1. ABSTRACT

Integrins are a large heterodimeric family of cell surface adhesion receptors that bind extracellular matrix and cell surface ligands. The extracellular ligand binding activity of integrins is a dynamic and highly regulated event involving the induction of conformational changes within the integrin structure. The adhesive properties of integrins can be controlled by altering the activation state of the integrin, either through conformational change or receptor clustering, using mechanisms that are regulated by intracellular proteins. In this review, we will discuss what is currently known about integrin structure and the ligand binding sites present within the receptor. In addition, the mechanisms by which the ligand binding event is regulated through conformational change will be addressed, and the potential role of intracellular cytoplasmic proteins will be discussed.

2. INTRODUCTION

The ability of a cell to bind various cell-membrane and extracellular matrix proteins through the integrins expressed on its cell surface plays a major role in a number of physiological processes such as inflammation, cell adhesion, migration, proliferation and differentiation.

Integrins are cell surface adhesion receptors composed of an alpha and a beta subunit which form a heterodimer. Seventeen alpha and eight beta subunits have been discovered to date that associate to form at least twenty two different receptors. These variations in subunit associations confer ligand binding specificities to the cell. Different integrins can recognise the same ligand and conversely integrins composed of a common subunit show different ligand binding specificities. To interpret this level of complexity, it is important to understand how integrins are capable of recognising and binding their ligands through sites present within the integrin. Consequently, much work has focused on the identification of ligand binding sites present within integrins.

Although it is unknown how these subunits associate together it is thought that they exist in different conformations according to their activation status: either inactive where they are unable to bind ligand, or active where they are capable of binding ligand. In addition, a cell responds to its environment through its ability to regulate the function of integrins by modulating their activity. Both divalent cations and monoclonal antibodies (mAbs) have been used as molecular tools to investigate the dynamic regulation of integrin-ligand binding. Similar conformational changes are induced in integrins through the interaction of intracellular factors and research has concentrated on the role of integrin cytoplasmic domains. Since the ability of the cell to control and modulate integrin structure will ultimately affect the extracellular ligand binding activity, the molecular basis of integrin activation has been the subject of intense recent investigation. In this review, we will discuss the known features of integrin structure and the ligand binding sites present within...
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integrins. In addition, we will address the effects of divalent cations and mAbs, and the role of proteins, involved in inside-out signalling, on the regulation of ligand binding of integrins.

3. INTEGRIN STRUCTURE

Integrins are heterodimers composed of an alpha and beta subunit. Each subunit has a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain (figure 1). The exception to this is the beta4 subunit which has an extended cytoplasmic domain containing four fibronectin (FN) type III-like domains (1). Several homologous domains have been identified within the integrin subunits and splice variants of the integrin cytoplasmic domain have been observed for some of the subunits (2).

All alpha subunits contain seven tandem repeats of about 60 amino acids at their N-terminus, and within three or four of these repeats there are potential metal binding sites of the general structure DxDxGxxD (single letter code, x represents any amino acid). The alpha subunits can be divided into two groups: those that contain an inserted or A-domain, and those with a post-translational proteolytic cleavage site. The A-domain module (approximately 200 amino acids long) is found in the alpha1, alpha2, alphaD, alphaE, alphah, alphaM and alphaX subunits and is homologous to the A-domain of von Willebrand factor (vWF). The proteolytic cleavage site of the alpha3, alpha5, alpha6, alpha7, alpha8, alpha1b and alphaV subunits is close to the transmembrane domain and is bridged by a disulphide bond. The alpha4 subunit is also cleaved but at a central location and is not disulphide bridged (3). There are also short conserved sequences in the integrin cytoplasmic domains and these are discussed later in this review. The beta subunit contains a highly conserved region of about 200 amino acids which has predicted structural similarity to the A-domain of the alpha subunit. It also contains a cysteine-rich region composed of four epidermal growth factor (EGF)-like repeats structurally related to those of laminin (4).

The overall size and shape of the integrin heterodimer has been resolved by electron microscopy, and observed to have a globular head and two tails (4). Using mAbs to bind to the rotary-shadowed integrin it has been possible to confirm that the tails contain the carboxyl terminal ends and the heads contain the amino terminal ends of the subunits (5). In addition, the two tails under some conditions were joined near their ends and could be separated by detergent suggesting they contain the transmembrane domains (4). In the presence of EDTA the structure dissociates into two similar “comma” shaped subunits each containing part of the globular head and a single tail (5).

3.1. Alpha Subunit A-Domain

The integrin structure has not yet been determined although individual domains have been studied. The three dimensional structure of the alphaM (6), alphal (7) and alpha2 (8) integrin A-domains and the homologous vWF A3 domain (9) have been elucidated by X-ray crystallography (figure 1, lower panel). This is the only region of integrin for which the structure is currently known. The A-domains are composed of five parallel and one anti-parallel beta strands surrounded by seven alpha helices forming a Rossmann fold (figure 2A). The integrin A-domains contain a metal ion-dependent adhesion site or MIDAS motif (6) which was observed to be modified in the vWF A3 domain (9). The MIDAS motif within the A-domain is a single cation binding site, which is disrupted in the vWF A-domain. In integrin A-domains the cation is coordinated by five amino acid side chains either directly or indirectly through a water molecule. Three of the co-ordinating residues are contained within the sequence DxSxS located in the loop between beta strand A and alpha helix 1, the other two co-ordinating residues are a threonine and an aspartate found on the loop between alpha helices 3 and 4, and the loop between beta stand D and alpha helix 5 respectively (figure 2A). The crystal structure of the alphaM A-domain complexed with Mg²⁺ has an unusual co-ordination of the cation involving a glutamate side chain from a neighbouring A-domain within the crystal. This co-ordination was suggested as a model for ligand binding, since the short acidic side chain of glutamate resembles that of aspartate which is essential in many integrin ligands (6).

The crystal structure of alphaM with Mn²⁺ has a water molecule completing the co-ordination sphere and imposes a structural shift in the C-terminal helix of the Rossmann fold. It has been proposed that the difference between the Mg²⁺ and Mn²⁺ containing crystal structures represent the active and inactive states of the integrin (10). In contrast, the crystal structure of the alphal A-domain had no major structure rearrangements whether in the presence of Mg²⁺ or Mn²⁺ or in the absence of cation (11).

3.2. Putative Beta Subunit A-Domain

The beta subunit putative A-domain also contains the DxSxS sequence (6) and has potential residues for the two other cation co-ordinating side chains conserved in beta1 (12, 13), beta2 (14, 15, 16), beta3 (17, 18, 19, 20), and beta5 subunits (figure 2B) (20). Structural similarity has been suggested between the putative beta A-domain and the alpha A-domain based on hydropathy plot analysis using sequence aligned around the DxSxS sequence (6). Structure predictions using similarly aligned sequence from alphal, alphah, beta3 and beta5 showed few of the structural elements seen in the alpha subunit to be present in the beta subunits (20). It is suggested that although the putative beta A-domain may not have identical structure to the alpha A-domain the MIDAS motif is still present. The conserved sequence of the putative beta A-domain has been proposed to have similarity to the vWF A-domain based on multiple sequence alignment and structural analysis (21). The beta subunit has been predicted to form a Rossmann fold / alpha-beta-alpha sandwich with two loop structures (between beta strands B and C, and beta strand D and alpha helix 5) protruding from the A-domain which are not present in the vWF or alpha subunit A-domains (figure 2B) (21). A murine gene (Pactolus) on alignment with the beta2 subunit to which it shares a high degree of homology (22), does not contain the sequence for the first of these loop structures. The absence of this loop is unlikely to disturb
Figure 1. Schematic structure of a generic integrin heterodimer. The A-domain present in some alpha subunits is represented by a horizontally hatched box. The putative beta A-domain is shown as a vertically hatched box. The cysteine-rich region of the beta subunit is represented by a crosshatched box. The upper panel shows the proposed structure for the N-terminal repeats (reproduced with permission from (24)). The seven beta sheets of the propeller domain are labelled as W1-W7. The lower panel shows the Rossman-fold structure of the alpha M A-domain (taken from (6)). The beta strand structure is represented by arrows, and alpha helices represented by ribbons. Cations are shown as colored spheres.
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Figure 2. Schematic representation of the alpha A-domain and the putative beta A-domain. (A) Alpha A-domain based on the known structures of the alpha M and alpha L A-domains (6, 7). (B) Beta A-domain based on both the known structure of the alpha M and alpha L A-domains (6,7), and structure prediction (21). Residues involved in co-ordinating metal ions are indicated by single amino acid code. Beta strands are represented as arrows and alpha helices as cylinders, with lines indicating connecting loops (reproduced with permission from (21)).

The overall structure suggesting that it may be an evolutionary addition to, or deletion from, the original structure (Tuckwell, D.S. personal communication). An alternative structure for the beta A-domain has been proposed using an alignment between the alphaM and the beta3 subunits. This predicted structure does not include the two looped out regions of Tuckwell et al. and therefore covers a reduced sequence length (19).

3.3. Alpha Subunit N-Terminal Repeats

The structure of a single repeat in the alpha subunit has been predicted as: an undefined region, loop, alpha helix and then a series of three beta strands connected by loops (23). The seven repeats could either be packed as individually folded structures or as a single structure forming part of the integrin globular head. An additional prediction has proposed that the N-terminal repeats form a beta propeller structure encompassing all seven repeats (24). The beta propeller is a cyclic structure composed of seven modules each comprising four beta strands (figure 1, upper panel). The original structure of the single repeat identified three of the beta strands within each module. The fourth beta strand is provided by the subsequent repeat and was initially proposed as the undefined region of the repeat. The prediction of a beta propeller in the alpha subunit and a Rossmann fold in the alpha subunit A-domain and the beta subunit putative A-domain has led to the proposal that integrins have similarity to G proteins (24). By superimposing the predicted structure of the alphaM subunit N-terminal domains on to the G protein crystal structure a flexible hinge is present between the beta propeller and the protruding A-domain of the integrin alpha subunit overlaid the switch II region in the G protein (24, 25, 26). The flexible hinge of the integrin alpha subunit or a loop of the beta subunit putative A-domain may function as an affinity switch leading to conformational changes between the subunits altering active site accessibility and modulating activity (27).

4. LIGAND BINDING SITES IN INTEGRINS

These models of integrin structure allow for a rationalisation of the data available concerning regions of integrin subunits implicated in ligand binding. It has been shown that several regions in both integrin subunits act together to form the ligand binding surface. Despite the variety of approaches used to localise the sites involved in ligand binding they are still not described with certainty. Studies so far have concentrated on the binding sites in integrin ligands. Short motifs including RGD of FN and vitronectin (VN), and LDVP and IDSP of FN and vascular cell adhesion molecule-1 (VCAM-1) respectively have been identified as the major binding sites along with so-called “synergy sites” and it seems likely that ligand interacts with integrin at two or more distinct sites within the heterodimer (28, 29). Examination of the family of beta1 integrins shows that while these integrins share the same beta subunit, the ligands bound differ widely: alpha2 beta1 binds collagen, alpha2 beta1 binds VCAM-1 site of FN at its LDVP site, alpha5 beta1 binds FN at its RGD site and alpha6 beta1 binds laminin. Similar observations can be made for the beta2 and beta3 integrins and from this it can be deduced that the alpha subunits play a major role in determining ligand specificity (30). Cross-linking, phage display, peptide inhibition, expression of subunit fragments, epitope mapping and site-directed mutagenesis studies have been used to identify potential ligand binding sites.

4.1. Sites in Alpha Subunits

For those alpha subunits which contain an A-domain, the isolated A-domains recapitulate most if not all of the ligand binding activity of the parent integrin. The A-domain of alpha2 binds specifically to type I collagen in a concentration and cation-dependent manner as does the complete receptor (31, 32). The A-domain of alphaM is an independent structural and functional unit which has many of the binding functions of the parent receptor, including the ability to bind iC3b, CD54, fibrinogen (Fg) and the neutrophil inhibitory factor (NIF) of canine hookworm (6).
Various studies have investigated which residues, in addition to those involved in the MIDAS motif, are involved in ligand binding.

Analysis of the alphaM A-domain using overlapping peptides identified a discrete 14 amino acid region (residues 232-245) which binds iC3b and inhibits the binding of iC3b to the alphaM A-domain as well as to full length alphaM beta2 (33). This region in the alphaM A-domain corresponds to beta strand D and a short segment of the following loop, and the importance of this loop between beta strand D and alpha helix 5 for ligand binding has been confirmed by two mutations in alphaM (figure 2A) (34). In addition, two mutations in this same loop of alphaL abrogate the binding of alphaL beta2 to intercellular cell adhesion molecule-1 (ICAM-1) (35). There is evidence that the loop from beta strand A to the alpha helix 1 and alpha helix 1 itself also play a role in ligand binding. The mutation of Met140 and Glu146 in alphaL reduced binding of alphaL beta2 to ICAM-1 (35) whilst the epitopes of anti-alphaL antibodies are also found in this region and anti-alpha2 antibodies are found to bind in an equivalent region of alpha2 (32, 36). Epitopes for other anti-alphaL antibodies are found to bind to the loop between alpha helices 3 and 4 (36). Finally, the binding of NIF to alphaM was mapped to four short peptides which contained loops from the top face of the A-domain (37). These results indicate that it is the top cation binding face of the A-domain that is involved with the binding of ligands, especially the loop between beta strand D and the alpha helix 5.

Although the majority of data point to a important role for the top of the A-domain, it is possible that sites other than those on the top face of the A-domain are involved in ligand binding. Despite a series of point mutations, the collagen-binding site of alpha2 beta1 has not yet been identified apart from residues involved in the MIDAS motif suggesting that collagen binds by a different mechanism (38). Alpha2 has an extra helix which protrudes from the MIDAS face to create a groove centred on the magnesium ion, and it is proposed that collagen binds within this groove via a glutamate residue (8). The binding sites of the human pathogen Echovirus-1 have been localised using mouse/human chimeras to two short, independent regions of alpha2 (39). They are in the sequences between beta strand C and alpha helix 3, and alpha helices 3 and 4 exposed on the surface of the A-domain at a site distinct from that which binds collagen. The adhesion of alphaL beta2 to ICAM1 was abrogated by the mutation of Pro192 in the loop between the alpha helices 2 and 3 of alphaL, although the antibody binding data suggest that the loss of binding was due to conformational changes in the A-domain (40). Mutation of the analogous Pro195 of alphaM reduced but did not abolish binding of alphaM beta2 to iC3b (41).

For those integrins which lack an A-domain, less is known about the binding sites on the alpha subunit. AlphaV/alphaIIb chimeras have been used to determine that the entire N-terminal third of the subunit is required for ligand binding. Substitution of the amino-terminal portion or divalent cation binding repeats alone did not change the specificity of alphaV (42). A peptide from the gamma chain of Fg cross-linked to the fifth N-terminal repeat of alphaIIb, and an RGD peptide cross-linked to two sites within the N-terminal repeats of alphaV (43, 44). An eleven amino acid peptide from the fifth N-terminal repeat of alphaIIb has been used to inhibit platelet aggregation and binding of Fg to platelets and purified alphaIIb beta3, confirming an important role for this region (45).

Epitope mapping has also been used to demonstrate the importance of the N-terminal repeats of the alpha subunits in ligand binding. Kamata et al. used interspecies chimeras to localise the epitopes of inhibitory mAbs to residues 108-268 of the alpha4 subunit (46). A similar study by Schiffer et al. located these epitopes to within residues 152-203 (47). Alanine scanning mutagenesis within this region revealed three residues, Tyr187, Trp188 and Gly190, which when altered inhibited the binding of VCAM-1 and CS-1 (a FN fragment) to alpha4 beta1 (48). Residues in the analogous region of the third N-terminal repeat of alpha5 and alphaIIb have also been shown to be critical for ligand binding (49). It is possible that these residues are not necessarily involved directly in ligand binding but are pivotal in maintaining the conformation of the ligand binding site.

Alpha4/alpha5 chimeras within the 108-268 region have also been used to locate the ligand binding site (50). Loops within the N-terminal repeats of alpha4 have been replaced with the corresponding loops in alpha5 and their binding properties determined. Swaps between loops in repeats 2 and 3 had an effect on binding to VCAM-1 and CS-1. Using the same loop swaps, inhibitory mAbs were found to bind in the loops between beta strands 2 and 3 of repeat 2 (residues 112-131), beta strands 4 and 1 (residues 151-164) and in beta strands 2 and 3 of repeat 3 (residues 186-191). These loops have been identified as being critical for ligand binding and are found to be adjacent and on the upper face in the beta-propeller model. This supports the model in which the upper face of the beta-propeller is believed to contain the active site (figure 1, upper panel) (24). However when a series of alanine scanning mutations were made in the loops of repeat 2, only a double mutation Tyr120Ala/Gly130Ala blocked the binding of ligand and mAbs. Other single and double mutations had no effect indicating that these residues have a structural role of the beta turn. However, these two residues are predicted to be in a loop, which, when swapped had no effect on ligand binding. Since a conservative change (Tyr-Phe, Gly-Gly) was made in this loop swap, it is possible that their role in ligand binding or in maintaining the structure of the binding site has been masked. In vitro translation of fragments of alphaL has been used to identify regions within the N-terminal repeats involved in binding ICAM-1. Consecutive peptides within this region have indicated two discontinuous areas (residues 458-467 and 497-516) as specific contact sites. It is possible however that binding sites in the intact receptor may differ from those identified from studies on a single subunit (51).
A study of the binding of alpha5 beta1 to the central cell binding domain of FN (CCBD) using inhibitory mAbs has also shed light upon the ligand binding activity of the alpha subunit (52). When integrin binds ligand, there is a conformational change in the receptor. Inhibitory antibodies are believed to function by an allosteric mechanism preventing the shape change from taking place. The binding of inhibitory mAbs to alpha5 beta1 was disrupted by the CCBD tryptic fragment which includes the RGD motif and synergy site, a CCBD tryptic fragment which lacks the synergy site, and a GRGD peptide. Epitope mapping has placed the epitopes of P1D6 and JBS5, two inhibitory antibodies, at the amino terminus of alpha5 confirming the important role of this region in ligand binding. The binding of P1D6 and JBS5 was inhibited by fragments containing the synergy site whilst the anti-beta1 antibody P4C10 was inhibited by fragments containing RGD only. It was proposed that the beta1 subunit is responsible for binding to RGD, whilst the alpha5 subunit is mainly responsible for binding to the synergy site, achieving a specificity and higher affinity of binding than could be conferred by RGD alone.

4.2. Sites in Beta Subunits

Ligand binding sites which so far have been identified in beta subunits are restricted to the putative A-domain. D’Souza et al. crosslinked an RGD peptide to stimulated platelets and identified a 63 amino acid region (residues 109-171) of the beta3 subunit which represents the region from beta strand A to beyond beta strand B in the predicted A-domain model (figure 2B) (21, 53). A phage display library of receptor sequences has been panned with RGD-containing FN fragments and a six amino acid sequence, WDDGWL, was subsequently found to inhibit RGD-dependent attachment to FN and VN (54). There is a similar KDDLW sequence in alpha helix 1 of the beta1 subunit adjacent to the MIDAS motif which is within the region identified by crosslinking. It is possible that this peptide is a structural mimic of the beta subunit RGD binding site. A direct role for this region has apparently been demonstrated using a peptide from this region (residues 118-131), encompassing parts of beta strand A and helix 1 which was able to inhibit alphalib beta3 binding and formed a complex with ligand or divalent cation with 1:1 stoichiometry (17).

Synthetic peptides from within the amino terminal 288 amino acids of beta3 have been tested for their ability to block alphalib beta3-Fg binding (55). Two overlapping peptides indicated an additional binding site within residues 204-229 (alpha helices 3 to 4), and a peptide corresponding to 211-222 blocked binding of FN, VN and vWF to alphalib beta3. Direct binding of the peptide 204-229 to Fg and FN was demonstrated by ELISA. This region is also highly conserved in other receptors and thus may be a universally important binding site. A study using beta3 derived peptides to inhibit the binding of Fg and albolabrin, a disintegrin which contains the RGD single cell recognition epitope to activated platelets, confirms that this region binds ligand through an RGD-dependent mechanism (56).

Highly diverse sequences within the putative A-domain of beta1 were swapped for those found in beta3 to locate the regions which confer ligand binding specificities (57). A region was identified (residues 187-193) between beta strands B and C which enabled chimeric alphaV beta1 to recognise Fg, vWF and VN which are usually recognised only by beta3. The reciprocal mutant blocked alphaV beta3 binding to these same ligands. In a similar study, the specificity of the beta5 subunit was changed to that of beta3 by exchanging 39 amino acids between beta strand B and alpha helix 2 (58). This suggests that this highly divergent sequence, which lies within regions previously identified as being critical for ligand binding, is a key determinant of integrin-ligand specificity. In another study, six amino acids of beta3 were replaced by the equivalent residues from beta1 (WSIQN→ENVKS in alpha helix 1) which resulted in an increase in the affinity for Fg (59).

A number of natural mutations in integrins have been found to block ligand binding. Glanzmann’s thrombasthenia is an autosomal recessive hereditary disorder of alphalib beta3 associated with the inability of the integrin to recognise macromolecular or synthetic peptide ligands. The cam variant of Glanzmann’s thrombasthenia results from a point mutation within the MIDAS (15). The residues Arg214 of the beta3 subunit and Asn224, Asp233, Asp267, Asp295 of the beta1 subunit have also been shown to be critical for ligand binding (13, 60).

It has been suggested that exposure of the ligand binding sites is facilitated by small movements between the subunits. This implies that the ligand binding site lies at or close to the interface between the alpha and beta subunits (61). Comparison of proteolytic digestion patterns of active and inactive conformers of alphalib beta3 have implicated such regions at the alpha/beta subunit interface in ligand recognition (62). It has been demonstrated that the alpha A-domain, putative beta A-domain and N-terminal repeats all contain regions critical for the binding of ligands. It remains to be seen how these structures are organised and how they interact together to form a coherent ligand binding pocket.

5. USE OF DIVALENT CATIONS AND MONOCLONAL ANTIBODIES TO PROBE CONFORMATIONAL CHANGES IN INTEGRIN LIGAND BINDING

Although sites in the integrin structure that are important in ligand binding have been pinpointed, the mechanisms that determine the ligand binding activity of a particular receptor have not been completely resolved. The complex regulation of integrin function appears to be a consequence of the dynamic structure of the integrin heterodimer. Even in the absence of complete structural data on integrins there is evidence to support the existence of structural changes within integrins that determine both the ligand binding capacity and the signalling ability of the receptor. Activation of the platelet integrin alphalib beta3 appears to result in a conformational change in the whole integrin that allows ligand binding to occur (63). Similarly,
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the differential expression of epitopes recognised by certain mAbs suggests that the integrin structure is dynamically altered (64, 65).

5.1. Role of Divalent Cations

The presence of various divalent cation binding sites in the alpha subunit N-terminal repeats, putative beta A-domain and the alpha A-domain reflects their importance as an integral part of the integrin structure. The role of divalent cations in integrin function is demonstrated by the lack of ligand binding upon removal of cations by chelating agents. Furthermore, divalent cations such as Mn$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$ have distinct effects on integrin function in vitro. The functional effects of divalent cations have been carefully dissected in the case of beta1 integrins. Generally, Mn$^{2+}$ confers high affinity binding properties on isolated integrins, whereas Ca$^{2+}$ inhibits ligand binding, with Mg$^{2+}$ playing a stimulatory role but to a lesser extent than Mn$^{2+}$. Studies on the FN receptor alpha5 beta1 have demonstrated the presence of at least three distinct classes of cation binding sites for Mn$^{2+}$, Mg$^{2+}$ and Ca$^{2+}$ (66). Cation binding, supported by Mn$^{2+}$ in a non-competitive manner implying distinct binding sites for these two cations. A second site that can bind Ca$^{2+}$ or Mg$^{2+}$ in a competitive manner, was observed. In addition, a third site that displayed high affinity for Ca$^{2+}$ was identified which has previously been termed an effector site (67).

Studies on a peptide derived from the putative beta3 A-domain have led to the proposal that ligand, divalent cation and integrin form a ternary complex which stabilises upon displacement of the divalent cation (17). A direct role for divalent cations in ligand binding was also suggested by structure determination of the alphaM A-domain which showed a Mg$^{2+}$ ion partially co-ordinated by a glutamate residue from an adjacent molecule in the crystal lattice (6). The role of the alpha subunit divalent cation binding motifs present in the N-terminal repeats of the alpha subunit is less clear. Although mutation of the three divalent cation binding motifs present in the alpha4 subunit resulted in decreased alpha4 beta1-mediated cell adhesion, it has subsequently been shown not to affect soluble ligand binding (68, 69). Whether these mutations affect the divalent cation binding capacity of the alpha4 subunit remains to be determined. An indirect role for the cation binding sites has been implied by the beta-propeller model of the N-terminal repeats of the alpha4 subunit, which located the cation binding motifs in loop regions on the opposite side of the domain to the putative ligand binding region (24). Despite the apparently conflicting evidence regarding the role of divalent cations in ligand binding, the presence of multiple cation binding sites in different regions of the integrin structure, suggests that both direct and indirect roles may be involved at different sites within the whole integrin.

5.2. Conformation-Dependent Epitopes

It has been proposed that divalent cations can modulate integrin function indirectly by inducing conformational changes in the integrin structure (61). The role of divalent cations as mediators of conformational change is supported by studies using mAbs that recognise conformation-dependent epitopes. Epitope mapping of anti-beta1 antibodies led to the discovery of a twelve amino acid sequence (residues 207-218) present in the putative beta A-domain that contains both epitopes for the stimulatory antibodies (TS2/16, 8A2, A1A5, 12G10) and inhibitory antibodies (mAb13, 4B4 and P4C10) (70, 71). Other conformation-dependent epitopes have been mapped to the cysteine-rich region of the beta subunit including 9EG7/7 (72), QE.2E5 (73), IB1B (74), and AG89 (75). The intervening region between the putative beta1 A-domain and the cysteine-rich region has also been shown to contain conformation-dependent epitopes for the antibodies 15/7 (76) and HUTS-4, -7, -21 (77). All these antibodies bind to sequentially distant regions of the beta subunit and exposure of their particular epitopes is regulated by divalent cations in a similar manner to that of ligand binding function. The distribution of these conformation-dependent epitopes throughout the beta1 subunit highlights the global conformational changes that occur during regulated ligand binding.

Studies on the FN receptor alpha5 beta1 have demonstrated that ligand, divalent cation and integrin form a ternary complex which stabilises upon displacement of the divalent cation (17). The conformational regulation of integrin function may also be the mechanism by which certain inhibitory antibodies prevent ligand binding. Previously, anti-integrin antibodies that blocked ligand binding function were presumed to act via a steric blocking mechanism, assuming that the antibodies bound at, or close to the ligand binding site. However, the localisation of both stimulatory and inhibitory antibodies within the twelve amino acid stretch of the beta1 subunit suggests steric blocking is unlikely. Indeed, the inhibitory antibody mAb13 has been shown to abrogate ligand binding to alpha5 beta1 by an allosteric mechanism (81). The allosteric mechanism of action suggests that the twelve amino acid region is involved in conformational changes that determine integrin ligand binding function. In an extension of these studies, mAb13 binding to alpha5 beta1 was shown to be regulated by divalent cations (as were other mAbs including 12G10), with the stimulatory divalent cation Mn$^{2+}$ reducing the affinity of mAb13 for the integrin, whereas Ca$^{2+}$ increased the affinity of mAb 13 (71).

The action of stimulatory and inhibitory antibodies, coupled with the regulation of ligand binding by divalent cations, has lead to a model of integrin-ligand binding that is dependent on conformational changes in the integrin structure (figure 3) (61, 82). In this model integrins can exist in active or inactive conformations, with the active conformation capable of binding ligand with high affinity. The conformational states exist in an equilibrium which can be shifted to the active form by the activating cation Mn$^{2+}$ and by stimulatory antibodies such as 12G10, or shifted to the inactive conformation by Ca$^{2+}$. Similarly, inhibitory antibodies such as mAb13 can induce conformational changes that prevent ligand binding by an
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Figure 3. Model of conformational changes in the integrin heterodimer that regulate ligand binding function. Shape changes in both the alpha (grey) and beta (black) subunits are proposed to occur in response to different divalent cation conditions. Potential ligand binding is represented as a cyan rectangle, with bound ligand shown as a blue rectangle. Potential epitopes for inhibitory monoclonal antibodies (mAbs) are represented as pink circles, with bound epitopes represented by red circles. Potential epitopes for stimulatory mAbs are represented by green circles. (a) Inactive integrin occupied by Ca\(^{2+}\), expresses the epitope for inhibitory mAbs (eg. mAb13), but not epitopes for stimulatory mAbs. (b) Active integrin conformer can be induced by Mn\(^{2+}\) or Mg\(^{2+}\) and has the potential to bind ligand. (c) Inhibited integrin conformer is bound by inhibitory anti-beta subunit mAb that induces shape change which prevents ligand binding. (d) Ligand occupied conformer is occupied by ligand and can be bound by stimulatory mAbs. Conformational changes may be propagated across the plasma membrane (single black line) altering the ability of the cytoplasmic domains to bind cytoskeletal/signalling molecules (82).

6. REGULATION OF LIGAND BINDING MEDIANED BY INSIDE-OUT SIGNALLING

It has been suggested that inside-out signalling regulates the adhesive function of integrins through the interaction of specific intracellular proteins with the integrin cytoplasmic tail. This contact may promote a structural change within the integrin that is transmitted across the plasma membrane to the extracellular domain. Integrins may also move laterally within the plasma membrane to cluster at distinct sites known as focal contacts which provide a link between the extracellular matrix proteins and the actin cytoskeleton. Therefore, the clustering effect may also play a relevant role in the regulation of ligand binding.

6.1. Motifs Within Cytoplasmic Domains

Sequences in the cytoplasmic domain control integrin affinity state and both subunits contribute to the affinity state maintenance (83, 84). The sequence GFFKR of the alpha subunit located a few amino acids after the transmembrane domain on the cytoplasmic side is thought to maintain an inactive state of the integrin. Mutations in this sequence, or its removal lead to constitutively active integrin (85, 86). The membrane proximal cytoplasmic sequence of the beta subunit may be all that is required for subunit association and integrin affinity state. Association between the GFFKR sequence of the alpha subunit and the KLLLxxxHDR sequence of the beta subunit has been studied by mutagenesis in the alphaIIb beta3 integrin. Mutagenesis of the arginine in the alpha subunit and aspartate in the beta subunit in the above sequences led to activation of the integrin whereas swapping the residues on the two subunits resulted in an inactive integrin. The formation of a salt bridge between these two residues of the alpha and beta subunits has been proposed to maintain the activity of the integrin (87).

Another conserved cytoplasmic domain sequence NPxY is found in many beta subunits within 30 residues of the transmembrane domain. In addition, a similar motif is located membrane distal on the cytoplasmic domain in the beta1, beta2 and beta3 subunits. These sequences are thought to be important in controlling integrin affinity by its involvement in inside-out signalling and its interaction with cytoskeletal components (88, 89).

6.2. Inside-Out Regulation by Cytoplasmic Proteins

A number of candidate proteins have been described that interact directly or indirectly with the integrin cytoplasmic domain. However, this review will concentrate on those that have been suggested to play a role in the regulation of integrin activity (table 1).

One such protein is cytohesin-1, a 47kDa cytoplasmic protein, which when overexpressed induced alphaL beta2-dependent binding to ICAM-1. It had no effect on alpha4 beta1-mediated adhesion, indicating that cytohesin-1 may specifically regulate beta2 integrins (90). Cytohesin-1 has been shown to be a guanine nucleotide-exchange protein for ADP-ribosylation factor (ARF) responsible for the formation of active ARF-GTP (91). How this is involved in the regulation of adhesion is currently under investigation.
### Table 1: Proteins which interact directly with integrin cytoplasmic domains and may play a direct role in inside-out signalling

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<th>CYTOPLASMIC PROTEINS</th>
<th>SIZE</th>
<th>INTEGRIN CYTOPLASMIC INTERACTION</th>
<th>NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytohesin-1</td>
<td>47kDa</td>
<td>beta2</td>
<td>A guanine nucleotide exchange protein. Overexpression induces alphal beta2 dependent adhesion.</td>
</tr>
<tr>
<td>ILK</td>
<td>59kDa serine threonine kinase</td>
<td>beta1, beta2, and beta3</td>
<td>Overexpression inhibited adhesion to laminin, fibronectin and vitronectin.</td>
</tr>
<tr>
<td>Beta3-endonexin</td>
<td>12.6kDa</td>
<td>beta3</td>
<td>Alternatively spliced. Induces high affinity state of alphalib beta3 by binding NITY motif.</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>60kDa</td>
<td>alpha subunits</td>
<td>Preferentially interacts with active alphal2 beta1 via GFFKR motif. Overexpression induces increased cell spreading and decreased motility.</td>
</tr>
<tr>
<td>CIB</td>
<td>25kDa</td>
<td>alphalib</td>
<td>Calcium binding protein.</td>
</tr>
<tr>
<td>ICAP-1</td>
<td>20kDa</td>
<td>beta1 only</td>
<td>Alternatively spliced. Binds via NPXY motif.</td>
</tr>
<tr>
<td>FAK</td>
<td>125kDa</td>
<td>beta subunits</td>
<td>Overexpression induces increased migration.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MEMBRANE-ANCHORED PROTEINS</th>
<th>SIZE</th>
<th>INTEGRIN CYTOPLASMIC INTERACTION</th>
<th>NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD98</td>
<td>125kDa</td>
<td>beta1 only</td>
<td>Stimulates beta1-dependent adhesion.</td>
</tr>
<tr>
<td>uPAR</td>
<td>55kDa cell surface receptor (no transmembrane domain)</td>
<td>beta1 and beta2</td>
<td>Preferentially interacts with activated integrin. Inhibits integrin function and promotes adhesion to vitronectin via a ligand binding site on uPAR.</td>
</tr>
<tr>
<td>IAP (CD47)</td>
<td>50kDa transmembrane protein</td>
<td>beta3</td>
<td>Thrombospondin-1 receptor. Possible roles in migration, calcium influxes and phagocytosis.</td>
</tr>
<tr>
<td>TM4SF proteins</td>
<td>Transmembrane proteins</td>
<td>beta1, alpha4 beta7, and alphal beta2</td>
<td>Almost 20 members. Possible roles in adhesion, motility and aggregation via integrins.</td>
</tr>
<tr>
<td>Rack1</td>
<td>36kDa</td>
<td>beta1, beta2 and beta5</td>
<td>Receptor for activated protein kinase C. Integrin interaction requires the presence of phorbol ester PMA.</td>
</tr>
</tbody>
</table>

Another cytoplasmic protein is integrin-linked kinase (ILK), a 59kDa serine-threonine kinase that is expressed ubiquitously, and is down regulated by adhesion to FN (92). ILK may function as a negative regulator of integrin-mediated adhesion possibly by phosphorylating integrin cytoplasmic domains. ILK has been shown to phosphorylate a beta1 cytoplasmic peptide \textit{in vitro} (92). It is likely that other phosphorylation events are involved in the regulation of integrin function. Ligand binding sites on alphalib beta3 become exposed on phosphorylation of the beta3 subunit (93). In addition, mutation of the beta3 cytoplasmic tail (Tyr747Phe) to prevent tyrosine phosphorylation abrogated alphalib beta3 mediated adhesion (94).

Beta3-endonexin has been shown to specifically interact with the cytoplasmic tail of the beta3 integrin subunit (95). Residue Ser752 and membrane distal residues 756-759 (NITY) in the beta3 cytoplasmic tail are known to be important for the interaction with beta3-endonexin (95, 96). Beta3-endonexin has been found to regulate the affinity state of alphalib beta3 when co-transfected into CHO cells as demonstrated by the ability to bind PAC-1, a ligand mimetic mAb (97).
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A number of calcium binding proteins are thought to play a role in the regulation of ligand binding activity of integrins. Calreticulin, a 60kDa intracellular calcium-binding protein, interacts with a highly conserved GFFKR motif present in all alpha subunit cytoplasmic domains, and in particular has been suggested to associate with active but not inactive alpha2 beta1 (98). The precise function of calreticulin has yet to be determined, although it may function by directly associating with integrins at the plasma membrane, maintaining the integrin in a high affinity state primed for ligand binding (98, 99). Another calcium binding protein is calcineurin which has been shown to regulate alpha5 beta1/FN interaction (100). In addition, neutrophil migration on VN was decreased by calcineurin inhibitors or intracellular calcium chelators. In the presence of these inhibitors, alphaV beta3 accumulated at the rear of the cell which subsequently became immobilized although restoration of calcium levels allowed migration to reoccur. The evidence suggests that calcineurin and calcium are important in integrin redistribution during cell migration (101). Calcium and integrin binding protein (CIB) is another calcium binding protein, with sequence homology to calcineurin and was identified using a yeast two-hybrid system to bind the cytoplasmic domain of alphaIIb specifically (102). Although the site of interaction is not yet known, the GFFKR region is unlikely to be involved.

Calpain, a calcium-dependent protease thought to cleave the cytoplasmic domains of integrins and cytoskeletal associated proteins, may also regulate migration, as shown using calpain inhibitors on alpha5 beta1 and alphaIIb beta3-mediated migration in CHO cells on FN and on Fg respectively. A reduction in calpain activity inhibited cell migration possibly by decreasing the rate of cell detachment from the substratum and stabilising the integrin-cytoskeletal linkage (103). Calpain cleaves the beta3 cytoplasmic domain near two NxxY sites, NPLY and NITY, (104) known to be important in the regulation of the affinity state of the receptor (88).

Integrin cytoplasmic domain-associated protein-1 (ICAP-1), another cytoplasmic protein, binds specifically to the beta1 cytoplasmic domain using the NPXY motif and a Val residue located towards the NH2-terminal (105). Whether this protein has an effect on the regulation of affinity or avidity modulation of integrins remains to be established.

There are several reviews which discuss the role of focal adhesion kinase (FAK) in the downstream signal transduction pathways triggered by integrins (106, 107, 108). FAK has been shown to play an important role in cell spreading, proliferation, and apoptosis (109, 110, 111, 112). FAK also appears to be involved in cell migration where over-expression of FAK in CHO cells resulted in increased migration on FN (113). In addition, cultured fibroblasts isolated from FAK null mice (114) and microinjection of the carboxyl fragment of FAK decreased cell migration (109). This suggests an important role for FAK in the regulation of integrin-ligand binding. Indeed, through peptide binding studies, the N-terminal domain of FAK has been shown to bind directly to the cytoplasmic tail of the beta1 subunit (115).

Cytoskeletal proteins including alpha-actinin, F-actin, filamin, paxillin, talin, tensin and vinculin have all been shown to associate directly or indirectly with integrin cytoplasmic domains where they may connect the actin cytoskeleton with the integrin cytoplasmic domain (116, 117, 118, 119). Interestingly, alpha-actinin has been shown to co-precipitate with the beta2 cytoplasmic domain from activated neutrophils specifically, suggesting it may function to modulate integrin avidity (120).

Although a number of these proteins have been implicated in the functional regulation of integrin activation, there is at present little evidence to support a direct interaction between regulatory cytoplasmic proteins and intact integrins. Instead, studies have mainly demonstrated an association of these proteins with synthetic or recombinant peptide forms of the cytoplasmic domains of individual integrin subunits. Since this does not necessarily reflect a physiologically relevant interaction, it will be important in the future to identify proteins that interact with the integrin as a heterodimer. In addition, evidence of the co-localisation of associated proteins with integrins would further support their role in regulating integrin function. Clearly, more research is required to establish the identity of cytoplasmic proteins that play an important role in inducing and regulating conformational changes within integrins.

6.3. Inside-Out Regulation by Membrane-Anchored Proteins

There are also a number of membrane-anchored proteins that may have a regulatory role on ligand binding activity (table 1). CD98 was identified using a strategy that depended on cloning a molecule that complemented the dominant suppression of a constitutively activated integrin. Antibodies crosslinking CD98 stimulated beta1-dependent adhesion (121), indicating that CD98 is involved in regulation of ligand binding activity of integrins, although the mechanism is still unknown.

Urokinase-type plasminogen activator receptor (uPAR) is a 55kDa heavily glycosylated cell surface protein that functions as a receptor to bind urokinase and VN at distinct sites (122). A direct interaction in vitro has been demonstrated between uPAR and beta2 integrins that is promoted by the active conformational state of the integrin and leads to the inhibition of adhesion to FN (123). uPAR also interacts with beta2 integrins activated by mAbs. This association was blocked by the addition of recombinant soluble uPAR or anti-uPAR mAb thereby inhibiting the alphaM beta2-dependent binding and demonstrating a specific interaction (123, 124). Whether the association is as a result of the activation of integrins or is responsible for the alteration in activation status is as yet unknown.

Another protein that is anchored to the cell membrane is integrin associated protein (IAP/CD47), a receptor for thrombospondin-1 (TSP-1), which spans the membrane several times (125). IAP has been implicated in modulating integrin functions in beta2 mediated neutrophil migration (126, 127) and alpha5 beta1 mediated...
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Phagocytosis in K562 cells transfected with alphaV beta3 (128). Binding of TSP-1 to IAP triggered alphaV beta3-mediated spreading in human melanoma; spreading was inhibited by the addition of anti-alphaV beta3 antibody and anti-IAP antibody (129). AlphaV beta3 ligand binding function has been shown to require IAP through direct interaction with the membrane-anchored IAP Ig variable domain. The membrane spanning and cytoplasmic domains of IAP were not required for integrin function (130).

The transmembrane 4 superfamily (TM4SF) consists of almost 20 homologous proteins with a structure that spans the membrane four times. Mainly through immunoprecipitation studies, TM4SF proteins appear to associate with beta1 integrins although CD81/alpha4 beta7 and CD9/alphaL beta2 complexes have been found. Although the mechanisms are still unclear, the functions of TM4SF proteins have been linked with integrin function in adhesion and cell motility (131).

6.4. Intracellular Signalling Pathways

A number of intracellular signalling pathways have been postulated to play an important role in exerting a regulatory effect on ligand binding activity of integrins. Phosphoinositide-3-kinase (PI 3-kinase) has been proposed as a downstream regulator of integrin activation in haemopoietic and endothelial cells. In haemopoetic cell lines, both mutation of the cytoplasmic motif of integrin regulators such as CD28 to prevent the association with PI 3-kinase and the use of PI 3-kinase inhibitor wortmannin results in the inhibition of beta1-mediated adhesion (132, 133).

A receptor for activated protein kinase C (PKC) named Rack1 has been shown to interact with the beta2 subunit cytoplasmic domain using the yeast two hybrid system (134). Rack1 was also shown to interact with other beta subunits such as beta1 and beta5 at a conserved membrane proximal region (resides 724-743) of the cytoplasmic domain. Interestingly, the interaction between full length Rack1 and beta integrins requires the presence of phorbol-myristate acetate (PMA), suggesting that PKC is involved. Whether it has a modulatory effect is not yet known although it may play a regulatory role since it interacts directly with integrins. Other signalling pathways may also act downstream following the interaction of integrins with more proximal proteins although the mechanisms involved have yet to be elucidated.

Activated H-ras has been shown to inhibit affinity modulation of alphaIIb beta3 induced by the co-expression of beta3-endoxin in CHO cells (97). The mechanism involved has yet to be deciphered, although it may involve a Raf-1-initiated ERK MAP kinase pathway. Activated H-Ras and Raf-1 kinase were found to suppress integrin activation as measured by the decrease in ability of CHO cells expressing an active alphaIIb beta3 chimera to bind PAC-1 (135). Antisense nucleotides targeting ERK1 and ERK2 expression resulted in a loss of cell migration, indicating that ERK1 and ERK2 MAP kinases play a role in the regulation of cell motility (136). In contrast, expression of a constitutively active R-Ras enhanced cell adhesion to extracellular matrix substrates by activation of the integrin, as determined by the ability to bind PAC-1 (137). Rho, a small GTPase, has also been implicated in the regulation of ligand binding activity. Alpha4 beta1 mediated adhesion in lymphoid cells induced using chemotactants was blocked by the use of Rho inhibitors although the precise mechanism is not yet clear (138).

7. PERSPECTIVE

Although X-ray crystallography and structure predictions have provided an insight into the structural domains of integrin subunits, the structure of an intact integrin has so far remained elusive. However, a number of ligand binding sites have been mapped within the extracellular regions of both alpha and beta subunits. Integrin-ligand binding is a highly regulated event with conformational changes determining the ligand binding potential of the receptor. It is clear that both subunits play a role in ligand specificity although how they interact with each other in the conformations predicted remains to be determined. Studies with anti-integrin mAbs and divalent cations have suggested the existence of both active and inactive conformations where only the active conformer is capable of ligand binding. It is apparent that the cytoplasmic tail, regulated by the interaction of specific intracellular proteins, is central to the signalling capacity of the integrin. Inside-out signalling may promote a structural change within the integrin that is transmitted through to the extracellular domain. Many different interacting cellular factors have been identified in the regulation of ligand binding activity. It is only now however, that researchers have started to investigate the actual mechanisms that may be involved. It may be that the essential mechanism of integrin activation may vary for different integrins, particularly with the discovery of proteins that demonstrate integrin subunit specificity. In addition, integrins may be activated by different signalling pathways that converge to use a common factor. It is not surprising then that the mechanisms of ligand binding regulation are likely to be complex given the co-ordinated control of specific integrin-ligand interactions required for cell function.

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Key words: Integrin, Structure, Ligand Binding Sites, Conformational Changes, Divalent Cations, Monoclonal Antibodies, Inside-Out Signalling

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