Structural Studies of Talin-Mediated Integrin Activation

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STRUCTURAL STUDIES OF TALIN-MEDIATED INTEGRIN ACTIVATION

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To my mother, Ayse, my brothers and sisters for their steady love and support during my education. To my husband, Bakytzhan and to my son, Mesud.
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ABSTRACT

Activation of heterodimeric (α/β) integrin transmembrane receptors by the cytoskeletal protein talin is essential for many important cell adhesive responses including cell-extracellular matrix contact, cell motility and survival. A key step in this process involves interaction of phosphotyrosine-binding (PTB) domain in the N-terminal head of talin (talin-H) with integrin β3 membrane-proximal cytoplasmic tails (β-MP-CTs). Compared to talin-H, intact talin exhibits low potency in inducing integrin activation. Using TROSY NMR spectroscopy, we found that the large C-terminal rod domain of talin (talin-R) interacts with talin-H and allosterically restrains talin in a closed conformation. Structural, mutational, and biochemical analyses demonstrate that talin-R specifically masks a region in talin-PTB where integrin β3-MP-CT binds and competes with it for binding to talin-PTB. The inhibitory interaction is disrupted by a constitutively activating mutation (M319A) or phosphatidylinositol 4,5-bisphosphate – a known talin activator. These data define a distinct autoinhibition mechanism for talin and suggest how it controls the dynamics of integrin activation and cell adhesion.
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ABBREVIATIONS USED

FERM domain, 4.1 protein, Ezrin, Radixin, Moesin domain

PTB domain, Phosphotyrosine Binding Domain

PIP2, Phosphatidylinositol 4,5-bisphosphate

PIPKI, Phosphatidylinositol Kinase I

ECM, Extracellular Matrix

CHO cells, Chinese Hamster Ovary cells

FACS, Fluorescence-Activated Cell Sorting

EGFP, Enhanced Green Fluorescent Protein

NMR, Nuclear Magnetic Resonance

HSQC, Heteronuclear Single Quantum Coherence

TROSY, Transverse relaxation optimized spectroscopy

SPR, Surface Plasmon Resonance

RIAM, Rap1-GTP-Interacting Adaptor Molecule
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CHAPTER I
INTRODUCTION

PART A. INTEGRIN ACTIVATION

Integrins

Integrins, heterodimeric cell surface receptors, are vital adhesion molecules that mediate cell-extracellular matrix (ECM) and cell-cell interactions for the homeostasis of animal species from drosophila to human (Hynes, 2002). They have critical roles in cell adhesion and migration which are crucial for embryonic development, hemostasis and angiogenesis. For example; in the presence of injury, the body’s primary action is to stop the bleeding by mediating platelet adhesion and aggregation through activating the fibrinogen receptors on platelets, integrin αIIbβ3. Also, during development migrating cells require activated integrins to be localized at the leading edge of the cell for the formation of proper cell adhesion. Similarly, during inflammation leukocytes adhere to and migrate across the endothelium through integrin activation (Moser et al., 2009).

Integrin family has more than 20 members and all of their structure and ligand binding mechanism exhibit similarity. Integrins are formed by non-covalent α and β heterodimers. The β1 chains, which form dimers with at least 9 distinct α chains, are
found to be on almost all vertebrate cells. \( \alpha 5\beta 1 \), for example is a fibronectin receptor, and \( \alpha 6\beta 1 \) is a laminin receptor on many type of cells. In contrast, \( \beta 2 \) chains form dimers with 4 types of \( \alpha \) chains, and expressed on the surface of white blood cells. Their role is mainly to mediate cell-cell, rather than cell-matrix interactions. The \( \beta 2 \) integrins enable white blood cells to attach firmly and cross the endothelium to reach at the sites of infection. Humans with leukocyte adhesion deficiency syndrome are unable to synthesize \( \beta 2 \) receptors in their white blood cells; as a consequence, they suffer from repeated bacterial infections leading to early death (Hynes, 2002). \( \beta 3 \) integrins are found on a variety of cells, including platelets which interact with fibrinogen during blood clotting, and humans with Glanzman’s disease, who are genetically deficient in \( \beta 3 \) integrins, suffer from severe bleeding (Hodivala-Dilke et al., 1999).

**Integrin Structure**

Integrins \( \alpha \) and \( \beta \) subunits have a large (more than 700 residue) N-terminal extracellular domain, a short (approximately 20 residues) transmembrane domain, and a short (13-70 residues) cytoplasmic tail (Figure1.1.). The crystal structures of the extracellular domain of \( \alpha \gamma \beta 3 \), without and with a bound RGD peptide have provided valuable information about integrin structure (Xiong et al., 2001; 2002). The extracellular domain of the \( \alpha \) subunit has a seven-bladed \( \beta \)-propeller domain, a thigh domain, and calf-1 and calf-2 domains. Some of \( \alpha \) integrins have an I domain which contains a conserved metal ion-dependent adhesion site (MIDAS) to bind divalent cations including \( \text{Ca}^{2+} \), \( \text{Mg}^{2+} \), and \( \text{Mn}^{2+} \) for proper integrin-ligand interaction. The \( \beta \) subunit is composed of an A domain,
Figure 1.1. *Structural organization and activation of integrins.* The crystal structure of the extracellular domain, a molecular model of the transmembrane region and NMR structures of the cytoplasmic tails have been adjoined to create a depiction of $\alpha_{IIb}\beta_3$. The equilibrium between a bent and an extended form of integrins are illustrated. The figure and legend is adapted from Ma et al, 2007.
which is the analog of the I domain of the \( \alpha \) integrin subunit, hybrid domain, PSI (plexin/semaphoring/integrin) domain, 3 EGF (epidermal growth factor) domains and a membrane-proximal \( \beta \) tail domain (\( \beta TD \)). The extracellular domain of \( \alpha/\beta \) heterodimer has a ligand binding domain and two legs. The ligand binding domain has a head-like structure and it is formed by the interaction between seven-bladed \( \beta \)-propeller of the \( \alpha \) subunit and the \( \beta A \) domain of the \( \beta \) subunit. The integrin \( \alpha v\beta 3 \)-ligand RGD peptide-Mn\(^{2+} \) ion complex structure reveals that ligand binds to an interface between \( \alpha \beta \) subunits, and has contact with a metal-occupied MIDAS in the integrin head. Both structural and biochemical analyses have provided snapshots of different conformational changes of integrins that occur during the transition from inactive to active state. In the resting state, integrins are in inactive state where they have a bent conformation. Agonist stimulation initiates structural changes across the whole integrin which leads to the conversion of the bent form of the integrins to an extended form with a higher-affinity ligand binding.

The structure of the short transmembrane (TM) domains is well-defined after two recent publications of the complex structure of the integrin \( \alpha IIb\beta 3 \) TM domain (Lau et al., 2009; Yang et al., 2009). The \( \alpha IIb \) TM domain is a 24-aa straight helix which occupies the whole plasma membrane, whereas the \( \beta 3 \) TM domain, a 30-aa a helix, is supposed to be tilted within the plasma membrane due to its longer helix length compared to membrane width. Both NMR structures of the integrin \( \alpha IIb\beta 3 \) TM domain (Lau et al., 2009; Yang et al., 2009) are in agreement on TM assembly but differ in CT portion. On the basis of the heterodimeric \( \alpha IIb\beta 3 \) TM structures, in the resting state, associated TM helices are stabilized by dimer interface. Either extracellular or intracellular signaling
destabilizes TM dimer interface, shifting the equilibrium to the dissociated state and allowing the extracellular domains to rearrange its conformation for high-affinity ligand binding.

In the integrin family, short α and β cytoplasmic tails show a high sequence homology, especially in the membrane proximal region. Nuclear magnetic resonance (NMR) studies revealed that a complex forms between αIIb and β3 CT membrane proximal helices (Vinogradova et al., 2002). This interaction is stabilized by hydrophobic and electrostatic interactions and a salt bridge between αIIbR995 residue and β3D723 residue. The mutational analyses confirm the existence of the clasp between two integrin subunit regions, which stabilizes the inactive integrins in the resting state. However, these interactions were not detected by others, suggesting that the clasp between the MP αIIb and β3 CT helices must be weak (Ulmer et al., 2001). The membrane-distal region of αIIbCT has a divalent ion binding site (Haas et al., 1996) and a turn formed by NRPP motif (Vinogradova et al., 2000). However, further investigation is needed to understand the significance of these structural elements. Almost all β tails have highly conserved two NXXY motifs which serve as recognition sites for proteins containing phosphotyrosine binding (PTB) domains (Calderwood et al., 2003). Integrin-binding proteins including talin and kindlins interact specifically with these motifs and regulate integrin activation.

**Regulation of outside-in and inside-out integrin signaling**

Integrin activation is known to be bidirectional, from outside-in and inside-out depending on intracellular and extracellular signals. To accomplish their bidirectional
functions, integrins are always in equilibrium between resting and activated states, and a series of coordinated and linked conformational changes take place during the transition from low-affinity to high-affinity state. Agonist-dependent intracellular signals initiate the “inside-out signaling” whereas extracellular ligand binding to integrins promotes “outside-in signaling”.

**Inside-out Signaling**

During inside-out signaling, integrin activation is tightly regulated by the changes in interactions and in the structures of the integrins. In the low affinity (inactive) state, integrins are associated by a ‘clasp’ between α and β subunits, thus preventing integrin activation. The clasp between membrane-proximal regions of α and β subunits has been shown to be stabilized by a salt bridge between a conserved arginine residue of α tail and an aspartate residue of β tail (Vinogradova et al., 2002). The integrin cytoplasmic tails also play an important role in regulating integrin affinity, especially by binding to cytoplasmic adaptor protein, talin (Calderwood et al., 1999; 2002; Tadokoro et al., 2003). Upon agonist stimulation, talin relieves from its autoinhibitory interaction, and only talin F3-PTB subdomain binds integrin β3 tails and results in integrin activation by a two-step interaction. In the first step, talin-PTB domain engages the membrane-distal (MD) part of the β3 integrin tail, which becomes ordered, but the α-β clasp remains intact. In the subsequent step, PTB engages the membrane-proximal part of the β3 tail, while maintaining the MD interaction (Wegener et al., 2007). This interaction destabilizes the clasp, relieving the salt bridge, and leads to additional electrostatic interactions between membrane lipids and F3 subdomain. The unclasping of integrin α and β tails leads to
separation of integrin TM domains, and formation of homomeric dimers and trimers of \( \alpha \) and \( \beta \) transmembrane helices (Li et al, 2001; 2003). Although there is no structural evidence, several biochemical studies suggest that homo-oligomerization in transmembrane domains provide a driving force for integrin activation and clustering (Li et al, 2004; Li et al, 2005).

**Outside-in Signaling**

Outside-in signaling is initiated by binding of adhesive ligands such as fibrinogen, von Willebrand factor (VWF) and fibronectin to integrin extracellular domain. These interactions lead to further conformational changes that are transmitted through transmembrane region to the cytoplasmic tails that causes irreversible integrin clustering. Structural and biochemical studies have revealed that in the resting platelets, Src and its regulatory kinase, Csk, are bound to \( \alpha \text{IIb}\beta3 \), and upon ligand binding, Csk dissociates from \( \alpha \text{IIb}\beta3 \), enabling Src and Syk protein tyrosine kinases to become activated (Obergfell et al., 2002). This results in the phosphorylation of downstream adaptor and effector molecules for actin polymerization. Thus, outside-in signaling is initiated by the activation of Src and Syk kinases, and further signaling is generated from \( \alpha \text{IIb}\beta3 \) to actin cytoskeleton for platelet activation. More biochemical and structural input is required for a better understanding of the outside-in signaling mechanism.

**Talin and its functions**

Talin is a high molecular weight cytosolic protein that was initially purified from human platelets and chicken gizzard (Molony et al., 1987). Discovered in high
concentrations at regions of cell-substratum contact (Burridge and Connell, 1983), talin has long been known to be a physical linker between the integrin and the actin cytoskeleton, which regulates a variety of cellular processes such as cell spreading, cell migration, and cell proliferation (Turner and Burridge, 1991; Calderwood et al., 2000). Recent advances have revealed an additional and especially important role for talin, by binding to integrin β CTs, talin was found to induce high affinity ligand binding of integrins, i.e., integrin activation (Tadokoro et al., 2003; Wegener et al., 2007) - a major step in integrin-mediated cell-ECM adhesion, migration, and numerous physiological and pathophysiological responses (Hynes, 2002).

Gene expression analysis revealed that there are two talin genes (Tln1 and Tln2) in vertebrates encoding 74% identical proteins (Monkley et al., 2001; Senetar et al., 2005). Although Tln2 is larger and have more complex gene than Tln1, most studies have focused on Tln1. Disruption of Tln1 gene in mice have arrested the gastrulation stage of the embryonic development due to absence of cell spreading, and focal adhesion (FA) assembly (Monkley et al., 2000), whereas N-terminal half of Tln2 is sufficient for embryonic development and mouse survival (Chen et al., 2005). Disruption of both Tln1 alleles in mouse embryonic stem cells have confirmed the previous knock-out studies that talin1 is required for proper cell spreading and FA assembly (Priddle et al., 1998). A recent talin1 depletion study revealed that only full-length talin can provide a physical linkage between the extracellular matrix and the actin cytoskeleton required for FA assembly and signaling (Zhang et al., 2008).
Talin Structure

Talin is a high molecular weight protein containing an N-terminal head (1-433, talin-H, 50 kDa) and a C-terminal rod domain (434-2541, talin-R, 220 kDa) (Figure 1.2.) in an equilibrium between monomeric and dimeric forms (Rees et al, 1990). Talin-H is globular, containing a FERM (41, ezrin, radixin, moesin) domain composed of three separate lobes, F1, F2, and F3 and an F0 subdomain with no homology to known domains. The F3 subdomain resembles a phosphotyrosine-binding (PTB) domain and binds NPxY motif on integrin β tails (Garcia-Alvarez et al., 2003). The attempts to crystallize the entire FERM domain was unsuccessful probably due to 30-aminoacid unstructured loop of F1 domain. The loop contains two phosphorylation sites (T144/T150) in platelet talin (Ratnikov et al., 2005), and psychological importance of these phosphorylations is not known. The linker region (401-481) between head and rod domains is cleaved in the intact talin by different proteases; the best known is the calpain-II (Rees et al., 1990). Although secondary structure predictions show the linker largely unstructured, numerous phosphorylation sites have been detected (Ratnikov et al., 2005), and how these phosphorylations affect the intact talin structure and functions needed to be studied. Talin-Rod is highly elongated, containing a series of helical bundle structures separated by linkers (McLachlan et al., 1994; Papagrigoriou et al., 2004; Fillingham et al., 2005; Gingras et al., 2008). Both the N-terminal region of the rod and the C-terminal half of the rod is made up of a series of five-helix bundles (Papagrigoriou et al., 2004; Gingras et al., 2008). However, the globular structure of the middle segment between two ends requires further investigation.
**Figure 1.2.** Domain structure of talin. The N-terminal talin head (1-400) contains a FERM domain comprising F1, F2, and F3 domains preceded by the F0 domain. The talin rod (482-2541) contains 62 amphipathic α-helices, the most C-terminal of which is required for talin dimerization (blue). The position of various ligand-binding sites is indicated, including the intramolecular interaction between F3 and the talin rod. Helices containing vinculin binding sites (VBS) are numbered-helices 4, 12, and 46 contain VBS1-3. The figure and legend is adapted from Critchley DR, 2009.
Binding Partners of Talin

The F3 or PTB domain binds the cytoplasmic tails of β-integrins (Calderwood et al., 2002), phosphotidylinositol 4-phosphate 5-kinase γ (PIPKγ) (Ling et al., 2002; Di paolo et al., 2002) and the hyaluronan receptor layilin (Wegener et al., 2008), whereas rod domain has multiple vinculin binding sites (Gingras et al., 2005), a recently identified a-synemin-binding site (Sun et al., 2008), two actin-binding sites (Hemmings et al., 1996) and a second integrin binding site (Xing et al., 2001; Tremuth et al., 2004) (Figure 1.2.). The C-terminus of the rod domain contains I/LWEQ motif that mediates the dimerization of the talin and facilitates the linkage between talin and F-actin (Gingras et al., 2008; Smith et al., 2007). The talin-head also contains an F-actin binding site (Lee et al., 2004), and recent studies have identified Wech proteins which are crucial for the integrin-cytoskeleton link (Loer et al., 2008). Finally, the talin head binds phospholipids and inserts into lipid bilayers (Goldman et al., 1992; Heise et al., 1991).

Regulation of talin-mediated integrin activation

Since integrin activation is tightly regulated, talin-integrin interaction has to be strictly controlled by different cellular mechanisms. Several studies show that there may be two or more mechanisms, which include autoinhibition of talin, phosphorylation, and regulatory interactions with different proteins, for the regulation of talin-mediated integrin activation. Autoinhibition, one of the mechanisms, is a widespread phenomenon in controlling protein functions, which has been shown to occur in multiple FERM-containing proteins (Pearson et al., 2000; Li et al., 2007; Lietha et al., 2007). These studies revealed that in the Ezrin, Radixin, Moesin (ERM) proteins ligand binding sites
located in the FERM domain are masked by intramolecular interactions. Talin also belongs to this ERM protein family, and has a FERM domain, creating a possibility that integrin binding sites could be masked by a similar fashion interaction. Calpain-II has been shown to cleave talin into a talin-H/talin-R mixture, thereby releasing talin-H for leading to enhanced binding to integrin (Yan et al., 2001). Talin-H and isolated PTB domain bind to β3 cytoplasmic tail with six-fold higher affinity than full-length talin (Calderwood et al., 2002; Yan et al., 2001). These studies support the possibility that integrin binding site is masked in intact talin, and conformational change is required for its exposure. What is the mechanism to trigger the conformational change of talin to expose its integrin binding site? One possible mechanism appears to involve phosphoinositide binding which lead to the unmasking of the ligand binding site located in the FERM domain in ERM proteins (Pearson et al., 2000; Hamada et al., 2000). Indeed, PIP2 has been shown to promote strong talin binding to the integrin β1 CT (Martel et al., 2001). Interestingly, the PIP2-producing enzyme, PIPKIγ, has been shown to be recruited to the integrin adhesion sites by talin (Ling et al., 2002; di Paolo et al., 2002) upon its phosphorylation by Src kinase (Ling et al., 2003), providing a mechanism for efficient PIP2/talin interaction. Thus, upon agonist stimulation, talin may recruit PIPKIγ to locally enrich PIP2, which in turn induces the conformational change of talin to expose its integrin binding site, promoting the high affinity talin binding to and activation of integrins. The effect of this positive feedback loop can be reversed by the accumulation of phosphorylated PIPKIγ which competes with integrin β tails for an overlapping binding site on talin PTB domain (Barsukov et al., 2003; de Pereda et al.,
and by Src kinase phosphorylation of β3 tail (Datta et al., 2002) which inhibits talin binding.

Phosphorylation is one of the mechanisms of regulation of talin-mediated integrin activation. NPxY motifs in integrin β tails are phosphorylated by Src family kinases which excludes integrins from focal adhesion and downregulates the cell adhesion (Wennenberg et al., 2000; Datta et al., 2002). Tails containing non-phosphorylatable NPxF motifs reverses the phenotype by retaining talin binding activity and integrin activation (Law et al., 1998). Structural studies support this mechanism that talin PTB-integrin NPxY interaction occurs only through acidic and hydrophobic interactions, because talin PTB lacks positively charged residues for binding to phosphorylated Tyr side chains of β3 tail (Garcia-Alvarez 2003). Also, the affinity of the talin PTB for phosphorylated β3 tail peptide is reduced compared to unphosphorylated one (Oxley et al., 2008), which is consistent with disruption of integrin binding by phosphorylation. Therefore, integrin phosphorylation may be a negative regulator of integrin activation by maintaining an inhibitory complex on inactive integrin tails or by blocking talin binding directly.

Recent studies show that autoinhibition and phosphorylation are not the only possible mechanisms, and talin is the final but not the only regulator of integrin activation. Kindlin family of proteins also binds to integrin tails, and talin depends on kindlins to increase integrin activation (Ma et al., 2008; Moser et al., 2008, 2009). However, how kindlin and talin cooperatively regulate integrin activation remains to be determined.
PART B. NMR SPECTROSCOPY IN STRUCTURAL BIOLOGY

General Overview of NMR Spectroscopy

NMR spectroscopy is an important complement to X-ray crystallography for determining three-dimensional structures of proteins and nucleic acids at atomic level. In 1945, it was discovered that the energy levels of atomic nuclei are split by an applied magnetic field. However, the field of NMR did not grow explosively until 1960 when pulsed Fourier-transform NMR and consequently multidimensional NMR spectroscopy are developed and applied in different disciplines such as chemistry and structural biology. In consequent years, more technical developments partially minimized the difficulties of NMR spectroscopy which result from low sensitivity of the technique and from highly complex content of the NMR spectra. The sensitivity and resolution of the NMR spectra (especially of extremely large molecules up to 1,000 kDa) are increased by the introduction of new spectroscopy techniques such as TROSY and CRINEPT. Also, progress in the theoretical and computational part of NMR, which includes computer programs for automated-assignment of backbone, side chains, NOE constraints of the proteins, leads to more efficient and faster utilization of information content of NMR spectra. Importantly, rapidly developing recombinant protein expression and purification techniques make easier and faster to study macromolecules such as large proteins and supramolecular assemblies, and membrane proteins. Especially, the isotopic labeling of proteins (uniformly or selectively) by the incorporation of isotopes such as $^{15}$N, $^{13}$C, $^2$H in proteins simplifies the multidimensional NMR spectra drastically.
The atomic nucleus has the intrinsic quantum property of spin. The overall spin of the nucleus is determined by the spin quantum number. Only nuclei with non-zero spin quantum number possess a magnetic moment and therefore are magnetically active. The phenomenon of nuclear magnetic resonance occurs when the magnetic moment of an atomic nucleus interacts with an external magnetic field and leads to a splitting of two energy levels which are aligned either with or against the magnetic field. The difference between the two energy levels is directly proportional to the gyromagnetic ratio of the nucleus and the strength of the external magnetic field. Therefore strong magnetic fields are necessary for NMR spectroscopy. Today, the spectrometers use powerful magnets having magnetic fields of 20T (tesla) (~900 MHz).

NMR spectroscopy is rapidly developed by the introduction of FT-NMR (Fourier Transform-NMR) which uses radio frequency (rf) pulses of different shapes, and durations in specifically-designed patterns or pulse sequences. In a pulsed NMR, the sample is magnetized in an external magnetic field, and a short but relatively strong radiofrequency is applied to all of the protons in the sample to excite them simultaneously. As the excited protons relax to their equilibrium positions, the emitted signal is recorded and collected by a computer and subjected to a Fourier transform mathematical analysis. The resulting frequency domain spectrum shows signal intensity as a function of frequency. In one dimensional NMR spectrum (especially for the proteins), the spectra are too complex for interpretation since most of the signals overlap with each other. The introduction of additional spectral dimensions (2D, 3D or 4D)
simplifies the spectra drastically. Therefore the invention of multidimensional spectra made a major impact on NMR spectroscopy and was awarded by a Nobel Prize.

Applications of NMR Spectroscopy in Structural Biology

In living organisms, most of the biological mechanisms are largely mediated by protein-protein interactions, which lead to conformational and dynamical changes in the proteins, and consequently chemical and physiological changes in the organism system. NMR spectroscopy is a powerful and highly-developed technique to study these biologically important macromolecules, and their interactions at the atomic resolution. Parallel developments of both multidimensional heteronuclear NMR experiments, and isotope labeling techniques enhanced the determination of solution structures of proteins and protein complexes up to a total molecular mass of 82kDa (Tugarinov et al., 2005).

The solution state of proteins is used in NMR experiments and that is particularly suitable for the proteins which do not provide diffraction quality crystals in X-ray crystallography. Most of NMR experiments can be carried out under similar conditions (similar pH, temperature, or solution) as the physiological conditions to study structure-function relationships of proteins whereas some crystal structures are determined under exotic solution conditions required for crystallization. Moreover, the fastest and relatively inexpensive $^1$H-$^{15}$N HSQC NMR experiment rapidly shows disordered or well-folded proteins on the NMR spectra and with which different protein constructs and solution conditions can be tested to improve protein folding. In addition, “foldedness” criteria and
crystallizability may be in correlation, so the data from HSQC experiment might help to construct new protein boundaries for crystallographic analysis.

NMR spectroscopy is not solely used for structure determination of macromolecules. It is also an excellent tool to quickly identify the binding interfaces of protein complexes using chemical shift perturbation mapping. This NMR approach works perfectly for weak protein-protein interactions ($K_d \leq 10^{-6}$); whereas to study the weakly bound protein complexes is very difficult in X-ray crystallography. In addition to binding analysis, another NMR approach, nuclear relaxation measurements are used to study protein dynamics which generally affect the mechanism of molecular function. Importantly, recent NMR application, transverse relaxation-optimized spectroscopy (TROSY), has opened the door to study larger proteins up to 1,000 kDa, more membrane proteins in detergent micelles, and larger oligonucleotides and their complexes with proteins.

**Protein Structure Determination by NMR Spectroscopy**

1. Sample Preparation
2. Data collection / Processing of NMR spectra
3. Sequential Backbone and Side-chain Assignment
4. Collection of Structural Constraints
5. NMR Structure Calculation
6. NMR Structure Refinement
The determination of NMR solution structure may be divided into six major steps, and each step will be explained briefly. At the first step, highly pure (≥90% purity) and usually ~250 ml of 1mM protein solution is prepared. If the molecular mass of the protein is greater than 10 kDa, protein labeling with $^{13}$C and $^{15}$N isotopes is required to prevent overlapping peaks on the spectra. To prepare such double-labeled protein, protein is expressed in M9 minimal media instead of LB media, and $^{15}$NH$_4$Cl and $^{13}$glucose are added into this media as nitrogen and carbon sources. As a second step, the sample is placed in NMR machine and a series of multidimensional NMR experiments are recorded, typically at temperatures 25 °C to 30°C. These data are processed by computer programs to get the NMR spectra. At the third step, backbone and side chain residues of the protein are sequentially determined using the spectra of triple resonance experiments. There are a lot of different triple resonance experiments which can not be covered in this short introduction. HNCA experiment which is a prototype for all triple resonance experiments is shown below. In HNCA spectra, the nitrogen atom of a given amino acid is correlated with C$\alpha$ of its own and C$\alpha$ of the preceding amino acid after the magnetization is transferred between 1H-15N-C$^\alpha$ atoms.
Using more triple resonance experiments like HNCA, both backbone and side-chain residues of whole amino acid sequence can be established, just like building a chain of dominoes. At the forth step, more experimental constraints from various NMR experiments (such as NOE for determining of interproton distances, RDC for determining relative orientation of protein parts) are used to calculate 3D NMR structure. The double arrow between 4th and 5th steps on the scheme indicates that NMR structure calculation is repeated many times using the analysis of structural constraints at the 4th step until most of the experimentally derived constraints is in agreement with a bundle of protein conformations. Finally, a family of calculated structures instead of one defined structure is refined to get a final ensemble.

\textbf{\textsuperscript{1}H-\textsuperscript{15}N Heteronuclear Single Quantum Spectroscopy (HSQC)}

HSQC is a two-dimensional NMR experiment that correlates the \textsuperscript{15}N frequency (chemical shift) with the directly attached \textsuperscript{1}H.

When \textsuperscript{15}N-labeled protein sample is used, \textsuperscript{1}H-\textsuperscript{15}N HSQC provides the chemical shift correlation of amide group\textsuperscript{15}N with its directly attached amide proton (\textsuperscript{1}H\textsuperscript{N}) for each amino acid of that protein (Figure 1.3.). In addition to backbone amide protons, the peaks
from NH$_2$ groups of the side chains of Asn, Gln and the aromatic $^1$H$^N$ protons of Trp and His are observed on the HSQC spectrum. Theoretically, the Arg side chain peaks are also observable on the spectrum; however, its side chain chemical shift value is outside of the spectrum limits, its peaks are folded and therefore they generally appear as negative peaks. Whereas, proline residues are not seen on the spectra since proline does not have amide proton on its cyclic structure.

**Figure 1.3.** $^1$H-$^{15}$N HSQC spectrum of a folded protein. The figure is adapted from this website: www.protein-nmr.org/uk
The position of each amino acid residue in the HSQC spectrum is dependent on the chemical environment of each amide of the protein; therefore every folded protein has a unique “fingerprint” pattern of peaks in the spectrum. Since chemical shifts are very sensitive to the environment, small changes in pH, temperature or binding to other molecules can induce chemical shift changes in the HSQC spectrum. For the determination of NMR structures of the proteins, HSQC is the first performed NMR experiment to check whether protein is well-folded or not, and whether have well-dispersed peaks for further $^{13}$C, $^{15}$N and/or $^2$H double and/or triple-labeling of the protein (which is expensive) for triple resonance experiments.

**Chemical Shift Perturbation Map**

The chemical shift perturbation mapping with HSQC experiment is the mostly used NMR method to detect binding interfaces of the protein complexes since its relatively short experiment time, high sensitivity and low sample (0.1-0.2 mM protein) requirement.

The electrons in a molecule surround the nuclei and create a small magnetic field which shields the nuclei slightly from the external magnetic field. Therefore, every nucleus has a different chemical environment. This effect is called “chemical shift”. The value of chemical shift ($\Delta$) in ppm (parts per million) is defined as:

$$\Delta = ((w_{\text{signal}}-w_{\text{reference}})/w_{\text{reference}})*10^6$$
The reference frequency for the ppm scale is defined as 0 ppm using the signal of the methyl groups of tetramethylsilan (TMS). In protein NMR, a substance called DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid) is used equivalently.

Different chemical groups have different chemical shifts which are very sensitive to the environment. This property is the major information source for NMR experiments. In proteins, for example, the signals of amide protons, aromatic and aliphatic protons can be distinguished by their chemical shifts, and these chemical shifts can be perturbed by changing their chemical environments.

In chemical shift perturbation mapping with $^1$H-$^{15}$N HSQC method, one protein is labeled with $^{15}$N-isotope, and unlabeled binding partner is titrated into a solution of the labeled protein. The chemical shift values of $^{15}$N and $^1$H$^N$ is changed upon addition of unlabeled binding partner if any interaction occurs, and these values are detected by NMR and used to map the binding interface. Only the residues whose chemical environment changes due to participating in binding or due to binding-induced conformational rearrangements change their chemical shifts, whereas the residues that are neither on the binding interface nor close proximity to the binding interface do not change their chemical shifts.

When protein A interacts with protein B, the binding is always in equilibrium between free and bound states, and this is defined as:

$$A + B \leftrightarrow AB \quad K_d = [AB]/[A][B]$$
In binding analysis by HSQC experiment, two sets of peaks appear in the HSQC spectrum: one set belongs to the free protein, and the other to the bound protein. When the binding is weak, the exchange rate between free and bound states is very fast, therefore only one peak is observed for each residue and its position is the averaged frequencies of the two states on the NMR spectra. In weak protein-protein interactions, small chemical shift changes occur and hence the binding interface can be qualitatively measured using the values of all perturbed and unperturbed residues if the assignment of the free protein is available. Also, equilibrium constant ($K_d$) can be estimated from the titration of $^{15}$N-labeled protein with its unlabeled binding partner. The strong protein-protein interactions often cause significant conformational and chemical shift changes. The exchange rate between free and bound states is slow in strong bindings, therefore averaged frequencies of two states cause broader peaks or completely to be disappeared on the NMR spectra, which makes the analysis of the interface less straightforward as in the fast exchange.

**Transverse relaxation optimized spectroscopy (TROSY)**

NMR spectroscopy is a powerful and useful method to study the structure determination of proteins that are difficult to crystallize, to map the binding interfaces of protein complexes, and to study intramolecular dynamics in macromolecules, reaction kinetics, and protein folding. However, NMR spectroscopy has been limited to small proteins (less than 25kDa) because of two main problems: First, large proteins and macromolecular assemblies have large number of resonances which causes overlapping peaks on the spectra, and this makes the analysis of the spectra very difficult. Second, in
The application of NMR to large proteins is normally limited by the fact that the width of the resonance lines in the spectrum is inversely proportional to the size of the protein. Larger proteins have slower tumbling times and consequently cause shorter relaxation times (T2). In other words, the NMR signal from larger molecules decays faster, and this results in weaker signal and broader lines in the NMR spectrum (Figure 1.4). Using TROSY, the effective relaxation can be reduced, enabling improved spectral resolution and improved sensitivity for large molecules. Technically, TROSY requires high magnetic fields to suppress transverse relaxation, and better sensitivity can be achieved using at least 700 MHz NMR machine for a 15-20 kDa protein. The full potential of TROSY can be enhanced by deuteration of large proteins.

Deuterium, also called heavy hydrogen, is a stable isotope of hydrogen. The deuterium contains one proton and one neutron in the nucleus, whereas common hydrogen nucleus contains no neutron. Therefore, deuterium has a different magnetic
moment from hydrogen, and deuterium oxide (D₂O) or heavy water is used in NMR spectroscopy frequently. Heavy water is not radioactive, and has physical properties similar to water except being 11% more dense. Small concentrations of heavy water are nontoxic for eukaryotes and bacteria can grow slowly in pure heavy water. In partially (70%-90%) or fully deuterated proteins, hydrogen source is exchanged with deuterium atoms, and when purified proteins are dissolved in H₂O solutions, only amide (NH) groups replace deuterons with protons. In this way, deuterated groups does not contribute to the NMR signal at the hydrogen frequency, and only protonated amide groups are observed by NMR which attenuates the relaxation rate and eventually improves spectral quality.
**Figure 1.4. NMR spectroscopy with small and large molecules in solution** (a) The NMR signal obtained from small molecules in solution relaxes slowly; it has a long transverse relaxation time (T2). A large T2 value translates into narrow line widths (Δ) in NMR spectrum after Fourier Transformation of the NMR signal. (b) By contrast, for larger molecules, the decay of the NMR signal is faster (T2 is smaller). This result both in a weaker signal measured after the NMR pulse sequence and in broad lines in the spectra. (c) Using TROSY, the transverse relaxation can be substantially reduced, which results in improved spectral resolution and improved sensitivity for large molecules. Figure and legend is adapted from Fernandez et al., 2003.
CHAPTER II

STRUCTURAL BASIS FOR THE AUTOINHIBITION OF TALIN FOR REGULATING INTEGRIN ACTIVATION

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ABSTRACT

Activation of heterodimeric (α/β) integrin transmembrane receptors by the 270 kDa cytoskeletal protein talin is essential for many important cell adhesive and physiological responses. A key step in this process involves interaction of phosphotyrosine binding (PTB) domain in the N-terminal head of talin (talin-H) with integrin β membrane-proximal cytoplasmic tails (β-MP-CTs). Compared to talin-H, intact talin exhibits low potency in inducing integrin activation. Using NMR spectroscopy, we show that the large C-terminal rod domain of talin (talin-R) interacts with talin-H and allosterically restrains talin in a closed conformation. We further demonstrate that talin-R specifically masks a region in talin-PTB where integrin β-MP-CT binds and competes with it for binding to talin-PTB. The inhibitory interaction is disrupted by a constitutively activating mutation (M319A) or by phosphatidylinositol 4,5-bisphosphate, a known talin activator. These data define a distinct autoinhibition mechanism for talin and suggest how it controls integrin activation and cell adhesion.

INTRODUCTION

Talin is a high molecular weight protein containing an N-terminal head (1–433, talin-H, 50 kDa) and a C-terminal rod domain (434–2541, talin-R, 220 kDa) (Figure 2.1A) (Rees et al., 1990). Talin-H is globular, containing a FERM domain composed of three lobes, F1, F2, and F3 or phosphotyrosine-binding (PTB) domain (Garcia-Alvarez et al., 2003); talin-R is highly elongated, containing a series of helical bundles separated by linkers (McLachlan et al., 1994; Papagrigoriou et al., 2004; Fillingham et al., 2005; Gingras et al., 2008). Discovered in high concentrations at regions of cell-substratum
contact (Burridge and Connell, 1983), talin has long been known to be a physical linker between integrins and the actin cytoskeleton, and a regulator of a variety of cellular processes such as cell spreading, migration, and proliferation (Turner and Burridge, 1991; Calderwood et al., 2000). Recent advances have revealed an additional and especially important role for talin: by binding to integrin β CTs, talin induces high affinity ligand binding to integrins, integrin activation (Tadokoro et al., 2003; Wegener et al., 2007), a major step in cell adhesion, migration, and numerous physiological and pathophysiological responses (for review, see Hynes, 2002). A surge of genetic, cell biological, and biochemical experiments has now established that talin F3, the PTB domain of talin-H, plays a dominant role in inducing the integrin activation (Calderwood et al., 2002; Tadokoro et al., 2003; Ma et al., 2006; Wegener et al., 2007). Structural analyses have shown that talin-PTB binds to both membrane-distal and membrane-proximal regions of integrin β CTs (Vinogradova et al., 2002, 2004; Garcia-Alvarez et al., 2003; Ulmer et al., 2003; Wegener et al., 2007), and the latter event causes integrin activation by unclasping the integrin α/β CT complex (Vinogradova et al., 2002, 2004; Kim et al., 2003; Wegener et al., 2007).

With rapidly accumulating in vitro (see review, Calderwood, 2004) and in vivo (Nieswandt et al., 2007; Petrich et al., 2007) data demonstrating the central role of talin in integrin activation, a fundamental question still remains: how is talin activity regulated? Previous functional analyses have suggested that talin activity may be autoinhibited for binding to integrins (Martel et al., 2001; Yan et al., 2001). However, this mechanism
remains largely speculative, and the structural basis for such autoinhibition and how it impacts integrin activation have not been established.

Here, we use structural, biochemical, and mutational analyses to pinpoint the molecular details of the talin regulation. Intact talin, but not its talin-H fragment, has low intrinsic integrin activating activity, directly supporting the notion that talin adopts a default inactive conformation. By performing extensive NMR-based structural analyses, we show that a middle segment of talin-R specifically interacts with talin-FERM and competes with integrin β CT for binding to talin-PTB. Mutations of key residues in talin-PTB involved in binding to integrin β CT revealed that talin-R specifically masks the integrin membrane-proximal β CT-binding site on talin-PTB that is key for controlling the cytoplasmic unclasping and activation of integrins. A structure-based mutation on talin-PTB, which does not affect talin-H/integrin interaction but disrupts the talin-H/talin-R interaction, leads to constitutive activation of full-length talin. Phosphotidylinositol 4,5-bisphosphate (PIP2), a known talin activator (Martel et al., 2001), disrupts the inhibitory talin-PTB/talin-R interaction. These results define a structural mechanism of the talin autoinhibition and suggest how it may serve as a specific cellular brake to control integrin activation.

MATERIALS AND METHODS

Sample preparation for NMR analyses: A pET15b vector containing a thrombin-cleavable N-terminal His-tag GSS(H)_6SSGLVPRGSHM was used to subclone the talin-H fragments, talin-F3/PTB (306-405), talin-F2F3 (206-405). A variant of pET15b, pET30a,
was used to clone larger talin-R fragments, talin-R 434-947 (R1), 944-1483 (R2), 1483-1653 (R3), 1654-1848 (R4), 1841-1983 (R5), 1984-2102 (R6), 2103-2229 (R7), 2225-2344 (R8), 2338-2541 (R9), and talin-Rm (1654-2344). A construct 1984-2344 encompassing R6-R8 was also made in the same way. The fragments were expressed in *E. coli* BL21 (DE3), and the cells were harvested and lysed using 10 mg/ml lysozyme. All fragments were purified using nickel columns and 250 mM imidazole in elution buffer (300 mM phosphate, 500 mM NaCl, pH 8.0). The eluted proteins were dialyzed with phosphate buffer (50 mM phosphate, 150 mM NaCl, 1 mM DTT, pH 7.0), and the His-tags were cleaved using thrombin. The proteins were then further purified by size exclusion chromatography on Superdex-200 (Amersham Bioscience). Talin-H was prepared as described before (Vinogradova et al., 2002).

To prepare $^{15}$N and/or $^{13}$C-labeled protein, the cells were grown in M9 minimal media containing $^{15}$NH$_4$Cl and/or $^{13}$C-glucose, and all other conditions were the same as for preparing the unlabeled proteins. To prepare $^{15}$N/$^{2}$H-labeled talin-PTB, cells were grown in M9 minimal media containing $^{15}$NH$_4$Cl and 90% $^2$H$_2$O. Point mutants of talin-PTB (M319A, L325R, W359A, S365D, S379R, and Q381V) were made using a QuickChange site-directed mutagenesis system (Stratagene, Inc) and prepared in the same way as the wild-type proteins. A point mutation (C336S) was also introduced in talin-PTB as reported previously (de Pereda et al., 2005) to improve protein stability.

The $\beta_3$ CT chimera peptide TIHDRKEFAKFEERARAKWVYSPLHY SAR (the sequence in bold is PIPKI$\gamma$ tail), $\beta_3$ CT chimera mutant (F730A), and PIPKI$\gamma$
(SWVYSPLHYSAR) were synthesized by our Biotechnology Core facility. For improved solubility, the KLLI sequence was not included at the N-terminus of the β3 CT since it is not involved in interacting with talin-PTB (Wegener et al., 2007). The β3 CT chimera binds tightly to talin-PTB (Kd~140 nM) in slow exchange, which was shown to mimic the β3 CT that has weaker binding to talin-PTB in intermediate exchange (Wegener et al., 2007).

**NMR spectroscopy:** All heteronuclear NMR experiments were performed as described by Bax and Grazsiek, 1993. All triple resonance NMR experiments for 1mM talin-F2F3 were performed on a Varian 600 MHz instrument equipped with a triple resonance probe and shielded z-gradient unit. These experiments were performed at 25°C, pH 7.0, in 150mM NaCl, 50 mM phosphate buffer, 1mM DTT. All HSQC-based experiments were performed on either Bruker Avance 600 or 900 MHz spectrometers using the cryogenic triple resonance probes.

15N-labeled and/or 15N/90%2H-labeled talin-PTB or talin-F2F3 (0.2 mM) was used with and without the talin-R fragments at 1:2 ratio in all HSQC experiments. For chemical shift mapping, TROSY-based HSQC (Pervushin et al., 1997) for talin-PTB or talin-F2F3 in complex with talin-RM were performed on a Bruker 900 MHz spectrometer equipped with a cryogenic triple resonance probe. Weighted chemical shift changes were calculated using the equation: \[ \Delta \delta_{\text{obs}[HN,N]} = [(\Delta \delta_{\text{HN}} W_{\text{HN}})^2 + (\Delta \delta_{\text{N}} W_{\text{N}})^2]^{1/2} \], where \( W_{\text{HN}} = 1 \) and \( W_{\text{N}} = 0.154 \) are weighting factors based on the gyromagnetic ratios of \( ^1\text{H} \) and \( 15\text{N} \). All the
spectra were processed with nmrPipe (Delaglio et al., 1995) and visualized with PIPP (Garrett et al., 1991).

**Surface Plasmon Resonance (SPR) Measurements:** SPR analysis was performed using a BIA Core 3000 instrument (Amersham Pharmacia Biotech). CM5 sensor chips were activated using the amine coupling kit from Amersham Pharmacia Biotech. Talin-RM was then immobilized to the activated surface. Talin-PTB and its mutants were each injected at the concentration range 0.1-5 \(\mu\)M and the flow rate was 20\(\mu\)l/min. For PIP2/talin-H interaction, biotinylated \(C_6\)-PIP2 (C-45B6a, echelon Biosciences, Inc) was immobilized to a biotin chip and talin-H was injected at 20\(\mu\)l/min. The surfaces of the sensor chips were regenerated by injection of 20mM NaOH. Experiments were performed at 25°C in 10 mM Hepes buffer, pH 7.4. All binding affinities were calculated using the BIAcore 3000 evaluation software (Biacore AB, Uppsala, Sweden).

**Plasmids and transfections:** The plasmid DNA encoding full-length mouse talin was kindly provided by Richard Hynes, MIT, and it was cloned into pEGFP-N1 vector (Clontech Lab, Inc) encoding a red-shifted variant of wild-type GFP using EcoRI and AgeI restriction sites. The final DNA had a C-terminal GFP fusion and was confirmed by sequencing. The full length talin M319A DNA mutant was prepared employing the QuickChange Site-Directed Mutagenesis Kit (Stratagene) using the cloned GFP-fusion talin DNA as a template. The resulting mutant DNA was also confirmed by sequencing.
**Integrin activation:** The effects of full-length talin, full length talin M319A, and talin-H on integrin activation were analyzed using CHO cells stably expressing integrin (\(\alpha_{IIb}\beta_3\)-CHO) and an activation-specific anti-\(\alpha_{IIb}\beta_3\) mAb (PAC1) as described previously (Ma et al., 2008). Briefly, \(\alpha_{IIb}\beta_3\)-CHO cells were transfected with EGFP-talin, EGFP-talin-H or EGFP vector alone as a control. Twenty four hours after transfection, the cells were harvested and stained with PAC1 followed by incubation with Alexa Fluor® 633 goat anti-mouse IgM conjugate. After washing, the cells were fixed and analyzed by flow cytometry on a FACSCalibur instrument (BD Scientific, Franklin Lakes, NJ). The PAC1 binding was analyzed only on a gated subset of cells positive for EGFP expression. The mean fluorescence intensities of PAC1 bound to the EGFP-talin or EGFP-talin-H expressing cells were compared to that of PAC1 bound to the control EGFP expressing cells. Three independent experiments were performed. T-student test was used for statistical analyses. \(p\) values <0.05 were considered statistically significant.

**RESULTS**

**Talin adopts a default low active state for regulating integrin activation**

It has been shown extensively that talin-H can activate integrins (Calderwood et al., 1999, 2002; Tadokoro et al., 2003; Kim et al., 2003; Ma et al., 2006; Bouaouina et al., 2007), and it is presumed that this activity is substantially blunted in full length talin. To directly compare the integrin activating activity of these two talin forms, we transfected vectors expressing full length talin or talin-H, all as EGFP constructs, into a CHO cell line stably expressing integrin \(\alpha_{IIb}\beta_3\) (\(\alpha_{IIb}\beta_3\)-CHO). The activation state of the \(\alpha_{IIb}\beta_3\) was then measured based on the binding to activation-specific anti-\(\alpha_{IIb}\beta_3\) antibody (PAC1).
**Figure 2.1. Effects of Full-Length Talin and Talin-H on Integrin Activation.** (A) Domain organization of full-length talin. The three lobes of talin-H FERM domain F1, F2, F3 or PTB and rod domain have been labeled. (B) Comparison of the activation of integrin αIIbβ3 by full-length talin and talin-H. CHO cells stably expressing the integrin αIIbβ3 were transfected with cDNAs for full-length talin or talin-H, each as EGFP constructs. The EGFP positive cells were gated and used to monitor PAC-1 binding to determine the extent of αIIbβ3 activation as previously described (Ma et al 2008,). The data are means ± S.D. from three independent experiments. **P<0.01.
To eliminate the influence of varied expression levels for talin-H and full length talin, only EGFP positive cells with similar expressions were gated for FACS analysis. The data summarized in Figure 2.1 are taken from three independent experiments and show that while talin-H induced substantial activation of αIIbβ3 activation, full-length talin was significantly weaker as an activator. Thus, our data provide direct functional evidence for the supposition that the integrin activation by full-length talin is dampened and is enhanced by events that change exposure of its head region.

**A middle segment of Talin-R (1654-2344) interacts with talin-PTB in talin-FERM**

Since talin-PTB (F3) in talin-H is solely responsible for binding to integrin β CTs during integrin activation (Calderwood et al., 2002; Garcia-Alvarez et al., 2003; Tadokoro et al., 2003; Wegener et al., 2007), we reasoned that talin-R might mask the integrin β CT binding site on talin-PTB, thereby preventing the talin-PTB function. To test this hypothesis, we set out to use NMR-based 2D ¹H-¹⁵N HSQC (heteronuclear single quantum correlation) experiment to examine the interaction between ¹⁵N-labeled talin-PTB and unlabeled talin-R fragments. 2D ¹H-¹⁵N HSQC is known to be an extremely sensitive technique for probing protein-target interactions with a wide range of affinities (Bonvin et al., 2005; Vaynberg and Qin, 2006; Takeuchi and Wagner, 2006).

The HSQC spectrum of a ¹⁵N-labeled protein contains many peaks, each of which is a correlation of a proton to its attached ¹⁵N within a particular residue in the protein. Some peaks may be shifted or broadened in the HSQC spectrum if the protein is bound to a target, which is an excellent indication of the binding interface. The peak broadening or
disappearance could be due to the size increase or intermediate rate exchange of the protein complex at the NMR time scale. Our strategy was that if any of the unlabeled talin-R fragments binds to $^{15}$N-labeled talin-PTB, some or all $^1$H/$^{15}$N amide signals of talin-PTB should be perturbed and broadened, which in turn provides information on the intramolecular interaction between talin-PTB and talin-R. Based on the available helical bundle structural information on talin-R (Papagrigoriou et al., 2004; Fillingham et al., 2005; Gingras et al., 2008) and a secondary structure prediction program (Bryson et al., 2005), we then dissected talin-R into nine consecutive fragments (R1:434-947, R2:944-1483, R3:1482-1653, R4: 1654-1848, R5: 1841-1983, R6: 1984-2102, R7: 2103-2229, R8:2225-2344, and R9: 2338-2541), where the division regions were predicted to be random coil or loop structures so the structural integrities of these fragments should be preserved. A series of HSQC spectra were collected for $^{15}$N-labeled talin F2F3 domain, which contains talin-PTB (F3), in the absence and presence of individual unlabeled talin-R fragments. Starting from the N-terminus of talin-R, we found that R1, R2, R3 had little effect on the HSQC spectrum of talin-F2F3 (Figure 2.2.A-2.2.C) whereas R4 (~21 kDa) caused significant line-broadening on talin-F2F3 (~22 kDa) (Figure 2.2.D), suggesting that R4 binds to talin-F2F3. From the C-terminus, R9 did not bind (Figure 2.2.E) but R8 caused significant line-broadening of talin-F3F3, suggesting that it also interacts with talin-F2F3 (Figure 2.2.F). These initial mapping data indicated that talin-R does interact with talin-F2F3 and the binding site appears to involve multiple regions including R4 and R8 but not the N-terminal R1-R3 and C-terminal R9. Based on these initial data, we then prepared another larger expression construct encompassing R4 and R8, i.e., 1654-2344 with a total molecular weight of ~76 kDa (termed talin-RM), which is well-folded as
Figure 2.2. The Interaction between $^{15}$N-Labeled Talin-PTB and Unlabeled Talin-Rod Fragments. 2D $^1$H-$^{15}$N HSQC of $^{15}$N-labeled talin-F2F3 in the presence (black) and absence (red) of (A) talin rod (R1:434-947) (B) talin rod (R2: 944-1483); (C) talin rod (R3:1482-1657); (D) talin rod (R4:1654-1848); (E) talin rod (R9: 2338-2541); and (F) talin rod (R8: 2225-2344).
assessed by its chemical shift dispersion pattern in the TROSY HSQC spectrum (Figure 2.3.). As predicted, talin-RM also bound to talin F2F3, as indicated by the substantial line-broadening and disappearance of talin F2F3 signals in HSQC (MW~100 kDa) (data not shown). By employing TROSY technique (Transverse Relaxation Optimized Spectroscopy) into HSQC, which is tailored for detecting NMR signals of large proteins and protein complexes (Pervusin et al., 1997), we were able to recover majority of the signals, some of which were significantly shifted due to binding (Figure 2.4.A).

To further understand the nature of this interaction, we performed backbone signal assignment of talin F2F3 using triple resonance NMR experiments, including HNCACB, CBCACONH, HNCA, HNCO, HC(CO)NH, and C(CO)NH (Bax and Grazsiek, 1993). Table A1 (see the appendix) lists the chemical shift assignments of this construct. Chemical shift mapping revealed that only NMR signals of F3 (PTB) but not F2 in talin F2F3 were either significantly shifted or broadened (Figure 2.4.B), thus supporting our hypothesis that talin-PTB is responsible for binding to talin-RM. To improve the spectral quality and to simplify the spectral analysis, we made $^{15}$N/$^2$H-labeled talin-PTB and performed its TROSY-HSQC in complex with the unlabeled talin-RM (total complex is ~90 kDa). Both deuteration and TROSY are known to dramatically reduce the line-broadening of the proteins, which led to an excellent and well-resolved spectrum of talin-PTB bound to unlabeled talin-RM (Figure 2.5.A). As expected, talin-RM caused significant chemical shift perturbation for talin-PTB (Figure 2.5.A). Surface plasmon resonance (SPR) experiment revealed that the dissociation constant ($K_D$) between talin-PTB and talin-RM is ~577 nM (Table 2.1 and Figure 2.5.B).
Figure 2.3. The TROSY HSQC Spectra of Talin-Rₘ. The signals are wellspread with a significant number of peaks resonating at ¹H>9ppm and <7.5ppm, which are indications of well-folded proteins. Note that unfolded proteins are known to have congested chemical shift dispersion with all signals resonating in the narrow ¹H spectral window (7.5-8.5 ppm). The sample concentration was 0.3 mM, and the data were acquired for 21 hours.
Figure 2.4. *The binding of talin-$R_M$ to talin-$F2F3$. (A)* 2D TROSY $^1$H-$^15$N HSQC of talin-$F2F3$ in the absence (black) and presence of unlabeled talin-$R_M$ (red). Well resolved peaks, which are either significantly broadened or shifted, are labeled in the free form talin-$F2F3$. *(B)* Chemical shift mapping of the binding to talin-$F2F3$. Only the resonances in F3 (306-405) are significantly perturbed. Signals that are diminished due to severe line-broadening are colored in grey.
Figure 2.5. The Interaction of Talin-PTB with Talin- RM (A). 2D TROSY 1H-15N HSQC of 15N/90%2H-labeled talin-PTB in the absence (black) and presence (red) of talin-RM. Significantly shifted peaks are labeled. The inset shows a representative peak that is shifted in the presence of talin-RM at 1:0.0 (black), 1:0.5 (pink), 1:1.0 (Cyan), and 1:2 (red) ratios.
Figure 2.5. (continued) *Talin-PTB/talin-RM interaction* (B) SPR measurement for the talin-PTB interaction with talin-RM. The dissociation constant (KD) between talin-PTB and talin-RM is 577 nM (Table 2.1). The affinities for other mutants measured are in Table 2.1.
Talin-RM and integrin membrane-proximal β3 CT compete for binding to an overlapping binding site on talin-PTB

To precisely map the talin-RM binding site on talin-PTB, we performed a series of NMR titration experiments. The TROSY-HSQC spectra were collected for the $^{15}$N/$^2$H-labeled talin-PTB in the absence and presence of increasing concentrations of talin-RM to obtain molar ratios of 1:0.0, 1:0.5, 1:1.0, and 1:2. This experimental design allowed us to trace the significantly perturbed signals (Figure 2.5.A). Figure 2.6.A shows the detailed chemical shift perturbation profile. Remarkably, the perturbation pattern was found to be similar to the previously reported one by integrin β3 CT or integrin membrane-proximal β3 CT segment fused to PIPKIγ peptide (Wegener et al., 2007) (Figure 2.6.A), suggesting that the talin-RM binding site on talin-PTB overlaps with that for integrin β3 CT.

Interestingly, chemical shift mapping revealed that talin-R4 (1654-1848) also induced very similar perturbation profile (Figure 2.7.A vs 7B), albeit with slightly reduced chemical shift changes and lower affinity ($K_D$~3.6µM, Table 2.1) than talin-RM ($K_D$~0.58µM, Table 2.1). On the other hand, a larger fragment containing C-terminal talin-RM (R6-R8, 1984-2344), induced different and very narrow-range of chemical shift changes peaking around 367-375 (Figure 2.7.C) with much lower affinity ($K_D$~78.0µM, Table 2.1) than talin-RM. Combining these two fragments in a single construct (talin-RM), however, yields higher affinity (0.58µM, see Table 2.1). These data suggest that R4 and R6-R8 synergistically bind to different regions in talin-PTB and that R4 plays more important role in binding to talin-PTB (see more data below). Figure 2.6.B highlights significantly shifted residues in talin-PTB upon binding to talin-RM and integrin β3 CT.
**Figure 2.6.** Mapping of the talin-$R_M$ binding site on talin-PTB. (A) $^1$H/$^{15}$N Chemical shift changes of talin-PTB upon binding to talin-$R_M$ as a function of residue number. As a comparison, the chemical shift changes of talin-PTB upon binding to $\beta3$ CT chimera is also reproduced using the chemical shift table deposited in the BioMagResBank (accession number 7150) by Wegener et al., 2007. The membrane-distal residues involved in binding to integrin $\beta3$ CT chimera are colored in red in the upper panel. Note that W359 changed dramatically by $\beta3$ CT chimera (the change was so big due to its bulky interaction with talin-PTB (Wegener et al., 2007) that a broken line was used to conserve space).
Figure 2.6. (continued) Mapping of the talin-$R_M$ binding site on talin-PTB. (B)

Significantly perturbed residues on talin-PTB by $\beta$3-CT chimera (left) and talin-$R_M$ (right) are highlighted using the structure of talin-PTB. The order of dark blue, blue, and light blue indicate the extent of the chemical shift changes with the dark blue having the most significant changes. The changes have some similarity indicating potential overlapping binding site for $\beta$3 CT and talin-$R_M$ but there are significant differences indicating the binding sites are not identical.
Figure 2.7. Chemical shift changes of talin-PTB in the presence of (A) talin-RM, (B) talin-R, (C) talin-R6-R8. Talin-R4 shows very similar perturbation pattern as talin-RM whereas talin-R6-R8 has very narrowly perturbed region peaking at 367-378. Talin-R6-R8 binds weaker than talin-R4 to talin-PTB (see Table 3.1) but the combined talin-R4 and talin-R6-R8 in a single talin-RM construct produces much higher affinity (see Table 3.1) suggesting that talin-R4 and talin-R6-R8 bind to different regions.
chimera, respectively, thus providing more direct view of how the two binding sites might overlap.

To more precisely evaluate how the integrin β3 CT binding sites on talin-PTB may be involved in binding to talin-RM, we introduced a series of structure-based talin-PTB point mutations L325R, W359A, S365D, S379R, Q381V, each of which has been previously shown to impair the talin-mediated integrin activation without affecting the structural integrity of talin-PTB (Wegener et al., 2007). L325R, S365D, S379R, and Q381V disrupted the integrin β membrane-proximal CT binding to talin-PTB whereas W359A abolished the integrin CT binding to talin-PTB by removing the bulky interaction of the membrane-distal CT with talin-PTB (Wegener et al., 2007). We also made M319A mutant. M319 is a surface-exposed hydrophobic residue that is not involved in interacting with either integrin β3 membrane-proximal region (Wegener et al., 2007) or membrane-distal region (Garcia-Alvarez et al., 2003), and thus its mutation to Ala had little effect on the interaction with integrin β3 CT chimera (Figure 2.8.). However, M319 is most significantly perturbed by talin-RM (Figure 2.6.A), suggesting that it may play a crucial role in interacting with talin-RM. Table 2.1 summarizes the K_D values of talin-RM binding to wild type talin-PTB, M319A, L325R, W359A, S365D, S379R, and Q381V as derived from SPR. As compared to the wild type talin-PTB, S365D, S379R, and Q381V exhibited markedly reduced binding to talin-RM, whereas L325R had very small effect. As expected, while M319A still binds to integrin β3 CT as wild-type talin-PTB (Figure 2.8.A vs 8.B), it had dramatically weakened interaction (~140 fold) with talin-RM and slightly weakened the interaction with talin-R4 (~10 fold) (Table 2.1).
**Table 2.1.** $K_D$ of the talin $R_M$ or smaller fragments binding to talin-PTB and talin-PTB mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Target</th>
<th>$K_D$ (M)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT talin-PTB</td>
<td>Talin-$R_M$</td>
<td>(5.77±1.35)$\times10^{-7}$</td>
</tr>
<tr>
<td>WT talin-PTB</td>
<td>Talin-R4</td>
<td>(3.60±0.29)$\times10^{-6}$</td>
</tr>
<tr>
<td>WT talin-PTB</td>
<td>Talin-R6-R8</td>
<td>(7.80±1.88)$\times10^{-5}$</td>
</tr>
<tr>
<td>PTB M319A</td>
<td>Talin-$R_M$</td>
<td>(8.09±2.38)$\times10^{-5}$</td>
</tr>
<tr>
<td>PTB M319A</td>
<td>Talin-R4</td>
<td>(4.23±1.32)$\times10^{-5}$</td>
</tr>
<tr>
<td>PTB L325R</td>
<td>Talin-$R_M$</td>
<td>(6.67±1.32)$\times10^{-7}$</td>
</tr>
<tr>
<td>PTB W359A</td>
<td>Talin-$R_M$</td>
<td>(5.29±1.39)$\times10^{-7}$</td>
</tr>
<tr>
<td>PTB S365D</td>
<td>Talin-$R_M$</td>
<td>(1.66±0.50)$\times10^{-3}$</td>
</tr>
<tr>
<td>PTB S379R</td>
<td>Talin-$R_M$</td>
<td>(6.25±1.55)$\times10^{-4}$</td>
</tr>
<tr>
<td>PTB Q381V</td>
<td>Talin-$R_M$</td>
<td>(3.14±0.80)$\times10^{-4}$</td>
</tr>
</tbody>
</table>

$^a$. All binding affinities were calculated by two independent measurements using the BiaCore 3000 evaluation software (BiaCore AB, Uppsala, Sweden). $\chi^2 <10$ were obtained for all the data, indicating good fit.
Figure 2.8: M319A binds to β3 CT chimera similarly as WT talin-PTB. (A) Overlay of M319A in the absence (black) and presence (cyan) of β3 CT chimera; (B) Overlay of WT talin-PTB in the absence (black) and presence (cyan) of β3 CT chimera. Comparison of (A) and (B) shows that integrin β3 CT chimera induced the same chemical shift changes for both M319A and WT talin-PTB.
The effects of S365D, S379R, and Q381V recapitulate those for binding to the integrin membrane-proximal β3 CT and indicate that this site significantly overlaps with that for talin-R$_{M}$ in the talin-PTB domain. To further confirm this conclusion, we prepared large quantities of two representative mutants in $^{15}$N/$^{2}$H-labeled form, S365D (reduced binding to talin-R$_{M}$ by $\sim$2.9x10$^{3}$ fold) and Q381V (reduced binding to talin-R$_{M}$ by $\sim$540 fold) (Table 2.1) and examined their chemical shift perturbation in the absence and presence of unlabeled talin-R$_{M}$. Consistent with the SPR data, the extent of the chemical shift changes was decreased for Q381V and much more decreased for S365D as compared to WT talin-PTB (Figure 2.9.).

Interestingly, W359A, which completely abolished the talin-PTB binding to integrin β3 CT by disrupting the bulky hydrophobic interaction between talin-PTB and membrane-distal β3 CT (Garcia-Alvarez et al., 2003; Wegener et al., 2007), had little effect on the K$_{D}$ of the talin-PTB/talin-R$_{M}$ interaction (Table 2.1). This observation, together with the above described effects of other mutants, suggested that the binding sites on talin-PTB for talin-R$_{M}$ and integrin β3 CT are overlapping but not identical. To further investigate this possibility, we performed HSQC-based competition experiments. As shown in Figure 2.10.A and 10.B, while the majority of signals disappear from $^{15}$N-labeled talin-PTB upon binding to the large talin-R$_{M}$ (total MW$\sim$90 kDa, K$_{D}$$\sim$577nM, see Table 2.1), these signals return upon addition of equal molar β3 CT chimera (β3 membrane-proximal CT fused to PIPK$\gamma$ peptide, MW$\sim$3.5 kDa, K$_{D}$$\sim$140nM, see Wegener et al., 2007), yielding a spectrum identical to that for $^{15}$N-labeled talin-PTB bound to the unlabeled β3 CT chimera (Figure 2.10.C). These data demonstrate that the
Figure 2.9. Chemical shift perturbation profiles for the WT talin-PTB and its mutants Q381V and S365D by talin-\(R_M\). Note that the chemical shifts are attenuated with the F3 Q381V but are still similar to WT, whereas chemical shifts are dramatically reduced with the F3 S365D mutant.
**Figure 2.10. NMR-based competition experiments.**

(A) 2D 1H-15N HSQC of 0.2mM 15N-labeled talin-PTB. (B) The same talin-PTB in the presence of 0.4mM unlabeled talin-RM. Majority of the signals disappeared as a result of large complex formation (~90kDa). (C) The same talin-PTB in the absence (black) and presence (red) of 0.4mM unlabeled talin-RM and 0.4mM β3 CT chimera. As compared to (B), addition of β3 CT chimera recovered all the peaks leading to a spectrum identical to that for 15N-labeled talin-PTB bound to the unlabeled β3-CT chimera (total MW~15 kDa), demonstrating that the β3 CT chimera effectively displaced talin-RM from binding to talin-PTB. (D) The same talin-PTB in the presence of 0.4mM unlabeled talin-RM and excess 0.8mM PIPK1γ peptide. In contrast to β3 CT chimera, excess PIPK1γ peptide did not compete with talin-RM.
small β3 CT chimera peptide competed with large talin-R_M for binding to talin-PTB. In contrast, excess PIPKI_γ peptide, which mimics the β3 membrane-distal CT binding and binds tightly to talin-PTB (K_D~270nM) (de Perera et al., 2005), did not recover the broadened signals at all (Figure 2.10.D), indicating that PIPKI_γ and talin-R_M do not have overlapping binding sites on talin-PTB. Since PIPKI_γ mimics the β3 membrane-distal CT for binding to talin-PTB (de Pereda et al., 2005; Kong et al., 2006; Wegener et al., 2007), our NMR data are in agreement with our SPR data on W359A mutant (Table 2.1) indicating that while the integrin membrane-proximal CT binding site is significantly masked by the talin-R_M, the β3 membrane-distal CT binding site for talin-PTB is not. Consistently, a F730A mutant of the chimera peptide, which has dramatically reduced membrane-proximal β3 CT binding to talin-PTB (Wegener et al., 2007), did not compete effectively with talin-R_M (Figure 2.11.A vs 11.B). Note that the β3 CT also binds to talin-R_M (Tremuth et al., 2004). However, such binding does not appear to interfere with the talin-PTB/talin-R_M interaction since β3 CT did not affect the talin-R_M interaction with talin-PTB W359A (Figure 2.12.). Note that W359A has the same affinity to talin-R_M as the WT talin-PTB (Table 2.1) but no binding to β3 CT (Wegener et al., 2007).

NMR-based competition experiments also revealed that talin-R4 (Figure 2.13.A) but not talin-R6-R8 (Figure 2.13.B) competed with β3 CT chimera for binding to talin-PTB. Since talin-R4 induced very similar chemical shift perturbation pattern as talin-R_M when binding to talin-PTB (Figure 2.7.), this finding suggests that talin-R4 plays a major role in masking the membrane-proximal CT binding site on talin-PTB. It also further supports our forgoing chemical shift mapping and affinity-based results that talin-R4 and
**Figure 2.11.** *Competition of β3 membrane-proximal CT and its mutant with talin-RM for binding to talin-PTB.* (A). HSQC of 0.2 mM 15N-labeled talin-PTB in the absence (black) and presence (red) of 0.4 mM talin-RM and 0.6mM WT β3 CT chimera, showing that WT β3 CT is very potent for competing with talin-RM for binding to talin-PTB, generating a spectrum of talin-PTB/β3-CT chimera complex (red); (B). HSQC of 0.2 mM 15N-labeled talin-PTB in the absence (black) and presence of 0.4 mM talin-RM (red), and presence of both 0.4 mM talin-RM and 0.6mM β3 CT F730A mutant chimera (green). As compared to (A), β3 CT F730A mutant did not effectively compete with talin-RM since majority of peaks are broadened (disappeared) as compared to WT β3 CT chimera in (A) despite the fact that the spectra were collected at the same experimental conditions.
Figure 2.12. Talin-RM/β3 CT interaction does not affect the talin-RM/talin-PTB interaction. (A) HSQC of W359A free form; (B) HSQC of 0.2mM 15N-labeled talin-PTB W359A in the presence of 0.4 mM talin-RM (black) showing the disappearance of signals due to the formation of the large complex with the same affinity as the WT talin-PTB/talin-RM (Table 2.1). Addition of excess (1mM) of β3-CT fused to MBP (cyan) had no effect on the spectrum.
Figure 2.13. Talin-R4 and Talin-R6-R8 bind to talin-PTB differently. (A) 1D trace of selective signal G394 from HSQC of 0.2mM 15N-labeled talin-PTB in the free form (black, see black arrow) and in the presence of 0.4mM Talin-R4 (red, see red arrow) and Talin-R4:β3 CT chimera (0.4mM: 0.6mM) (blue, see blue arrow). Addition of Talin-R4 dramatically reduced the signal of talin-PTB G394 (see red arrow) due to line-broadening of the complex but addition of β3 CT chimera resumed the signal intensity and shifted the signal to the β3 CT bound position (see blue signal), indicating β3 CT chimera replaced the talin-R4 from talin-PTB, showing that β3 CT chimera disrupted the talin-PTB/talin-RM interaction. (B). The same 1D trace as (A) for 0.2mM 15N-labeled talin-PTB in the free form (black) and in the presence of 0.4 mM Talin-R6-R8 (red, see red arrow) and Talin-R6-R8:β3 CT chimera (0.4mM:0.6mM) (blue, see blue arrow). In contrast to (A), β3 CT chimera had little effect on the talin-PTB/talin-R6-R8 interaction (the signal is shifted by binding to β3 CT but still has the same height as that bound to talin-R6-R8).
talin-R6-R8 bind to different regions of talin-PTB. Figure 2.14.B summarizes the surface depiction of the talin-PTB structure in which potential key residues directly involved in binding to talin-RM are highlighted based on the chemical shift mapping, mutagenesis and competition data. The binding surface is compared to that for the talin-PTB/integrin β CT complex (Figure 2.14.A) (Wegener et al., 2007), which shows that the integrin membrane-proximal β3 CT binding site significantly overlaps with that for talin-RM, thus providing a view of how talin-R may sterically suppress the integrin β CT binding to talin-PTB in the closed conformation of talin.

To further functionally evaluate the significance of the talin autoinhibition, we examined the talin M319A activity in integrin activation. Since this mutant still maintains the integrin binding (Figure 2.8) but has dramatically reduced the talin-PTB/talin-RM affinity (Table 2.1), we speculated that the full length talin M319A could be constitutively active in activating integrin. As shown in Figure 2.15., M319A indeed dramatically enhanced talin induced integrin αIIbβ3 activation as compared to the wild type talin. Furthermore, such an enhancement could be abolished by incubation of RGDS peptide, a ligand binding inhibitor for β3 integrins (data not shown), indicating its specificity. Thus, this observation offers a very strong support for our hypothesis. Note that the M319A-induced integrin activation is slightly more potent than talin-H. While the precise mechanism for this higher potency remains to be determined, one can envision two possibilities: (1) full-length talin M319A has higher affinity to integrin than talin-H alone, thus leading to the more potent integrin activation. In addition to talin-H, isolated talin-R also binds to integrin β CT (Xing et al., 2001; Yan et al., 2001; Tremuth et al., 2004) at the membrane-distal region (Tremuth et al., 2004), but not the membrane-
Figure 2.14. Talin autoinhibition and its effect on integrin activation. (A) Surface representation of talin-PTB domain with the integrin β3 CT binding site highlighted. The integrin binding site was based on Garcia-Alvarez et al., 2003 and Wegener et al., 2007. The integrin membrane-proximal β3 CT binding residues are colored in green and the membrane-distal β3 CT binding residues are colored in red. (B) The same view as (A) but with the talin-RM binding site highlighted. Residues whose mutations impaired the talin-RM binding are colored in green whereas other potential binding residues are colored in cyan based on their significant chemical shift perturbation in Figure 3.6 and competition data in Figure 3.10.-3.13. Notice the significant overlap of the integrin β3 membrane-proximal binding site in green as compared to that for talin-RM in (B) also in green.
Figure 2.15. Comparison of the activity of integrin αIIbβ3 by full-length talin, full length talin M319A, and talin-H activation. Note that M319A is more potent than talin-H probably due to its higher affinity for integrin β3 CT than talin-H due to synergistic talin-H/integrin (unmasked) and talin-R/integrin interactions. **P<0.01; *P<0.05.
proximal site (E.G. and J.Q., unpublished data). Thus, the constitutively open conformation of M319A may have higher affinity for integrin than talin-H alone due to both talin-H (M319A)/integrin and talin-R/integrin interactions. (2) Membrane anchoring of talin is important for integrin activation (Vinogradova et al., 2004; Wegener et al., 2007). It is possible that the open conformation of M319A has stronger capacity to anchor to the membrane than talin-H, resulting in more potent integrin activation.

Conformational activation by PIP2

Given the above findings that talin-R interacts with talin-PTB and restrains talin in a closed conformation, an obvious question is how this closed conformation is opened. A well-known talin activator is phosphatidylinositol 4,5-bisphosphate (PIP2), which has been shown to promote strong talin binding to integrin β CT (Martel et al., 2001), resulting in the formation of a ternary PIP2/talin/integrin complex in living cells for mediating integrin activation and clustering (Cluzel et al., 2005). Our SPR experiment revealed that talin-H, but not talin-RM, can potently bind to biotinylated PIP2 with high affinity (K_D~89.2±1.25nM, Figure 2.16.A). The biotinylated PIP2 was mounted on a biotin sensor chip, and such positioned PIP2 should mimic the PIP2 anchored to the membrane. Since talin-H contains FERM domain and multiple FERM domains have been shown to bind to PIP2 involving PTB/PH subdomains (Hamada et al., 2000; Bompard et al., 2003; Cai et al., 2008), we wondered if talin-PTB is involved in binding to PIP2. Our HSQC-based mapping experiment revealed that PIP2 can indeed interact with talin-PTB in a site-specific manner with the most significant perturbation around 370-378 (Figure 2.16.B), which overlaps with that for talin-RM (Figure 2.16.D). Consistently, SPR
experiment demonstrated that PIP2 can indeed disrupt the talin-\(R_M\)/talin-PTB interaction in a concentration-dependent manner (Figure 2.17.A). Such competition was further confirmed by HSQC experiment where soluble \(C_4\)-PIP2 was able to compete with talin-\(R_M\) for binding to talin-PTB (Figure 2.17.B). Since PIP2/talin-H interaction has been shown not to interfere with the talin-H/integrin interaction (see Figure 6 in Martel et al., 2001) and instead it induces conformational change of talin for high affinity integrin binding (Martel et al., 2001), our findings lead to a mechanism by which PIP2 binds to talin-PTB and sterically displaces the inhibitory talin-R to expose the integrin binding site for the effective talin/integrin interaction.
Figure 2.16. The Interaction of PIP2 with Talin. (A) SPR data showing the PIP2/talin-H interaction. KD was estimated to be (89.2±1.25)x10^-9 M. (B) Chemical shift perturbation data showing that soluble C4-PIP2 specifically binds to talin-PTB. Note that the chemical shift changes are small indicating that the affinity between soluble C4-PIP2 and talin-PTB is weaker than longer carbon chain PIP2 bound to talin-H.
Figure 2.16. (continued) The PIP2/talin interaction (D) Surface representation of talin-PTB domain with potential PIP2 binding site highlighted based on the chemical shift mapping of significantly perturbed residues (Green, most significantly shifted, Cyan, next significantly shifted).
**Figure 2.17.** PIP2 disrupts the inhibitory talin-PTB/talin-$R_M$ interaction. (A) SPR data showing that C$_8$-PIP2 (echelon Bioscience, Inc) suppresses the talin-PTB interaction with talin-$R_M$ in a concentration dependent manner. Talin-$R_M$ was immobilized to the activated surface. Talin-PTB mixed with increasing amount of PIP2 was each injected at the flow rate of 20µl/min. (B). Overlay of HSQC spectra of $^{15}$N-labeled talin-PTB showing that soluble C$_4$-PIP2 (soluble at high concentration in 150mM NaCl, 50mM phosphate buffer, pH 7.0) recovers many signals of talin-PTB (red), which were otherwise broadened/disappeared in the presence of talin-$R_M$ (black) (talin-PTB:talin-$R_M$:PIP2 = 0.2mM:0.4mM:2.0mM).
DISCUSSION

A combination of NMR spectroscopy, mutagenesis, and biochemical experiments has revealed how talin-FERM, via its PTB subdomain, interacts with talin-R to restrain talin in an inactive/autoinhibited conformation. Autoinhibition is a widespread phenomenon in controlling protein functions, which has been shown to occur in multiple FERM-containing proteins (Pearson et al., 2000; Li et al., 2007; Lietha et al., 2007). However, only the talin FERM domain can directly mediate integrin activation (Tadokoro et al., 2003) by binding to the integrin membrane-proximal β CT and inducing integrin cytoplasmic unclasping (Vinogradova et al., 2002; Wegener et al., 2007). The masking of the integrin membrane-proximal β CT binding site in talin-FERM by talin-R is quite unique as compared to other autoinhibitory FERM-containing proteins, which utilize FERM domains to autoinhibit the functions of other parts of proteins (Pearson et al., 2000; Li et al., 2007; Lietha et al., 2007). For example, focal adhesion kinase (FAK) utilizes its N-terminal FERM F2 domain to mask the C-terminal kinase active site (Lietha et al., 2007) whereas moesin uses its N-terminal FERM F2 and F3 subdomains to mask its C-terminal actin binding site. Thus, we have unraveled a novel autoinhibition mechanism for talin in which other parts of the molecule talin-R, control the function of the FERM domain in integrin activation.

Our cell-based experiments provide direct functional evidence to support the mechanism that the full-length talin was a poor activator of integrin αIIbβ3, whereas talin-H was substantially more potent (Figure 2.1.). These data are also consistent with
the report of Han et al, (2006) showing that talin must be activated to display its integrin activating activity. Furthermore, talin M319A mutation, which disrupts the talin-PTB/talin-R interaction but not talin-PTB/integrin interaction, constitutively activated integrin $\alpha_{IIb}\beta_3$ (Figure 2.15.). These data provides strong functional evidence for the talin autoinhibition in regulating integrin activation. The autoinhibitory domain was mapped to the middle region (1654-2344) of talin-R and its affinity for talin-PTB was found to be within the submicromolar range as assessed by SPR using purified components (Table 2.1). We note that the intramolecular interaction involving the two domains in the intact talin is expected to be even stronger by placing the binding surfaces within close proximity within the same molecule, and may provide the tight control of the talin activity needed to prevent, spontaneous integrin activation. Interestingly, despite its inhibitory conformation that prevents the talin-PTB/integrin membrane-proximal $\beta$ CT contact, full-length talin can still bind weakly to integrin $\beta$ CT (the affinity is about six-fold weaker than that of the talin-H/integrin interaction, see Yan et al., 2001), suggesting that the integrin binding site on talin is not completely masked. Our data are consistent with this observation in that the integrin membrane-distal CT binding site on talin-PTB does not appear to be significantly masked by talin-R. Isolated talin-R was also shown to bind to integrin $\beta$ CT (Xing et al., 2001; Yan et al., 2001; Tremuth et al., 2004) at the membrane-distal (Tremuth et al., 2004) but not the membrane-proximal side (Goksoy and Qin, data not shown), which may contribute to the full length talin/integrin interaction.

Based on our observations, we propose a model for how talin controls integrin activation via a closed (autoinhibitory) form to trigger conformational change (Figure
2.18.). In the closed state, the integrin membrane-proximal β CT binding site on talin-H is masked by talin-R, although talin can still weakly associate with integrin β CT via its unmasked sites. Upon activation by cellular stimuli, talin undergoes conformational change so that talin-PTB can access the integrin membrane-proximal β CT, which leads to the integrin α/β CT unclasping and inside-out activation. Since talin also binds to actin via the C-terminal end of talin-R (outside talin-RM) (Gingras et al., 2008), the enhanced talin/integrin interaction, consequence to the talin conformational change, may alter the strength of the integrin-actin linkage. In this manner, cells can undergo dynamic cytoskeleton remodeling, movement or shape change in coordination with integrin activation and ligation.

A dynamic equilibrium exists between monomeric and dimeric talin, which only shifts to the dimeric state at high talin concentration (>3µM) (Molony et al., 1987), and the dimeric state may strengthen the integrin-actin linkage for regulating cell adhesion and migration. Although the exact dimerization topology for the full length talin is not clear, recent crystallographic studies of the C-terminal actin binding module (2300-2541) revealed a dimer structure and have suggested that the full length talin dimer may exist in three possible conformations (Gingras et al., 2008): parallel, tail-to-tail V-shaped or tail-to-tail antiparallel fashion. Earlier EM studies (Goldman et al., 1994) suggested that the two globular heads (talin-Hs) are on two opposite ends of the talin dimer and suggested a head-to-tail antiparallel dimer, which raises a possibility that the middle talin-RM in one subunit interacts with talin-H in the other. However, such head-to-tail antiparallel is
incompatible with the crystallographic studies (Gingras et al., 2008) and thus the intermolecular autoinhibition model is less likely to occur in cells.

What is the mechanism to trigger the conformational change of talin to expose its integrin membrane-proximal $\beta$ CT site \textit{in vivo}? As illustrated above, one mechanism appears to involve PIP2 which has been shown to promote strong talin binding to the integrin $\beta_1$ CT (Martel et al., 2001). Interestingly, the PIP2-producing enzyme, PIPKI$\gamma$, has been shown to be recruited to the integrin adhesion sites by talin (Ling et al., 2002; di Paolo et al., 2002), providing a mechanism for efficient PIP2/talin interaction. Our NMR and biochemical data have indicated that while the PIPKI$\gamma$ binding to talin does not affect the autoinhibitory conformation of talin (Figure 2.10.D, i.e., PIPKI$\gamma$ peptide did not interfere with the talin-R/talin-PTB interaction), the PIPKI$\gamma$ product, PIP2, does disrupt the inhibitory talin-R/talin-PTB interaction (Figure 2.17.). Thus, upon agonist stimulation, talin may recruit PIPKI$\gamma$ to locally enrich PIP2, which in turn induces the conformational change of talin to expose its integrin membrane-proximal $\beta$ CT site, promoting the high affinity talin binding to and activation of integrins. This model is supported by several independent \textit{in vivo} observations: (i) integrin $\alpha_{IIb}\beta_3$ activation was found to be directly associated with the increased PIPKI$\gamma$ activity and PIP2 levels in platelets (Hinchliffe et al., 1996). (ii) PIP2 level is associated with talin on activated platelets (which requires $\alpha_{IIb}\beta_3$ activation) but not on resting state platelets (Heraud et al., 1998); and (iii) a ternary complex involving PIP2, talin, and integrin $\alpha\nu\beta_3$ was formed in the living cells to mediate the integrin $\alpha\nu\beta_3$ activation and clustering (Cluzel et al., 2005).
Another emerging mechanism for the talin activation involves RIAM (Han et al., 2006). Using a genetic approach, it was shown that RIAM may form a supramolecular complex with talin and Rap1 to activate integrins. It remains to be determined whether RIAM changes the conformation of talin in this supramolecular complex, leading to its enhanced interaction with and activation of integrins. Finally, calpain has been shown to cleave talin into a talin-H/talin-R mixture, thereby releasing talin-H for leading to enhanced binding to integrin (Yan et al., 2001). However, activation of integrin αLβ2 by ionomycin was found not to involve calpain-mediated talin cleavage (Dreolini and Takei, 2007). RIAM-mediated integrin activation also does not involve calpain-dependent talin cleavage (Han et al., 2006). Most data implicate that calpain is involved in integrin outside-in signaling (Schoenwaelder et al., 1997; Hayashi et al., 1999; Franco et al., 2004).

In summary, we have demonstrated the structural basis of the autoinhibition for talin in regulating integrin activation. Our findings, together with previous structural data (Vinogradova et al., 2002; Garcia-Alvarez et al., 2003; Wegener et al., 2007), now provide a view of how a series of conformational changes occur on the intracellular face for talin-mediated integrin activation (Figure 2.18.).
Figure 2.18. The model for the talin activation in inducing the integrin activation. In the closed state, the integrin membrane-proximal β CT binding site (blue bar) on talin-H is masked by talin-R, although talin can still weakly bind to integrin β CT via unmasked sites. Upon activation by some cellular factor such as PIP2 or RIAM, talin undergoes conformational change so that talin-PTB can access to the integrin membrane-proximal β CT and induces the integrin α/β CT unclasping and integrin activation. Note the ternary complex involving PIP2/talin/integrin has been indicated by Martel et al., 2001 and Cluzel et al., 2005.
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CHAPTER III
TOWARDS STRUCTURE DETERMINATION OF TALIN-ROD4/ TALIN-PTB COMPLEX

INTRODUCTION

Talin has long been known as a crucial regulator of a variety of cellular processes such as cell spreading, migration, and proliferation. Its importance has been investigated by many in vivo, in vitro and structural studies in recent years. On checking the Pubmed for “talin”, nearly 1100 papers have been appeared that directly or indirectly related to talin research. This promising field has been enriched by recent studies that revealed an additional and very important role for talin. Talin binds to integrin β cytoplasmic tails, and induces integrin activation by changing the integrin conformation for high affinity ligand binding. In this major step, talin F3, or PTB domain of talin-H is the dominant player in inducing integrin activation. In our recent paper, we used structural, biochemical and mutational analyses to investigate the molecular details of talin autoinhibition mechanism for the regulation of integrin activation. By performing NMR-based structural analyses, we showed that a middle segment of talin rod domain (talin-RM) specifically interacts with talin-PTB domain for an autoinhibited
conformation. We further demonstrated that talin-RM specifically binds a region in talin-PTB where integrin \( \beta \) membrane proximal CT binds and competes with talin-RM for binding to talin-PTB. Several studies show that this overlapping binding site on talin-PTB is the key region for controlling the cytoplasmic unclasping and activation of integrins.

Our studies have revealed that talin-RM fragment on talin rod domain is required for talin head-rod interaction and autoinhibition of talin. To precisely minimize the binding site of talin-RM from 70 kDa to 20-22kDa, we subcloned smaller fragments of this construct and purified the labeled proteins and used for HSQC experiment to observe the binding of talin-PTB to these smaller fragments. The chemical shift mapping revealed that R4 (1654-1848) showed a very similar perturbation profile as talin-RM despite the slightly reduced chemical shift changes (Figure 2.7). SPR experiment also confirmed the binding by showing lower affinity (~3.6 \( \mu \text{M} \)) than talin-RM (0.58\( \mu \text{M} \)) (Table 2.1). Moreover, we did a NMR-based competition experiment to understand whether this binding is competed by \( \beta 3 \) CT, and as shown in figure 2.13, \( \beta 3 \) CT chimera replaced talin-R4 from talin-PTB, indicating that R4 is the major binding site for talin-PTB and this region is enough to mask the integrin binding site on talin-PTB.

To understand the structural details of the interaction of talin-R4 with talin-PTB at atomic level, the talin-R4 structure has to be solved by NMR. Solving the 3-D structures of proteins consists of many steps; some of them have to be repeated many times to acquire an accurate structure. The first step is the preparation of labeled pure sample (\(^{13}\text{C},\)
\(^{15}\)N labeled talin-R4 protein) for triple resonance experiments. The second step is the data acquisition by NMR which takes 10-15 days to be completed. The following steps include processing the data, the assignment of backbone and side chain resonances, the generation of NOE restraints and structure calculations. Each of these steps may take time from 1 week to few months to be completed, which totally depends on the quality of NMR spectra (well-dispersed peaks vs overlapped peaks) and the size of protein (90 amino acid vs 200 amino acid).

In this chapter, the ongoing study of structure determination of talin-R4, and especially published talin-R4 structure (by Critchley group) and the differences in the results will be discussed in details.

METHODS AND MATERIALS

**Sample Preparation for NMR experiments:** Talin R4 (1654-1848) is subcloned in pET-30a vector containing Factor-Xa cleavable N-terminal His tag. To prepare \(^{15}\)N, \(^{13}\)C-labeled talin-R4 protein, the cells were expressed in E. coli BL21(DE3) and grown in M9 minimal media containing \(^{15}\)NH\(_4\)Cl and \(^{13}\)C-glucose at 37 °C for 3 hrs with 1mM IPTG. The cells were harvested by centrifugation and resuspended in 100 ml lysis buffer (50mM NaPO\(_4\), 300mM NaCl, %0.001 detergent) containing 10mg/ml lysozyme, and shaked at 4 °C for overnight. The double-labeled talin R4 protein was purified on nickel column using elution buffer (50mM NaPO\(_4\) pH 8.0, 300mM NaCl, and 250mM imidazole). The protein was then cut by Factor Xa to remove 5 kDa His-tag. The buffer is
exchanged into a lower salt content buffer (50 mM NaPO₄, 150 mM NaCl, 1 mM DTT, pH 7.0) and further purified by gel filtration using this buffer. The pure fractions of the protein was collected and concentrated for NMR experiment. The protein concentration was measured by Nano-drop, and 0.9 mM talin-R4 protein was used for triple resonance experiments.

For intermolecular NOE experiments, ¹⁵N-labeled talin-R4 and ¹⁵N-labeled talin-F3 proteins were grown in %100 D₂O containing M9 minimal media. The proteins were purified in the same way as previous proteins.

**ELISA Assay:** 96 well Maxisorp F96 Immunoplates (NalgeNunc) were coated with 1 μM wild type Talin-F3 in 100 μl of 50 mM carbonate buffer (pH 9.6) overnight at 4°C and blocked with 3% BSA in 1XPBS. Selected wells were incubated with either Talin-R4 alone or Talin-R4 preincubated with various amounts of wild type Talin-F3 or F3M319A mutant or ovalbumin as a control. Because Talin-R4 bears S-Tag, the binding was detected by HRP-conjugated anti-S-tag antibody (Pierce) and TMB substrate (Sigma). The reaction was stopped by sulfuric acid and absorbance was measured by a 96-well spectrophotometer at 450 nm.

**Surface Plasmon Resonance Experiments:** CM5 sensor chips were activated using the amine coupling kit from Amersham Pharmacia Biotech. Talin-R₄ was then immobilized to the activated surface. Talin-PTB and its mutant were each injected at the concentration range 0.1-5 μM and the flow rate was 20 μl/min. The surfaces of the sensor
chips were regenerated by injection of 20mM NaOH. Experiments were performed at 25°C in 10mM Hepes buffer, pH 7.4 on a BIA Core 3000 Instrument (Amersham Pharmacia Biotech). All binding affinities were calculated using the BIAcore 3000 evaluation software (Biacore AB, Uppsala, Sweden).

RESULTS

3D NMR Structure of Talin-R4 Protein

It is well-known that talin is the final activator of integrins upon agonist stimulation, and in the absence of signaling; integrins have a bent conformation in inactive state. However, little was known about talin conformation in the resting state. Using structural, and biochemical analyses, we showed that full length talin has an autoinhibited conformation in the inactive state by binding of talin-PTB to a middle fragment of talin rod. We were able to minimize the binding site from 70kDa to 20 kDa using NMR experiments. To elucidate the structural details of talin-R4/talin-PTB interaction, double-labeled talin-R4 protein was purified and used for NMR triple resonance experiments for solving its 3D structure.

During the backbone assignment of Talin-R4, Critchley group from UK published the NMR structure of talin R4 (1654-1822) on J. Biol. Chem. (Goult et al, 2009). Their both talin-R4 structure and HADDOCK modeling studies confirm our published results except one of the talin-PTB mutants. As on Figure 3.3, talin-R4 consists of five antiparallel amphipathic α-helices forming a bundle which is stabilized by hydrophobic
interactions. The topology of the bundle is similar to previously solved talin 482-655 fragment (Papagrigorio et al., 2004) and two bundles of talin 1974-2293, which contains integrin binding site-2 (Gingras et al., 2009).

**Structural Analysis of the Complex between Talin R4 and Talin-PTB**

The interaction between the talin-R4 (1654-1848) and talin-PTB was studied by collecting $^1$H, $^{15}$N HSQC spectra of $^{15}$N-labeled talin 1655–1822 in the presence of unlabeled F3 at 1:0, 1:0.5, 1:1 and 1:2 ratios. The chemical shift perturbation map (Figure 3.1) shows that mostly helix 4 and helix 1 are involved in binding to talin-PTB which is similar to those observed by Goult et al (Figure 3.3). They also show that this domain binds to talin-PTB predominantly via surface-exposed residues on helix 4. They also report the binding affinity of talin-R4/talin-PTB interaction as 20 µM using NMR which is similar to our SPR binding affinity (3.6µM).

Both their chemical shift perturbation map (Figure 3.1) and competition experiment and HADDOCK modeling of the complex confirm our hypothesis that talin-R4 rod domain binds and masks the known binding site in talin-PTB for the β3-integrin membrane proximal CT site whereas the β3-integrin membrane-distal CT site on talin-PTB is not masked by talin-R.

**Biochemical Studies of the Complex between Talin R4 and Talin-PTB**

We previously examined the activity of talin M319A mutant in integrin activation and found that this mutant still maintains the integrin binding, but has dramatically
reduced the talin-PTB/talin-RM affinity (~140 fold). To evaluate the effect of talin-PTB M319A mutation for binding to talin-R4, we did two SPR experiments. On the first experiment, binding analysis showed that talin-R4 binds to Talin-PTB M319A with a lower affinity (42 µM) (Table 2.1). On the second SPR experiment (data not shown), we immobilized talin-R4 protein on chip, and flowed through both WT talin-PTB and M319A mutant proteins. This spectrum confirms the SPR binding affinity that there is a remarkable binding difference between WT and M319A talin-PTB. In addition to NMR and BiaCore data, ELISA assay also shows that M319 of talin-PTB is a critical residue for the interaction of talin-PTB with talin-R4. Talin F3 M319A mutant protein couldn’t compete with coated WT F3 for binding to talin-R4 (Figure 3.1.). Although our results confirm that there is a dramatic difference in binding of wild-type and mutant talin-PTB to talin-R4, Goult et al. did not observe any difference in affinity from NMR Kd measurements, interestingly their M319A talin-PTB mutant binds with a higher affinity (14 µM).

To partially map the binding sites and to check whether amide proton of M319 of talin F3 involves in direct interaction with talin-R4 residues, we purified %100 D2O grown, 15N-labeled and unlabeled WT talin-F3 and R4 proteins for intermolecular NOE experiment. The intermolecular NOEs were recorded by collecting two spectra, one of 15N-labeled talin R4 in complex with the unlabeled talin F3 domain, and another of 15N-labeled F3 in complex with unlabeled talin-R4. However, we couldn’t acquire a significant result from this experiment.
Figure 3.1. *M319 is a critical residue for the interaction of talin-PTB with talin-R4.* ELISA assay showing that talin F3 M319A mutant protein couldn’t compete with coated WT F3 for binding to talin-R4 in a concentration dependent manner. 1 µM wild type Talin-F3 were coated in 100 µl of 50 mM carbonate buffer (pH 9.6) overnight at 4°C and blocked with 3% BSA in 1XPBS. Selected wells were incubated with either Talin-R4 alone or Talin-R4 preincubated with various amounts of wild type Talin-F3 or F3M319A mutant or ovalbumin as a control. Since Talin-R4 bears S-Tag, the binding was detected by HRP-conjugated anti-S-tag antibody (Pierce) and TMB substrate (Sigma).
Figure 3.2. Chemical shift perturbation map of talin R4 (1655-1822) in the presence of talin F3
Figure 3.3: Mapping the F3 FERM subdomain binding site in talin 1655–1822. C, weighted shift map obtained from the $^1$H, $^{15}$N HSQC spectra of talin 1655–1822 on the addition of F3. D and E, residues in talin 1655–1822 that are significantly perturbed following the addition of F3 are highlighted on the talin 1655–1822 structure. The most significant shifts (>0.2 ppm) are shown in dark blue, and the residue numbers are labeled; smaller perturbations (>0.07 ppm) are shown in light blue. F, residues in talin F3 (PDB 2H7D) that are significantly perturbed following the addition of talin 1655–1822 are highlighted on the F3 structure. This figure and legend is adapted from Goult et al, 2009.
DISCUSSION

Our previous NMR spectroscopy, mutagenesis, and biochemical experiments have revealed how talin-FERM, via its PTB subdomain, interacts with talin-R$_M$ (1654-2344) to restrain talin in an inactive/autoinhibited conformation. Within these region, we found two PTB binding site, one of which (R4:1654-1848) has a higher affinity for talin-PTB (Kd 3.6 μM) than the other fragment (R6/8: 1984-2344, Kd 78 μM). The affinity difference between large talin R$_M$ (0.6μM) and its smaller constructs suggests that although the main binding site spans talin-R4 region, additional lower affinity interactions with other domains in the vicinity may occur, and these interactions might increase the overall affinity of the head-rod interaction.

We have started to determine the solution structure of talin-R4 followed by its complex with talin-PTB. However, Goult et al. solved and published the NMR structure of talin-R4 (1655-1822) before us. According to their HADDOCK modeling of the complex, the talin-PTB binding site comprises two main interaction areas on talin-R4. Tyr-377 of talin-F3 docks into a small hydrophobic pocket at the top of the helical bundle. The second binding site (residues 316-326 of F3), also called F3 activation loop, have many basic residues, and binds to a cluster of negatively charged residues on helix 4 of talin-R4. Our chemical shift perturbation map (Figure 3.2) also shows that helix 4 is the main binding site of talin-PTB which is in agreement with their NMR results.
Using NMR and biochemical analyses, we proposed a model (Figure 2.18) for how talin controls integrin activation. In the closed state, the integrin membrane-proximal β CT binding site but not membrane-distal β CT binding site on talin-H is masked by talin-R. Importantly, PIPKIγ90 binding site in talin-PTB is exposed even in the autoinhibited form of talin (Figure 2.10.D). Upon activation by cellular stimuli, PIPKIγ90, which is activated by talin, then translocates the complex to the plasma membrane where PIP2 synthesis is locally enriched. PIP2 then induces the conformational change of talin to expose its integrin membrane-proximal β CT, which leads to the integrin α/β CT unclasping and inside-out activation. Our proposed model is similar to those observed by Goult et al.

Although our results and conclusions are confirmed by the structure and model of the talin-R4/PTB complex (Goult et al. 2009), one of their results is not in agreement with our published and unpublished results. They claimed that M319 of talin-PTB is not involved in direct binding to talin-R4 and interestingly, M319A talin-PTB mutant binds talin-R4 with a higher affinity (14 μM). The calculation of dissociation constant from NMR titration experiment may not be highly reliable because of few reasons. During NMR titration, one labeled protein is diluted with small increments of the binding partner on each time, and during this process some of the sample is lost, which may affect the K_d measurement, especially for weak interactions. Secondly, K_d calculation is totally depends on the shift difference between bound and unbound ligand and especially big proteins (≥ 15 kDa) have crowded and overlapped peaks on NMR spectra which makes difficult to analyze their chemical shift values unambiguously. Finally, the common
concept in the scientific field is to repeat the same experiment with different *in vivo* (if applicable) and *in vitro* techniques to confirm the result. Using NMR chemical shift mapping, BiaCore, ELISA, and integrin activation assay techniques, we confirm that M319 of talin-PTB is critical for the inhibitory interaction of talin head-rod, and M319A mutation disrupts this interaction leading to constitutively activated integrins.

In conclusion, the studies of talin-R4/talin-PTB interaction validate the structural basis of autoinhibition, and provide a foundation for further structural studies. Importantly, the 3D-complex structure of talin-R4/talin-PTB solved by NMR or X-ray crystallography instead of modeling will show the structural details of the complex, and this may correct the inconsistent results.
CHAPTER IV
GENERAL DISCUSSION AND FUTURE WORK

The integrin have been studied over the fifty years at cellular, molecular and atomic level to better understand cellular responses such as cell adhesion, migration, and survival. Until this date, more than 150 components and more than 650 connections have been described in integrin signaling. (Zaidel-Bar et al. 2007) However, more clinical and basic research studies are required to appreciate the biological importance of each component in integrin signaling.

In this thesis, we showed the molecular and structural details of talin regulation using different NMR spectroscopy techniques. Although talin is too big (270 kDa) to study with NMR, we successfully dissected talin into more than ten fragments without disrupting their structural integrity. To study whether there is a head/rod interaction in talin, we used fast and effective NMR method, HSQC to examine the protein-protein interactions. Importantly, using deuterated talin fragments and TROSY method, we were able to get well-resolved NMR spectra of large protein complexes and that enabled us to
map the binding interfaces unambiguously. Also, we successfully used NMR as a tool for different competition experiments, and for the detection of binding defective mutants.

We have unraveled a distinct autoinhibition mechanism for talin and a possible release mechanism of its inhibited conformation by PIP2 using structural, biochemical, mutational and cell-based techniques. Although a ternary PIP2/talin/integrin complex in living cells has been reported, the mechanism how talin translocates to the membrane for such a ternary complex has not been established yet. We showed that even in the autoinhibited form of talin, PIPKγ binding site on talin is not masked by the intramolecular interaction, and PIPKγ, which is activated by talin, is recruited to the membrane, where PIP2 is locally synthesized. Locally enriched PIP2 then relieves autoinhibited conformation of talin by competition, and exposes the integrin binding site on talin-H.

Another mechanism for talin regulation has been defined recently in which talin/Rap1A/RIAM complex binds to and activates the integrin. According to this mechanism, activated GTP bound Rap1 translocates to the plasma membrane, where it interacts with RIAM leading to the recruitment of talin for integrin activation (Han et al, 2006). Two important questions need to be answered. Han et al. showed that talin/Rap1A/RIAM complex does not require calpain-cleavage or phosphorylation of talin for integrin activation. Then, how talin is released from its autoinhibited conformation? Does RIAM can lead a conformational change in talin to expose its integrin binding site? Also, talin activity is tightly regulated by both PIPKγ/PIP2 and
Rap1/RIAM but it is not known in which manner they work. Do they work synergistically, simultaneously or mutually exclusive?

For years, talin was regarded as the only and final activator of the integrin; however, recent studies have indicated that the kindlin family proteins (kindlin-1,-2, and -3) cooperate with talin for the regulation of integrin conformational shift from low to high-affinity state (Ma et al, 2006; Moser et al, 2008). Biochemical studies showed that kindlins also interact with integrins, but unlike talin, they bind only to the membrane-distal region of integrin β cytoplasmic tails (Ma et al, 2008; Montanez et al, 2008); therefore kindlins cannot activate integrins without talin, and talin itself is not sufficient to induce integrin affinity. Although all these studies have opened a venue in the integrin field, more questions remain to be answered. The binding modes of kindlin and talin during integrin activation have to be investigated since their binding sites on integrins partially overlap. Kindlins also have lipid binding sites like talin; therefore their activity may also be regulated by phosphoinositide signals. It may worth to investigate lipid-mediated kindlin regulation and its effects on talin and integrin activation. In general, more functional and biochemical studies are required to understand the kindlin activity and its signaling mechanism.

In conclusion, the structural studies of talin-mediated integrin activation make significant contribution to the scientific field by answering the question of how talin activity is regulated for efficient integrin activation. The researches will continue in advance to answer more questions.
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APPENDIX
APPENDIX A

Table A1: F2F3 chemical shift assignment table is electronically deposited in the Biological Magnetic Resonance Data Bank with accession number 15792.
APPENDIX B

Table B1. Chemical shift assignment values of talin R4 (1655-1822) free and talin R4/talin F3 complex.

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Figure B1. Backbone assignment of R4 (1654-1848). Numbered squares are zoomed on the next pages.
Figure B1. (continued) Zoomed squares are shown for better peak resolution.
Figure B2. Chemical shift perturbation map of talin-F3 WT and M319A mutant upon addition of talin-$R_M$. 