Molecular Environment of Integrins


Structural basis for ligand recognition by RGD (Arg-Gly-Asp)-dependent integrins

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Abstract

Since the discovery of the RGD sequence motif as the essential cell attachment site in Fn (fibronectin), RGD-dependent ligand recognition by integrins has been the major focus of many integrin researches. Although many integrins recognize RGD-containing ligands, it is believed that residues outside the RGD motif provide specificity as well as high affinity for each integrin–ligand pair. These ‘secondary’ sites are generally assumed to interact directly with the α subunit of integrin, whereas the RGD motif binds primarily to the β subunit. This necessitates that the integrin-ligand interface comprises a relatively large, or even scattered, area. Molecular electron microscopy and single-particle analysis were performed on a headpiece fragment of integrin α5β1 in the presence and absence of bound ligand (Fn fragment), and revealed a marked shape change of the β subunit hybrid and I-like domains that is linked with the ligand docking. Furthermore, electron microscopy images revealed a focal rather than a large contact area at the α5β1–Fn interface, raising a question about ‘2-site docking model’. Kinetic analysis of real-time binding data showed that the synergy site greatly enhances k_on but has little effect on the stability or k_off of the complex, suggesting that the synergy site exerts its positive effect on α5β1 binding by facilitating the initial encounter, rather than by contributing to the protein-protein interaction surface. Thus the ligand recognition mechanism by integrins needs further refinement through more structural analyses of the complexes as well as kinetic analysis of binding data.

The integrin family of cell-adhesion receptors comprises distinct 24 αβ heterodimers that recognize glycoprotein ligands in the extracellular matrix or on cell surfaces [1]. Integrins and their ligands play fundamental roles in all events that involve cell adhesion, detachment and migration, the hallmarks of multicellular organisms. Many members of the integrin family, including α5β1, α8β1, α11β3, αVβ3, αVβ5, αVβ6 and αVβ8, recognize an Arg-Gly-Asp (RGD) motif within their ligands. These ligands include Fn (fibronectin), fibrinogen, vitronectin, von Willebrand factor and many other large glycoproteins. In fact, peptides containing this motif can efficiently block these integrin–ligand interactions [2]. It is the residues outside the RGD motif, however, that provide specificity as well as high affinity for each integrin–ligand pair. α5β1 integrin and Fn form a prototype integrin–ligand pair [3,4]. This receptor–ligand pair is functionally very important, since it mediates Fn fibril formation and governs extracellular matrix assembly, which is vital to cell function in vivo [5]. The interaction between α5β1 and Fn is fundamental for vertebrate development, since lack of α5β1 or Fn results in early embryonic lethality [6–8]. In addition to the RGD sequence present in Fn type III module 10 of Fn, a set of residues present in FnIII module 9 (synergy site) contribute to the high-affinity recognition by α5β1 [9–14].

The crystal structure of the extracellular domain of αVβ3 integrin [15] has established a basis to think about integrin function on the atomic level. The subsequent structure of αVβ3 in complex with a ligand-mimetic peptide [16] provided a first glimpse as to how integrins recognize the RGD tripeptide motif, where arginine and aspartic acid side chains bridge integrin α and β subunits at the centre of the ligand-binding pocket. However, the structure provided little insights into how integrins achieve specificity and high affinity in recognizing physiological protein ligands. For example, the bound ligand mimetic cyclic peptide contacts a limited set of residues in αV and β3, burying only approx. 350 Å^2 (1 Å = 0.1 nm) surface area [16]. The contact was made almost exclusively via an essential RGD sequence; other chemical moieties did not comprise an interface. The peptide–integrin interface contained very little hydrophobic

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Abbreviations used: CPK, Corey–Pauling–Koltun; EM, electron microscopy; Fn, fibronectin.

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Figure 1 | Residues important for the recognition of protein ligands

Structure of αVβ3 [CPK (Corey–Pauling–Koltun) model, αV in blue and β3 in magenta] in complex with ligand mimetic peptide (cyclo-RGD-(d)-N-methyl-V, yellow stick model) is shown. Residues corresponding to the ones in αIIbβ3, which are known to be important for fibrinogen binding, shown in green, and ones that correspond to α5β1 residues involved in Fn recognition are in red [24]. MIDAS (metal-ion-dependent adhesion site) and ADMIDAS metals are shown in white.

interaction and was comprised mostly of electrostatic interactions involving positively charged arginine and negatively charged aspartic acid in the RGD motif. In physiological integrin–ligand interactions, however, many more residues in α and β subunits are involved in ligand recognition (Figure 1) and residues outside the RGD motif in the ligand make essential contribution to the association. Therefore it was essential to obtain structural information regarding the integrin in complex with protein ligand.

So far, no atomic resolution structure is available for non-I domain integrin in complex with protein ligands. Molecular EM (electron microscopy) of a complex between a recombinant α5β1 headpiece fragment and an Fn fragment resulted in an approx. 25 Å resolution structure [17]. In the absence of ligand, the projection average of the α5β1 headpiece was remarkably similar to the crystal structure of the extracellular domain of αVβ3 (Figure 2A). The globular head is asymmetric with a large density corresponding to the large β-propeller domain of the α subunit and a smaller density corresponding to the I-like domain of the β subunit. The α5 thigh domain and the β1 hybrid domain protrude from the longer axis of the globular head in an obtuse and an acute angle respectively. Therefore the unliganded α5β1 headpiece assumes a conformation referred to as ‘closed’, which was previously visualized by EM of αVβ3 containing the complete extracellular domain [18]. The very high percentage of particles in the closed conformation (>90%) confirmed that this is the most stable conformation of the unliganded α5β1 headpiece, even in the absence of the tailpiece.

In contrast with the unliganded integrin headpiece fragment, a complex of the same fragment and the Fn7-10 fragment had a three-legged appearance, with the α-tail, β-tail and the Fn fragment each forming one of the three legs (Figure 2B). There was a striking difference in the conformation of integrin before and after ligand binding. All of the α5β1 headpieces with a bound Fn fragment assumed the open conformation, with a nearly 80° swing out of the β-tail compared with the closed conformation. This unambiguously proves that there is a direct link between ligand binding and the opening of the angle between the I-like domain and the hybrid domain, as was suggested earlier [18]. The random conical tilt approach was used to calculate a three-dimensional reconstruction of the negatively stained α5β1 headpiece and its complex with Fn fragment (Figure 2C). The resulting
density map of the headpiece revealed all the expected integrin domains and allowed for a good fit with the segment of the αVβ3 crystal structure that corresponds to the headpiece. The large conformational change in the β subunit hybrid domain is evident in the liganded integrin, necessitating the rotation of the hybrid domain as a rigid body away from the remainder of the molecule by an angle of approx. 80° to adjust the crystal structure to the EM map (Figure 2C, liganded).

An unexpected feature of the complex is that the binding of the Fn7-10 fragment to the integrin headpiece was restricted to a single contact point at the end of the Fn7-10 fragment. The remaining segments of Fn molecule showed no defined orientation with respect to the integrin headpiece and radiated out from it in many different directions (Figure 2B). Because of this, FnIII modules other than Fn10 were averaged out in the projection averages, leading to the presence of only the Fn10 in the three-dimensional reconstruction (Figure 2C, liganded). The lack of stable interaction between Fn9 and integrin is inconsistent with the notion that the synergy site in the 9th FnIII module directly binds to the β-propeller domain of the α5 subunit [12,14]. Real-time surface plasmon resonance binding assays using wild-type and the R1374A/P1376A/R1379A mutant Fn9-10 (∆SYN Fn9-10) revealed that the deletion of the synergy site mainly affected the association phase (15-fold decrease in $k_{\text{on}}$), whereas the dissociation phase was only slightly affected (1.8-fold increase in $k_{\text{off}}$) [17]. In general, mutations in residues that are part of protein–protein interfaces affect mostly the dissociation rate with minimal effects on the association rate [19–21]. Therefore the EM observations and kinetic measurements of the α5β1–Fn interactions do not support the two-site binding model, which proposes that the RGD loop and the synergy site latch simultaneously on to widely separated binding pockets on the β1 and α5 subunits respectively. Instead, they suggest that FnIII module 9 supports integrin binding in an indirect fashion, although its contribution to the overall affinity is substantial.

A model of the α5β1 integrin headpiece created on the basis of the crystal structure of αVβ3 revealed that there is an extensive stretch of acidic surface on the top of the α/β interface, where the ligand-binding site is located (Figure 3, left). It is important to note that five out of eight mutations in the Fn9-10 fragment reported to disrupt synergy effect are arginine to alanine mutations [12]. Aside from Asp1495, which is part of the RGD sequence, the Fn9-10 fragment contains several conserved acidic residues on the same face as the RGD loop, namely Glu1372, Asp1373, Glu1453 and Glu1462 in the human sequence (Figure 3, right). When nearby basic residues are mutated to alanine, negative potential would dominate because of these acidic residues, leading to the decreased association rate by an electrostatic repulsion from the acidic integrin surface. Furthermore, if the synergy site forms part of the Fn–α5β1 interface in the final complex, as the two-site model proposes, there must be a specific complementary site on the α5 subunit. However, the effect of the synergy site is universal rather than α5-specific, because a surface plasmon resonance binding assay using recombinant soluble αVβ1 showed a similar decrease in the affinity when the synergy site in Fn9 is mutated (J. Takagi, unpublished work). Therefore it is probable that the basic residues in the Fn9 module do not make direct contact in the final complex but mediate
long-range electrostatic steering, thus contributing to the overall affinity.

Many integrin heterodimers share RGD-recognizing capability while showing ligand specificity. Traditionally, this ‘fine-tuning’ of ligand specificity has been attributed to a complementary binding between α subunit and a second site on ligand molecule, exemplified by the α5 and the synergy site in Fn9. The above discussion does not rule out the possibility that residues other than RGD motif contribute directly to the interface with integrin, but such residues may constitute continuous contact surface together with the RGD site. In the Fn10–α5β1 complex visualized by EM, orientation of Fn10 relative to α5β1 is roughly fixed. Therefore it is possible that there are essential residues for integrin binding in Fn10 yet to be identified. Previous work relied on a cell-adhesion assay when determining the effect of certain mutations on the integrin binding activity of Fn fragments. However, the cell-adhesion assay is susceptible to factors other than the affinity for integrins, such as the stability of the mutant recombinant protein [11,22,23], and is not suitable for quantitative analysis of the kinetic behaviour of the binding. Protein–protein binding analysis using purified recombinant integrins and ligand proteins used in the studies described above will be more appropriate for this purpose. Such experiments are required for more precise, residue-by-residue understanding of the integrin–ligand interface, until the atomic resolution structure of the integrin–ligand complex becomes available.

References

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