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αIIbβ3: structure and function

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Summary

During the past decade, advanced techniques in structural biology have provided atomic level information on the platelet integrin αIIbβ3 activation mechanism that results in it adopting a high-affinity ligand-binding conformation(s). This review focuses on advances in imaging intact αIIbβ3 in a lipid bilayer in the absence of detergent and new structural insights into the changes in the ligand-binding pocket with receptor activation and ligand binding. It concludes with descriptions of novel therapeutic αIIbβ3 antagonists being developed based on an advanced knowledge of the receptor's structure.

Keywords

electron microscopy; integrin alphaIIbbeta3; platelet; thrombosis; x-ray crystallography

αIIbβ3 structure in a lipid bilayer

αIIbβ3 is the paradigmatic integrin receptor, with studies of its structure and function, including inside-out signaling, ligand binding, and outside-in signaling, leading the way in understanding the biology of the entire family of receptors [1]. It is required for normal hemostasis as demonstrated by the lifelong bleeding diathesis suffered by patients with Glanzmann thrombasthenia, who lack the receptor or receptor function on a genetic basis [1]. It is also a validated drug target because it plays a central, non-redundant role in uncontrolled platelet aggregation leading to thrombosis and ischemic cardiovascular disease [2]. Some genetic variants of αIIbβ3 are immunogenic and lead to the alloimmune disorders neonatal alloimmune thrombocytopenia and post-transfusion purpura [3]. This review will focus on recent structural findings related to the conformation of αIIbβ3 in a lipid bilayer and the changes in the ligand-binding pocket with ligand binding, along with implications of the latter for the development of novel αIIbβ3 antagonists for clinical use.

A number of αIIbβ3 structures have been reported, and the results have dramatically improved our understanding of the relationship between structure and function. These include (i) crystal structures of the unliganded αIIbβ3 ectodomain and the liganded αIIbβ3 headpiece [4–8], (ii) NMR structures of the transmembrane and cytoplasmic domains of

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Disclosure of Conflict of Interests

In accord with federal law and institutional policies, B. Coller has royalty interests in abciximab (Centocor) and the VerifyNow assays (Accumetrics). Rockefeller University has applied for patents on the RUC compounds.

αIIb and β3 [9–12], (iii) rotary shadowing and/or negative stain 2-dimensional (2D) images of αIIbβ3 in detergent, with or without ligands or mAbs [13–15], (iv) transmission electron cryomicroscopy (cryo-EM) of αIIbβ3 in detergent [16], (v) small-angle neutron scattering (SANS) of αIIbβ3 in detergent [17], (vi) small-angle x-ray scattering (SAXS) of αIIbβ3 in detergent [18], (vii) cryoelectron tomography of αIIbβ3 in liposomes [19], (viii) electron tomography of activated αIIbβ3 in detergent [20], and (ix) negative stain EM of αIIbβ3 in lipid bilayer nanodiscs, with and without the talin head domain [19,21,22]. While each structure has provided very valuable information, the data are not concordant. In particular: (i) the crystal structure of the unliganded receptor does not fit snuggly in the cryo-EM, SAXS, or SANS maps (Fig. 1); (ii) the 3D reconstruction of the cryo-EM maps shows the head domain pointing away from the putative membrane, whereas the reconstructions of the SANS and SAXS images were interpreted as being most consistent with the crystal structure oriented so that the head points toward the membrane; and (iii) the cryo-EM data suggest that the lower legs are bent, whereas the crystal structure of the ectodomain and the SANS and SAXS studies were interpreted as consistent with the straight, parallel, and adjacent lower leg conformations seen in the crystal structure [4].

Nanodisc technology enabled studies of intact, purified αIIbβ3 in a lipid bilayer in the absence of detergent, an important intermediate step in understanding the conformation of the receptor in a platelet membrane. Ye *et al.* [19] performed 2D EM imaging of αIIbβ3 in nanodiscs and found a compact structure for the receptor in the absence of activation (Fig. 1); the addition of the talin head domain resulted in an increase in the percentage of receptors adopting either a fully extended conformation or a slightly less compact shape. They proposed a model in which the head domain points down toward the membrane. Our laboratory built on these pioneering studies and analyzed more than 12 000 pairs of tilted and untilted negative stain images of unactivated full length αIIbβ3 in nanodiscs to develop a 3D reconstruction of the electron density map at 20.5 A resolution (Fig. 1) [22]. We then obtained a first approximation of a 3D molecular model of αIIbβ3 in the nanodisc by molecular docking of the αIIbβ3 ectodomain crystal structure domains into the EM electron density map, using the binding of monoclonal antibodies with known epitopes to guide the process (Fig. 1). The most striking feature of our 2D images and the resulting 3D reconstruction was the orientation of the head domain away from the membrane. In addition, we did not find the leg domains to be straight and parallel; instead, there was a bend between the calf-1 and calf-2 domains of αIIb and a coiled path for the β3 IEGF-2–4 domains, with the latter entering into relatively few contacts with neighboring structures rather than lying in a cleft between the β3 headpiece and the αIIb lower leg. These data suggest an important role for the region that links the distal calf-2 and β-tail domains to their respective TM domains in transmitting the conformational changes in the TM domains associated with inside-out activation. This interpretation is supported by studies demonstrating that alanine mutations of key residues in the β3 I-EGF-4 and β-tail domains produce constitutively active receptors [23]. The 3D reconstruction also suggests that receptor extension requires changes in multiple interdomain articulations rather than a change in a single fulcrum in each subunit. Advances in cryo-EM technology, especially improvements in detectors, should allow for higher resolution images of unliganded and liganded αIIbβ3, providing better domain fitting and insights into how inside-out signaling initiates the conformational

changes responsible for ligand binding. The resulting predictions will, however, require independent confirmation through mutagenesis and functional studies.

Ligand binding to the RGD binding pocket

The pioneering crystallographic studies of Xiong *et al.* [24] on RGD peptide binding to αVβ3 provided the first atomic resolution description of ligand binding to a β3 integrin receptor, but the study was limited because the ligand was soaked into the crystal. Xiao *et al.* [5] provided the first description of ligand binding to the αIIbβ3 headpiece at the atomic level based on crystal structures, including the mechanisms of binding of the two small molecule αIIbβ3 antagonists in clinical use, eptifibatide and tirofiban (Fig. 2A). The crystal structures of the unliganded αIIbβ3 ectodomain [4], the complex of αIIbβ3 with the fibrinogen γ chain dodecapeptide [7], and intermediate states of αIIbβ3 between the closed, inactive conformation and the open, active conformation [8] provided a wealth of information on the changes that accompany ligand binding. Three metal ions in the β3 I domain play important roles in ligand binding, the MIDAS, SyMBS, and ADMIDAS, and each is discussed below. When crystalized in the presence of both Ca^{2+} and Mg^{2+}, Ca^{2+} occupies both the SyMBS and ADMIDAS and Mg^{2+} occupies the MIDAS [6]. The central features of ligand binding are the reorganization of the amino acid residues that coordinate the MIDAS and ADMIDAS metal ions (Fig. 3) and a swing-out motion of the β3 hybrid and PSI domains away from the β3 I domain and the αIIb subunit as a result of a pistonlike displacement of the β3 I domain α7-helix that causes a 62° reorientation between the β3 I and hybrid domains and a 70Å separation between the knees of the αIIb and β3 subunit legs (Fig. 4) [5].

The MIDAS

The events at the MIDAS that accompany ligand binding can be briefly summarized as follows [4,5,7]: (i) The ligand's positively charged arginine or lysine binds to the negatively charged Asp residue 224 in αIIb. (ii) Although both Mg^{2+} and Ca^{2+} can support ligand binding to αIIbβ3, the only crystal structures available have Mg^{2+} in the MIDAS. The Mg^{2+} is held relatively weakly in the MIDAS by four water molecules, the S121 hydroxyl oxygen, and one of the two E220 carboxyl oxygens (Fig. 3, top panel), as evidenced by the need for millimolar concentrations of Mg^{2+} to support ligand binding and platelet aggregation [25]. The weak binding of the Mg^{2+} to the β3 MIDAS residues allows it to retain more of its electropositivity to capture the ligand through one of the latter's Asp carboxyl oxygens [26]. (iii) The ligand carboxyl oxygens not only help coordinate the Mg^{2+}, but also interact with the backbone nitrogens of Y122 and S123 in the β1-α1 loop in the β3 I domain and the side-chain of N215, which also participates in coordinating the SyMBS (Fig. 2, bottom panel). These interactions stabilize conformations in which the β1-α1 loop moves closer to the MIDAS and thus trigger the swing-out motion described above. (iv) The αIIbβ3 headpiece undergoes extension away from the legs, either before or after the swing-out motion (Fig. 4).

Factors other than the presence of a ligand carboxyl affect the swing-out process as not all RGD-based antagonists induce major conformational changes [27]. One possible explanation for the differences comes from studies of the binding of fibronectin fragments to

αVβ3, in which a high-affinity fragment with a Trp substitution for Ser after the RGD sequence did not induce a major conformational change with binding [28]. The crystal structures of the complexes showed that the Trp interacted with β3 Tyr122 in the β1-α1 loop and thus prevented the motion of the loop toward the MIDAS. In addition, Tyr1446 in the fibronectin fragment now interacted with the ADMIDAS metal ion via a water molecule [28].

The SyMBS

The MIDAS is connected to the synergistic metal ion-binding site (SyMBS [4]; termed ligand-associated metal ion-binding site [LIMBS] in αVβ3) [24], which contains a Ca^{2+} ion in crystal structures when the receptor is crystalized in the presence of both Ca^{2+} and Mg^{2+}. Unlike the MIDAS Mg^{2+}, the SyMBS Ca^{2+} is relatively inaccessible to solvent [29]. It is required for ligand binding to αIIbβ3 [29], but its precise role is still under investigation. Variations in SyMBS coordinating residues among integrin β subunits can alter both ligand binding and the impact of pH on metal ion binding to the SyMBS [30]. In fact, differences in the reported presence or absence of divalent cations in the SyMBS (LIMBS) in crystal structures of αIIbβ3 and αVβ3 most likely reflect technical differences in the pH and divalent cation concentrations used during crystal formation [31]. β3 E220 plays an important role in both the SyMBS and MIDAS as one of its carboxyl oxygens coordinates the Ca^{2+} in the SyMBS and the other coordinates the Mg^{2+} in the MIDAS (Fig. 3). The coordination of the SyMBS metal ion by one of the E220 oxygens probably reduces the strength of binding of the other E220 oxygen to the MIDAS metal ion, thus enhancing the MIDAS metal ion's electropositivity and its ability to bind a ligand's carboxyl oxygen [26]. The SyMBS and LIMBS residues make contact with the integrin αIIb and αV subunits, respectively, and so differences between the αIIb and αV contacts may contribute to differences in ligand binding between the two receptors despite their sharing the same β3 subunit [32].

The ADMIDAS

In the unliganded crystal structures of αVβ3 and αIIbβ3, the ADMIDAS metal ion is coordinated by the residues from the βb1-α1 loop, the α1 helix, and the β6-α7 loop, including the carbonyl oxygen of S123, carboxyl oxygens of D126 and D127, and the carbonyl oxygen of M335 (Fig. 3) [4,33]. In contrast to the RGD-based ligands that bind to the β3 subunit of αVβ3 and αIIbβ3 by one of the ligand's carboxyl group oxygens completing the coordination of the metal ion of the MIDAS, the fibrinogen c chain C-terminal dodecapeptide (HHLGGAKQAGDV) binds to αIIbβ3 by a combination of the coordination of the MIDAS metal ion by the Asp carboxyl and the coordination of the ADMIDAS metal ion by the C-terminal Val carboxyl through an intermediate water molecule [7]. The importance of the latter interaction is supported by the observation that amidation of the C-terminal Val carboxyl reduces the affinity of the peptide for the receptor by more than 80% [34].

Ligand binding leads to reorganization of the ADMI-DAS, with the ADMIDAS metal ion, the β1-α1 loop, and the α1 helix moving closer to the MIDAS; the coordination of the ADMIDAS ion by the M335 carbonyl being replaced by a carboxyl oxygen of D251; and

the movement of the β6-α7 loop further from the ADMI-DAS. The reorientation of D251 away from the MIDAS and toward the ADMIDAS has been proposed to reduce the binding of the MIDAS metal ion, thereby increasing its electropositivity and thus its ability to bind the ligand Asp [35,36]. The movement of the β6-α7 loop is associated with a downward motion of the α7 helix and the dramatic swing-out motion of the hybrid domain [5]. The movement of the β1-α1 loop toward the MIDAS is also thought to contribute to enhanced ligand binding by orienting the two backbone nitrogens in residues β3 Y122 and S123 so that they interact with the ligand's carboxyl oxygen that does not coordinate the MIDAS metal ion and the ligand's carboxyl oxygen that does coordinate the metal ion, respectively (Fig. 3).

Based on studies of ligand binding to αVβ3 and other integrin receptors, the ADMIDAS has been considered a negative regulatory site responsible for integrin inhibition by high concentration of Ca^{2+} [37–39]. Unlike αVβ3, however, αIIbβ3 -mediated binding of fibrinogen is not inhibited by Ca^{2+} [25]. Studies conducted with other integrin receptors or the isolated β3 βA (I-like) domain identified variable effects of altering the coordination of the ADMIDAS metal ion, with alterations in surface expression and receptor conformation [40], enhanced or reduced adhesion to immobilized ligands [35,36,41,42], decreased lymphocyte migration *in vitro* and abnormal lymphocyte homing *in vivo* [43], diminished adhesion to immobilized ligand [44], and diminished ability to bind soluble ligand [45]. In contrast, a β3 D251A mutation did not affect either αIIbβ3 surface expression or binding of the ligand-mimetic antibodies PAC-1 and OPG2 [46]. β3 D126A and D127A mutations in αIIbβ3 did not decrease surface expression of the receptor or cell adhesion to immobilized fibrinogen in the presence of Ca^{2+} and Mg^{2+} [47]. An isolated recombinant β3 βI domain containing a D126A mutation in the ADMIDAS was, however, reported to have decreased binding of soluble fibrinogen [48]. It has been proposed that the β3 residue Ala252, and the corresponding Ala in β1, distinguish these β integrin families from the β2 and β7 families. The latter have instead an Asp residue that enhances the electronegativity near the MIDAS, reduces ligand binding affinity, and defines the response to the loss of the ADMIDAS coordinating residues [35,36].

While reconciling these data is challenging, especially the dramatic difference in cation preference for ligand binding to αVβ3 and αIIbβ3 despite their sharing the same β3 subunit that contains the cations directly engaged in ligand binding, it is likely that the ability of the fibrinogen γ chain dodecapeptide (which interacts with αIIbβ3, but not αVβ3) to bind to both the MIDAS and ADMIDAS metal ions [7] is important. Crystal structures of α5β1 in the presence and absence of Ca^{2+} demonstrate that loss of the ADMIDAS Ca^{2+} facilitates the movement of the β1-α1 loop toward the MIDAS in response to the binding of an RGD peptide, leading to higher ligand binding affinity as the ligand Asp carboxyl gains additional interactions with the backbone nitrogens in the loop [39]. As αVβ3 interacts with the ^{572}RGD^{574} sequence in the fibrinogen a chain rather than the γ chain dodecapeptide [49], loss of the αVβ3 ADMIDAS Ca^{2+} would be expected to enhance its affinity for fibrinogen; in contrast, the effect of loss of the ADMIDAS Ca^{2+} in αIIbβ3 would reflect the balance from gaining higher affinity for the Asp carboxyl that binds to the MIDAS, but losing the interaction of the terminal Val carboxyl with the ADMIDAS Ca^{2+}. Support for this interpretation comes from the studies of the binding of the high-affinity fibronectin

fragment to αVβ3 because the binding of this fragment was not inhibited by Ca^{2+} in association with it developing a water-mediated interaction with the ADMI-DAS metal ion [28]. Further support comes from studies of the monoclonal antibody AP7, which contains an RGDGGN sequence in its heavy chain CDR3 region [38]. This antibody binds to both αIIbβ3 and αVβ3; Ca^{2+} inhibits its binding to αVβ3, but not αIIbβ3. Changing the sequence to RGDGGA resulted in no effect on the its binding to αVβ3 or its inhibition by Ca^{2+}, but led to complete loss of binding to αIIbβ3, presumably due to loss of Asn-mediated binding to the ADMIDAS.

The structural basis of new αIIbβ3 antagonists

Three αIIbβ3 antagonists have been approved for human use in the USA, starting with abciximab, the chimeric Fab fragment of the murine monoclonal antibody 7E3, in 1994, followed in 1998 by eptifibatide, modeled on the KGD sequence, and tirofiban, modeled on the RGD sequence. These drugs have demonstrated efficacy in reducing death and ischemic complications of percutaneous coronary artery interventions in a large number of randomized studies [2], but they are associated with an increased risk of major bleeding and thrombocytopenia. As a result, their use is restricted to situations in which there is a high risk of thrombosis. Attempts to develop orally active αIIbβ3 antagonists based on the RGD sequence failed because the agents were not efficacious and several were associated with increased mortality [50,51]; they also caused thrombocytopenia [52–54]. The R(K)GD-based drugs all bind by the same fundamental mechanism in which there are two major points of attachment, one via a positively charged residue interacting with the αIIb D224 and the other via a ligand aspartic acid carboxyl oxygen coordinating the MIDAS Mg^{2+} (Fig. 2A). As a result, all of these agents induce conformational changes in the receptor and induce the receptor to adopt a high-affinity ligand-binding state, that is, they are partial agonists. Thus, it has been hypothesized that the increased mortality with the oral agents was due to their ‘priming’ the αIIbβ3 receptor to adopt a high-affinity ligand-binding state, resulting in platelet aggregation [50,51]. In fact, eptifibatide and tirofiban also prime the receptor, which may limit their efficacy [21,55–57]. Thrombocytopenia produced by both the oral and intravenous agents may also result from their inducing conformational changes that expose regions of the receptor to which some patients have preformed antibodies [52–54]. Thus, there are theoretical reasons to try to develop αIIbβ3 antagonists that do not induce receptor extension and swing-out.

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This small molecule antagonist does not induce the conformational changes in the receptor produced by RGD-based compounds [58]. Molecular docking suggests that while it binds to αIIb D224, its carboxyl does not engage the MIDAS metal ion; rather it appears to form a salt bridge with β3 Arg 165 and a hydrogen bond with Tyr 166 [59].

Activation-specific single-chain antibodies

Schwarz *et al.* [60,61] produced human single-chain antibodies specific for activated αIIbβ3 and showed that they do not induce LIBS epitope expression or platelet adhesion to fibrinogen. These antibodies inhibit platelet thrombus formation *in vitro* and in animal

models with less prolongation of the bleeding time than other αIIbβ3 antagonists. They almost all contain an RXD sequence, which would be expected to induce the active conformation of the receptor, so it is likely that the surrounding amino acids modify the binding so as to prevent the conformational change.

The RUC compounds

We identified RUC-1 (Fig. 2B) by screening 33 264 small molecules for their ability to inhibit platelet adhesion to fibrinogen [57,62]. It inhibits ADP-induced platelet aggregation with an IC50 of ~13 μm and soluble fibrinogen binding to platelets and purified αIIbβ3, but does not inhibit ligand binding to αVβ3, GPIb, or α2β1. Unlike tirofiban and/or eptifibatide, RUC-1 binding does not induce conformational changes in the β3 subunit detectable by LIBS mAbs, Stokes radius changes, or electron microscopy, and pretreatment of purified αIIbβ3 with RUC-1 does not enhance fibrinogen binding ('priming') [6,57]. Molecular docking and x-ray crystallography indicated that RUC-1 is unique among αIIbβ3 antagonists in binding exclusively to αIIb; moreover, it does not induce the rearrangement of the β3 βI subunit or the extensive hybrid domain swing-out we previously found associated with the binding of RGD-based agents [5,6]. With Dr. Craig Thomas' medicinal chemistry group at NIH, we synthesized RUC-2 (Fig. 2C), which is ~100-fold higher in affinity than RUC-1 but equally selective for αIIbβ3 compared to αVβ3 [21]. It has antithrombotic properties in the carotid artery FeCl3 injury model using mice developed by Poncz's group expressing human αIIβ and mouse β3 [62]. It also does not induce major conformational changes in β3 as judged by mAb binding, light scattering, or gel chromatography, nor does it prime the receptor to bind ligand [21]. Unlike eptifibatide and tirofiban, neither RUC-1 nor RUC-2 induces extension of intact αIIbβ3 molecules inserted into lipid bilayer nanodiscs [21]. X-ray crystallographic analysis of the RUC-2-αIIbβ3 headpiece complex in 1 mM Ca^{2+}/5 mM Mg^{2+} at 2.6 Å revealed that RUC-2 binds to αIIb the way RUC-1 does, but in addition, it binds to the β3 MIDAS residue E220, thus displacing Mg^{2+} from the MIDAS [21]. Thus, RUC-2 locks the receptor in an inactive conformation by displacing the Mg^{+} required for ligand binding, eliminating the ability of a ligand Asp carboxyl to bind and initiate the movement of the β1-α1 loop. More recently, we synthesized a much more water-soluble derivative of RUC-2, RUC-4, and it shows very similar binding and antithrombotic properties. It currently is under development for the prehospital therapy of ST segment elevated myocardial infarction [63].

Altering intracellular signaling of a αIIbβ3

Although space limitations do not permit a detailed description of this topic, an alternative approach to inhibiting αIIbβ3 is to alter the intracellular signaling that leads to activation of αIIbβ3. There have been dramatic advances in understanding the structural details of this process, providing a wide range of potential targets [59,64–66].

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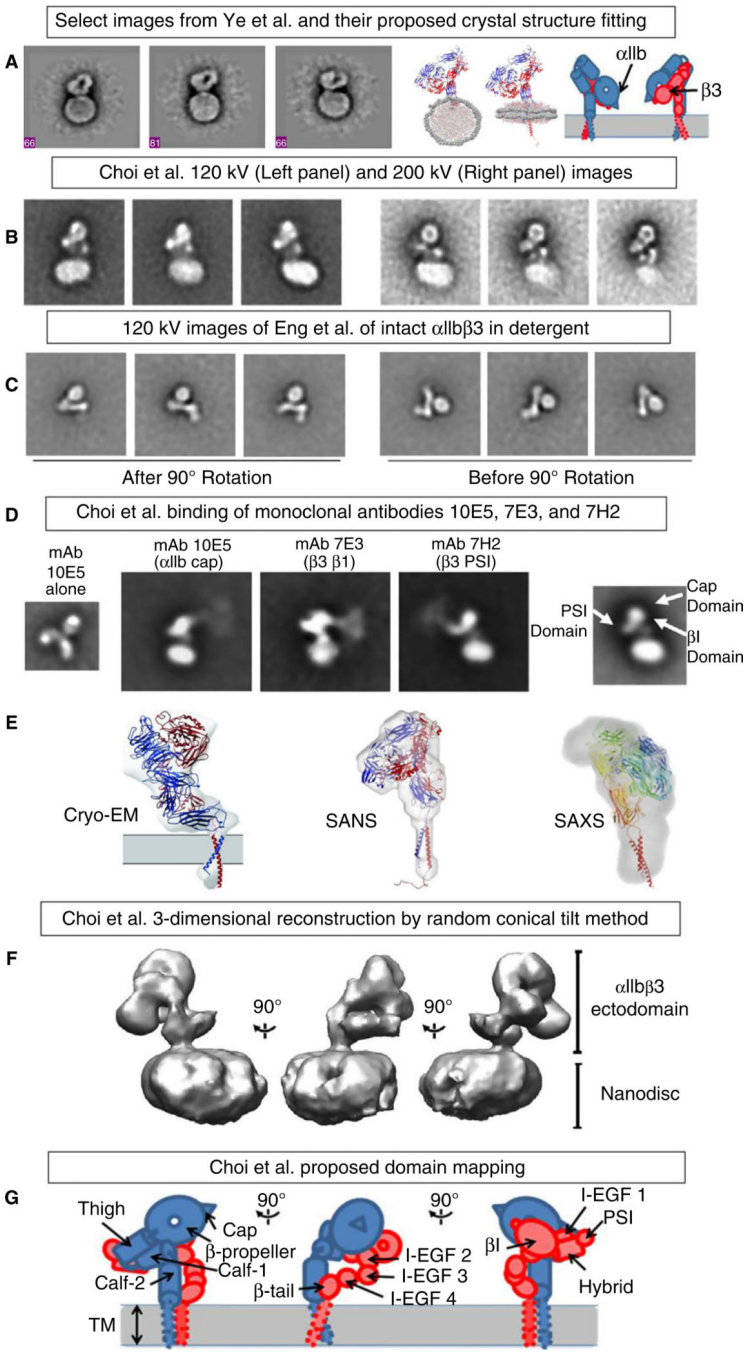

Fig. 1.

(A) Ye *et al.*'s [19] negative stain EM images of αIIbβ3 nanodiscs and model. (B) Choi *et al.*'s [22] negative stain EM images at 120 (left) and 200 kV (right). (C) Negative stain EM images of Eng *et al.* [18] after (left) and before (right) rotating to facilitate comparison with Choi *et al.*'s images. (D) Choi *et al.* negative stain EM images of αIIbβ3 nanodiscs reacted with mAbs 10E5 (αIIb cap), 7E3 (β3 βI), and 7H2 (β3 PSI). (E) Cryo-EM [16], small-angle neutron scattering (SANS) [17] and small-angle x-ray scattering (SAXS) [18] maps with

fitted crystal structures. (F, G) Choi *et al.* 3D reconstruction map and model (Reproduced from Refs [16–19] and [22] with permission).

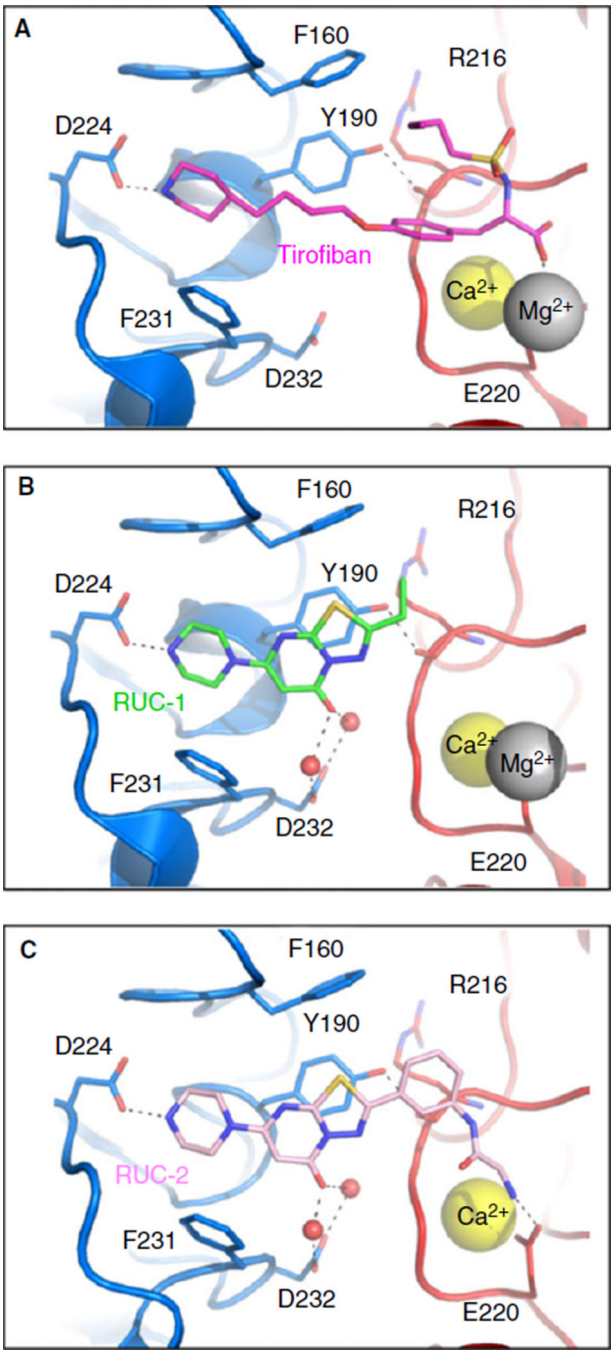

Fig. 2.

Crystal structures of (A) tirofiban, (B) RUC-1, and (C) RUC-2 in complex with αIIbβ3 (PDBs 3NIF, 2VDM, and 3T3M, respectively). RUC-2's interaction with E220 leads to loss of the MIDAS Mg^{2+} (silver ball).

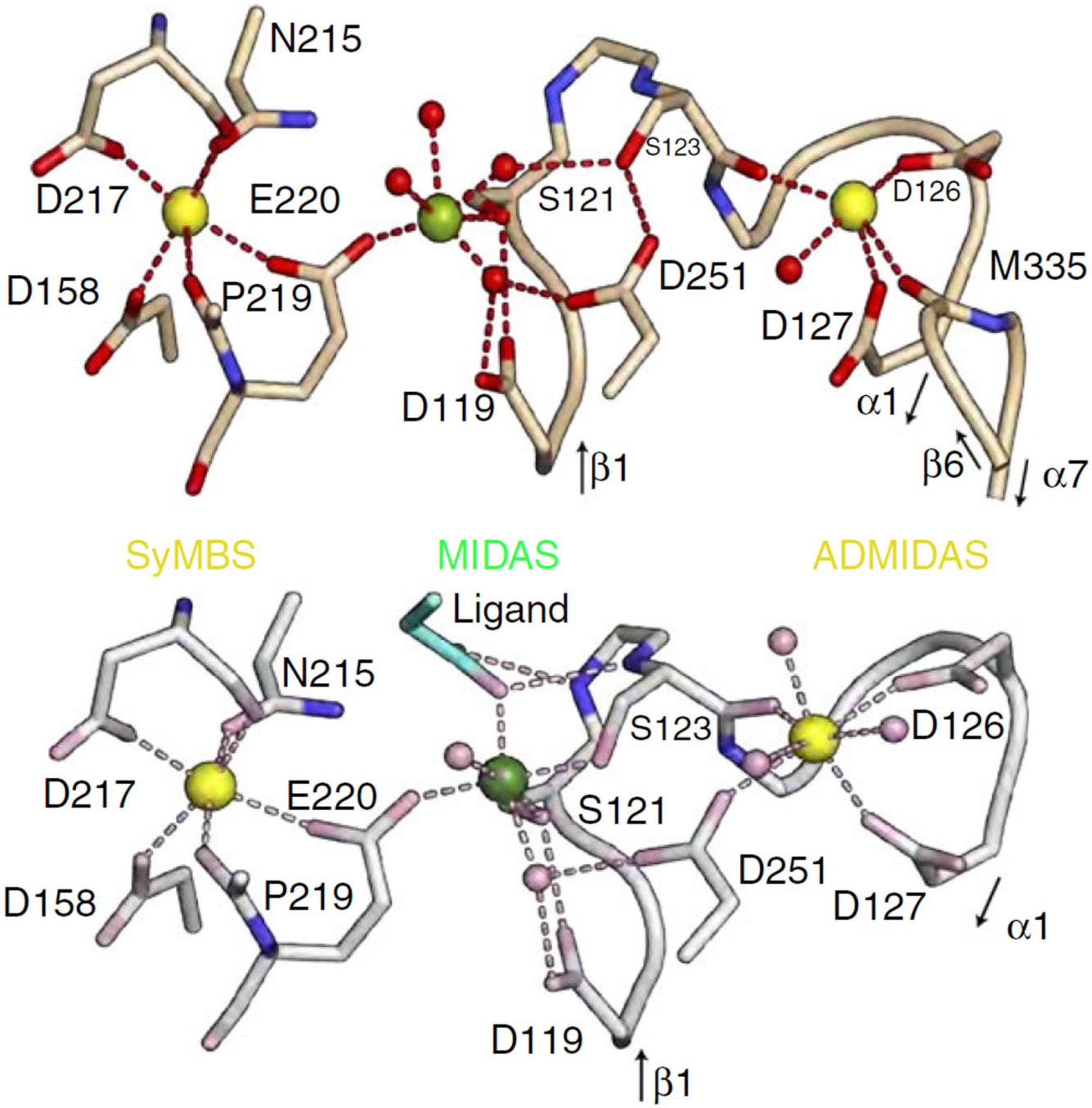

Fig. 3.

Metal ion coordination sites in the β3 βI domain in unliganded-closed αIIbβ3 (upper panel) and liganded-open αIIbβ3 (lower panel) [4]. Ca^{2+} ions are gold and Mg^{2+} ions are green. Note that only the ligand Asp carboxyl is depicted and that it interacts with the two backbone nitrogens in the β1-α1 loop and stabilizes a conformation in which the ADMIDAS is closer to the MIDAS, setting in motion a series of events that lead to both swing-out of the β3 hybrid domain and extension of the αIIbβ3 headpiece away from the leg regions (Reproduced from Ref. [4] with permission).

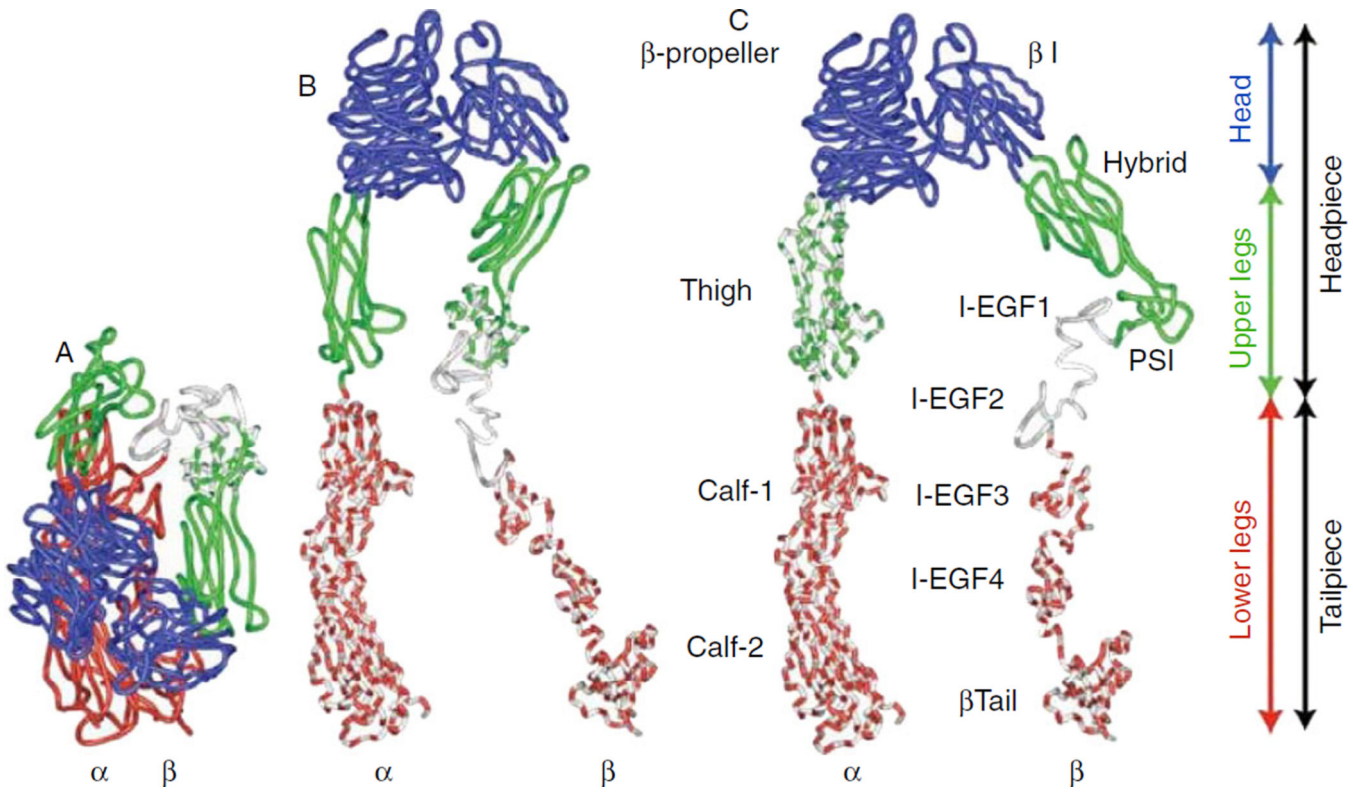

Fig. 4.

αIIbβ3 in its compact, bent conformation (A) and models of its structure after extension (B) and after both extension and swing-out of the β3 hybrid domain (C). (Reproduced from Ref. [5] with permission).