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Biochemical Pharmacology 64 (2002) 805–812

Biochemical Pharmacology

Divalent cations and the relationship between αA and βA domains in integrins

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Received 31 January 2002; accepted 19 March 2002

Abstract

Integrins contain either one or two von Willebrand factor A-like domains, which are primary ligand and cation binding regions in the molecules. Here we examine the first structure of an A domain of a β subunit, in αvβ3 and compare it to known A domain structures of α subunits. Ligand binding to immobilized αvβ3 domain is stimulated by Ca^{2+} rather than inhibited by it. Biochemical, cell biological and structural evidence suggests that the A domain is a major site of ligand interaction in αvβ3. The Arg–Gly–Asp based inhibitor cilengitide (EMD 121974) inhibites ligand interaction with transmembrane-truncated αvβ3 in the presence of either Ca^{2+} or Mn^{2+} ions, and does so with similar kinetics. The αvβ3 structure reveals that both the αA and βA domains share common structural cores. But, in contrast to αA, the βA domain has three cation binding sites, that are involved either directly or indirectly in ligand binding. Structural alignment of αA and βA domains reveals additional loops unique only to the βA domain and much evidence support that that these loops are important for ligand binding specificity and for the interaction between α and β subunits. Since the position of these loops are evolutionary conserved but their primary sequence varies between the various βA domains, they represents potential targets for dissecting functional diversity among integrins.

Keywords: Integrin; αvβ3; αA domain; βA domain; Crystal structure

1. Introduction

Integrins are a family of heterodimeric class I transmembrane signal transducing proteins that control many vital processes during embryonal development and during conditions as diverse as cancer, inflammation, cardiovascular disease, rheumatoid arthritis, wound healing and microbial infection. They depend on divalent cations for their ligand binding activity, and can be activated by intracellular or extracellular stimuli (reviewed in [1]). Given their ubiquitous importance, it is hardly surprising that integrins form a substantial field of research, with MEDLINE showing over 30,000 citations since their naming in 1986 [2]. Despite this mass of data, we know little about how integrins function at a molecular level. This is unfortunate, because integrins appear to be excellent therapeutic targets. Drug development in the absence of molecular structure is usually more-or-less informed, but always extremely expensive, guesswork. We have attempted to discover the three-dimensional structure of intact integrins.

There are two major series of integrins, those where the α subunit contains an extra inserted region of ~180 amino acids, the αA domain [3,4], and those that do not (Fig. 1). The αA domains from both β2 and β1 series integrins have been crystallised, and directly bind to ligand in a divalent cation dependent manner [5,6]. The structural elucidation of αA domains led to a model where a A-like domain was located to the β subunit [7]. The presence of this domain has now been confirmed within the structure of an intact non-αA domain integrin, αvβ3 [8]. Both the A and A-like domains share structural homology to von Willebrand factor A

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Abbreviations: ADMIDAS, Adjacent to the Metal Ion Dependent Adhesion Site; LIMBS, Ligand associated Metal Binding Site; MIDAS, Metal Ion Dependent Adhesion Site; VEGF-A, Vascular endothelial growth factor-A.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01142-5

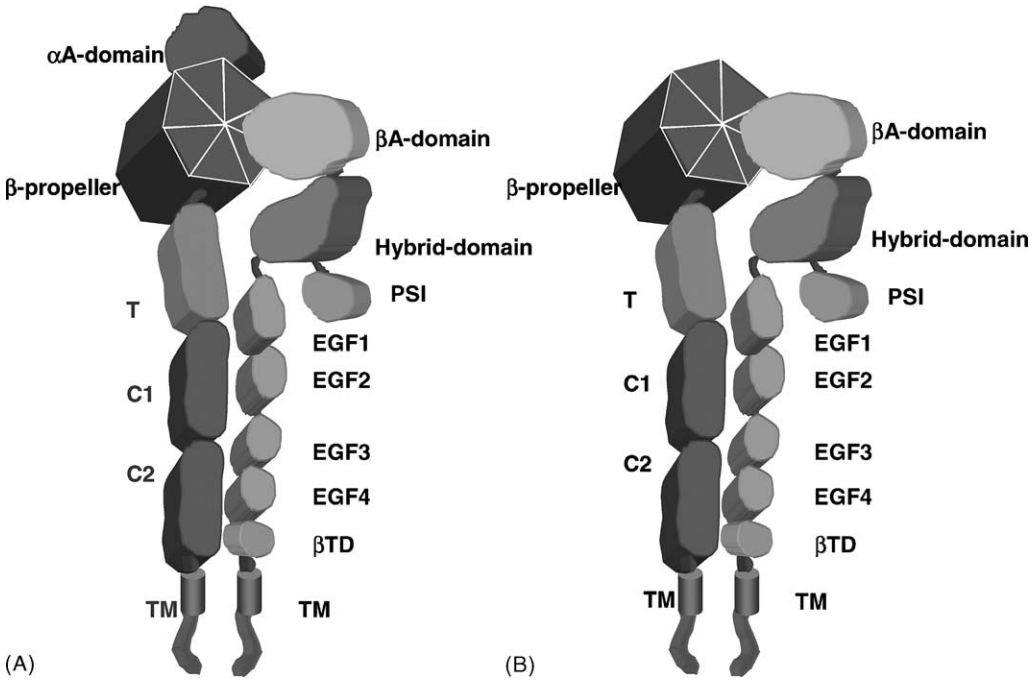

Fig. 1. Schematic representation of the structure of integrin α and β subunits. The α subunits (left chains) are in two classes, those with an additional αA domain (A) and those without (B). All β subunits (right chains) appear to have an βA domain. The α subunits N-terminal has a 7-fold repeat, formed into a β-propellor, followed by a thigh (T) and two calf domains (C1, C2), a single pass transmembrane helix and a small cytoplasmic domain. The β subunits have an N-terminal PSI domain which is linked via an S-S bond to the EGF1 domain and an IgG-like domain into which is inserted the βA domain, to form an IgG-A hybrid domain, C-terminal of the hybrid, are 4 EGF-like domains (EGF1-4) followed by a single pass transmembrane helix, and a short (excepting β4) cytoplasmic domain. In some integrins, including αv, the α chain is cleaved in domain C2 to produce an N-terminal heavy and a C-terminal light chain. The αA-domain probably lies on the face of the β-propeller adjacent to the βA domain.

domain and hence the terms αA and βA domains. Integrins can, therefore, be divided into two classes, those with one or those with two A domains. For integrins with two A domains, it is the αA domain that mainly participates in ligand binding: little is known of the role of the A domain in the β subunit [5,6,9]. The structural conservation between αA and βA domains is so great that integrins with a single A domain in the β subunit presumably also use that domain in the main ligand binding site. The ligand binding mechanisms of the αA domains have been extensively studied. Since A domains in each subunit display common structural folds, we examined how this homology could help us to understand the as yet unknown binding mechanism of the βA domain.

Integrin αvβ3 lacks an αA domain, and is an interesting target in cancer, osteoporosis, rheumatism, macular degeneration and cardiovascular disease [10,11]. It is a promiscuous receptor for ligands containing the prototype integrin recognition sequence NH2–Arg–Gly–Asp–COOH. The recombinant extracellular domains of human αvβ3, surprisingly, have similar ligand binding properties to the full length molecule [12]. The αvβ3 is unusual amongst the integrins as being usually found in an activated state, i.e. it can bind its ligands from the extracellular matrix under physiological conditions without additional prior activation—and this ligand-binding state is retained when the molecule is isolated. It appears that in some cases αvβ3 in situ can be further activated in response to extracellular stimuli like VEGF-A [13]. This is in marked contrast, for example, to integrins of the β2 series, or to αIIbβ3, which must be activated before they bind ligands [1]. There is excellent evidence that activation of integrins generally requires change in conformation: for integrins with an αA domain, the activation involves a large realignment on the surface of the A domain involving the α7 helix, which descends ∼1 nM and moves away from the surface of the helix [14,15]. The homologous helix in the non-αA domain integrins has so far only been visualised once, and lies in a position that would correspond to the activated rather than the inactive form of an equivalent αA domain [8]. Furthermore, a release of constraints at the C-terminal of the α–β complex can transform inactive to active states in α5β1 [16], and the form of αvβ3 we examined was unconstrained at the C-terminal in solution. Thus the biochemical and structural indicators suggested that the crystal form of human αvβ3 is likely to be capable of binding ligand (active).

In this commentary we consider the relationship between αA and βA domain [8]—and examine how the A domains of the αA and βA domain integrins may function—especially with respect to divalent cations.

2. Materials and methods

Recombinant human αvβ3 integrin was produced as described [12]. Extracellular matrix proteins were purified

and labelled with biotin [17]. Ligand binding to isolated integrins was measured as previously detailed, using integrins adsorbed to 96-well plates, and biotinylated ligands [12]. Ligand inhibition assays and inhibitory IC_{50} were measured as detailed [18]. The αvβ3 inhibitory peptides cyclic (Arg–Gly–Asp-D–Phe–Val) (EMD 66203) and cyclic (Arg–Gly–Asp-D–Phe–[N-Me]–Val) (EMD 121974-cilengitide) [17] and the αvβ6-inhibitor EMD 221935 [18] and inactive cyclic (Arg-ß–Ala–Asp-D–Phe–Val) (EMD 135981), were the gift of Dr. A. Joncyzk (Merck KGaA). Cation dependency assays will be detailed elsewhere (Welge *et al.*, manuscript in preparation). Briefly, concentrated cations, serially diluted in Tris buffered saline (TBS) were added to integrin coated plates, followed by biotinylated ligands diluted in TBS. After 3 hr at 37° bound ligand was measured [12].

3. Results and discussion

3.1. αvβ3 binds ligands under the conditions of crystallisation

The recombinant water soluble αvβ3 bound ligands vitronectin, fibrinogen and fibronectin in the ionic conditions under which it was crystallised (Fig. 2). Furthermore, peptidic inhibitors of αvβ3, like cyclic (Arg–Gly–Asp-D–Phe–[N-Me]–Val) were equally active, and control peptides were inactive under these conditions.(Table 1) [17]. This suggests that the 'snapshot' seen in the crystal structure probably represents a ligand-competant form of αvβ3 integrin [8].

In millimolar concentrations Ca^{2+} suppresses ligand binding both to αA containing integrins like α1β1, α2β1 [19,20] and to integrins lacking αA, like α5β1 [21]. The mechanism of this suppression for α5β1 involves Ca^{2+} competing for Mn^{2+} or Mg^{2+} binding sites. Ca^{2+} has also been reported to inhibit αvβ3 [22], however, micromolar amounts of calcium are proadhesive.

We find that vitronectin, fibronectin and fibrinogen bind to our immobilized αvβ3 preparations in millimolar Ca^{2+} (Fig. 2). Ligand binding assays measure affinity or avidity; binding or integrins in solution to immobilized ligands generally reflects changes in affinity [22] whereas the

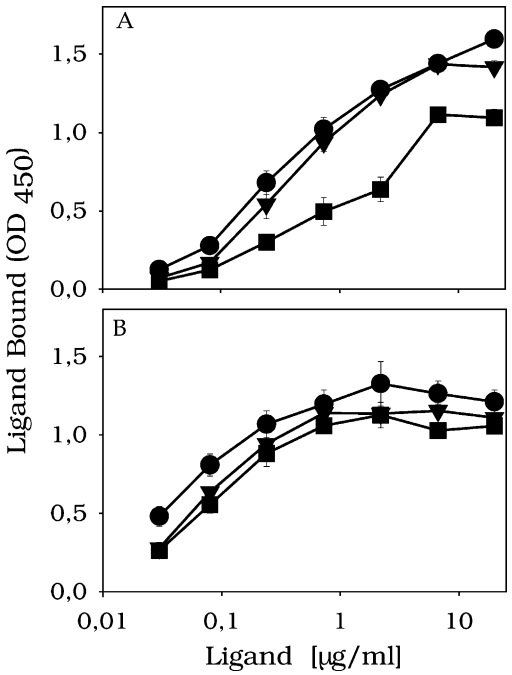

Fig. 2. Integrins αvβ3 is active under ionic conditions used in crystallisation. The binding of biotinylated ligands to immobilised integrin αvβ3 was measured (A) in the presence of 5 mM calcium ions in crystallisation buffer (100 mM NaCl, 100 mM MES: pH 6.0) or (B) at physiological pH and salt (150 mM NaCl, 50 mM Tris–Cl: pH 7.4). Vitronectin (circles), fibronectin (squares) or fibrinogen (triangles).

reverse (i.e. our assay) is sensitive to alterations in avidity (i.e. multiple low affinity interactions). The different effects of millimolar Ca^{2+} on ligand binding to the same integrin may be explained by these differences. Interestingly, the αvβ3 crystal structure shows a Ca^{2+} (or Mn^{2+}) ion at a site adjacent to the metal-ion-dependent-adhesion-site (MIDAS) but no metal ion at MIDAS itself, when crystals are generated in 5 mM Ca^{2+} or Mn^{2+} buffer, respectively. MIDAS is clearly occupied by Mn^{2+} as well when bound to cilengitide in Mn^{2+}-containing buffer, with a third Mn^{2+} bound in βA at a novel ligand-associated-metal-binding-site (LIMBS) [39]. In Ca^{2+}-containing crystallization buffer, Ca^{2+} is poorly bound to MIDAS even when ligand is present and no Ca^{2+} is found at LIMBS, suggesting that the LIMBS cation is bound secondarily to the occupation of MIDAS by cation. These data

Table 1
Integrin αvβ3 can be inhibited with similar kinetics under mock physiological conditions and under conditions used in crystallisation

Inhibitor	Specificity	Conditions; IC_{50} (nM)			
		pH 7.4 + Ca^{2+}	pH 6.0 + Ca^{2+}	pH 7.4 + Mn^{2+}	pH 6.0 + Mn^{2+}
c(RGDfV)	αvβ3	13	19	9.6	9.2
c(RGDf–[N-Me]-V)	αvβ3/αvβ5	1.8	1.8	3.8	3.3
c(R-ßA-DfV)	Null	>10,000	>10,000	>10,000	>10,000
c(RTDLDSLRT)	αvβ6	>10,000	>10,000	>10,000	>10,000

Integrin inhibition assays, using biotinylated vitronectin as ligand (1.5 µg/mL) were performed in normal (150 mM NaCl pH7.4 in Tris–Cl), or crystallisation buffer (100 mM NaCl pH 6.0 in MES), in the presence of 5 mM calcium or 2 mM manganese ions (cf. [8]). The IC_{50} for inhibition of ligand binding is shown. Note that the buffer and salt conditions have little effect on the sensitivity to inhibition of the integrin.

suggest that βA contains a low affinity Ca^{2+} site (in MIDAS) and a high affinity site at ADMIDAS. The former may facilitate low affinity binding in assays sensitive to avidity, but may be insufficient (or inhibitory) for high affinity interaction. The function of the metal ions at ADMIDAS and LIMBS is unclear; structurally, they appear to rigidify the ligand-binding interface in βA. The integrin in the crystals can bind a peptide ligand and show that it is ligand-competent.

3.2. Integrins have either one or two A domains

Nine of the known eighteen α subunits contain an additional domain, the αA domain, looping out from a predicted seven-bladed β-propeller of the α subunit. The αA domain was first isolated for biochemical ligand binding studies from the integrin Mac-1 (CD11b/CD18 or αMβ2) by Arnaout and colleagues. Since the αA domain was not found in all integrins, it was a surprise to find that it could bind ligand in a manner similar to that of the intact receptor, suggesting that it is a pivotal ligand binding site.

The first αA domain crystal structure was determined from integrin Mac-1 (CD11b) and was shown to adopt a classical dinucleotide-binding fold previously only observed in intracellular proteins, such as the α subunits of the heterotrimeric G proteins [4]. There were two interesting observations associated with this finding. First, the αA domain contains a conserved novel cation binding site designated the MIDAS. Mg^{2+}, and in subsequent structures, Mn^{2+} were co-ordinated by a conserved Asp-X–Ser-X–Ser motif and two residues that are not contiguous in sequence, Thr209 and Asp242. Secondly, based on a secondary structure prediction of a ∼240-amino acid conserved domain of the integrin β subunit revealed that this sequence also resemble an A domain [6], a suggestion confirmed with solution of the structure of αvβ3.

3.3. Ligand binding induces conformation change from 'closed' to 'open' form of αA domain

Recent structural determinations and studies of numerous A domains have advanced our understanding of their ligand binding mechanism. There is strong evidence that the metal ion at MIDAS is obligatorily coordinated by an acidic residue of the ligand and thus participates directly in ligand binding. In the Mac-1 crystal structure, the octahedral coordination of the Mg^{2+} ion was completed by a Glu side chain from the A domain of a neighboring molecule in the crystal [3]. Based on the conservation of the Arg–Gly–Asp motif in many integrin ligands, and the fact that all integrin–ligand interactions are strictly dependent on divalent cations, Arnaout, Liddington and colleagues proposed that the observed crystal contact represented a ligand mimetic [23]. Further, structural elucidation of A domains from Mac-1 with Mn^{2+} as the metal ion [23], from LFA-1 with no metal ion or with either Mg^{2+} or Mn^{2+} ion [6,24], and from α1 and α2 chains [25,26], suggests that A domain exists in one of two conformations, 'open' or 'closed', depending on the position of the C-terminal α7 helix.

When the A domain is bound to a ligand-mimetic, α7 undergoes a dramatic descent of 10 Å, leading to an 'open' conformation as compared to unligated 'closed' form. The issue of whether the two conformations truly represent ligand-occupied and -unoccupied states, and whether the metal ion is in fact replaced by acidic residue from the ligand was resolved with the solution of the structure of the α2 A domain in complex with a triple helical peptide derived from collagen I [6]. This structure confirmed the predictions of Lee *et al.* [3,23] and provided the first definitive evidence for a central and direct structural role for integrin metal ions in binding ligand. Comparison of the ligand-occupied and -unoccupied A domain structures indicates that ligand binding forces rearrangement of residues co-ordinating the metal such that a direct metal bond to Asp254 (equivalent to Asp242 in CD11b) is lost while a direct bond to Thr221 (equivalent to Thr209 in CD11b) is gained. These subtle changes in metal-coordination are linked to more extensive secondary and tertiary changes that causes the C-terminal α7 helix to descend and transform the structure from a 'closed' into an 'open' conformation.

Thus, structural transitions propagate from the 'top' (MIDAS) surface of the domain, where ligand binds, to the opposite pole, near where the A domain interfaces with the rest of the α subunit. Conformational changes upon ligand binding were also recently detected in an NMR study using the LFA-1 A domain and its ligand ICAM-1 [27]. Evidence increasingly suggests that the lower surface of A domain is regulating ligand binding. In the closed form, the helix α7 has an extra N-terminal turn and is tethered to the domain body through hydrophobic contacts that include insertion of a conserved isoleucine from α7 into a socket-for-isoleucine (SILEN) site. Arnaout and colleagues [28] identified mutations that remove this contact in the hydrophobic pocket and shift α7 by 10 Å, which switch the A domain to the 'open' high affinity form. The importance of this site is further supported by the finding of A domain peptides that block activation [29] and mutations that lead to constitutive activation of Mac-1 [30], which map to this lower surface. The crystal structure of the LFA-1 A domain, in complex with the inhibitor lovastatin, reveal that the inhibitor binds in SILEN at the bottom of the domain near the C-terminal helix [31]. It seems, therefore, that this region of A domain is important for allosteric control of ligand binding.

3.4. Prediction of common structural features between αA and βA domains

The mid-segment of the integrin β subunit has been implicated in the ligand and cation binding functions of the β3 integrins. This region has been predicted to share

structural folds similar to the αA domain based upon similarities in their hydropathy profiles, secondary structural predictions and mutational analyses [3,32–34]. For integrins that have only one A domain, for example αvβ3, recent studies have implicated the presence of not one, but two, functionally distinct classes of ion binding sites using ligand binding reaction into association and dissociation steps [22] and subsequent surface plasmon resonance [35,36].

Since βA domain shares similar structural fold to those of integrin αA domain and G protein α subunit, it is interesting to examine if βA domain may undergo conformational change upon ligand binding or activation in a way similar to that observed for both the integrin αA domain and G protein. Furthermore, by determining the common structural core shared between αA and βA domains, one can detect structure features that are unique to each of the two domains. The detected structural differences may reveal interesting insights into the functional difference between them, allowing us to gain a better understanding of how two domains with a common structural fold, both present at the N-terminal regions of their respective subunits, have evolved, perhaps under different functional and structural constraints, to interact with a diverse range of ligands.

3.5. αvβ3 crystal structure reveals novel structural differences between αA and βA domains

The solution of the αvβ3 structure [8] allows detailed structural comparisons between βA and αA domain structures. The αvβ3 crystal structure confirms that the A-like domain in the β subunit indeed adopts a dinucleotide-binding fold. Though both the αA and βA domains share weak-to-no sequence similarity, both domains share many interesting similarities at the structural level. As in the αA domain, a MIDAS motif occupies a crevice at the top surface above the central β-strands. The side chains that form the MIDAS motif are equivalent in positions in both the αA of CD11b and βA of αvβ3. Thus the overall geometry of the metal ligand co-ordination is similar to that of αA. However, in contrast to αA, a metal ion is not found at MIDAS in the unligated state; instead a metal ion is found at ADMIDAS. When βA is ligated, the metal ion coordination in αA and βA is almost identical, and a third ion at LIMBS, unique to βA, is bound.

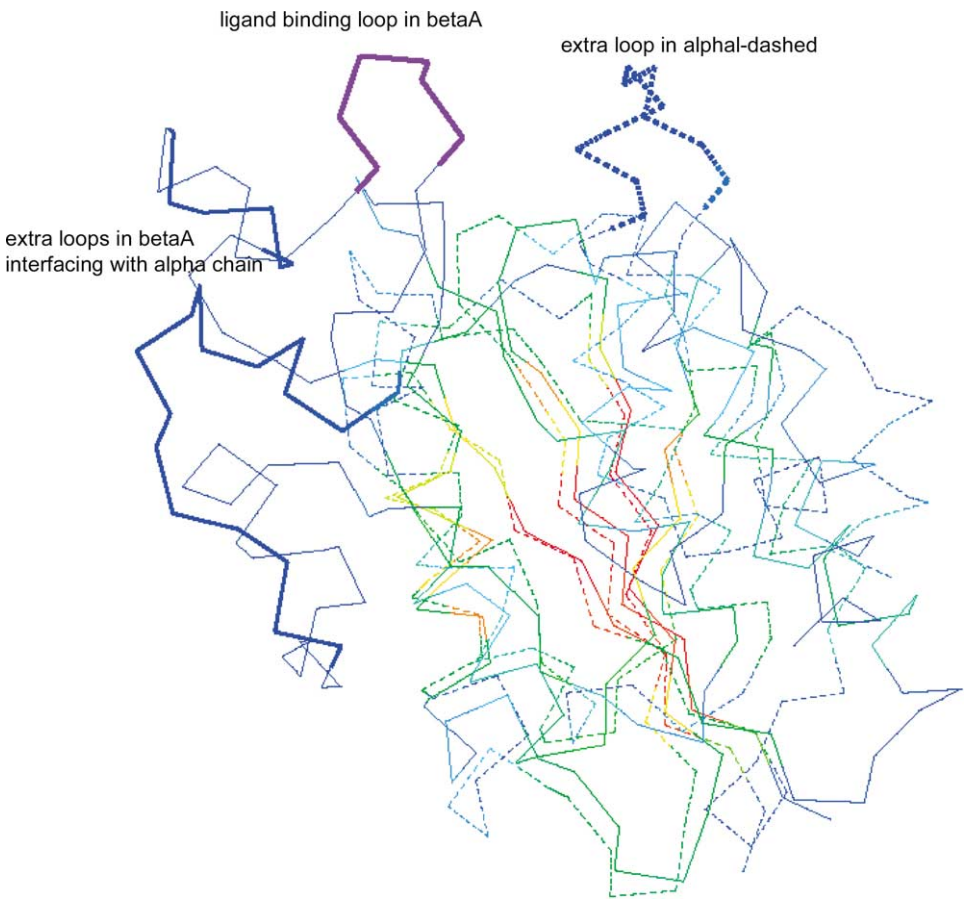

Fig. 3. Wire-frame diagram of a structural-based superimposition of αA and βA domain from integrin α2β1 (dashed lines) and αvβ3 (solid lines) subunits. The colour coding highlights the degree of structural conservation [38], with red being most similar, and blue having no corresponding structure at all. The main differences are in peripheral loops. The two additional loops in βA are serving two separate functions. One is to interface with the α chain (bold blue lines, left), the other is putatively the interaction with the protein ligand(s) (magenta, top left). The extra loop in αI (bold dashed lines, top right) is involved in collagen binding.

3.6. αvβ3 crystal structure resembles an activated form of integrin

With the exception of the two large insertions in the βB-βC and βD-β5 loops, βA domain structure can be superimposed reasonably well on that of the αA domain. The structural superimposition (Fig. 3) facilitates the evaluation of the position shift in α7 of βA and allows direct comparison with the corresponding positions of α7 of αA domain in both the 'open' and 'closed' forms. This analysis clearly shows that the position of α7 of βA domain is at the 'open' form, and this includes the additional similarities such as the lack of an extra turn at the top of α7, and that the α7 is positioned similarly relative to the body of the domain. Despite the lack of ligand binding, these structural features suggest that the βA domain of the αvβ3 structure resembles and is more consistent with the open form of αA domain. The fact that βA domain can exist in an open form without ligand binding, unlike the αA domains, suggests that there are perhaps differences in the mechanism of regulation between the αA and βA domains.

3.7. Specific loops distinguish αA from βA domains

Beside the common structural cores shared between the αA and βA domains, multiple alignment of protein sequences of all human αA and βA domains (data not shown) indicates that βA domains have additional unique insertion(s) not present in the αA domain and *vice versa*. Based on the structural alignment between the αA domain of α2 subunit and βA domain of β3 subunit (Fig. 3), the βA domains has two additional unique loops (insertions at βB-βC and βD-β5 loops) that are not found in the αA domains. Only a single unique loop can be detected in the αA domain that is not found in the βA domain. To study if these additional loops are conserved among members of αA and βA domains, homology modeling of all human αA and βA domains was performed (data not shown). Structural alignments of these domains clearly show that all share common structural cores, and generally there are two additional loops among members of βA domains that are conserved in their insertion positions into the structural cores, and which are absent among members of αA domains. The fact that

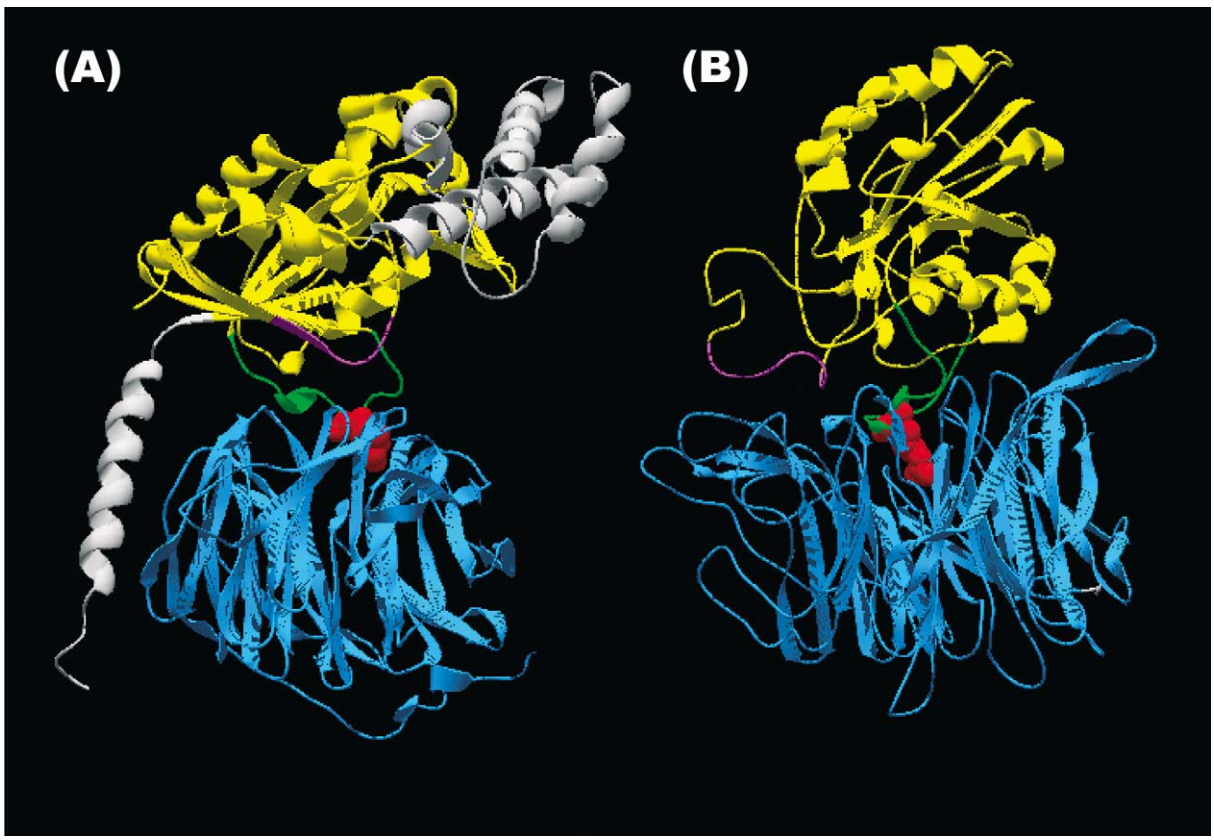

Fig. 4. Ribbon diagram highlighting the common structural elements involved at the G-protein (G-α/G-β) and the integrin (βA/propeller) interfaces. The G-protein and integrin are shown in (A) and (B), respectively. The dinucleotide-binding folds and the propeller of each proteins are shown in yellow and blue respectively. Two loops from each structural fold contribute to the interface. The first loop type is in green and contains the helices at the central location that form the core of the binding interface (3_{10} and switch II helix for integrin and G-protein, respectively). The side chains of residues (Arg261 for integrin and Lys210 for G-protein) that project into the central cavity of respective propeller are represented as Van der Waal. The second additional loop regions that contribute to the interface and interact with the periphery of the propellor structure is shown as pink. G-protein co-ordinates used were derived from pdb file 1GP2.pdb. In this figure, G-α starts with Ser2 and ends with Asn340. The G-β N-terminal helix (Leu5–Arg32) and the G-γ subunit are not shown. Integrin protein co-ordinates are from [8].

these additional loops are conserved in positions among βA domains suggests their biological significance. Indeed, for the unique loop inserted at βB-βC, previous studies have demonstrated that a swap of a single 39-residues segment of β3 subunit (aa164–202, which covers the entire unique loop at βB-βC) into the backbone of β5 enabled the resulting hybrid β subunit to adopt a ligand binding specificity expected of β3 instead of β5 subunit [37]. The fact that they are of slightly variable lengths but unique in protein sequence among members of β subunits strongly suggest that these loops could play an important role in ligand binding specificity, at least for those integrins with only one A domain.

Detailed analysis of the interface between the βA domain and propeller domain structure of αv subunit highlights regions of βA domain that may be important for αv and β3 subunit interaction. The unique βD-β5 loop projects a 3_{10} helix into the central cavity of the propeller structure. Additionally, part of the unique βB-βC loop also contributes side chains that interact with the peripheral of the propeller structure (Fig. 4). It is conceivable that since these loops are unique in protein sequence among members of β subunits, they may play a role in defining the interaction specificity between α and β subunits. For αA domain of α2 subunit, there is a unique loop not predicted to be present among βA domains (Fig. 3). The loop has a short C helix in the closed structure but this helix fully unwinds in the open conformation. Analysis of this region in the complex of the A domain and collagen suggests that the opening of this region create the space needed for the docking of collagen onto the upper surface of A domain [6]. In short, these unique loops detected through structural alignments of integrin A domains are interesting candidates for further studies to clarify the structural basis for the functional diversity observed amongst integrins.

In conclusion, αA and βA domains in integrins are remarkably similar in structure, but not primary sequence, and have distinct structural variations related to function. In both, the A domains are a major divalent cation binding site, which modulates the ligand interaction with the integrin. With the identification of the cation binding sites in an unliganded and a liganded integrin, the biological rational for the subtleties in ion specificity between integrins can now be investigated.

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