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Molecular Dynamics Models of Integrin Clustering and Activation Mechanisms

By

Mehrdad Mehrbod

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of the
University of California, Berkeley

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Abstract

Molecular Dynamics Models of Integrin Clustering and Activation Mechanisms

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Doctor of Philosophy in Bioengineering

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Integrins are α/β transmembrane receptors that mediate cell-matrix and cell-cell adhesion, comprised of multi-domain, massive ectodomains, single-pass, transmembrane domains, and short, floppy cytoplasmic domains. They link the extracellular matrix or counter-receptors on other cells with the contractile cytoskeleton, mediating the transduction of mechanochemical signals across the plasma membrane and playing critical roles in a host of cellular functions, such as migration, cell traction, motility, platelet aggregation, and leukocyte transmigration.

Functionally, integrins are switch-like proteins that can take at least three different functional states: inactive, active, and ligand-bound. Integrin function is dependent upon allosteric conformational changes in its structure. Integrins are, by default, in an inactive (low affinity) state and can be activated via interacting with cytoplasmic proteins (e.g. talin) and/or engaging with extracellular ligands (e.g. fibrinogen). Integrin activation triggered by a cytoplasmic signal is called inside-out signaling, while outside-in signaling is defined as ligand-integrin binding followed by conformational changes in the transmembrane and cytoplasmic domains. Association of the integrin with the ligand induces quaternary changes in the integrin, leading to cell signaling and dynamic cell adhesion. However, atomistic details of these conformational changes as well as mechanisms of integrin clustering are not fully understood.

This study employs molecular dynamics techniques to provide detailed, mechanistic answers for a few key questions on integrin $\alpha\text{IIb}\beta 3$ function, a platelet-specific integrin member that plays a critical role in thrombosis. It is highly debated whether integrin transmembrane domains form homo-oligomers, leading to focal adhesion growth. This study suggests that homo-oligomerization of the β -subunit potentially regulates integrin clustering, as opposed to the α -subunit, which appears to be a poor regulator for the clustering process. Two distinct hypotheses are proposed to explain the atomic mechanism of integrin activation and how conformational changes triggered by cytoplasmic/extracellular proteins are propagated across the integrin structure: The switch-blade and the deadbolt model. To reconcile these apparently-contradictory models for integrin activation, this work investigated the mechanism of integrin $\alpha\text{IIb}\beta 3$ inside-out activation triggered by interactions with the cytoplasmic protein talin, and its outside-in activation as a result of exposure to the soluble RGD ligand. Finally, it was shown that the integrin α -subunit head domain regulates integrin-ligand binding affinity indirectly via inducing conformational changes in a key metal ion binding site (named LIMBS) in the $\beta 3$ subunit head domain. Hence, it was concluded that the difference between ligand binding affinities of integrin $\alpha\text{IIb}\beta 3$ and $\alpha\text{V}\beta 3$ is attributed to the larger attraction between the αV subunit head domain and the metal ion binding site LIMBS.

Dedication

***Dedicated to the kindest father and teacher,
Aba Salih Al-Mahdi (A.A.T.F)***

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Nomenclature

Symbols

D	Diffusion Coefficient
F	Helmholtz Free Energy
G	Gibbs Free Energy
k_a	Association Constant
k_B	Boltzmann Constant
M	Number of Sample Systems
n	Number of Samples
T	Temperature
t	Time
V	Instantaneous Speed
W	External Work
x	Position

Definition

Greek Symbols

β	$1/k_B T$
Δ	Difference
σ^2	Position Variance

List of Abbreviations

β TD	β Tail Domain
ADMIDAS	Adjacent to Metal Ion-Dependent Adhesion Site
C α	Carbon- α
DAG	Diacylglycerol
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FAK	Focal Adhesion Kinase
FERM Domain	4.1 Protein Ezrin, Radixin Moesin Domain
FnIII10	10 th type III Fibronectin
H-bond	Hydrogen Bond
ILK	Integrin-Linked Kinase
IMC	Inner Membrane Clasp
IP3	Inositol trisphosphate
LIMBS	Ligand-Induced Metal Binding Site
mAb	Monoclonal Antibody
MD	Molecular Dynamics
MIDAS	Metal Ion-Dependent Adhesion Site
MP	Membrane Proximal
NMA	Normal Mode Analysis
OMC	Outer Membrane Clasp
PAR-1	Proteinase-Activated Receptor 1

PDB	Protein Data Bank
PH Domain	Pleckstrin Homology
PKC	Protein Kinase C
PME	Particle Mesh Ewald
POPC	1-Palmitoyl-2-Oleoyl-sn-glycero-3-Phosphocholine
PSI	Plexin Semaphorin Integrin
PTB Domain	Phosphotyrosine-Binding Domain
PI(4,5)P2	Phosphoinositole-4,5-bisphosphate
RIAM	RAP1–GTP-Interacting Adaptor Molecule
RMSD	Root-Mean-Square Deviations
SMD	Steered Molecular Dynamics
VMD	Visual Molecular Dynamics
TMC Domain	Transmembrane-Cytoplasmic Domain

Chapter1

Introduction: Integrins

Integrins are transmembrane, heterodimeric proteins composed of large extracellular domains, single-pass transmembrane helices, and short cytoplasmic tails [1]. Thus far, 25 different members of the integrin family have been identified, all of which are formed by non-covalently binding of an α -subunit and a β -subunit (see Fig.1.1). Each integrin subunit is composed of a massive, extracellular domain (about 800 amino acids long), a helical transmembrane domain (about 20 amino acids), and a short, unstructured cytoplasmic domain. In mammalian cells, at least 18 α -subunits (i.e. $\alpha1$ -11, αV , αD , αE , αL , αM , αX , and αIIb) and 8 β -subunits (i.e. $\beta 1$ -8) have been identified whose distinct combinations construct a family of integrin members with a variety of ligand-binding properties as well as different interactions with focal adhesion proteins [2].

Integrins are believed to transfer mechanical signals bidirectionally into and out of the cell. This family of proteins are named as “integrins” because they act to integrate the cytoskeletal filaments and the extracellular matrix (ECM) [3]. Integrins play critical roles in a host of cellular functions, such as migration, cell traction, motility and survival, platelet aggregation, and leukocyte transmigration [4,5]. At the tissue scale, abnormal function of integrins can result in aberrant development or diseases such as atherosclerosis, bleeding disorders, leukocyte adhesion deficiencies, skin blistering, and several types of cancer [6]. Therefore, integrins may be effective targets in a variety of adhesion-related therapies [5,7].

Integrin function relies on the allosteric conformational changes in its structure. However, details of these conformational changes have remained ambiguous [8,9]. This chapter will introduce different models proposed for activation of several integrin members via tailoring integrin function and its conformation with a focus on one integrin compartment at a time.

1.1. Integrin Activation

Integrins are switch-like proteins that can take at least three different functional states: inactive, active, and ligand-bound [5,10,11]. To bind physiologic ligands, integrins should be activated [12,13]. Integrin activation is defined as the process through which the affinity of integrin for the ECM ligand is enhanced by one to a few orders of magnitude. Integrin activation mediates the cell migration and assembly of the ECM, thereby regulating events such as tumorigenesis and metastasis [5,14]. Blockade of integrin activation may therefore be useful in anti-adhesive therapies [5,15,16]. The biophysical significance of integrin as a gateway for signal transduction

into and out of the cell and its high potential in drug design and therapeutics has motivated an intensive research field seeking to decipher details of the integrin activation.

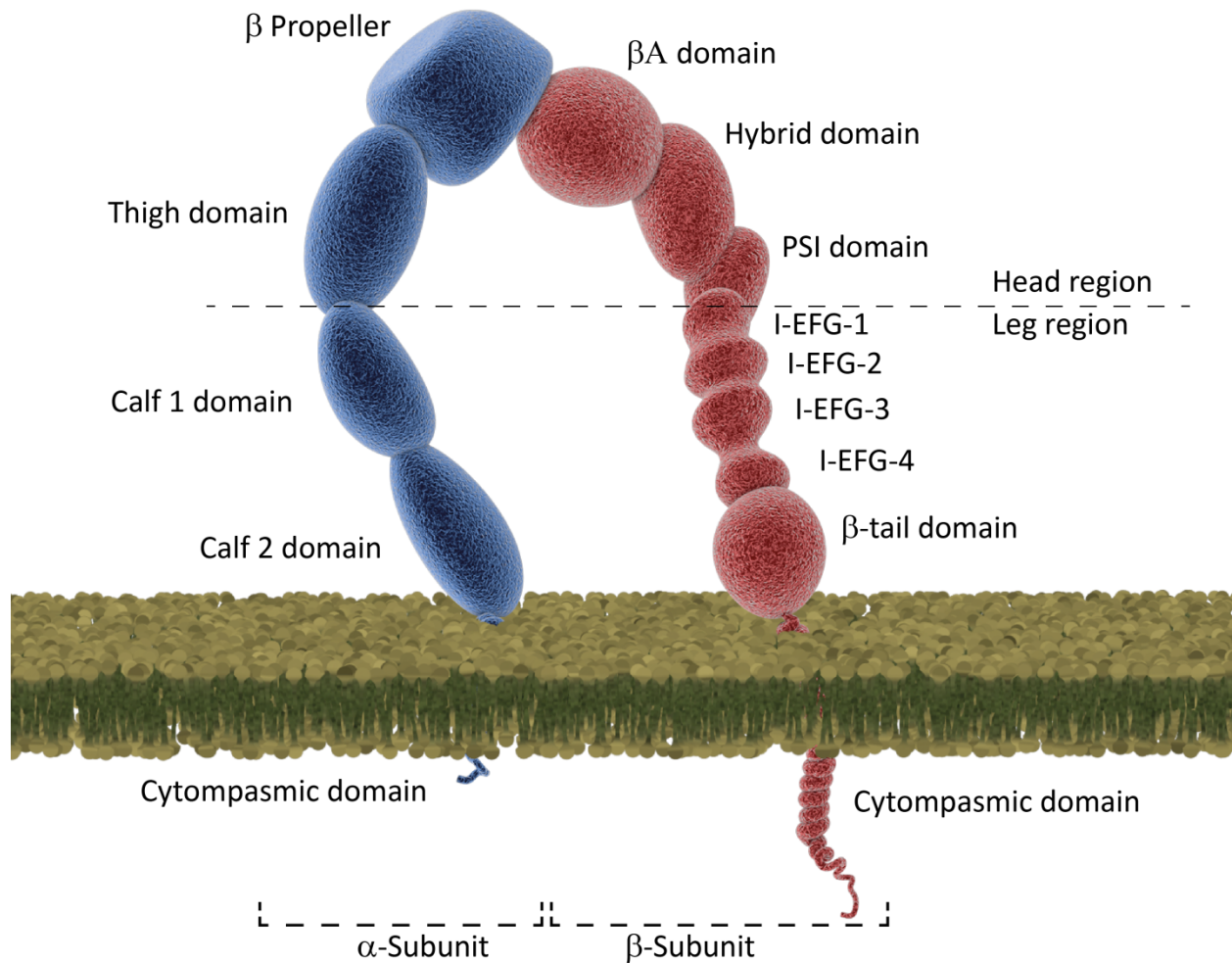


Figure 1.1: A schematic view of integrin $\alpha\text{IIb}\beta 3$ in its fully stretched conformation. Integrin members are composed of two non-covalently-bound subunits with large, multi-domain extracellular compartments, single-pass transmembrane helices, and short, floppy cytoplasmic domains.

Integrins are shown to undergo rapid conformational changes (within less than a second) to switch to their active conformation [17,18]. The activated integrin along with the focal adhesion protein talin and plasma-membrane-bound phosphoinositide-4,5-bisphosphate (PI(4,5)P2) putatively form the smallest adhesive unit that could link the cell to ECM [19]. The integrin activation process is temperature-dependent and reversible [20,21]. Surprisingly, numerous

studies carried out to investigate integrin activation have conferred different activation mechanisms for different members of the integrin family. Each integrin type as well could be activated via different pathways that involve specific, corresponding conformational changes. Integrin activation processes are broadly categorized as “outside-in” and “inside-out” pathways. Integrins could be activated via exposure to an extracellular signal, such as binding to ECM ligands or certain monoclonal antibodies (mAbs), or exposure to Mn^{2+} [12,22,23]; this process is termed as the outside-in activation. On the other hand, cytoplasmic proteins, such as talin and kindlin, can also induce the active status of integrins, which is referred to as the inside-out activation [22].

1.2. Integrin Ectodomain

The extracellular segments of integrin α - and β -subunits have up to ~1100 and ~800 residues, respectively, which together form a massive, ligand-specific structure for the cell that links with the ECM mechanically [23]. The N-termini of the α - and β -subunits interact non-covalently to form a selective interface for multiple ligands, such as fibronectin, fibrinogen, collagen, and vitronectin at the farthest region of the stretched integrin from the plasma membrane, called the “headpiece.” The integrin headpiece is connected to the transmembrane helices via multi-domain “legs” in either of α - and β -subunits.

Headpiece

The integrin headpiece consists of a β -propeller from the α -subunit and a vWFA Rossmann fold (βA) from the β -subunit. The structure of the integrin head domain highly resembles that of G-proteins. Integrin's βA and β -propeller domains are found to be the structural equivalents of the nucleotide binding ($G\alpha$) and propeller ($G\beta$) domains of G-proteins [12]. Half of integrin α -subunits, including the αL , αM , and αX subunits, contain an additional vWFA domain of ~200 amino acids that is inserted between β -sheets 2 and 3 of the β -propeller domain, which is named the αA or I domain [18,23,24] (see Fig. 1.2). The αA domain is the major ligand-binding site in integrin members that possess it [25,26]. The αA domain takes two distinct conformations, closed and open, corresponding to the low and high affinity states of the domain for the physiological ligand in αA -containing integrins [25,27,28,29,30].

In αA -lacking integrins (e.g. $\alpha V\beta 3$ and $\alpha IIb\beta 3$), RGD-containing ligands interact with a groove formed between the βA and β -propeller domains [31,32,33]. Experimental studies showed the ligand binding to the integrin headpiece induces an opening between the βA and hybrid domains in integrin whose reported magnitudes differ, ranging from 10° to 80° [34,35,36,37,38,39,40]. The swing-out of the hybrid domain away from the α -subunit pulls the $\alpha 7$ helix of the βA domain downward, favoring the upward movement of the $\alpha 1$ helix and shifting the βA to an active state (see Fig. 1.3) [34,39].

However, this opening between the βA and hybrid domains has not been observed in the crystal structure of the liganded $\alpha V\beta 3$ [33]. Molecular dynamics (MD) simulations have been employed to resolve this controversy. In their MD simulations, Puklin-Faucher et al. placed the crystal structure of the RGD-containing 10th type III fibronectin (FnIII10) adjacent to the $\alpha 1$ helix

of the β A domain in integrin α V β 3 head domain. They equilibrated the system in a box of explicit water and showed that the β A-hybrid domain hinge opens spontaneously upon ligand binding [41]. Hence, they suggested the closed hinge observed in the crystal structure of the α V β 3-ligand complex might have been caused by the structure obtained by soaking unliganded integrin crystals, in which integrins are already constrained, into RGD peptides [41]. Nonetheless, this implies that the β A-hybrid domain hinge opening is not necessary for ligand binding though it might occur post ligand binding. More recent molecular dynamics studies indicated that a hydrophobic T-junction between the middle of the α 1 helix (L133 and L134) and end of the α 7 helix (L333) on the β A domain forms upon ligand binding to integrin α V β 3 [42]. A slight inward movement of the α 7 helix induces the β A-hybrid domain hinge opening afterward (see Fig. 1.4) [42].

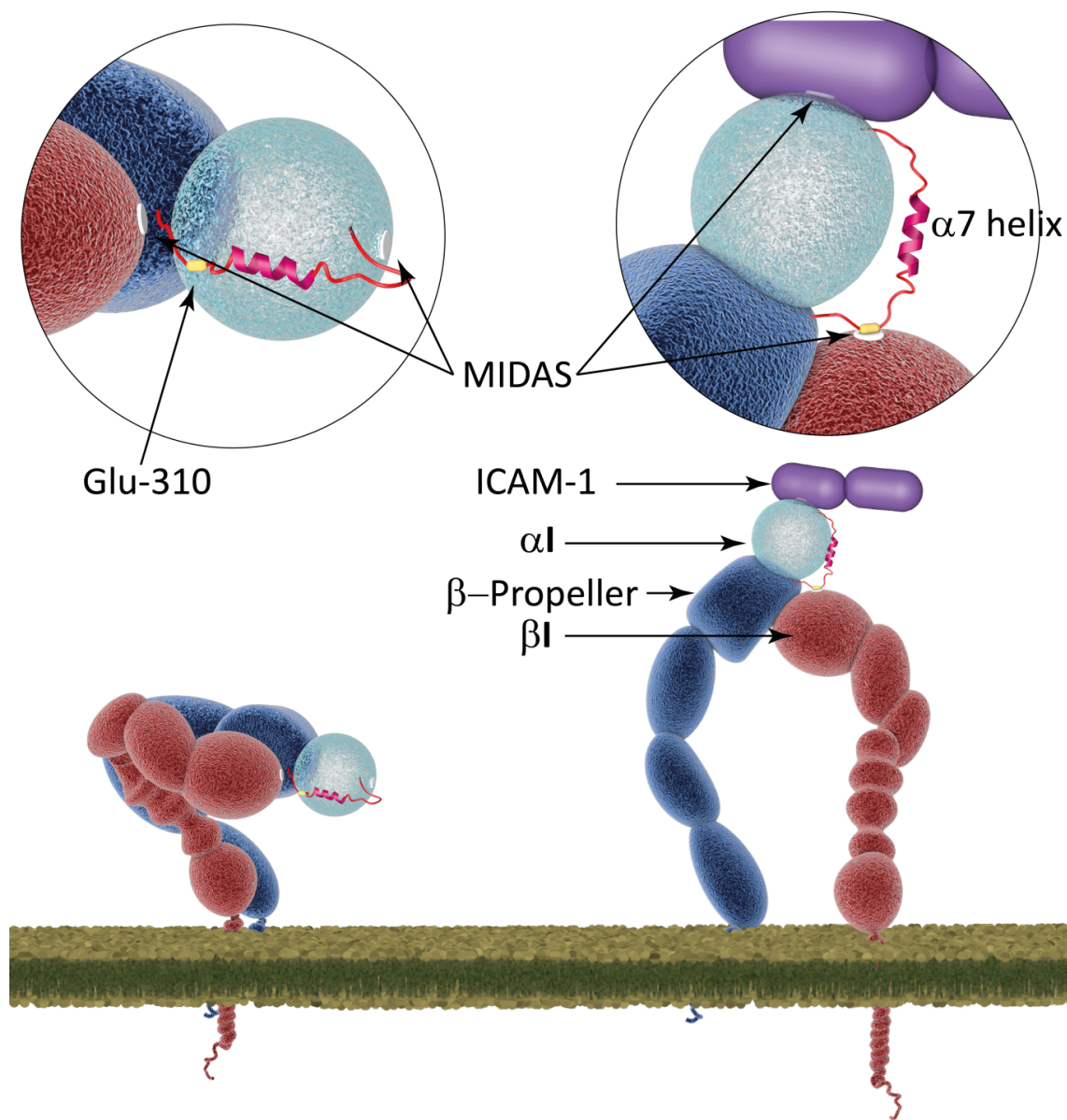


Figure 1.2: Conformational changes in the αA domain of integrin $\alpha\beta 2$ that lead to integrin activation. The αA domain is the major ligand-binding site in integrin members that possess it. The αA domain takes two distinct conformations: closed (left) and open (right), corresponding to the low (bent) and high affinity (stretched) states of integrin for the physiological ligand in αA -containing integrins. Upon stretching the integrin ectodomain, the $\alpha 7$ helix (shown in pink) is pulled downward via interaction of Glu310 with the βA domain. There are three divalent metal ion binding sites on the integrin βA domain. The first one, termed as the metal ion-dependent adhesion site (MIDAS), is coordinated with Mg^{2+} or Mn^{2+} , while the other two cation binding sites, called adjacent to MIDAS (ADMIDAS) and ligand-induced metal binding site (LIMBS), are mapped to the opposite sides of MIDAS and coordinated with Ca^{2+} or Mn^{2+} . The active MIDAS of the βA domain can bind to Glu-34 of ICAM-1 and mediate cell adhesion [43].

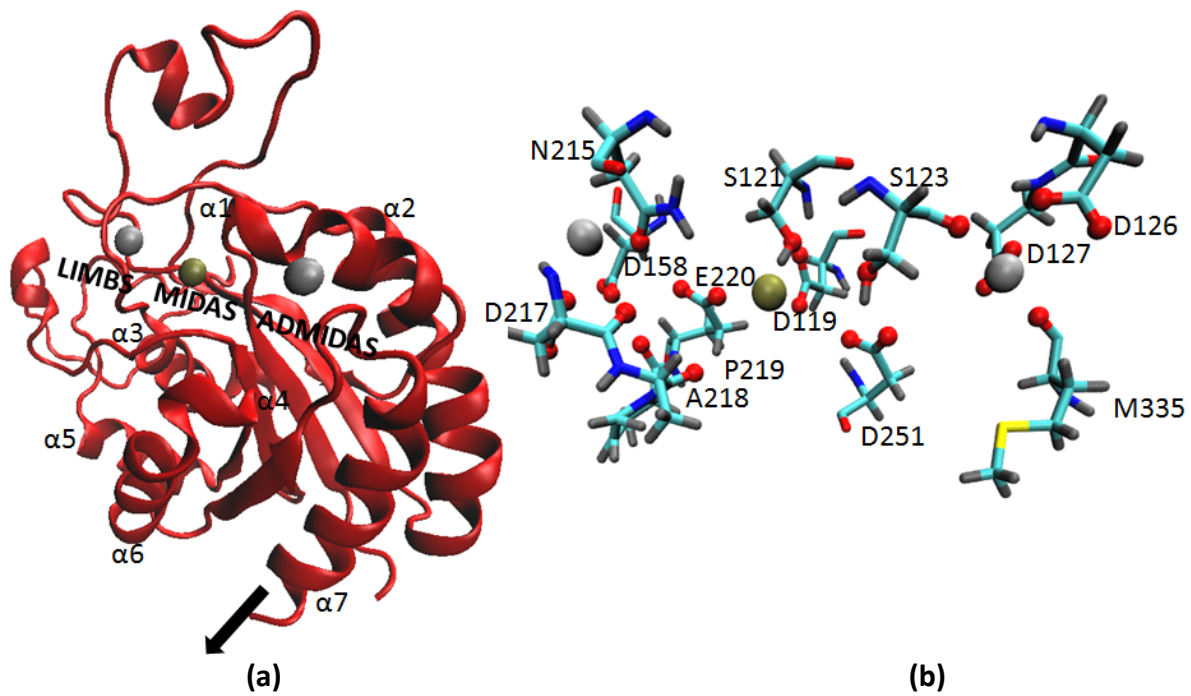


Figure 1.3 (a) βA domain of integrin $\beta 3$ (residues 109 to 354 from PDB ID: 3FCS) with divalent metal cation binding sites. While LIMBS and ADMIDAS are occupied by Ca^{2+} , MIDAS is occupied by Mg^{2+} . Helix $\alpha 7$ is known to move significantly downward upon ligand binding. (b) Details of divalent cation coordination by ion pockets of the βA domain. All ion pockets are formed by oxygen atoms (shown in red spheres) that form ionic bonds with the cation [44].

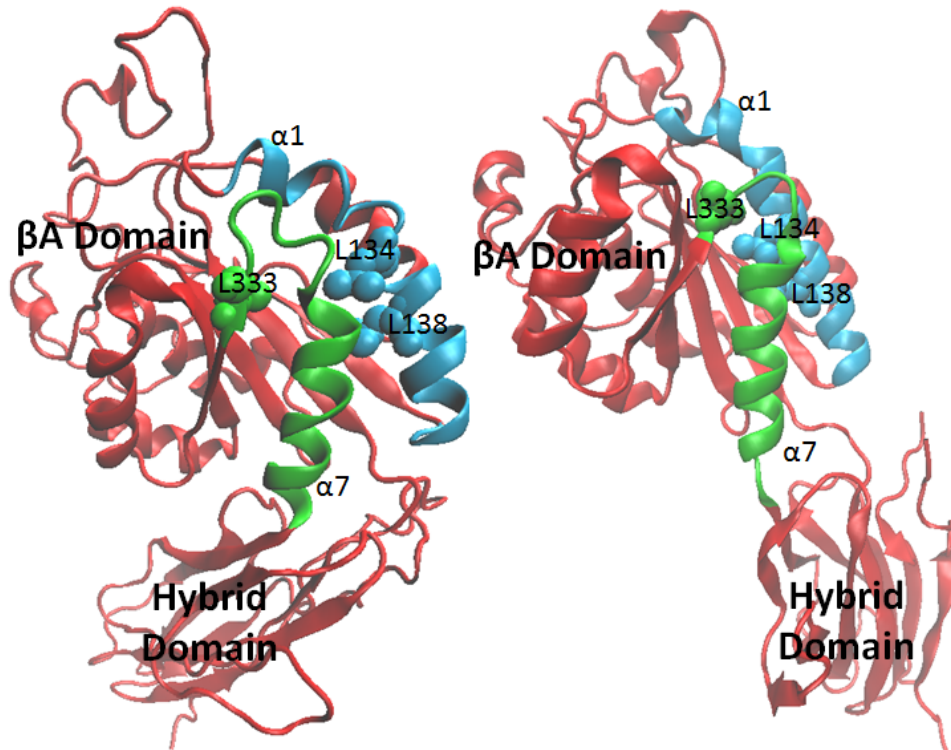


Figure 1.4: The hinge formed by the β A and hybrid domains is closed in the crystal structure of the liganded α V β 3 (PDB ID: 1JV2, shown on the left), while experimental studies showed ligand binding to the integrin headpiece induces the opening between the β A and hybrid domains in integrin α V β 3 and α IIb β 3 by up to 62°. A hydrophobic T-junction between the middle of the α 1 helix (L133 and L134) and end of the α 7 helix (L333) on the β A domain forms upon ligand binding to integrin α V β 3. A slight inward movement of the α 7 helix induces the β A-hybrid domain hinge opening afterward [42].

Divalent Cation Coordination Sites

Binding of divalent cations with the integrin head domain (i.e. β A and β -propeller domains on the extracellular side) is reported to regulate the integrin function. These binding sites can act as ligand antagonists as well as ligand specificity modifiers [31]. There exist three divalent metal ion binding sites on the integrin β A domain. The first one, termed as the metal ion-dependent adhesion site (MIDAS), is coordinated with Mg^{2+} or Mn^{2+} , while the other two cation binding sites, called adjacent to MIDAS (ADMIDAS) and ligand-induced metal binding site (LIMBS), are mapped to the opposite sides of MIDAS and coordinated with Ca^{2+} or Mn^{2+} [33,34,45] (see Fig. 1.3). The MIDAS pocket is formed by shared oxygen atoms of a DxSxS group of residues, while mutating any of these contributing residues within integrin β 1, β 2, β 3, and β 5 subunits ablates ligand binding.

High concentrations of Ca^{2+} reinforces the bent, inactive state of integrin α V β 3, while in presence of Mn^{2+} , a cation that activates integrins, most integrin α IIb β 3 molecules have been observed to be in an extended conformation [35]. The LIMBS pocket has been shown to act as a positive regulator of ligand binding in low Ca^{2+} concentrations while the ADMIDAS mediates the negative regulatory effects at high Ca^{2+} concentrations, which are compensated by Mn^{2+} replacement [46]. Binding of a Mn^{2+} ion with the ADMIDAS is known to induce a conformational

change resulting in an active form of the integrin [47]. It is proposed that the RGD ligand and divalent cation share a common binding pocket on the integrin $\beta 3$ head domain on the interface of the βA and β -propeller domains [31,32]. Mutations of integrin $\alpha 4\beta 7$ that affect the coordination of the MIDAS and LIMBS have suggested that while the normal function of the LIMBS is necessary for rolling of leukocytes, the presence of the MIDAS is indispensable for both rolling and firm adhesion of leukocytes [46].

Beta3 integrins have two types of cation-binding sites: a ligand-competent high-affinity site that must be occupied for the ligand to bind, most likely the MIDAS, and a low-affinity Ca^{2+} -binding inhibitory site, likely to be ADMIDAS. Mn^{2+} activates integrins by competing with Ca^{2+} at the ADMIDAS. Thus, ADMIDAS appears to have a dual role in the $\beta 3$ integrin function, stabilizing the unliganded state (in the Ca^{2+} -bound form) as well as the liganded state (by stabilizing the MIDAS metal) [23]. It remains controversial whether ligand binding is required for occupancy of the LIMBS [33,48,49,50]. As opposed to the structure of integrin $\alpha \text{IIb}\beta 3$ [49], $\alpha 4\beta 7$ [51], and $\alpha 5\beta 1$ [52], whose cation binding sites are fully occupied, the crystal structure of integrin $\alpha \text{V}\beta 3$ shows a discrepancy in occupancy state among different crystal structures [50,53]. It has been recently hypothesized that the pH in which integrin structure is crystallized may have an important effect on the occupancy of the metal ion binding sites [50].

In αA -domain-containing integrin members, the top surface of the αA domain possesses a metal ion-dependent adhesion site (MIDAS) that is located closely to the ligand binding site [25,27]. A conformational change at the MIDAS that regulates ligand binding is linked to a significant movement of the α -helix that connects the bottom of the αA domain to the β -propeller domain [25,27,30,54]. In the closed state, a metal ion (Mn^{2+}) is coordinated by a DxSxS MIDAS motif from the βA - $\alpha 1$ loop, a T209 from the $\alpha 3$ - $\alpha 4$ loop (loop 2) that hydrogen bonds to a metal-bound water, and a D242 from the βD - $\alpha 5$ loop (loop 3) that directly coordinates the metal ion producing the Y- H_2O -metal-D closed MIDAS configuration [23]. Upon switching of the αA domain to the active conformation, all three MIDAS-forming loops rearrange. Concurrently with this change, the F- $\alpha 7$ loop undergoes a major restructuring that is associated with a downward movement of the $\alpha 7$ helix. The rearranged contacts of the F- $\alpha 7$ loop with the hydrophobic core lead to a slight inward pull of the $\alpha 1$ helix, resulting in the open MIDAS configuration Thr-metal-water-Asp. This change is linked to an inward movement of loop 1 and in position of loop 3 bearing D242 that allows T209 to coordinate directly the divalent metal ion (Mg^{2+} or Mn^{2+}) while D242 hydrogen-bonds to a metal-bound water molecule. The resulting increased electrophilicity of the MIDAS attracts a Glu side chain from the ligand to provide the sixth metal coordination group, replacing a water molecule [23].

Integrin Grasps Ligands

Binding of the integrin ectodomain with ligands on a stiff substrate is essential for most integrins to form a mechanical linkage between the ECM and cytoskeleton [55,56,57], which is critical for force generation in focal adhesions and focal complex maturation [56,58,59]. Integrins are ligand-specific receptors that recognize several distinct motifs [31]. Regarding their specific ligands, integrins are broadly categorized into four groups (Fig. 1.5): (1) Integrins that recognize ligands containing RGD sequences, such as fibronectin, fibrinogen, and

vitronectin. This group encompasses most intensively studied integrin members (i.e. α IIb β 3, α V β 3, and α 5 β 1); (2) Integrin members that bind collagen, which recognize the minimal motif GFOGER (Gly-Phe-Hydroxyproline-Gly-Glu-Arg); (3) Leukocyte-specific integrins among which the β 2-containing subgroup (i.e. α L β 2, α M β 2, α X β 2, and α D β 2) is mostly investigated; (4) Integrins that engage with laminin receptors.

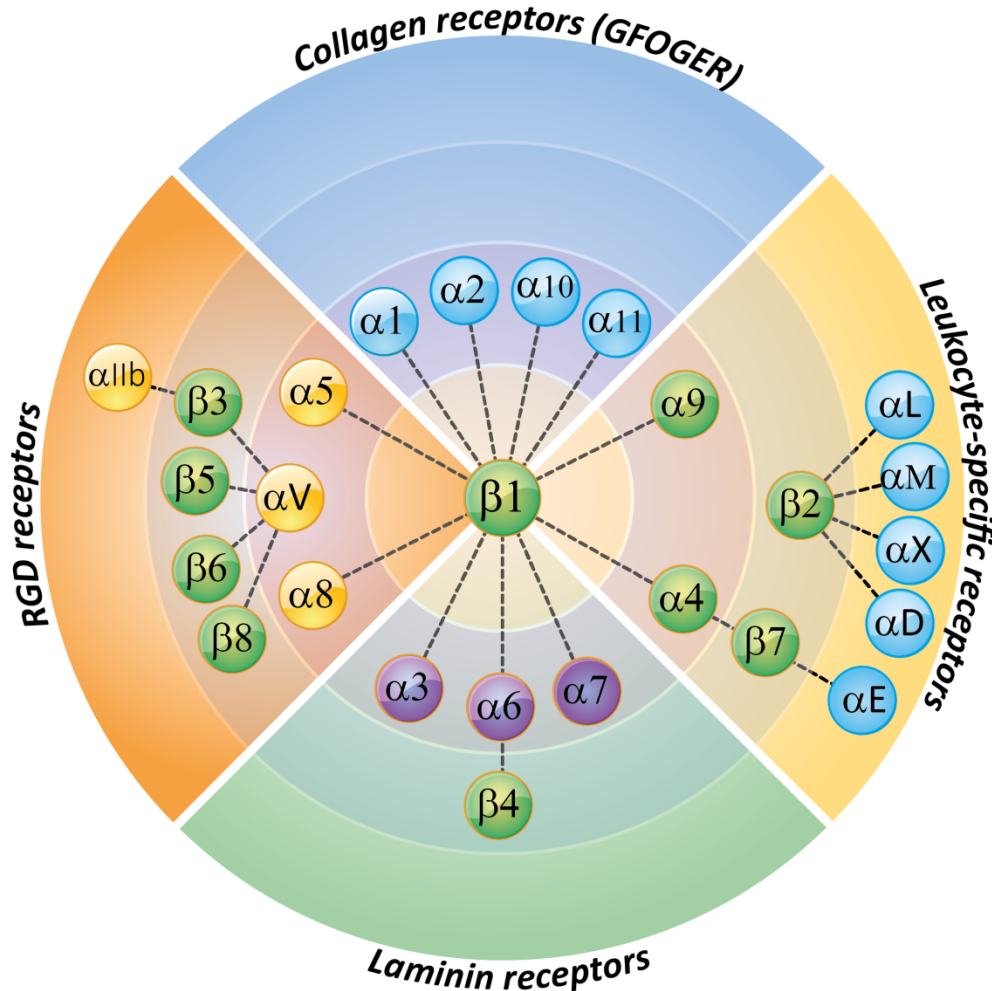


Figure 1.5: Different integrins as formed by combining various α - and β -subunits. Regarding ligands that integrins recognize, integrins are broadly categorized into four major groups. The figure is adapted from Barczyk et al. 2010 [3] with modifications.

Binding of most studied fixed ligands occurs at the integrin headpiece within a crevice formed by the β -propeller of the α - as well as β A of the β -subunit via a β A loop called the SDL loop [60]. Ligand binding occurs at the activated state of the integrin and accompanies with a conformational change in the headpiece. In the RGD ligand-bound integrin β 3, the ionic contact of the F- α 7 loop with the α 1 helix via M335 is broken, which leads to an inward pull of this helix, as occurs with liganded α A domains, along with a large inward displacement of the cation at the ADMIDAS with the MIDAS carboxyl oxygen of D251, now replacing the carbonyl oxygen of M335 [44]. This new conformation of the ADMIDAS favors its occupation with Mn^{2+} rather than Ca^{2+} . The MIDAS is subsequently occupied by Mn^{2+} . As in α A, the residue Asp of the ligand

provides another oxygen to the MIDAS, while the residue Arg of the ligand is anchored at the β -propeller, bringing the β -propeller and β A domains closer together. The position of MIDAS is stabilized further through the repositioned ADMIDAS and via linking to the LIMBS cation. The LIMBS cation is coordinated by the carboxyl oxygen of E220, the side chains of D158, N215, and D217, and the carbonyl oxygens of D217 and P219 [23,33].

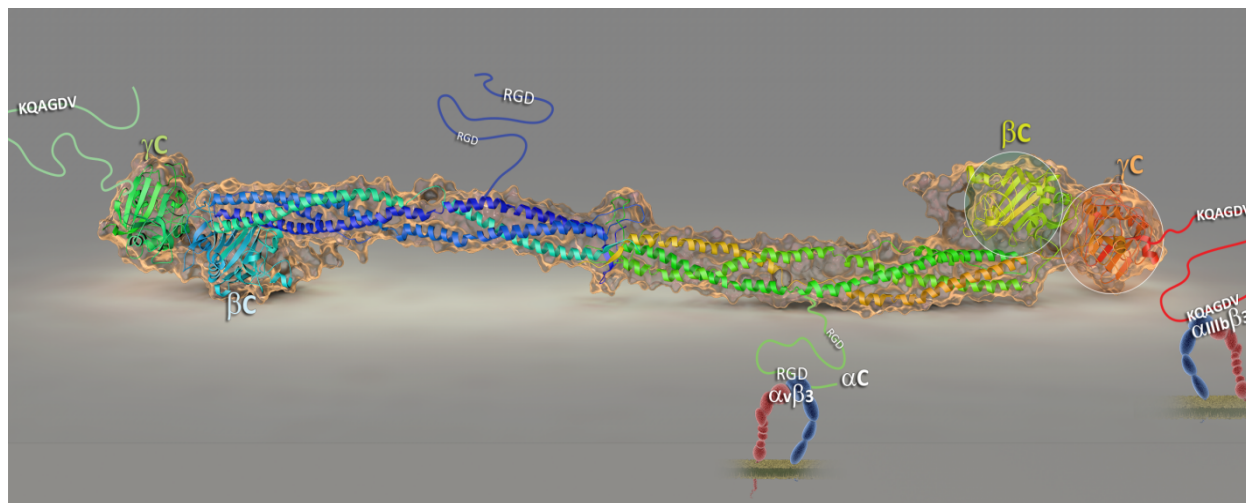


Figure 1.6: While integrin α V β 3 binds RGD sequences of fibrinogen, integrin α IIb β 3 binds a KQAGDV sequence at the γ C domain of fibrinogen [49].

Leg Segments (Stalk Region)

The leg segment of the integrin ectodomain is composed of the Ig-like thigh domain and β -sandwich domains Calx-1, and Calx-2 in the α -subunit, and the hybrid, plexin semaphorin integrin (PSI) domain, EGF1 to 4, and β -Tail domain (β TD) in the β -subunit (see Fig. 1.7) [10,12,25,34,61]. A divalent metal cation (Ca^{2+} or Mn^{2+}) binding site is present between the thigh and Calx-1 domains of α V and α IIb subunits in both the unliganded and liganded integrin structures. At the base of the propeller, blades 4–7 each contain a divalent metal ion coordinated in a β -hairpin loop, which can be occupied by Ca^{2+} or Mn^{2+} [23].

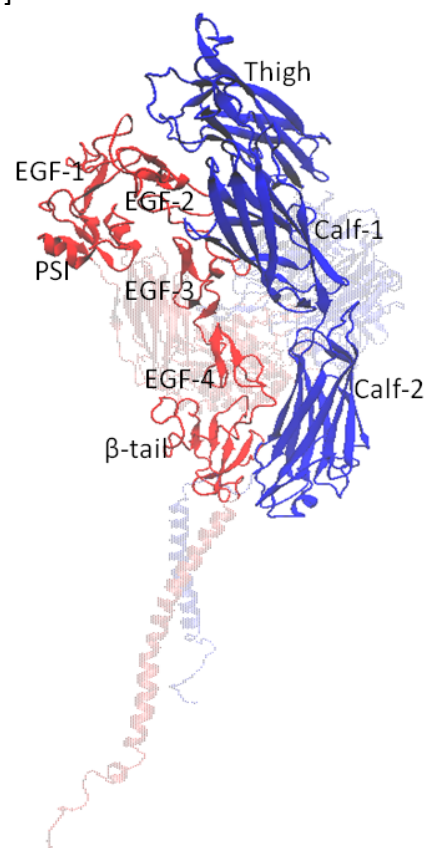
In most integrin family members the inactive state corresponds to a geometry in which the extracellular domain is folded over itself, forming an $\sim 135^\circ$ angle between the ectodomain head and leg domains [12]. For most integrin members, extension of the ectodomain appears to concur with integrin activation. It has been shown for integrin α V β 3 and α IIb β 3 that upon activation the ectodomain head piece swings about a hinge-like region located between Calx-1 and thigh domains of the α -subunit (α -genu), and between EGF-1 and EGF-2 domains of the β -subunit (β -genu) [62]. On the other hand, the crystal structure of integrin α V β 3 in complex with the ligand demonstrates a sharp kink at the ectodomain at the “knees” of the α V (α -genu) and β 3 (β -genu) [10]. The NMR structure of integrin β 2 subunits indicates that epitopes that are exposed in activated integrins are buried in the bent conformation [62].

Regarding the conformational changes leading to integrin α V β 3 activation, a number of studies have suggested contradictory models. Early electron microscopy studies proposed that integrin

activation requires an extension of the ectodomain [63,64,65]. However, a later study by Adair et al. revealed that the bent form of integrin, once considered inactive, could bind ligands [66].

The cysteine-rich, lower leg of integrin β -subunits, including the β TD and EGF1-4 domains, plays a critical role in regulating the ectodomain via interacting with the head domain. Several monoclonal antibodies (mAbs) that activate integrin or bind activated integrins are mapped to the integrin β -subunit lower leg [18]. Activation of leukocyte-specific integrins α L β 2, α M β 2, α X β 2 via mutations that disturb interactions between transmembrane domains of α - and β -subunits as well as exposure to Mn^{2+} , greatly enhance integrin interactions with mAb KIM127 via exposing the β 2 subunit lower leg [18]. The mAb LIBS2, which increases affinity of integrin α IIb β 3 for fibrinogen, binds to the β TD of the β 3 subunit [64]. The mAb TASC, which enhances cell adhesion to laminin, and the integrin-activating mAb QE.2E5 are mapped to the EGF domains of integrin β 1 subunit [67,68], while integrin-activating mAbs CBR LFA-1/2, KIM185, and MEM48 bind to the lower leg of integrin β 2 subunit [69,70,71]. We combined the crystal structures of integrin α IIb β 3 ectodomain (PDB ID: 3FCS [49]) and transmembrane-cytoplasmic domains (PDB ID: 2KNC [46]), embedded in a lipid bilayer, to explore effects of soluble RGD peptides in the vicinity of the integrin α IIb β 3. This study indicated that an RGD peptide is able to fully dissociate β A (on the headpiece) and β TD of integrin β 3 via competing between the Arg of the RGD peptide and K350 (on the β A domain) that forms a self-inhibitory interaction with S673 and S674 (on the β TD) in the inactive state of integrin [72].

Figure 1.7: Integrin leg segment (shown in solid colors) is composed of a thigh domain and β -sandwich domains Calf-1, and Calf-2 in the α -subunit (blue), and the Hybrid, plexin semaphorin integrin (PSI) domain, EGF1 to 4, and β -Tail domain (β TD) in the β -subunit (red).



Currently, there are two major models that attempt to relate conformational changes of integrins to their activation state, namely the “switch-blade” and the “deadbolt” models.

The Switch-blade Model

According to the switch-blade model, integrins take on three distinct conformations, corresponding to their inactive, active, and ligand-bound states. The inactive state of integrin features a bent-over configuration wherein the molecule shows a low affinity for ECM ligands [62] (Fig. 1.8). Negatively stained images of soluble integrin α V β 3, α L β 2, and α X β 2 show bent structures corresponding to a low affinity of ligand binding, when the C-termini are clapsed or samples are within a buffer with high concentration of Ca^{2+} [8,11]. Also, in the inactive state, the inner and outer membrane clasps hold the transmembrane domains of integrin together [2]. The bent form of integrin was inferred to be

inactive as it was abundantly formed in the presence of the inhibitory cation Ca^{2+} . Also, in the bent conformation of integrin, the head domain faces down toward the membrane, thereby is unlikely to bind the ligand [62]. Integrin activation results in a stretched conformation of the ectodomain and breakage of the inner and/or outer membrane clasps, most likely via presence of a competing molecule (e.g. talin and kindlin) that occupies these binding sites on the transmembrane domain of integrin β -subunit [5,73]. Activated integrin is known to have a considerably higher affinity for ECM ligands [35]. The ligand-bound state takes place when the integrin head domain engages in an interaction with an ECM molecule (e.g. fibronectin and fibrinogen). The ligand-bound conformation differs from the non-bonded active state in that the integrin head domain introduces an opening between the α - and β -subunits when in the ligand-bound state [12,43] (Fig. 1.8).

Several lines of evidence corroborate the switch-blade model for integrins. Firstly, recombinant integrin $\alpha 5 \beta 1$, when a disulfide bridge clasped the membrane-proximal α - and β - legs together, does not bind immobilized ligands [65]. Secondly, the Stokes radius of integrin $\alpha \text{V} \beta 3$ ectodomain in buffers containing Ca^{2+} or Mn^{2+} has been estimated at 5.7 nm and 6.0 nm, respectively, and the respective electron microscopic images revealed a bent conformation for up to 80% of the Mn^{2+} -bound integrins that were extended. Addition of a cyclic RGDfV (Arg-Gly-Asp-[D]Phe-Val) peptide in Ca^{2+} or Mn^{2+} buffers shifted the Stokes radius to about 6.4 nm and the vast majority of the molecules observed were extended in this case [12]. Finally, a mutation that holds the headpiece against the legs of integrin $\alpha \text{V} \beta 3$ via a cysteine bridge results in an inactive integrin [35].

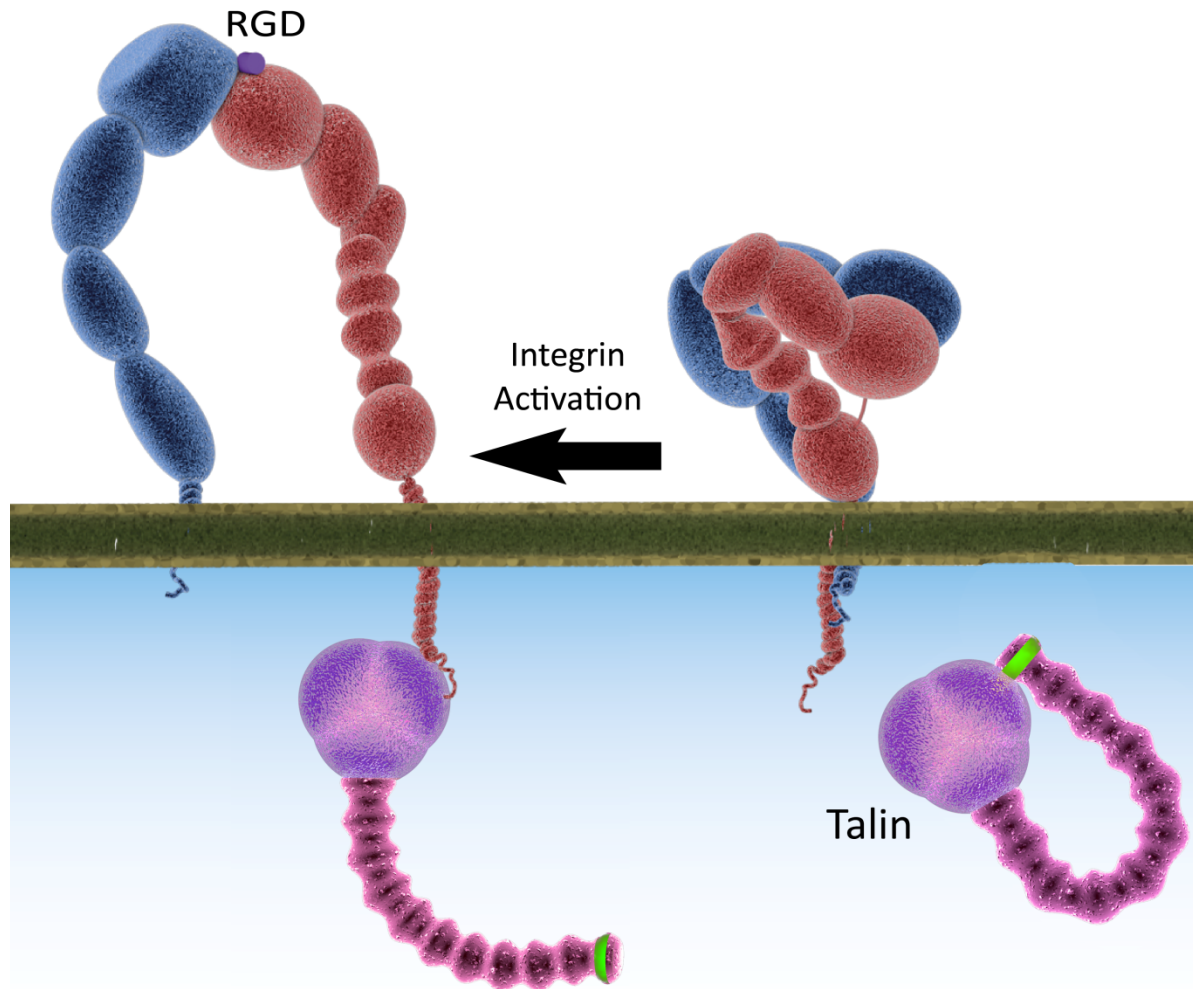


Figure 1.8: The switch-blade model for integrin activation suggests that the inactive conformation of integrin is featured by a bent-over configuration (right) wherein the molecule shows a low affinity for ECM ligands. Also, in the bent conformation of integrin, the head domain faces down toward the membrane and, hence, unlikely to bind the ligand. Integrin activation results in a stretched conformation of the ectodomain and breakage of the inner and/or outer membrane clasps (left), most likely via the presence of a competing molecule such as talin (shown in pink) that occupies these binding sites on the transmembrane domain of integrin β -subunit. Activated integrin is known to have considerably higher affinity for ECM ligands.

However, folded or partially-unbent integrin has also been observed to bind a number of ligands [39,74]. The bent form of integrin $\alpha V\beta 3$ is reported to bind fibronectin [66]. Inspection of mAb epitope maps for inactive and active integrin $\alpha IIb\beta 3$ indicated that either state could assume a bent conformation [75]. Furthermore, addition of Mn^{2+} , which activates integrins directly by interaction with the cation coordination sites in the βA domain, did not show a change in its height, indicating a lack of full extension [76]. FRET studies on integrin $\alpha 4\beta 7$ indicated a 5nm extension of the integrin ectodomain in presence of Mn^{2+} , and a 2.5nm movement away from the plasma membrane when stimulated by chemokines [39,77]. This is in agreement with FRET studies of integrin $\alpha V\beta 3$ and $\alpha IIb\beta 3$ in living cells [53,78]. Cryo-electron tomographic studies also demonstrate that $\alpha IIb\beta 3$ maintains its height in reconstituted membranes after activation by Mn^{2+} [39,76].

These results suggest that there are cases in which the integrin could be activated without being fully stretched out. It is argued that clasping the head to leg compartment might rather hinder tertiary and quaternary conformational changes needed for activation instead of merely blocking the ectodomain swing-out [12]. Finally, binding of different cyclic peptide ligand mimics to integrin $\alpha\text{IIb}\beta 3$ on intact platelets induced distinct conformational changes, suggesting that various ligands initiate different conformational changes [79]. Therefore, the full stretch of the ectodomain could be a consequence of ligand binding.

The Deadbolt Model

The deadbolt model suggests a significantly smaller conformational change for activation to be initiated, wherein a hairpin loop between β -strands C and D of the β tail domain (i.e. the CD loop of the β TD), located on β -subunit of integrin $\alpha\text{V}\beta 3$, proximal to the plasma membrane, functions as a “deadbolt” that links the β TD to the $\alpha 7$ helix in the ligand-binding β A domain. Upon release of the CD loop, the integrin is activated, as the bonding between the β TD and β A domains on the integrin head domain is lost [12,23,80] (Fig. 1.9). Subsequently a downward displacement of the $\alpha 7$ helix induces the active conformation of the β A [27,81]. According to the deadbolt model, the inside-out activation is transmitted to the ectodomain through either piston-like [2], seesaw-like [82], sliding [83], or rotational [84] movement of the transmembrane helices to unlock the adjacent β TD deadbolt.

Nonetheless, the structure of the $\alpha\text{IIb}\beta 3$ integrin ectodomain did not show the deadbolt interface [49]. Additionally, deletion of the CD loop in the β TD, did not activate integrin $\alpha\text{IIb}\beta 3$ or $\alpha\text{V}\beta 3$ [85]. Several hypotheses have been put forward to explain these discrepancies. It is shown that the structure of the $\beta 3$ integrin transmembrane domain can be different between detergent micelles and phospholipid bilayers [86], the latter more closely mimicking the plasma membrane [5]. Moreover, image selection bias is a potential problem in electron microscopy studies [8,66]. Cryo-electron tomography of lipid-embedded $\alpha\text{IIb}\beta 3$ integrin exhibits an average height of 11nm, much less than the 19nm height expected of a fully extended integrin [10,76]. In order to shed light on early stages of integrin activation in atomistic details, we have simulated interactions of integrin $\alpha\text{IIb}\beta 3$ with soluble RGD sequences. Interestingly, we recognized a key interaction group between K350 in the β A domain, with S673 and S674 in the β TD. We observed the full detachment of the β TD and β A domains as a result of the Asp side of the RGD peptide competing for Ser674 in β TD [72]. Thus, we suggested that removing the key interaction between β A and β TD potentially releases the integrin head domain, allowing integrin to bind the RGD ligand. Nonetheless, our model does not necessarily exclude possibility of opening of the ectodomain following the activation.

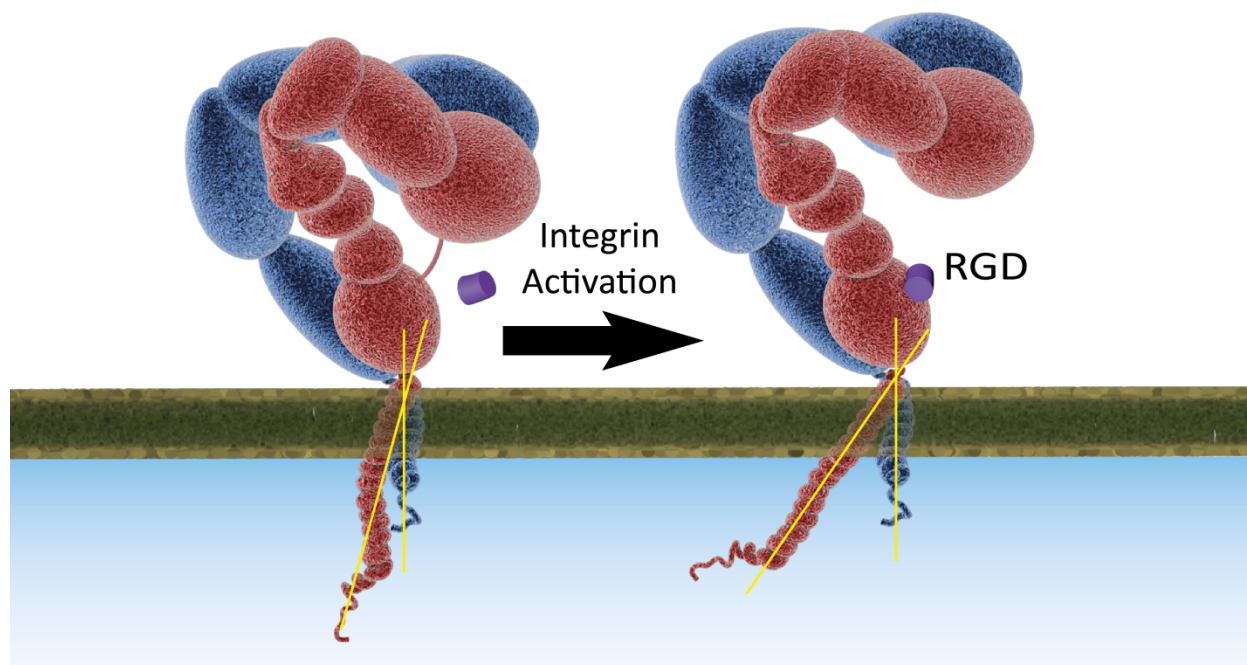


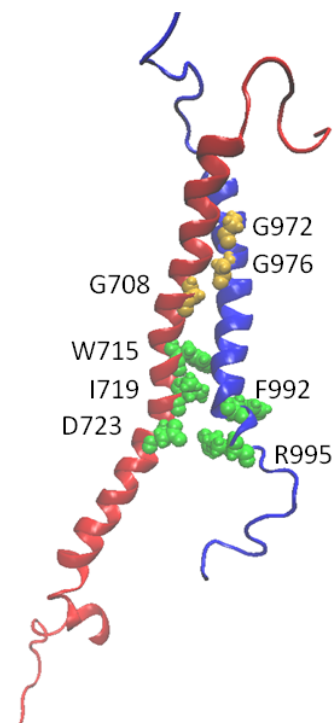
Figure 1.9: The deadbolt model suggests that integrin could also be activated via separation of the β TD and β A domains as a result of ligand binding even if it is not fully stretched. Upon release of the β TD from β A, a rotational movement of the transmembrane domains leads to an increase in the original angle formed between the α - and β -transmembrane domains of integrin.

1.3. Integrin Transmembrane Domains

In addition to their role in bridging the extracellular environment and cytoplasm, the helical transmembrane domains of integrins play a critical role in regulating the integrin function [87]. Integrin α - and β -subunit transmembrane domains are non-covalently bound in the inactive state [7]. The structure of the α -transmembrane segment reveals a linear 24-residue α -helix. The β transmembrane segment of integrin consists of a linear 29-residue α -helix that is tilted inside the membrane, forming an angle of about 25° with normal to the membrane [2,5] (Fig. 1.10). In the inactive state, there are two groups of interactions between the transmembrane domains of integrin α - and β -subunits [1,88]: A group of hydrophobic α - β interactions between transmembrane domains of integrin, called the outer membrane clasp (OMC) along with a group of charged and hydrophobic residues binding the α and β transmembrane domains, called the inner membrane clasp (IMC) [7,9,89] (Fig. 1.10). In integrin α IIb β 3, the OMC is formed by packing interactions at G972 and G976 of α IIb with G708 of β 3, while the IMC is composed of hydrophobic interactions (i.e. F992 & F993 on α IIb with W715 & I719 on β 3) and a salt bridge (i.e. R995 on α IIb with D723 on β 3) [2,9,82,90]. A GFFKR sequence in the transmembrane domain of the α -subunit located at the interface of the plasma membrane and cytoplasm is engaged with the β 1, β 2, β 3, and β 7 subunits to form the IMC [91,92,93]. Breaking the salt bridge R995-D723 in β 3 and the corresponding residues in β 1 and β 2 leads to integrin activation. Although the crystal structures of transmembrane domains are solved for human integrin α IIb β 3 [2], α L [94], and α 1 [95], the sequence of events linking conformational changes

in the transmembrane domains to integrin activation is yet to be cleared. Several experimental and computational studies indicate that disrupting either of the IMC or OMC causes integrin activation [2,72,96]. Although destabilizing IMC via mutations is known to trigger integrin activation [82,90], mutations that removed the salt bridge in the IMC did not show any significant activating effect on integrin $\beta 1$ [97]. Measuring resonance energy transfer between fluorescence dyes associated with the cytoplasmic domains of integrin $\alpha L\beta 2$ indicated a separation upon activating integrins via ligand binding or interacting with cytoplasmic proteins [98]. Furthermore, release of an artificial clasp that was replaced with the interactions of the transmembrane domains of integrin $\alpha 5\beta 1$ resulted in an approximately 14nm separation of the stalks and increased binding to fibronectin [65].

Figure 1.10: In the inactive state, there are two groups of interactions between the transmembrane domains of integrin α - (blue) and β -subunits (red): A group of hydrophobic α - β interactions between transmembrane domains of integrin, called the outer membrane clasp (OMC) (orange) along with a group of charged and hydrophobic residues binding the α and β transmembrane domains, called the inner membrane clasp (IMC) (green). In integrin $\alpha IIb\beta 3$, the OMC is formed by packing interactions at G972 and G976 of αIIb with G708 of $\beta 3$, while the IMC is composed of hydrophobic interactions (i.e. F992 & F993 on αIIb with W715 & I719 on $\beta 3$) and a salt bridge (i.e. R995 on αIIb with D723 on $\beta 3$) [2].



Separation of the α - and β -transmembrane helices and extension of the ectodomain are the two well-known features of the active and ligand-bound states in some members of the integrin family. It has been shown that disrupting the interactions between the transmembrane domains is sufficient to induce the active conformation of integrin $\alpha IIb\beta 3$ [22,99,100,101]. Homomeric interactions of integrin αIIb and $\beta 3$ seemingly compete with the heterodimeric interactions that maintain the integrin in the inactive state. Thus, it is suggested that integrin subunit homo-oligomerization stabilizes integrins in their active state [99]. Interestingly, Litvinov et al. observed that disrupting the interactions between the transmembrane helices in $\alpha IIb\beta 3$ integrins either by activating the molecule through a monoclonal antibody (PT25-2) or by residue mutations in transmembrane helices ($\beta 3$ G708N or αIIb G972N) have similar effects on the maximum force between the integrin and ligand [22]. This led researchers to propose a switch-like model for integrin activation. Furthermore, molecular dynamics and biochemical analyses showed that talin head domain likely activates integrin via disrupting the IMC by attaching to the $\beta 3$ subunit [72,102]. In another study, blocking transmembrane separation through generating inter-subunit disulfide bonds hindered adhesion-induced outside-in signaling [103]. Therefore, it could be proposed that separation of integrin $\alpha IIb\beta 3$ transmembrane domains is necessary and sufficient for integrin activation. Interestingly though, it has also been shown that outside-in activation of integrin $\alpha L\beta 2$ could still occur in presence of Mn^{2+} and mAb even when the transmembrane domains are locked by crosslinking and even in case the transmembrane domains are free to

separate, activation with Mn^{2+} does not require transmembrane dissociation [98,104,105]. Hence, it could be argued that the outside-in activation is not necessarily dependent upon transmembrane helix dissociation. As the ligand binding site is far from the OMC, an indirect, entropic destabilization of the OMC is proposed to occur via dissociation of the α - β lower leg assembly in the ectodomain upon outside-in activation [2]. Later, our molecular dynamics simulations showed that as a result of integrin $\alpha IIb\beta 3$ activation with soluble RGD peptides, the β tail domain retracts backwards such that the crossing angle between αIIb and $\beta 3$ transmembrane helices was increased in a lever-like motion, weakening the IMC via elevating the distance between the interacting residues [72].

Conformational changes in the transmembrane domains of integrin that lead to the separation of cytoplasmic domains and membrane-proximal regions of the transmembrane domains upon integrin activation remain ambiguous. Mainly, there are two models of transmembrane signal transduction supported by electron microscopy and biochemical data [21,31,65]. Both models agree that a scissor-like movement takes place after ligand binding and that this movement separates the cytoplasmic domain, however, they differ in the position of the hinge region. The first model suggests that the hinge region is located in the transmembrane region, whereas, the second one proposes a hinge region close to the extracellular compartment [84]. Examining validity of these models experimentally would be challenging as conformational changes take place within a fraction of a second and in a nanometer-scale embedded in the plasma membrane *in vivo*. Molecular dynamics simulations showed that the hinge-like motion is triggered most likely through an increase of the crossing angle of the transmembrane domains of integrin [72,84].

In line with computational studies, changes in the crossing angle of α - and β transmembrane domains of integrin $\alpha IIb\beta 3$ followed lateral separation of the transmembrane domains are predicted upon activation [90,99,104,106]. In such case, the C-terminal GFF motif on αIIb subunit may take on a helical conformation [89,107]. Alternatively, the five-residue hydrophobic stretch C-terminal to the first charged residues on the intracellular side of the $\beta 3$ subunit, K716, may repartition into the cytosol, leading to a straight $\beta 3$ transmembrane helix and a more parallel $\alpha IIb\beta 3$ transmembrane association. Moreover, membrane embedding of the transmembrane helices may also change at the extracellular face in a piston-like sliding motion, and thereby, transmit the force to the integrin ectodomain [2].

Integrin Clustering Models

Integrin-mediated adhesions are reinforced in two ways: by changing the ligand affinity of a single integrin, or by recruiting more integrins already available on the plasma membrane to form a cluster [108]. Integrin clustering is defined as interactions of hetero-dimers to shape lateral assemblies that eventually lead to focal complex formation [5,19]. Both physical and chemical properties of the ECM affect the integrin clustering. The density and rigidity of matrix ligands and their affinity for integrins are important determinants of the integrin cluster size and shape [109]. In an arrayed ECM, integrin clustering is largely impaired if the inter-ligand distance exceeds 60-70 nm or ligand density falls below $\sim 200\text{-}300\ \mu\text{m}^{-2}$. It is noteworthy that the minimum ligand density required for integrins to form clusters is less for disordered ligands

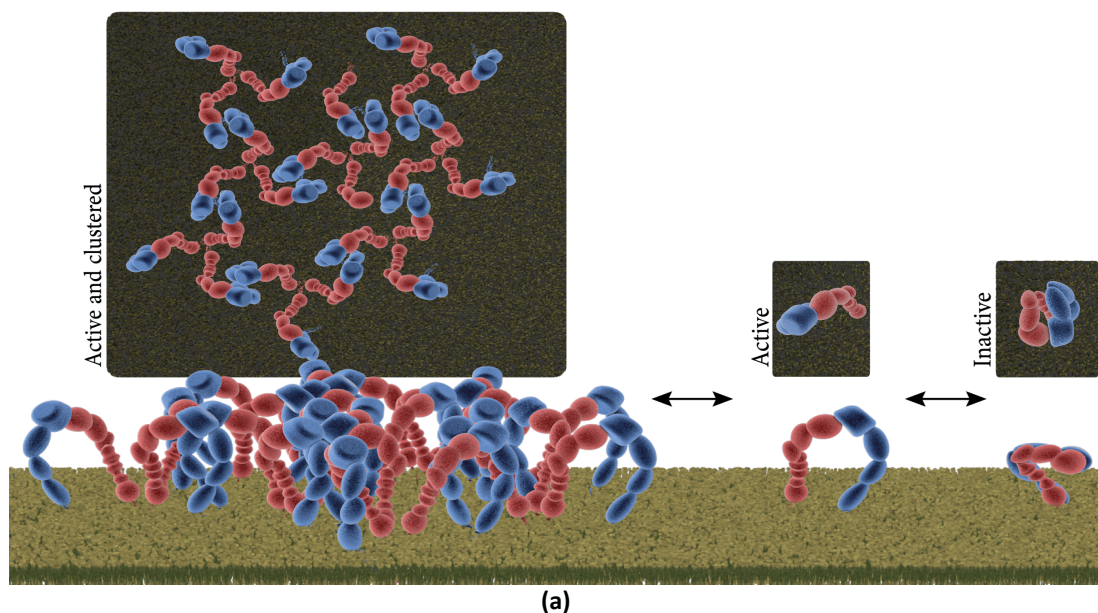
[110]. Many of current cell adhesion assays reflect the combined effects of single integrin conformation and integrin cluster regulation on adhesion strength, while distinguishing the two effects remains challenging [111,112].

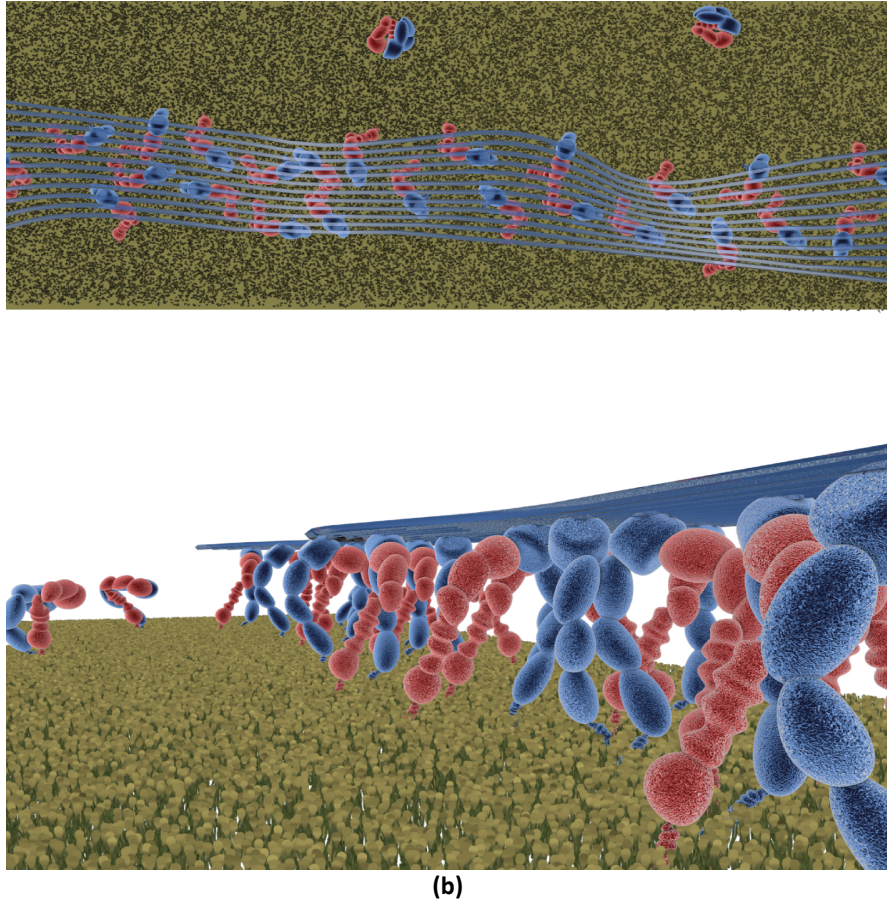
Bidirectional integrin signaling involves conformational changes in the hetero-dimer, integrin clustering, and the assembly of a large intracellular adhesion complex known as “adhesome” [73,113,114]. It has been known for two decades that integrin activity is regulated by its conformational changes [34,42]. Although there is a strong correlation between integrin clustering and activation, their interplay has remained enigmatic [19,100,101,114,115,116]. Some researchers proposed that integrin $\alpha\text{IIb}\beta\text{3}$ activation, which triggers the clustering process, is regulated by homo-dimerization of α - and homo-trimerization of β -subunits via interactions at their transmembrane domains [100,101,114,116]. This model suggests that there is a GxxxG region on transmembrane domain of α -subunits that may play a role in homo-dimerization of α -subunits after separation of α - and β -transmembrane domains [99] (Fig. 1.11a). In contrast to this hypothesis, a series of Cys mutagenesis scanning experiments has suggested that neither inactive nor ligand-bound integrin $\alpha\text{IIb}\beta\text{3}$ form homomeric association [115]. In human platelets, binding of Integrin $\alpha\text{IIb}\beta\text{3}$ to multivalent but not monovalent ligand was increased by thrombin activation and in CHO cells, talin regulated binding of integrin $\alpha\text{IIb}\beta\text{3}$ to multivalent but not monovalent ligands [117]. Therefore, it has been hypothesized that integrin clustering takes place upon binding of several integrins to multimeric ligands, and integrin clustering is independent of the homo-oligomerization of the transmembrane-cytoplasmic domains [115] (Fig. 1.11b). Intriguingly, there is also evidence demonstrating that it is hetero-dimerization of different integrin molecules that triggers the clustering phenomenon [9,115].

Generally, the process of focal adhesion maturation consists of three stages: (1) Microclustering (complex size $<0.3\text{ }\mu\text{m}$), (2) Myosin II-mediated maturation (size: $0.3\text{--}1.0$), (3) Focal adhesion stabilization [118]. A number of studies indicated that focal adhesions alter their size as a result of changes in the force they sustain [42,119], a process known as “reinforcement” [120]. For focal adhesions larger than $1\mu\text{m}^2$, the traction force at the focal adhesion increases with its size almost linearly [119]. In U2OS cells, the focal adhesion traction stress does not exceed $\sim 60\text{ Pa}$ in the absence of Myosin II. It has been shown that during the Myosin II-mediated focal adhesion maturation there is a strong correlation between the focal adhesion size and traction force [118]. Another study provided evidence that the focal adhesion area increases almost linearly with the cytoskeletal traction upon focal adhesion maturation [119]. Additionally, the force-surface area correlation highly depends on the contact point distance from the cell edge [118]. Integrin-ligand bond is categorized as a catch bond. Meaning that the integrin-ligand bond life-time is increased when the bond sustains a dissociating force [39,59]. Steered molecular dynamics simulations [41,49] and experimental studies [111,121] suggest that force can contribute to integrin activation and extension. This model is corroborated by the fact that integrin clustering has not been observed in cells lacking the talin rod domain, thereby being unable to bind integrins to the actin cytoskeleton, though solely talin head domain is sufficient to activate integrins [122,123].

Current experimental activation/clustering models do not provide an explanation for the force-recruitment correlation in integrins. Molecular dynamics studies were used to estimate the free energy of homo-oligomerization for α IIb- and β 3-subunits embedded in a computational model of the lipid bilayer, raising the possibility of recruiting more β -subunits in the focal adhesion in an oligomerized form via linking them to F-actins as they are further stretched [124]. This is aligned with the observation in which low concentrations of cytochalasin D (i.e. an actin polymerization inhibitor) increased the number of adhered leukocytes, whereas higher concentrations decreases the adhesion ratio of leukocytes [125]. This could be an effect of the tradeoff between the release of β 2 integrins from the actin cortex to diffuse over the plasma membrane and the lack of aligning F-actins to form the clusters as release of integrin α L β 2 from the cytoskeleton is reported to cause rearrangement of integrins and clustering in leukocytes.

Focal adhesion proteins that directly interact with integrin could also be important role players in integrin clustering. Homo-dimerization of talin with two binding sites for the cytoplasmic β -tail of integrin is suggested to cause integrin clustering via bringing integrin β -tails together when connected to the actin cytoskeleton [6,126]. The structure of α -actinin and filamin also show multiple integrin binding sites, suggesting possible roles in integrin clustering. α -actinin is an anti-parallel homodimer and thus there are two integrin binding sites on opposite sides of a single molecule. Simultaneous association of α -actinin to two integrins prevents their separation [127]. Similarly, several repeats of the filamin monomer bind integrin that could bring a number of β -tail together [128]. The common trait of these focal adhesion proteins that raise the possibility of them being regulators of integrin clustering is their multiple binding sites for the integrin β -tail as well as the fact that they directly link the actin cytoskeleton, which may recruit more integrins upon an increase in the level of tension in the cytoskeleton [87]. Focal adhesion kinase (FAK) is also shown to be a modulator of the integrin cluster size [129]. Upon tyrosine phosphorylation, binding of FAK with the cytoplasmic tail of clustered integrin β 1 and β 3 is increased, potentially at the N-terminal region of FAK [130].





(b)

Figure 1.11: Mainly, two models are suggested for integrin clustering: (a) According to the integrin homo-oligomerization model, upon activation of integrin, its α - and β -subunit transmembrane domains are separated, while integrin clustering occurs as a result of dimerization of activated α -subunits and trimerization of activated β -subunit transmembrane domains. (b) Nonetheless, binding of several integrins to multivalent ligands without formation of homo-oligomers is deemed the driving force for integrin clustering by the multivalent ligand model.

1.4. Integrin Cytoplasmic Domains

Integrin inside-out activation is initiated by interactions of focal adhesion proteins (e.g. talin and kindlin) with the cytoplasmic tail of the integrin β -subunit. Interactions of integrin cytoplasmic domains with each other or cytoplasmic proteins lead to the long-range allosteric rearrangements of the integrins that underlie activation [64,131]. The transmembrane domain of integrin α -subunits ends at a GFF sequence, forming the IMC interface, followed by a KR motif in all α -subunits but $\alpha 8$, $\alpha 9$, $\alpha 10$, and $\alpha 11$, which maps inside the cytoplasm, playing a crucial role in integrin activation [2,5]. The cytoplasmic tail of integrin β -subunit is home to a variety of focal adhesion proteins that either directly activate integrin or regulate its function [87].

Out of several cytoplasmic proteins that interact with α - and β -subunit cytoplasmic domains, only talin and kindlin are thus far believed to directly activate integrin [5]. Almost all β -tails have two well-defined motifs that are part of a canonical recognition sequence for

phosphotyrosine-binding (PTB) domains [132], consisting of a membrane-proximal NPxY motif and a membrane-distal NxxY motif. These motifs are binding sites for multiple integrin-binding proteins, including talin and the kindlin [6].

Talin Activates Integrin

Talin is a ~270-kDa protein with a globular, N-terminal head region and a flexible, C-terminal tail domain. The N-terminal head domain is composed of a FERM (4.1 protein ezrin, radixin moesin) domain, which consists of F1, F2, and F3 subdomains, as well as an F0 subdomain [133,134,135,136]. Talin head and rod domain are connected through a flexible linker containing a Calpain 2 cleavage site [87]. Talin orthologs have been identified in all multi-cellular eukaryotes studied. Vertebrates encode two talin isoforms, named talin1 and talin2 [137,138].

Talin is the most well known focal adhesion activator of the integrin, believed to be the downstream target of activating cascades that finally lead to integrin activation [106]. Talin rests natively in an auto-inhibited state, wherein its PTB subdomain is blocked via an interaction with the C-terminus [139]. Upon receipt of an activating signal, talin head domain is separated from its rod domain. Binding of phosphatidylinositol (4,5)-bisphosphate (PIP2) to talin is deemed responsible for activation of talin [39,139] (Fig. 1.12). Activation of talin and its recruitment to the plasma membrane via binding to PIPKly enables talin to readily recognize the integrin cytoplasmic β -tail [134]. Binding of the ~50-kD talin head domain to the integrin β -tail is deemed critical for integrin activation [102] (see Fig. 1.12) as knock-down of talin-1 and talin-2 have been shown to hinder interactions of integrin α IIb β 3, α V β 3, and α 5 β 1 with their extracellular ligands [88,140,141,142], whereas the over-expression of talin's N-terminus activates integrins [143]. Talin interacts with the cytoplasmic tail of integrin β -subunit via a phosphotyrosine-binding (PTB) subdomain within the talin FERM domain [133]. PTB subdomains in other cytoplasmic proteins are also binding sites for the β -subunit cytoplasmic tail of the integrin. The PTB subdomain of talin associates with the membrane-proximal NPxY/F motif present in most integrin β -tails [132,134].

Binding of talin to β -subunit tails through a PTB-like interaction and engaging a membrane-proximal β -subunit NPxY/F tail site leads to integrin activation because of three events that follow it: (1) Positioning basic patches on talins for an extended electrostatic interaction with the phospholipid head groups of the plasma membrane, (2) favoring the formation of a stable, continuous helix that extends out of the transmembrane and membrane-proximal portions of the β -subunit, enabling talins to change the stable crossing angle on the β -subunit transmembrane domain, (3) rendering talins capable of forming a second interaction with the β -tail that disrupts the key Arg-Asp interaction of the IMC by forming a salt bridge with the β -subunit Asp [5,88,106], unlike other PTB-containing cytoplasmic proteins (Dok1, tensin and Numb) that only bind the NPxY/F motif [6,133,144]. Compelling evidence has been put forward that talin head domain activates integrin via competing with the inner membrane clasp [2,72,89,96,106]. Destabilizing the key IMC interaction between R995 of α IIb and D723 of β 3 as a result of talin1 interaction with the β 3 tail domain leads to integrin activation [5]. Similarly,

talin2 interacts with β 1D cytoplasmic domain, disrupting the corresponding key Arg-Asp electrostatic interaction [145]

Nevertheless, how this disruption results in the extension or conformational changes of integrin and how signals from the tails are transmitted to the ectodomain remain ambiguous. Recent studies demonstrate that in addition to the NPxY/F motif, talin interacts with other membrane proximal regions on β -integrin tail which might explain its unique ability to activate integrin [106]. Whether talin is sufficient for integrin activation or it requires other focal adhesion proteins, particularly kindlin, also remains controversial [102]. The talin head domain has been shown to induce separation of the integrin cytoplasmic tails in integrin α L β 2, increasing the integrin affinity for the ligand [98], while talin1-depleted cells across several species have been unable to respond to activation stimuli properly [88,140,142,146,147,148]. These pieces of evidence led scientists to suggest talin is necessary and sufficient for integrin activation. In contrary, some studies propose the inadequacy of talin and an essential role of kindlin in cooperation with talin in integrin activation and function [149,150,151,152].

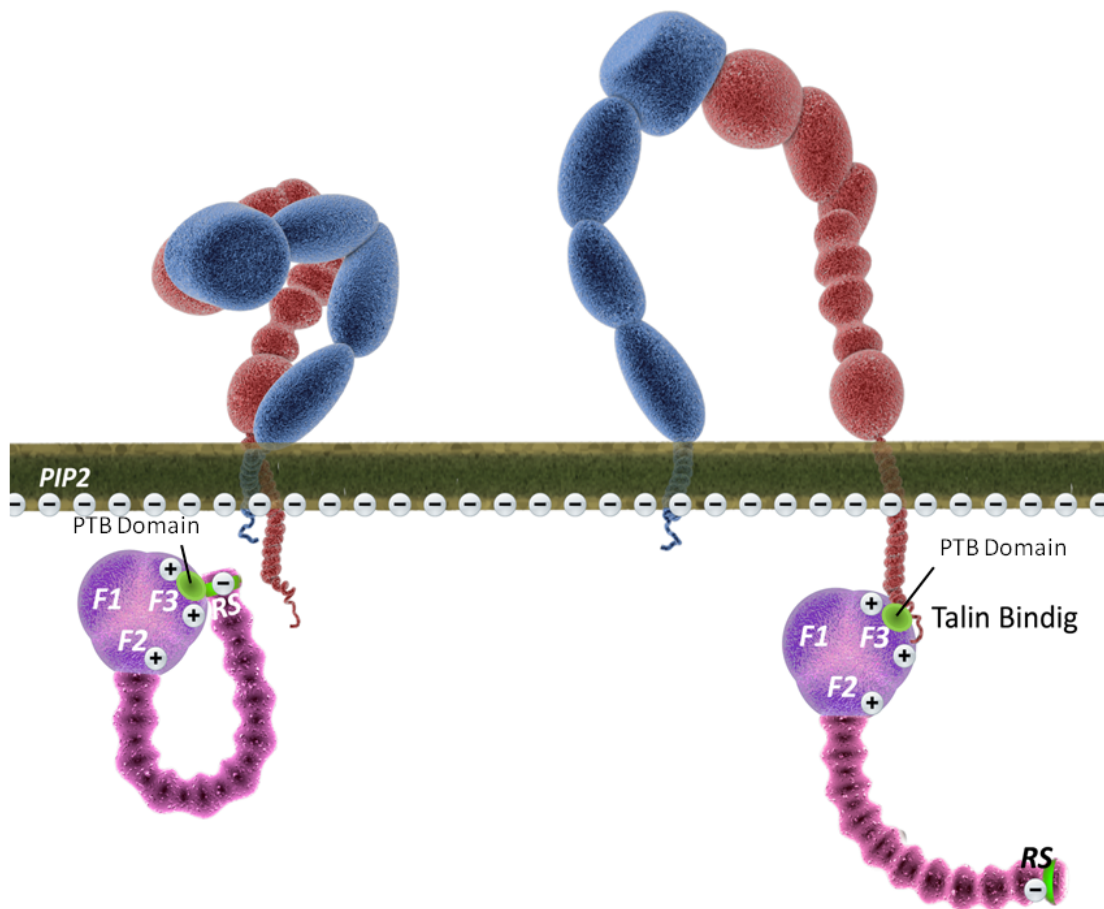


Figure 1.12: Schematic view of integrin activation by talin. (left) Integrin and talin are both in an inactive state, where integrin ectodomain is folded with α - and β -transmembrane domains bound together. Talin is composed of an N-terminal head connected to an extended helical bundle known as the rod domain. In the resting state, talin is auto-inhibited via an interaction between its F2/F3 domains and RS subdomain located at the end of the rod domain. The talin RS domain of the inactive talin binds to F3 domain, preventing the F3 domain from accessing the β -

tail of integrin. (right) To bind integrins, talin should be activated. One of known activators of talin is PIP2 (phosphatidylinositol 4,5-bis-phosphate). Integrin binding occurs at talin FERM (Four-point-one-protein/Ezrin/Radixin/Moesin) domain, which consists of F1, F2, and F3 domains. The FERM domain of talin is positively charged, whereas, its RS domain is negatively charged. In PIP2-rich regions of the plasma membrane, interactions of the negatively-charged head of PIP2s attracts and recruits the talin head domain to the membrane and the RS domain is repelled, detaching from F3 domain. Finally, the F3 domain binds to the β -subunit cytoplasmic tail, dissociating the integrin cytoplasmic tails and integrin activation [153]. Protein Calpain also activates talin via severing its head domain [153].

Kindlin Cooperates with Talin

Kindlin is another focal adhesion protein family, consisting of kindlin1, kindlin2, and kindlin3, known to play an important role in integrin activation. Similar to talin, the 76-kDa kindlin structure contains a FERM domain, with a PTB subdomain, which binds the membrane-distal NxxY/F motif of integrin β 1, β 2, and β 3 subunit tails, whereas talin binds the membrane-proximal NPxY/F motif of the integrin β -tail [6,149,151,154,155,156,157,158]. The FERM domain of kindlin is distinguished from that of talin in that the FERM domain possesses a pleckstrin homology (PH) domain that splits the FERM domain into two portions. Kindlin activation and recruitment at integrin sites is likely promoted by PIP3 binding to its PH domain [6,159]. Although kindlin is not sufficient to activate integrins, it seemingly cooperates with talin to facilitate its function [6,149,151,156]. Kindlin1 and 2 are linked indirectly to the actin cytoskeleton via binding to integrin-linked kinase (ILK) and migfilin [6]. Genetic and siRNA depletion of kindlin1, 2, and 3 in mice and cells provided experimental proof that kindlins are crucial for integrin activation because the conformational change of integrins from the low- to high-affinity state does not occur in the absence of kindlins [149,151,157,160].

Currently, three hypotheses exist regarding the cooperative function of talin and kindlin to activate integrin: (1) Kindlin binds the membrane-distal NxxY motif of the integrin β -tail, facilitating the binding of talin to the membrane-proximal NPxY motif, which finally releases kindlin and activates integrin, (2) talin and kindlin likely associate with the β -tail simultaneously as their binding sites are separate, (3) binding of talin and kindlin occurs each at the β -tail of a separate integrin. Upon formation of a nano-cluster of integrin tails, talin and kindlin are brought close together and interact to activate integrin (see Fig. 1.13). Further studies are required to shed light on the molecular-scale cooperation of talin and kindlin to activate integrin [6,161].

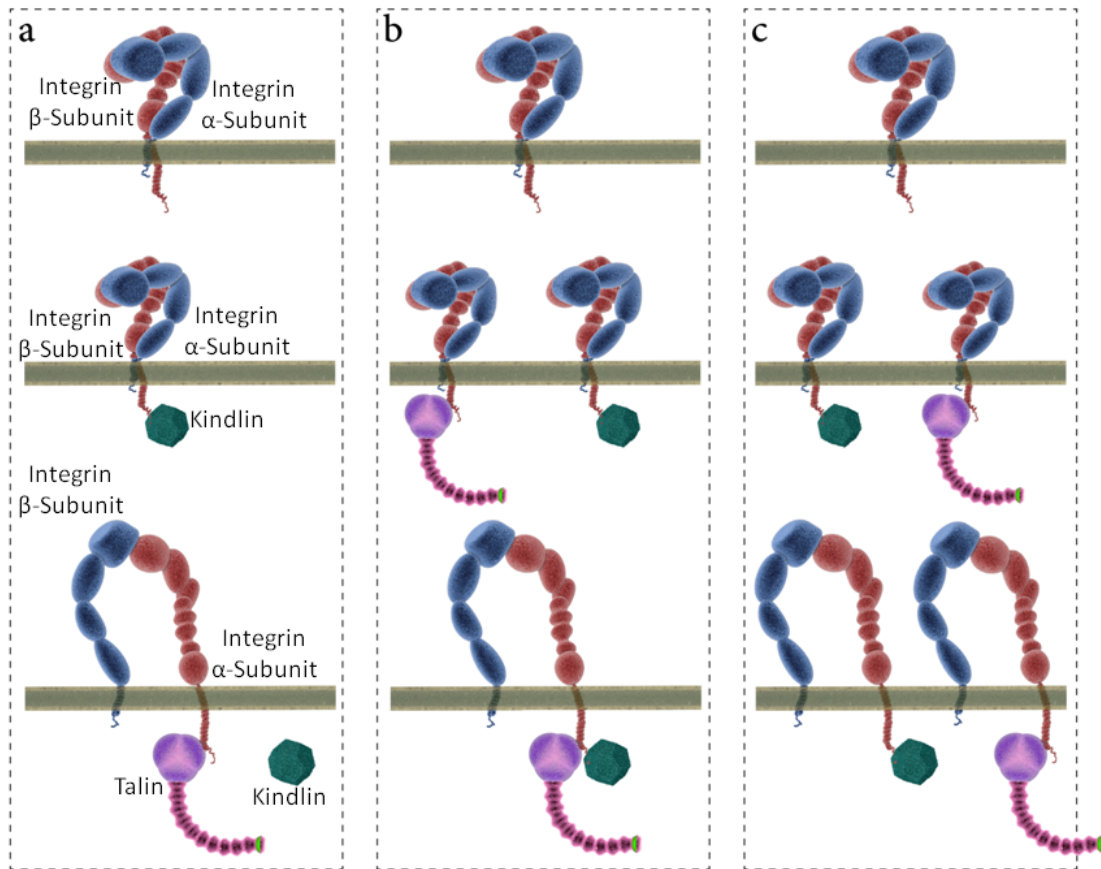


Figure 1.13: Current models for talin-kindlin cooperative mechanisms of integrin activation. (a) Kindlin (green) binds the membrane-distal NxxY motif of the integrin β -tail, facilitating the binding of talin (pink) to the membrane-proximal NPxY motif, which finally releases kindlin and activates integrin. (b) Talin and kindlin may associate with the β -tail simultaneously as their binding sites are separate. (c) Binding of talin and kindlin occurs each at the β -tail of a separate integrin. Upon formation of a nano-cluster of integrin tails, talin and kindlin are brought close together and interact to activate integrin [6].

Integrin Negative Regulators

Negative regulators of integrins are broadly divided into two categories: direct and indirect regulators. As an indirect negative regulation could take place at any step of integrin activation cascades for various integrin family members, reviewing it would be outside the focus of this work. However, a few examples of the proteins that hinder activation via interacting with the cytoplasmic domains of integrin are briefly discussed here.

Generally, Tyr-phosphorylation of the NPxY/F motif of the β -subunit tail domain, triggered by the Src family kinases blocks talin1-binding via interrupting acidic and hydrophobic interactions between the NPxY/F motif and talin FERM domain, hindering the integrin inside-out activation [162]. The caveats are β 1 integrins in which Tyr-phosphorylation apparently has no functional significance [97]. Additionally, the Tyr-phosphorylation favors the interaction of cytoplasmic proteins with PTB subdomains that do not activate integrins, such as the one in Dok1 [132,163].

Phosphatidylinositol phosphate kinase type 1 γ 90 is also known to compete with β -subunit tails for binding to talin1 [164,165]. Whether filamin A and FAK also serve to inhibit integrin activation is still under debate.

Cytoplasmic proteins that bind to integrin α -subunit tails could also affect integrin activation. CIB1 is shown to inhibit integrin α IIb β 3 activation via associating with the α IIb subunit tail and interfering with talin1 binding to integrin β 3 [166]. Finally, RAPL regulates integrin α L β 2 integrin-mediated adhesiveness of leukocytes via binding to α L tail domain [167,168].

1.5. Dissertation Objectives

The overall goal of this thesis is to develop a detailed understanding of integrin function linked to its conformational changes, using molecular dynamics techniques. To achieve these overall goals, we set the following specific objectives:

1. Evaluate two major models proposed for the integrin clustering process and propose a new model to integrate the experimental evidence and computational studies.
2. Shed light on atomic events that lead to integrin activation by talin and soluble RGD ligand, using a molecular model.
3. Investigate the potential role of integrin α -subunit head domain on ligand binding via coordinating the metal cation at the ion binding site LIMBS, using a descriptive model of integrin.

1.6. Structure of Thesis Document

The objectives discussed in the previous section will be met and demonstrated using the following structure:

- In Chapter 2, to pursue our first objective, we examine possibility of integrin homo-oligomerization within the plasma membrane of the cell. As explained earlier in this chapter, there is a debate on whether integrin α - and β -transmembrane domains form homo-oligomers, forming integrin clusters that would act as the nucleation point for focal adhesion formation and later maturation.
- In Chapter 3, the second objective is fulfilled via all-atomic simulations of integrin α IIb β 3 inside-out and outside-in activation pathways via interacting with a well-established cytoplasmic protein (i.e. talin) and an extracellular activator (i.e. fibrinogen).
- Chapter 4 approaches the third objective by comparing effects of cation binding site LIMBS, located on the α -subunit-facing side of integrin β 3 head domain, on RGD-ligand binding in integrin α IIb β 3 and α V β 3, indicating the significance of the α -subunit head domain in determining ligand specificity.
- Chapter 5 concludes the results of this study and suggests a road map for the future.

Chapter2

Integrin Oligomerization and Clustering

It remains controversial whether the homo-dimerization of integrin transmembrane domains occurs following the integrin activation (i.e. when integrin ectodomain is stretched out) or if it regulates integrin clustering. This chapter focuses on molecular dynamics modeling approaches to address these questions in molecular details and sheds light on the crucial effect of the plasma membrane. Conducting a normal mode analysis of the intact $\alpha\text{IIb}\beta\text{3}$ integrin, it is demonstrated that the ectodomain and transmembrane-cytoplasmic domains are connected via a membrane-proximal hinge region, thus merely transmembrane-cytoplasmic domains are modeled in this chapter of the study. By measuring the free energy change and force required to form integrin homo-oligomers, this study suggests that homo-oligomerization of the β -subunit potentially regulates integrin clustering, as opposed to the α -subunit, which appears to be a poor regulator for the clustering process. If α -subunits are to regulate the clustering, they should overcome a high-energy barrier formed by a stable lipid pack around them. Finally, an outside-in activation-clustering scenario is speculated, explaining how further loading the already-active integrin may affect its homo-oligomerization and focal adhesion growth.

2.1. Introduction

Bidirectional integrin signaling involves conformational changes in the hetero-dimer, integrin clustering, and assembly of a large intracellular adhesion complex [73,113,114]. Integrin clustering is defined as the interaction of hetero-dimers to shape lateral assemblies that lead to focal complex formation [5,19]. It has been known for two decades that integrin activity is regulated by its conformational changes [34,42]. Although there is a strong correlation between integrin clustering and activation, how one leads to the other has remained elusive [19,100,101,114,115,116]. A body of evidence has proposed that integrin $\alpha\text{IIb}\beta\text{3}$ activation, which triggers the clustering process, is regulated by α - and β -subunit homo-oligomerization [100,101,114,116]. In opposition to this hypothesis, a series of Cys mutagenesis scanning experiments has suggested that neither inactive nor ligand-bound $\alpha\text{IIb}\beta\text{3}$ integrins form homomeric association [115]. Instead, it has been hypothesized that integrin clustering occurs as a result of binding of several integrins to multimeric ligands, and the transmembrane-cytoplasmic (TMC) domains do not play any significant role in the process [115]. Additionally, there is evidence that demonstrates it is hetero-dimerization of different integrin molecules that triggers the clustering phenomenon [9,115].

Studies indicate that focal adhesions alter their size as a result of changes in the force they sustain [42,119], a process termed “reinforcement” [120]. It has been reported that for focal adhesions larger than $1\mu\text{m}^2$, traction force at the focal adhesion increases with its size linearly [119]. Current activation/clustering models do not provide an explanation for the force-recruitment correlation in integrins. In contrast, the switch-blade model, which is a widely-accepted functional model for integrin, limits the integrin molecular conformations to three

modes, namely passive, active, and ligand-bound [34,35,43]. The passive mode is corresponding to a bent integrin structure while integrin activation is marked by a global conformational change, which results in a stretched structure with a separation of α - and β transmembrane-cytoplasmic domains from each other. Finally, an opening between the α - and β -subunit heads gives rise to a ligand-bound conformation [35,43]. Once the ligand-bound conformation is achieved, it is unclear how the reinforcement phenomenon takes place under larger loads.

The effect of the plasma membrane on integrin activation/clustering has been largely neglected in previous experimental works, whereas, molecular-level, computational studies of integrin have been mostly focused on simulating the ectodomain [42,80,112]. In this chapter, all-atom molecular dynamics techniques are presented to conclude that the plasma membrane surrounding the integrin transmembrane domains plays an important role in the process of activation/clustering by forming a lipid pack around the transmembrane domains. Furthermore, by performing normal mode analysis on the integrin α IIb β 3, it is shown that the integrin ectodomain and TMC domains swing about a hinge-like region that links them together. Finally, we hypothesize a potential integrin clustering scenario that explains the seemingly contradictory results for integrin clustering mechanism, namely homo-oligomerization-based and multivalent ligand-based clustering models, elucidating the unexplained force-area correlation in focal adhesions as well. One of the major determining factors for feasibility of a reaction is the free energy change profile over the reaction coordinate. Therefore, we finally asked if integrin subunits were to oligomerized, how the free energy of the system would change as the two monomers come close.

2.2. Computational Procedures

Normal Mode Analysis: Integrin α IIb β 3 ectodomain (PDB ID: 3FCS chains A and B [49]) and transmembrane-cytoplasmic domains (PDB ID: 2KNC [169]) were downloaded from the Protein Data Bank (PDB) and combined to build the full-length integrin molecule, assuming a covalent bond between residues Cys959 and Gly955 in the α -subunit, and Gly684 and Gly690 in the β -subunit. The natural vibration frequencies of the full-length integrin molecule were determined using the normal mode analysis (NMA) software WEBnm@ [170]. The normal mode matrix, which is a function of integrin molecular structure, shows natural movements in flexible molecular regions and little movement in rigid regions. WEBnm@ uses the MMTK [171] software internally and computes natural frequencies using Hinsen's computational methods [172], which calculates approximate normal modes by determining the eigenvectors of the matrix of second derivatives of potential energy with respect to displacement of the C α atoms of each residue. The potential energy function used for this calculation utilizes a Hookian potential between C α atoms within an 8Å cutoff distance. Because NMA represents movements resulting from the overall structure, the use of C α atoms is sufficient for NMA calculations [173].

Molecular Dynamics: All molecular dynamics simulations are carried out with the program NAMD2.7 [174], using the CHARMM27 force field for lipids and CHARMM22 for proteins [175]. The transmembrane-cytoplasmic domains of the integrin α IIb β 3 molecule are taken from Protein Data Bank (PDB ID: 2KNC). Integrin subunits are embedded in a 1-palmitoyl-2-oleoyl-sn-

glycero-3-phosphocholine (POPC) model of the lipid bilayer, using the software VMD [176]. System geometries have been defined by TCL code scripting within VMD while physical properties have been defined in a configuration file used by NAMD. In all simulations, overlapping lipid chains are removed afterward and the membrane is solvated in an explicit water box with ionic NaCl concentration of 8mM [177]. In all simulations integrin is assumed to be in an activated state as transmembrane-cytoplasmic domains of α - and β -subunits are apart. The temperature and pressure of the system are held constant at 1atm and 310K, using Langevin's piston and Hoover's method [174]. Coordinates, energy, and steering forces were recorded every 1ps or 10ps and the time step was 2fs. The cutoff distance for non-bonded interactions was 1.2nm. For all simulations, particle mesh Ewald (PME) method was used for electrostatic force calculations [174]. The cutoff distance for non-bonded interactions was 1.2nm. The hydrogen atom bond length was constrained using SHAKE method. SHAKE method fixes bond lengths between large atoms and hydrogen atoms, preventing unnecessary calculation of irrelevant interactions [178].

In α - α and β - β homo-oligomerization simulations, the two monomers were initially positioned 5nm apart within the lipid bilayer and the system was solvated and ions were randomly added to the system afterward. Subsequently, the system was minimized for 4ps and equilibrated for 0.5ns. The results were visualized using VMD [176]. The steered α -monomer was pushed at C α of residues Ala956, Gly972, Ala 986, and Gly1007, whereas β -monomer is forced at C α of residues Val695, Gly708, Ala728, and Ala742 with a constant velocity of either 0.025Å/ps or 0.0025Å/ps. The other monomer is either fixed at the same atoms or free to move.

To observe the α -dimerization interaction, two α -dimers are placed initially 2nm apart and equilibrated for 1ns. After bonds are formed and the system reached equilibrium, one monomer is constrained at the same four atoms while the other is pulled on from the atoms corresponding to the constrained one, away from the fixed monomer. β -trimerization is scrutinized by primarily placing three transmembrane-cytoplasmic β -domains on vertices of an equiangular triangle each 2nm away from its adjacent monomers. The system is then equilibrated for 1ns until the trimerization interaction assumes an equilibrium state. Then, one monomer is pulled while others are constrained. This was continued until the steered monomer was completely separated from its neighboring β -monomers. All dimerization and trimerization simulations were equilibrated for 1.5ns to confirm the stability of interactions.

Plasma Membrane Density Calculations: The density of the cytoplasmic compartments of lipid chains were measured for the entire length of the plasma membrane along the line that connected the two subunits embedded in the membrane. A 100x50Å and a 100x150Å lipid patch were used to embed α - and β -domains in, respectively. The lipid patches and integrin subunits were minimized and equilibrated. Nine (each one 100ps after the previous one) and five (each one 500ps after the previous one) snapshots of the system in equilibrium were averaged for α - and β -domains, respectively.

Free Energy Calculations: In order to calculate the free energy profile of the α - α and β - β homo-oligomerization of cytoplasmic domains, we invoked Jarzynski method [179]. This method is a

powerful tool for calculating free energy profiles along reaction coordinates. It is applicable to non-equilibrium processes on the energy landscape of the system. The method employs the following formula to relate the free energy change of the system to the external work done on the system:

$$e^{-\beta \cdot \Delta F} = \langle e^{-\beta W} \rangle \quad (2.1)$$

where β , ΔF , and W are $1/k_B T$, change in free energy, and external work, respectively. Provided the number of samplings is extremely high, the formula applies independent of the process speed. Nonetheless, reasonable approximations have been made using Jarzynski method for steered molecular dynamics, when the system has been sampled only 10 times. Therefore, we minimized and equilibrated the system 10 times including two integrin TMC subunits, 5nm apart, embedded in a patch of plasma membrane. Afterwards, one subunit was steered toward the other from its cytoplasmic tail, while the other subunit was left unconstrained. The identical process of steering was repeated once starting from each of the 10 equilibrated systems. In order to avoid the bias toward the samples with smaller numbers, the free energy was calculated using the second order expansion of Eq. 2.2, as follows:

$$\Delta F = \frac{1}{M} \sum_{i=1}^M W_i - \frac{\beta}{2} \times \frac{M}{M-1} \left[\frac{1}{M} \sum_{i=1}^M W_i^2 - \left(\frac{1}{M} \sum_{i=1}^M W_i \right)^2 \right], \quad (2.2)$$

where M and W_i are number of samples and the work corresponding to the i -th sample.

2.3. Results

Normal Mode Analysis of Full Integrin: Normal mode analysis is a powerful method to detect softer regions in proteins and has proved useful in determining physiologically-relevant motions of proteins as well as their compartmental breakpoints [180,181,182,183]. Our normal mode analysis of the full-length integrin α IIb β 3 suggested that the protein's structure consists of a rather rigid ectodomain, hinged to two cantilever-like transmembrane-cytoplasmic (TMC) domains (see Fig. 2.1). Modes 7 to 10, corresponding to lowest non-zero deformation energies, show a clear rotation of the ectodomain and TMC domains about the hinge-like region. The soft linker region between the ectodomain and TMC domains hinders strong mechanical integrity of TMC domains with the extracellular domain. As expected, under an instantaneous excitation, the ectodomain and TMC domains swing about the soft region in opposite directions at any given time. As mode number increases, the molecule deformation energy is elevated rapidly, and consequently, contribution of higher modes to the molecule movement is insignificant (Fig. S1) [184]. Additionally, no homomeric interactions are reported to occur between integrin ectodomains [115]. Therefore, in order to reduce the computational cost of our simulations, in all molecular dynamics simulations solely the TMC α - and/or β -domains are modeled at this phase of the study.

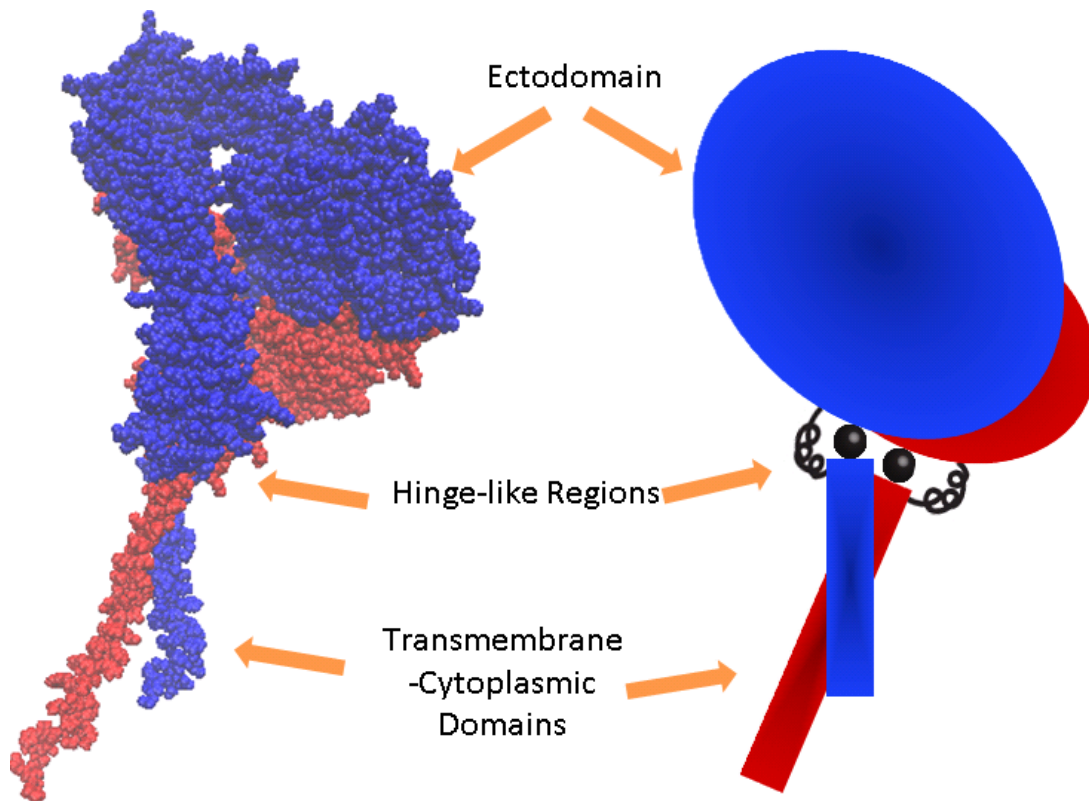
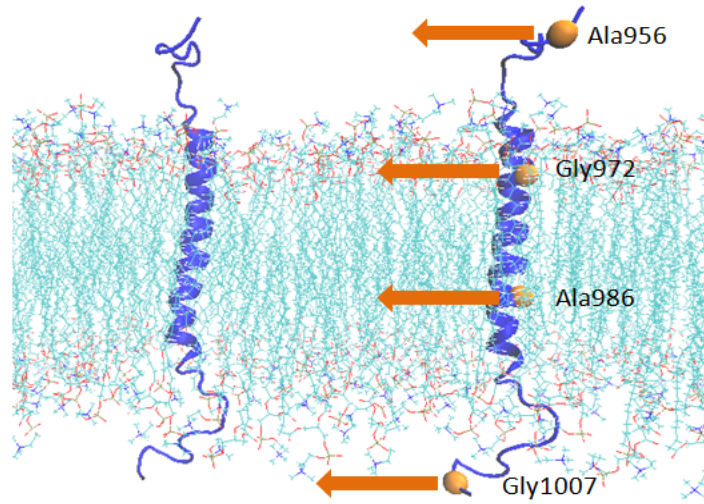


Figure 2.1: (left) Full-length integrin $\alpha\text{IIb}\beta\text{3A}$ dimer consists of an α -subunit (blue) and a β -subunit (red). (right) A purely mechanical model is suggested, illustrating how TMC domains are hinged to the more rigid ectodomain while rotational springs represent the weak bending stiffness at joints. See also Figure S1.

Integrin α - α Homo-oligomerization: The first step to our molecular dynamics simulations was aimed at modeling the homomeric interactions between the two α -subunits when they are in an active state (i.e. α - and β -subunits are distant enough not to interact significantly). In order for integrin α -subunits to initiate focal adhesion formation through their homo-oligomerization, α -subunits should be able to readily bind/unbind each other. Two identical crystal structures of integrin $\alpha\text{IIb}\beta\text{3}$ TMC α -subunits (Gly955 to Glu1008) were carefully embedded in a plasma membrane patch, 5nm apart from one another. The system was minimized and equilibrated for 0.5ns and steered molecular dynamics (SMD) was exploited to drag one of the α -subunits toward the other for 1.2ns with a constant velocity of $0.025\text{\AA}/\text{ps}$, while the other monomer was left unconstrained. Importantly, the free monomer also started to move in the same direction soon after the steered one did. Furthermore, the overall force required to direct the steered monomer toward the free subunit showed an abrupt increase at distance 5.1nm (see Fig. 2.2c), and electrostatic as well as van der Waals energies of the interaction between the two monomers were insignificant during a major part of the simulation (data not shown). This indicated that the two biomolecules did not ‘see’ each other throughout the simulation. Some minor positive interaction energy spikes were observed, which, based on the trajectory of the simulation, can be attributed to

interactions between a GAMG sequence located at the very end of the extracellular side of the steered α -subunit, where it is truncated off the ectodomain (Fig. S2). Conducting the same simulation but without the GAMG motif demonstrated almost no interaction energy at any time (see Fig. S2). This negative control suggests that the emerging energy spikes were an artifact of the separation of the integrin transmembrane and extracellular domains that had left this flexible linker region unconstrained, allowing the GAMG sequence to reach out to the other monomer. Another site of interaction was the hydrophobic patch on the cytoplasmic side of the free monomer, which also emerged from the plasma membrane. We further explored the phenomenon by repeating the simulation using a geometry in which the steered monomer's orientation is 90° rotated about its longitudinal axis relative to the free molecule. This time the free monomer maintained a distance of $\sim 4\text{nm}$ (data not shown).



(a)

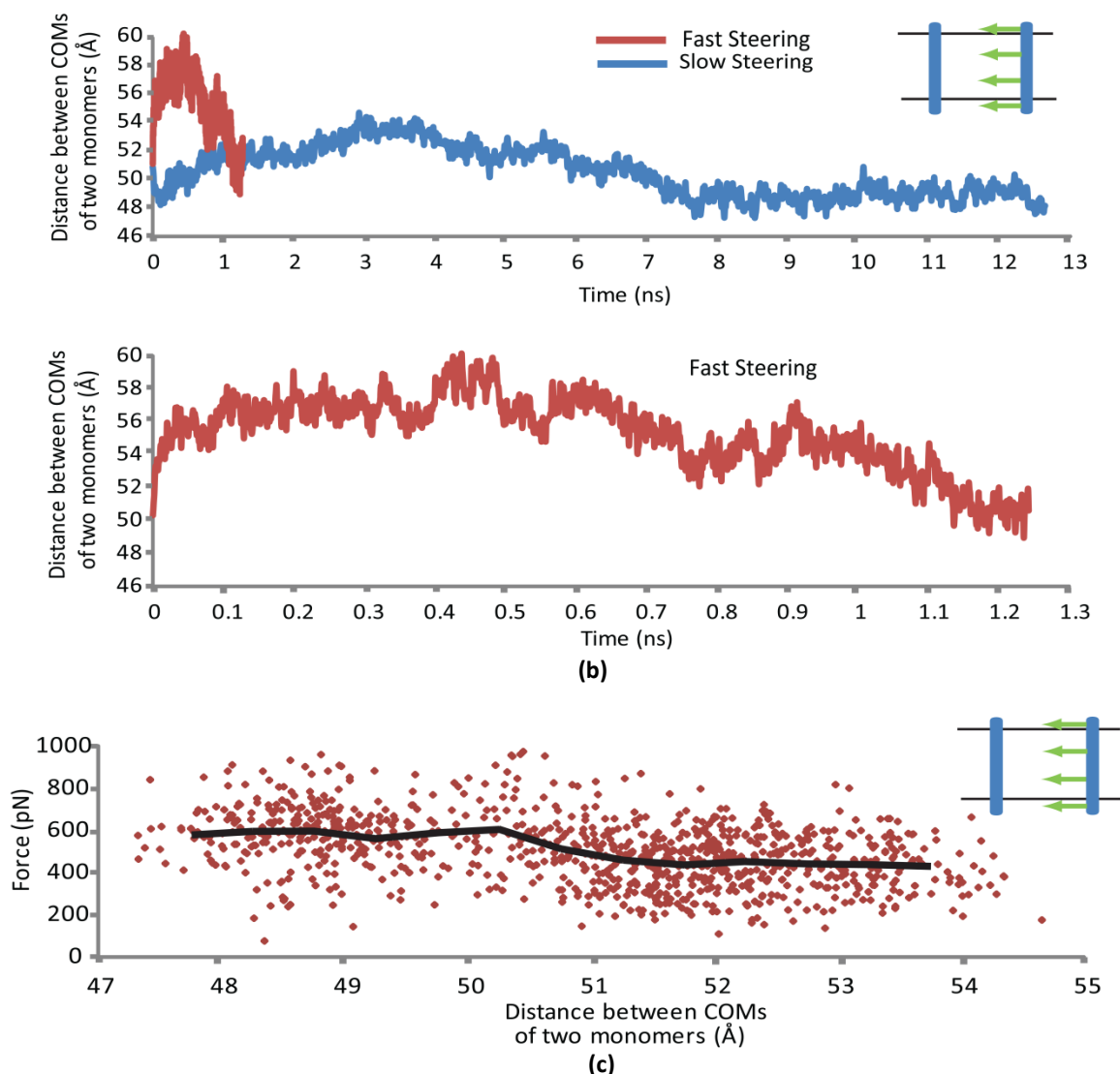


Figure 2.2: (a) Two transmembrane-cytoplasmic domains of integrin α -subunit are embedded in a lipid bilayer patch (shown by scattered points). The orange spheres represent the steered atoms being pulled along the green arrows. (b) Distance between centers of mass of the two α -subunit monomers as a function of time for two different pulling rates. The slow steering velocity is $0.0025\text{\AA}/\text{ps}$ whereas the fast steering velocity is $0.025\text{\AA}/\text{ps}$. The diagram legend shows how one α -monomer (blue) is pushed toward the other free one while both are embedded in a lipid bilayer. Diagrams for the two steering speeds are juxtaposed in the upper diagram to show that the final distance between the monomers is not dependent upon steering speed. The lower diagram provides more details for the slow speed diagram. (c) The pulling force needed to steer one monomer toward the other is plotted. When monomers are more than 5.1nm apart, about 400pN is required to drag the molecule. This ramps up as the monomers further approach toward each other, and eventually levels off when the final distance is attained. The heavy black line is a window-averaged form of the data with the window size of 0.5nm .

To observe if the pulling rate has a significant effect on results, the simulation was performed for a longer time scale with a pulling velocity as low as $0.0025\text{\AA}/\text{ps}$. The results were, in general, similar to the first simulation except that the fluctuations of the distance between the

monomers fell in a narrower band. Nevertheless, as Fig. 2.2b depicts, the distance in both cases eventually approaches the same value (5-5.1nm), which is the closest distance between centers of mass that the two monomers can reach without any constraints applied. We call this distance “final distance” hereinafter. The increase in the force level required to maintain the constant pulling rate also occurs at the distance of 5 to 5.1nm.

Mimicking α -dimerization appeared unlikely if α -subunits are not constrained. Thus in the next round of simulations we fixed the non-steered monomer at the same four atoms (C α of residues Ala956, Gly972, Ala 986, and Gly1007). The steered monomer was pulled on toward the unconstrained one at the same four atoms as before, which were selected such that they divide the monomer into similar length spans, and fixed atoms with smallest side chains are chosen to reduce the potential error caused by steering. The steered monomer approaching the constrained one, the pulling force rose again when the monomers were $\sim$ 5.1nm apart. The four-point SMD was deemed incapable of mimicking the full dimerization process. The pulling mechanism used in our simulations assumed a dummy point that moved in the space with a constant velocity and pulled the steered points on the molecule, using dummy springs that are linking those points to the dummy point. In fact, when the α -monomer was being pulled from four points, the two points at the two ends of the monomer fell inside the water box while the two points in the middle were embedded in the lipid bilayer. As a result, when the lipid packs around the two monomers started to overlap, the two embedded points experienced a substantially higher resistance than the ones moving in the water box, and therefore fell behind the lipid embedded points. This initially led to unfolding of the steered monomer. We avoided this problem by releasing the two end points when the steered monomer approached the fixed one. Hence, the simulation was continued one more time with only two, membrane-embedded steering residues (i.e. Gly976 and Ala986) (Fig. 2.3). These two complementary simulations, therefore, conferred a profile of the overall force required to tow a single TMC α -domain toward the other constrained one. An overall SMD force of as much as 1.7nN is required to eventually bind the two α -domains.

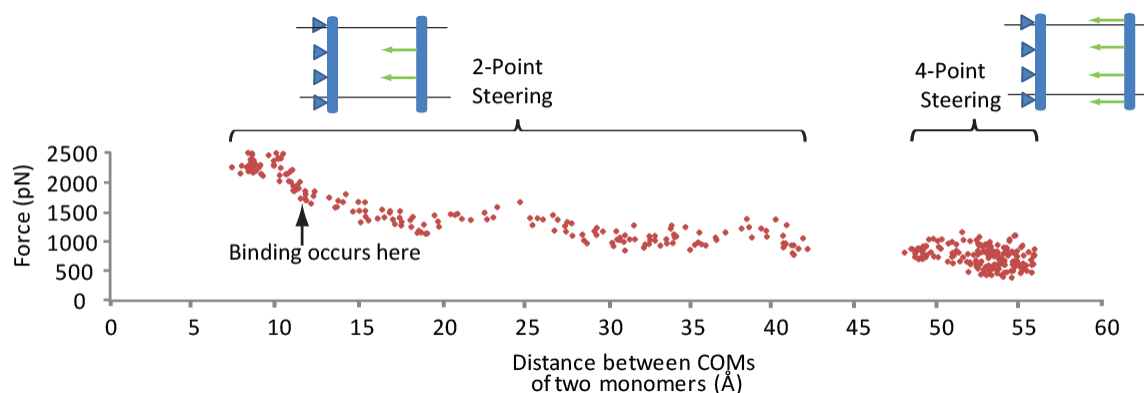


Figure 2.3: Results of SMD simulation of one α -monomer as the other is constrained. The force required to drag the monomer rises gradually as the distance decreases, when the monomers are further than 5.1nm apart from one another. Results of the 4-points and 2-points SMD are juxtaposed.

Lipid Packing Phenomena: Observing the system trajectory from the top view (perpendicular to the membrane patch), it appeared that two cylinder-like batches of lipid chains surrounding the

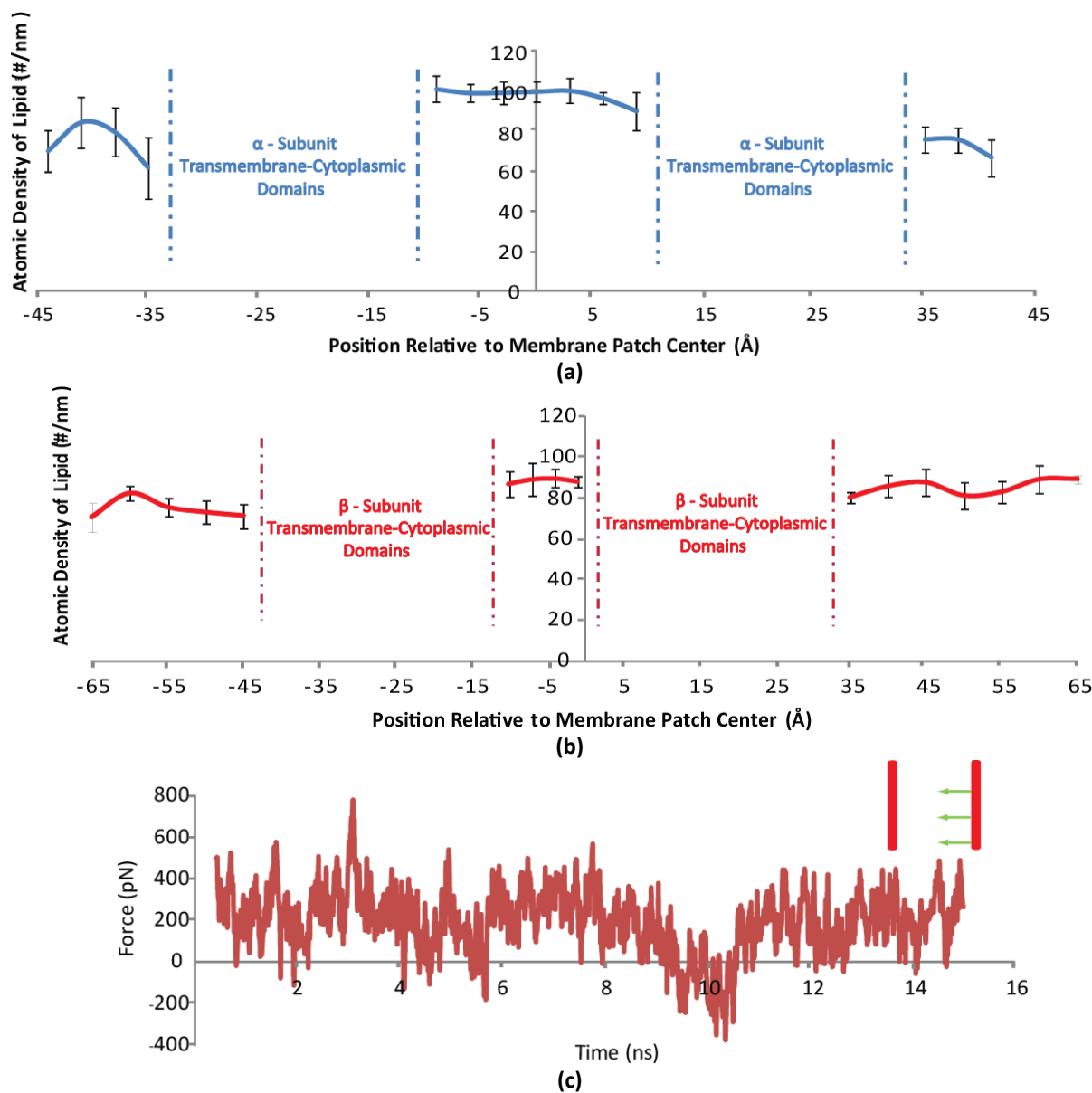
α -subunits moved along with the monomers. As shown in Fig. S3a, although a portion of lipid chains originally located in the proximity of the monomer disperses in different directions, the majority of lipid molecules within at least 10Å of the transmembrane domain are more energetically favorable to remain attached to the monomer. In order to further quantify the phenomenon, we measured the distance between the monomer's center of mass and three lipid chains located at the rear side of the moving monomer, along the line that connects the two monomers, with different initial distances from the monomer. Intriguingly, the lipid chain that was originally located as close as ~12Å to the monomer's center of mass almost kept a constant distance with the monomer, which means it moves with a constant velocity along with the monomer. Nonetheless, the farther the lipid chain located from the monomer, the more slowly the lipid chain was dragged behind the monomer (Fig. S3b).

It was speculated that "lipid packing" around the monomers might be the underlying reason for why the free monomer was "repelled" from the steered monomer. Thus, the atomic density (number of atoms per volume) of lipid around both α - and β -subunits was measured as a function of the lipid position, relative to the membrane patch center. Interestingly, as depicted in Fig. 2.4a and 2.4b, the atomic density of lipid in the region surrounding the two α -subunits is approximately 20% higher than the density of the free areas on the two sides of the membrane patch. However, the lipid arrangement around β -subunits does not show this much of a contrast. Although the density of the lipid between the two β -subunits is also higher than other regions, it appears that formation of a significantly denser lipid chain conformation between α -subunit TMC domains is more favorable. A major portion of the transmembrane domain of α -subunit consists of hydrophobic residues, which makes it favorable for lipid chains to form a denser pack in the region defined by two α -subunits. Although it is challenging to verify this phenomenon experimentally, computational approaches allowed us to investigate a molecular model of this system in detail.

To verify potential effects of the lipid bilayer, a control simulation was performed exclusively with two TMC α -subunits with no surrounding membrane. Interestingly, this time the repulsion of the free monomer was not observed, rather the two monomers simply approached each other and eventually associated together (see Fig. 2.4c). The pulling force remained constant until $t=900\text{ps}$, when the interaction between the two monomers began. The pulling force reached a negative minimum at $t=1000\text{ps}$ when the helix surfaces were in contact and the hydrophobic attraction between the monomers was so strong that the steered monomer was effectively pulled back to keep the velocity constant (see Fig. 2.4c). Subsequently, the steered helix compelled the free one to move along with it, elevating the force back to the level exhibited prior to the interaction. It should be noted that the minimum separation reached by the α -monomers was ~2nm. Therefore, the average effective radius of each monomer, considering the steric repulsion caused by side chains, could be estimated to be ~1nm.

As mentioned earlier, a portion of the lipid side chains surrounding each monomer traveled along with the α -helix. In the simulation where a TMC α -domain was directed toward another free one, the van der Waals interaction energy between the two lipid cylinders, each with external radius of 2.5nm, was computed (see Fig. 2.4d). The final distance in this case was

5.1nm. As the system is geometrically symmetric relative to the monomers, it could be concluded that a lipid cylinder of 2.55nm forms around each membrane-embedded α -monomer. Nonetheless, it is noteworthy that the cylinder is not perfect, meaning its radius differs along different directions. Since the effective radius of each α -monomer (considering the steric repulsion caused by side chains) is more than 1nm the lipid cylinder average radius is estimated to be 1.5nm. The result indicated a rise in the interaction level between the two semi-cylinders until $t=1000\text{ps}$. The interaction energy continued fluctuating about a plateau afterwards. At this moment, the two monomers were at their final distance and the steered monomer pushed off the free one via their lipid cylinders (Fig. 2.4d).



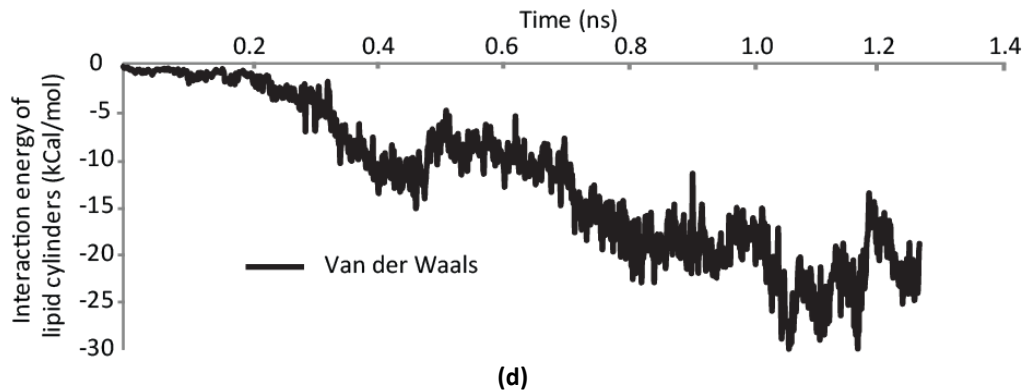


Figure 2.4: Lipid packing is suggested to have a major effect on integrin homo-oligomerization (a) The lipid density of the cytoplasmic compartment of the plasma membrane in the area enclosed by the two α -domains is significantly higher than the density of regions outside α -domains by $\sim 20\%$. (b) The lipid dense region forms between β -domains too. However, the contrast between the regions defined by the two β -domains with outside is much lower. (c) The steered and free α -monomers readily interact when the membrane is absent. Pulling force remains constant until $t=0.8\text{ns}$ when the interaction between the two monomers begins. The pulling force reaches a minimum at $t=1\text{ns}$ when the helix surfaces are in contact and the hydrophobic attraction replaces the required pulling force gradually, until it eventually takes a negative amount. The legend illustrates the steered α -monomer approaching the free one while both move outside the lipid bilayer. (d) van der Waals interaction energy between lipid cylinders around the two α -monomers is a measure of their contact. It takes a maximum at $t=1\text{ns}$ which is indicative of the full interaction between the lipid cylinders.

These results implied that the repulsion is caused by lipid packing around monomers. Given the highly hydrophobic nature of the membrane-embedded residues of the TMC α -integrin, when the two monomers are far enough from each other, local interactions between their surrounding lipid chains and their hydrophobic residues occupy their hydrophobic patches' binding sites, preventing the buried residues in the two monomers from interacting. Furthermore, these hydrophobic interactions form a dense pack of lipid chains around each monomer. Once the two lipid packs overlap, they are forced away due to steric repulsion. The friction of the two lipid packs with their surrounding lipids creates a barrier for each movement step of the pack. Therefore, the free monomer is pushed off intermittently rather than being shifted smoothly. Therefore, if the two monomers are to dimerized, external, mechanical work is needed to overcome the energy barrier that maintains the packed lipid chains together, letting the monomers pass through.

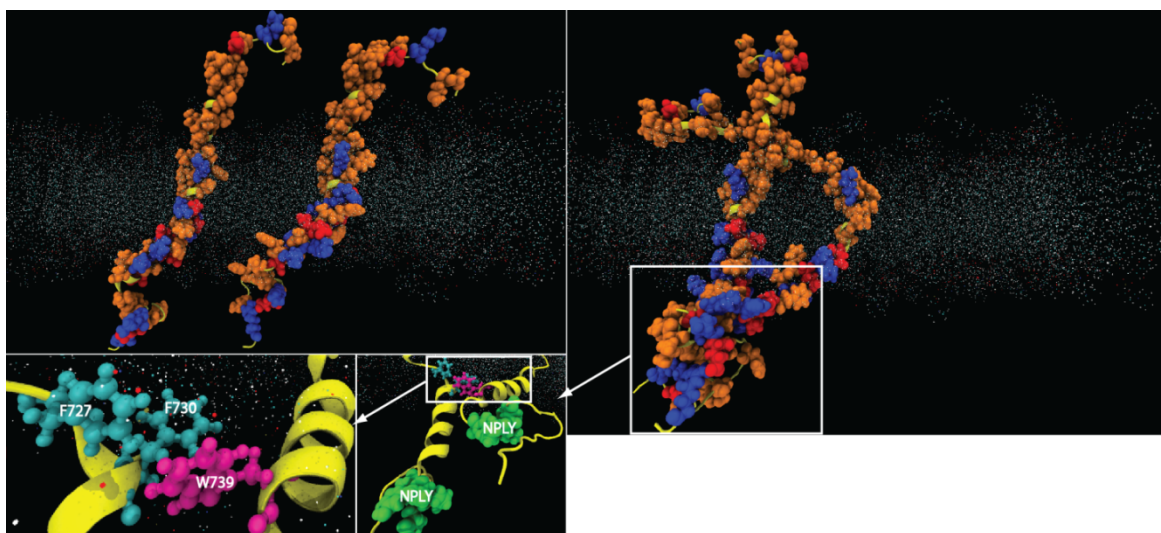
α -dimerization: As proposed by some researchers, to regulate integrin clustering/activation the TMC α -domains should be able to readily associate and detach from one another [100,116]. A dimerization test was carried out in order to characterize the α -domain homo-oligomerization process. Two α -domains were positioned in a plasma membrane patch as close as 2nm apart. The system was minimized and equilibrated for 2ns. Interestingly, at this time, the two monomers bonded to each other fairly rapidly ($\sim 50\text{ps}$ after the simulation started). Afterward, one monomer was fixed (at the same four points), while the other monomer was pulled away with a constant velocity ($0.025\text{\AA}/\text{ps}$). The force increased linearly with the steered monomer moving away from the fixed one. A maximum force of 1.6nN was required to completely separate the monomers.

Integrin β - β Homo-oligomerization: Another crucial element of integrin activation/clustering is β -subunit, which is another candidate reported to be an initiator for integrin clustering and focal adhesion formation consequently. We probed the effect of outside-in signaling on the TMC β -domain by conducting a simulation of two TMC β -domains (Gly684 to Thr762), one constrained at four atoms (C α of Val695, Gly708, Ala728, and Ala742) while the other one is steered (at the same four points) toward it with a constant velocity of 0.025 Å/ps. Regions of the β -domains falling outside the membrane interacted first, trapping the system in a stable conformation. The stability of the oligomerized state was confirmed by removing the steering force after the dimer was formed and the system was equilibrated for 3ns. It was observed that the system did not change its conformation significantly after this period of equilibration.

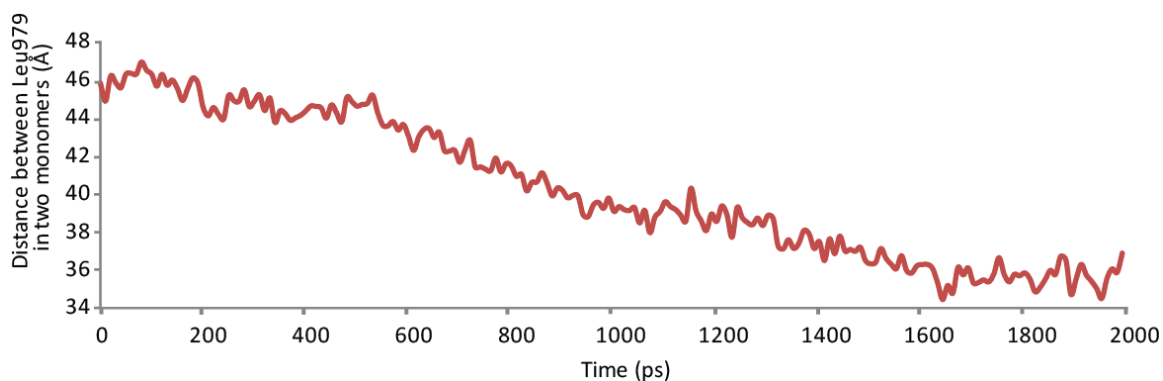
The process of β -dimerization took place before the monomers paired up completely, leaving some lipid chains trapped in between (Fig. 2.5a). In fact, the lipid packed around the monomers partially ruptured at 1.5nN and let the β -monomer through. Lower density of hydrophobic residues in the cytoplasm-proximal side of the transmembrane domains forms a region of narrow lipid packing, further facilitating the interaction between the cytoplasmic domains of the β -monomers. Membrane-proximal, cytoplasmic residue W739 on the steered monomer and residues F727 and F730 on the constrained monomer interacted with the plasma membrane strongly enough to introduce a kink between the cytoplasmic and transmembrane domains of each monomer that signifies the partially hydrophilic region of the transmembrane protein within the cytoplasmic leaflet (Fig. 2.5a). Residue W739 of the steered monomer and residues F727 and F730 of the constrained monomer form hydrophobic bonds, shaping a stable conformation.

The relative position of C α of Leu718 residues on the two monomers are depicted in Fig. 2.5a, which represents the geometric center of the lipid-embedded portions of the monomers. The distance drops linearly down to 36Å and remains constant afterward, at a level close enough to allow the cytoplasmic and extracellular regions of the transmembrane domains to interact.

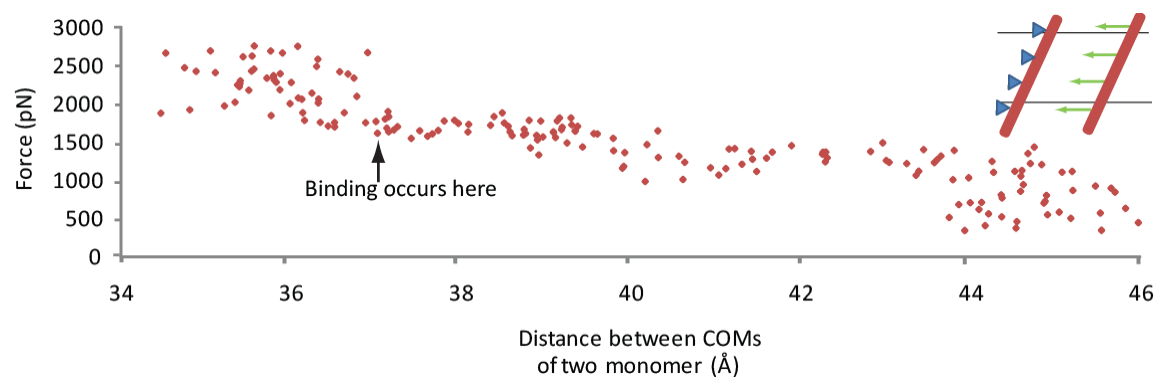
β -trimerization: As β -domains are often reported to form trimers in focal adhesions, we placed them on vertices of an equiangular triangle with the side length of 2nm. Trimerization occurred quickly within 50ps. Again, we exploited steered molecular dynamics to separate one of the β -subunits from the trimer, exerting forces on four atoms (C α of Val695, Gly708, Ala728, and Ala742). The steered monomer separated from other two at a maximum of 1.3nN. In contrast to TMC α -domains, which have an entirely hydrophilic membrane-embedded compartment, these residues are sparse on the TMC β -domains cytoplasm-proximal compartment (starting from D723). By narrowing the lipid pack barrier, this forms a weak spot where the lipid barrier cuts open in a zipper-like fashion. In other words, the steered β -domain detachment initiates at the cytoplasm-distal region. As the steered molecule is further forced other bonds break one at a time.



(a)



(b)



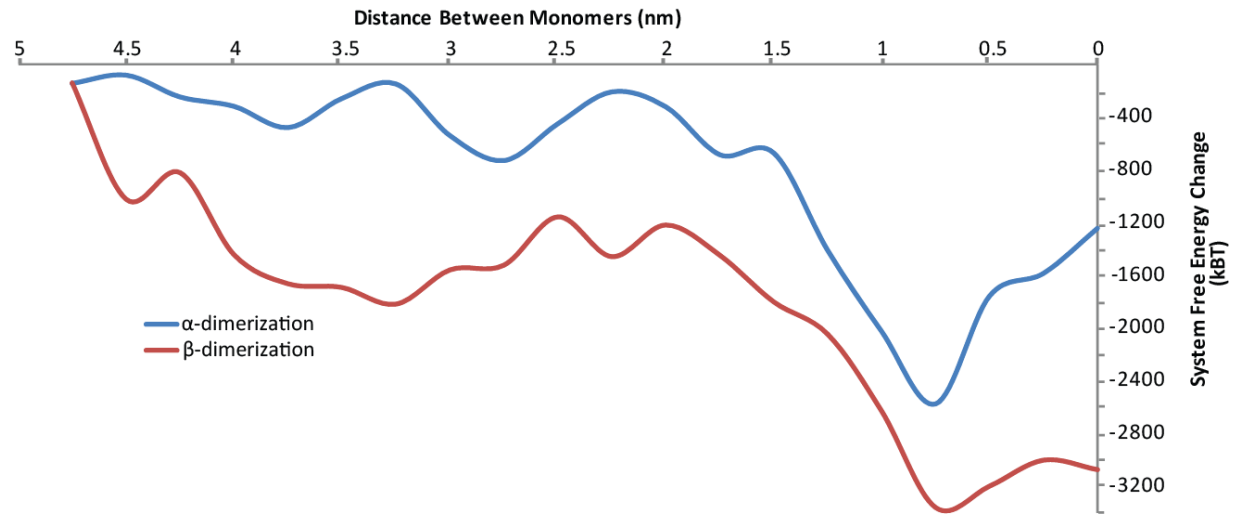
(c)

Figure 2.5: (a) (top left) Two β -subunits are placed 5nm apart from one another (helix backbone: yellow; hydrophobic residues: orange; acidic residues: red; basic residues: blue) and equilibrated in a plasma membrane patch (point representation). Membrane-proximal, cytoplasmic residue W739 on the steered monomer and residues F727 and F730 on the constrained monomer interact with the plasma membrane strongly enough to introduce a kink between the cytoplasmic and transmembrane domains of each

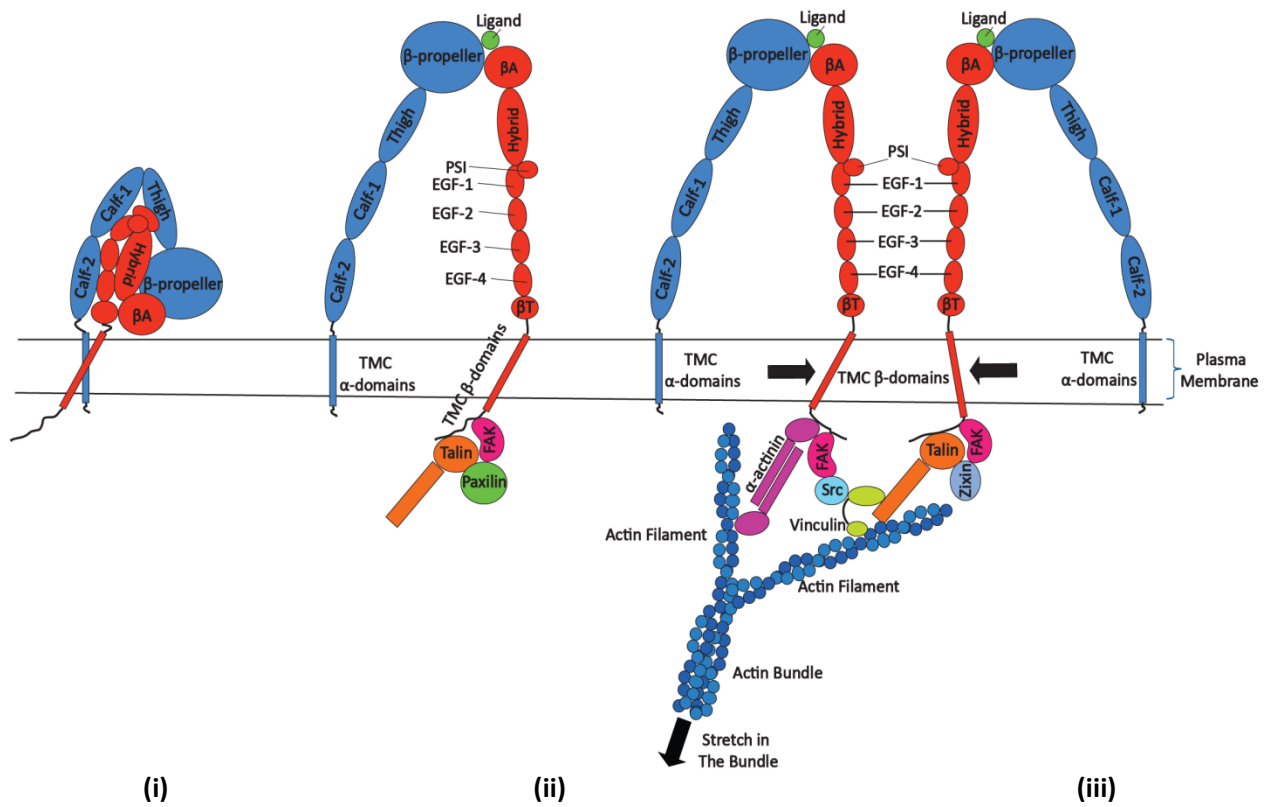
monomer. (top right) Steered residue W739, and constrained residues F727 and F730 form hydrophobic bonds, shaping a stable conformation. (inset) The NPLY sequences, where talin-integrin interaction occurs, are highlighted. (b) Distance between the centers of mass of the monomers diminishes linearly until the two monomers interact at distance 3.6nm. (c) The SMD force increases as the steered monomer comes closer, and the interaction eventually occurs at 1500pN. The legend illustrates the steered β -monomer (red), being pulled toward the constrained monomer while they are embedded in a lipid bilayer.

Free Energy of Dimerization: In order to further evaluate possibility of integrin subunit homo-oligomerization we analyzed the free energy change of the system as one TMC subunit approached the other for either of α - and β -subunits (see Fig. 2.6a). To model the system more accurately, rather than solely bringing the monomers close to each other, we included the effects of the linkage of β -subunits to the cytoskeleton through talin. We started from an initial step where two β -subunits are embedded in the plasma membrane patch, 5nm apart. Then, we exerted the steering force only on the cytoplasmic domain of the moving monomer (i.e. Ala750-Thr762), while the other monomer was left to diffuse freely. The steering speed was kept constant at 0.025 Å/ps and the two cytoplasmic domains dimerized after 2ns. The same protocol was employed to measure the free energy difference for α -dimerization.

To calculate the free energy of interaction we invoked the Jarzynski method for non-equilibrium transitions in the system phase space [179]. This method has been able to closely estimate the free energy difference in molecular dynamics simulations by sampling the system for a limited number of times (e.g. 10 times) [185]. To achieve reliable results from the Jarzynski method, sufficient number of identical systems should be minimized and equilibrated separately before they are steered [186]. We minimized and equilibrated the system for either of α - and β -subunit homo-oligomerization profiles 10 times for 0.5ns, each starting from the same initial state. The systems were steered subsequently and the free energy was evaluated using a second-order expansion of the Jarzynski equality [179,185]. The free energy profile of the system is shown in Fig. 2.6a. The free energy profiles of α - as well as β -subunit association are plotted against each other. The free energy of the system decreases gradually as either of the moving α - or β -subunit approaches the free subunit. The effect of the lipid pack disturbance is seen at the few local peaks that appear between 2nm and 3.5nm for both subunits, and finally, the free energy drops drastically as a result of the two monomers interacting and forming dimers. Interestingly, the global free energy minima for the oligomerized states of the α - or β -subunits closely coincide. However, the α -monomer indicates a sharper drop in the free energy with about 10% higher free energy difference between its peak and valley. Furthermore, α -monomers require about twice as much activation energy as do β -monomers to overcome local free energy maxima. The free energy barrier that should be overcome by β -subunits to homo-oligomerize is about 400k_BT. Although this value is too high for two free β -subunits to overcome, the mechanical work caused by their linkage to the cytoskeleton is likely to push β -subunits over this free energy bump, which would lead to oligomerization. On the other hand, α -subunits would require as high as 800k_BT of activation energy to overcome the oligomerization energy barrier. Considering that no significant mechanical linkage between α -subunits and the cytoskeleton has been reported, the homo-oligomerization seems thermodynamically unlikely.



(a)



(b)

Figure 2.6: While β -subunit homo-oligomerization could be a candidate mechanism underlying integrin clustering and focal adhesion formation, α -subunits are not as likely to form oligomers. (a) The free energy profiles of α - as well as β -subunit association are plotted against each other. The free energy of the system decreases gradually as either of the moving α -subunit or β -subunit approaches their free counterparts. The effect of the lipid pack disturbance is seen at the few local peaks that appear between

2ns and 3.5ns for both subunits, and finally, the free energy drops drastically as a result of the two monomers interacting and forming a dimer. Interestingly enough, the global free energy minimum for the oligomerized state for α - and β -monomers coincides. However, the α -monomer indicates a sharper drop in the free energy with about 10% higher free energy difference between the peak and valley. Furthermore, α -monomers require about twice as much activation energy as do β -monomers to overcome local free energy maxima. (b) A schematic for a potential integrin activation and clustering scenario (i) In an inactive integrin molecule the transmembrane α - and β -domains are tightly bonded. (ii) Once integrin is activated, it forms a micro-cluster of focal complex proteins around it. (iii) After the focal complex matured to a focal adhesion the clusters grow and bind to actin filaments. If the tension in an actin filament is increased, its sub-filaments come closer to one another, bringing their corresponding β -domains together, thereby increasing the focal adhesion area by recruiting more β -integrins in trimers.

2.4. Discussion

Although the role of integrin activation in integrin clustering and vice versa has been studied intensively over the past decade, the phenomenon still remains ambiguous in many aspects [101,115,116]. Some researchers suggested that monomer homo-oligomerization of transmembrane integrin α - and β -subunits triggers integrin clustering [101,116]. However, others provided evidence that homo-oligomerization of integrin subunits does not significantly take part in the process of integrin clustering [115]. Because real-time monitoring of single integrins proved challenging, previous studies utilized assays that involved collecting integrins from cells that are already lysed [100,101,115,116], which implied neglecting effects of the plasma membrane. This study aimed at investigating effects of an expressive model the plasma membrane on the integrin clustering phenomenon via a full-atomic simulation.

Molecular dynamics has been widely recruited to model mechanistic behaviors of cytoskeletal proteins [174]. Particularly, Kalli et al. in their recent work provided evidence that molecular dynamics simulations are able to closely mimic the hetero-dimerization of transmembrane domains of integrin, when embedded in a lipid bilayer [187]. With no experimental techniques currently available to obtain dynamic, atomic-level insights into the integrin activation pathway, we employed steered molecular dynamics (SMD) simulations to derive these insights computationally. Because integrin has a massive structure, usually only the portion of integrin that is relevant to the problem is truncated and modeled in molecular dynamics simulations [42,43]. Also, it is believed that the membrane-proximal segment of the ectodomain is a soft region that links the transmembrane domain and the ectodomain. We conducted a normal mode analysis on the full-length integrin molecule to show the presence of a hinge-like region that minimizes the mechanical integrity of the extracellular and TMC domains of the integrin [2,73]. Thus, we circumvented the extremely high computational cost of simulating an intact integrin embedded in a membrane, by including only the TMC domains in our simulations.

Inactive integrin α - and β -subunits are locked by two transmembrane interactions: Outer membrane clasp (OMC) and inner membrane clasp (IMC). The GXXXG motifs are thought to be sufficient for keeping the two hetero-dimers in contact [188]. Also these sequences allegedly play a significant role in the process of homo-oligomerization [116]. It has been demonstrated that transmembrane α -domains in an activated integrin tend to form dimers, while transmembrane β -domains are more inclined toward homo-trimerization [100,101,116].

Fluorescence microscopy studies demonstrated that there is no interaction between α - and β -cytoplasmic tails in the active state, and they are at least 10nm apart [98]. Therefore, in all simulations we mimicked the ligand-bound mode by employing non-bonded TMC α - and/or β -domains as opposed to the inactive case where these domains are in a hetero-dimerized state. Ectodomains are associated with the ECM so they could not have a significant effect on homo-oligomerization [115].

In fact, diffusion of transmembrane proteins, including integrins, occurs significantly slower *in vivo* compared to how rapidly it would occur in artificial lipid bilayers. This is suggested to be caused by the physical blocking of their cytoplasmic tails by the cortical cytoskeleton [189]. Lepzelter and Zaman proposed the value of $0.25\text{nm}^2/\mu\text{s}$ for the free diffusion coefficient of integrin dimers [189]. Assuming a 2D random walk on the membrane, we can employ the Green's function of diffusion to correlate the integrin position variance from the movement origin with the diffusion time as follows:

$$\sigma^2 = 4Dt, \quad (2.3)$$

where σ^2 , D , and t are respectively the position variance, diffusion coefficient, and diffusing time. Assuming a normal distribution, a single integrin traveling from the origin will fall within 3σ of the origin at all times with 99.7% certainty. The time scale is 2ns for most of our simulations. Substituting these values in Eq. 2.3 yields a standard deviation of 0.0447nm, consequently, $3\sigma=0.1342\text{nm}$. Dividing this by the time scale of 2ns gives a characteristic velocity of 0.0671nm/ns. However, this is the molecule average speed from its hypothetical origin, rather than its instantaneous speed at each walking time step, which is what is “mechanically sensed” by its surrounding. Indeed, the instantaneous speed could be much greater than this. According to the central limit theorem, if the molecule takes steps of size Δx each in time span of Δt , variance of the position could be calculated as:

$$\sigma^2 = \frac{t}{\Delta t} (\Delta x)^2 \quad (2.4)$$

Comparing Eq. 2.3 and 2.4, noting that $\frac{\Delta x}{\Delta t}$ is simply the instantaneous (time step) speed for small time steps, and rearranging, we are left with the following expression for the instantaneous speed:

$$V = 2\sqrt{\frac{D}{\Delta t}}, \quad (2.5)$$

in which we denote the instantaneous speed with V . Substituting $0.25\text{nm}^2/\mu\text{s}$ and $\Delta t=2\text{fs}$ (the time step used in all simulations), the instantaneous speed is $V=22.36\text{nm/ns}$. We used a pulling velocity of 2.5nm/ns , which is an order of magnitude less than the average instantaneous speed in order to avoid sudden sharp movements of the steered monomer that would otherwise impose a significant impulse to the free monomer.

Our simulations showed that a high level of hydrophobicity in the transmembrane α -domain combined with its perpendicular-to-membrane orientation forms a lipid pack of $\sim 1.5\text{nm}$ thick around its effective surface. In addition, in the case where two α -subunits are to approach one another, the density of lipid chains was shown to be significantly higher in the region between the subunits. Although highly neglected in previous studies, this lipid shield forms a significant

energy barrier of about $800k_B T$ that should be overcome if two α -subunits are to dimerize. Hence, unless there is an external mechanical load that forces the monomers through the lipid pack, the lipid barrier simply hinders α -dimerization. Because, to the best of our knowledge, no mechanically effective binding of α -subunits with cytoskeletal proteins has been reported, it is unlikely for α -subunits to form homo-dimers even if they are in an active/ligand-bound state. Studies that reported α -subunit homo-dimerization have, in fact, observed integrins after the cell had been lysed, i.e. the plasma membrane had been removed. To examine validity of our results, we mimicked the experiments carried out by Li et al. [100] by observing the dimerization process in the absence of the plasma membrane (see Fig. 2.4). Our results showed that under such circumstances homo-oligomerization readily occurs. Transmembrane α -domain dissociation simulations corroborate this conclusion. Even if the homo-dimerization occurred, α -subunits would require $\sim 25\%$ higher magnitude of force to dissociate than would their β -counterparts. Thus, α -subunit homo-oligomerization does not seem to be a readily-available regulating event in integrin clustering. Although Wang et al. also performed homo-oligomerization and Cys scanning analyses in the absence of the cell plasma membrane, they used full-length integrin versus TMC domains used by Li et al [100,115]. Insofar as integrin TMC domains are highly hydrophobic, in a hydrophilic milieu, it is likely that the integrin TMC domains have bonded to hydrophobic patches on the integrin ectodomain, thereby blocking the homo-oligomerization sites on the TMC-domains. This could explain why homo-oligomerization was not observed by Wang et al.

On the other hand, transmembrane β -domains are α -helices longer than α -domains that also span the plasma membrane. They possess a large number of hydrophobic residues but the subtlety lies within a cytoplasm-proximal region of them. TMC β -domains are different from α -subunits in three distinct ways. First, TMC β -domains feature longer cytoplasmic regions that are less affected by the lipid packing phenomenon as their cytoplasmic compartment is significantly longer; second, in the resting as well as ligand-bound states β -domains maintain a 25° angle with the membrane surface normal, as opposed to α -domains that remain perpendicular to the membrane [2,7,86]; and finally, in β -domains the membrane-embedded region is not fully hydrophobic which gives rise to a narrowing of the lipid pack adjacent to the β -domains in the cytoplasmic leaflet of the plasma membrane. Unlike transmembrane α -domains that are more or less uniformly hydrophobic throughout their membrane-embedded length, a transmembrane β -domain length span of $\sim 2\text{nm}$ in proximity of the cytoplasm experiences a combination of hydrophobic and hydrophilic residues that weakens the lipid packing effect around it. This region, starting from the residue D723, appears to rotate against the main body of the monomer shortly after it was equilibrated and this forms a kink that signifies this region. The rotation is caused by another hydrophobic interaction between the residue W739 and the plasma membrane. This interaction acts as a trigger for homo-dimerization. Although 1.5nN of steering force was required to overcome the lipid pack and promote homo-oligomerization, this event could be considered as feasible physiologically since it is widely established that talin head domains bind to the NPLY sequence on the β -cytoplasmic domain, a key bond that links integrins to actin filaments. Other focal adhesion proteins such as α -actinin and kindlin are also capable of linking β -cytoplasmic domains to the cytoskeleton [5,190].

Calculating the average density of the lipid bilayer around α - and β - TMC domains provides a reliable measure to validate the results on responses of these two monomers when embedded in the plasma membrane (Fig. 2.4a and 2.4b). Intriguingly, the average density of lipid reaches its highest in the region between the α -domains ($\sim 100 \text{ \#}/\text{nm}^3$), presenting a dense area of lipid that potentially hinders α -homo-oligomerization. Similar phenomenon occurs for β -domains except that the difference between the area enclosed by and outside the β -domains is no more than a few percents. It is noteworthy that the reported densities are averaged over five microstates of the system for each data point. Another piece of evidence was put forward by estimating the free energy changes along the reaction coordinate of β - β cytoplasmic domain homo-oligomerization. Importantly, an overall loss of free energy on the system was observed as the moving monomer approached the free one until it reached an energy bump at 2.5nm. After the dimerization occurred, a drastic decrease in the system free energy was seen. This showed that the reaction is favorable and feasible if a source of energy exists that injects as much as $400k_B T$ into the system such that it could overcome the energy barrier. As the average load sustained by an actin filament is reported to be $\sim 50\text{pN}$ [59], an actin stretch of $\sim 34\text{nm}$ would be sufficient to provide the amount of energy required for homo-oligomerization.

Cluzel et al. reported that activated integrin, immobilized ligands, presence of monomeric talin head domain, and phosphoinositole-4, 5-bisphosphate (PIP2) are necessary factors for integrin micro-clustering [19]. Presence of actin network, however, is not critical for *de novo*-formed integrin clusters. Along the same line, Tan et al. did not observe the linear regime of focal adhesion area growth with the force they exerted to the substrate, for nascent focal adhesions (i.e. less than $1 \mu\text{m}^2$ in area) [119]. Therefore, since homo-oligomerization is dependent on the presence of actins, it seems reasonable to assume neither TMC α - nor β -domains play an important role in promoting clustering in newly-formed focal adhesions. Nevertheless, TMC β -domains might be capable of mechanically regulating the clustering process when the focal adhesion is matured. Previous molecular dynamics simulations mapped out details of vinculin and α -actinin activation that is highly involved in the process of maturation [182,183,191,192,193]. An increase in the stretch level of the cytoskeleton, and particularly actin filaments that are already bonded to talin and other focal adhesion proteins and consequently to integrin β -subunits, might lead to a lateral gathering effect that brings β -subunits closer, thereby making it more probable for them to homo-oligomerize (Fig. 2.6b). Thus, one could hypothesize a model to link integrin activation and clustering as follows (see Fig. 2.6b). Individual active integrins absorb focal adhesion proteins, including talin, vinculin, etc., and eventually bind actin filaments. As the focal adhesion matures, adjacent protein hubs (~ 40 - 50nm apart) [194] gradually grow in size and unify. Increasing the tension in actin bundles, and consequently actin filaments that form the bundles, causes monomers to be aligned and dragged toward each other more vigorously. Homo-oligomerization interactions reinforce the binding by overcoming lipid barriers, which assembles larger numbers of integrin subunits in the focal adhesion.

Although current activation models explain how three conformations of integrin are associated with different signaling states, they fall short on elucidating alterations in the surface area of

focal adhesions as the tension in focal-adhesion-binding actins grows in a 2D cell culture regime [5,34,73,101,195,196]. Our speculated model, however, explains the phenomenon as follows. The higher the tension in an actin bundle grows, the more β -integrin homo-trimerization interactions likely occur. This in turn increases the area of the focal adhesion in a 2D culture, which implies that a larger number of active integrins are recruited if an elevated magnitude of force is to be borne by the corresponding actin bundle. The extended ectodomain of integrin and presence of a hinge-like region between the ectodomain and TMC domains enables integrin β -cytoplasmic tails to homo-oligomerize while their ectodomains are bonded to adjacent ECM ligands. The GXXXG sequences are binding sites for hetero-trimerization. As long as the cytoskeletal tension is present and integrins are stabilized in a homo-trimeric interaction, they are not expected to return to their resting state, where α - and β -domains are bonded at their IMCs and OMCs.

In summary, our simulations showed that TMC α -domains are unlikely to contribute to the process of integrin clustering. However, TMC β -subunits' geometry and composition make them an important candidate to drive the integrin clustering process. Previous experiments implicitly neglected energetic effects of the plasma membrane, obtaining apparently contradictory outcomes with lysed cells. Hence, we suggest future experimental studies of the field focus on clustering of integrin β -subunits over the course of the focal adhesion formation and collapse either *in vivo* or within artificial lipid bilayers. Furthermore, in order to obtain a thorough understanding of the focal adhesion growth, effects of other environmental factors, such as extracellular and cytoplasmic ion concentration, on integrin clustering should be examined.

Chapter3

Integrin Inside-out and Outside-in Activation

Integrin $\alpha\text{IIb}\beta 3$ is a member of the integrin family of transmembrane proteins present on the plasma membrane of platelets. Integrin $\alpha\text{IIb}\beta 3$ is widely known to regulate the process of thrombosis via activation at its cytoplasmic side by talin and interaction with the soluble fibrinogen. It is also reported that three groups of interactions restrain integrin family members in the inactive state, including a set of salt bridges on the cytoplasmic side of the transmembrane domain of the integrin α - and β -subunits known as the inner membrane clasp, hydrophobic packing of a few transmembrane residues on the extracellular side between the α - and β -subunits that is known as the outer membrane clasp, and the key interaction group of the βA domain (located on the β -subunit head domain) with the βTD (proximal to the plasma membrane on the β -subunit). However, molecular details of this key interaction group as well as events that lead to detachment of the βTD and βA domains have remained ambiguous. In this chapter, molecular dynamics methods are implemented to take a comprehensive outside-in and inside-out approach at exploring how integrin $\alpha\text{IIb}\beta 3$ is activated. First, we show that talin interaction with the membrane-proximal (MP) and membrane-distal regions of integrin cytoplasmic-transmembrane domains significantly loosens the inner membrane clasp (IMC). Talin also interacts with an additional salt-bridge (R734-E1006), which facilitates integrin activation through the separation of the integrin α - and β -subunits. The second part of our study classifies three types of interactions between RGD peptides and the extracellular domains of integrin $\alpha\text{IIb}\beta 3$. Finally, it we show that the interaction of Arg of the RGD sequence may activate integrin via disrupting the key interaction group between K350 on the βA domain and S673/S674 on the βTD .

3.1. Introduction

Integrin $\alpha\text{IIb}\beta 3$, which is the major integrin type expressed on the surface of platelets, plays a critical role in platelet aggregation and blood clotting [197,198]. Therefore, integrin $\alpha\text{IIb}\beta 3$ malfunction is marked by several diseases such as thrombosis in myocardial infarction, ischemic stroke, peripheral arterial disease, and Glanzmann thrombasthenia bleeding disorder [198,199]. Integrin $\alpha\text{IIb}\beta 3$ is activated via inside-out and/or outside-in signaling mechanisms, and these two pathways are reported to be discrete while having mutual effects on each other [2,73,200].

The inside-out activation of integrins is defined as the process of activation that is triggered by a cytoplasmic signal and leads to increased affinity of the integrin head domain for the ligand. Inside-out activation processes modulate integrin $\alpha\text{IIb}\beta 3$ affinity for extracellular ligands (i.e. fibrinogen) through binding of key cytoplasmic proteins (see Fig. 3.1). Among the various proteins that bind integrins and link them to the actin cytoskeleton, talin and kindlin are reported to activate integrin via associating with the cytoplasmic tail of integrin β -subunit [5,89]. The most well known inside-out activation cascade for integrin $\alpha\text{IIb}\beta 3$ is triggered by binding of cleaved thrombin to proteinase-activated receptor 1 (PAR1) located on the plasma membrane of human platelets. This binding leads to phospholipid hydrolysis, which results in the

generation of inositol trisphosphate (IP3) and diacylglycerol (DAG), and an increase in cytosolic free Ca^{2+} . The elevated concentration of Ca^{2+} and DAG, which in turn activates CALDAG-GEFI and protein kinase C (PKC), converts RAP1 from a GDP-bound to a membrane-attached, GTP-bound form. Activation of RAP1 leads to recruitment of its effector, RAP1–GTP-interacting adaptor molecule (RIAM), and its binding partner, talin 1, to the plasma membrane. This facilitates access of talin to the integrin $\beta 3$ tail and talin-induced activation of integrin $\alpha \text{IIb} \beta 3$ [5]. Two major interactions attach the transmembrane domains of the α - and β -subunits: the Outer Membrane Clasp (OMC) embedded within the lipid bilayer and the Inner Membrane Clasp (IMC) located near the membrane-proximal, cytoplasmic tail between the integrin subunits [73]. Upon release of these clasps, the transmembrane-cytoplasmic domains of integrin are separated and activated, causing the extracellular domains to become more receptive to ECM ligand binding. Binding of the integrin $\beta 3$ subunit to talin-1 is one of major factors that disrupts the interactions between the two integrin subunits, separating the transmembrane-cytoplasmic domains [89]. Current study provides molecular-resolution simulations that demonstrate how talin head domain binding to the transmembrane-cytoplasmic domain of the integrin $\beta 3$ subunit mechanically loosens the inner membrane clasp.

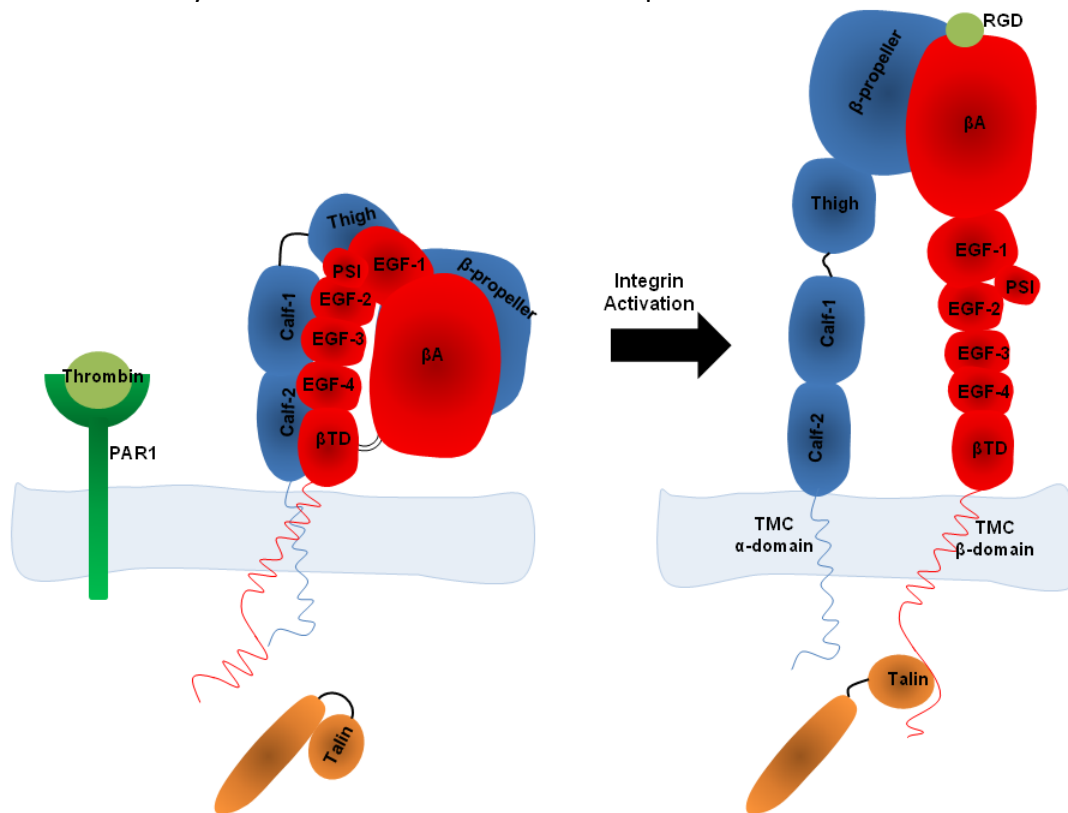


Figure 3.1: A schematic of one of main pathways of integrin $\alpha \text{IIb} \beta 3$ inside-out activation. Binding of thrombin to proteinase-activated receptor 1 (PAR1) causes separation of the talin head and tail domains. Consequently, the talin head domain associates with the $\beta 3$ subunit cytoplasmic domain, activating integrin $\alpha \text{IIb} \beta 3$.

On the other hand, the outside-in activation is identified by interactions of the integrin extracellular head domain with the ECM ligand or divalent cations that lead to partial unfolding

of the extracellular domain and separation of integrin subunits. The integrin $\beta 3$ subunit is known to be the major regulator of activation for integrin $\alpha \text{IIb}\beta 3$ and $\alpha \text{v}\beta 3$ [10,34]. Binding of divalent cations with the integrin head domain (i.e. βA and β -propeller domains on the extracellular side) is reported to determine the integrin activation state. There exist three divalent metal ion binding sites on the integrin βA domain. The first one, termed as the metal ion-dependent adhesion site (MIDAS) is coordinated with Mg^{2+} , while the two other cation binding sites, called the adjacent to MIDAS (ADMIDAS) and ligand-induced metal binding site (LIMBS) located on the opposite sides of MIDAS, are coordinated with Ca^{2+} [33,34,45]. High concentrations of Ca^{2+} reinforce the bent, inactive state of integrin $\alpha \text{v}\beta 3$, while in presence of Mn^{2+} , a cation that activates integrins, most integrin $\alpha \text{IIb}\beta 3$ molecules observed by electron microscopy were in an extended conformation [35]. It is proposed by some researchers that the RGD ligand and divalent cation share a common binding pocket on the integrin $\beta 3$ head domain on the interface of the βA and β -propeller domains [31,32]. Importantly, external force has also been shown to directly activate certain types of integrin and prolong their ligand-bound lifetime (20-21). Last but not least, the integrin head domain interaction with the ligand at the metal ion-dependent binding sites is shown to activate integrins though its details are not fully agreed upon yet. Also, whether an interaction between the CD loop of the βTD and the $\beta 6$ - $\alpha 7$ loop of the βA domains regulates integrin activation remains highly controversial [12,85]. Hence, we explored potential effects of RGD-binding to integrin $\alpha \text{IIb}\beta 3$ through positioning two soluble RGD peptides proximal to the metal ion-dependent binding sites and one near the βA - βTD domains interface.

Two mechanistic models are widely invoked to describe the behavior of integrins: the switch-blade and the deadbolt model [62]. According to the switch-blade model, integrins take on three distinct conformations, corresponding to their inactive, active, and ligand-bound states. The inactive state of integrin is featured by a bent-over configuration wherein the molecule shows a low affinity for ECM ligands (see Fig. 3.1) [62]. Also, in the inactive state, inner and outer membrane clasps hold the transmembrane domains of integrin together [2]. Integrin activation results in a stretched conformation and breakage of the inner and outer membrane clasps, most likely via presence of a competing molecule (e.g. talin and kindlin) that occupies these binding sites on the transmembrane domain of the integrin β -subunit [5,73]. Activated integrin is known to have a significantly higher affinity for ECM ligands [35]. Finally, the ligand-bound state takes place when the integrin head domain engages in an interaction with an ECM molecule (e.g. fibronectin and fibrinogen). The ligand-bound conformation differs from the non-bonded active state in that the integrin head domain introduces an opening between the α - and β -subunits when in the ligand-bound state [12,43]. Alternatively, the deadbolt model suggests a significantly smaller conformational change, wherein a hairpin loop between β -strands C and D of the $\beta 3$ tail domain (i.e. the CD loop), located on the β -subunit proximal to the plasma membrane, functions as a deadbolt. Upon release of the CD loop, the integrin molecule is activated, as the bonding between the βTD (proximal to the plasma membrane) and the βA domains on the integrin head domain is lost [12,23,80].

Despite numerous experimental studies conducted to shed light on the elaborate mechanism of integrin $\alpha \text{IIb}\beta 3$ activation from inside-out as well as outside-in perspectives, the atomistic

details of either of these mechanisms have remained ambiguous [5,200]. In this study, we developed molecular dynamics models to probe details of inside-out and outside-in activation of integrin $\alpha\text{IIb}\beta\text{3}$ via integrin-talin interactions. It has been shown previously that outside-in signaling pathways of integrin $\alpha\text{IIb}\beta\text{3}$ are spatially distinct from those of inside-out activation in platelets [200]. Hence, this study chose to dissect two of the most established pathways for integrin $\alpha\text{IIb}\beta\text{3}$ activation in molecular details: Talin binding to the cytoplasmic domain of the β3 subunit to represent the inside-out activation, and RGD binding to the ectodomain to simulate the outside-in activation. First, we estimated the strength of the integrin transmembrane bonds (i.e. inner and outer membrane clasps) and showed that the presence of the talin head domain bonded to the cytoplasmic domain of the integrin β -subunit markedly loosens the inner membrane clasp such that a lower external force is required to separate the transmembrane domains of the α - and β -subunits. We focused our attention on the talin-integrin β3 interaction as the last step of a major cascade of events that leads to integrin activation to show, in molecular details, the contribution of this critical event in dissociating the integrin transmembrane domains. Furthermore, using the full-length crystal structure of integrin including the ectodomain and transmembrane-cytoplasmic domains for the first time to the best of our knowledge, we identified a number of potential binding sites of the soluble RGD ligand to the integrin head domain. Based on these results, three likely mechanisms are proposed for integrin-ligand binding. Finally, we explored the deadbolt model in atomic details, mapping out three key residues that act as the “deadbolt”.

3.2. Computational Procedures

Interactions of RGD Peptides with Full-length Integrin: All molecular dynamics simulations were carried out with the program NAMD [174], using CHARMM27 force field. The integrin $\alpha\text{IIb}\beta\text{3}$ ectodomain (PDB ID: 3FCS chains A and B [49]) and the transmembrane-cytoplasmic domains (PDB ID: 2KNC [46]) were obtained from the Protein Data Bank (PDB) and combined to build the full-length integrin $\alpha\text{IIb}\beta\text{3}$ structure (except residues 954-959 of the α -subunit and 684-690 of the β -subunit), assuming covalent bonds between residues C959 and G955 on the α -subunit and G684 and G690 on the β -subunit. The integrin structure was embedded in a patch of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) lipid bilayer with dimensions of 152x113Å, using the software VMD [176]. In all simulations, lipid chains overlapping with the transmembrane domain were removed afterward. PDB files of three RGD peptides were manually produced and positioned in proximity of the integrin head domain as shown in Fig. 3.2. The system was solvated afterward in a water box with an ionic KCl concentration of 150mM [177].

The temperature and pressure of the system were held constant at 1atm and 310K, using Langevin's piston and Hoover's method [174]. The time step was taken as 2fs. The cutoff distance for non-bonded interactions was 1.2nm. For all simulations, particle mesh Ewald (PME) method was used for electrostatic force calculations [174]. The cutoff distance for non-bonded interactions was 1.2nm. The hydrogen atom bond length was constrained using SHAKE method. The SHAKE method fixes bond lengths between large atoms and hydrogen atoms, preventing unnecessary calculation of irrelevant interactions [178]. The system was minimized, at 2000

steps, and equilibrated six times with RGD peptides present and once with no RGD peptide in the system as control, each time for ~13ns.

Integrin–Talin Binding: The crystal structure of the transmembrane and cytoplasmic domains of integrin $\alpha\text{IIb}\beta 3$ was embedded in a patch of POPC. The first step in our simulations was to induce the talin-1 FERM head domain to bind to integrin $\alpha\text{IIb}\beta 3$, which are known to bind to each other [88,106]. A model was created, using the molecular graphics software VMD, with the transmembrane-cytoplasmic regions of integrin $\alpha\text{IIb}\beta 3$ (PDB ID: 2KNC [169]) embedded in a POPC lipid bilayer [176]. The Talin-1 FERM head domain (PDB ID: 3IVF [135]) was placed with the F3 (PTB-like) domain, facing the integrin $\beta 3$ subunit with a 27Å distance between the center of mass of the F3 domain (L320) to $\beta 3$ residue W739. The system was solvated, then neutralized and ionized to a concentration of 150mM NaCl. The system underwent minimization (2000 steps) and equilibration for 7ns, before each center of mass in the F0-F3 talin domains was given a nudge for 1ns ($k = 0.07\text{kcal/mol}/\text{\AA}^2$; velocity = 17.5Å/ns) in the direction that minimizes the mentioned 27Å distance. Following that, the system was allowed 28ns to equilibrate. At the end of the total 36ns, we nudged the F0 domain of talin for 1ns ($k = 0.06\text{kcal/mol}/\text{\AA}^2$, velocity = 12.5Å/ns) to tilt the F3 domain of talin towards the integrin $\beta 3$ subunit. This was performed to promote the membrane-proximal interactions between F727 and F730 on the $\beta 3$ subunit to the S1-S2 loop of talin F3 [106]. Following that, the system was equilibrated for 9.5ns.

Analysis of Integrin-talin Interactions Using Full-length Integrin Structure: Having obtained a full-length integrin $\alpha\text{IIb}\beta 3$ structure, we placed it inside a membrane, solvated and ionized it with 150mM NaCl, and ran a minimization and equilibration simulation for 6ns. Next, with our equilibrated full-length integrin structure, a similar model consisting of integrin and talin-1 FERM head domain was built, with a similar orientation and initial distance maintained between the integrin $\beta 3$ subunit and F3 domain of talin-1. This model was minimized and equilibrated for 4ns, ten times to get a random sample of velocities and trajectories. From the simulations, we measured the final distance (at ~4ns) between R734-E1006, between the αIIb and $\beta 3$ extracellular domains, between the βTD and βA domains, and between the center of masses of selected regions of the $\beta 3$ subunit of integrin and talin-1 F3 domain. Finally, the Spearman's tests of dependence were performed to further pinpoint which region of the $\beta 3$ subunit tail is correlated to the weakening of this additional interaction and whether the degree of talin binding to the integrin $\beta 3$ subunit affected integrin extracellular conformation. (Details for the comparison of the inner membrane clasp interactions and other results in integrin versus integrin-talin systems are included in supporting materials.

3.3. Results

Interactions between the Soluble RGD and Integrin Potentially Activates Integrin $\alpha\text{IIb}\beta 3$: In order to probe likely binding sites of the soluble ligand, RGD sequences were placed at three locations proximal to the integrin head domain previously reported as potential binding spots of integrin for fibrinogen [12,34,45]. Depicted in Fig. 3.2 are locations of the three soluble RGD ligands relative to the integrin molecule. Two RGD peptides were positioned in vicinity of the

interface between the β -propeller domain of the α -subunit and the β A domain of the β -subunit while another RGD peptide was located in proximity of the pocket formed by the β A and β TD domains of the β -subunit. Fibrinogen is a large macromolecule with a low translational and rotational diffusion coefficient [201]. Therefore, identifying the potential binding sites of the fibrinogen on the integrin head domain requires an excessive amount of computational time, i.e. several seconds or even longer, which is beyond the time scale of current molecular dynamics models. Hence, merely a functional part of the fibrinogen molecule, i.e. the RGD sequence, was located close to the integrin head domain and allowed to diffuse freely.

The integrin molecule embedded in the lipid bilayer was solvated in a water box, minimized in 2000 steps of 2fs, and equilibrated for no less than 13ns. The three RGD peptides diffused throughout the water box until they formed interactions at various regions on the integrin head domain. The simulation was repeated seven times, corresponding to a total of ~104ns. RGD peptide 3 (RGD3) interacted with various binding sites located on the integrin head domain and/or within the inner pocket formed by the bent molecule (see Fig. S4). However, RGD peptide 1 and 2 (RGD1 and RGD2) combined interacted with a more limited number of binding sites on the interface of the β A and β -propeller domains. Intriguingly, RGD3 was confined to the β A- β TD pocket region in all simulations, whereas RGD1 and RGD2 remained associated with the head domain only in half of the simulations.

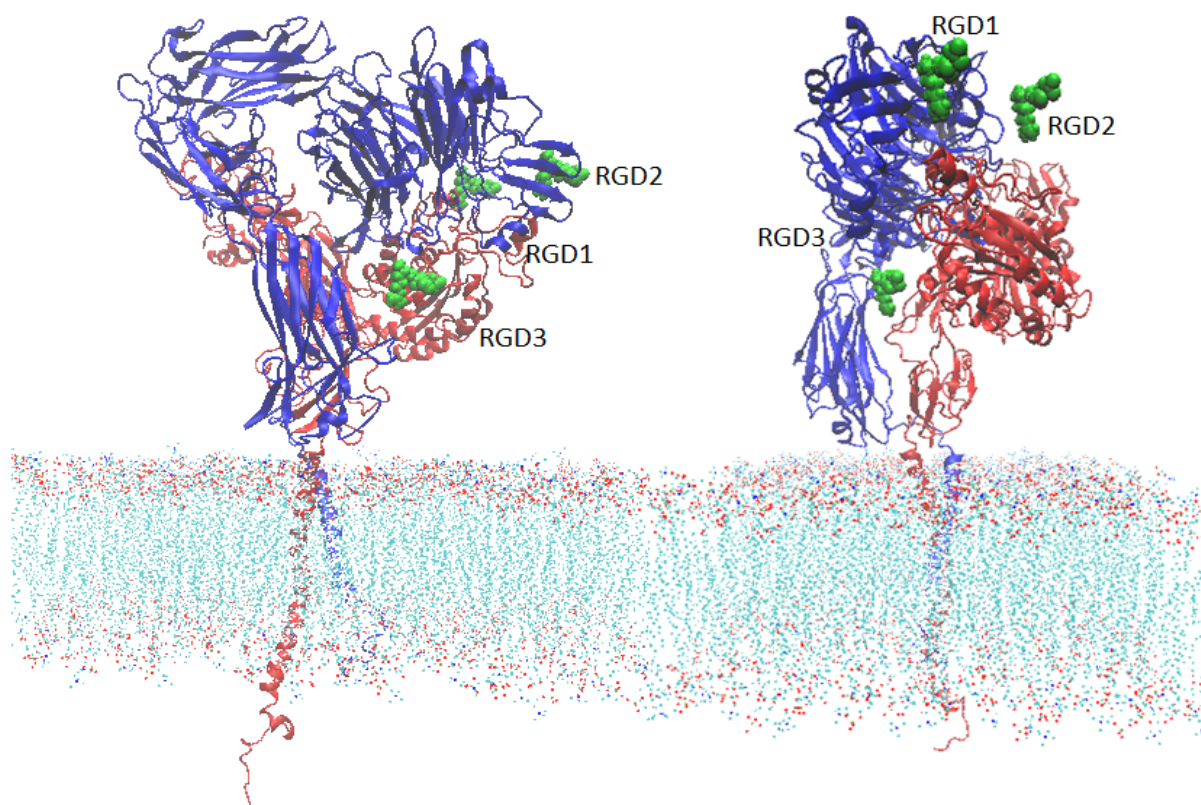


Figure 3.2: Crystal structure of integrin α IIb β 3 embedded in the plasma membrane. Three RGD peptides (green) are positioned in potential binding regions of integrin. The only RGD sequence that interacted

with the integrin molecule was the one located near the β TD- β A pocket (RGD3). The α IIb and β 3 subunits are depicted in blue and red, respectively. The plasma membrane is shown in gold.

Interactions that lasted for a minimum of ~ 3 ns after their initial formation were called “permanent bonds” as opposed to those that formed only intermittently, termed “temporary bonds” hereinafter. Depicted in Fig. S4 is the distance between interacting residues of integrin within the β A- β TD pocket region and RGD3. It is noteworthy that interactions between the RGD peptide and extracellular domain of integrin are mainly salt bridges, as the RGD peptide is equipped with a strong basic residue and a highly acidic one (i.e. Asp and Arg) with long side chains at its two ends that are separated by a small hydrophobic spacer (i.e. Gly). For this reason, all permanent bonds shown in Fig. S4 are of ~ 4.5 Å bond length, which is the characteristic length of a salt bridge [202,203].

According to the nature of bonds in the simulated trajectories, integrin-RGD bindings were classified into three major groups; the first group (i.e. type1 bond) comprised interactions with only a single, permanent interaction between the RGD peptide and integrin as demonstrated in Fig. S4a and S4b. Permanent bonds were formed as a result of type1 bonds with minimal fluctuations in all cases. The bond distance between the Arg of RGD3 and D817 on the Calf2 domain of the α -subunit is presented in Fig. S4, whereas Fig. S4b shows the bond distance between the Arg of RGD3 and D127 on the β A domain of the β -subunit.

The second type of binding that occurred in two of our simulations included bindings in which one side of the soluble RGD peptide interacted permanently with the β A domain on the integrin head domain, while the other end only showed temporary engagement with membrane proximal domains of integrin. In one simulation, the Arg side of the soluble RGD formed a permanent bond with E312 on the β A domain (see Fig. S4c). This interaction served as an anchorage for the ligand while the other side interacted temporarily with K650 on the β TD. Another permanent interaction formed between the Arg of RGD3 and D232 of the β -propeller domain of the α IIb subunit, whereas the Asp side of RGD3 formed a temporary interaction with N313 on the β A domain (see Fig. S4d).

The other, and the most complex, type of interactions involved one permanent binding site and two temporary interactions on the integrin molecule that competed with each other. This group of interactions featured one end of the free RGD peptide tethering to the β -subunit permanently with other short-term bindings also took place at the other end of the RGD peptide. For instance, Fig. S4e shows a permanent bond between the Arg of RGD3 and D628 on the Calf1 domain of the α -subunit (blue) as well as the temporary bonds between the Asp of RGD3 with R897 on the Calf1 domain of the α -subunit (green), and K600 on the linker region between the EGF-1 and β TD (red).

Although partial detachments were seen in a few of the simulations, a full separation of the β TD and β A domains was only observed in one out of six RGD-included simulations, when RGD3 interacted with three binding sites as depicted in Fig. S4f. A permanent, polar interaction (as shown in blue in Fig. S4f) was formed between the Arg of RGD3 and E671 on the β TD.

Importantly, it was observed that the electrostatic interactions of S673 and S674 on the β TD with K350 serve as a key for activation of integrin α IIb β 3, as depicted by Fig. 3.3. This simulation was run for ~21ns. The key residues on the β TD (i.e. S673 and S674) continued to interact with K350 until $t=7.5$ ns (see Fig. 3.3c). However, as the Arg of the RGD peptide reached the vicinity of S674 at $t=7.5$ ns, the Arg of the RGD peptide competed with K350 for S674 and the key interaction was highly disturbed (Fig. 3.3d). Finally, the key interaction was fully removed as the Asp of the RGD peptide engaged in a competitive interaction with K600 and K611, pulling the β TD away from the β A domain (Fig. 3.3e). As a result of the RGD peptide replacing K350 on the β A domain, the β TD swung open, separating from the β A domain (see Fig. 3.3a and 3.4b). The open conformation of the β TD appeared to be stable insofar as the β TD remained open for another 4.5ns until the end of the simulation, as shown in Fig. S5. Finally, we repeated the simulation one more time without any RGD peptides included. As expected, the β TD and β A domains remained attached in the absence of an RGD peptide.

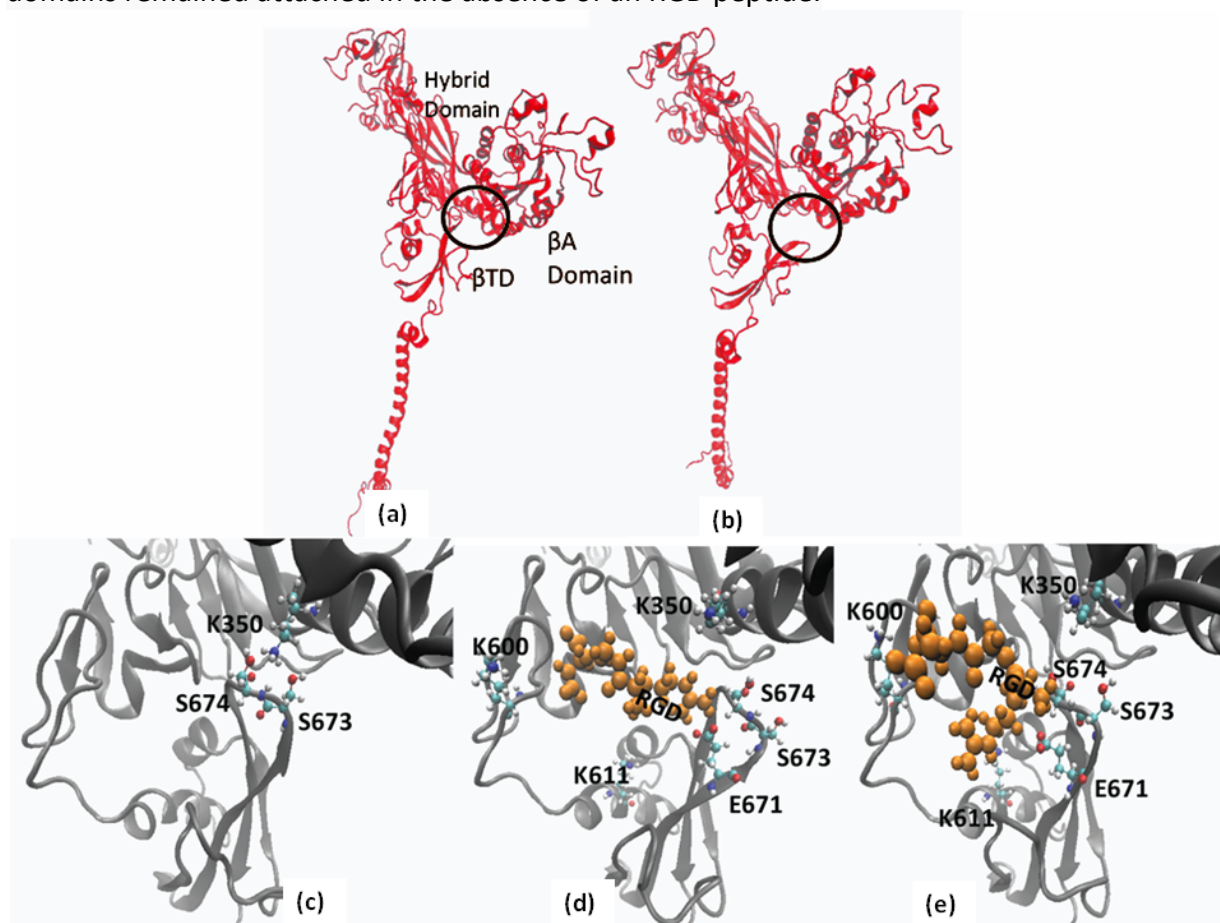
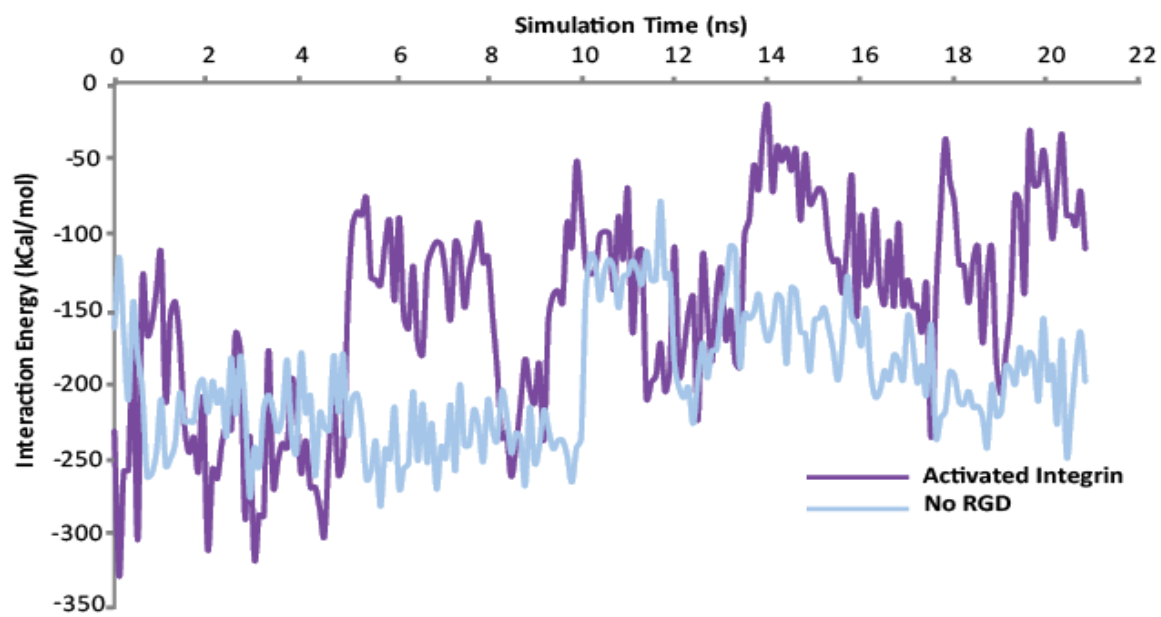
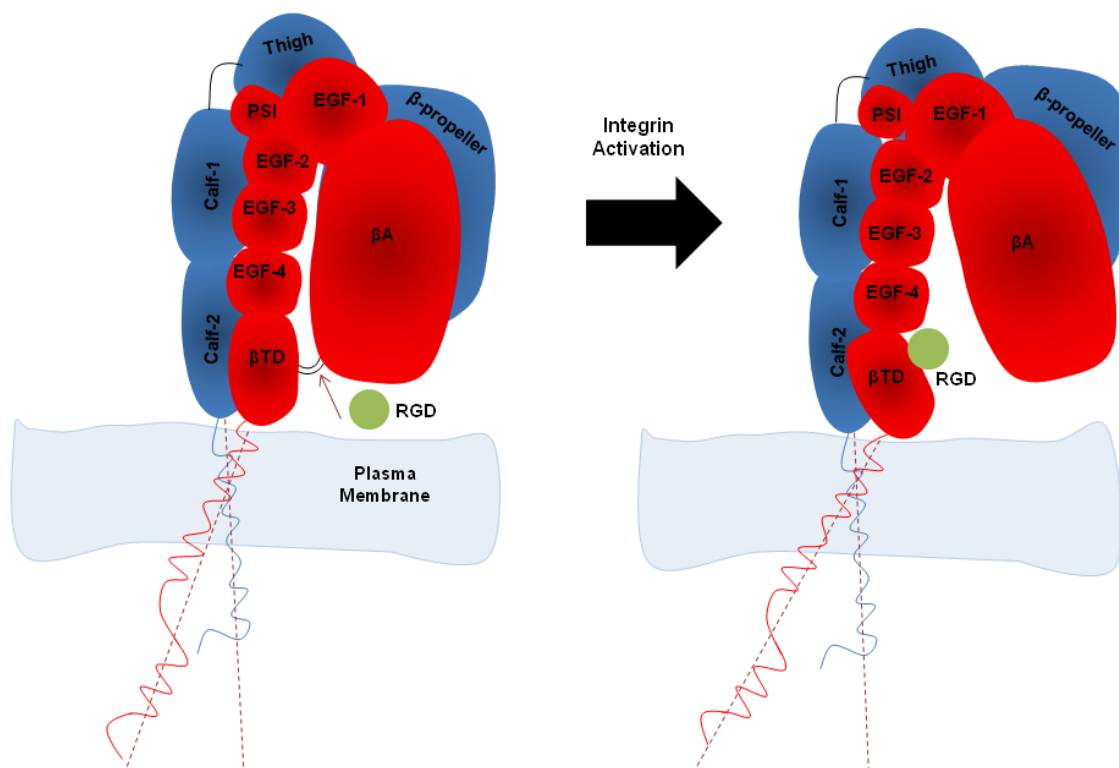


Figure 3.3: Arg of RGD disrupts the β A- β TD interactions via competing with K350 on β A domain for S673. (a) β A and β TD domains are attached in the inactive, bent state of integrin. (b) The β TD swings open and separates apart from β A domain, which leads to integrin activation. (c) Focusing further into the β TD- β A pocket shows the key interaction between K350 and S673/S674 that keeps integrin inactive. (d) The RGD peptide approaches S674 and interacts with it, disrupting the key interaction between β TD and β A domains. (e) While Arg of the RGD peptide is bound to E671 and S674, Asp of the RGD peptide switches back and forth between K600 and K611. This competitive interaction on the Asp side of the RGD peptide shifts the β TD further toward EGF3/4 domains and apart from β A domain.



(a)



(b)

Figure 3.4: (a) Interaction energy between integrin transmembrane-cytoplasmic domains of α - and β -subunits as the simulation progresses for the integrin activation case as well as for the simulation with no RGD peptide. The initial 5ns of the simulation demonstrates the pre-equilibration phase, where both systems show similar interaction energies of ~ 250 kCal/mol. This energy finally reaches a stable state at ~ 200 kCal/mol in the absence of an RGD peptide. However, for the case where separation between integrin β TD and β A domains occurs, the energy of interaction between the transmembrane-cytoplasmic domains of integrin significantly drops to ~ 100 kCal/mol. This difference highlights a potential underlying mechanism that could loosen and eventually break apart the electrostatic interaction that holds the transmembrane-cytoplasmic domains of integrin together, as a result of an RGD-induced outside-in activation. (b) A schematic of integrin α IIb β 3 immediately after activation via RGD binding to the β TD. Integrin β TD and β A domains are bound by the key interaction group. Insertion of an RGD peptide into the β TD- β A pocket leads to a separation between β TD and β A domains that somehow unbends integrin, while loosens the electrostatic interaction between integrin transmembrane domains via rotating the transmembrane domain of β -subunit.

We observed that the dissociation of the β TD and β A domains caused the β TD to snap back, which induced a slight rotation in the β -subunit transmembrane domain (about 3° over the course of our simulations) in a lever-like motion. This rotation increased the angle between the α - and β -subunit transmembrane domains. We conducted energy analyses to measure the electrostatic energy between the α - and β -subunit transmembrane domains for the case where there was no RGD peptide as well as for the case where the integrin activation was observed. Interestingly, the electrostatic energy of interaction appeared to decrease dramatically after the β TD and β A domains separated (see Fig. 3.4a). A tiny rotation of the long, β -subunit transmembrane domain increased the distance between acidic and basic residues on the α - and β -subunit transmembrane domains that considerably weakened the inner membrane clasp.

Integrin – Talin Binding: To study the effect of talin on integrin activation, we first needed to create two separate conditions: the experimental condition, integrin with talin bound, and the control, integrin alone. To create our experimental condition, we promoted the binding of talin to integrin through a series of equilibration and steering simulations. After the 48ns of equilibration and nudging, we observed binding between integrin and talin. Our results match that of the suggested mode of binding in literature [73].

First, hydrophobic packing is evident between conserved residues and motifs, W739 and NPLY (744 to 747), on the integrin β 3, and residues on the F3 domain of talin. The identified residues on the F3 domain of talin that bind to W739 on the integrin β 3 subunit are the residues R358 and A360 on the S5 β -sheet on talin, which form a pocket for W739 [134]. Additionally, the NPLY motif interacts with these S5 residues and additional residues, A389, Q390, A393, and I398, on the final H1 helix of the F3 domain (Fig. 3.5). Finally, after the nudges to tilt talin, as expected, F727 on the β 3 subunit embedded itself into the talin S1-S2 loop region strand, consisting partly of residues K318, M319, and K320 [106].

Using the integrin-talin and integrin-alone models, we randomized the initial velocities in 12 repetitions, equilibrated for 5ns, and pulled apart the IMC for 5ns. The Inner Membrane Clasp (IMC) of integrin changed its conformation upon binding talin. Three parameters were used to quantify the state of the IMC. The average distance between the carbon- α atoms of D723–R995 and E726–R995 shows that talin induces a bulge in the IMC even prior to the steered

separation. The mean distances, as indicated by distance “d” in Fig. S6, for both the integrin-talin and integrin alone conditions were 9.8Å and 13.5Å, respectively, and this difference was significant using a permuted two-sample, one-tailed, t-test ($p < 0.0003$) (see supporting materials). As a result of this increase in distance, the interaction energy between the acidic $\beta 3$ and basic αIIb IMC residues was also diminished. The mean nonbonding energy for the integrin-talin systems was -93.4kcal/mol and for the integrin-only system was -133.4kcal/mol (Fig. 3.6), and again this difference was significant ($p < 0.002$). More interestingly, in all 12 simulations, the interaction energy between the IMC residues was never stronger than -129.7kcal/mol in integrin-talin simulations, whereas the strongest observed energy was -163.6kcal/mol in integrin-only simulations.

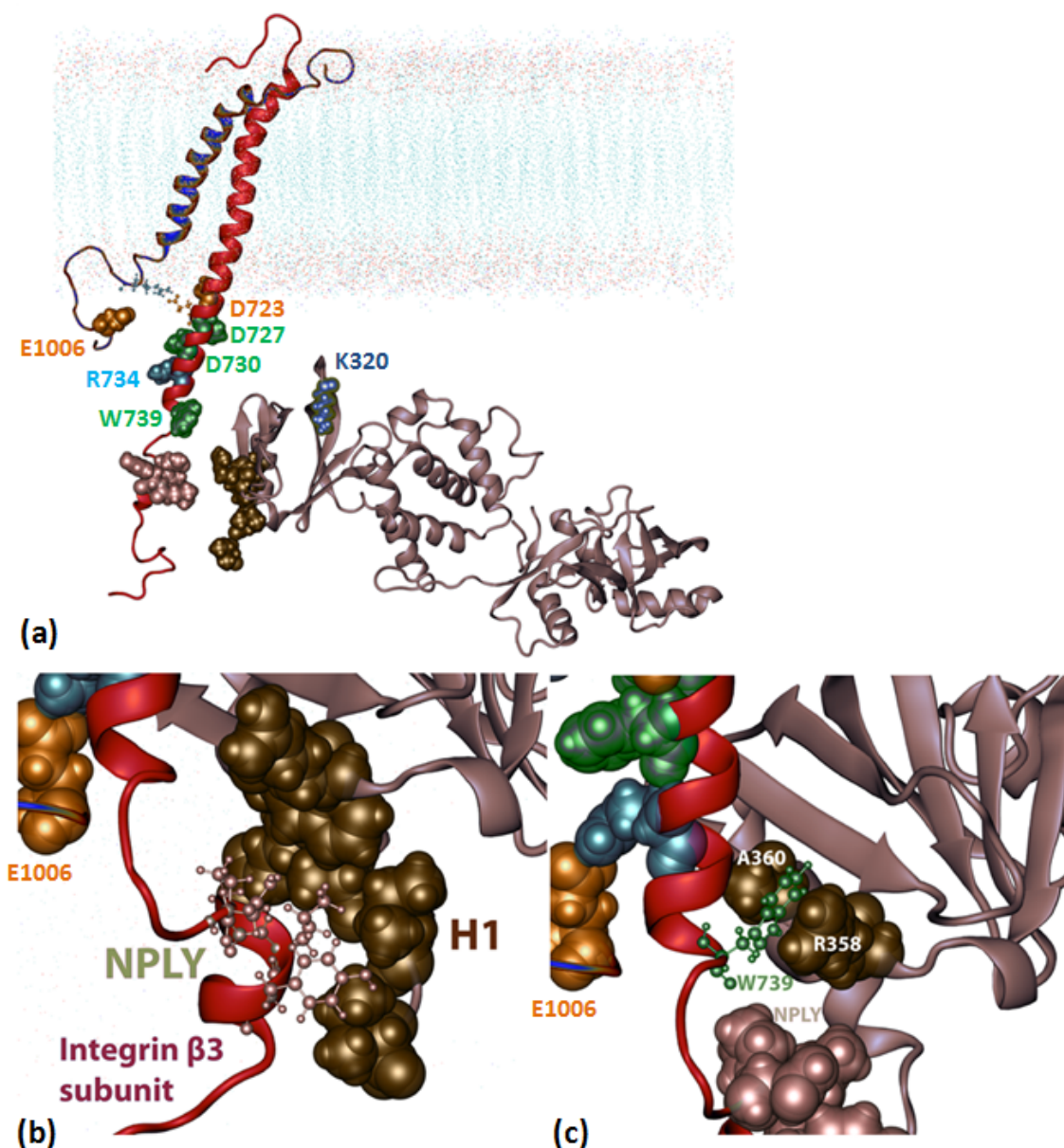


Figure 3.5: (a) Initial simulation position of integrin $\alpha IIb\beta 3$ transmembrane-cytoplasmic domains (PDB 2K9J) and Talin-1 FERM domain (PDB 3IVF). For the new-cartoon representations, in blue is αIIb -subunit

of integrin and in red is the $\beta 3$ -subunit. In pink is talin-1 FERM domain. For van der Waals sphere representations, in orange are the acidic residues E1006 (on αIIb) and D723 (on $\beta 3$) integrin, in cyan are the basic residues and R734 (on $\beta 3$), in green are the residues F727, F730, and W739 (from top going down on $\beta 3$), in pink is the NPLY motif, in brown are residues R358, A360, A389, Q390, A393, and I398 on talin F3 domain, and on the talin in blue is K320 of the S1-S2 loop on talin-1 F3 domain. Note that the CPK representation in cyan (R995, αIIb) and orange (E726, $\beta 3$) are tightly bound to hold the IMC together. These highlighted residues play an important role as discussed in literature and is shown by our simulations. (b + c) Talin-1 F3 domain (F0-F2 not shown) binding with NPLY motif and W739 on integrin $\beta 3$ subunit. The left rendering (b) highlights NPLY motif binding to H1 helix; while the right rendering (c) highlights W739 binding in the pocket formed by R358 and A360 in talin. Time taken is at 24ns after the start of the simulation.

The last parameter investigated was the force required to break apart the IMC. As expected, with talin bound to integrin, the force required to break the IMC was lower than without talin. The mean force required, given the spring constant of $k = 0.05 \text{ kcal/mol/\AA}^2$ and the constant-pulling velocity $10 \text{ \AA/ns}$ of the carbon- α atom of R995 while fixing the carbon- α atoms of D723/E726, was $224.6 \pm 9.75 \text{ pN}$ with talin bound and $249.8 \pm 12.2 \text{ pN}$ with integrin only (mean $\pm$ standard error). This difference was slightly significant ($p < 0.04$). These differences in initial energies, distances, and forces between the integrin-talin and integrin-alone conditions were confirmed with the difference in work of approximately 1000 kcal/mol required to separate the IMC, up to $16 \text{ \AA}$ of separation. In addition, with the integrin-talin simulations, a basic residue, K320, on talin's S1-S2 loop interacted with the integrin $\beta 3$ IMC residue D723 before and after the breakage of the IMC, with interaction energies of -75.2 kcal/mol and -84.5 kcal/mol , respectively.

One interesting difference between the integrin-talin versus the integrin-only system is that there is an additional salt bridge interaction between R734 on the $\beta 3$ and E1006 on the αIIb subunit with the integrin-talin system. Quantitatively, we observed that the distance between the most terminal carbons on R734 and E1006 was significantly different between the integrin-talin ($5.25 \text{ \AA}$) and the integrin only ($16.1 \text{ \AA}$) conditions, with $p < 0.0003$ (see supporting materials).

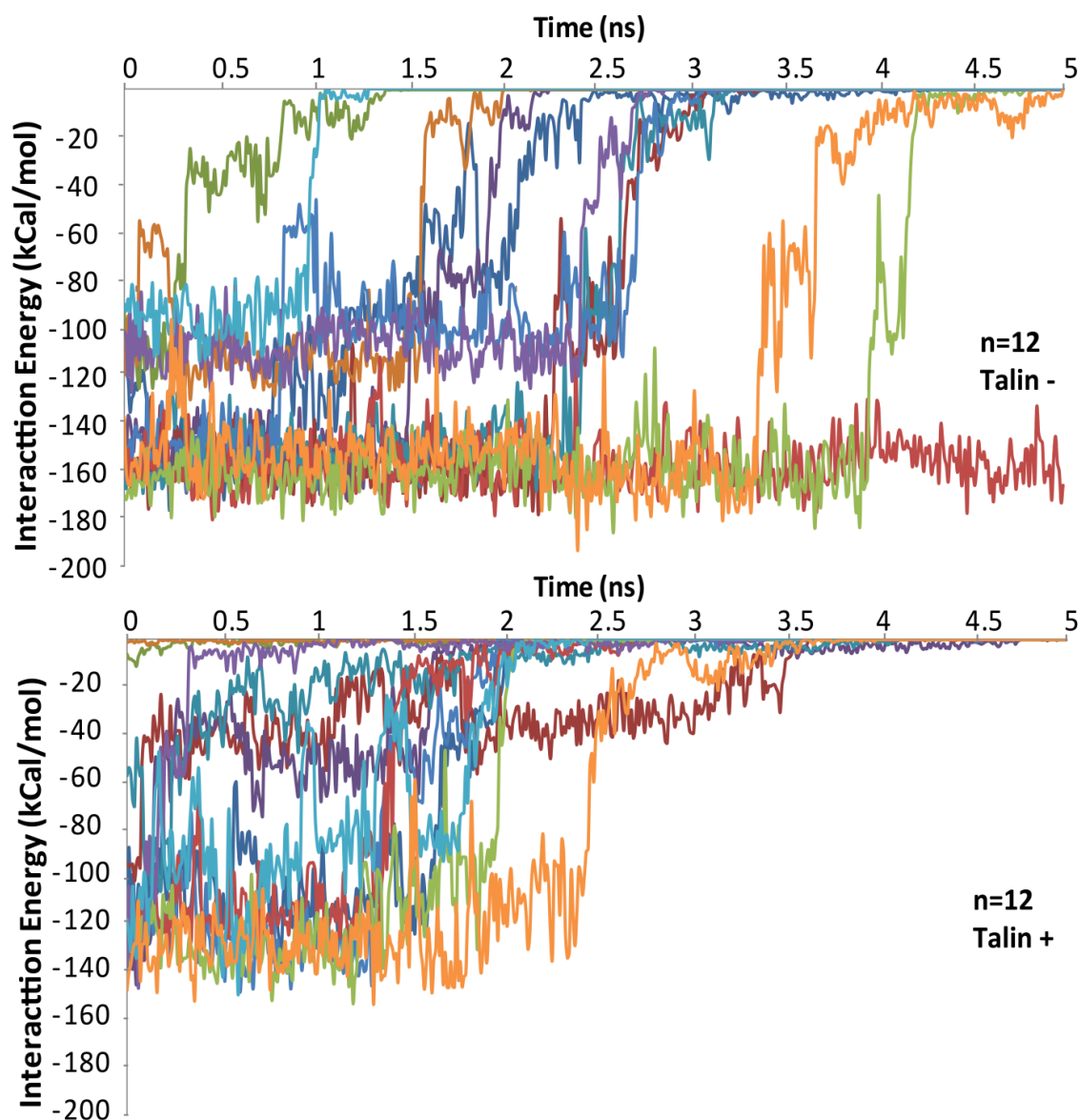


Figure 3.6: (a, b) Total non-bonding (van der Waals and electrostatic) energy between the IMC residues R995 on the α IIb, and D723 and E726 on the β 3 subunits for the steered molecular dynamics simulations, where the α IIb R995 carbon- α atom was pulled ($k = 0.05 \text{ kcal/mol/\AA}^2$, velocity = $10 \text{ \AA/ns}$) and the β 3 D723 and E726 carbon- α atoms were fixed. The colored lines represent the raw data from each of the 12 trials per condition, whereas the dark black line indicates the average of these trials. The two vertical dark gray bars within the first 100ps indicate the averaged region to obtain the initial non-bonding energy prior to performing SMD. The graph on the left (a) is the integrin-alone condition, while the right (b) is with talin bound to integrin.

Effect of Talin binding on Full Integrin Structure: Using the full-length integrin structure, we assessed the effects of the F3 domain of talin binding to specific local regions on the integrin β 3 cytoplasmic tail. Focusing on this additional interaction, R734-E1006, we found an overall negative correlation between the center of mass distances of the integrin β 3 cytoplasmic tail to the talin F3 domain and distance between R734 and E1006. In other words, as the talin F3 domain approaches the integrin tail, the distance between R734 and E1006 enlarges, which also

results in a lower interaction energy. Upon closer analysis (see Table S2), a stronger correlation is observed when selecting the dependent variable as the center of mass of a subdomain of the cytoplasmic $\beta 3$ subunit, specifically the region from the NPLY motif onwards (termed NPLY-T762), to measure the distance to the center of mass of the F3 domain of talin ($\rho = -0.82$, $p = 0.0038$). This suggests a greater significance in the membrane-distal binding of the $\beta 3$ integrin to the F3 domain of talin-1. Similarly, we also examined the potential conformational changes in the integrin extracellular domain followed by the F3 domain of talin binding to the integrin $\beta 3$ cytoplasmic tail. The degree of talin binding did not show any significant effects to alter integrin extracellular conformation (Table S3).

3.4. Discussion

Although integrin has been a subject of intensive study over the past two decades, molecular details of its function have remained ambiguous [3,12,62]. As shown in Fig. 3.1, interaction of thrombin proteinase-activated receptor 1 (PAR1) with thrombin triggers the integrin $\alpha IIb\beta 3$ activation pathway, which finally leads to thrombosis [2,5,204]. Therefore, understanding how integrin $\alpha IIb\beta 3$ functions paves the road for deciphering pathways that regulate thrombosis [204,205]. We have successfully used molecular dynamics techniques to model mechanosensitive focal adhesion proteins [124,182,183,191,192,193,206,207]. Here, we performed a series of molecular dynamics simulations to observe how exposing integrin $\alpha IIb\beta 3$ to soluble RGD peptides as well as the talin head domain may affect the integrin structure. Integrin $\alpha IIb\beta 3$ is a highly studied member of the integrin family, which is present in platelets, enabling them to interact with soluble fibrinogen (a specialized ligand of integrin $\alpha IIb\beta 3$ that exists abundantly in blood stream). It is widely known that soluble fibrinogen binds the head domain of integrin $\alpha IIb\beta 3$, initiating the blood clotting process by forming a network of fibrinogen ligands with platelets trapped inside it [205,208,209].

Approaching integrin $\alpha IIb\beta 3$ from the intracellular perspective, we have uncovered new biomolecular, mechanistic details on the inside-out activation process by talin. Although it is known that talin is required for inside-out activation of integrin, the specific mechanism of how integrin is activated by talin is uncertain [40]. Our results could be verified by the current working model that suggests talin binding to the cytoplasmic tail of the $\beta 3$ subunit would occur firstly at the membrane-distal region, which then promotes a membrane-proximal (MP) interaction to take place between talin and integrin $\beta 3$ subunit. This MP interaction is where the mechanism of activation arises [39]. Our data suggests a similar mechanism, in that $\beta 3$ subunit F727 embedding in the talin S1-S2 loop may help the inner membrane clasp (IMC) salt bridge to loosen its grip because this allows the basic K320 residue to energetically compensate for the IMC salt bridge loss. Furthermore, in the timescale of our simulations, we did not observe significant conformational changes in the integrin extracellular domains though previous work suggested that breakage of the IMC salt bridge by talin is necessary but not sufficient to fully activate integrin [39].

Additionally, we observed a salt-bridge interaction between the most membrane distal regions of the αIIb and $\beta 3$ integrin subunits with residues E1006 and R734, respectively. Interestingly,

this interaction appeared in two of the four separate simulation conditions, forming in the integrin-talin bound simulations using only the cytoplasmic-transmembrane domains of integrin, and in the integrin-alone simulations using the full-length integrin $\alpha\text{IIb}\beta 3$. We make two propositions, the first of which states that this interaction may be an artifact of using only the cytoplasmic-transmembrane domains of integrin. Secondly, the default presence of this interaction using full-length integrin suggests that talin plays an even more important role in integrin activation. This artifact may cause the force and work required to separate the IMC under the integrin-talin conditions to be overestimated. Without this interaction, the integrin-talin conditions may be even more favorable towards IMC separation and integrin activation. More interestingly, it seems that the membrane-distal interactions of talin and integrin are important for more than solely providing an anchor for the MP interactions to weaken the IMC [39]. Instead, we propose that the binding of talin to the membrane-distal region of the $\beta 3$ subunit cytoplasmic tail weakens this salt bridge E1006-R734 interaction, which is the first step in integrin inside-out activation.

Integrin is also activated via association with an extracellular ligand, a process called outside-in activation. It is reported that integrin $\alpha\text{IIb}\beta 3$ has a high affinity for the RGD sequence of fibrinogen [3,21,198]. Interactions between integrin $\alpha\text{IIb}\beta 3$ and fibrinogen are reported to occur on the interface of the β -propeller domain of the αIIb and the βA domain of the $\beta 3$ subunit. Particularly, RGD peptides are shown to associate with a region of the $\beta 3$ defined by D109 and E171 as well as another region limited to S211 and G222 [31,32]. Nonetheless, a number of studies showed that the integrin $\alpha\text{IIb}\beta 3$ head domain interacts solely with a C-terminal region of fibrinogen (i.e. γC) rather than the RGD sequences on the fibrinogen α -subunit [34,45]. To address this controversy making best use of the computational time, instead of modeling the entire fibrinogen molecule, we placed two RGD sequences at regions of the integrin $\alpha\text{IIb}\beta 3$ head domain that had been reported to have high affinity for fibrinogen [3,34]. An RGD peptide was positioned in vicinity of the βTD - βA domain interface, another sensitive region that is suggested to contribute to integrin activation, to explore its potential interactions especially ones that could regulate integrin function. The small size of RGD peptides conferred upon them high diffusivity and capability to sweep the space in vicinity of the sensitive areas of integrin $\alpha\text{IIb}\beta 3$. We initiated our simulations with inactive integrin $\alpha\text{IIb}\beta 3$, wherein the βA domain of the integrin head domain is attached to the membrane-proximal βTD . This forms a pocket-like configuration between the integrin head domain and membrane-proximal region, which is geometrically appropriate for the long, coiled-coil molecule of fibrinogen to insert into. Because the RGD motif of fibrinogen is located on the surface of the γ -subunit of fibrinogen, it has high potency for detecting RGD-sensitive regions of integrin $\alpha\text{IIb}\beta 3$.

The association constant for the integrin-peptide interaction is:

$$k_a = \frac{[IP]}{[I][P]}, \quad (3.1)$$

where $[IP]$, $[I]$, and $[P]$ are concentrations of integrin, free RGD peptide, and integrin-RGD complex, respectively. The association constant is related to the free energy of association as follows:

$$k_a = e^{\frac{-\Delta G}{k_B T}} \quad (3.2)$$

where ΔG , k_B , and T are the free energy of association per mol, Boltzmann constant, and temperature, respectively. Our simulations indicated that for the highly dynamic RGD binding sites in the β TD- β A pocket, 10ns is a reasonable time scale for bonds to form and break down at least once (see Fig. S4). As the free energy per mol of a significant electrostatic bond in amino acid is typically taken as 5 kCal/mol [210,211], substituting that value for ΔG in Eq. 3.2 and solving at $T=310K$ yields $k_a \sim 0.06 \text{mol}^{-1}$. According to the Ergodic hypothesis in statistical thermodynamics, for a stochastic system, any average of thermodynamic parameters over the entire volume of the system at any instant in time equals the average of the same parameter taken over a long time span at a single point in the system [212]. Hence, as a first approximation, we can assume the free concentrations of integrin and peptide (denoted as [I] and [P] in Eq. 3.1) are proportional to the sum of time spans that the system experiences the unbound state. On the other hand, the concentration of the complex (denoted by [IP] in Eq. 3.1) is proportional to the accumulative time period that the system spends in the bound state. Therefore, the minimum association constant for significant bond formations ($k_a=0.06 \text{mol}^{-1}$) is corresponding to a binding time of at least 3ns within a simulation scale of 10ns. We defined bindings that remained associated for more than $\sim 3\text{ns}$ as permanent bonds and bindings with life spans any shorter than 3ns as temporary bonds.

Our simulations highlighted several binding sites for the RGD peptide on integrin $\alpha\text{IIb}\beta_3$, most of which were either inside or proximal to the β A- β TD pocket. Binding sites of integrin $\alpha\text{IIb}\beta_3$ for RGD peptides in this region fell into three distinct categories, wherein permanent interactions always form at the Arg end of the RGD peptide. This could be attributed to the highly basic side chain of the Arg that facilitates formation of permanent salt bridges. The first category featured bonds formed between the Arg of the RGD peptide and integrin. Although this group included permanent bindings, these bindings did not contribute to detachment of the β A and β TD domains within our simulation time because they were not able to disturb the key interaction (i.e. K350 with S673 and S674). In the second type of interactions, the Arg of the RGD peptide formed a permanent bond with integrin $\alpha\text{IIb}\beta_3$ while the Asp of the RGD peptide formed a temporary bond, which was highly fluctuating and unstable. Finally, the third and most complex type of RGD peptide-integrin interactions were composed of a permanent interaction of the Arg of the RGD peptide with integrin $\alpha\text{IIb}\beta_3$ at one end and a competitive, temporary interaction of two other residues with the Asp of the RGD peptide at the other end. Within the time scale of our simulations ($\sim 13\text{ns}$), one out of two observed, type3 interactions fully dethatched the β A domain from the β TD, which according to both switch-blade and deadbolt models is equivalent to integrin activation.

After about 7.5ns of equilibration and free diffusion of the RGD peptide in vicinity of the β A- β TD pocket, as depicted in Fig. 3.3, the key β A- β TD domain interactions (i.e. K350 with S673 and S674) were disrupted as a result of the Arg of the RGD side chain taking over the OH-group on the side chain of S673. About 1ns after the key interaction was disrupted, the β TD swung open and the conformational change continued as the Asp of the RGD peptide was engaged in two competing interactions with K600 on the EGF3-EGF4 domain linker region and K611 on the

EGF4 domain of the $\beta 3$ subunit (see Fig. 3.3e). These competing interactions shifted the RGD peptide more towards the EGF3 and EGF4 domains, which furthered the distance between the βA and βTD domains. It is worthwhile mentioning that since Ser residues do not have significant avidity for Lys, the two Ser residues together still do not maintain K346 on the βA domain in a permanent salt bridge (i.e. a bond distance of $\sim 4\text{\AA}$). Hence, this rather weak interaction combined with the strong basic identity of Arg side chains may explain how integrin $\alpha IIb\beta 3$ is activated by a soluble, RGD-containing ligand (e.g. fibrinogen).

It has been shown that fibrinogen binds an extensive interface between the βA and β -propeller domains of the integrin $\alpha IIb\beta 3$ head domain proximal to the metal ion-dependent sites (i.e. MIDAS and ADMIDAS), which leads to integrin activation [34,45,213]. Aligned with previous studies, RGD1 and RGD2 interacted with the crevice between the βA and β -propeller on the head domain [31,32]. However, we did not observe any significant conformational changes induced in integrin as a result of RGD binding to the head domain. This is aligned with a more recent study that showed functional associations of fibrinogen with integrin occur at the RGD-lacking γC peptide of fibrinogen [45]. Our study shed light on another important binding site for the RGD motif inside the βA - βTD pocket that activates integrin. Our simulations indicated that the pocket formed by the βTD and βA domains of integrin is home to several binding spots for the RGD peptide. Short-term interactions between these binding spots and the RGD peptide increase the residence time of the soluble RGD peptide near the pocket, which escalates odds of an interaction occurring between the RGD peptide and the key bonds between the βTD and βA domains (i.e. K350 with S673 and S674), which would lead to integrin activation via disrupting the key interactions.

Two major mechanisms are reported to “unlock” the integrin inhibitory interactions. While the switch-blade model proposes a drastic change, from bent to fully-stretched conformation upon activation, the deadbolt model suggests more local conformational changes in the molecule due to dissociation of the βTD and βA domains [13,23,34,42]. The switch-blade model suggests that the interaction of the integrin cytoplasmic domain with particular focal adhesion proteins leads to a 135° rotation of the integrin head piece relative to the tail piece. This rotation causes the βA - β -propeller interface to face away from the plasma membrane, thereby becoming available to interact with the ligand [35,200]. The switch-blade model is corroborated for different members of integrin family by a number of previous studies. First, when recombinant integrin $\alpha 5\beta 1$ was artificially constrained with disulfide bonds in a bent conformation it did not associate with immobilized ligands [65]. For integrin $\alpha v\beta 3$, a double mutation that held the head domain against the leg domains via a Cys bridge hindered activation [35]. In addition, a significant increase in the protein Stokes radius has been recorded for cases where integrins were exposed to RGDFV peptides or Mn^{2+} (i.e. integrin activators) versus the case where they are in a Ca^{2+} buffer, which is an activation inhibitor [35]. However, integrin $\alpha IIb\beta 3$ activation paradigm appears to remain controversial. As detailed monoclonal antibody epitope maps for inactive and active integrin $\alpha IIb\beta 3$ *in vivo* demonstrated similar compact forms consistent with the bent conformation [12,75]. Furthermore, binding of different ligand mimics to integrin $\alpha IIb\beta 3$ in intact platelets has shown distinct conformational changes in the receptor [79].

Another mechanistic explanation for integrin activation was given by the deadbolt model. The deadbolt model assumes that the elongated CD loop of the β TD locks the β A domain via forming bonds with it in the inactive state of the native structure, although the atomistic details of these interactions are not currently clear [12]. It is important to note that the deadbolt model does not necessarily exclude a subsequent ligand-induced switch-blade-like swing-out. Interestingly enough, our simulations demonstrated that the disruption in the interactions between the β TD and β A domains could be a trigger for integrin activation, recognizing electrostatic interactions of K350 on the β A with S673 and S674 on the β TD as a potential regulatory lock.

We observed that the dissociation of the β TD and β A domains was succeeded by the β TD snap-back, which caused the β -subunit transmembrane domain to slightly rotate (about 3°) in a lever-like motion. This increase in the angle between the α - and β -subunit transmembrane domains is also reported by previous electron microscopy and biochemical data as a potential activating mechanism for integrin α IIb β 3 [21,31,40,84]. However, another model is proposed for a number of other integrin family members (e.g. α 5 β 1) that suggests the hinge region for the scissors-like motion of integrin, upon activation, is located on the interface of the α - and β -subunit head domains [65]. To validate our simulations, we measured the electrostatic energy between the α - and β -subunit transmembrane domains for the simulation with no RGD peptides and compared it to the one in which the integrin activation had been observed. Intriguingly, the electrostatic energy of interaction appeared to decrease dramatically after the β TD and β A domains separated. A slight rotation of the long, transmembrane domain of the β -subunit could considerably increase the distance between charged residues on the α - and β -subunit transmembrane domains, which would weaken the inner membrane clasp (IMC). Therefore, we believe RGD interaction with the β TD could be a potential trigger for the complete separation of the transmembrane domains. Although this set of data proposes a key interaction that might function as a regulating “deadbolt,” the loosening of the transmembrane domain clasps as a result of the β TD- β A domain separation suggests subsequent, more drastic conformational changes, and explains how ligand-binding could lead to the transmembrane domain separation as proposed by the switch-blade model. On the other hand, within the time scale of our simulations, we did not observe any significant conformational changes in the ectodomain nor did we see any decreases in the β A- β TD interaction energy upon talin binding with the β 3 cytoplasmic tail. This suggests that outside-in activation via RGD association with the key residues embedded in the β A- β TD pocket occurs more rapidly than inside-out activation followed by talin binding. It is also noteworthy that distinct activation mechanisms and conformational changes might be at work for different combinations of integrin-ligand interactions.

Chapter 4

Role of α -Subunit Headpiece in Regulating the LIMBS Ion

Integrin α IIb β 3 and α V β 3 show distinct ligand binding affinities while sharing the same β -subunit. The aspartate in the prototypical integrin-binding motif Arg-Gly-Asp binds the integrin β A domain of the β -subunit through a divalent cation at the metal-ion-dependent adhesion site (MIDAS). An auxiliary metal ion at a ligand-associated metal-ion binding site, LIMBS, stabilizes the metal ion at MIDAS. LIMBS contacts distinct residues in the α -subunits of the two β 3 integrins α IIb β 3 and α V β 3, but a potential role of this interaction on stability of the metal ion at LIMBS in β 3 integrins has not been explored. Equilibrium molecular dynamics simulations of fully hydrated β 3 integrin ectodomains revealed strikingly different conformations of LIMBS in unliganded α IIb β 3 versus α V β 3, the result of stronger interactions of LIMBS with α V, which reduce stability of the LIMBS metal ion in α V β 3. Replacing the α IIb-LIMBS interface residue F191 in α IIb (equivalent to Trp179 in α V) with Trp strengthened this interface and destabilized the metal ion at LIMBS in α IIb β 3; a W179 to Phe mutation in α V produced the opposite effect. Consistently, a F191W substitution in cellular α IIb β 3 and a W179F substitution in α V β 3 reduced and increased, respectively, the apparent affinity of Mn^{2+} to the integrin. These findings offer an explanation for the variable occupancy of the metal ion at LIMBS in α V β 3 structures in the absence of ligand, and provide new insights into the mechanisms of integrin regulation.

4.1. Introduction

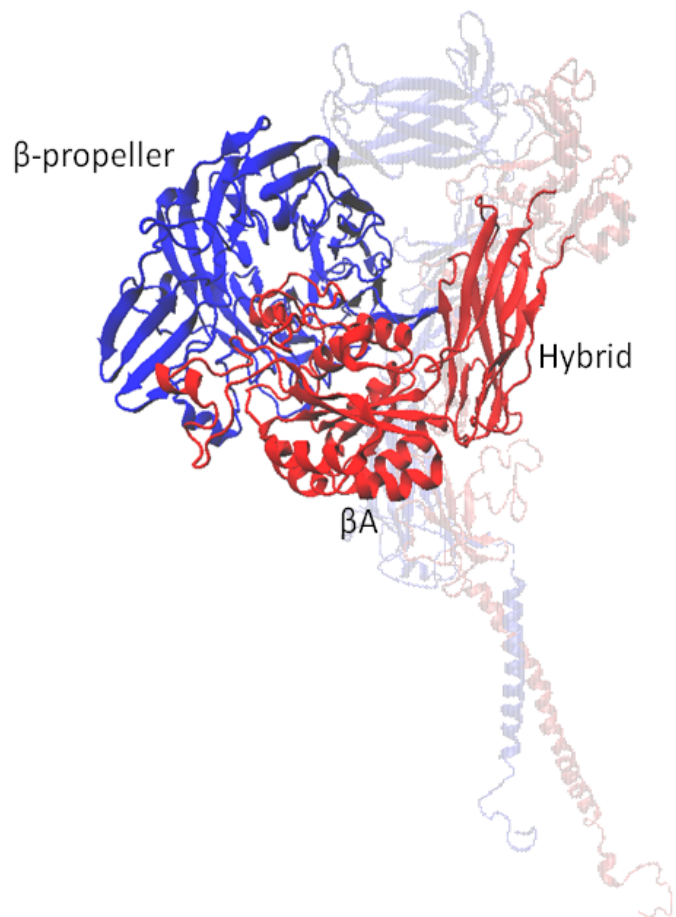
The ligand-binding compartment of integrins consists of a seven-bladed β -propeller domain from the α -subunit that associates non-covalently with a GTPase-like domain, β A, from the β -subunit (see Fig. 4.1) [10]. Contacts between the cytoplasmic tails, legs and head are believed to hold the integrin in an inactive state (unable to bind physiologic ligands). Binding of talin and kindlin to the β -cytoplasmic tail triggers conformational changes in the ectodomain that switch the head domain into the active (ligand-competent) conformation (inside-out signaling) [6]. Ligand binding triggers conformational changes that are propagated through the plasma membrane to the tails, leading to outside-in signaling as mentioned in detail in Chapter 1.

Integrin-ligand interactions are regulated in a complex manner by divalent cations [214,215,216,217]. Whereas Mn^{2+} and, to a lesser extent, Mg^{2+} stimulate ligand binding, Ca^{2+} is typically known to be inhibitory cation. The ligand-binding face of the β A domain is decorated by three metal ion binding sites: a *metal-ion-dependent adhesion site* (MIDAS), flanked on one side (facing the α -subunit's Propeller domain) by a *ligand-associated metal binding site* (LIMBS), and on the opposite side by an *Adjacent-to-MIDAS* (ADMIDAS) [33]. A ligand aspartate completes the octahedral metal coordination of an Mg^{2+} (or Mn^{2+}) at MIDAS in ligand-bound integrins. Ca^{2+} but not Mg^{2+} binds preferentially at LIMBS and ADMIDAS in physiologic buffer conditions, and both sites can coordinate Mn^{2+} . The metal ion at LIMBS stabilizes the one at MIDAS [33,218,219], thus acting as a positive regulator of ligand binding to integrins, whereas

the ADMIDAS metal ion can stabilize alternate inactive and active conformations of the integrin [74].

Figure 4.1: Integrin α IIb β 3 head domain consists of a seven-bladed β -propeller domain from the α -subunit that associates non-covalently with the β A domain, from the β -subunit connected to the hybrid domain.

In the β 3 subunit, the LIMBS loop residues R216, D217, and A218 contact residues in the α -subunit propeller domain (Fig. 4.2), but a potential role of the α -subunit in regulating metal ion occupancy in the β A domain has not been explored. In this communication, we carried out computational and functional studies on the two β 3 integrins α V β 3 and α IIb β 3. Our studies reveal an important role of the α -subunit in regulating metal ion stability at LIMBS in β 3 integrins. The significance of this finding is discussed.



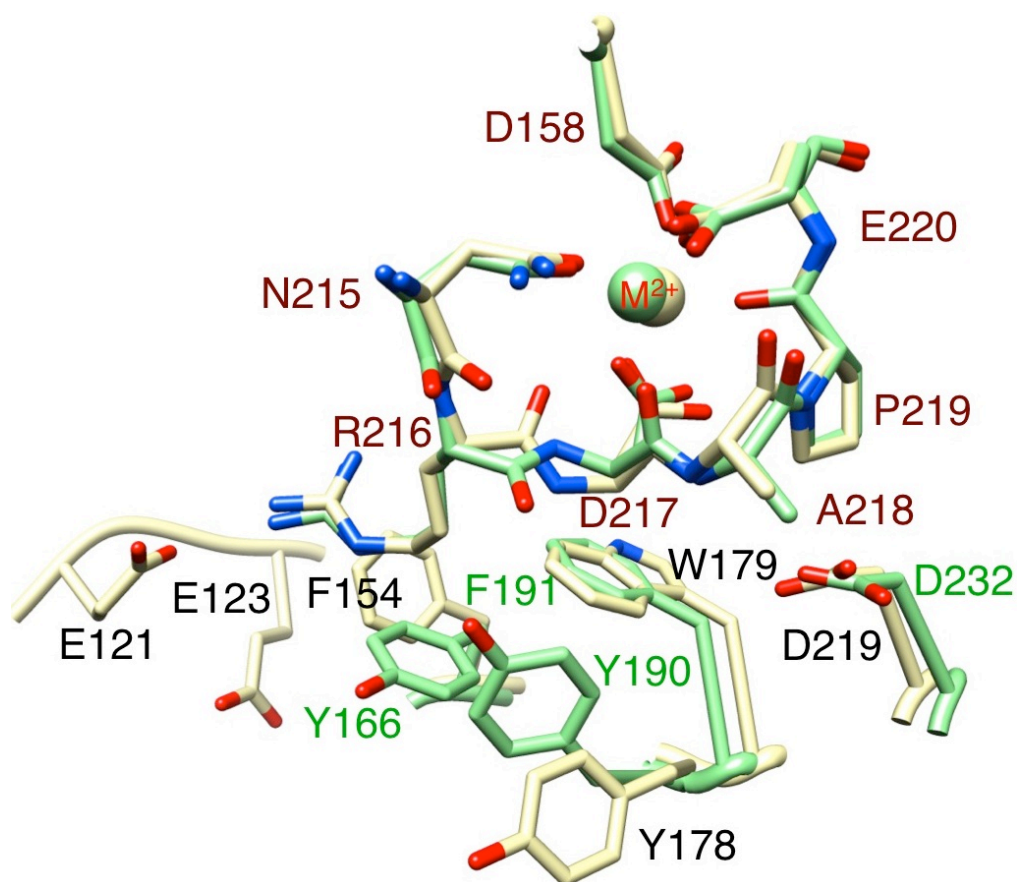


Figure 4.2: α -subunit residues facing LIMBS in β_3 integrins. Ribbon diagram showing the residues from α IIb and α V-facing LIMBS loop residues R216-A218. The β_3 subunits of unliganded α IIb β_3 (green, 3fcs.pdb) and α V β_3 (yellow, 4g1e.pdb) ectodomains were superposed with Chimera. The metal ion (M^{2+}) at LIMBS (sphere) has the color of the respective integrin. α IIb and α V residues are labeled in green and black, respectively, and the LIMBS residues are labeled in red.

4.2. Computational Procedures

Molecular Dynamics Simulations Design: The crystal structures of the ectodomains of α IIb β_3 (pdb 3FCS) [49] and α V β_3 (pdb 4G1E) [50] were downloaded from the Protein Data Bank (PDB). In α IIb β_3 , LIMBS, MIDAS and ADMIDAS, were occupied by Ca^{2+} , Mg^{2+} and Ca^{2+} respectively. Since only LIMBS was occupied (by Ca^{2+}) in α V β_3 structure, Mg^{2+} and Ca^{2+} were respectively placed at MIDAS and ADMIDAS such that their initial distance (i.e. before minimization) from all the proximal oxygen atoms were between 2.4-4Å. Mutations were made to the native structures using the software Swiss-Pdb Viewer 4.1.0 [220]. All non-protein atoms were removed from integrin α IIb β_3 and α V β_3 structures, leaving α - and β -subunits with nearly 24,000 atoms for each, including hydrogens. Proteins were solvated in water boxes of sizes 168x169x207 Å (for α V β_3) and 180x144x226Å (for α IIb β_3), adding approximately 183,000 and 185,000 water molecules to α V β_3 and α IIb β_3 , respectively, and ionized with 150mM of KCl. To investigate the effects of F191 in α IIb and W179 in α V on coordinating divalent cations at LIMBS, six distinct combinations of cation type and mutation for α IIb β_3 and four for α V β_3 were tested.

To examine the effects of each cation arrangement, the $\alpha\text{IIb}\beta 3$ and $\alpha\text{V}\beta 3$ structures were equilibrated with LIMBS-MIDAS-ADMIDAS occupancies of Ca^{2+} - Mg^{2+} - Ca^{2+} and Mn^{2+} - Mn^{2+} - Mn^{2+} .

Molecular Dynamics Simulations: Molecular dynamics simulations of the integrin ectodomain were performed with NAMD cvs_20130828 software package [174]. The CHARMM27 force field parameter [221] was used to model the protein. The TIP3P model [222] was used for water molecules. Structures were visualized with Visual Molecular Dynamics (VMD) [176] or Chimera [223]. The crystal structure of the ligand-free forms of $\alpha\text{IIb}\beta 3$ and of $\alpha\text{V}\beta 3$ ectodomains were used without modification, except for the manual placement of metal ions at LIMBS and ADMIDAS in $\alpha\text{V}\beta 3$, and removal of the sugar and water molecules before solvation. All simulations were carried out at the computing facilities of the National Energy Research Scientific Computing Center (NERSC). Periodic boundary conditions were applied in all three directions. Afterward, the entire system was minimized for 20,000 steps, followed by 20 nanoseconds (ns) of equilibration. A time step of 2fs was used in all simulations. The temperature and pressure of the systems were held constant at 1 atmosphere and 310K (NPT), using the Langevin's piston and Hoover's method, as successfully used for modeling integrins [72,80,124,174]. We performed 20 nanoseconds of MD simulation for each run, and the trajectories were used for all analyses. The cutoff distance for non-bonded interactions was 1.2 nm, and the Particle Mesh Ewald (PME) method was used for electrostatic force calculations [174]. All B-factors were set at zero. The hydrogen atom bond length was constrained using the SHAKE algorithm [224].

Structure Analysis: Two parameters, root-mean-square deviations (RMSD) of the LIMBS metal ion and the energy of interaction of the metal ion with the LIMBS pocket, were used to evaluate stability of the metal ion at LIMBS. The RMSD of a single atom is a measure of its distance at each time step from the ion's initial position. Hence, higher RMSDs represent fluctuations with larger amplitudes, and less stable ion-pocket bonds. Higher interaction energies reflect higher bond stability between the ion and LIMBS, or between the α -subunit and the LIMBS loop comprising residues R216, D217 and A218. To let systems equilibrate for a considerable time span before starting to take samples for RMSD measurements, RMSD values for the ion were averaged over time steps between $t=16$ -20ns for all simulations ($n=40$). Energies of interaction between the divalent ion and the LIMBS pocket were estimated using the Langevin Dynamics. As this energy of interaction did not show significant fluctuations throughout the simulations, values for energy were averaged over $t=0$ -20ns ($n=200$).

4.3. Results

LIMBS Structure and Stability of the Metal Ion in $\beta 3$ Integrins: Measurements of the energy of interaction of αV and αIIb subunits with the α -subunit-facing LIMBS loop residues R216-D217-A218 revealed a stronger (2-3 fold) interaction of αV with the LIMBS loop when compared with αIIb . This difference was first detected at 8 ns of simulations and maintained through 20ns (Fig. 4.3A).

Molecular dynamics simulations after a few nanoseconds of equilibration showed that Ca^{2+} coordination at LIMBS in $\alpha\text{IIb}\beta 3$ comprised 6 “primary” oxygens (i.e. the two carboxyl oxygens of D158 and D217, one carboxyl oxygen of E220, and the carbonyl oxygen of P219) (Fig. 4.3B), which hold a mean distance of 2.2-2.3Å from the encapsulated cation for the entire simulation, along with 2 “secondary” oxygens (i.e. the carbonyl oxygens of D217 and A218), whose mean distance from the cation was no more than 4.5Å over the whole trajectory. The primary oxygens remained in close contact with the Ca^{2+} , forming highly stable bonds with a mean length of 2.2-2.3Å and fluctuation amplitude below 0.5Å. The primary coordinating oxygens formed an octahedral arrangement, with a planar surface formed by OD2 of D158, OE1 of E220, OD1 of D217, and the carbonyl oxygen of P219, with OD1 of D158 and OD2 of D217 at the top and bottom of the plane, respectively (Fig. 4.3B), restricting cation fluctuations. Replacing Ca^{2+} with Mn^{2+} in LIMBS, MIDAS and ADMIDAS of $\alpha\text{IIb}\beta 3$ increased the energy of interaction of Mn^{2+} with LIMBS by 5-8%, but had a minimal effect on RMSD (Table 1).

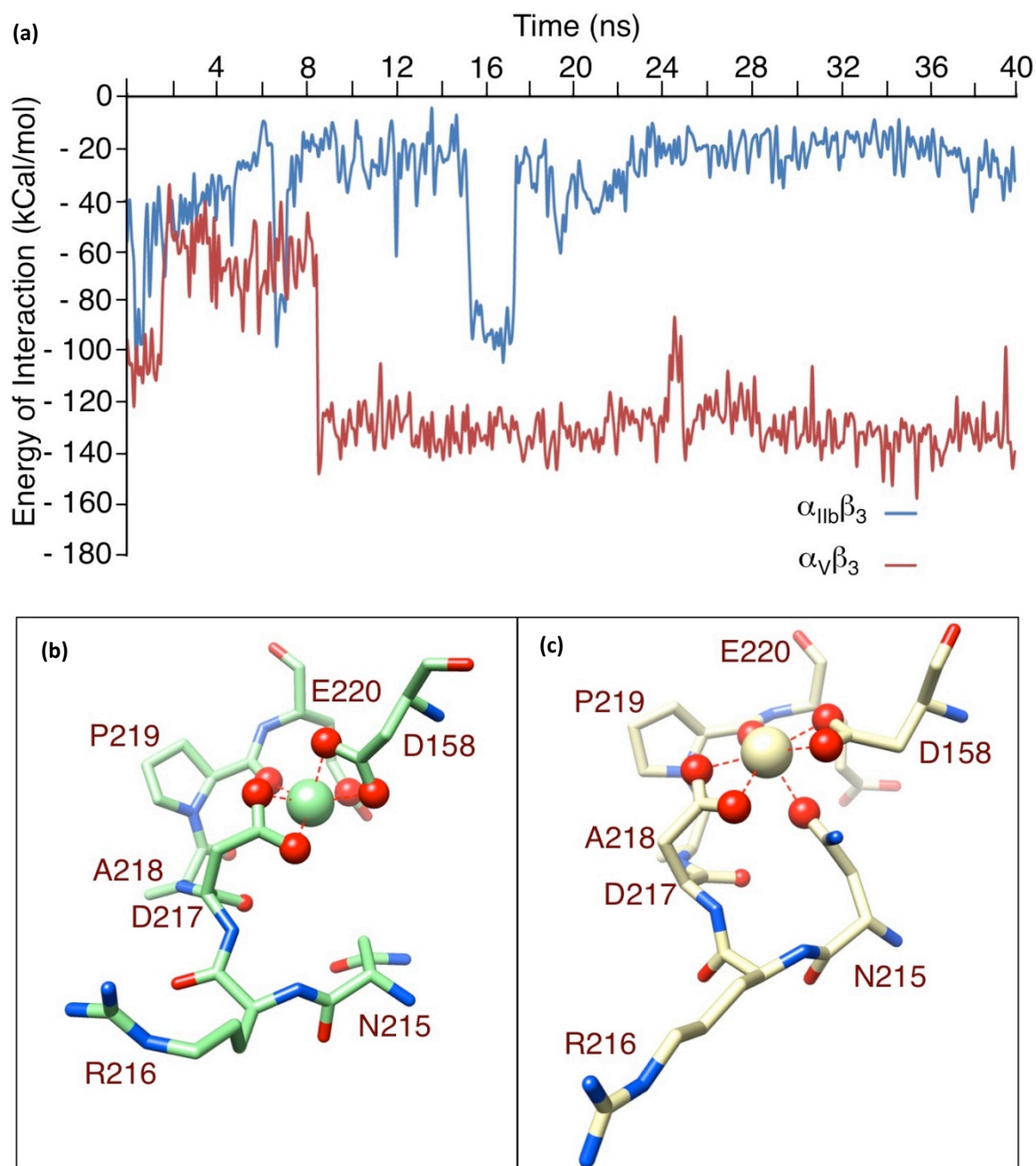


Figure 4.3: α -subunit/LIMBS interaction energies and LIMBS conformations in β_3 integrins. (a) Computed energy of interaction between α_{IIb} and α_V subunits and LIMBS loop residues R216-A218. The two trajectories appear to equilibrate after around 8ns of simulation. The transient peak seen afterwards (at ~ 16 ns) most likely represents a high-energy local minimum state that the system occasionally takes rather than simply a random fluctuation. (b, c) Snapshots of molecular dynamics simulations at $t=20$ ns, showing structures of LIMBS in $\alpha_{IIb}\beta_3$ (b) and $\alpha_V\beta_3$ (c), in the same orientation, with the LIMBS Ca^{2+} shown as a large sphere in each case. In $\alpha_{IIb}\beta_3$, the LIMBS metal ion contacts six primary oxygens (magnified red spheres) and two secondary oxygens (small red spheres)(b). In $\alpha_V\beta_3$, the LIMBS metal ion also contacts six primary oxygens but only one secondary oxygen (c). Note that the side chain of R216 is stretched out in (c) vs. in (b), and that the five primary oxygens in $\alpha_V\beta_3$ (c) lie in one plane, in contrast to the octahedral arrangement of the primary oxygens in $\alpha_{IIb}\beta_3$ (b).

Integrin and $\beta 3$ metal ion occupancy state		$\alpha \text{Ib}\beta 3$ (Ca^{2+} - Mg^{2+} - Ca^{2+})			$\alpha \text{Ib}\beta 3$ (Mn^{2+} - Mn^{2+} - Mn^{2+})			$\alpha \text{V}\beta 3$ (Ca^{2+} - Mg^{2+} - Ca^{2+})		$\alpha \text{V}\beta 3$ (Mn^{2+} - Mn^{2+} - Mn^{2+})	
Integrin mutation		Wild Type	F191W	D158N	Wild Type	F191W	D158N	Wild Type	W179F	Wild Type	W179F
RMSD ($\text{\AA}$)	Mean	2.0	8.3	14.5	2.7	11.8	4.7	12.3	6.1	11.0	4.7
	Standard Deviation	0.5	1.2	1.2	1.4	1.1	1.1	0.7	0.7	0.8	1.0
	P-Value *		<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Energy of Interaction between LIMBS and metal ion (kCal/mol)	Mean	859	865	620	930	913	752	775	732	779	780
	Standard Deviation	20	14	14	17	23	15	15	13	15	16
	P-Value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

* All P-values were compared to wild-type $\alpha \text{Ib}\beta 3$ (Ca^{2+} - Mg^{2+} - Ca^{2+}) structure.

Table 4.1 Summary of mean RMSD and interaction energy values for tested $\beta 3$ integrins

In contrast, the LIMBS pocket is distorted towards a planar shape in $\alpha \text{V}\beta 3$ (Fig. 4.3C), the result of the stronger interaction that pulled the LIMBS loop towards αV , changing metal ion coordination at this site. MD simulations showed that 6 “primary” oxygens coordinate Ca^{2+} (the two carboxyl oxygens of D158, D217, the carbonyl oxygen of P219 and the carboxyl oxygen of N215), but only one “secondary” coordinating oxygen, the carbonyl oxygen from D217 (Fig. 4.3C). The carboxyl oxygens of D158 and D217 as well as the carbonyl oxygen of P219 all form one planar surface that surrounds the cation, with only the side chain oxygen of N215 interacting with the cation at the bottom of the plane (in Fig. 4.3C). These changes made Ca^{2+} at LIMBS significantly less stable in $\alpha \text{V}\beta 3$ when compared to $\alpha \text{Ib}\beta 3$, as reflected by the significantly higher RMSD and lower energy of interaction of the metal with LIMBS (Table 1). Replacing Ca^{2+} with Mn^{2+} in LIMBS, MIDAS and ADMIDAS of $\alpha \text{V}\beta 3$ had minimal effects on RMSD or on the energy of interaction of Mn^{2+} with LIMBS (Table 1).

Structural Basis for the Stronger Interaction of αV with the LIMBS Loop: In $\alpha V\beta 3$, all three LIMBS loop residues were involved in more extensive interactions with αV : R216 side chain was engaged in strong ionic bonds with E121 and E123, its carbonyl oxygen occasionally H-bonded the side chain of Y178 (H-bond probability 0.5%). R216 also formed Van der Waals contacts with F154, and with the indole group of W179 (Fig. 4.4A, B). In addition, the main and side chains of D217 formed Van der Waals contacts with the indole group of W179, and the side chain of A218 contacted the carboxyl oxygen of D219 in αV . These interactions stretched the LIMBS loop towards αV , distorting LIMBS.

In contrast, interaction of αIIb with LIMBS loop residues R216-D217-A218 was primarily limited to R216: The side chain of R216 formed intermittent H-bonds with the hydroxyl group of Y190 (H-bond probability 5.0%), and its carbonyl oxygen contacted the side chain of Y190 (Fig. 4.4B). R216 side chain also formed occasional ionic interactions with E123, but made no contacts with F191 (Fig. 4.4C, D). Additionally, D232 made intermittent Van der Waals contacts with the side chain of A218.

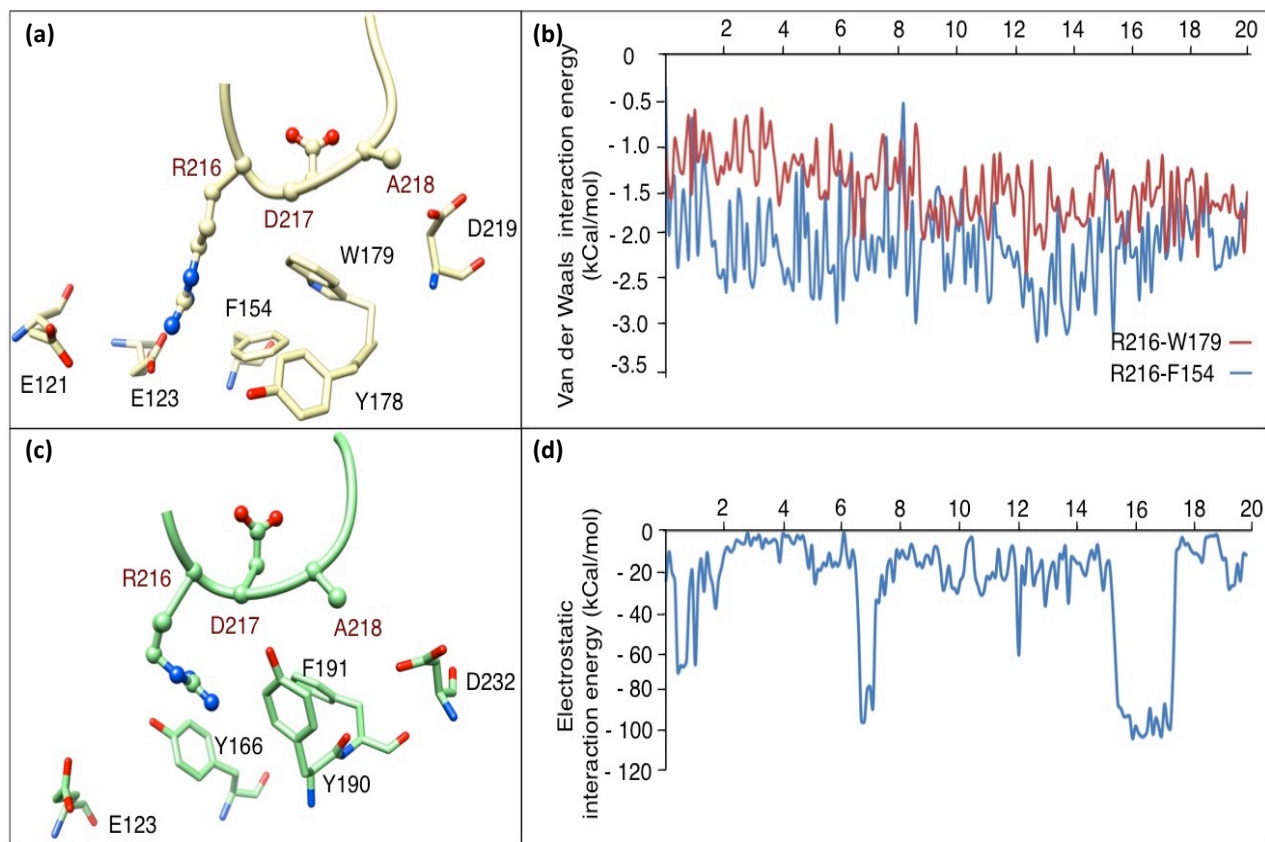


Figure 4.4: α -subunit residues interacting with the LIMBS loop. (a) Snapshot of MD simulations at 20ns showing interactions of the LIMBS loop R216-A218 residues (shown as ball and stick) with α V subunit residues (shown as stick). The structures in (a) and (c) are shown in the same orientation after superposing LIMBS of each, and LIMBS residues are labeled red (also in Figures 4.5B and 4.6B). In α V, the LIMBS loop interacts with α V's F154, Y178, W179, and D219, E121 and E123. (b) MD simulations showing Van der Waals energy of interaction between R216 of the LIMBS loop with W179 and F154 of the α -subunit. (c) Snapshot of MD simulations at 20ns showing interactions of the LIMBS loop R216-A218 residues with α IIb subunit residues (shown as stick). In α IIb interactions are limited to the corresponding residues Y166, Y190, F191, D232 and a transient interaction with E123. (d) Electrostatic energy of interaction between R216 of the LIMBS loop and E123 of the α IIb subunit. Occasional jumps to higher energy levels represent ionic bonds between R216 and E123. The energy peaks at t=7ns and 16ns show sharp increases to the same value of about 100kCal/mol, suggesting that the ionic bond occurs at a local energy minimum that the system continues to take, while the R216-E123 bond spends most of the simulation time in a lower electrostatic energy state (i.e. longer bond distance).

Effects of Modifying α IIb and α V on Stability of the Metal Ion at LIMBS: Of the α -subunit residues contacting the LIMBS loop residues R216-D217-A218, the side chains of F191 in α IIb and W179 in α V, are superimposable (Fig. 4.2). We evaluated the impact of interchanging these two residues on stability of the metal ion at LIMBS in the two β 3 integrins. As an internal control, we measured the effects of destabilizing the metal ion at LIMBS through the D159/N substitution (which removes one of the main coordinating oxygens from the metal ion). MD simulations showed that implementing the D158N mutation, yielded a significant increase in RMSD ($\sim$ 2-fold) and a reduction in the energy of interaction (Table 1), both reflecting destabilization of the metal ion at LIMBS.

Implementing the F191W substitution in α IIb significantly increased the energy of interaction of the LIMBS loop with the bulkier W191 in α IIb^{F/W}, when compared to F191 in α IIb (Fig. 4.5A). Snapshots of the structure at t=20ns showed that the side chain of R216 of β 3 releases its interaction with Y190 and forms Van der Waals contacts with the indole group of W191 in α IIb^{F/W} (compare Fig. 4.5B with Fig. 4.4B), pulling the LIMBS loop region towards the α IIb^{F/W} propeller domain. With W191 and Y190 pulling the LIMBS loop in the same direction, A218 is brought closer to D219 to form more contacts, increasing the energy of interaction of α IIb^{F/W} with the LIMBS loop, and deforming the octahedral shape of the pocket. These movements displaced the oxygens forming the LIMBS pocket from their native pattern towards a more planar configuration. While the energy of interaction of Ca²⁺ or Mn²⁺ with the LIMBS pocket in the α IIb^{F/W} β 3 structure did not change, RMSD increased by $\sim$ 4-fold (Table 1). Hence, it appears that the energetic component of the free energy remains unchanged upon applying F191W, whereas, the entropic component is highly changed upon deformation of the pocket as represented by a 4-fold increase in RMSD.

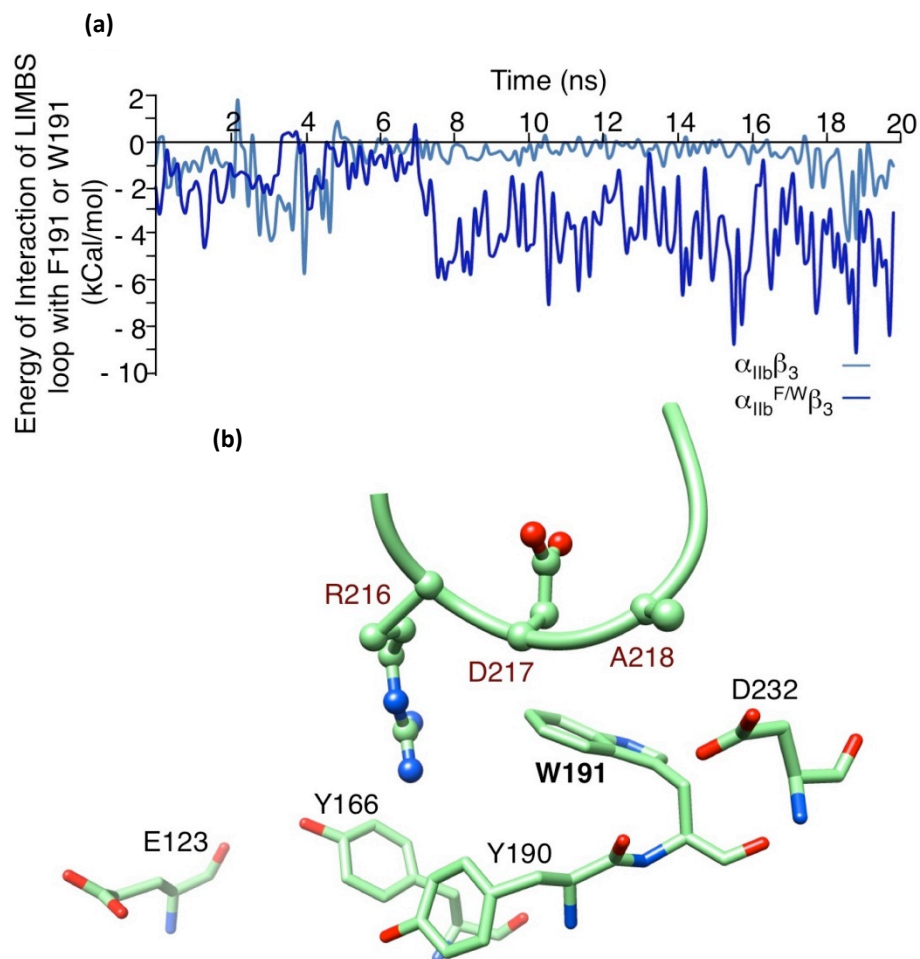


Figure 4.5: Effect of F191W change in $\alpha_{IIb}\beta_3$ on interaction energies and shape of LIMBS. (a) Computed energy of interaction between LIMBS loop residues R216-A218 and W191 in $\alpha_{IIb}^{F/W}$. (b) Snapshot at t=20ns of the interactions of LIMBS loop with $\alpha_{IIb}^{F/W}$ residues Y166, Y190, W191, D232 and E123. Mutating F191 to Trp in $\alpha_{IIb}^{F/W}$ enhanced interactions of the larger W191 side chain with the LIMBS loop, especially with R216, modifying the conformation of the LIMBS pocket.

Implementing the W179F substitution in $\alpha_V^{W/F}$ did not change the energy of interaction of the LIMBS loop with $\alpha_V^{W/F}$ (Fig. 4.6A). It reduced however fluctuations of the Ca^{2+} or Mn^{2+} at LIMBS (RMSD reduced by ~ 2 -fold, Table 1), the result of removing the contacts between W179 and the LIMBS loop (Fig. 4.6B). The W179F substitution did not significantly change the interaction energy of the metal ion with LIMBS (Table 1), since it did not affect the other interactions of α_V with the LIMBS loop, particularly with R216 (Fig. 4.6B).

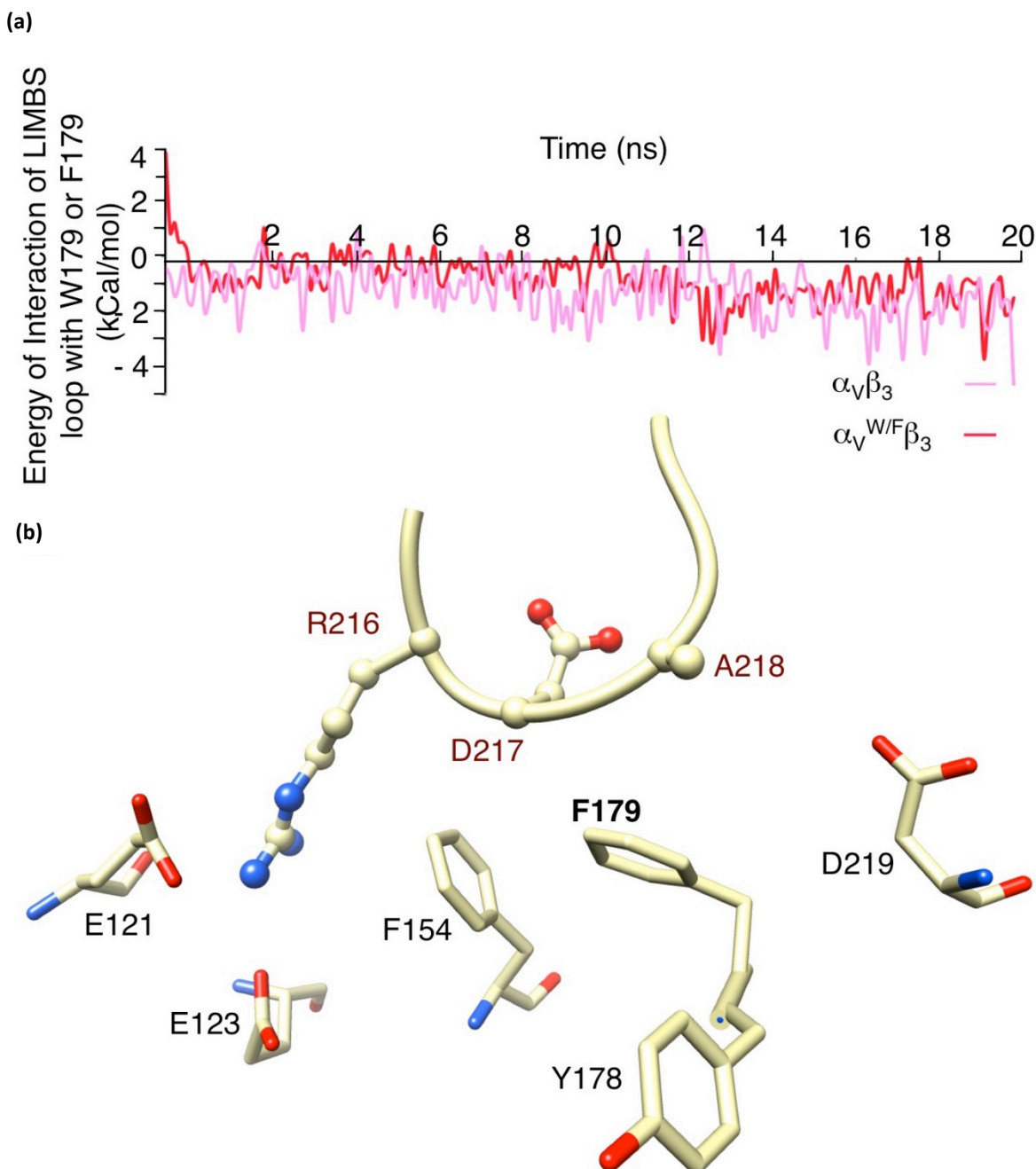


Figure 4.6: Effect of W179F in $\alpha_V\beta_3$ on interaction energies and shape of LIMBS. (a) Computed energy of interaction between the LIMBS loop residues R216-A218 and F179 in $\alpha_V^{W/F}$. Mutating W179 to Phe does not change the energy of interaction with the LIMBS loop significantly. (b) Snapshot at $t=20$ ns of interactions of the LIMBS loop with α_V subunit residues E121, E123, F154, F179, and D219. The W179F mutation weakens interaction of LIMBS loop with $\alpha_V^{W/F}$, reshaping the LIMBS pocket. The structure is shown in the same orientation as in Figure 4.5B.

4.4. Discussion

In this chapter, we provided computational evidence that the α -subunit plays an essential role in determining metal ion coordination at LIMBS in β_3 integrins. By combining these two

approaches, we demonstrated the following: 1) Interaction of the LIMBS loop with αV is more extensive than with αIIb , 2) Metal ion coordination at LIMBS after 20ns of equilibration becomes planar in $\alpha V\beta 3$. 3) Changing the αIIb -LIMBS loop interface residue F191 to Trp destabilized the metal ion at LIMBS, while a W179 to Phe mutation in αV produced the opposite but weaker effects.

The higher energy of interaction of αV with the LIMBS loop residues R216-A218 was directly related to the more extensive contacts αV made with this loop, when compared with αIIb . The stronger contacts increased fluctuations of the metal ion at LIMBS in $\alpha V\beta 3$, as reflected by the ~4-fold increase in RMSD, and also increased the mean distances between the coordinating oxygens and the metal ion at LIMBS, as reflected by the reduction in total energy of interaction of the metal ion with the pocket. These observations may offer an explanation for the variable occupancy of LIMBS by metal ion in crystal structures of unliganded $\alpha V\beta 3$ ectodomains, where LIMBS was metal occupied in one (4g1e.pdb, used in this study) [50] but not in four other unliganded $\alpha V\beta 3$ ectodomain structures [16,33,53,225]. The lack of metal occupancy at LIMBS was attributed to unfavorable crystallization conditions [50]. However, the LIMBS is metal-ion occupied in $\alpha V\beta 3$ under the same crystallization conditions when $\alpha V\beta 3$ is ligand-bound [33,226]. The data produced in our study suggest that variability in LIMBS occupancy by metal is the result of the different contacts the LIMBS loop makes with αV in contrast to αIIb . In presence of ligand, E220 of the βA domain provides an additional primary oxygen, stabilizing the metal ion at LIMBS. In the absence of ligand, this stabilizing influence is lost, and the metal ion is freer to escape LIMBS. This scenario is reflected in the higher RMSD and lower energy of interaction of the metal ion with LIMBS in integrin $\alpha V\beta 3$ when compared to $\alpha IIb\beta 3$ (Table 1).

Integrin $\alpha V\beta 3$ is widely expressed in tissues including bone, where it mediates dynamic cell adhesion [227,228]. The majority of Mn^{2+} in the body is sequestered in bone [229] to levels that approach the K_d of $\alpha V\beta 3$ for Mn^{2+} [216], suggesting that Mn^{2+} plays an important role in regulating $\alpha V\beta 3$ function in this tissue. In contrast, $\alpha IIb\beta 3$ is solely expressed on circulating platelets in blood containing high levels of its physiologic ligands, mainly fibrinogen, and mM concentrations of the divalent cations Ca^{2+} and Mg^{2+} . Maintaining $\alpha IIb\beta 3$ in a dormant, inactive state in this environment is therefore essential to prevent pathologic thrombosis. This phase of our study provides insights into how regulation can be tailored to the particular environment where an integrin is expressed. Occupancy of LIMBS, MIDAS and ADMIDAS by metal ions in unliganded $\alpha IIb\beta 3$, may explain the rapid ligand association rates to activated $\alpha IIb\beta 3$ [216]. This potential proactivation tendency at the ligand-binding site must be counteracted by energy barrier(s) to activation elsewhere to effectively keep $\alpha IIb\beta 3$ in an inactive state on circulating resting platelets. One such barrier may exist in the integrin leg segments, between the α -subunit Calf-2 domain and the $\beta 3$ -subunit EGF-like 4 and βTD domains [230]. Disruption of this interface rendered $\alpha IIb\beta 3$ as susceptible to Mn^{2+} -induced ligand binding as $\alpha V\beta 3$ [230]. In $\alpha V\beta 3$, where this Calf-2/IE4- βTD barrier is weak or absent [230], a relatively stronger αV -LIMBS interface may help favor the inactive $\alpha V\beta 3$ conformation, thus limiting stable occupancy of the metal ion to conditions when ligand is also accessible.

Chapter 5

Concluding Remarks and Future Works

5.1. Conclusion

Integrin forms the mechanical linkage between the cytoskeleton and ECM, serving as the backbone for clustering focal adhesion proteins. Although it is equivocally reported that integrin signaling is dependent upon allosteric conformational changes of its structure that initiate at certain points and propagate over the entire structure, the sequence of events leading to integrin activation and extensive conformational changes remains elusive in many aspects. Integrins have been studied intensively in the past three decades. With the advent of computational resources and more elaborate techniques becoming available to researchers, computational models are employed increasingly to reconcile apparently contradictory experimental pieces of evidence. This study scrutinized integrin function driven by conformational changes at integrin compartments from different angles, using all-atomic molecular dynamics simulations.

In Chapter 2, the two major models for integrin clustering were tested and contrasted. The first model suggested that integrin α - and β -subunit transmembrane domains form homo-oligomers, as a result of separation upon integrin activation. In contrast, the second model rejects formation of integrin homo-oligomers in the focal adhesion and attributes integrin clustering to integrin binding with multivalent ligands. It was shown that α - and β -transmembrane domains of integrin α IIb β 3 require excessive amounts of external work to be able to form homodimers within the membrane. Nonetheless, β -subunit homo-oligomerization may still be feasible due to its reported mechanical linkage with the actin cytoskeleton. It should not be overlooked that the effect of the viscous environment of the plasma membrane on integrin activation/clustering mechanisms is remarkable, thus studying the integrin behavior outside the membrane or in a cell lysate might yield inaccurate results. The absence of the cell membrane in the cell lysate could lead to unwanted conformational changes that could alter the integrin function as it is shown that the IMC structure in phospholipid bicelles differs from that of the same region of the isolated cytoplasmic domains in aqueous solution [89]. We suggest future experimental studies of the field focus on clustering of integrin β -subunits over the course of the focal adhesion formation and turn-over either *in vivo* or within artificial lipid bilayers.

Chapter 3 focused on inside-out versus outside-in activation mechanisms for integrin, using the most widely-established cytoplasmic and extracellular players for integrin α IIb β 3 (i.e. talin head domain and RGD sequence respectively). The activated state of integrin is commonly characterized by three major dissociations: breaking of the inner and outer membrane clasps (IMC and OMC), and unbinding of the β A and β TD [2,5,12,98]. The integrin α IIb β 3 function was explained via exploring how these key interaction groups are disrupted in specific pathways. Our observations of the transmembrane-cytoplasmic domains of integrin α IIb β 3 showed that

the IMC is loosened with the presence of talin, most likely through membrane-proximal interactions which result from the energetic compensation of the weakened IMC by K320 on the S1-S2 loop of talin. We also found, using the full-length crystal structure we built, that talin has a larger role in integrin activation by disrupting an additional stable clasp (R734-E1006) through the initial membrane-distal interactions that anchors talin to integrin [73]. Furthermore, the full-length crystal structure of integrin α IIb β 3 was used to explore binding sites of soluble RGD ligands and their ability to activate integrin α IIb β 3 or maintain it in the activated state. Three major groups of bindings with two specific binding sites corresponding to each group were found. According to our simulations, the major group of interactions that maintains integrin α IIb β 3 in the inactive, bent state occurs between K350 on the β A domain, and S673 and S674 on the β TD. Interestingly, we observed the full dissociation of the β TD and β A domains when this interaction group was disrupted and eventually broke as a result of a competition between the Arg of the RGD peptide with S674. Therefore, we proposed this molecular scenario as a potential trigger mechanism for outside-in activation of integrin α IIb β 3 by soluble RGD ligand.

Different members of the integrins family show distinct responses to agonists. While integrin α V β 3 shifts to the high-affinity states when exposed to Mn^{2+} , integrin α IIb β 3 is not as sensitive to this metal ion. Interactions between leg domains of integrin α IIb β 3 are stronger than those of α V β 3, which may explain why the default state of α IIb β 3 integrin on platelets is completely inactive, whereas α V β 3 integrin is constitutively active on many cell types [39,230]. Integrin α X β 2 is harder to activate than other β 2 integrins [18]. Although integrin is generally known to unbend upon activation but degree and mechanism of activation seem to depend on the integrin member as well as ligand [39]. Chapter 4 of this work was dedicated to comparing metallic cation coordination patterns at LIMBS between integrin α IIb β 3 and α V β 3. Our results clearly indicated that the LIMBS conformation as well as cation binding affinity with the pocket is significantly different between the two integrin family members though they share the same β -subunit. The integrin α -subunit headpiece interacts more strongly with the α -subunit-facing loop of the LIMBS pocket in integrin α V β 3, deviating the LIMBS conformation from an octahedral conformation towards a planar one. The more or less planar arrangement of oxygens around the cation now allows for higher fluctuations of the cation, thereby lower affinity with the pocket.

Indeed, many properties of integrins are inferred from analyzing a few members of the integrin family (e.g. α IIb β 3, α v β 3, α L β 2) while accuracy of generalizing these traits to all integrin members is questionable [1]. Fundamentally different and sometimes contradictory results that are presented using various studies regarding integrin activation and clustering indicates that different members of the integrin family may follow distinct activation pathways, therefore, experimental and computational results obtained from one integrin type could not be generalized to the entire family unless compelling evidence exists.

5.2. Roadmap for Future Work

Integrin is a crucial protein that forms the focal adhesion interface against mechanical signals. Although it has been researched abundantly over the past three decades details of its activation and clustering still remains controversial. This study attempted to examine a few well-known hypothetical models for activation and clustering of integrin $\alpha\text{IIb}\beta 3$. This work could be continued and expanded from different angles. As explained earlier in this chapter, activation/clustering mechanisms could be considerably different across members of the integrin family. Thus, we are far from generalizing the proposed models to all integrin family members. By publication of new crystal structure for other integrin members further studies could be aimed at comparing these mechanisms between different integrin members computationally.

Additionally, this study suggested a model for integrin clustering as the focal adhesion matures and sustains larger forces. However, current computational resources would not allow modeling the entire focal adhesion with atomic details. Hence, this model could be tested either via fluorescence microscopic studies *in vivo* or using a coarse-grained model of the focal adhesion *in silico*.

Two examples of integrin activation upon interaction with talin from the cytoplasmic side and soluble RGD ligand from the extracellular side were scrutinized in this work. For the interest of computational cost, this study was solely focused on the final stages of activation in either case, and involved only the integrin and main activator protein. An interesting avenue for the future studies would be engaging more proteins in the simulation. For example, adhesion proteins such as kindlin, focal adhesion kinase, filamin, and α -actinin are also known to interact with integrins, shifting integrin either toward activation or a more stable inactive state. Investigating triple complexes of integrin and talin with any of these adhesion proteins would be highly impactful.

Finally, mapping out details of integrin interactions with different extracellular ligands and how the interaction affinity is regulated with different divalent metal ion occupancies in the integrin head domain ion pockets would be critical. This study indicated the important effect of the integrin α -subunit head domain on the ion pocket LIMBS at the β -subunit head domain, which could be expanded via addition of different extracellular ligands and measuring their interaction affinity with the integrin head domain at various physiologically-relevant metal ion occupancies.

Appendices

Supplementary Figures

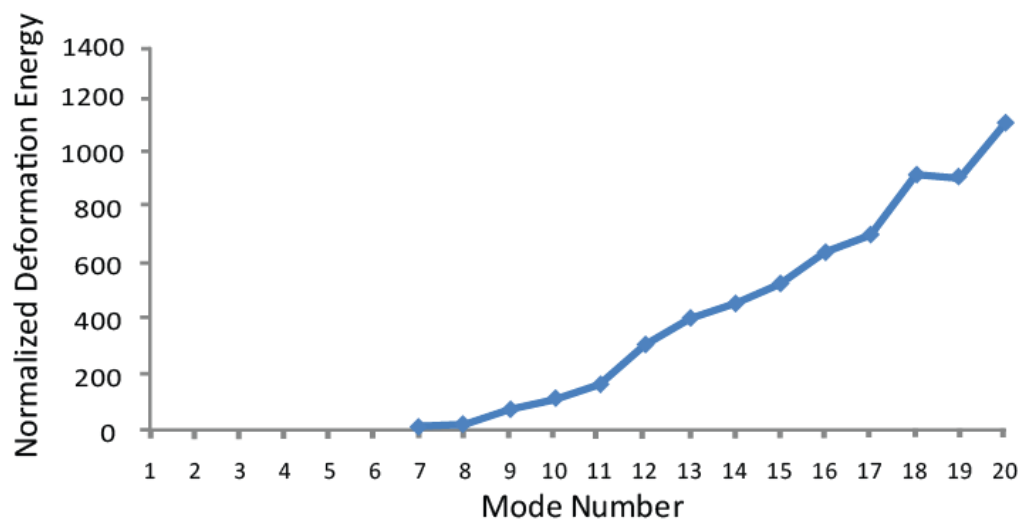
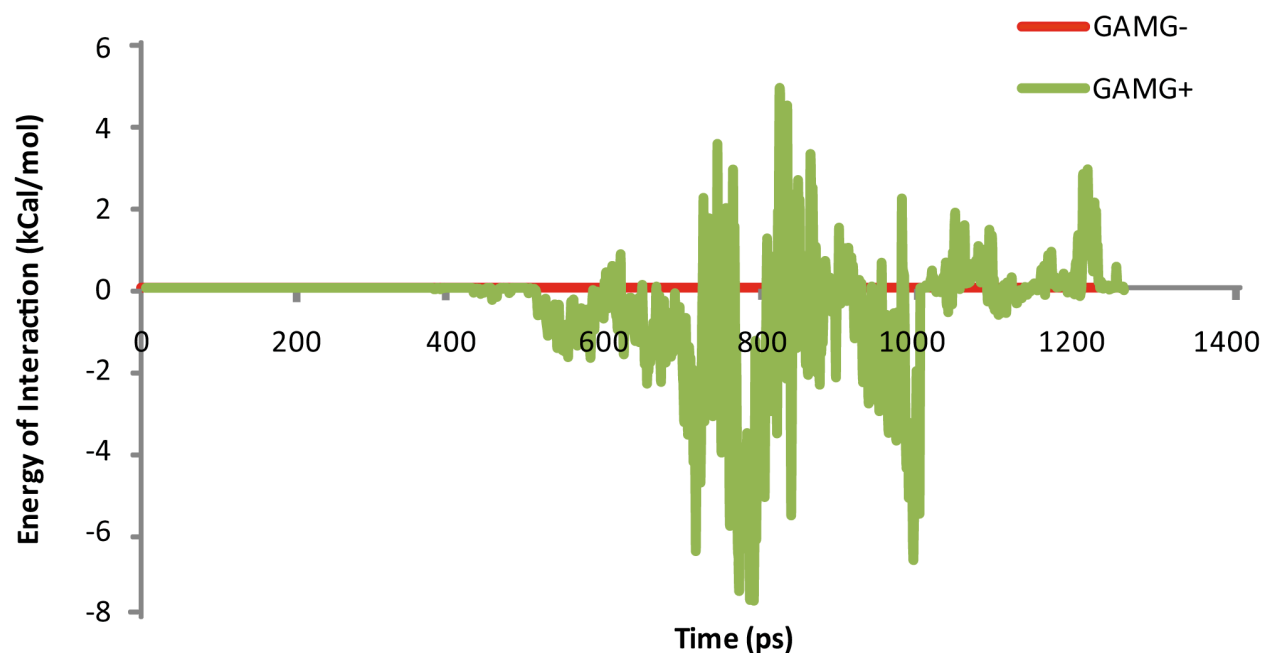
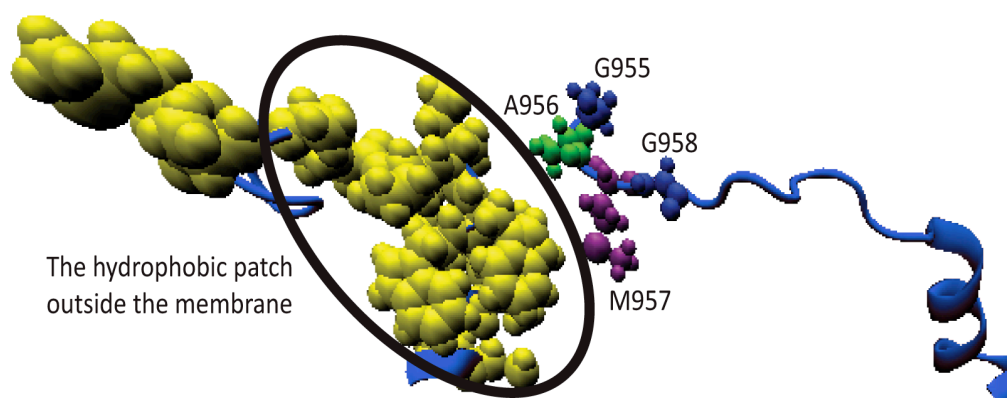


Figure S1: Modal deformation energy increases rapidly with the mode number, making higher modes have much smaller contribution to the protein deformation.



(a)



(b)

Figure S2: Some minor interactions occur when one integrin α -subunit is dragged towards the other. (a) Interaction energy between the two monomers does not show any significant changes as the two monomers approach one another. Positive energy magnitudes are indicative of interactions between hydrophobic groups of the monomers that fall outside the plasma membrane. The steering force and interaction energies all correspond to the slow steering rate. In presence of the GAMG sequence there are a number of energy spikes whereas almost no interaction is observed in the absences of the GAMG sequence. (b) Upper parts of each α -monomer are chopped off in order to illustrate the interaction site more clearly. The two α -monomers are depicted when they have already reached their final distance. Hydrophobic interactions of the GAMG sequence (Gly: blue, Met: purple, and Ala: green) with a hydrophobic region (yellow) of the free monomer is illustrated.

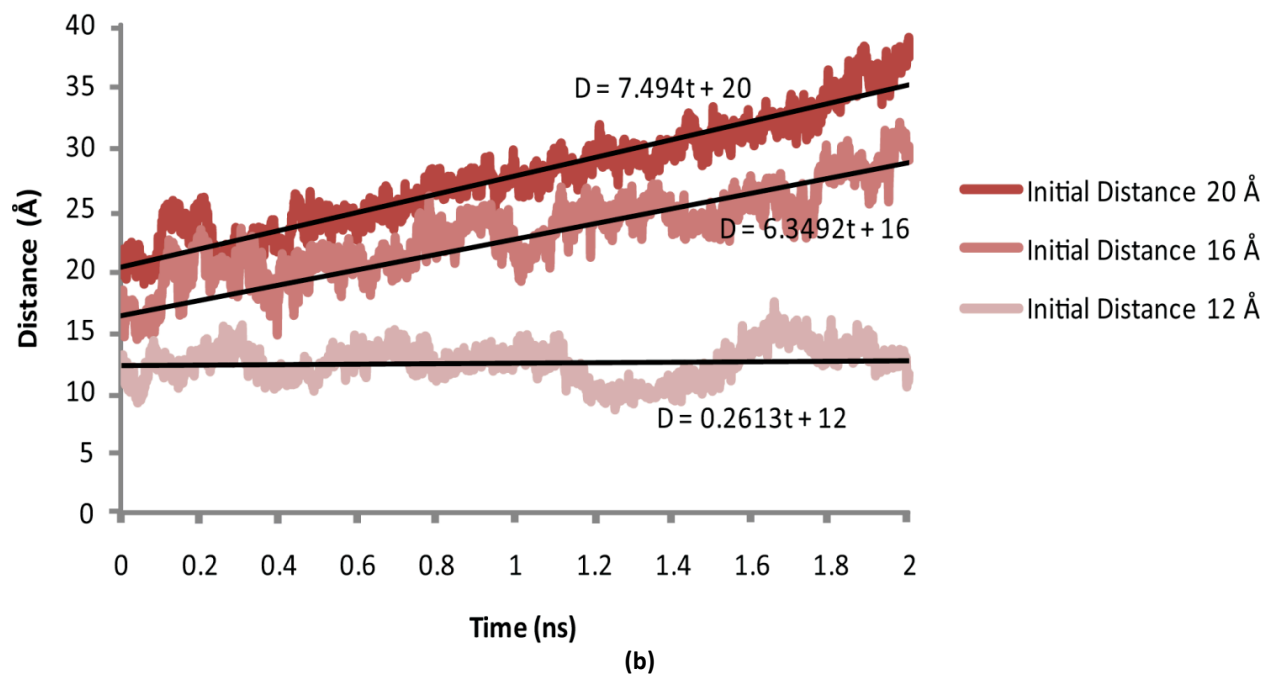
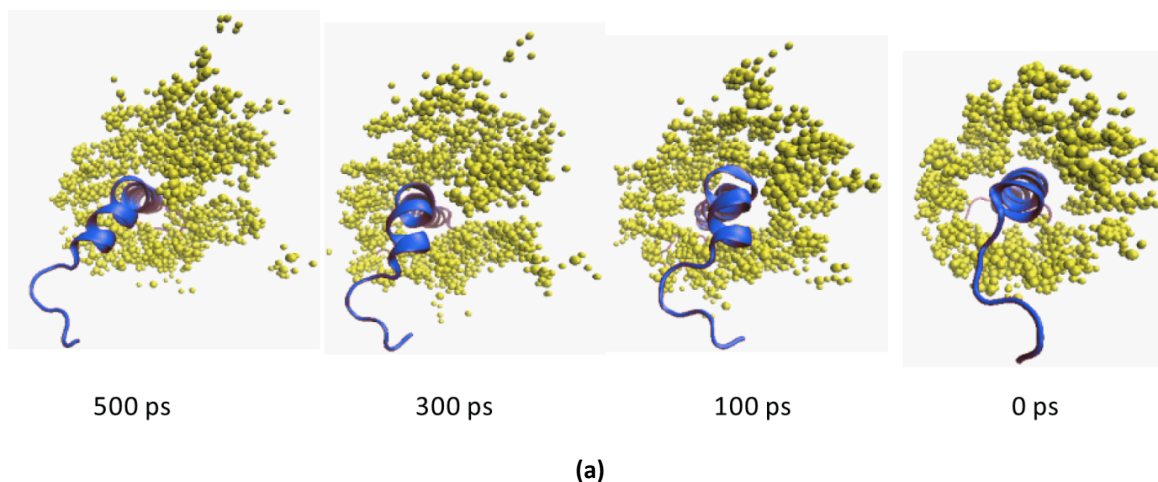
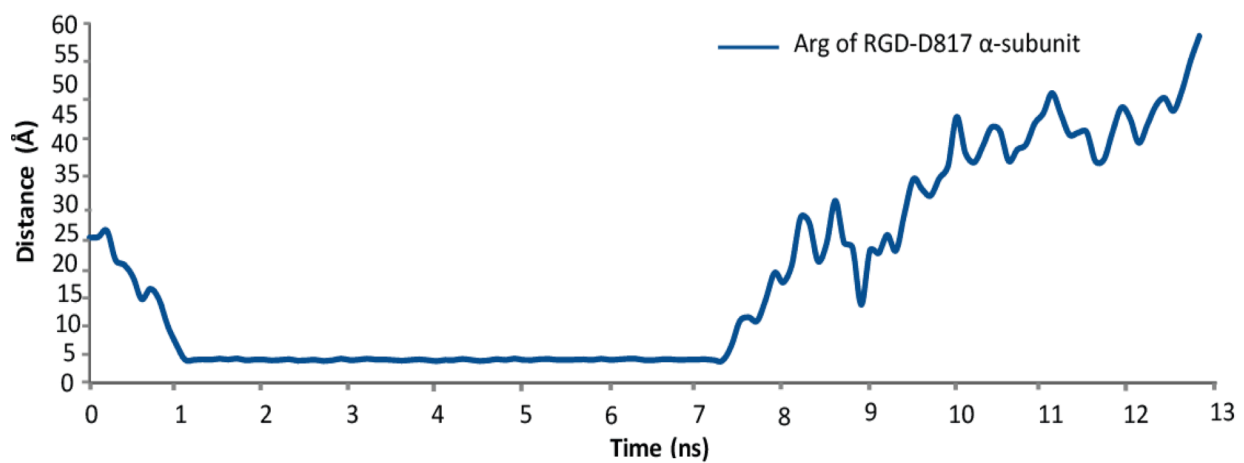
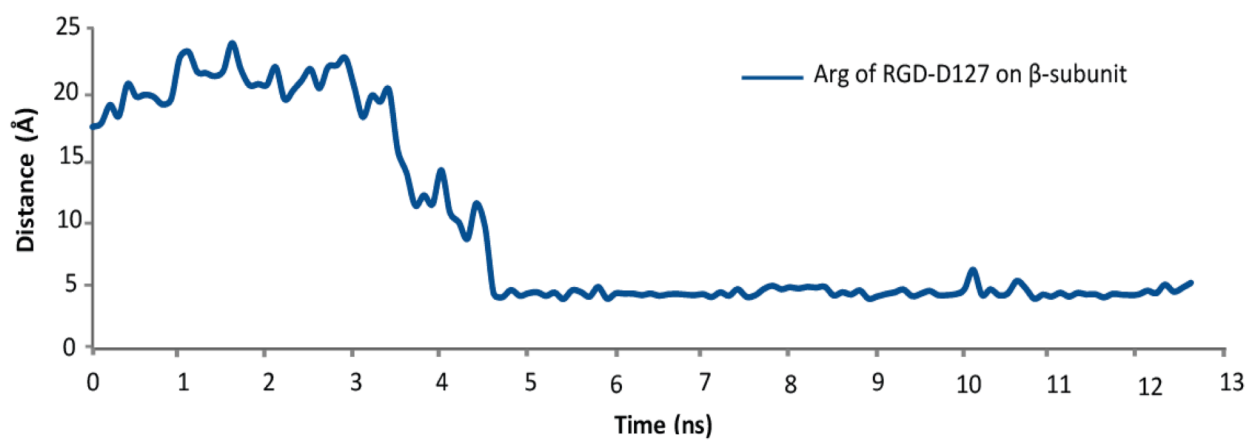


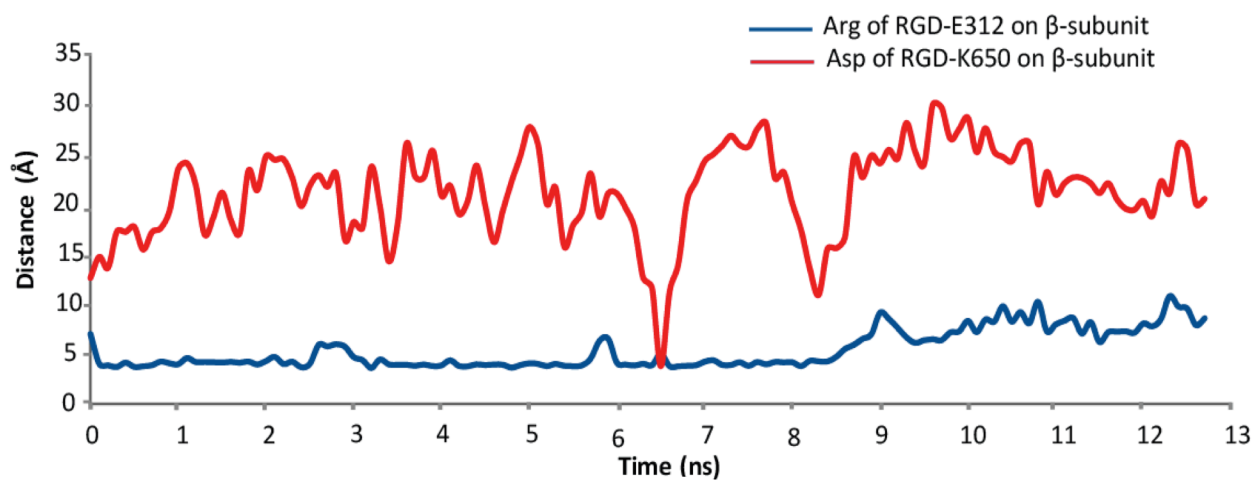
Figure S3: A lipid pack forms around the transmembrane domain which moves along with the monomer. (a) Lipid atoms initially located within 10Å of the α -monomer are visualized and monitored over the course of the simulation. Although some lipid chains are dispersed away as the monomer moves along, the major part of the lipid pack compartment remains attached to the monomer. (b) Shows the distance between the monomer's center of mass and three lipid chains located at different distances at the rear side of the moving monomer along the line that connects the two monomers. The graph clearly shows that lipid chains that are closer to the monomer move along with the monomer, whereas, the ones that are initially more distant fall behind the moving monomer more rapidly.



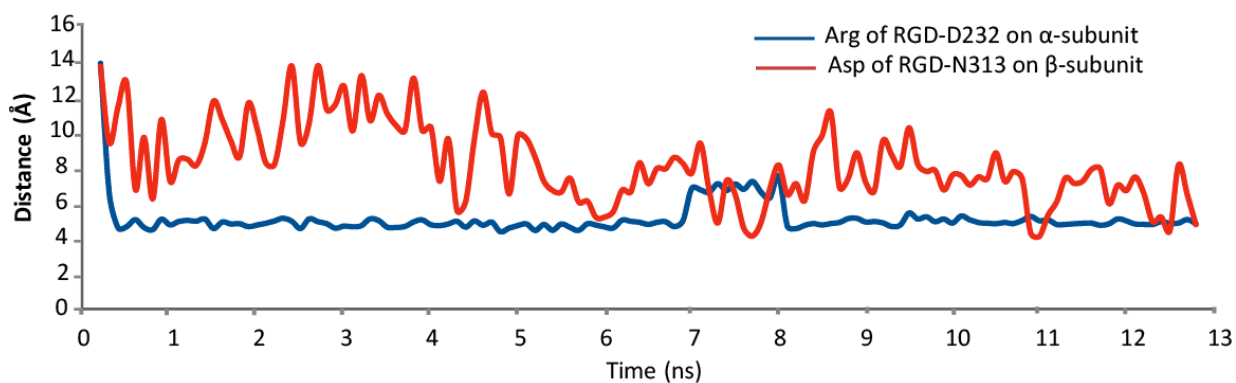
(a)



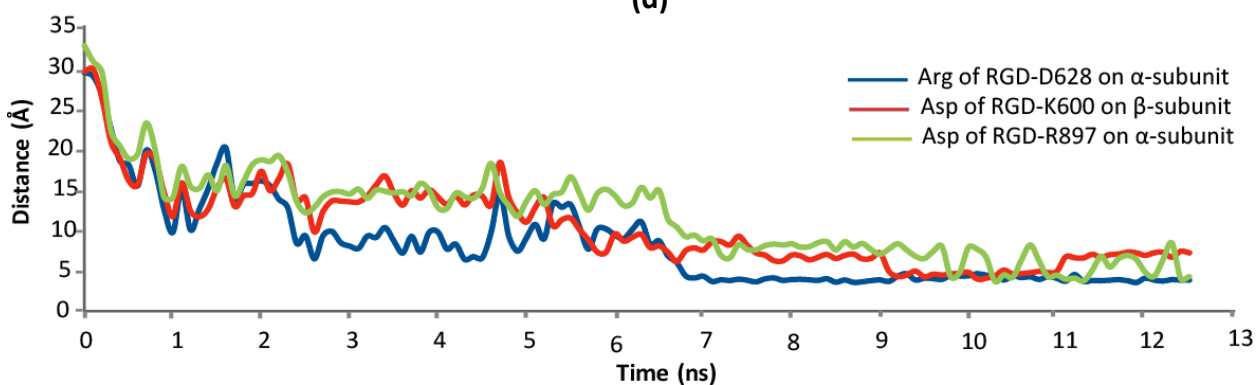
(b)



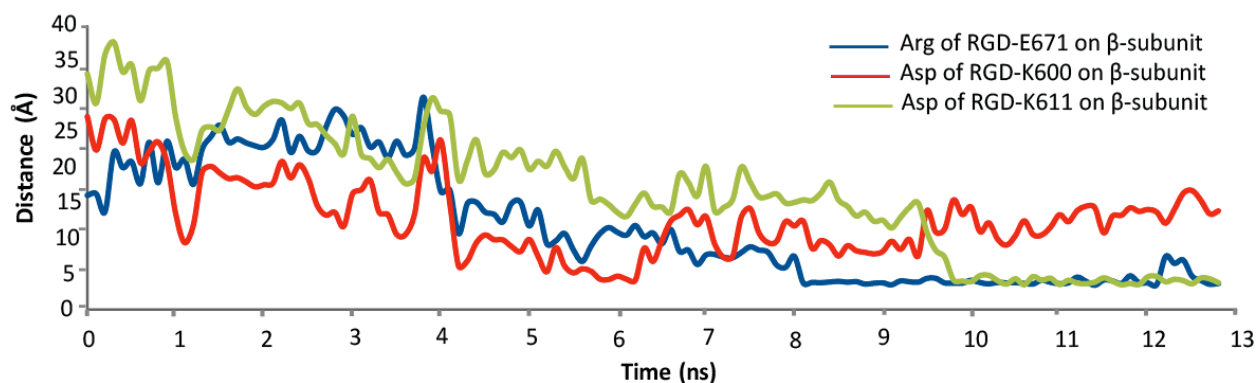
(c)



(d)

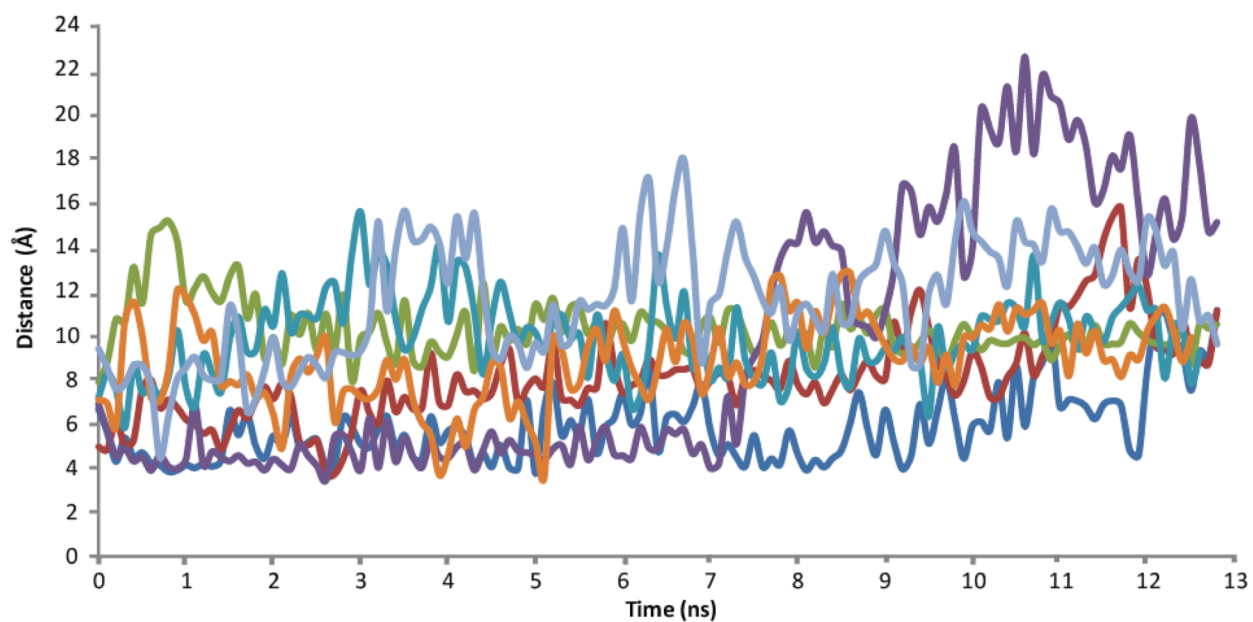


(e)

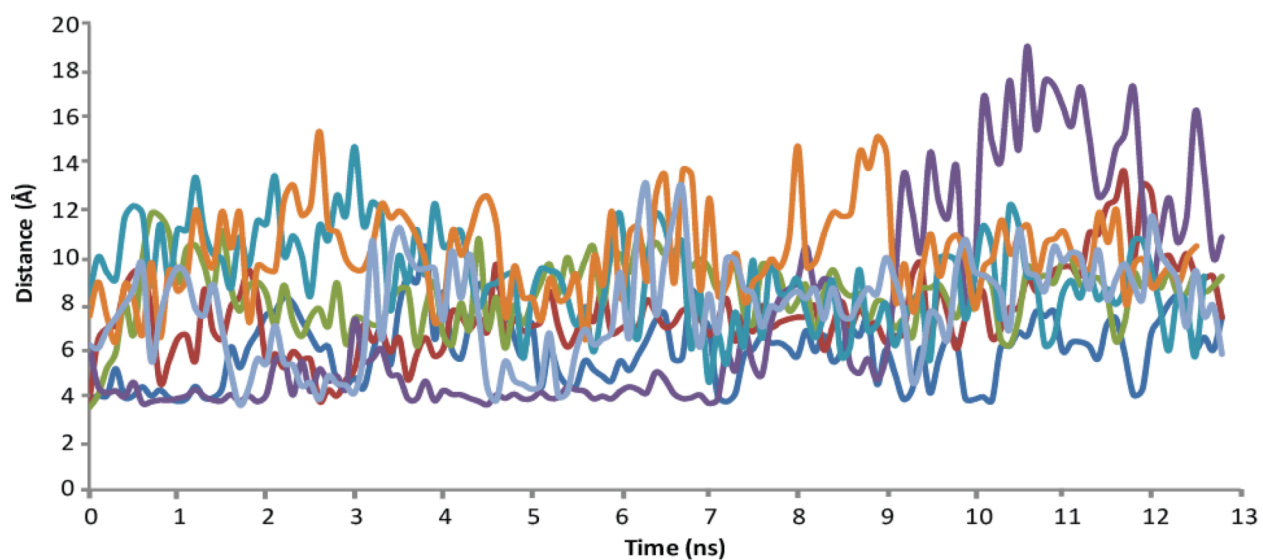


(f)

Figure S4: Distance between interacting residues of integrin with the RGD peptide for RGD-included runs. (a) and (b) represent the type1 interaction with only one permanent bond between the Arg of the RGD peptide and integrin. (c) and (d) are corresponding to the type2 interaction, wherein the Arg of the RGD interacts permanently with integrin while the Asp of the RGD peptide interacts only temporarily with another single residue of integrin. (e) and (f) show the cases where the Arg of the RGD permanently bound to a residue of integrin and the side chain of the Asp of the RGD switched back and forth between two other residues of integrin, which is called type3 binding.



(a)



(b)

Figure S5: Disruption of the key interaction group K350-S673/674 detaches the β TD from the β A domain. (a) Distance between S673 and K350 as a function of the simulation time for six RGD-included as well as one non-RGD run (light blue). The simulation that led to β A- β TD dissociation is shown in purple. The interaction is disrupted at ~ 7.5 ns (b) Distance between S674 and K350 as a function of the simulation time for six RGD-included as well as one non-RGD run (light blue). The simulation that led to β A- β TD detachment is shown in purple.

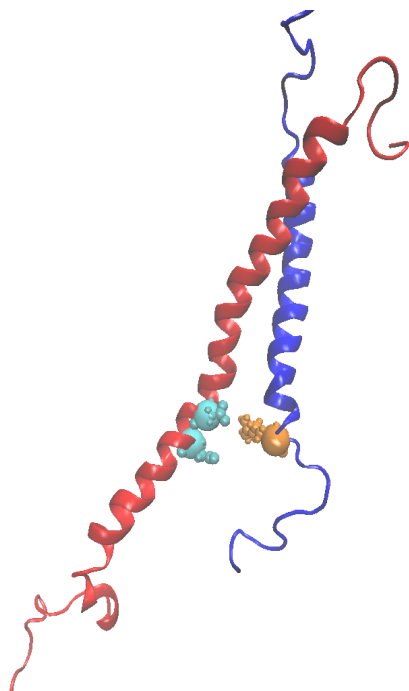


Figure S6: The electrostatic bonds formed by R995 (orange) on the α -subunit with D723 and E726 (cyan) on the β -subunit were subjected to tensile forces. Carbon- α of D723 and E726 (orange VDW representation) were fixed while Carbon- α of R995 (cyan VDW representation) was steered.

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