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COMMENTARY

Getting integrins into shape: recent insights into how integrin activity is regulated by conformational changes

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INTRODUCTION

Integrins are a large family of cell-adhesion receptors, with a conserved αβ heterodimeric structure (Hynes, 1992). They play crucial roles in normal physiological processes, such as development, wound healing and immune system function. Integrins also participate in the pathogenesis of a wide variety of diseases, making them attractive targets for therapeutic intervention (Horton, 1996).

Over the past decade, a great deal of effort has been directed towards understanding the molecular mechanisms of integrin-ligand interactions. However, while integrin recognition sequences in many of their ligands are now well defined, we currently only have a preliminary understanding of the nature and location of ligand binding sites within integrins (Newham and Humphries, 1996). It has also been known for several years that the affinity of integrin-mediated cell adhesion can be dynamically regulated (Hynes, 1992); however the molecular basis of these changes has yet to be elucidated. Here I briefly summarise what is currently known about ligand binding sites in integrins, and discuss how ligand-binding activity may be modulated. I propose a model for integrin regulation that assumes they behave as allosteric proteins, and then describe recent results that support this model and shed new light on the extracellular conformational changes by which integrin activity is altered. I will not discuss here the role of integrin cytoplasmic domains and cell signalling in the regulation of integrin activity; for this the reader is referred to three excellent recent reviews (Sastry and Horwitz, 1993; Williams et al., 1994; Schwartz et al., 1995).

INTEGRIN STRUCTURE

The N-terminal portion of integrin α subunits comprises seven homologous, tandemly repeated domains of about 50 amino acids (Fig. 1). Repeats 4-7 (or in some integrins 5-7) contain cation-binding sequences, similar to the EF-hand motif found in Ca^{2+}-binding proteins such as calmodulin. Seven integrin α subunits (α1, α2, αE, αL, αM, αX, and αD) contain a domain of ~200 amino acids inserted between the second and third N-terminal repeats. This domain is homologous in sequence to the 'A' domains of von Willebrand factor, and has been shown to contain a single cation binding site (Michishita et al., 1993; Lee et al., 1995a; Qu and Leahy, 1995).

The β subunit contains a region of ~240 amino acids near its N terminus that is highly conserved between different β subunits. This region may also have an A-domain-like structure with a cation binding site (see below). The C-terminal portion of the β subunit contains a number of cysteine-rich repeats with homology to EGF modules.

WHERE ARE THE LIGAND BINDING SITES?

To understand the process of integrin activation it is first necessary to define the regions of the α and β subunits that participate in ligand recognition. During the past 5-10 years, many studies including determination of the cross-linking sites of peptide ligands, localisation of the epitopes of inhibitory monoclonal antibodies (mAbs), analysis of mutant integrins, and expression of recombinant integrin fragments have provided many important advances in our understanding of integrin function.

For those integrin α subunits that contain an A-domain, there is now a wealth of evidence to implicate this region in ligand recognition (for a review see Tuckwell and Humphries, 1996). Recombinant A-domains recapitulate most, if not all, of the ligand binding repertoire of the parent molecule. Although the majority of α subunits do not possess an A-domain, all β subunits contain a conserved sequence, which, based on hydropathy plots (Lee et al., 1995a) and other structure prediction techniques (D. S. Tuckwell, personal communication), appears to have an A-domain-like fold. For β3 integrins the primary binding site for RGD peptides lies within this region (D'Souza et al., 1988; Smith and Cheresh, 1988; Pasqualini et al., 1995). In addition, mutations in this domain of several β subunits have been shown to lead to loss of ligand binding (Takada et al., 1992; Bajt et al., 1995; Loftus et al., 1990). Recently, a recombinant fragment containing part of the

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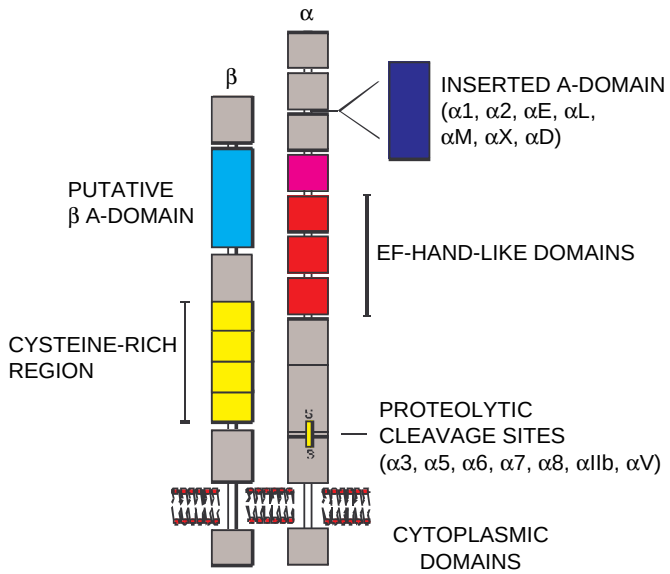

Fig. 1. Schematic diagram of integrin structure (adapted from Newham and Humphries, 1996). See text for further details.

putative A-domain of the β3 subunit was also shown to bind directly to fibrinogen (Alemany et al., 1996).

Less is known about ligand binding sites in α subunits that lack an A-domain. A peptide from the γ-chain of fibrinogen cross linked to the fifth N-terminal repeat of αIIb, and an RGD peptide cross linked to multiple sites within the N-terminal repeats of αV (D'Souza et al., 1990; Smith and Cheresh., 1990). Recently, in a study of αV/αIIb chimeras, the complete N-terminal third of αIIb (repeats 1-5) was found to be required to confer the ligand binding specificity of αIIbβ3 to αVβ3 (Loftus et al., 1996).

Further attempts to map sites involved in ligand binding have been made by localising the epitopes for mAbs that inhibit integrin function. For α subunits with an A-domain, these antibodies localise at or close to this domain. For the α4 subunit (which lacks this domain) these antibodies map mainly to the third N-terminal repeat (Schiffer et al., 1995; Kamata et al., 1995). Significantly, this region is close to the corresponding position of the A-domain in α subunits that possess this sequence. Point mutations in a short hydrophobic portion of this part of α4 block ligand binding to α4β1 (Irie et al., 1995). Similar results were also obtained for α5β1 (Irie et al., 1995). For the β1 subunit, all mAbs that inhibit integrin function lie within a very short sequence (residues 207-218) within the putative βA-domain (Takada and Puzon, 1993); intriguingly this region also contains the epitopes of several mAbs that stimulate integrin function.

Taken together, these studies suggest that multiple sites in the N-terminal region of both α and β subunits co-operate in ligand recognition. Hence, the ligand-binding pocket is likely to be made up of elements of both α and β subunits, which although distantly located in the primary sequence, are brought into close proximity by the spatial folding of the polypeptide chains.

HOW IS INTEGRIN ACTIVITY REGULATED?

Several lines of evidence suggest that ligand binding sites on integrins are cryptic and become exposed only when integrins are activated. First, comparison of the proteolytic digestion patterns of active and inactive conformers of αIIbβ3 suggests that sequences implicated in ligand recognition lie at the subunit interface (Calvete et al., 1992). Second, naturally occurring mutations in the β2 and β3 A-domains suggest that this region is involved in heterodimer formation, as well as ligand recognition (Back et al., 1992; Lanza et al., 1992). Third, a mAb against β3 residues 109-128 (a site involved in recognition of the RGD sequence) shows very low binding to αIIbβ3 unless the integrin is activated (Andrieux et al., 1991). These data imply that the ligand binding sites lie at, or close to, the interface between the α and β subunits, and that subtle motions between the two subunits could regulate exposure of ligand binding sites (Calvete, 1994). Support for this suggestion comes from fluorescence energy transfer experiments, which show that there is a change in the spatial separation or orientation of the αIIb and β3 subunits upon platelet activation (Sims et al., 1991). In addition, there is a marked increase in the proteolytic sensitivity of the active form of αIIbβ3, consistent with a more open structure (Kouns et al., 1992; Calvete et al., 1994). Reducing agents such as dithiothreitol have also been shown to activate integrins (Kouns et al., 1994; Davis and Camarillo, 1993), presumably by releasing structural constraints imposed by disulphide bonds. Furthermore, many mAbs have been described that distinguish between inactive and active forms of integrins (reviewed by Diamond and Springer, 1994; Faull and Ginsberg, 1995). These antibodies recognise regions of the subunits which become exposed on integrin activation, some of which have been shown to lie near sites involved in subunit-subunit interactions (Kouns et al., 1994; Calvete,1994). Alteration of α and β subunit interactions by replacing the β2 subunit in human αXβ2 with its chicken counterpart resulted in a constitutively active integrin (Bilsland et al., 1994).

In summary, current evidence supports the hypothesis that activation represents a conformational transition in the integrin, involving relaxation of subunit contacts and leading to the exposure of ligand-binding sites. Significantly, it is well known that the activity of allosteric proteins is frequently regulated by changes in the interactions between subunits (Perutz, 1989).

AN ALLOSTERIC MODEL OF INTEGRIN ACTIVATION

I have proposed a model for the regulation of integrin activity based on the assumption that integrins are allosteric proteins and can exist in distinct conformational states, with a conformational equilibrium between the inactive (I_1) and active states (I_2) (Mould et al., 1996; Fig. 2). This is essentially the same as the two-state model of allosteric protein function, where there is a conformational equilibrium between a low-affinity quaternary state ('T' state) and a high-affinity quaternary state ('R' state) (Monod et al., 1965). Based on the observation of two distinct conformational states of the αM A-domain, a similar model for the regulation of integrin function has been proposed (Lee et al., 1995b). However, in classical theory of allosteric proteins the conformation of the R state is close to that of the ligand-occupied state, whereas it is clear that integrins undergo considerable conformational changes in

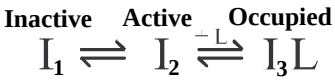

Fig. 2. Allosteric model of the regulation of integrin activity. Three major conformational states of an integrin can be distinguished: I_1, I_2 and I_3, corresponding to the conformations of the inactive, active and ligand-occupied states, respectively. The I_1 state is incompetent to bind ligand because ligand-binding sites are not exposed. Ligand binding sites are exposed in the I_2 state. In this model, integrin activity is regulated by shifting the position of the conformational equilibrium between I_1 and I_2. Binding of ligand (L) to the I_2 state induces changes in the integrin conformation, producing the I_3 state; these ligand-induced conformational changes may be analogous to the large substrate-induced conformational changes described in kinase enzymes (Anderson et al., 1979).

response to ligand occupancy (see below). Since the conformation of an active (i.e. competent to bind ligand) and the ligand bound states do appear to be significantly different, an I_3 (ligand-occupied) conformation is included in the model (Fig. 2). It should also be pointed out that, at least for some integrins, there could be intermediate states between I_1 and I_2. For example αIIbβ3 on unstimulated platelets can recognise small RGD peptide ligands but not soluble fibrinogen; this is probably best described as a partially active, rather than an inactive state of αIIbβ3.

WHAT ROLE DO DIVALENT CATIONS PLAY?

Integrin function is completely inhibited in the presence of EDTA, demonstrating that divalent cations are essential for integrin function. It is clear that cation binding and ligand binding are intimately linked because all of the regions of the integrin implicated in ligand recognition lie at or close to cation-binding sites. However, the precise role of divalent cations in integrin-ligand interactions is still uncertain. The initial observation that the cation-binding sequences in α subunits differed from classical EF-hand sequences in that they lacked an essential oxygenated residue at the -z position led to the suggestion that an aspartate (D) in integrin recognition sequences, such as RGD and LDV, could provide this missing residue to complete the co-ordination geometry of the divalent ion (Corbi et al., 1987; Humphries, 1990). Hence, in this model the divalent ion would act as a bridge between ligand and integrin. Some support for such a model has recently been obtained from the crystal structure of the A-domain of αM (Lee et al., 1995a). This structure showed that one co-ordination position of the cation-binding site in the A-domain was filled by a glutamate residue from a neighbouring molecule. This glutamate residue was suggested to be analogous to an oxygenated residue in integrin ligands (Lee et al., 1995a; Bergelson and Hemler, 1995). A similar model has also been proposed in which cation, ligand and receptor initially form a quaternary complex in which ligand is bridged to the integrin through the cation, and cation is subsequently displaced from the ligand-binding site (D'Souza et al., 1994).

One weakness of the above models is that they fail to explain why different divalent cations have markedly different effects on ligand recognition. For example, Mn^{2+} is a potent activator of many integrins, whereas Ca^{2+} generally inhibits integrin

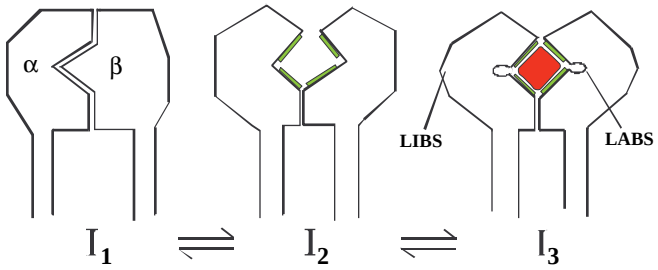

Fig. 3. Schematic model of the modulation of integrin affinity through conformational changes: a summary. Ligand binding sites (green rectangles) are hidden in the inactive (I_1) state but become exposed in the active (I_2) state. In the absence of divalent cations essentially all the integrin is in the I_1 state. Occupancy of cation-binding sites by Mn^{2+} or Mg^{2+} shifts the equilibrium between I_1 and I_2 in favour of the I_2 state. For most integrins, Ca^{2+} is inhibitory for ligand binding and appears to shift the equilibrium in favour of the I_1 state. The position of this equilibrium can be further influenced by activating mAbs, and presumably also by cell-specific factors in vivo. Recognition of ligand by the active integrin causes further conformational changes, including the increased exposure of ligand-induced binding site (LIBS) epitopes and the reduced exposure of ligand-attenuated binding sites (LABS) epitopes. Antibodies that recognise LIBS epitopes shift the conformational equilibrium in favour of the ligand-occupied (I_3) state and thereby stimulate ligand binding. Antibodies that recognise LABS epitopes shift the conformational equilibrium in favour of the unoccupied (I_2) state and thereby inhibit ligand binding. Only the portion of the ligand that lies within the ligand-binding pocket is illustrated above (red polygon). Note that the actual conformational changes involved in the regulation of integrin function are likely to be more subtle than those depicted here.

function. In addition, it is clear that multiple cation-binding sites (rather than a single one) regulate integrin function (Masumoto and Hemler, 1993; Mould et al., 1995a). As an alternative (or complementary) model, I propose that a major role for divalent cations is to directly induce a conformational change required for exposure of ligand binding sites, and thereby to shift a conformational equilibrium between inactive and active states in favour of the active state. Much of the evidence for this model comes from studies of the effects of divalent cations on the binding of mAbs that activate integrin function (Dransfield et al., 1992; Mould et al., 1995b; Bazzoni et al., 1995). These studies show that the binding of these mAbs is increased by Mn^{2+} (and to a lesser extent by Mg^{2+}), but is decreased by Ca^{2+}. Hence, we suggested that both Mn^{2+} and Mg^{2+} support ligand binding because they shift the conformational equilibrium between inactive and active states in favour of the active state; however, Mn^{2+} supports higher affinity binding than Mg^{2+} because it shifts this equilibrium further towards the active state (Mould et al., 1995b)*. Conversely, Ca^{2+} may induce a conformation change that is inhibitory to ligand binding, and so shift the conformational equilibrium between inactive and active states in favour of the inactive state.

*Based on a simple allosteric model, with the assumption that the inactive state has negligible affinity for ligand, the apparent dissociation constant of ligand binding $K_D = (1+N)K_R$, where N is the ratio of inactive to active states ($[T]/[R]$), and K_R is the dissociation constant for ligand binding to the active state. The observed difference in apparent affinity between Mg^{2+}- and Mn^{2+}-supported ligand binding can be explained if Mn^{2+} is better than Mg^{2+} at inducing the conformational change required for ligand recognition, and hence decreases N more than Mg^{2+}, giving a lower value for K_D (higher apparent affinity).

In further support of a largely indirect function for divalent cations in integrin-ligand interactions, several recombinant integrin fragments or peptides have been shown to support ligand binding in a divalent-cation independent manner (Kamata and Takada, 1994; Pasqualini et al., 1995; Alemany et al., 1996). Hence, divalent-cation binding to integrins may play a role akin to that of Ca^{2+}-binding to EF-hand containing proteins such as calmodulin; i.e. to induce a conformational change required for their ligand-binding function (Strynadka and James, 1989).

Recent studies of inhibitory anti-α5 mAbs have shown that the affinity of binding of these mAbs to α5β1 is increased by divalent cations, particularly by Mn^{2+} (manuscript in preparation). Since the epitopes recognised by these mAbs probably lie proximal to sites involved in ligand binding (see below), these data provide further evidence that cation-binding induces conformational changes that correlate with the competence of the integrin to bind ligand.

HOW IS INTEGRIN FUNCTION STIMULATED OR INHIBITED BY MONOCLONAL ANTIBODIES?

Antibodies that stimulate integrin function fall into two main classes: (i) those that bind selectively to the active (I_2) state; and (ii) those that bind selectively to the ligand-occupied (I_3) conformation, known as anti-ligand induced binding site (LIBS) mAbs (reviewed by Williams et al., 1994; Faull and Ginsberg, 1995). The stimulation of integrin function by these mAbs is readily explained by the proposed allosteric model, since reagents that preferentially bind to the active state will stabilise this state and shift the conformational equilibrium between inactive and active states in favour of the active state, thereby promoting ligand binding. Reagents that preferentially bind to the I_3 state will favour this conformation and promote ligand recognition by stabilising the interaction of receptor with ligand. There also appear to be other activating mAbs which do not bind selectively to either the I_2 or I_3 state, it is possible that these mAbs may directly induce a conformational change in the integrin that favours ligand binding.

In addition to stabilising active or ligand-occupied states, some activating mAbs are able to release integrins from a constitutively inactive state. We have recently shown that a sub-population of purified or cell-surface α5β1 is locked in an inactive (I_1) conformation (i.e. not capable of attaining an I_2 conformation). This sub-population can be rescued from a permanently inactive state by activating mAbs, such as 9EG7 and 12G10 (Mould et al., 1996). Such inactive pools of integrin could provide cells with an additional means of regulating their adhesiveness in vivo (Yednock et al., 1995).

Many antibodies have been described that inhibit integrin function, although the mechanism of this inhibition is unclear. Recently, we have examined if a function blocking antibody to the β1 subunit, known as mAb 13, is a direct competitive inhibitor or an allosteric inhibitor of α5β1. Our results indicate that mAb 13 and ligand do not compete directly for binding to α5β1, but that mAb 13 recognises a site that is strongly attenuated by ligand occupancy. We proposed that mAb 13 acts as an allosteric inhibitor because it binds with much lower affinity to the ligand-occupied state than to the unoccupied (I_2) state, and hence destabilises ligand binding by shifting a conformational equilibrium in favour of the unoccupied state. Preliminary studies of several other inhibitory anti-β1 or anti-α5 mAbs suggest that these antibodies also act mainly as allosteric inhibitors of integrin function (manuscript in preparation). Our studies highlight the danger of attempting to localise ligand binding sites on integrins by mapping epitopes of inhibitory mAbs, because these antibodies may recognise sites attenuated by ligand occupancy, rather than sites directly involved in ligand recognition. Nevertheless, inhibitory antibodies probably recognise sites proximal to the ligand-binding domains because they lie in the same regions of the subunits identified by other techniques as containing ligand-binding sequences. We hypothesised that these mAbs recognise sites attenuated during the conformational adaptation of the integrin to ligand, and have termed these ligand-attenuated binding sites (LABS) (Mould et al., 1996). The epitope recognised by mAb 13 lies within a region of the β1 subunit (residues 207-218) that contains the epitopes of other inhibitory anti-β1 mAbs and also those of several antibodies that activate β1 integrins (Takada and Puzon, 1993). This region of the β1 subunit is probably close to the interface between α and β subunits and may be crucially involved in regulating access to ligand binding sites. Indeed, many of the epitopes for activating mAbs lie on integrin β subunits, implying that this subunit plays the major role in the regulation of integrin activity.

HOW DO MUTATIONS AFFECT INTEGRIN-LIGAND INTERACTIONS?

Many natural and artificial mutant integrins have been described; the majority of these are constitutively inactive. Some of these mutations may be at sites directly involved in ligand binding, however, the effect of many of these can probably be explained by allosteric inhibition of integrin function. For example, although the major ligand binding site in αMβ2 lies in the αM A-domain, mutations in the β2 subunit abolish cell adhesion (Bajt et al., 1995). A possible interpretation of this finding is that β2 mutations could lock this subunit into an inactive (I_1) conformation and prevent the transition of the whole integrin into the active (I_2) state, in which ligand binding sites on the α subunit become exposed*. Similarly, a mutation in a region of the β3 subunit that is not directly involved in ligand recognition causes loss of fibrinogen binding by αIIbβ3 (Kouns et al., 1994).

On the other hand, a constitutively active form of αIIbβ3 has been produced by replacing six amino acids in the sequence of β3 with an equivalent sequence from the β1 subunit (Bajt et al., 1992). Although these changes were highly conservative, the mutant receptor bound fibrinogen and fibrinogen peptides with greatly enhanced affinity compared to the wild-type integrin. Although it cannot be ruled out that these changes could have directly altered a sequence involved in ligand recognition, it seems more likely that interactions between α and β subunits were changed, resulting in enhanced exposure of ligand-binding sites.

*I have assumed here that the α and β subunits change conformation in a concerted manner to form the active complex; however, a sequential model in which each subunit can be in either an inactive or active conformation could also explain these results (Lee et al., 1995b).

In other well-studied allosteric systems single point mutations can radically alter the equilibrium between inactive and active states, even where the residues affected play no role in ligand recognition (Perutz, 1989). Hence it is likely that for many previously described 'inactive' mutant integrins the defect in integrin function lies not in the alteration of ligand-binding sites, but rather in the destabilisation of the active state or the stabilisation of the inactive state, resulting in a failure to undergo the conformational change necessary to expose ligand-binding sites. In support of this suggestion, the ligand-binding capacity of some mutant integrins can be restored by the addition of Mn^{2+} and/or activating mAbs (see e.g. Masumoto and Hemler, 1993; Muñoz et al., 1996).

SUMMARY AND FUTURE PERSPECTIVES

Although we are still lacking 3-D structural information on whole integrins, a good deal of progress has been made in identifying ligand-binding sites and in understanding the conformational changes that these receptors undergo in response to activation and ligand occupancy (summarised schematically in Fig. 3). The allosteric model for the regulation of integrin function proposed here may be an oversimplification, but nevertheless provides a working hypothesis on which to base further experiments.

In the future, more detailed mapping of ligand binding sites is required, and it will be important to test more carefully if mutated integrins are truly defective in ligand binding or instead are locked in an inactive state. Further localisation of the epitopes for inhibitory mAbs, while not accurately identifying the position of ligand binding sites, may provide valuable new information on regions of the α and β subunit proximal to these sites. Integrin sequences that are conformationally altered by divalent cation occupancy also need to be more exactly defined in order to understand how these changes relate to ligand-binding competence. Precise localisation of the epitopes for activating mAbs should also provide further insights into the conformational changes required for ligand recognition.

Finally, a fuller understanding of the molecular mechanisms by which integrin activity is regulated could provide a rational basis for the future design of novel integrin agonists or antagonists for use in the treatment of adhesion-related human disorders.

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