Regulation of platelet β3 integrins

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In this issue of the journal, Jayo *et al.* report a heterozygous *de novo* L718P mutation in the β3 integrin cytoplasmic domain of a woman with a life-long history of severe mucocutaneous bleeding.[1] Although aspects of the patient's platelet function differ from those of patients with classic Glanzmann's thrombasthenia, the location of the mutation focuses attention on the importance of cytoplasmic domains in regulating αIIbβ3 function.

Integrins are a family of heterodimeric adhesion receptors that reside on cell surfaces in a finely-tuned equilibrium between resting low affinity and active high affinity conformations.[2] This equilibrium is particularly important for platelets. When platelets encounter vascular damage, the integrin αIIbβ3 is rapidly shifted from its inactive to its active conformation, enabling it to bind soluble ligands such as fibrinogen and von Willebrand factor and initiate platelet aggregation.[3] However, on circulating platelets, αIIbβ3 is maintained in its inactive conformation to prevent the spontaneous formation of intravascular platelet thrombi.

Crystal structures of the extracellular portion of αIIbβ3,[4] and of the homologous integrin αvβ3,[5] revealed that the molecules in the crystals were severely bent, whereas electron microscopy of the active molecules revealed extended structures, implying that large conformational changes occur upon αIIbβ3 and αvβ3 activation.[6,7] This global rearrangement is initiated by signals generated in the platelet cytoplasm. The signals are then transmitted across the platelet plasma membrane via the αIIbβ3 and αvβ3 transmembrane helices to extracellular ligand binding sites. Because integrins lacking transmembrane and cytoplasmic domains are constitutively active, these domains appear to constrain integrins in their resting conformations.

Two loci of protein-protein interaction which exert constraining effects on integrin activity have been identified. The first locus consists of a unique α/β transmembrane domain heterodimer that results from the packing of complementary small and large side chains on neighboring helices.[8] In the case of αIIbβ3, this packing places the αIIb transmembrane helix motif G---G---L in juxtaposition to the β3 transmembrane helix motif V---I---G (Figure 1). This transmembrane helix packing arrangement, conserved across the entire integrin family, results in helix-helix interactions whose strength is appropriate for a system that undergoes rapid conformational switching. Thus, single point mutations that disrupt the transmembrane heterodimer are sufficient to cause αIIbβ3 activation.[9]

The second locus involves the integrin cytoplasmic domains. Because deletion of the conserved membrane-proximal αIIb cytoplasmic domain sequence GFFKR or the conserved β3 cytoplasmic domain sequence LLITIHD causes αIIbβ3 activation,[10] it has been proposed that these sequences interact to form an activation-constraining 'clasp', a feature of which may be a stabilizing salt-bridge between R995 in αIIb and D723 in β3.[11] However, under physiological circumstances, αIIbβ3 activation occurs when intracellular proteins such as talin and kindlin-3 bind to highly conserved portions of the β3 cytoplasmic domain and cause cytoplasmic domain separation.[12]

To determine a structure for the activation-constraining clasp, Vinogradova *et al.* used nuclear magnetic resonance

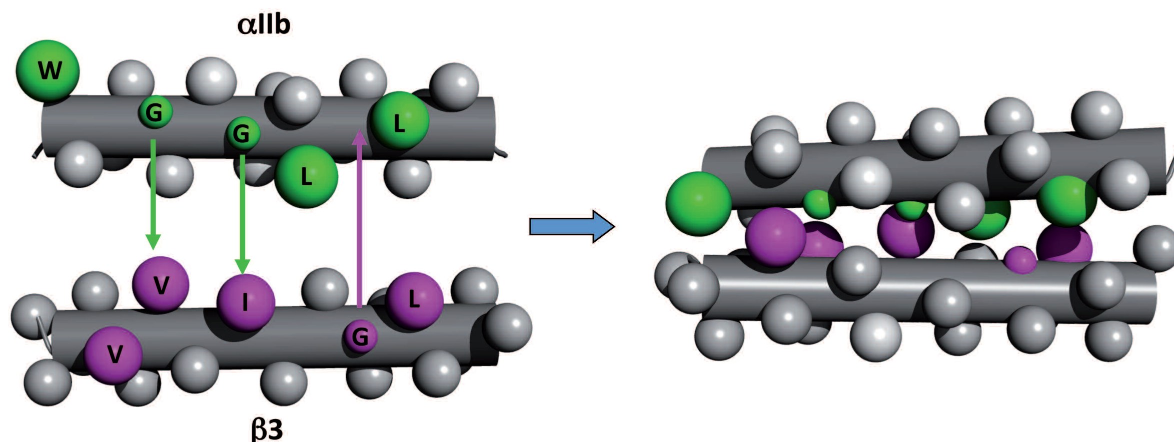

Figure 1. Model of the reciprocal "large-small" integrin transmembrane heterodimer interface. The integrin heterodimer is represented as an idealized pair of helices with large spheres denoting large hydrophobic residues and small spheres representing small polar residues. G, glycine; L, leucine; V, valine; I, isoleucine; and W, tryptophan.

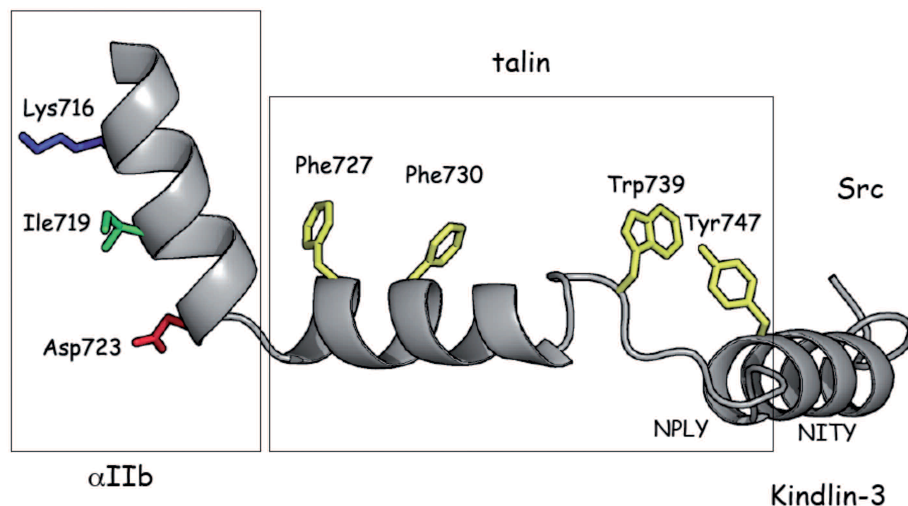

Figure 2. Ribbon diagram of the β3 cytoplasmic domain. The regions of the domain that interact with αIIb, talin, kindlin-3 and Src are indicated. The side chains of the residues that interact with αIIb are shown in blue, green, and red. The side chains of the hydrophobic residues that are either embedded in the membrane or interact with talin are shown in yellow.

(NMR) to study interactions between αIIb and β3 cytoplasmic domain peptides, either as full-length native peptides or as fusions with maltose-binding protein.[13,14] Calculated structures revealed an N-terminal α/β interface containing both hydrophobic and electrostatic interactions, including an electrostatic interaction between the guanidyl of αIIb R995 and the carboxyl of β3 D723. Subsequently, when NMR was performed in the presence of dodecylphosphocholine micelles to mimic a membranous environment, β3 residues 716-721 were found to be embedded in lipid. Lastly, NMR was performed using a mixture of β3 peptide and the talin FERM domain. The talin FERM domain binds to two regions of the β3 cytoplasmic domain centered on residues 739 and 747 and phenylalanine residues 727 and 730. Under these conditions, NMR chemical shifts for β3 residues T720-D723 were perturbed, suggesting that talin binding to the β3 cytoplasmic domain may physically disrupt the membrane-proximal clasp.

Surprisingly, it has been difficult to detect the αIIb/β3 clasp experimentally. Thus, neither Ulmer *et al.* who used NMR to study the structure of the αIIb and β3 cytoplasmic domains tethered by a coiled-coil[15] nor Li *et al.* who analyzed the interaction of the αIIb and β3 transmembrane and cytoplasmic domain polypeptides dissolved in detergent micelles detected their heteromeric association.[16]

To obtain a structure for the αIIbβ3 cytosolic domain heterodimer as it might exist in resting αIIbβ3, Metcalf *et al.* introduced cysteines at αIIb residue 987 and β3 residue 712 and dissolved the resulting disulfide-crosslinked construct in dodecylphosphocholine micelles for NMR experiments (*unpublished data*). While the αIIb cytoplasmic domain was found to be intrinsically disordered, the β3 cytoplasmic domain showed considerable structure, consisting of a proximal helix contiguous with the transmembrane helix and two distal helices (Figure 2). The proximal helix extended to residue D723 and was followed by a hinge at residue R724. This hinge allowed the proximal helix and the first distal helix to pack together at an angle of 110°, bringing the two distal helices into proximity to the membrane bilayer. Lys716 and Ile719, located on the same face of the proximal helix, interacted with the αIIb cytoplasmic domain, perhaps allowing residue D723 to interact electrostatically with αIIb R995. The first distal helix extended from residues K725 to A737 and was followed by a flexible linker spanning residues 738-743 and the second distal helix beginning at residue N744 through I757. The remainder of the cytoplasmic domain, consisting of its extreme C-terminus, was flexible and unstructured. It is noteworthy that the two distal helices are amphipathic and consequently, may interact with the membrane bilayer. However, they are also dynamic and likely available in the cytosolic compartment for binding to cytoplasmic signaling proteins such as talin and kindlin-3.

These structural studies provide a background for understanding the pathogenesis of the bleeding disorder of the patient reported by Jayo *et al.*[1] The mutated residue, L718, is located in the proximal β3 cytoplasmic domain helix. Replacing it with proline likely 'breaks' the helix at this point, leading to either of two diametrically opposed outcomes: either the mutation activates αIIbβ3 by disrupting the interaction between αIIb and β3 or it inhibits αIIbβ3 activation by uncoupling talin and kindlin-3 binding to the β3 cytoplasmic domain and cytoplasmic domain separation. In Chinese hamster ovary cells, L718P did cause spontaneous αIIbβ3 activity, enhanced αIIbβ3 clustering, and disruption of ordered lipid domains. But, in platelets, where it was expressed heterozygously with normal β3, the mutation was associated with impaired platelet aggregation and decreased ligand binding to αIIbβ3. Platelets from individuals heterozygous for Glanzmann's thrombasthenia express 50% of the normal amount of αIIbβ3 and aggregate normally.[17] Thus, in this case, either αIIbβ3 containing the L718P mutation impairs the function of the normal co-expressed αIIbβ3 (a dominant-negative effect) or additional abnormalities contribute to the patient's bleeding diathesis.

Previously, Peyruchaud *et al.* reported the case of a patient with an R995→Q mutation in αIIb that was predicted to cause constitutive αIIbβ3 activation by disrupting the electrostatic interaction between αIIb R995 and β3 D723.[18] However, the mutant αIIbβ3 was not constitutively active and the patient's thrombasthenia-like phenotype

was most likely due to a decreased amount of αIIbβ3 on the platelet surface. Subsequently, Ruiz *et al*. described a patient with thrombasthenia whose C560→R mutation in β3 locked αIIbβ3 in its high affinity conformation.[19] Although agonist-stimulated platelet aggregation was impaired, platelet microaggregates formed spontaneously in stirred platelet suspensions, ligands such as fibrinogen bound spontaneously to the mutant αIIbβ3, and fibrinogen was present on the surface of circulating platelets. Nonetheless, the patient presented with a thrombasthenic phenotype because of a substantially reduced amount of platelet surface αIIbβ3.

The patient Jayo *et al.* studied, unlike typical patients with thrombasthenia, was slightly thrombocytopenic and had platelets that were smaller than normal.[1] Consequently, although the amount of αIIbβ3 on the platelet surface was slightly decreased, the density of αIIbβ3 was likely normal or nearly so. Further, unlike typical thrombasthenic platelets, there was a marked decrease in αIIbβ3-independent platelet secretion, suggesting that other platelet function abnormalities were contributing to the patient's mucocutaneous bleeding. Thus, this case underscores the complexity of platelet function and the difficulty in extrapolating from effects seen in tissue culture cells expressing recombinant αIIbβ3 to platelets where αIIbβ3 is normally expressed.

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