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Signaling of integrin lower leg and transmembrane domains

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SIGNALING OF INTEGRIN LOWER LEG AND TRANSMEMBRANE DOMAINS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Biological Sciences

by

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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ............................................................................................................. ii

LIST OF FIGURES .................................................................................................................. iv

ABSTRACT ............................................................................................................................... vi

CHAPTER ONE: GENERAL INTRODUCTION ........................................................................ 1

CHAPTER TWO: DISSOCIATION OF THE α-SUBUNIT CALF-2 DOMAIN AND THE β-SUBUNIT 1-EGF4 DOMAIN IN INTEGRIN ACTIVATION AND SIGNALING ........................................ 28

CHAPTER THREE: TEST OF THE INTEGRIN TRANSMEMBRANE DOMAIN HOMOOLIGOMERIZATION DURING INTEGRIN LIGAND BINDING AND SIGNALING ................................................................. 56

CHAPTER FOUR: SUMMARY AND GENERAL DISCUSSION ................................................ 83

VITA ......................................................................................................................................... 87
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The mammalian integrin receptor family</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Integrin architecture</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>Crystal structure of integrin αIIbβ3</td>
<td>6</td>
</tr>
<tr>
<td>1.4</td>
<td>Structure of the integrin αIIbβ3 transmembrane (TM) and cytoplasmic domains</td>
<td>9</td>
</tr>
<tr>
<td>1.5</td>
<td>The switchblade model</td>
<td>10</td>
</tr>
<tr>
<td>1.6</td>
<td>Conformational change and allosteric transmission by βI domains</td>
<td>12</td>
</tr>
<tr>
<td>1.7</td>
<td>Predicted model of integrin TM activation</td>
<td>16</td>
</tr>
<tr>
<td>1.8</td>
<td>Working models of the integrin outside-in signaling</td>
<td>18</td>
</tr>
<tr>
<td>2.1</td>
<td>Mutations in the αIIbβ3 structure.</td>
<td>32</td>
</tr>
<tr>
<td>2.2</td>
<td>Expression and immunoprecipitation of wild-type and mutant αIIbβ3 integrins</td>
<td>38</td>
</tr>
<tr>
<td>2.3</td>
<td>Ligand-binding activity of wild-type and mutant αIIbβ3 integrins.</td>
<td>41</td>
</tr>
<tr>
<td>2.4</td>
<td>Exposure of the LIBS1 epitope.</td>
<td>44</td>
</tr>
<tr>
<td>2.5</td>
<td>Cell adhesion and spreading</td>
<td>47</td>
</tr>
<tr>
<td>3.1</td>
<td>Sequences of the αIIb and β3 TM regions</td>
<td>63</td>
</tr>
<tr>
<td>3.2</td>
<td>Integrin αIIb TM domains do not form homooligomers before and after soluble ligand binding</td>
<td>65</td>
</tr>
<tr>
<td>3.3</td>
<td>The homomeric disulfide bond of the αIIbW967C was formed during biosynthesis.</td>
<td>66</td>
</tr>
<tr>
<td>3.4</td>
<td>Integrin β3 TM domains do not form homooligomers before and after soluble ligand binding</td>
<td>67</td>
</tr>
<tr>
<td>3.5</td>
<td>Exposure of the LIBS1 epitope.</td>
<td>69</td>
</tr>
<tr>
<td>3.6</td>
<td>Integrin TM domains do not form homooligomers during inside-out activation</td>
<td>70</td>
</tr>
<tr>
<td>3.7</td>
<td>Cell adhesion and spreading of randomly selected TM cysteine mutants</td>
<td>72</td>
</tr>
<tr>
<td>3.8</td>
<td>Integrin clustering of selected TM cysteine mutants</td>
<td>74</td>
</tr>
</tbody>
</table>
Figure 3.9 Integrin TM domains do not form homooligomers after cell adhering to the immobilized fibrinogen
ABSTRACT

Integrin conformational changes mediate integrin activation and signaling triggered by intracellular molecules or extracellular ligands. Even though it has been shown that TM and/or cytoplasmic α and β domains associate in the resting state and separation of these domains is required for integrin signaling, it is still not clear how this signal is transmitted from the transmembrane domain through two long extracellular legs to the ligand-binding headpiece. In addition, integrin TM homomeric association was also observed. But the role of this interaction remains elusive. In this work, the platelet integrin, αIIbβ3, has been used to elucidate the roles of integrin lower leg and TM homomeric association in integrin signalling.

We first addressed whether the separation of integrin αβ lower leg is critical for integrin activation and outside-in signaling. Using a disulfide bond to restrict dissociation of the α-subunit Calf-2 domain and β-subunit I-EGF4 domain, we were able to abolish integrin inside-out activation and outside-in signaling. In contrast, disrupting the interface by introducing a glycosylation site into either subunit activated integrins for ligand binding through a global conformational change. Our results suggest that the interface of the α-subunit Calf-2 domain and β-subunit I-EGF4 domain is critical for integrin bidirectional signaling.

Formation of the TM homooligomers was observed in micelles and bacterial membranes previously, and it has been proposed that this homomeric association is important for integrin activation and clustering. We then addressed whether integrin TM domains form homooligomers in mammalian cell membranes using cysteine mutagenesis scanning method. Our results show that TM homomeric interaction does not occur before or after soluble ligand binding, or during inside-out activation. In addition, even though the cysteine mutants and the heterodimeric disulfide-bounded mutant could form clusters after adhering to immobilized ligand, the integrin TM domains do not form homooligomers, suggesting that integrin TM homomeric association is
not critical for integrin clustering or outside-in signaling. Therefore, the integrin TM homooligomerization is not required for integrin activation, ligand binding and signaling.
CHAPTER ONE:
GENERAL INTRODUCTION
**Introduction**

Integrins are a large family of cell adhesion receptors that mediate cell-cell, cell-extracellular matrix (ECM), and cell-pathogen interactions. These receptors are principal transmembrane proteins in that they connect the ECM with the actin cytoskeleton and a variety of signalling molecules. As they integrate the extracellular and intracellular environments by transmitting signals bidirectionally across the plasma membrane [1, 2], they were given the name “integrin”. Cooperated with other proteins, integrins play critical roles in many biological processes including hemostasis, inflammation, immune responses, development and cancer.

Integrins are usually in low affinity state under physiological conditions. When cells are stimulated by external agents, specific intracellular molecules impinge on integrin cytoplasmic domains, resulting in its conformational change and thus leading to increase of affinity for extracellular ligands. This process is called inside-out signaling, which is the unique feature of integrin receptors compared to other adhesion receptors. On the other hand, ligand binding transduces signals from the extracellular environment to the cytoplasm and activates many intracellular signaling pathways, a process known as outside-in signaling.

Integrins are expressed in all animals investigated and are of critical importance to them. In vertebrates, 18 α-subunits and 8 β-subunits have been identified, forming at least 24 distinct αβ pairs (Fig. 1.1). Each of the 24 integrins has a specific, nonredundant function [3]. In this study, we investigated the bidirectional signalling of the most intensively studied integrin αIIbβ3.
Figure 1.1 The mammalian integrin receptor family. 18 α and 8 β subunits form 24 heterodimers. α subunits with gray hatching or stippling have inserted α I domain. The figure is adapted from the review [3].

Biology of αIIbβ3

αIIbβ3, also known as glycoprotein IIb/IIIa, is expressed on the surface of platelets and their precursor megakaryocytes [4]. The formation of the αIIbβ3 complex from both αIIb and β3 subunits is necessary for the receptor to be expressed on the cell surface [5]. The assembly occurs in the endoplasmic reticulum (ER), and then the complex is transported to the Golgi for post-translational processing [6, 7]. Mature αIIb contains 1008 amino acids and β3 has 762 amino acids [8]. As the main platelet integrin, αIIbβ3 mediates platelet aggregation and thus is essential for thrombosis and hemostasis. In unstimulated platelets, αIIbβ3 is in the inactive, low affinity state. The activation of the receptor occurs through inside-out signalling, in which the platelets are stimulated by external agonists such as adenosine diphosphate (ADP), thrombin and thromboxane A2, resulting in conformational change and thus facilitating the major ligand fibrinogen binding. Platelets are therefore cross-linked through fibrinogen, causing platelet
aggregation. There are approximately 80,000 αIIbβ3 copies on the membrane of each platelet [9]. Dysfunction or shortage of αIIbβ3 can result in the bleeding disorder Glanzmann thrombasthenia [7]. The antagonists to αIIbβ3 such as abciximab, eptifibatide and tirofiban were prescribed for the prevention of thrombosis. But the therapy of Glanzmann thrombasthenia remains unsatisfactory due to the fact that binding to platelets by these drugs can cause conformational changes of αIIbβ3, resulting in paradoxical thrombosis [7]. Investigation of the structure and function of αIIbβ3 will lead to additional and improved ways to prevent or treat the pathological consequences of αIIbβ3 dysfunction.

**αIIbβ3 Structure**

**Domain Organization and Overall Structure**

Integrins are heterodimeric glycoproteins composed of two distinct noncovalently associated α and β subunits, each with a large extracellular domain, a single spanning transmembrane (TM) domain and a short cytoplasmic domain. Two groups of integrins have been identified: one containing an extra von Willebrand factor type A domain (α I domain) in their α subunits; whereas the other including αIIbβ3 lacking this domain (Fig. 1.1). As a typical α I–lacking integrin, αIIb subunit extracellular portion consists of four domains: an N-terminal seven-bladed β–propeller domain and three β–sandwich domains: the thigh, calf-1 and calf-2 domains. The β3 extracellular portion contains eight domains: an N-terminal β I domain, a hybrid domain, a cysteine-rich PSI (Plexin-Semaphorin-Integrin) domain, four epidermal growth factor-like (EGF-like) domains and a membrane proximal tail domain (βTD) (Fig. 1.2). The β–propeller domain from the α subunit and the β I domain from the β subunit assemble to form a “headpiece”. Two “legs” are formed by the remaining extracellular domains of the two subunits (Fig. 1.2b). Initially, αIIbβ3 structure obtained by electron microscopy (EM) revealed a large
globular head domain of approximately 80 Å diameter followed by a long rigid stalk comprising two flexible tails approximately 170 Å in length [10, 11]. Later on, X-ray crystal structures of the extracellular domains of the integrin αVβ3 and αIIbβ3 revealed that the legs were severely bent at the “genu” or knee (located between the Hybrid and Calf-1 domains in the α, and between EGF1 and EGF2 in the β), generating a V-shaped topology in which the head domain was closely juxtaposed to the membrane-proximal portions of the stalks [12, 13] (Fig. 1.3, 1.5(1)). Mutational and EM studies of different integrins established that the bent conformation represents the physiological resting state [14, 15].

Figure 1.2 Integrin architecture. (a) Organization of domains within the primary structures. Some α subunits contain an I domain inserted in the position denoted by the dotted lines. Cysteines and disulfides are shown as lines below the stick figures. (b) Schematic of the course of the α and β subunit polypeptide chains through domains from the N to C termini. The figures are adapted from the review [16].

The α-subunit Extracellular Domains

The N-terminal β-propeller domain is formed by seven-fold ~60 amino acid repeats arranged like blades of a propeller. Each repeat or blade contains a four-stranded β sheet. The seven-bladed β-propeller was initially predicted by computational methods [17] and later confirmed by crystal structures [12, 13, 18, 19]. The β-propeller forms the the α subunit head domain and provides a critical interface with the β-subunit (Fig. 1.3). On the bottom face of β-
propeller, four Ca\(^{2+}\) ions are chelated by loops in blades 4-7 [13]. Interestingly, the crystal structure of αIIbβ3 headpiece reveals a cap sub-domain that comprises four insertions in the β-propeller. Although probably not involved in allosteric regulation, the cap contributes functionally to ligand binding as demonstrated by mutagenesis studies [20]. The marked variation in the length and sequence of the inserts among α subunits suggests a role of the cap in determining ligand binding specificity [19].

![Figure 1.3 Crystal structure of integrin αIIbβ3.](image)

The thigh, calf-1 and calf-2 domains, which constitute the remaining portion of the α-subunit extracellular domains, are three immunoglobulin-like β-sandwich domains orderly arranged after the β-propeller domain and comprise the leg of the α-subunit, with the thigh domain in the upper leg and calf-1 and calf-2 domains in the lower leg (Fig.1.2b, 1.3). These three domains have similar folds and are longer than typical Ig-like domains. A small Ca\(^{2+}\)-binding loop located between the thigh and calf-1 domains represents the α-subunit genu (Fig. 1.3).
The β-subunit Extracellular Domains

The β-subunit organization is more complicated than that of the α-subunit. The β I domain is a highly conserved domain with ~240 residues and adopts a Rossmann fold, in which α-helices and β-strands alternate in the secondary structure and a central β-sheet is surrounded by α-helices in the tertiary structure. Additionally, the β I domain contains two additional segments: one forms the interface with β-propeller and the other is known as the specificity-determining loop because of its role in ligand binding. The interacting interface between β I domain and β-propeller is much greater than any other domain-domain interface in integrins. In addition, the β I domain contains three metal ion-binding sites: the metal ion dependent adhesion site (MIDAS); SyMBS (synergistic metal ion binding site) and ADMIDAS (adjacent to MIDAS), which shares some coordinating residues with the MIDAS. The β I domain MIDAS appears to directly bind the ligand.

The β I domain is inserted in the β-sandwich hybrid domain, which is in turn inserted in the PSI domain (Fig. 1.2a). The hybrid domain makes extensive contact with the β I domain. Its structure resembles the I-set Ig domains [21]. The PSI domain consists of a two-stranded antiparallel β sheet flanked by two short helices [22]. The inserted topology of the β I domain plays a critical role in its allosteric regulation and signaling, as discussed below.

PSI domain connects the hybrid domain with the lower leg of β-subunit, which contains four I-EGF domains and a β-tail domain (Fig. 1.2b). The αVβ3 crystal structure revealed that I-EGF3-4 domains assume the structure of a classic I-EGF fold and contain rich disulfide bonds typical for EGF domains [12]. Recently, crystal structure of the entire extracellular domain of αIIbβ3 in a physiologically resting state has been solved [13] (Fig. 1.3). In this structure, there is a highly acute bend between the I-EGF domains 1 and 2 (the β “knee”). In contrast, I-EGF
domains 2, 3 and 4 extend in an almost straight orientation. I-EGF4 domain is followed by β-tail domain, which consists of a four-stranded β sheet and contacts with β I domain in the αVβ3 crystal structure but not in the αIIbβ3 crystal structure [12, 13].

Transmembrane and Cytoplasmic Domains

In contrast to the crystal structures of integrin extracellular domains, the structural studies of transmembrane and/or cytoplasmic domains produce conflicting results, in which the α and β transmembrane and/or cytoplasmic fragments are either dissociated or associated with different interfaces [23, 24-32]. Although early work failed to detect the association of the α and β transmembrane and/or cytoplasmic domains [23, 24], later studies, which demonstrated the interactions of the α and β subunits in this region [25-32], have been widely accepted. Despite the fact that residues associate differently in various data, the GXXXG motif from the transmembrane domains and GFFKR motif from the cytoplasmic domains were generally considered to contribute to the association. Last year, a newly developed method combining disulfide scanning with Rosetta computational modeling has been used to solve the structure of αIIbβ3 TM and cytoplasmic domains [30]. Since the structure is obtained based on experimental data using intact integrins with the extracellular and cytoplasmic domains that regulate TM association on the mammalian cell surface, we believe that it most likely represents the physiological structure in the resting state. In this structure, the αIIb GXXXG motif and their β3 counterparts of the TM domains associate with a ridge-in-groove packing (Fig. 1.4A). The αIIb TM α-helix extends beyond the 23-residue TM hydrophobic segment and then Gly-991 of GFFKR is a turn which changes the TM right-handed α-helix to a left-handed one, making Phe-992 and Phe-993 sit in the interface of αIIb and β3 at the membrane/cytoplasm interface (Fig. 1.4B), and thus, this motif is critical for α/β association. A salt bridge between αIIb Arg-995 and
\( \beta_3 \) Asp-723 was proposed previously based on mutagenesis data [33]. In the structure, Arg-995 is close to both Asp-723 and Glu-726, consistent with this electrostatic interaction. However, there are a variety of different conformations of the side chains of Arg-995 and Asp-723, indicating that this salt bridge is not absolutely necessary for the association.

![Figure 1.4 Structure of the integrin \( \alpha_{IIb}\beta_3 \) transmembrane (TM) and cytoplasmic domains.](image)

(A) The interface between two associating TM domains on the cell surface.
(B) Cytoplasmic fragment association of integrin \( \alpha_{IIb} \) and \( \beta_3 \) subunits in the Disulfide/Rosetta structure. The figures are adapted from [30].

**\( \alpha_{IIb}\beta_3 \) Activation**

The activation of \( \alpha_{IIb}\beta_3 \) is tightly regulated through inside-out signalling. As described above, upon stimulation of external agents, \( \alpha_{IIb}\beta_3 \) undergoes conformational rearrangement and facilitates ligand binding. Understanding the conformational changes of the overall structure and individual domains of \( \alpha_{IIb}\beta_3 \) is crucial to unravel the mechanism of its signalling transduction and to successfully design a target drug. Two different models of \( \alpha_{IIb}\beta_3 \) activation, the switchblade model and the deadbolt model, have been proposed to interpret the overall
conformational rearrangement of integrins [15, 19, 34]. I will describe the first model in detail, and discuss the second one later.

The Switchblade Model

The “switchblade” model proposes that the bent conformation represents the physiological resting state, and upon activation integrins undergo a large global conformational change that results in a fully extended conformation and a switchblade-like hybrid domain swing-out [15, 19], leading to the conformational change at the ligand binding headpiece, especially the β I domain, therefore increasing affinity for ligands (Fig. 1.5). This marked change in tertiary structure is supported by X-ray crystallography [19], electron microscopy (EM) [14, 15, 35, 36], solution X-ray scattering [37], antibody epitope mapping [38], and mutational studies [15, 16, 37, 39-44].

Figure 1.5 The switchblade model. Domain rearrangement of integrins during activation. The β subunit lower legs are flexible and are therefore shown in what may be the predominant (solid representation) and less predominant (dashed lines) orientations. The figures are adapted from the review [16].
Conformational Change of β I Domain

The structure of the β I domain was first solved in the context of αVβ3 extracellular domains in the absence of ligand [12]. Subsequent mutagenesis studies [41-43, 45, 46] and the structure of the αIIbβ3 headpiece co-crystallized with different ligand mimetic drugs [19] revealed conformational change in the open, high affinity state of the β I domain. With the higher resolution (2.55Å) of αIIbβ3 complete ectodomain crystal structure [13], more detailed conformational changes in the β3 I domain can be obtained by superimposition of the headpieces from this physiologically resting, unliganded, closed structure with the high-affinity, liganded, open structure (Fig. 1.6). The inward movement of the β1-α1 loop and the α1 helix is tightly coupled with the downward displacement of the β6-α7 loop and the α7 helix from the resting state to the active state. This linkage is critical for propagation of conformational signals from the ligand binding pocket to the other integrin domains and vice versa. The coordination of the Met335 backbone carbonyl in the β6-α7 loop to the ADMIDAS metal ion (Ca$^{2+}$ in physiologic condition) in the low-affinity conformation is broken in the high-affinity conformation. The breaking of this coordination in turn enables the inward movements of β1-α1 loop and the ADMIDAS Ca$^{2+}$ toward the MIDAS metal ion (Mg$^{2+}$ in physiologic condition) (Fig. 1.6), which is the major difference between the high and low-affinity conformations of the β I domain ligand binding site, consistent with earlier findings [18, 19].

The Swing-out of the Hybrid Domains

Since the β I domain is inserted into the hybrid domain and they have extensive contact to each other, the activation and movement of the β I domains will inevitably cause conformational changes of the hybrid domain. As a consequence of the inserted topology of the β I domain into the hybrid domain, the downward displacement of the α7-helix in the high-
affinity, liganded crystal structure results in complete remodeling of the interface between these domains, leading to the swing-out of the hybrid domain [19] (Fig. 1.6). Compared to the closed conformation, the hybrid domain swings out about 60°, resulting in the separation of the knees of the α and β subunits by 70 Å (Fig. 1.6). The two conformations of the integrin headpiece were supported by EM studies of integrin αVβ3 [15] and α5β1 [35, 47] and a range of other studies [40, 42, 47-49].

Figure 1.6 Conformational change and allosteric transmission by β I domains. Superposition of closed and open structures of the β I domains and their linkages to hybrid and PSI domains. (From PDB ID codes 3FCS and 2VDL). Nonmoving segments of backbone are shown as a gray worm. The moving segments of the backbone and the MIDAS metal ions are cyan (closed) and red (open). The direction of movement is indicated with arrows.
Separation of the Lower Legs

Crystal [12, 13, 18] and EM [15] structures provide direct evidence that in the resting state the membrane-proximal portion of the extracellular domains of the α and β subunits are in close juxtaposition. Enforced association of the two stalks with acid/base coiled coils renders integrin low affinity, whereas release of these constraints promotes high affinity ligand binding [50]. Introducing a 10-residue flexible spacer between the extracellular domain and the TM domain enhanced αMβ2 ligand binding on the cell surface [51]. Crystal structure of the open αIIbβ3 headpiece [19] and EM structure of the entire αVβ3 extracellular domains [15] confirmed that the two stalks separate during integrin activation or ligand binding. The lower β leg in the averaged EM images of the open conformation tended to disappear, suggesting that the β lower leg is highly flexible and varied in conformations among individual particles [15]. Therefore, even though the crystal structure of the open αIIbβ3 headpiece indicated that the swing-out of the hybrid domain results in a 70 Å separation at the knees [19], the distance between the two C-terminal stalks in the open conformations may vary, and this variation will result in a spectrum of different conformations. The stalk separation is a key step for integrins to transmit signals bidirectionally across the plasma membrane.

The Models of TM and Cytoplasmic Domain Activation

The TM and cytoplasmic domains are key for integrin inside-out and outside-in signaling, since both signals must be conveyed across the plasma membrane. Several models have been proposed to interpret how signals are transmitted across the cell membrane. These models are distinguished by the positional changes of the TM domains in the lipid bilayer. In the ‘piston’ model, either the α subunit, the β subunit, or both subunits move vertically in the membrane for integrin activation [52]. The ‘twist’ and ‘scissor’ models propose that a fulcrum,
formed by association of the α and β subunits within or nearby cell membrane, must occur for signal transduction, based upon which the α and β TM domains are either ‘twisted’ or undergoing a scissor-like movement [52, 53]. Recently, the separation of TM and cytoplasmic domains has become widely accepted, which is discussed as follows.

Many studies showed that deletions or mutations in the α and β subunit TM and cytoplasmic domains, which are expected to destabilize α/β association, activate integrins [33, 54-57]. FRET study shows that in the resting state the integrin α and β subunit cytoplasmic domains are close to one another, but undergo significant spatial separation upon inside-out activation induced by phorbol ester or talin head domain or outside-in signaling induced by ligand binding [58]. NMR studies of the integrin cytoplasmic tails suggest that their association is weak, with significant differences observed between published structures [25-27], or that association is undetectable [24]. These studies imply that the cytoplasmic interaction is modest and/or transient. Binding of intracellular proteins such as RAPL [59] or the talin head domain [60-62] to the integrin cytoplasmic tails induces tail separation and activate integrins for ligand binding [25]. The structural basis for binding of talin head domain and filamin to the integrin β cytoplasmic domain resulting in integrin activation has been demonstrated by NMR studies [61-64]. The separation rather than rearrangement of TM domains was further supported by mutagenesis studies, in which introduction of disulfide bridges to prevent or reverse separation abolished the activating effect of cytoplasmic mutations [28], whereas mutations that disrupt the TM interface activate integrins [29, 65, 66].

The tilting of the β3 helix within the membrane may provide another possible mechanism for integrin activation. The comparisons of the Disulfide/Rosetta structure [30] with the NMR structures of isolated αIIb and β3 TM/cytoplasmic domain fragments [67, 68], which are
believed to represent the physiologically active state, gave some clues about the mechanism [69] (Fig. 1.7). The isolated αIIb and β3 TM/cytoplasmic NMR structures are similar with the Disulfide/Rosetta structure. The dissociated β3 TM domain solved by NMR appeared to be a 30-residue linear α-helix extended into the cytoplasm, and instead of the 23 TM residues in the Disulfide/Rosetta complex structure, 29 residues appeared to be embedded in the bicelle core. In the NMR structure, β3 Lys-716 is followed by a 5-residue hydrophobic segment (L717-LITI721), and the continuous helix spanning the TM and juxtamembrane segments could undergo a substantial tilt in the membrane, with snorkeling of the Lys-716 side chains into the polar environment [67]. By contrast, the β3 helix embedded in lipid membrane in the Disulfide/Rosetta complex structure is significantly shorter, suggesting that after dissociating from the αIIb helix, the β3 helix is tilted with an angle of 20-30° due to inserting of 5-6 additional hydrophobic residues to the hydrophobic lipid environment. The tilting of the β3 helix can be caused by force transmission from the actin cytoskeleton since the integrins are activated by force applied to the actin cytoskeleton that binds to the β cytoplasmic domain [13]. This tilting of the β3 helix may be important for integrin activation and signaling.

Interestingly, the structure of integrin αIIbβ3 TM and cytoplasmic domain complex was also solved by NMR in the presence of phospholipid bicelles, and it was found to have a similar interhelical interface to the Disulfide/Rosetta structure [31]. However, the NMR structure was solved using an artificial hydrogen-bond constraint between the αIIB(R995)- β3(D723). The presence of the salt bridge was based on the fact that mutations of either residue affected the helix-helix interaction as monitored by NMR. We propose that this electrostatic interaction is important for the priming of helix-helix interaction. After forming more stable helix-helix interaction, the salt bridge is probably not critical for further stabilization. Therefore, the NMR
structure might represent an “intermediate” or “transient” state between the physiological resting state (represented as the Disulfide/Rosetta structure) and the dissociated active state (represented as the NMR structure of the isolated monomers as discussed below) (Fig. 1.7). It is interesting that the NMR structures of the complex have almost identical structures and angles within the membrane to the isolated monomers, and there were substantial amounts of $\alpha$IIb and $\beta$3 monomers present in the solution used for determining the NMR complex structures. These observations confirm our hypothesis that the NMR structure of the complex is an intermediate or “transient” state.

Figure 1.7 Predicted model of integrin TM activation. (I) The resting state represented as Disulfide/Rosetta structure; (II) the “intermediate” or “transient” state represented as NMR structure of integrin $\alpha$IIb$\beta$3 TM and cytoplasmic domain peptides (PDB ID 2K9J); (III) the activated state represented as monomeric $\alpha$IIb and $\beta$3 NMR structures (PDB ID codes 2K1A and 2RMZ). The charges shown in II and III are proposed to be important for the initial association of $\alpha$ and $\beta$ subunits. The outer bounds of the hydrophobic, interface, and polar region of the membrane are shown as black, red, and green lines, respectively.
Homooligomerization of TM Domains

Although the heterodimeric association of \( \alpha/\beta \) TM domains in the resting state has been widely accepted, the homomeric association of isolated integrin TM fragments was also proposed [23, 70, 71, 72]. In 2001, Li et al. failed to detect the heterodimeric association between TM \( \alpha \) and \( \beta \) fragments in micelles using various methods including ultracentrifugation and NMR study. Instead, they observed the \( \alpha \) homodimers and \( \beta \) homotrimers [23]. Later, \( \alpha \)IIb and \( \beta \)3 TM helices were confirmed to form homooligomers in bacterial membranes using TOXCAT assay, with the similar interfaces as in heterodimers [70, 72]. Asparagine mutagenesis in this region of \( \beta \)3 subunit indicated that M701N and G708N can activate integrin for ligand binding. Furthermore, G708N was believed to induce \( \beta \) TM homotrimers, integrin clustering and phosphorylation of FAK [73]. Thus homooligomers were proposed to contribute to integrin activation and clustering. Combined with the mutagenesis study of \( \alpha \)IIb TM domain, a push-pull model was proposed in which disruption of the \( \alpha/\beta \) heterodimeric association of TM domains pushes the integrin to the activated state, whereas homooligomerization pulls the equilibrium toward activation [66]. Although integrin TM homooligomerization received support from computer modeling [74], it has not been observed using intact integrins on mammalian cell surface. Furthermore, the \( \beta \)3 G708N mutant was later found to increase ligand binding affinity as a consequence of increased affinity rather than valency [29]. Therefore, it is necessary to further test whether integrin TM domains form homooligomers during integrin activation and signaling.

The Deadbolt Model

As an alternative or supplemental to the switchblade model, the “deadbolt” model posits that the association between the \( \beta \) I domain from the headpiece and \( \beta \)-TD domain from the lower leg of \( \beta \) subunit is key to keep the integrin in the resting state, whereas dissociation of this interface results in release of the constraint of \( \beta \) I domain involved in ligand binding and allows it to undergo a subtle conformational change to shift the integrin to a high-affinity ligand binding state. Thus the extension is not critical for initial integrin activation, but rather a post-ligand-
binding event in deadbolt model [34]. The model was also supported by many data [36, 75-78]. Additional structural analysis is necessary to evaluate these two models.

**αIIbβ3 Outside-in Signaling**

Outside-in signaling of αIIbβ3 is triggered by extracellular ligand binding, which promotes actin polymerization, cytoskeleton reorganization, and further cell spreading [7]. αIIbβ3 can bind several Arg-Gly-Asp (RGD)-containing ligands including fibrinogen, von Willebrand factor (vWF), vitronectin, fibronectin and thrombospondin. The ligand binding to αIIbβ3 involves specific regions on the headpiece from both αIIb and β3 subunits [19]. It is believed that integrin conformational rearrangements in outside-in signaling may occur in a similar way as in inside-out signaling [15, 50, 58, 79]. In addition, the lateral association (clustering) of integrin heterodimers, which occurs as a consequence of multivalent ligand binding [80, 81], was shown to play a major role in outside-in signaling [82]. Although the exact mechanism for integrin clustering remains unclear, it seems to regulate activation of several kinases including FAK, Src and Syk [82-85]. Furthermore, the activated kinases phosphorylate the substrates leading to intracellular signaling (Fig.1.8)

![Diagram of integrin outside-in signaling](modified from [28]).
An Overall Perspective

Numerous studies from different labs suggest that integrin bidirectional signaling across the plasma membrane is accomplished by coupling extracellular conformational change to an unclasping and separation of the α and β TM and cytoplasmic domains. Based on the above description, we can summarize the basic conformational changes of integrins in their bidirectional signaling. For inside-out signaling, when cells are stimulated by agonists such as von Willebrand factor (VWF), thrombin and ADP, specific intracellular proteins, for example talin, can interact with integrin β cytoplasmic domains through its FERM domain [86], further initiating the separation of the cytoplasmic and TM domains. This event will destabilize the extracellular α/β tail interface, concomitantly perturbing the tail/head interface and facilitating the hybrid domain swing-out, which is coupled directly to the downward movement of the β I domain α7 helix and thus the MIDAS rearrangement [15, 38]. There must exist an equilibrium of different integrin conformational states in this process. The intracellular protein binding triggers the change of the integrin bent conformation toward the more extended conformation. Integrin conformational rearrangements may occur in a similar way for outside-in signaling [15, 50, 58, 79]. Ligand binding stabilizes integrin in the extended conformation with open headpiece and two separate legs, resulting in the separation of the two cytoplasmic tails. Then, multivalent ligand binding brings several integrins close to each other, leading to integrin clustering, and kinases are recruited and activate each other, leading to intracellular signaling.

Work presented in this dissertation demonstrates that disruption of α/β association, specifically, separation of two extracellular lower legs (αIIb calf-2 domain and β3 I-EGF4 domain) that induces a global conformational change of integrins is critical for transmitting the bidirectional signals. Furthermore, the integrin TM homooligomerization was tested in order to
understand TM signaling and clustering. In contrast to previous work [23, 70, 73], we did not
detect any homooligomers except for αW967 mutant under various activating conditions. Our
results therefore showed that disrupting integrin heterodimeric association of the
TM/cytoplasmic domains and the extracellular legs, but not the formation of homomeric
association, is critical for integrin activation and signaling.

References


CHAPTER TWO:
DISSOCIATION OF THE \( \alpha \)-SUBUNIT CALF-2 DOMAIN
AND THE \( \beta \)-SUBUNIT I-EGF4 DOMAIN IN INTEGRIN
ACTIVATION AND SIGNALING
**Introduction**

Integrins are heterodimeric cell adhesion receptors that transmit signals bidirectionally across the plasma membrane. Together with other proteins, they mediate cell-cell and cell-extracellular matrix interactions and communication. As functionally important signalling molecules, they regulate a variety of cellular processes including growth, migration, differentiation, and survival. Integrins are normally inactive on the surface of the cell. When external agents stimulate cells, specific intracellular signals impinge on integrin cytoplasmic domains resulting in changes in structure and ligand-binding affinity in the integrin extracellular domain. In turn, binding of multimeric ligands triggers outside-in signalling, leading to several cellular processes including cell spreading and kinase activation. Thus, integrin activation and signalling are dependent on specific allosteric conformational changes in the integrin on the cell surface.

Integrin α and β subunits each have a large extracellular domain, a single transmembrane (TM) domain, and a short cytoplasmic domain (except the β4 subunit). The association of the α and β subunit TM/cytoplasmic tails is critical for maintaining integrins in the low-affinity state, whereas intracellular signals that destabilize αβ TM/cytoplasmic association result in integrin activation [1-7]. Recently, structures of both the complex and the isolated monomers of the TM/cytoplasmic domains were reported [8-11]. These structures show that in the resting state, ridge-in-groove packing of the TM domain and the GFFKR motif in the α subunit cytoplasmic domain are important for αβ association. Alternatively, integrins can be activated through the
binding of intracellular molecules such as talin [12], which dissociates the αβ TM/cytoplasmic domains and leads to a conformation with high affinity for ligands [2, 7, 13, 14].

However, the mechanism of how activation signals are transmitted from the TM domain through two long extracellular legs to the ligand-binding headpiece remains elusive. Two different models have been developed in attempts to define this mechanism. The “deadbolt” model has been proposed in which interaction at a small interface between the β-tail domain (βTD) CD loop (the deadbolt) and the β I domain α7 helix in αVβ3 ectodomain structure is critical for stabilizing integrins in the low-affinity state [15]. The inside-out signal causes the βTD CD loop to move away from the βI domain, enabling the α7 helix of the βI domain to displace from the ligand-binding pocket. Thus, integrins assume high affinity for ligands even in the bent conformation [16]. This model suggests that overall conformational change is not critical for initial integrin activation, but rather a post-ligand-binding event. The model was supported by transmission EM studies of the integrin αVβ3 extracellular domain in complex with a fibronectin fragment [17], and a number of other studies [18-21]. However, one study that was designed to directly test this model found that deleting the CD loop residues, or mutating these residues to Ala had no effect on integrin ligand binding [22].

By contrast, the other model, the “switchblade” model, has gained more experimental support. This model proposed that upon inside-out activation, the integrin extracellular domains rearrange through a “switchblade”-like movement to extend and assume high-affinity conformations for ligands [23]. It suggests that this extension of extracellular integrin domains is critical for integrin activation and signaling, since it preferentially places the ligand-binding
site away from the surface of the cell favoring ligand accessibility. At the same time, extension enables hybrid domain swing-out, thus pulling the βI domain α7 helix through a crankshaft-like displacement, converting the headpiece from the closed, low-affinity state to the open, high-affinity state [24]. This marked change in tertiary structure is supported by X-ray crystallography [25], electron microscopy (EM) [14, 23, 26, 27], solution X-ray scattering [28], antibody epitope mapping [29], and mutational studies [23, 28, 30-36]. However, the role of the two extracellular legs on integrin signaling remains elusive. Although the separation of the upper legs was observed by EM and crystal structures [23, 25], conformational change of the two lower legs is less defined. Patients with mutations in calf-1 and calf-2 domains of αIIbβ3 showed Glanzmann thrombasthenia [37], implying the significance of this region. Recently, a disulfide bond introduced into α5β1 βTD and Calf-2 domain to restrict the leg separation blocked integrin extension and signaling [38].

In this chapter, we tested the role of integrin lower leg separation on integrin activation and signaling by introducing mutations that either prevent or disrupt the interface between the α-subunit Calf-2 domain and β-subunit I-EGF4 domain (Fig. 2.1). Our results showed that a disulfide bridge that prevents separation of this interface completely abolished integrin inside-out activation and outside-in signaling. In contrast, introduction of an N-glycan that disrupts this interface resulted in high-affinity conformations. The results indicate that the separation of the αβ legs is required for integrin activation and outside-in signaling.
Figure 2.1 Mutations in the αIIbβ3 structure. The Calf-2 domain is in pink, the I-EGF4 domain is in green, and the βTD domain is in cyan. A. The mutations are located one domain N-terminal to the βTD CD loop (in salmon). The ligand-binding βI domain is in marine and the α7-helix is in purple. Mutated residues shown with spheres are residues R751 (magenta) and N753 (red) of αIIb, and Y594 (orange) and T603 (splitpea) of β3. B. The αIIb_R751 (magenta) and β3_T603 (splitpea) are close to each other, and mutating them to cysteines was expected to form disulfide-linked heterodimer. C. The αIIb N753 (red) and β3 Y594 (orange) are located at the interface, and introduction of a N-glycosylation site to these positions was predicted to disrupt αβ association.
Experimental Procedures

Cell Culture

HEK 293T cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA) and was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) with 1X nonessential amino acids, 1X penicillin/streptomycin, 1X L-glutamine, 1X sodium pyruvate (100X stock solutions were purchased from Invitrogen) at 37°C in 5% CO₂ atmosphere. The total medium was changed every other day.

Plasmid Construction, Expression, and Immunoprecipitation

Plasmids with sequences encoding full-length human αIIb and β3 were subcloned into pEF/V5- HisA and pcDNA3.1/Myc-His (+), respectively [23]. The αIIb mutants F992A/F993A (activating GAAKR mutant, denoted as α*), F755T, and α*R751C and the β3 mutants Y594N/D596T and T603C were made using site-directed mutagenesis with the QuikChange kit (Stratagene, La Jolla, CA). Constructs were transfected into HEK293T cells (American Type Culture Collection, Manassas, VA) using a FuGENE transfection kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. To detect the expression levels of αIIb and β3 by flow cytometry staining, twenty-four or forty-eight hours after transfection, cells were detached and suspended in Tris-buffered saline (TBS) supplemented with 5mM Ca²⁺. Then 10 μg/ml of following monoclonal antibodies: AP3 (nonfunctional anti-β3 mAb, American Type Culture Collection), 7E3 (anti-β3 mAb), and 10E5 (anti-αIIb mAb, kindly provided by B. S. Coller, Rockefeller University, New York, NY) were incubated on ice for 30 min with the suspended cells separately, followed by staining of FITC conjugated anti-mouse IgG on ice for another 30 min. After wash, the samples were stored in phosphate-buffered saline (PBS) and
were subjected to FACS scans using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) from LSU School of Veterinary Medicine. To characterize disulfide-bond formation and glycosylation, twenty-four hours after transfection, the HEK293T cells were metabolically labeled with $[^{35}\text{S}]$ cysteine/methionine for 1.5 h before adding chase medium containing 500 μg/ml of cysteine and 100 μg/ml of methionine, and cells were cultured for 17 h and lysed in lysis buffer containing 20 mM Tris-buffered saline, pH 7.4 (TBS), supplemented with 1 mM Ca$^{2+}$, 1% Triton X-100, and 0.1% Nonidet P-40 [3]. The lysates were immunoprecipitated with 1 μg of anti-β3 mAb AP3 and protein G-sepharose at 4°C for 1 h, eluted with 0.5% SDS. After the addition of 1% Nonidet P-40, the protein was treated with or without 500 units of PNGase F (New England BioLabs) at 37°C for 1h. Material was subjected to 7.5% nonreducing SDS-PAGE and fluorography [3].

**Two-Color Ligand Binding Assay on HEK293T Transfectants**

Soluble binding of ligand mimetic IgM PAC-1 (BD Biosciences, San Jose, CA) and Alexa Fluor 488-labeled human fibrinogen (Enzyme Research Laboratories, South Bend, IN) was determined as previously described [31]. Briefly, transfected cells suspended in 20 mM HEPES-buffered saline, pH 7.4 (HBS) supplemented with 5.5 mM glucose and 1% bovine serum albumin were incubated on ice for 30 min with PAC-1 or Alexa Fluor 488-conjugated human fibrinogen in the presence of either 5 mM EDTA, 5 mM Ca$^{2+}$, 100 μM Ca$^{2+}$/1 mM Mn$^{2+}$ plus 10 μg/ml activating mAb PT25-2 (anti-αIIb, kindly provided by M. Handa, Keio University Hospital, Tokyo, Japan) [39], or 1 mM Mn$^{2+}$ plus 10 μg/ml activating mAb LIBS-1 (anti-β3, kindly provided by M. H. Ginsberg, Scripps Research Institute, La Jolla, CA) [40]. For PAC-1 binding, cells were washed and stained with FITC-conjugated anti-mouse IgM on ice for another 30 min before being subjected to flow cytometry. Cells were also stained in parallel with Cy3-
conjugated anti-β3 mAb AP3. Binding activity is presented as the percentage of the mean fluorescence intensity (MFI) of PAC-1 or fibrinogen staining after background subtraction of the staining in the presence of EDTA, relative to the MFI of the AP3 staining.

**Ligand-Induced Binding Site (LIBS) Epitope Expression**

LIBS epitope expression was measured as previously described [31]. Briefly, transfected cells suspended in HBS supplemented with 5.5 mM glucose and 1% bovine serum albumin were incubated with or without 50 µM GRGDSP peptide in the presence of 1 mM Mn$^{2+}$ plus 10 µg/ml anti-LIBS antibody. After incubation on ice for 30 min, cells were washed and stained with FITC-labeled anti-mouse IgG on ice for 30 min. The stained cells were subjected to flow cytometry, and LIBS epitope expression was expressed as the percentage of MFI of anti-LIBS antibody relative to MFI of the conformation-independent anti-β3 mAb AP3.

**Cell Adhesion Assays**

Cell adhesion on immobilized human fibrinogen was assessed by the measurement of cellular lactate dehydrogenase (LDH) activity as previously described [41]. Briefly, cells suspended in HBS supplemented with 5.5 mM glucose and 1% bovine serum albumin and 1 mM Ca$^{2+}$ with or without 1mM DTT were added into flat bottom 12-well plates ($1 \times 10^5$ cells/well) precoated with 20 µg/ml fibrinogen and blocked with 1% bovine serum albumin. After incubation at 37°C for 1 h, wells were washed three times with HBS supplemented as indicated above. Remaining adherent cells were lysed with 1% Triton X-100, and LDH activity was assayed using the Cytotoxicity Detection Kit (Roche Applied Science) according to the manufacturer's instructions. Cell adhesion was expressed as a percentage of bound cells relative to total input cells.
Cell Spreading and Microscopy

Glass bottom 6-well plates (MatTek Corporation, Ashland, MA) were coated with 20 µg/mL human fibrinogen in phosphate-buffered saline at pH 7.4 (PBS) overnight at 4°C, and then blocked with 1% BSA at room temperature (RT) for 1 h. The transiently transfected HEK293T cells were detached with trypsin/EDTA, washed three times with DMEM, and seeded on fibrinogen-coated plates with or without 1mM DTT. After incubation at 37°C for 1 h, cells were washed three times with PBS and fixed with 3.7% formaldehyde in PBS at RT for 10 minutes for microscopy.

Differential interference contrast (DIC) imaging was conducted on a Leica TCS SP2 spectral confocal system coupled to a DM IRE2 inverted microscope with a 63X oil objective. For the quantification of cell spreading area, outlines of 100 randomly selected adherent cells were generated, and the number of pixels contained within each of these regions was measured using ImageJ software (Bethesda, Maryland).

Results

Mutations of αIIbβ3 Extracellular Membrane-Proximal Stalk Stabilizes or Disrupts αβ Leg Association

To test whether the conformational rearrangements of integrin lower legs are important, we designed mutations at the interface between the αIIb-subunit Calf-2 domain and the β3-subunit I-EGF4 domain (Fig. 2.1). To mimic integrin inside-out activation, site-directed mutagenesis was used to mutate two phenylalanine residues in the GFFKR motif of the αIIb cytoplasmic domain to alanines (αIIb_F992A/F993A/β3, denoted α*/β). Cysteine residues were introduced into the α*/β construct to test the effects of a disulfide-bridged mutant on integrin inside-out signaling [3]. The distance between Cβ atoms of αIIb-subunit Arg751 and β3-subunit
Thr603 in the αIIbβ3 crystal structure is 4.1 Å [42]. Therefore, cysteine residues introduced to replace these two residues (α*_R751C/β3_T603C, denoted α*751C/β603C) were expected to form a disulfide bond.

In addition to introducing this disulfide clasp to prevent the αβ dissociation, we also designed mutations to disrupt this interface to determine whether disrupting the αβ leg association affected ligand binding. N-glycosylation sites were introduced on the αIIb-subunit Calf-2 domain and on the β3-subunit I-EGF4 domain. In the crystal structure, the αIIb_N753 and β3_Y594 residues are at the interface between the Calf-2 and I-EGF4 domains (Fig. 2.1C) and were predicted to be important for the αβ association. Therefore, introducing an N-glycan chain to either residue was expected to disrupt the αβ association. The following mutants were constructed to test this hypothesis: αIIb_F755T/β3 (denoted F755T/β, resulting in N-glycosylation of N753 in αIIb) and αIIb/β3_Y594N/D596T (denoted α/(Y594N/D596T), resulting in N-glycosylation of Y594N in β3).

Expression of Wild Type and Mutant αIIbβ3 on HEK293T Cells

To determine the expression of wild type and mutant αIIbβ3, wild type and four mutated αIIb and β3 subunits were co-transfected into HEK293T cells and subjected to immunostaining flow cytometry (Fig. 2.2A). Two anti-β3 antibodies AP3 and 7E3, which recognize the β3 I and hybrid domains, respectively, and one anti-αIIb antibody 10E5, which recognizes the β-propeller domain, were used to monitor cell surface expression. Wild-type and mutant integrins bound to the three antibodies (Fig. 2.2A), suggesting that they adopted a native conformation on the cell surface. To exclude the possible contribution of endogenous αV in HEK293T cell lines, β3 integrin alone was transfected into the cells, and none of these three antibodies bound (Fig.
2.2A), suggesting that this cell line does not express endogenous αV integrin. Indeed, no αV expression was detected by using the anti-αV antibody LM609 (data not shown).

Figure 2.2 Expression and immunoprecipitation of wild-type and mutant αIIbβ3 integrins. A. Immunofluorescent flow cytometry. HEK293T transfectants were labeled with AP3 (anti-β3), 7E3 (anti-β3), and 10E5 (anti-αIIb). Thick and thin lines show labeling of the αIIbβ3 transfectant and the mock transfectant, respectively. B. Immunoprecipitation. Lysates from 35S-labeled HEK293T cell transfectants were immunoprecipitated with mAb AP3. Precipitates were subjected to nonreducing 7.5% SDS-PAGE and fluorography.
Nonreducing SDS-PAGE of $^{35}$S-labeled, immunoprecipitated receptors showed that in the activating mutant (Fig. 2.2B, lane 2), the $\alpha^*$ and the $\beta$ subunits migrated in a similar pattern to the wild-type receptor (denoted $\alpha/\beta$, Fig. 2.2B, lane 1). In comparison, the receptors with the pair of cysteine mutants $\alpha^*_R751C/\beta_T603C$ formed a disulfide-linked receptor (Fig. 2.2B, lane 3), and the efficiency of the disulfide-bond formation was close to 100%. The $\beta$ subunit of $\beta$-glycosylated mutant $\alpha/(Y594N/D596T)$ (Fig. 2.2B, lane 4) migrated slightly slower than that of the wild type (Fig. 2.2B, lane 1), whereas the $\alpha$IIb subunit from this glycosylation mutant migrated in a similar pattern to the wild type $\alpha$IIb subunit, suggesting that there was an additional glycan chain added only to the $\beta$-subunit. For the $\alpha$IIb-glycosylation mutant F755T/\beta (Fig. 2.2B, lane 5), the $\beta$ subunit migrated in a similar pattern to the wild-type $\beta$ (Fig. 2.2B, lane 1), whereas the mutated $\alpha$IIb subunit (Fig. 2.2B, lane 5) migrated slightly slower than its wild-type counterpart (Fig. 2.2B, lane 1), consistent with the presence of an additional glycan chain. Furthermore, these differences between the wild-type and glycosylation mutants disappeared on deglycosylation by PNGase F (Fig. 2.2B, lanes 6-8), confirming the attachment of extra glycan chains.

**Separation of the $\alpha$-subunit Calf-2 Domain and the $\beta$-subunit I-EGF4 Domain Is Required for Integrin Inside-out Signaling**

Integrin inside-out signals are transmitted from the cytoplasmic/TM domains to the extracellular domains, leading to the conformational change of the ligand-binding headpiece, resulting in high-affinity ligand binding. To study the role of the separation of the $\alpha$IIb-subunit
Calf-2 domain and the β3-subunit I-EGF4 domain in integrin activation, two-color flow cytometry was used to determine the binding of the soluble ligand-mimetic antibody PAC-1 and fibrinogen to the wild-type and mutant receptors on the HEK293 cell surface [4]. As shown in Figure 3A, the expression level of receptors was monitored by the Cy3-labeled anti-β3 antibody AP3. The Cy3-AP3 fluorescence intensity was divided into four domains that represented specifically labeled receptors. The R1 and R2 domains contained cells designated as positive expressers. Ligand binding affinity was monitored by the FITC-labeled PAC-1. Cells located in the R2 and R4 domains are those with high-affinity ligand binding. In the presence of Ca$^{2+}$, very few cells with the wild-type αIIbβ3 (α/β) were located in the R2 domain, and most positive expressers were in the R1 domain, indicating that wild-type αIIbβ3 bound very little ligand-mimetic PAC-1 antibody. This is consistent with a low-affinity conformation under these physiological conditions. In the presence of Mn$^{2+}$ and activating antibodies PT25-2, most positive expressers shifted to the R2 domain, indicating that wild-type αIIbβ3 bound PAC-1 with high affinity (Fig. 2.3A). When the GFFKR motif of the αIIb was mutated to GAAKR, the mutant receptor (α*/β) bound PAC-1 with high affinity even in the presence of Ca$^{2+}$, since most positive expressers were located in the R2 domain (Fig. 2.3A). The addition of the PT25-2 activating antibody did not change this pattern (Fig. 2.3A), suggesting that the GAAKR mutation mimics integrin inside-out activation. When a disulfide bond was introduced to this activating mutant (α*$^{751C}$/ β603C), the ligand binding affinity in the presence of Ca$^{2+}$ was reversed and a
Figure 2.3 **Ligand-binding activity of wild-type and mutant αIIbβ3 integrins.** A. Flow cytometry of dot plots. B and C. Quantified soluble ligand-binding affinity. Cells were incubated with PAC-1 (A-B) in the presence of 5 mM Ca$^{2+}$ or 10 µg/ml PT25-2 plus 1 mM Ca$^{2+}$, or FITC-fibrinogen (C) in the presence of 5 mM Ca$^{2+}$ or 10 µg/ml LIBS1 plus 100 µM Ca$^{2+}$ and 1 mM Mn$^{2+}$ as indicated. Binding activities were determined by flow cytometry and expressed as described in Materials and Methods.
majority of the positive expressers were located in the R1 domain (Fig. 2.3A), indicating that preventing separation of the αβ legs by a disulfide bond abolishes the integrin inside-out activation. In contrast, the two glycosylation mutants α/(Y594N/D596T) and F755T/β bound PAC-1 with high affinity in the presence of Ca^{2+} alone, with most positive expressers located in the R2 domain (Fig. 2.3A). The addition of the PT25-2 activating antibody did not influence their ligand binding, suggesting that these two mutants constitutively bound ligand with maximal affinity (Fig. 2.3A).

Figure 2.3B quantifies this data by measuring the MFI of FITC-labeled PAC-1. The results confirmed that the wild-type receptor bound PAC-1 only in the presence of activating conditions, whereas the GAAKR mutant bound PAC-1 constitutively even in the presence of Ca^{2+} alone (Fig. 2.3B). The disulfide-bonded receptor reversed the GAAKR-induced inside-out activation, but did not abolish the activating antibody-induced ligand binding. When N-glycan chain was introduced into the αβ interface of either subunit, receptors bound PAC-1 constitutively (Fig. 2.3B). Soluble fibrinogen binding was also carried out and similar results and conclusion were obtained (Fig. 2.3C). Taken together, these experiments suggest that separation of the αβ lower legs is required and sufficient for integrin inside-out activation.
Disruption of the Interface between the α-subunit Calf-2 Domain and the β-subunit I-EGF4 Domain Causes a Global Integrin Conformational Change

Priming and ligand binding induce αIIbβ3 conformational changes that expose the LIBS epitopes. LIBS epitopes are at the interfaces between the headpiece and tailpiece and between the α and β legs so that they are buried in the bent conformation but exposed in the extended conformation [23, 29]. To investigate the conformational state of the αIIbβ3 mutants, binding of anti-β3 LIBS mAb LIBS1 [43] was analyzed. The LIBS1 bound poorly to wild-type αIIbβ3 in the presence of Ca\(^{2+}\) alone. The binding significantly increased when Mn\(^{2+}\) and the ligand mimetic peptide GRGDSP were added (Fig. 2.4), suggesting that the ligand mimetic peptide stabilizes integrins in the more extended conformation. The GAAKR mutant (α*/β) bound LIBS1 better than the wild type in the presence of Ca\(^{2+}\) alone, suggesting that the mutation mimicking inside-out signaling shifts the integrin towards a more extended conformation. Addition of Mn\(^{2+}\) and GRGDSP peptide further increased binding of the GAAKR mutant to LIBS1, indicating that this mutation cannot stabilize integrins in the fully extended and open state. In comparison, introducing the disulfide-bond into this mutant (α*751C/β603C) slightly decreased LIBS1 binding in the presence of Ca\(^{2+}\) (Fig. 2.4), suggesting that the disulfide-bridge reverses the conformational change induced by inside-out activation. In contrast to the wild-type and the GAAKR mutant receptors, the two glycosylation mutants (α/Y594N/D596T and F755T/β) bound LIBS1 in the presence of Ca\(^{2+}\) (Fig. 2.4), suggesting that separation of the two legs stabilizes integrins in a more extended conformation. Thus, this conformational change could explain their high-affinity ligand binding.
Figure 2.4 Exposure of the LIBS1 epitope. Cells were stained with anti-LIBS antibody LIBS1 in the presence of 5 mM Ca$^{2+}$ or 1 mM Mn$^{2+}$ plus 50 µM RGD peptides (GRGDSP). LIBS epitope exposure was determined as the percentage of MFI of anti-LIBS1 antibody relative to non-functional anti-β3 mAb AP3. Error bars are standard deviation (SD).

Separation of the α-subunit Calf-2 Domain and the β-subunit I-EGF4 Domain Is Crucial for Cell-Adhesion and Spreading

We further determined how separation of the α-subunit Calf-2 domain and the β-subunit I-EGF4 domain affects outside-in signaling by assaying cell adhesion and spreading. HEK293T cells transiently transfected with wild-type and mutant αIIbβ3 were seeded on fibrinogen-precoated dish surfaces at 37°C for 1 h. The amount of adherent cells was assessed by quantifying the cellular lactate dehydrogenase (LDH) activity. The results showed that in contrast to previous studies in CHO transfectants [32, 41], all mutants with higher affinity for soluble ligands adhered to immobilized fibrinogen similarly to the wild-type cells. This suggests that the αIIbβ3 integrins in HEK293T cells are more active than similar integrins in CHO cells.
In contrast, HEK293T cells transfected with a TM disulfide-bonded αIIbβ3 (α968C/β693C) showed much less adhesion than those with a wild-type receptor (Fig. 2.5A). It is not surprising that the disulfide bonded mutant (α*751C/β603C), which restricts separation of the αβ legs, exhibited less adhesion compared to the activating mutant (α*/β) (Fig. 2.5A), since the adhesion strength is dependent not only on the affinity of the receptors, but also on the spreading of the cells on the immobilized ligands. As shown below, the disulfide-bonded mutants had defective cell spreading, resulting in easier detachment of cells during washing. When these two disulfide-bonded mutants were treated with DTT, the cell adhesion ability was recovered to the similar level of the wild type with DTT.

To test if separation of the αβ lower leg can affect cell spreading, HEK293T transient transfectants were coated on immobilized fibrinogen at 37°C for 1 h, followed by fixation and microscopic analysis. Cells transfected with wild-type receptor demonstrated cell adhesion and cell spreading (Fig. 2.5B). Previously, the TM disulfide-bonded αIIbβ3 (α968C/β693C) in CHO transfectants exhibited defective spreading, indicating that separation of the TM domains is required for integrin outside-in signaling [41]. We confirmed that this disulfide-bonded mutant in HEK293T cells exhibited similar defective spreading on fibrinogen (Fig. 2.5B), and even though some cell could adhere to the immobilized fibrinogen, they remained round and did not change size. The cell area was quantified, and showed that the disulfide-bonded mutant had a significant decrease in adherent cell size comparing to that of the wild type (Fig. 2.5C). Thus, this mutant was used as a negative control (Fig. 2.5B and C). As we discussed in our previous paper [41], failure of outside-in signaling of the disulfide-bonded mutant was not likely due to
the failure to bind ligands, since the mutant could bind ligands with similar level as the wild-type (Fig. 2.3). The GAAKR mutant had little effect on cell spreading or on cell shape and size. By comparison, the disulfide bonded mutant (α*751C/ β603C) had defective spreading (Fig. 2.5B and C). Most adherent cells remained round and stayed the same size (Fig. 2.5B). To demonstrate that the defect in spreading was due to the disulfide linkage, we treated the cells with 1 mM DTT. Such treatment has shown to reduce the majority of engineered disulfides in the mutant receptor and rescued cell spreading of the TM linkage (Fig. 2.5B and C) [41]. Similarly, the DTT treatment of the disulfide-bonded integrin α*751C/ β603C led to a rescue of cell spreading (Fig. 2.5B and C). The effect of DTT treatment was unlikely due to the direct effect on ligand binding affinity because DTT had little effect on wild-type cell adhesion and spreading (Fig. 2.5). The quantitative adherent cell area of the disulfide bonded mutant cells decreased by greater than 30% of that of the wild-type cells and of the GAAKR mutant cells. This suggests that separation of the α/β lower leg is crucial for cell spreading. HEK293T cells transfected with either glycosylation mutant could adhere to immobilized fibrinogen and demonstrated substantial spreading (Fig. 2.5C). However, more glycosylation mutant cells than wild-type cells remained round (Fig. 2.5B), suggesting that high-affinity mutants may have some defective effect on outside-in signaling probably due to their effect on cell detachment. Further research is required to determine the exact molecular mechanism of this defect.
Figure 2.5 Cell adhesion and spreading. A. Adhesion of HEK293T transfectants in the presence of 1mM Ca\textsuperscript{2+} with or without DTT (1mM) to surfaces coated with 20 µg/ml fibrinogen. The amount of bound cells was determined by measuring LDH activity as described in Materials and Methods. Data are representative of three independent experiments, each in triplicate. B. DIC images of HEK293T transfectants after adhering to immobilized fibrinogen at 37°C. a: α/β; b: α/β + DTT; c: α*/β; d: α*751C/β603C; e: α*751C/β603C + DTT; f: α*(Y594N/Δ596T); g: F755T/β; h: α968C/β893C; i: α968C/β893C + DTT. The images are representatives of three independent experiments. Scale bar represents 10 µm. C. Quantification of the areas of adhering/spreading cells as described in Materials and Methods. Error bars are SD. *** P <0.001.
Discussion

When cells are activated, binding of intracellular molecules such as talin, dissociates the αβ TM/cytoplasmic domains and leads to integrin activation. The current study demonstrates that separation of the interface between the α-subunit Calf-2 domain and the β-subunit I-EGF4 domain is required for both integrin inside-out activation and outside-in signaling. It suggests that the dissociation of the αβ TM/cytoplasmic domains is coupled with the dissociation of the extracellular αβ lower legs, specifically, the interface between the α-subunit Calf-2 domain and the β-subunit I-EGF4 domain. The mechanism by which this dissociation affects the conformational change of the upper legs and ligand-binding headpiece leading to high-affinity ligand binding remains to be determined.

When disulfide was introduced to the calf-2 and I-EGF4 interface, the mutant receptor could be activated from outside by external reagents such as Mn²⁺ and activating antibodies. In addition, LIBS epitope was exposed upon RGD binding, suggesting that the disulfide-bonded mutant could adopt an extended conformation. Similar results were obtained when a disulfide bond was introduced to the αIIbβ3 TM domain [3, 41]. This is probably because of the highly flexible β3 leg as suggested in the crystal structure [42]. Interestingly, when a disulfide bond was introduced to the α5β1 calf-2 and βTD interface, the mutant α5β1 failed to be extended. The discrepancy may be due to the varied role of different interface on integrin activation, or due to different integrin families. While the β3 integrins must response rapidly to environment [44], changing their conformation within seconds from their default low affinity state to high affinity state, the β1 integrins do not require a rigid control of their affinity [38].

It is still controversial whether integrin extension is required for integrin activation and signaling. A cryoelectron tomography study showed that the αIIbβ3 remains the same height.
after Mn\(^{2+}\) activation [20]. However, for particles the size of integrins, cryo-EM cannot distinguish between a particle in two different orientations or two different conformations [45]. Since preparations of integrins, including αIIbβ3, often contain a mixture of particles with different conformations [23, 42], the intermediate αIIbβ3 conformation may have resulted from averaging particles together in extended and bent conformations [24]. In addition, it has been shown that the activation of αIIbβ3 by Mn\(^{2+}\) is limited [46], and the presence of Mn\(^{2+}\) might not induce any global conformational changes, but may result in integrin aggregation, which would complicate the samples for EM study. Recently, fluorescence lifetime imaging microscopy of integrin αVβ3 on live cells indicated that integrins were in the bent conformation and no extension occurred upon activation [21]. But similar to the cryo-EM image [46], this study may just represent an average of all conformational states of integrins on the cell surface, and the method might not be sensitive enough to monitor conformational change of a small portion of integrin molecules. On the other hand, negative-staining EM studies of integrin αVβ3 [23], αIIbβ3 [42], and αXβ2 [27, 47] showed that a substantial amount of integrin molecules were in the extended conformation. Sklar and colleagues used FRET to measure the distance between an FITC-labeled ligand-mimetic peptide bound to integrin α4β1 as the donor and a plasma membrane dye as the acceptor [48], and showed that integrins extend about 50 Å converting between the resting and Mn\(^{2+}\)-activated states. This distance is much less than the fully extended conformational change, which will result in an approximately 200-Å change. Therefore, the study suggests that full extension is not necessary for integrin activation. Recently, Blue et al. [49] introduced a disulfide bond between the αIIb calf-1 and thigh domain to limit integrin extension. They showed that this mutant had reduced ability to bind large ligands, suggesting that integrin extension is important for ligand accessibility. Most interestingly, Ye et al. (2010) utilized negatively-stained EM to study the conformational states of integrin αIIbβ3 embedded in
phospholipid nanodiscs activated by the talin head domain. They showed that about 22% of integrins in nanodiscs in the presence of the talin head domain were in the extended conformation in the absence of other membrane proteins [14]. Binding of the talin head domain is sufficient to shift the equilibrium towards extension, even though not all molecules are stabilized in the fully extended state. Taken together, these studies suggest that when integrins are activated by intracellular signals, equilibrium of different integrin conformers is shifted toward a more extended state. However, the full extension of activated integrins may not be necessary for ligand binding on the cell surface.

In the present study, the inside-out activation by GAAKR mutation only slightly exposed the LIBS1 epitope (Fig. 2.4), and addition of a ligand-mimetic peptide further exposed the epitope, suggesting that this inside-out activation does not induce full extension of all integrin molecules. Inside-out activating signals may act by shifting the equilibrium, but not stabilizing integrins in the fully extended state, which represents an extreme conformation. However, this equilibrium shift is sufficient to initiate ligand binding. Regardless of the extent of integrin extension, separation of the TM/cytoplasmic tails [3, 13, 14, 50] and dissociation of the lower αβ legs as observed in the present and previous [38] studies are required for the transmission of the inside-out signals to the ligand-binding headpiece. This observation is consistent with a previous crystallography study of the complete ectodomain of integrin αIIbβ3 [42], which suggested that breathing motions at the lower α and β legs might be a pathway for integrin extension, and this motion shifts equilibrium towards a more extended and higher affinity states. EM studies of three integrin families showed that the legs of the extended integrins are often crossed at the α and β genu region [14, 23, 27, 42], suggesting that more structural assessments are needed to determine how signals are conveyed between the headpiece and the lower legs.
Outside-in signaling is induced by binding of integrins to multimeric ligands, which results in integrin conformational change and clustering, both of which are critical for signaling. A number of studies showed that binding of ligands or ligand-mimetic peptides stabilizes integrins in the more extended conformation [23, 27, 42, 47]. However, it is not known whether integrins assume a fully extended conformation on the cell surface upon ligand binding in the physiological conditions. It is evident from the present study and previous publications that dissociation of the lower αβ legs and the TM/cytoplasmic tails is required for this signal transduction.

In conclusion, our study strongly suggests that a global conformational change is required to transmit integrin inside-out activation. Introduction of a glycan chain to dissociate the αβ lower legs lead to both a high-affinity ligand binding state and a global conformational change, whereas introduction of a disulfide bond to restrict the dissociation abolishes both inside-out and outside-in signaling. Thus, this interface lies within the critical pathway of integrin bidirectional signaling.

References


CHAPTER THREE:
TEST OF THE INTEGRIN TRANSMEMBRANE DOMAIN
HOMOOLIGOMERIZATION DURING INTEGRIN
LIGAND BINDING AND SIGNALING
Introduction

Integrins are cell adhesion molecules that are essential for many biological functions such as cell migration, survival and differentiation. These functions are accomplished by integrin bidirectional signalling across the cell membrane. Inside-out activation occurs when specific intracellular molecules such as talin and kindlin bind to the integrin cytoplasmic domain, leading to the integrin conformational change and therefore high affinity for extracellular ligands. Then, binding of multimeric extracellular ligands results in outside-in signalling that is critical for many cellular processes. It has been shown that integrin bidirectional signal transduction requires integrin structural change and lateral distribution (clustering).

Integrins are type I transmembrane (TM) proteins consisting of two non-covalently associated α and β subunits, each with a large extracellular domain, a single spanning TM domain and a short cytoplasmic domain. Recent structural studies have greatly advanced our understanding of how integrin assumes conformational change during inside-out activation [1-4]. Even though relatively short, the integrin TM/cytoplasmic domains play a crucial role in this process. It has been shown that the association of α and β subunit TM/cytoplasmic domains is critical for stabilizing integrins in the resting state [5-8]. When induced by binding of the β subunit cytoplasmic domain through talin or other intracellular molecules, the TM/cytoplasmic domains separate, driving integrin extension and shifting the ligand-binding α/β headpiece to an open, high-affinity conformation [4, 9]. Recently, the structure of the TM/cytoplasmic domains in the resting state was proposed based on Rosetta computational modeling and experimental data using intact integrins on mammalian cell surface [8]. In this structure, the αIIb GXXG
motif and their β3 counterparts of the transmembrane domains associate with ridge-in-groove packing, and the αIIb GFFKR motif and the β3 Lys-716 in the cytoplasmic segments play a critical role in the α/β association. The structures of the complex and monomeric α and β subunit TM/cytoplasmic domains have also been solved by NMR [10-12]. These studies have shed light on structural basis of integrin TM/cytoplasmic domain signaling across the plasma membrane [13].

In contrast to the role of the heterodimeric TM/cytoplasmic domain association and dissociation, that of homooligomerization of integrin TM domains in integrin signaling remains elusive. In 2001, NMR study in this region by Li et al. failed to detect the heterodimeric association between the α and β subunit TM/cytoplasmic domains using TM/cytoplasmic fragment peptides in micelles. Instead, they observed that the α and β fragments tend to form homodimers and homotrimers, respectively [14]. Later, αIIb and β3 TM helices were confirmed to form homooligomers in bacterial membranes using TOXCAT assay [15, 16]. The α and β homomeric interactions have been widely studied by computational modeling [17-19]. These studies showed that the homooligomerization interface and the heterodimerization motifs largely overlap, but it seems that homomeric interaction is less specific than the heterodimeric interaction [16]. In 2003, asparagine mutagenesis study in the TM region of β3 subunit (with most experiments on the mutation β3_G708N) suggests that TM homooligomerization contributes to integrin activation and clustering [20]. However, the mutation β3_G708N, which was reported to enhance trimerization in detergent and increase ligand binding avidity in the transfected CHO cells [20], was later found to activate the integrin by changing ligand binding
affinity rather than valency [7]. Furthermore, mutations that disrupted homodimerization of integrin TM domains, which also disrupted heterodimerization since two interfaces overlap, were shown to activate integrins for ligand binding, suggesting that TM domain separation is sufficient to activate integrins [21]. Therefore, it was proposed that integrin TM homooligomerization is not a critical step for inside-out activation, but instead, it may help to stabilize the integrin in the high affinity state [15].

It has been shown that integrin outside-in signaling requires both conformational change and clustering of integrins. However, the mechanism of how integrins transmit these signals across the plasma membrane through the TM/cytoplasmic domains remains unknown. More specifically, it is unclear whether integrin TM homooligomerization plays any role in integrin clustering and signaling, even though it has been proposed that it provides structural basis for this process. Importantly, although α and β TM homooligomerization was found in micelles and bacterial cell membrane [14, 16, 20-22], it has never been observed in mammalian cell membrane using full-length integrins. In this paper, we tested whether integrin TM domains form homooligomers in mammalian cell membrane using disulfide scanning of the intact integrin αIIbβ3. Our results showed that integrin TM domains do not form homooligomers before or after soluble ligand binding or during integrin bidirectional signaling.

**Experimental Procedures**

**Plasmid Construction and Transient Transfection**

Plasmids coding for full-length human αIIb and β3 were subcloned into pEF/V5-HisA and pcDNA3.1/Myc-His (+), respectively [23]. Amino acid substitution in TM and TM proximal
regions to cysteine was made using site-directed mutagenesis with the QuikChange kit (Stratagene). Constructs were transfected into 293T cells (American Type Culture Collection, Manassas, VA) using a Fugene transfection kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

**Immunoprecipitation**

Twenty-four hours after transfection, 293T cells were treated with 20 μg/ml 2-Bromopalmitate (2-BP), metabolically labeled with \[^{35}\text{S}\] cysteine/methionine for 1.5 h before adding chase medium containing 500 μg/ml of cysteine and 100 μg/ml of methionine, and cells were cultured 17 h overnight [6]. Then cells were detached and suspended in Tris-buffered saline (TBS) (10^6 cells in 100 μl) supplemented with 5 mM Ca^{2+}, 1 mM Mn^{2+} with 3mg/ml fibrinogen or 1 mM Mn^{2+} with 50 μM RGD peptide and incubated at room temperature for 30 minutes. Then, saponin was added to a final concentration of 0.02%, and 20 μM CuSO₄/100 μM o-phenanthroline was added by 10-fold dilution from stock solutions, and cells were incubated on ice for 10 min. Oxidation was quenched by adding an equal volume of TBS containing Ca^{2+} and 5 mM N-ethyl maleimide. Cells were lysed by addition of an equal volume of 2% Triton X-100 and 0.1% NP-40 in the same buffer for 10 min on ice. Cell lysate was centrifuged and immunoprecipitated with 1 μg of anti-β3 mAb AP3 and protein G Sepharose at 4 °C for 2 h. After three washes with lysis buffer, precipitated integrin was dissolved into 0.5% SDS sample buffer and subjected to nonreducing 7.5% SDS-PAGE and fluorography [6].

For αIIb W967C mutant, 2 mM DTT was added to the \[^{35}\text{S}\]-labeled cells and incubated at 37°C for 10 min, washed twice with TBS and then oxidized by Cu-phenanthroline on ice for
10 min. Oxidation was quenched by adding an equal volume of TBS containing Ca$^{2+}$ and 5 mM N-ethyl maleimide. Cells were lysed and integrins were immunoprecipitated as described above.

To test whether integrins form homomeric disulfide bond after adhering to the immobilized fibrinogen, $[^{35}S]$-labeled cells were seeded on the surface of 6-well plates pre-coated with immobilized fibrinogen and incubated at 37°C for 1hr. After washing twice with TBS, adherent cells were incubated on ice with Cu-phenanthroline and saponin and quenched with 5 mM N-ethyl maleimide. Cells were lysed and integrins were immunoprecipitated as described above.

**Ligand-induced Binding Site (LIBS) Epitope Expression**

LIBS epitope expression was measured as described [24]. Briefly, transfected cells suspended in HBS supplemented with 5.5 mm glucose and 1% bovine serum albumin were incubated either with 5 mM Ca$^{2+}$, 3mg/ml fibrinogen or 50 μM Gly-Arg-Gly-Asp-Ser-Pro peptide (GRGDSP) in the presence of 1 mM Mn$^{2+}$ at 37°C for 15min, and then 10 μg/ml anti-LIBS1 antibody was added. After incubation on ice for 30 min, cells were washed and stained with FITC-labeled anti-mouse IgG on ice for 30 min. The stained cells were subjected to flow cytometry, and LIBS epitope expression was expressed as the percentage of MFI of anti-LIBS antibody relative to MFI of the conformation-independent anti-β3 mAb AP3.

**Cell Spreading, Integrin Clustering and Microscopy**

Glass bottom 6-well plates (MatTek Corporation, Ashland, MA) were coated overnight at 4°C with 20 μg/ml human fibrinogen in phosphate-buffered saline at pH 7.4 (PBS), followed by blocking with 1% BSA at room temperature (RT) for 1 h. The transiently transfected HEK293T cells were detached by trypsin/EDTA, washed three times with DMEM. Cells were seeded on fibrinogen-coated dishes. After incubation at 37°C for 1 hour, cells were washed 3 times with
PBS and fixed with 3.7% formaldehyde in PBS at RT for 10 minutes, or for integrin clustering, 10μg/ml anti-β3 mAb AP3 was added at room temperature for 30 minutes, followed by staining with 10μg/ml FITC-conjugated goat anti-mouse IgG for 30 minutes at room temperature before fixation. 400nM cytochalasin-D was added as control before seeding.

Differential interference contrast (DIC) imaging was conducted on a Leica TCS SP2 spectral confocal system, coupled to DM IRE2 inverted microscope with 63X oil objective. For the quantification of cell spreading area, outlines of randomly selected 100 adherent cells were generated and the number of pixels contained within each of these regions was measured using ImageJ software (Bethesda, Maryland).

**Cell Adhesion**

Cell adhesion on immobilized human fibrinogen was assessed by the measurement of cellular lactate dehydrogenase (LDH) activity as described [25]. Briefly, cells suspended in HPS supplemented with 5.5 mM glucose, 1% bovine serum albumin and 1 mm Ca²⁺ were added into flat bottom 12-well plates (1 × 10⁵ cells/well) that had been precoated with 20ug/ml fibrinogen and blocked with 1% bovine serum albumin. After incubation at 37 °C for 1 h, wells were washed three times with HBS supplemented as indicated above. Remaining adherent cells were lysed with 1% Triton X-100, and lactate dehydrogenase activity was assayed using the Cytotoxicity Detection Kit (LDH) (Roche Applied Science) according to the manufacturer's instructions. Cell adhesion was expressed as a percentage of bound cells relative to total input cells.

**Results**

**Integrin TM Domains Do Not Form Homooligomers Before or After Soluble Ligand Binding**
Previously, cysteine mutagenesis and heterodimeric disulfide scanning were used to successfully identify the integrin αIIb and β3 TM interface in mammalian cell membrane [6, 8]. Here we used the single cysteine mutations of the αIIb and β3 TM region (αIIb residues 965-995 and β3 residues 691-723, Fig. 3.1) and applied the similar method to determine if the αIIb or β3 TM domain forms homooligomers during integrin signaling. As suggested by TOXCAT assay [15, 16] and predicted by computer modeling [17-19], the αIIb TM helix forms homodimers, and the β3 TM helix forms homotrimers, with similar residues (for example GXXXG motif in the αIIb subunit) in their interface as observed in the heterodimeric interface. If this homomeric interface is actually formed in the mammalian cell membrane, we expected that some single cysteine mutations of these residues would form homodimeric disulfide bonds in the presence of an oxidation catalyst such as Cu-phenanthroline. In addition, 2-BP was used to block cysteine palmitoylation, which can inhibit disulfide formation; saponin was used to increase the permeability of Cu-phenanthroline and the efficiency of disulfide bond formation as described previously [8]. To confirm the efficiency of disulfide bond formation in the presence of Cu-phenanthroline, several cysteine pairs (one from α and the other from β) at the TM or TM proximal region, which have been shown to form heterodimeric disulfide bonds [6, 8], were used as control.

Fig. 3.1 Sequences of the αIIb and β3 TM regions. Segments predicted as TM are boxed. Residues used for cysteine scanning in this study are indicated by heavy dots.
As shown in Figure 3.2, the cysteine pair α971C/β697C formed a heterodimeric disulfide bond in the presence of Cu-phenanthroline, as described previously [6]. By contrast, none of the single cysteine mutants of αIIb TM regions formed homomeric disulfide bonds under these conditions except for αIIb W967C (Fig. 3.2). As reported previously [6, 7], αIIb W967C, when cotransfected with wild-type β3, formed a homomeric disulfide bond in the resting state, and therefore, the αIIbβ3 integrin associated to form a disulfide-bridged tetramer (αIIbW967C/β3)_2. This residue is located outward, away from the heterodimeric interface or predicted homodimeric interface [6, 7]. When DTT was added, the disulfide bond was reduced. After DTT was removed and Cu-phenanthroline was added to catalyze disulfide formation, only trace amount of homomeric disulfide was formed on the cell surface (Fig. 3.3A). We further traced the disulfide-bond formation of αIIb W967C mutant after ^35S_ labeling by lysing cells and immunoprecipititating the protein at different time points (Fig. 3.3B). After 30 minutes of labeling, αIIb precursor was formed. Then after 1 hour, mature αIIb subunit was formed and trace amount of disulfide-bonded αIIb was observed. In 1.5 hours, a significant amount of disulfide-bonded αIIb was formed. This suggests that the homomeric disulfide bond is formed during biosynthesis and post-translational modification. Since this disulfide bond of the αIIb mutant is formed during biosynthesis and in the resting state in which heterodimeric TM association is not affected, we excluded this mutant for the following studies. Except for this cysteine mutant, none of the other 30 αIIb cysteine mutants formed a homomeric disulfide bond (Fig. 3.2), neither did any of the 34 β3 cysteine mutants (Fig. 3.4). These results suggest that the αIIb and β3 TM helices do not form homomeric association when αIIbβ3 is in the resting state.
Figure 3.2 Integrin αIIb TM domains do not form homooligomers before and after soluble ligand binding. Except for αIIbW967C, none of the cysteine mutants of the αIIb TM regions formed homomeric disulfide bonds before or after soluble ligand binding.
Figure 3.3 The homomeric disulfide bond of the αIIbW967C was formed during biosynthesis. A. Formation of the homomeric disulfide bond after DTT treatment. The $^{35}$S-labeled cells were treated with or without 2 mM DTT at 37°C for 10 min. After washing with TBS, DTT treated cells were incubated with or without Cu-Phenanthroline. B. Tracing the formation of disulfide bond of αIIb W967C mutant after labeling. The cells were lysed at different period of time after $^{35}$S-labeling and integrin αIIbβ3 was immunoprecipitated and subjected to 7.5% non-reducing SDS-PAGE.
Figure 3.4 Integrin β3 TM domains do not form homooligomers before and after soluble ligand binding. None of the cysteine mutants of the β3 TM regions formed homomer disulfide bonds before or after soluble ligand binding.
To determine if the $\alpha$ and $\beta$ TM domains form homooligomers after soluble ligand binding, we used Mn$^{2+}$ to activate integrin $\alpha$IIb$\beta$3 followed by binding of soluble fibrinogen and ligand-mimetic RGD peptide. It has been shown that ligand binding induces integrin conformational changes that expose the LIBS (for Ligand-Induced Binding Site) epitopes, which are located at the interfaces between the headpiece and tailpiece and between the $\alpha$ leg and $\beta$ leg so that they are buried in the bent conformation but exposed in the extended conformation [26, 27]. We used anti-$\beta$3 LIBS mAb LIBS1 [28] to determine the conformational states of the $\alpha$IIb$\beta$3 in the presence of Mn$^{2+}$ with fibrinogen or RGD. The LIBS1 bound poorly to wild type $\alpha$IIb$\beta$3 in Ca$^{2+}$ (Fig. 3.5), suggesting that it is in the bent conformation. The binding significantly increased when fibrinogen with Mn$^{2+}$ or RGD with Mn$^{2+}$ were added (Fig. 3.5), suggesting that these ligands bound to integrins, and they stabilized integrins in the more extended conformation. Disulfide scanning was used to test whether $\alpha$ and $\beta$ TM domains form homomeric interface after integrin bound to soluble fibrinogen and RGD peptide under the same condition, and no homomeric disulfide formation was observe for any mutants except the $\alpha$W967C as described above (Fig. 3.2, 3.3, 3.4), suggesting that after ligand binding, even though integrins are stabilized in the more extended state and probably with two separating legs and TM/cytoplasmic tails, homooligomerization does not occur for TM/cytoplasmic tails in mammalian cell membrane.
Fig. 3.5 Exposure of the LIBS1 epitope. LIBS epitope exposure was determined as the percentage of MFI of anti-LIBS1 antibody relative to non-functional anti-\(\beta3\) mAb AP3. Error bars are standard deviation (SD).

**Integrin TM Domains Do Not Form Homooligomers During Inside-out Signaling**

Upon the stimulation of external agents such as thrombin and ADP, the intracellular molecules (talin, kindlin etc.) can bind to the cytoplasmic domain of integrins, disrupting the association of cytoplasmic domains and further triggering a cascade of inside-out signaling events [29, 30]. Previously we have shown that dissociation of the TM heterodimers is critical for integrin activation [6]. In addition to the TM heterodimeric association and dissociation, the TM homomeric association was proposed to play an important role in this process [20, 21]. Here, we designed experiments to test whether integrin TM region form homooligomers during inside-out activation. It is well known that the GFFKR motif in the \(\alpha\)IIb cytoplasmic domain is crucial for maintaining integrin in the resting state. When the two phenylalanines of the GFFKR motif are mutated to alanines (GAAKR mutant), integrins are activated to bind ligands with high affinity. Thus, this GAAKR mutant can be used to mimic integrin inside-out activation [6]. We confirmed that the mutant bound fibrinogen constitutively; in addition, it bound LIBS1 much
better than the wild type in Ca\textsuperscript{2+} condition (Fig. 3.5), indicating that the mutation shifts integrin towards more extended conformation, well mimicking inside-out signaling. We made 6 single cysteine mutants using this GAAKR construct, and co-transfected with the wild-type β3 integrin. None of the αIIb integrin cysteine mutants formed a homomeric disulfide bond (Fig. 3.6A). Similar results were obtained when 34 β3 single cysteine mutants were co-transfected with the αIIb GAAKR mutant; none of these mutants formed a homomeric disulfide bond (Fig. 3.6B). These results strongly suggest that during integrin inside-out activation across the mammalian cell membrane, TM homomeric association is not formed. Therefore, the TM homooligomerization does not play any role in this process.

![Figure 3.6 Integrin TM domains do not form homooligomers during inside-out activation. A. Cysteine mutations of the αIIb GAAKR mutant were co-transfected with wild type β3 integrin. No homomeric disulfide bond was formed for any of the αIIb cysteine mutants. B. Cysteine mutations of the β3 TM region were co-transfected with the αIIb GAAKR mutant. No homomeric disulfide bond was formed for any of the β3 cysteine mutants.](image-url)
**Integrin TM Domains Do Not Form Homooligomers After Adhering to Immobilized Fibrinogen**

After binding to immobilized ligands, integrins on the mammalian cell surface will transmit outside-in signaling. We carried out a series of experiments to address whether TM homomeric association is formed during this process. We randomly chose two αIIb and two β3 cysteine mutants and used them for cell adhesion and cell spreading assay. HEK293T cells transiently transfected with wild-type and mutant αIIbβ3 were seeded on fibrinogen-precoated dish surfaces at 37°C for 1 h. The amount of adherent cells was assessed by quantifying the cellular lactate dehydrogenase (LDH) activity. All selected mutants could adhere to the immobilized fibrinogen similarly to the wild-type receptor, whereas the disulfide bounded mutant (α968C/β693C) showed much less adhesion, and DTT treatment which was used to disrupt the disulfide bond was able to recover its adhesion to the similar level to the wild-type with DTT (Fig. 3.7A). To test if the single cysteine mutations could affect cell spreading, HEK293T transient transfectants were coated on the immobilized fibrinogen at 37°C for 1 h, followed by fixation and microscopic analysis. Cells transfected with all single cysteine mutants demonstrated cell spreading to the similar level to those transfected with the wild-type receptor. By contrast, the disulfide bounded mutant (α968C/β693C) showed no spreading, and DTT treatment restored cell spreading (Fig. 3.7B-C). These results confirmed that dissociation of the TM heterodimers is required for integrin outside-in signaling as described previously using CHO cell transfectants [25]. It also showed that the single cystein mutations in this region do not affect the integrin adhesion to immobilized fibrinogen, nor do they affect outside-in signaling.
Figure 3.7 Cell adhesion and spreading of randomly selected TM cysteine mutants. A. Adhesion of HEK293T transfectants in the presence of 1mM Ca\(^{2+}\) to surfaces coated with 20 µg/ml fibrinogen. The amount of bound cells was determined by measuring LDH activity as described in Materials and Methods. Data are representative of three independent experiments, each in triplicate. B. DIC images of HEK293T transfectants after adhering to immobilized fibrinogen at 37°C. The images are representatives of three independent experiments. Scale bar represents 10 µm. C. Quantification of the areas of adhering/spreading cells as described in Materials and Methods. Error bars are SD. *** P <0.001.
It is widely believed that lateral association (i.e. “clustering”) of integrin heterodimers, which occurs as a consequence of multivalent ligand binding [31, 32], plays a major role in outside-in signaling (see review [33]). It was also shown that ligand binding can directly lead to and stabilize separation of integrin cytoplasmic domains [34], and this integrin conformational change is critical for outside-in signaling as well [25]. However, it remains unclear whether the integrin TM homomeric interaction after the TM helix separation is critical for integrin clustering. To assess formation of integrin clustering, HEK293T transfectants were allowed to adhere to fibrinogen-coated substrates followed by fixation and staining with fluorescent anti-\( \alpha\)IIb\( \beta\)3 antibodies. Under these conditions, wild-type integrins and all cysteine mutants could be readily detected in clustered patterns (Fig. 3.8). Interestingly, although disulfide bounded mutant is defected with cell spreading, it formed the similar cell clustering as others, in contrast to the negative control in which cytochalasin-D was used to disrupt the actin polymerization linked integrin clustering (Figs. 3.7-3.8).

We then further determined, under the same condition, whether the integrin TM domains formed homooligomers in the plasma membrane. HEK293T cells transfected with a variety of \( \alpha\)IIb and \( \beta\)3 cysteine mutants were seeded on pre-coated fibrinogen surface at 37°C for 1 hr. After cells fully adhered and spread on immobilized fibrinogen, Cu-phenanthroline was added to catalyze disulfide bond formation. Four cysteine mutation pairs, \( \alpha\)968C/\( \beta\)693C, \( \alpha\)971C/\( \beta\)697C, \( \alpha\)972C/\( \beta\)697C and \( \alpha\)955C/\( \beta\)723C, were used as control to confirm the efficiency of oxidation. Under this condition, four cysteine pairs formed disulfides efficiently. By contrast, none of the \( \alpha\)IIb or \( \beta\)3 TM cysteine mutations formed a homomeric disulfide bond (Figs. 3.9). Since these integrins adhered to the immobilized fibrinogen, clustered on the cell surface and transmitted outside-in signaling leading to cell spreading, the results suggest that homomeric association of
the integrin TM domains is not important for integrin functions. Similar results were obtained when CHO cells were used (data not shown), suggesting that integrin TM/cytoplasmic domains do not form homomer interaction during integrin outside-in signaling in mammalian cells.

**Figure 3.8 Integrin clustering of selected TM cysteine mutants.** Confocal microscopy studies of integrin clustering on the cell surface. Cells expressing αIIbβ3 wild type and mutants were seeded on fibrinogen-coated surface for 1h at 37°C in the presence or absence of cytochalasin-D (Cyto D). Attached cells were then stained with anti-β3 mAb AP3 in the presence or absence of Cyto D for 30 minutes at room temperature, followed by staining with FITC-conjugated goat anti-mouse IgG for 30 minutes at room temperature. After fixation, cells were subjected to confocal microscopy.
Figure 3.9 Integrin TM domains do not form homooligomers after cell adhering to the immobilized fibrinogen. A. None of the αIIb cysteine mutants formed homomeric disulfide bond after adhering to the immobilized fibrinogen. B. None of the β3 cysteine mutants formed homomeric disulfide bond after adhering to the immobilized fibrinogen.
Discussion

It has been shown that TM heterodimeric association stabilizes integrins in the resting state; when integrins are activated by intracellular signals, two TM/cytoplasmic tails separate, leading to conformational change of the ligand-binding extracellular regions, resulting in high affinity for ligands [6-8, 12, 35, 36-39]. Therefore, equilibrium between the dissociated monomers and associated heterodimers of the TM domains is critical for integrin inside-out activation. Based on the observation that integrin TM helices formed homooligomers using recombinant peptides in detergent [14, 20, 22], the GALLEX assay in bacteria [39], the TOXCAT assay in bacteria [15, 16] and the computational modeling [17-19], TM homomeric association has been suggested to be important for integrin activation [20-22]. Mutagenesis studies on the proposed TM homomeric interface residues showed that these mutations could lead to integrin activation, suggesting that heterodimer dissociation is sufficient to activate integrins [21]. Therefore, a push-pull model was proposed in which after dissociation of the TM heterodimer, integrins are stabilized by TM homomeric association [21].

Here our experiments on mammalian cell transfectants using full-length integrin αIIbβ3 showed that the αIIb and β3 TM domains do not form any homomeric association in the resting state, nor do they form after soluble ligand binding. The only mutant that formed a homomeric disulfide is αIIb_W967C, as reported previously [6, 7]. We found that this homomeric disulfide was formed during biosynthesis and in the integrin resting state, and dissociation of the TM heterodimer is not required for its formation. Therefore, we do not think that the same homomeric association occurs during integrin inside-out activation or outside-in signaling. We found that except for this mutant, none of the other 30 αIIb and 34 β3 cysteine mutants formed a homomeric disulfide bond before or after binding to soluble ligands. In addition, during integrin inside-out activation, the integrin TM domains do not form any homomeric association that can be detected by disulfide scanning. Integrins must be regulated to be activated and de-activated
quickly in mammalian cells, even within seconds. It has been shown that detachment of integrin from ligands in the trailing edge is critical for integrin functions [40], and mutations that constitutively active integrins result in malfunctions [41-44]. Therefore, it is unlikely that integrin TM domains form more stable homomeric association during inside-out signaling.

Although integrin clustering is known to participate in integrin signaling pathways [45-48], the exact mechanisms of integrin clustering remain elusive. One mechanism proposes that the integrin TM homooligomerization promotes integrin clustering [20]. Studies have established that extracellular ligand binding triggers integrin conformational changes that promote oligomer assembly through TM fragments [49-51]. In this study, we observed that after adhering to immobilized fibrinogen integrins clustered on the cell surface, but we did not detect any integrin TM homomeric interaction under this condition. Interestingly, the heterodimeric disulfide-bonded integrin formed clusters, suggesting that even the dissociation of integrin α/β TM domains is not required for integrin clustering. Therefore, our study suggests that integrin clustering is induced by bringing several integrins physically close together by multimeric ligands, and the TM homomeric association is not required for this process.

Even though the TM separation is not required for integrin clustering, it is critical for integrin outside-in signaling [25]. We showed that all cysteine mutants could adhere and spread on the fibrinogen-coated surface, suggesting that they could transmit signals into the cells after binding to immobilized ligands. However, we did not observe any TM homomeric disulfide formation in the same condition, indicating that the TM homomeric association does not occur during integrin outside-in signaling. The reason why integrin outside-in signaling requires the TM separation but not homomeric association is unknown. Based on the previous FRET studies [34], we assume that ligand-induced TM separation is likely coupled to cytoplasmic domain separation, implying that α-β cytoplasmic domain interactions somehow constrain or inhibit kinase activation [7, 25, 29, 34, 52]. One possibility is that separation of TM and cytoplasmic
domains induced by multimeric ligand binding is required for kinases or other intracellular proteins to bind integrin cytoplasmic tails. On the other hand, some studies have shown that certain tyrosine kinases, such as Src, associate constitutively with integrin αIIbβ3 in platelets, and platelet adhesion to fibrinogen causes a rapid increase in Src activation [53]. Thus, another possibility is that the association of the integrin TM and cytoplasmic domains somehow constrains the activity of integrin tail-bound kinases, whereas dissociation of the TM and cytoplasmic domains releases these constraints. In either case, binding of multimeric ligands brings integrins together, and at the same time, induces dissociation of the TM/cytoplasmic domains, resulting in kinase auto-phosphorylation and activation. The current study strongly suggests that during this process, the TM homomeric association does not play any role in kinase activation.

In conclusion, our study shows that the integrin TM homomeric association does not occur in mammalian cell membranes before or after soluble ligand binding, during inside-out activation or outside-in signaling. Therefore, conformational change induced by intracellular signals or extracellular ligands, more specifically, separation of TM and cytoplasmic domains, but not the homomeric association is critical for integrin bidirectional signaling.

References


CHAPTER FOUR:
SUMMARY AND GENERAL DISCUSSION
Integrin signaling is often accompanied by its conformational rearrangement. Understanding the biologically relevant conformational changes is important to unravel the mechanisms of integrin signaling, and to further provide effective cues for drug design for integrin-involved diseases. Although significant progress has been made in integrin structural studies and its conformational changes in the past decade, many aspects, especially the conformational alteration coupled with bidirectional signaling, need to be further investigated in more details.

To address the roles of integrin lower leg in integrin signaling, we have introduced a disulfide bond and two glycosylation sites into the interface between the α-subunit Calf-2 domain and β-subunit I-EGF4 domain to either clasp or disrupt the association of this region. Then we determined the effect of these mutants on ligand binding and outside-in signaling. In addition, conformational states and cell adhesion were also tested (Chapter 2). Our results showed that restricting the association by disulfide bond abolished the inside-out activation and outside-in signaling, whereas disrupting the interface by glycosylation mutations activates the integrins by a global conformational change. Therefore, separation of integrin lower leg is required for inside-out activation and outside-in signaling.

In chapter 3, we used cysteine scanning mutagenesis method to test the possibility of homomeric interaction under the context of the full length integrin in mammalian cells under physiological and activating conditions. Our results showed that TM domains do not form homooligomers in the mammalian cell membrane, which is in contrast with previous data obtained in micelles and bacterial membranes in which the homooligomers were observed using TM fragments. Further studies showed that disulfide-bonded integrin heterodimer is able to aggregate to form clusters, even though its bidirectional signaling is blocked. It is generally believed that integrin outside-in signaling is controlled by conformational change and clustering. Our results show that integrin TM homomeric association does not play any role in integrin
clustering. Furthermore, the integrin TM domains do not form homooligomers after adhering to immobilized ligands. Taken together, the homooligomerization through TM helices is not important for αIIbβ3 inside-out activation and outside-in signaling. We think that binding of multimeric ligands brings integrins physically close to each other, leading to clustering; at the same time, ligand binding triggers integrin leg separation, resulting in kinase activation.

For extracellular domains, two models have been proposed to address the possible conformational changes upon inside-out activation: The “switchblade” model posits the full extension is necessary for ligand binding, whereas in the “deadbolt” model, the extension is the post-ligand binding event. Both models have received various supports. Studies have established that integrins can exist in several states: low, intermediate, and high affinity states. The coexistence of the two conflicting models may suggest that some intermediate conformations could be enough to fulfill the mission of ligand binding at least in some signaling occasions. Additional characterization of the biologically relevant conformational change is needed to fully understand the story.

Currently, we are designing the experiments to examine these two models using FRET. A FITC or Alexa Fluor®488 labeled antibody that binds to integrin headpiece and a dye that labels the plasma membrane will be used as a donor-acceptor pair to determine the distance between integrin headpiece and the plasma membrane using wild type and various mutant integrins under physiological and activating conditions. Since this distance reflects the extent of integrin extension, with different constructs under various conditions, we will be able to determine the dynamics of conformational change in different scenarios. The FRET will also be used under the conditions of inside-out activation, in which the talin head domain will be co-transfected with the integrins, or the external activators such as ADP and thrombin will be added to induce the platelet activation. No change of FRET signal upon addition of the activators would support the
deadbolt model; while a loss of FRET signal would be consistent with the switchblade model; a FRET signal change in between would lead us to propose a new model.

As far as the TM and cytoplasmic domains are concerned, although it is known that the separation of TM is required for integrin signaling, the detailed mechanism needs to be further investigated. Studies have shown that separation of the two associated subunits is caused by either talin binding to two positions of integrin cytoplasmic fragments in succession or by talin binding to cytoplasmic domain and inner membrane respectively. However, it was also proposed that the lateral force caused by actin polymerization results in TM separation. Interestingly, it was found that β TM fragment is tilted in the membrane bilayer in the active state. The tilting of β TM domain may play an important role in the activation. More research is needed to define this phenomenon. In addition, β cytoplasmic domains were considered as the scaffold for integrin signaling, since a variety of intracellular proteins are directly or indirectly involved in connection with this region. Much work remains to be done to uncover the intracellular signaling events.

In conclusion, our work presented in this dissertation has shed new light on the mechanism of how integrins transmit signals bidirectionally across the plasma transmembrane. However, many signaling events coupled with conformational rearrangement remain unclear. Future investigation is necessary to advance our understanding of integrin signaling.
VITA

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