps using conformation-specific antibodies or FRET. We also do not know exactly how the bent extracellular part of an integrin is connected to the membrane. What is the significance of the fact that most integrins lacking an α-I/A domain are cleaved near the base of their α subunit legs (in the calf2 domain), whereas none of those with an α-I/A domain is so cleaved? We know essentially nothing about the transmembrane domain structures and their interactions. They are always assumed to be helical but are they and, if so, how long are they? The results of Armulik et al. (1999) suggest that the TM segments may be longer than necessary for a straight, perpendicular α helix and could even move in and out of the membrane to some degree. There are intriguing conservations in primary sequence among integrin TM domains; what do they mean? Do the α and β TM segments interact? Do other integral membrane proteins known to interact with integrins perhaps interact within the membrane? Could this affect activation and signaling? Possible candidates for such interactions and effects include IAP/CD47 (Brown and Frazier, 2001), tetraspa-

nins (Hemler, 2001), CD98 (Fenczik et al., 1997; Kolesnikova et al., 2001), and others (Hemler, 1998; Hughes and Pfaff, 1998), importantly including the growth factor receptors with which integrins cooperate in signal transduction (Figure 2).

The role of cytoplasmic domain separation in integrin signaling seems well established, but we need to know more about exactly what happens. What are the precise structures of the cytoplasmic domains in the inactive and active states? If the talin head PTB domain binds β tails and activates integrins, as seems clear, do other proteins with PTB domains do the same or do some of those proteins only bind a previously activated tail? Do some such proteins prefer a phosphorylated NPxY sequence? There are typically two NPxY sequences in β tails. Do both work analogously? Do different proteins bind different ones? What about the many other proteins that have been reported to bind to integrin cytoplasmic domains? It seems likely that, like anti-integrin antibodies, there will be activating, activation-specific, and inhibitory interactors among them. Plausible models exist for activation of talin to allow it to bind to β tails (Figure 6), but how is that controlled? Evidence exists for small GTPases as intermediates in pathways leading to integrin activation (Zhang et al., 1996; Hughes et al., 1997; Schoenwaelder and Burridge, 1999; Katagiri et al., 2000; Bos et al., 2001; Bertoni et al., 2002). Phosphatidyl inositols are also likely to be significant, since they activate many of the proteins that might interact with integrin tails (talin, vinculin, ERM proteins) and many of the proteins are phosphorylated, so they could be regulated by kinases and phosphatases. Somewhere in this network of regulators must be the mechanisms by which integrins regulate each other.

We should also not forget that active integrins typically cluster in the plane of the membrane, and this "avidity modulation" of cell adhesion has long been a competitive model (Bazzoni and Hemler, 1998) with the "affinity modulation" models that I have reviewed here. Although it is clear that affinity modulation of integrins plays a central role in regulating their functions, that certainly does not exclude clustering from also making major contributions; the two are not mutually exclusive and usually occur in concert (Hato et al., 1998). Could the conformational changes intrinsic to the allosteric, bidirectional control of integrins' affinities and signaling also regulate their ability to cluster? Clustering could be via integrin-integrin interactions, regulated interactions with integrin-associated proteins, altered associations with lipid domains in the membrane, or contributions of any or all of these, not to mention the well-established cytoskeletal interactions of integrins (Schoenwaelder and Burridge, 1999). There are hints in the literature about all of these possibilities; progress would be greatly enhanced by a better understanding of the transmembrane domains of integrins.

It is clear that these fascinating and important receptors have many secrets yet to be discovered. The structural information has made sense of a lot of prior data and offered possible answers to some long-standing questions about integrin functions. It has also raised or, rather, refocused many additional questions and models, a few of which I have touched upon. We can expect that the rapid advances in understanding these receptors will continue in the next few years as these questions and others are addressed, incorporating structural information along with the cell biological data and new techniques such as proteomic analysis of complexes and real-time imaging of molecules and their interactions in situ. One hope is that the insights obtained will lead to better therapeutic agents targeting integrins in human diseases as diverse as thrombosis, hemorrhage, inflammation, atherosclerosis, osteoporosis, cancer, and infectious diseases. It is, after all, the biological importance of these receptors that makes them particularly interesting; the elegance of their allosteric signal transduction mechanisms is an extra bonus.

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