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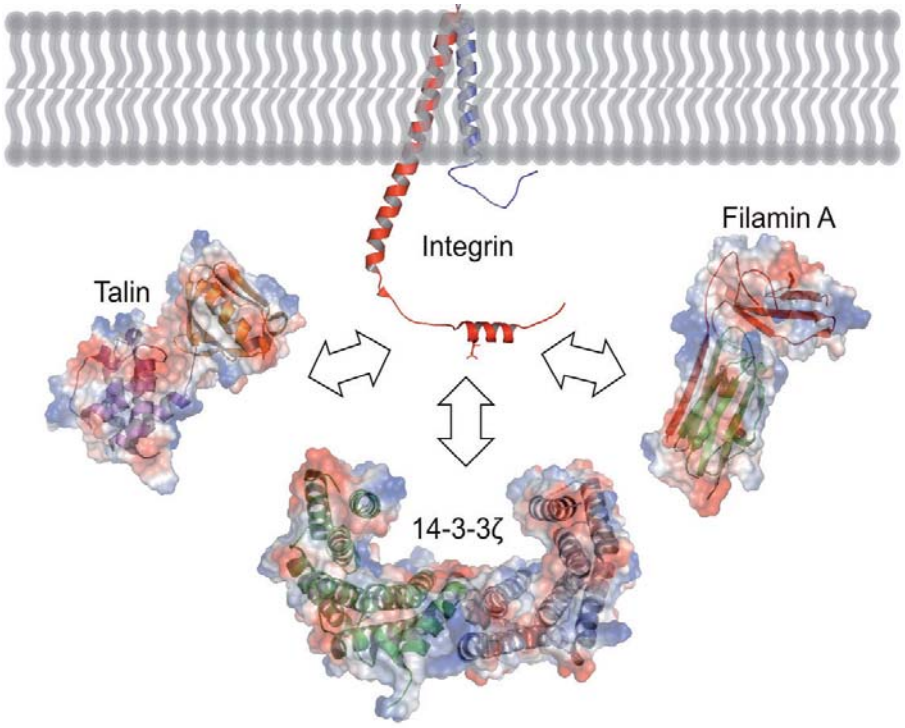
Heikki Takala

Three Proteins Regulating Integrin Function – Filamin, 14-3-3 and RIAM



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I may not have gone where I intended to go, but I think I have ended up where I needed to be.
-Douglas Adams

ABSTRACT

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Three proteins regulating integrin function – filamin, 14-3-3 and RIAM
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Integrins are transmembrane adhesion receptors important in cell adhesion, migration and survival. Integrin activation is regulated by cytoplasmic protein–protein interactions. Talin activates integrins by binding to integrin cytoplasmic tails. As dozens of other cytoplasmic proteins interact with integrins, their mutual interactions and function in integrin cytoplasmic adhesion structures have to be carefully regulated. This is achieved, *e.g.*, by protein recruitment, competitive binding, alternative splicing and protein phosphorylation. The Rap1-GTP interacting adapter molecule (RIAM) recruits talin to integrins with Rap1 GTPase, thereby facilitating integrin activation. Filamins compete with talin for integrin binding. This is expected to be particularly important for a splice variant-1 (var-1) of filamin A (FLNa) that lacks a segment with an autoinhibitory role in integrin binding. The cytoplasmic tail of β2 integrin is phosphorylated on a threonine residue upon inside-out activation events, which is accompanied by 14-3-3 binding. In this thesis, the structural and functional properties of RIAM, FLNa var-1 and 14-3-3ζ were studied. RIAM contains a Ras-association (RA) domain followed by a pleckstrin homology (PH) domain. We showed that the RA domain is responsible for the binding to Rap1, whereas the PH domain accounts for the stability of the protein. FLNa var-1 lacks a segment of 41 residues. We showed that this absence causes domain 19 to be unstructured and unable to bind ligands, but it does not seem to significantly affect the binding properties of domain 21. The crystal structure of 14-3-3ζ in complex with the phosphorylated β2 integrin peptide reveals at the atomic level how this interaction is dependent on the phosphate moiety. The same phosphorylation abrogates FLNa binding but does not affect the binding of talin. However, talin binding to the phosphopeptide is outcompeted by 14-3-3ζ, which is explained by the partially overlapping binding sites on the integrin.

Keywords: 14-3-3ζ; cytoplasmic interactions; filamin A variant-1; integrin; phosphorylation; RIAM; talin.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals.

I Takala H. & Ylänne J. 2011. Binding Properties and Stability of the Ras-association Domain of Rap1-GTP Interacting Adapter Molecule (RIAM). Submitted manuscript.

II Pentikäinen U., Jiang P., Takala H., Ruskamo S., Campbell I.D. & Ylänne J. 2011. Assembly of a Filamin Four-domain Fragment and the Influence of Splicing Variant-1 on the Structure. *Journal of Biological Chemistry* 286: 26921-26930.

III Takala H.,* Nurminen E.,* Nurmi S.M.,* Aatonen M., Strandin T., Takatalo M., Kiema T., Gahmberg C.G., Ylänne J. & Fagerholm S.C. 2008. Beta2 Integrin Phosphorylation on Thr758 Acts as a Molecular Switch to Regulate 14-3-3 and Filamin Binding. *Blood* 112: 1853-1862.

*Equal contribution

RESPONSIBILITIES OF HEIKKI TAKALA IN THE THESIS ARTICLES

Article I I conducted all experimental work, including DNA cloning, protein expression and purification. I did the pull-down experiments with the purified proteins. I conducted the proteolysis assays in cells and *in vitro*. I prepared all the figures. I planned and wrote the article with Prof. Jari Ylänne.

Article II I conducted the affinity measurements of the protein constructs. I prepared related mutant constructs and participated in purifying the proteins. I participated in preparing the figures and writing the article.

Article III I expressed and purified the 14-3-3ζ protein, and crystallized it in the complex with the synthetic integrin peptide. I processed the diffraction data, solved and refined the crystal structure of the 14-3-3ζ/peptide complex. I purified and conducted interaction assays with 14-3-3ζ and talin together with Dr. Susanna Fagerholm. I participated in writing the article and preparing the figures.

All the studies in this thesis were performed under the supervision of Prof. Jari Ylänne.

ABBREVIATIONS

ABD	actin-binding domain
ABS	actin-binding site
ADAP	adhesion and degranulation-promoting adapter protein
β-TD	β-tail domain
CHO	Chinese hamster ovary
Dok1	docking protein 1
ECM	extracellular matrix
F-actin	filamentous actin
FA	focal adhesion
FERM	band four-point-one/ezrin/radixin/moesin
FLNa	filamin A
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GTPase	guanosine triphosphatase
IBS	integrin-binding site
ICAP-1α	integrin cytoplasmic domain-associated protein-1α
ICAM	intercellular adhesion molecule
I-EGF	integrin epidermal growth factor
Ig	immunoglobulin
MIDAS	metal-ion-dependent adhesion site
MRL	Mig-10/RIAM/Lamellipodin
NMR	nuclear magnetic resonance
PDB	Protein Data Bank
PH	pleckstrin homology
PIP2	phosphatidylinositol 4,5-bisphosphate
PIPKIγ	phosphatidylinositol 4-phosphate 5-kinase γ
PSI	plexin/semaphorin/integrin
PTB	phosphotyrosine-binding
RA	Ras-association
RAPL	regulator for cell adhesion and polarization enriched in lymphoid tissues
RIAM	Rap1-GTP interacting adapter molecule
SKAP-55	55-kDa src kinase-associated phosphoprotein
TCR	T-cell receptor
TM	transmembrane
VBS	vinculin-binding site
var-1	variant-1
VASP	vasodilator-stimulated phosphoprotein

1 INTRODUCTION

Integrins are important proteins in several biological processes that involve development, immunity and hemostasis. These transmembrane receptors mediate the signals and mechanical force between the cytoskeleton and the extracellular surroundings. Cells require integrins for adhesion and migration, and their function has to be carefully regulated (Hynes 2002). Integrin activation and signaling are regulated by intracellular protein-protein interactions and phosphorylation.

This doctoral thesis deals with integrins and their intracellular binding partners. First, the current literature is reviewed. The experimental part focuses on three proteins that regulate integrin interactions with talin, the main integrin activating factor and linkage to actin cytoskeleton. Rap1-GTP interacting adapter molecule (RIAM) has a role in talin recruitment to integrins, filamin A (FLNa) competes with talin for integrin interaction and 14-3-3ζ binds to an integrin after phosphorylation events. The structural aspects and binding properties of RIAM (I) and FLNa var-1 (II) were investigated with various biochemical methods. The crystal structure of 14-3-3ζ in complex with a phosphorylated integrin was determined, and the effects of phosphorylation and 14-3-3 binding were studied (III).

2 REVIEW OF THE LITERATURE

2.1 Integrins

Integrins are transmembrane adhesion receptors that mediate interactions and signals between a cell and the extracellular matrix (ECM) or another cell. Integrins also act as a transmembrane mechanical link by binding between extracellular contacts and intracellular cytoskeleton. Integrins play a critical role in many biological events, including development, immunity, hemostasis and cancer, thus acting as important therapeutic targets in cancer, inflammation and thrombosis. At the cellular level, integrins have a profound role in cell migration, cell adhesion, differentiation, cell cycle progression and apoptosis (Hynes 2002, Gahmberg et al. 2009).

Integrins are found only in the metazoa. In mammals, 18 different α subunits and 8 β subunits can form 24 distinct integrin dimers. Each integrin dimer has its specific ligand specificity and nonredundant function. For example, αIIbβ3 is important in platelet function whereas αLβ2 in leukocyte function. According to the α subunit, integrins can be divided into laminin receptors, collagen receptors, leukocyte-specific receptors, α4/α9-containing receptors and arginine-glycine-aspartic acid (RGD) receptors (Johnson et al. 2009). For example, the RGD receptors can bind to fibronectin and vitronectin, and the leukocyte-specific integrins mediate cell-cell adhesion by recognizing immunoglobulin (Ig)-superfamily counterreceptors, such as intercellular adhesion molecules (ICAMs) (Humphries et al. 2006).

Integrins can transmit signals bidirectionally. As many other receptors, integrins transmit signals from the outside of the cell to the cell interior via an outside-in signaling cascade. The engagement of extracellular ligand can result in changes in cytoplasmic regions and further downstream events. These events include changes in actin cytoskeleton arrangement, kinase signaling cascades, gene expression and the cell cycle. Secondly, integrin affinity for extracellular ligands can be regulated from inside the cells. In this inside-out signaling, intracellular signals lead to interactions of cytoplasmic proteins with integrins. The

cytoplasmic interactions lead to changes in the integrin extracellular parts, leading to an increase in integrin affinity for extracellular ligands (Ginsberg et al. 2005, Abram & Lowell 2009, Harburger & Calderwood 2009).

Cell adhesion involves the coordinated assembly and disassembly of integrins into adhesion-based mechanosensory structures. These structures include focal complexes, focal adhesions (FAs) in adherent fibroblasts, hemidesmosomes in epithelial cells, immunological synapses in T-lymphocytes, podosomes in macrophages and invadopodia in cancer cells (Shattil et al. 2010). In these complexes, integrin cytoplasmic parts are linked to the cell cytoskeleton and signaling cascades via various proteins (Zaidel-Bar et al. 2003, Legate & Fässler 2009). There are three described stages in the development of the cultured cell-ECM contacts: focal contacts, focal adhesions (FAs) and fibrillar adhesions. Each stage has its characteristic protein composition that consists of integrins and associated proteins (Zamir et al. 1999, Geiger et al. 2001). The complexity of the adhesion structures enables the modulation of cell adhesion and signaling.

2.1.1 Integrin structure

The integrins are type I transmembrane proteins that span plasma membrane once (Fig. 1). They form large non-covalent heterodimers (about 280 Å long) consisting of one α subunit (150-180 kDa) and one β subunit (about 90 kDa). Each integrin subunit consists of a large N-terminal extracellular domain (approximately 800 residues), a single transmembrane (TM) α-helix (approximately 20 residues) and a short cytoplasmic domain or tail (usually 13-70 residues).

Ectodomains

Since the first breakthrough crystal structures of a complete αVβ3 integrin ectodomain (Xiong et al. 2001, Xiong et al. 2002), only a few integrin heterodimer structures have been determined. These include resting structures of αIIbβ3 (Zhu et al. 2008), a more complete αVβ3 (Xiong et al. 2009) and αXβ2 (Xie et al. 2010). All of the structures are roughly similar, except the αXβ2 structure includes an inserted (α-I) domain. The integrin α chain ectodomain consists of β-propeller, thigh, calf-1 and calf-2 domains; the β chain ectodomain consists of plexin/semaphorin/integrin (PSI), hybrid, β-I, four epidermal growth factor (EGF), and β-tail (β-TD) domains (Luo et al. 2007). Additionally, nine of the 18 α subunits also contain an α-I domain, also named the von Willebrand factor A domain (Lee et al. 1995). The crystal structures reveal that the ectodomain can be considered an ovoid head standing on two legs (Fig. 1A). Accordingly, the integrin dimers can be divided structurally into three parts (head, upper legs, lower legs) or into two parts (headpiece, tailpiece).

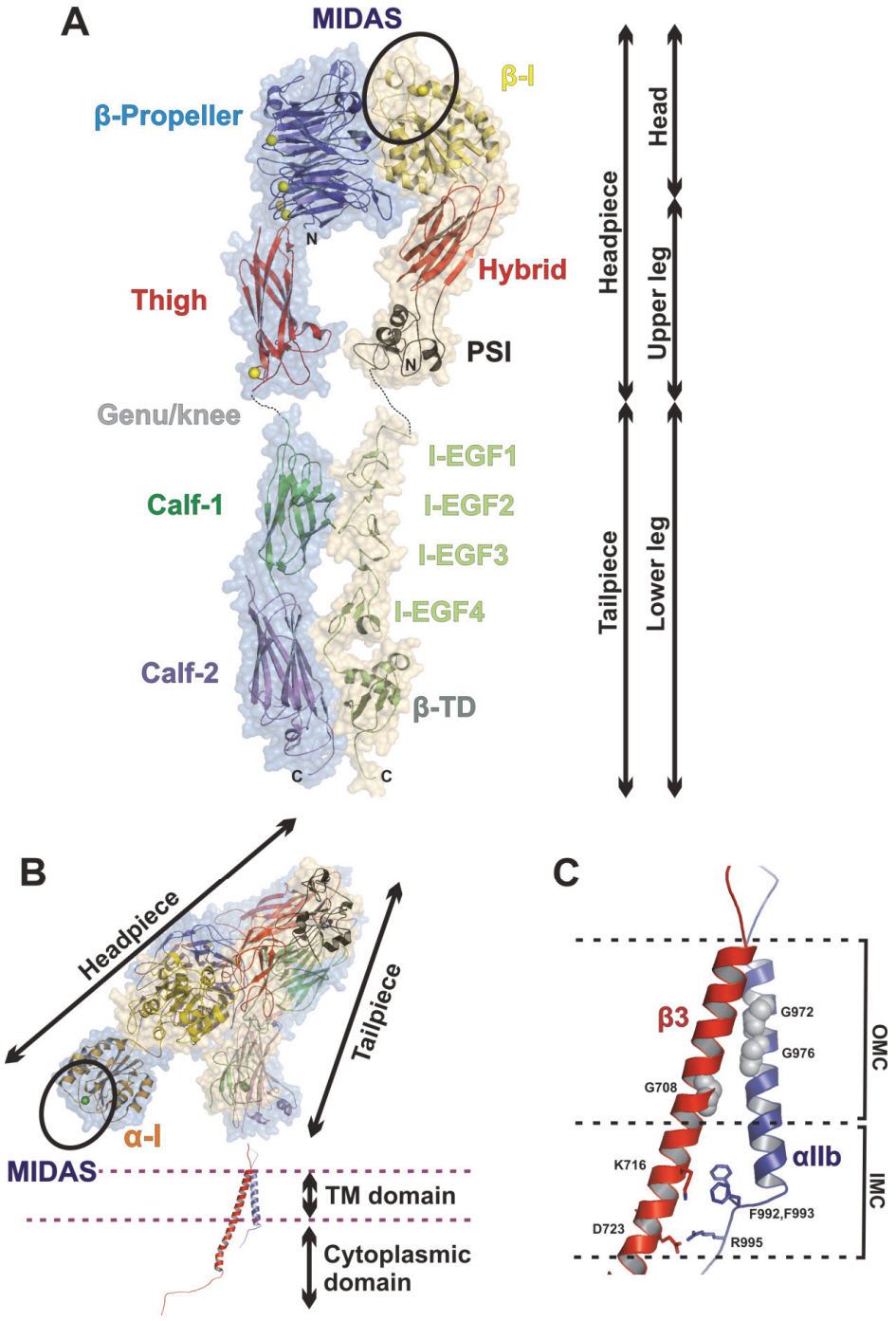

FIGURE 1 Structure of integrin extracellular and transmembrane domains. A. The extracellular parts of αVβ3 integrin (Protein Data Bank (PDB) code: 3IJE) (Xiong et al. 2009). The domain names and the metal-ion-dependent adhesion site (MIDAS) are explained in the text. Genu/knee denotes the bending point. The two alternatives for naming structural units are shown on the right. The

bent PDB structure is split into a headpiece and a tailpiece and presented as an extended for the sake of clarity. B. The bent ectodomain of αXβ2 that contains an α-I domain (PDB code: 3K6S) (Xie et al. 2010). A model of the transmembrane (TM) domain of αIIbβ3 (Lau et al. 2009) fused with integrin cytoplasmic parts (Vinogradova et al. 2002) is also included. C. The transmembrane domain of αIIbβ3 (PDB code: 2K9J) (Lau et al. 2009) where key residues of the outer membrane clasp (OMC) and the inner membrane clasp (IMC) are indicated. The integrin β chain is extended after D723 with a cytoplasmic solution structure (PDB code: 1M8O) (Vinogradova et al. 2002). Figure was prepared by using the PDB coordinates referred.

When present, the α-I domain is the major or only ligand-binding site in integrins. The domain consists of a Rossman fold with a central β-sheet surrounded by seven α-helices (Lee et al. 1995). On the upper surface of the domain resides a divalent cation-binding site where Mg^{2+} is coordinated by three loops. The residues coordinating the metal constitute a conserved metal-ion-dependent adhesion site (MIDAS). The MIDAS and the residues on the surrounding surface bind ligands in a cation-dependent way (Emsley et al. 2000). An α-I domain can exist in three conformations (closed, intermediate, open) that differ in coordinating the MIDAS site. The integrin α subunit head always contains a seven-bladed β-propeller domain. The domain has seven four-stranded antiparallel sheets that are radially arranged as blades. When included in the integrin, the α-I domain is inserted between two β-sheets of the β-propeller (Luo et al. 2007).

The integrin β subunit head consists of a β-I domain that is inserted into a loop of the hybrid domain. The β-I domain is homologous to the α-I domain with a central six-stranded β-sheet surrounded by eight α-helices. β-I domains have two conformations (closed, open). The β-I domain has three metal-binding sites. In addition to the MIDAS, there is a Ca^{2+} ion-binding site adjacent to the MIDAS (ADMIDAS). The third metal-binding site, the Ca^{2+}-binding ligand-induced metal ion-binding site (LIMBS) or synergistic metal ion binding site (SYMBS), resides near the β-propeller. In integrins that lack an α-I domain, the ligand binds to a crevice between the α and β subunits (Takagi et al. 2003, Adair et al. 2005). The ligand interacts with a MIDAS within the β-I domain and with the β-propeller domain (Xiong et al. 2002, Xiao et al. 2004). The propeller domain and the β-I domain form a large interface, and this interface is the main extracellular contact between the integrin chains (Xiong et al. 2001).

The leg region of the integrin α subunit comprises three β-sandwich domains. The upper leg consists of an Ig-like thigh domain, and the lower leg contains two domains called calf-1 and calf-2. In the β subunit, the hybrid domain forms the upper portion of the upper leg. The hybrid domain has an Ig-like fold with extensive contacts with the β-I domain. Surprisingly, the hybrid domain actually is an insertion in the PSI domain – a second component of the upper leg segment (Xiao et al. 2004). The rest of the flexible leg comprises four cysteine-rich integrin epidermal growth factor (I-EGF) domains, and a β-tail domain (β-TD).

In the ectodomain crystal structures, the legs are in a bent conformation (Fig. 1B) (Xiong et al. 2001). This same conformation is also determined by other

structural methods (Takagi et al. 2002). The bending point, termed the knee or genu, resides between the thigh and calf-1 domains (α-knee), and between the β integrin I-EGF1 and I-EGF2 domains (β-knee). The structures of the complete resting or bent ectodomains reveal a defined, well-packed interface between the lower leg parts. This α/β stalk interface (Kamata et al. 2005), together with an interface between I-EGF2 and the thigh domain (Xiong et al. 2009), can stabilize the resting state of integrins (Ulmer 2010). In addition, interactions between the β-TD and the head part can have a stabilizing role. As will be discussed later, this bent or resting conformation represents a low-affinity state of integrins (Luo et al. 2007).

Transmembrane domains

In a nuclear magnetic resonance (NMR) model of the αIIbβ3 integrin TM complex, both α- and β-chains form membrane-spanning helical structures that were at first thought to constitute a coiled-coil-like arrangement (Vinogradova et al. 2002, Gottschalk 2005). In the present asymmetric model (Fig. 1C), the α-chain helix is approximately perpendicular to the plasma membrane while the longer β chain is tilted by 25°. These conserved TM helices form two interacting association elements. The first element is termed the outer membrane clasp (Partridge et al. 2005, Lau et al. 2009). The outer membrane clasp is an interface with glycine-centered packing interactions where the central residues include αIIb G972 and G976, and β3 G708.

The second element, the inner membrane clasp, has hydrophobic and electrostatic bridges. The inner membrane clasp has an αIIb chain backbone reversal (a G-cap motif in GFF sequence) that packs a successive pair of hydrophobic phenylalanines (F992, F993) against the β TM helix residues. Additionally, the β3 K716 participates in the interface by interacting with αIIb (Zhu et al. 2009). This arrangement promotes an electrostatic interaction between β3 D723 and αIIb R995 (Lau et al. 2009), which is referred as the D723-R995 salt bridge. The salt bridge and the clasps are postulated to stabilize the association of the membrane proximal regions and thus the inactive state of integrins (Hughes et al. 1996, Partridge et al. 2005). However, the role of this salt bridge is not necessarily applicable to all integrins (Czuchra et al. 2006).

Cytoplasmic domains

The integrin cytoplasmic tails have highly conserved motifs that are important for integrin function (see Fig. 7 in chapter 2.3 below). These involve membrane-proximal GFFKR and HDR(R/K)E motifs of the α and β subunits, respectively, that have a role in integrin subunit association, as discussed above (Peter & O'Toole 1995, Hughes et al. 1996, Vinogradova et al. 2002). After these conserved sequences, the integrin β3 cytoplasmic domain consists of a stable helix contiguous with the TM helix. This helix is followed by a β-turn at the first conserved NPxY motif (residues 744-747) (Ulmer et al. 2001, Li et al. 2002, Vinogradova et al. 2002, Metcalf et al. 2010). The NPxY motif is followed by a Ser/Thr-rich sequence that contains a threonine triplet in some integrins. This region usually adopts an α-helical structure (residues 748-755) that is surround-

ed by unstructured regions (Li et al. 2002, Vinogradova et al. 2004, Ma et al. 2006). This C-terminal helix is followed by a second conserved β-turn motif (NxxY).

Some NMR studies indicate that the cytoplasmic tails of αIIb and β3 interact weakly (Li et al. 2002, Vinogradova et al. 2002, Ulmer et al. 2003), and this interaction influences the conformation of the β3 tail (Vinogradova et al. 2002, Metcalf et al. 2010). However, the results regarding the exact structure of cytoplasmic tails and the mutual interactions are not consistent. This is probably due to the transient and weak nature of the interactions measured (Ulmer et al. 2001, Wegener & Campbell 2008).

2.1.2 Integrin activation

Regulation of cell adhesion, migration and assembly—thus, integrin activation—is essential for virtually all cells. For example, normal integrin function is pivotal in the cell migration during multi-cellular organism development (Bökel & Brown 2002). In addition, the switch from the non-adherent to adherent cells is of great importance in blood cells. In platelets, the adhesion and aggregation in response to injury require rapid fibrinogen receptor (αIIbβ3 integrin) activation. This enables platelets to stop bleeding in a controlled way. Also at sites of inflammation, leukocyte integrins (*e.g.*, αLβ2) are activated in a coordinated way in order to get cells to adhere and migrate across the endothelium to the infected tissues, or in the T-cell interactions in immunological synapses (Abram & Lowell 2009). Consequently, abnormal integrin function can result in developmental defects, bleeding disorders (*e.g.*, arterial thrombosis and Glanzmann's thrombasthenia) or leukocyte adhesion deficiencies (LADs) (Abram & Lowell 2009).

Integrin conformation reflects its activity state. Integrins are thought to exist in three major conformational states: in low-affinity (inactive), high-affinity (active or primed) and ligand-occupied states. The transition from low- to high-affinity state is referred to as integrin activation (Calderwood 2004). This phenomenon can result from integrin clustering and from the changes in integrin conformation. Integrins are unique among adhesion molecules in that their adhesiveness can be regulated from inside the cell. In this inside-out signaling, stimuli received by other receptors start intracellular signals that alter integrin affinity for an extracellular ligand. In the outside-in signaling, extracellular ligand binding can induce integrin signaling that is conveyed directly from the integrin extracellular domain to the cytoplasm. Ligand-bound integrins recruit additional proteins to the cytoplasmic domains, which provide a link to a repertoire of signal transduction pathways and mechanical connection to the cytoskeleton (Abram & Lowell 2009).

The key event in inside-out integrin activation is the talin binding to the cytoplasmic β subunit of integrin (Tadokoro et al. 2003). In addition, the kindlin family of proteins is found to be essential integrin co-activators (Karaköse et al. 2010), as will be discussed later. The binding of talin disrupts the D723-R995 salt bridge and reorients the β TM helix. The altered tilt or movement of the β

TM helix disrupts the TM clasp (Anthis et al. 2009). The outcome is the separation of the integrin TM domains and cytoplasmic tails (Kim et al. 2003), and destabilization of the αβ stalk interface between the legs. This then leads to rearrangements in the extracellular thigh-knee interface (Ulmer 2010, Anthis & Campbell 2011).

The conformational changes accompanying integrin leg separation are believed to lead to long-range conformation changes that propagate all the way to the integrin ligand-binding headpiece. According to the current understanding, integrin extracellular domains adopt an extended conformation in the high-affinity state, whereas in the low-affinity state the domains are in a bent conformation. In the bent conformation, the integrin extracellular headpiece is closely associated with the tailpiece (Fig. 1B) (Xiong et al. 2009). There are two proposed structural models in integrin activation, called switchblade and deadbolt models. In the switchblade model, integrins bind to extracellular ligands only when in extended conformation. This more accepted model assumes that integrins have three conformations: bent, extended and extended with an open headpiece (Takagi et al. 2002, Askari et al. 2009). In contrast, the deadbolt model proposes that integrins extend only after ligand binding. The lifting of the constraints, which α-I experiences by a deadbolt interface with β-TD, could induce activation without integrin linearity (Xiong et al. 2003, Adair et al. 2005).

In general, the inactive integrin seems to adopt more compact conformation, although both bent and extended versions have been reported (Ye et al. 2010). Still, there are plenty of reports in which an active or ligand-bound integrin does not necessarily need to be in fully extended (Xiong et al. 2009). There is a correlation between the activity and unbending, although the degree of extension depends on the agonist and integrin (Askari et al. 2009). Ultimately, the integrin affinity for ligand depends on whether the integrin headpiece is in the open or closed conformation (Xiao et al. 2004).

The integrin activation is accompanied by changes in the β-hybrid domain orientation. In the bend state, the leg domains interact with each other and with the hybrid domain, which prevents the outward swing movement (Takagi et al. 2002). Upon activation, the β-hybrid domain of integrin is predicted to swing open, and the hybrid and PSI domains act as a rigid lever that relays this motion between the legs and the head. In the head, this swing-out pulls the α7 helix of the β-I domain downwards, which then favors the α1 helix to move upwards. These changes cause the β-I domain conformation to change from low-affinity to high-affinity, which includes some reorganization of the metal binding sites (Xiao et al. 2004). Structurally similar rearrangements are also seen in α-I domains. These include rearrangements in the MIDAS coordination as well as movements of α1- and α7-helices (Emsley et al. 2000, Takagi et al. 2002, Takagi et al. 2003). The allosteric activation signal is thought to be transmitted between β-I and α-I by an invariant glutamic acid. The Glu residue resides between the α-I and β-propeller domains, and scientists have proposed that it acts as an intrinsic ligand for the β-I MIDAS site (Xiao et al. 2004, Luo et al. 2007).

2.2 Integrin binding proteins – structure

2.2.1 Talin

Talin is a large (~270 kDa) cytoplasmic protein that activates integrins and links them to actin cytoskeleton. Two talin genes in vertebrates code two talin isoforms, talin1 and talin2. Talin2 is predominantly associated with stable adhesion structures in striated muscle and brain; talin1 is widely expressed in more dynamic adhesions of motile cells and is the only isoform in platelets (Senetar et al. 2007). Talins are composed of an N-terminal head domain (~50 kDa) and a C-terminal flexible rod domain (~220 kDa) (Fig. 2). A talin can form an antiparallel homodimer, and exists in extended and globular conformational states. The talin head has reported binding sites for, *e.g.*, integrins, phosphatidylinositol 4-phosphate 5-kinase γ (PIPKIγ), focal adhesion kinase (FAK), layilin and filamentous actin (F-actin); the talin rod has binding sites for vinculin, integrin and F-actin (Critchley & Gingras 2008).

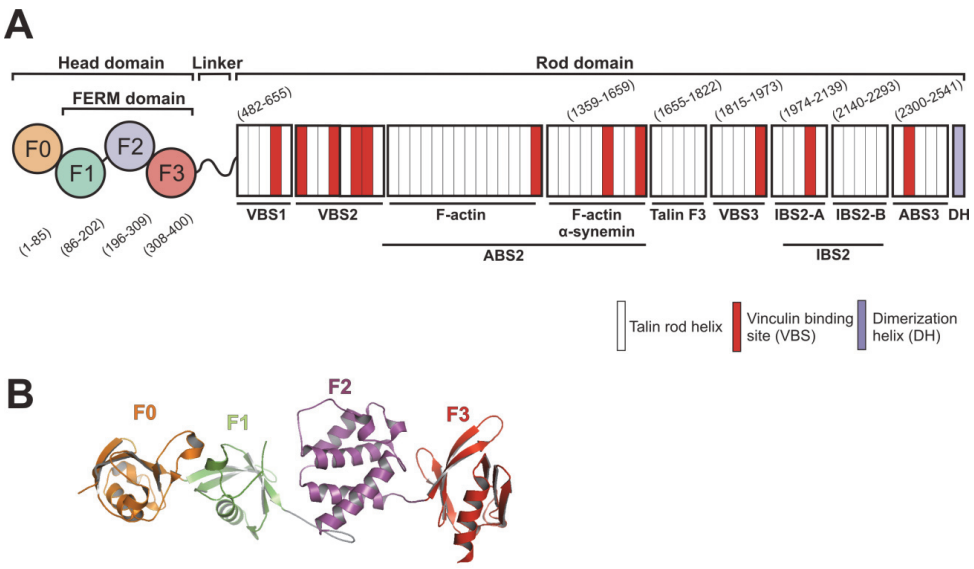

FIGURE 2 Overall structure of talin and the head domain. A. The schematic structure of the talin monomer, presented as in (Gingras et al. 2010). The binding sites of some proteins are indicated as well as residue ranges. Vinculin binding sites are colored red. B. Extended crystal structure of the complete talin head domain that includes a FERM domain (PDB code: 3IVF) (Elliott et al. 2010).

Talin head

The talin N-terminal head domain (residues 1-433) contains a band four-point-one/ezrin/radixin/moesin (FERM) domain subdivided into F1, F2 and F3 domains (Hamada et al. 2000, Pearson et al. 2000) that is preceded by the F0 domain (Fig. 2B). Both F0 and F1 domains have ubiquitin-like folds including a

five-stranded β sheet and an α-helix (Goult et al. 2010). The F2 domain contains a core of four α-helices and a short linker similar to the structure of the acyl-coenzyme A-binding protein. The F3 has a fold similar to other phosphotyrosine-binding (PTB) or pleckstrin homology (PH) domains and consists of a sandwich of two orthogonal antiparallel β sheets followed by a C-terminal α-helix (Garcia-Alvarez et al. 2003).

Talin has some unique characteristics compared to other FERM superfamily members. The F0 domain is characteristic of talin, and the specific feature of the domain is an extra helical turn not found in other ubiquitin-like folds. The F1 subdomain has an unstructured loop insertion of 30 residues. This F1 loop binds negative lipid bilayers, which induces helix formation. In a proposed fly-casting model (Goult et al. 2010), the loop draws talin closer to the plasma membrane while interacting with membrane phospholipids. The F2 and F3 domains also contain a phosphoinositide binding ridge that regulates talin autoinhibition, facilitates talin membrane association and supports integrin clustering (Saltel et al. 2009).

The consensus structure of FERM domains is characterized by a globular clover-shaped arrangement with independently folded F1-3 domains (Pearson et al. 2000). Surprisingly, the crystallized talin head adopts an extended conformation with linear domain arrangement (Fig. 2B) (Elliott et al. 2010). The network of close contacts between interfaces F0-F1 and F2-F3 stabilize the domain orientations. In contrast, the F1-F2 interface is relatively loosely packed. This F1-F2 flexibility and certain properties of F3 subdomain explain the non-canonical arrangement of the talin head (Elliott et al. 2010).

Talin head interactions

Talin is the first cytoplasmic protein characterized as directly interacting with integrins (Horwitz et al. 1986). It is shown to bind strongly with β1A, β1D, β2, β3 and β5 and weakly to β7 integrin tails (Calderwood 2004). This interaction takes place via the F3 subdomain of the head fragment that is usually sufficient for activating integrins in cells (Calderwood et al. 1999, Calderwood et al. 2002). Integrin activation also requires a stable β turn formed by an unphosphorylated integrin NPxY motif (Calderwood et al. 2002, Calderwood et al. 2003). Talin may be a general integrin activator because the motif for talin binding is well conserved among integrin β tails and talin F3 binds most integrins (Calderwood et al. 2003). Talin F3 forms an extensive binding interface with β integrin tails. This interface consists of membrane-distal and membrane-proximal regions of interaction (Vinogradova et al. 2002, Garcia-Alvarez et al. 2003, Wegener et al. 2007, Anthis et al. 2009). The structural details (and thus affinities) of how different integrins bind to talin vary (Anthis et al. 2010), but the main interactions are somewhat similar.

The membrane-distal interaction involves β3 residues ^{739}WDTANNPLYDEA^{750} (Fig. 3). Talin F3 interacts with the membrane-proximal NPxY motif by way of a canonical PTB domain (Pearson et al. 2000). Bound integrin ^{744}NPLY^{747} forms a reverse turn where the tyrosine Y747 side chain projects into an acidic binding pocket of talin F3 (Garcia-Alvarez et al.

2003). This binding pocket is strongly basic in other canonical PTB domains, and it coordinates the phosphate moiety of phosphotyrosine. Consequently, integrin binding is abrogated by NPxY phosphorylation in favor of other PTB protein interactions. Talin F3 also has a distinctive pocket that is occupied by a conserved β3 tryptophan (W739). In addition to NPxY, this aromatic residue is a key player in integrin interaction (Garcia-Alvarez et al. 2003), and it is located between the membrane-distal and membrane-proximal parts of the talin binding interface. When compared to other talin-binding NPxY-peptides, the integrins have two extra residues between the conserved tryptophan and the NPxY motif. This residue insert reduces the binding affinity to a more biologically optimal one (Anthis et al. 2010).

The membrane-proximal integrin-talin interaction is mainly hydrophobic and a unique feature not encountered in other PTB proteins. This interaction has also been shown to be critical in integrin activation (Wegener et al. 2007). When bound to the talin head, the integrin tail adopts a helical conformation extending from β3 A737 to the β TM domain (Anthis et al. 2009, Metcalf et al. 2010). A flexible loop of talin F3 forms an elongated, largely hydrophobic interface that interacts with two β3 phenylalanine side chains (F727 and F730) (Wegener et al. 2007).

When bound to integrin, the extended talin head may orient to enable favorable electrostatic contacts with acidic membrane phospholipids (Elliott et al. 2010). These contacts involve positive patches on talin F2 (Anthis et al. 2009) and F3 (Wegener et al. 2007, Ye et al. 2010) and the basic F1 loop discussed above (Goult et al. 2010). Talin F3 binding to integrin membrane-proximal region causes the β tail helix to tilt in respect to the membrane bilayers. In addition, the F0 domain is required for maximal integrin activation, but the exact mechanism is still unclear (Bouaouina et al. 2008, Goult et al. 2010). These membrane interactions of F0-3 domains are important for full integrin activation and clustering (Bouaouina et al. 2008, Saltel et al. 2009).

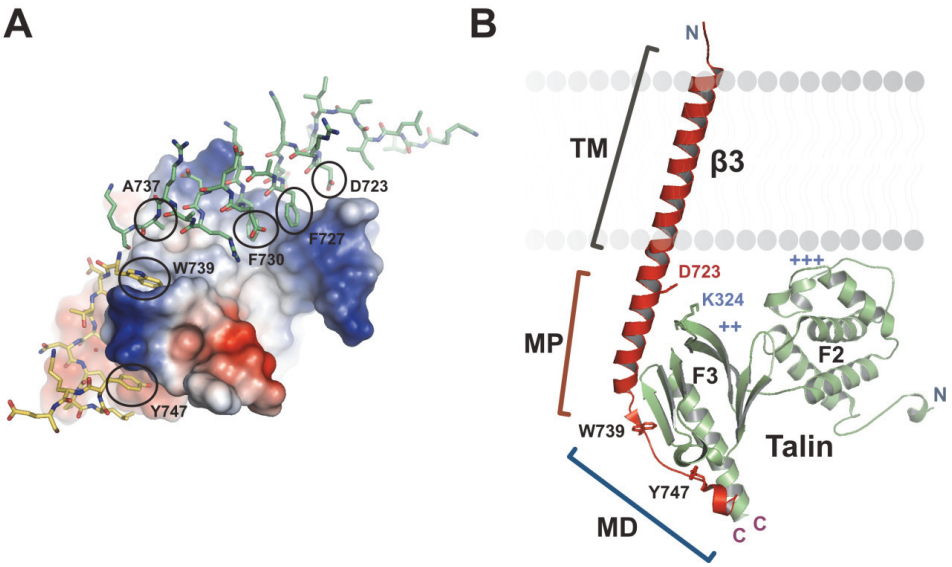

FIGURE 3 Structure of talin in complex with the β integrin tail. A. Talin F2-F3 domain pair in complex with β3 integrin. The figure is prepared by superimposing the crystal structure of the membrane-distal (MD) interaction (PDB code: 1MK7, yellow) (Garcia-Alvarez et al. 2003) and the solution structure of membrane-proximal (MP) interface (PDB code: 2H7E, green) (Wegener et al. 2007). Key residues are indicated. B. Crystal structure of talin2 F2-F3 in complex with the β1D integrin peptide (PDB code: 3G9W) (Anthis et al. 2009). The TM region is taken from the structure of αIIbβ3 (PDB code: 2K9J) (Lau et al. 2009). Residues are named as in β3 integrin and talin1.

In the current inside-out activation model, talin initially binds to the NPxY motif and subsequently engages the membrane-proximal helix of the integrin β tail (Fig. 3) (Campbell & Ginsberg 2004). As discussed above, this destabilizes the integrin α-β subunit interactions leading to integrin activation. The D723-R995 salt bridge is disrupted by a new salt bridge between the F3 K324 and β3 integrin D723 (Anthis et al. 2009). In addition, the tilt induced by the talin interaction disrupts the inner membrane clasps leading to changes in the TM interface (Lau et al. 2009). Indeed, it has recently been shown that talin binding to a single αIIbβ3 dimer in a phospholipid layer is sufficient to induce integrin extension (Ye et al. 2010).

In addition to integrins, talin FERM is shown to bind layilin and an enzyme variant called phosphatidylinositol phosphate kinase type Iγ-90 (PIPK1γ-90). Both PIPK1γ-90 and layilin bind to the F3 subdomain with a canonical PTB-like interaction. These interactions differ only subtly from the integrin membrane-distal NPxY interaction. Consequently, the binding of these three proteins to talin is mutually exclusive. The affinities, however, differ quite substantially as the PIPK1γ binding is the strongest and the integrin binding the weakest (Barsukov et al. 2003, Garcia-Alvarez et al. 2003, de Pereda et al. 2005, Wegener et al. 2007, Anthis et al. 2010).

Talin has three reported actin-binding sites (ABS1-3): one in the head and two in the C-terminal rod domain (Fig. 2A). The ABS1 resides on the F2 and F3 subdomains of FERM. The actin binding probably does not exclude integrin or PIPK1γ binding to the F3 subdomain, which could allow the formation of ternary complexes with actin (Lee et al. 2004). In addition, phosphatidylinositol 4,5-bisphosphate (PIP2) binds to a site spanning the F2 and F3 subdomains. This binding disrupts the interaction between the talin rod and head regions (Martel et al. 2001, Saltel et al. 2009) but does not interfere with the talin-integrin interaction (Goksoy et al. 2008). This PIP2 binding has a role in talin activation, as will be discussed later.

Talin rod

The talin rod region consists of a total of 62 amphipatic α-helices (Fig. 2A). These helices form mainly five-helix bundles with intervening four-helical bundles (Roberts & Critchley 2009). The rod region starts with a five-helix bundle (residues 482-655) followed by a four-helix bundle (residues 656-786) that has unique topology with multiple vinculin-binding sites (VBSs) (Izard et al. 2004, Papagrigoriou et al. 2004). The middle region of the rod (residues 1359-1659) consists of two unusual bundles with a four-helix bundle inserted into one loop of the five-helix bundle. This region contains an actin-binding site, two VBSs and a binding site for α-synemin (Gingras et al. 2010).

In addition to the three initially found vinculin-binding sites (VBS1-3), around eight amphipatic talin α-helices are predicted to bind a hydrophobic pocket in the vinculin D1 (Vd1) domain (Gingras et al. 2005). In intact VBSs of talin, the key residues involved in the Vd1 interaction are buried within the hydrophobic core of the talin helical bundle. Consequently, the uncovering of these cryptic VBSs may require regional unfolding of talin rod bundles, for example, by mechanical force or phosphorylation (Izard et al. 2004, Papagrigoriou et al. 2004).

A five-helix bundle of talin rod (residues 1655-1822) binds to the F3 of the talin head and partially masks the integrin-binding site (Calderwood 2004, Goksoy et al. 2008). This results in globular autoinhibited conformation of talin. The head-rod interaction may also inhibit the association of the FERM domain with the membrane interface (Goult et al. 2009).

The C-terminal part of the talin rod (residues 1974-2293) also contains a second integrin-binding site (IBS2) (Gingras et al. 2009). This can be divided into two five-helical bundles, called IBS2-A and IBS2-B, both of which are required for strong integrin interaction. The binding site is located within the membrane-proximal region of the β integrin tails that includes the HDR(R/K)E motif. The talin rod affinity for integrins is substantially lower than the head's (Yan et al. 2001). Unlike the FERM domain, IBS2 is not able to activate integrins but probably contributes to integrin clustering (Tremuth et al. 2004, Gingras et al. 2009).

The C-terminus of talin has a five-helix bundle (residues 2300-2482) that resembles a talin/HIP1R/Sla2p actin-tethering C-terminal homology (THATCH) core domain (Gingras et al. 2008). This bundle contains a conserved

actin-binding site (ABS3) on a hydrophobic surface of its helices 3 and 4 that is flanked by positively charged residues. The bundle is followed by a C-terminal helix that is responsible for talin dimerization. Together, the talin C-termini form a dimer that can bind to F-actin (Gingras et al. 2008).

2.2.2 Filamin

Filamins (FLNs) are large members of actin-binding proteins that were the first actin cross-linking proteins to be found in non-muscle cells (Stossel & Hartwig 1975). They are found in *Dictyostelium* amoebas and all over the animal kingdom but not in plants. In vertebrates, there are three filamin isoforms, filamin A (FLNa), filamin B and filamin C. Filamins A and B are ubiquitously expressed, whereas filamin C is mainly muscle-specific. The three isoforms have a largely conserved domain structure and both common and specific binding partners (Stossel et al. 2001, van der Flier & Sonnenberg 2001).

Human FLNa (or ABP-280) is a large (240-280 kDa) elongated protein that forms a Y- or V-shaped homodimer with 80-90° branching angle (Fig. 4) (Nakamura et al. 2007). The FLNa subunit is composed of an N-terminal actin-binding domain (ABD) followed by a rod region that consists of 24 Ig-like domains or repeats (van der Flier & Sonnenberg 2001). They form an immunoglobulin-like β sandwich fold of two antiparallel β sheets that are usually composed of seven β strands (named A-G). The first sheet is composed of strands A, B, E and D; the second sheet consists of strands C, F and G (van der Flier & Sonnenberg 2001, Pudas et al. 2005). Two flexible hinge regions separate the domains into two segments: rod1 (domains 1-15) and rod2 (domains 16-23). The dimerization of filamins occurs through the most C-terminal domain, FLNa24 (Pudas et al. 2005).

In electron micrographs, FLNa rod1 appears as an extended chain with end-to-end domain composition whereas rod2 has a more compact structure (Nakamura et al. 2007). Filamin is shown to have more than 90 binding partners that include intracellular signaling molecules, ion channels, enzymes, transcription factors and membrane receptors (Nakamura et al. 2011). While the ABD and flexible rod1 bind F-actin, most of the filamin partner interactions occur within the rod2 region (Nakamura et al. 2007). More than 50 binding partners are reported to bind rod2. FLNa facilitates signal transduction between the molecules by binding multiple partners with this region (Ohta et al. 2006).

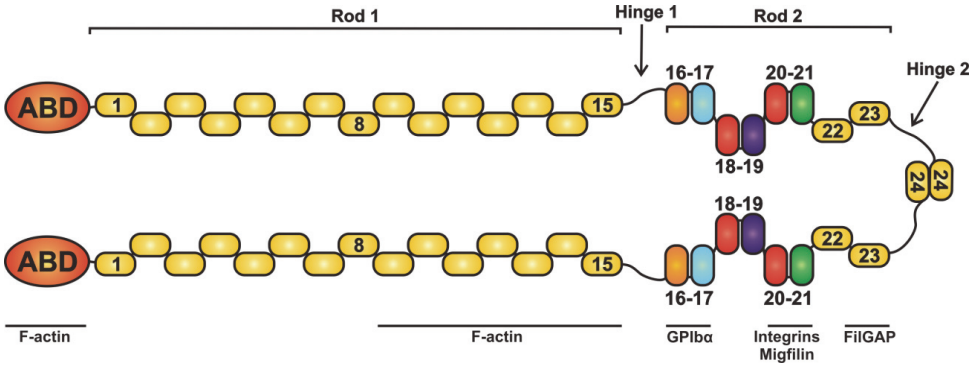

FIGURE 4 Overall schematic structure of filamin A dimer, adapted from (Stossel et al. 2001). The binding sites of some proteins are included.

Filamin ABD consists of two helical calponin homology domains (CH1 and CH2) with three actin-binding sites. There are several patient mutations reported in the ABD (Clark et al. 2009, Ruskamo & Ylänne 2009). The amino acid sequence places ABD in the α-actinin or spectrin superfamily. Calmodulin regulates the FLNa-F-actin interaction in a calcium-dependent way (Nakamura et al. 2005). A second actin-binding site of FLNa resides at the domains 8-15 of rod1 region, and this distal half of rod1 facilitates the F-actin binding of ABD (Nakamura et al. 2007).

Dimerization of FLNa is pivotal for the actin-crosslinking and F-actin branching activity, which is consistent with the engagement of the N-terminal part with F-actin (Nakamura et al. 2007). The dimerization domain (FLNa24) adopts a seven-stranded Ig-like fold with a unique dimerization interface. This extensive interface involves the strands C and D of both FLNa24 monomers. The D strands extend the β-sheet of both monomers to form a single large β-sheet, whereas the β sheets formed by CFG strands remain separate (Pudas et al. 2005, Sjekloca et al. 2007, Seo et al. 2009). Although FLNa dimerizes in an anti-parallel fashion, the whole filamin dimer is predicted to run parallel (Fig. 4).

The ultrastructure of the rod2 dimer appears as three globular units that form an L-shaped structure (Nakamura et al. 2007). The compactness of the rod2 region is explained by the unique arrangement of the domains, where the even-numbered domains form pairs with the next odd-numbered domain. This arrangement forms pairs FLNa16-17, FLNa18-19, FLNa20-21 and FLNa22-23 (Heikkinen et al. 2009). The FLNa16 and FLNa17 domains interact side-by-side, and the FLNa22-23 adopts a more elongated configuration without interdomain interactions (Heikkinen et al. 2009).

Unexpectedly, the crystal structure of FLNa19-21 revealed a novel type of arrangement between domains 20 and 21 (Fig. 5A) (Lad et al. 2007). The N-terminal A-strand of FLNa20 extends from the domain and interacts with the groove formed by strands C and D (the CD face) of the FLNa21. In this way, FLNa20 extends the β sheet made by strands C, G and F of FLNa21. This kind of interaction causes non-sequential domain order. The FLNa19 is followed by

FLNa21 in a linear fashion, whereas the FLNa20 lies on top of FLNa21 roughly perpendicular to the long axis. In addition, FLNa18-19 forms an interdomain complex similar to FLNa20-21 but with some differences in relative domain orientations (Heikkinen et al. 2009). This domain arrangement provides nonlinear configuration and thus a compact fold of the rod2.

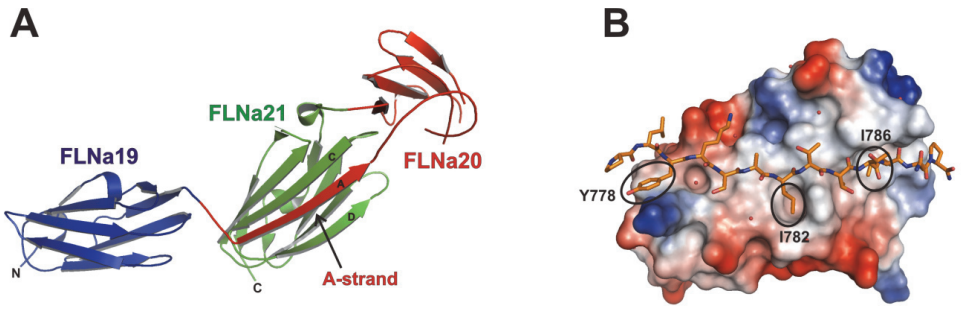

FIGURE 5 Structure of FLNa rod 2 domains and a complex with β7 integrin. A. Crystal structure of FLNa19-21 (PDB code: 2J3S) (Lad et al. 2007). The autoinhibitory A-strand is flanked by C- and D-strands as indicated. B. Crystal structure of FLNa21 in complex with β7 integrin peptide (PDB code: 2BRQ) (Kiema et al. 2006). Key binding residues are marked.

Filamin - integrin interaction

Filamin has been shown to bind β1A, β2, β3 and β7, but poorly to β1D integrin cytoplasmic tails (Sharma et al. 1995, Pfaff et al. 1998, Calderwood et al. 2001, Travis et al. 2004). The integrin β cytoplasmic tail binds mainly to filamin domain 21 and weaker to domain 19 (Kiema et al. 2006). The crystal structure of FLNa21 in a complex with the integrin β7 cytoplasmic tail fragment reveals that the interacting part of the integrin includes residues ^{776}PLYKSAITTTINP^{788} (Fig. 5B) (Kiema et al. 2006). These residues reside at the conserved segment between the two NPxY sequences. The bound integrin forms a β-strand that extends the β sheet formed by the CFG strands of FLNa21. The binding site consists of the CD face of the FLNa that is otherwise occupied by the A-strand of the FLNa20 domain (Lad et al. 2007). In addition to the main-chain interactions, the contacts are mainly hydrophobic. Three residues (Tyr778, Ile782 and Ile786) contribute the most to the interaction surface, and these residues are identified as important for filamin binding (Zent et al. 2000, Calderwood et al. 2001, Kiema et al. 2006).

Small variations of the interacting residues modify the affinity of the integrin-filamin interaction. These changes include the sequence variations in integrin β tails (Calderwood et al. 2001) as well as changes in binding residues in FLNa19 or other filamin domains (Kiema et al. 2006). The FLNA21/β7 complex can be regarded as a general model for multiple integrin-filamin interactions, and the specificity of the interactions is determined by the hydrogen bonding and hydrophobic interactions of the side chains (Kiema et al. 2006). Other rod2 interactions are formed in a similar way, as seen in the complex structures of FLNa17 and glycoprotein Ibα (GPIbα), FLNa21 and migfilin, and FLNa23 and

filamin A-associated Rho guanosine triphosphatase-activating protein (FilGAP) (Nakamura et al. 2006, Ohta et al. 2006, Lad et al. 2008, Nakamura et al. 2009). Each binding interface consists of a β-strand of nine residues that positions itself in the groove of the CD face. Alternating residues face this groove in hydrophobic fashion.

In FLNa19 and FLNa21, the A-strand of the preceding even-numbered domain binds to the same site as the ligand. This leads to competitive binding and is the basis for filamin autoinhibition. For example, the sequence of the FLNa20 A-strand sequence is suboptimal compared to β7 integrin but does compete for FLNa21 binding. Indeed the disruption or truncation of this interaction between FLNa20 A-strand and FLNa21 enhances the binding of integrin (Lad et al. 2007). The CD faces of the domains 17 and 23 are not masked by an A-strand. Instead, the non-bound A-strand allows free interaction with other proteins, such as glycoprotein Ibα (GPIbα) and FilGAP (Heikkinen et al. 2009).

2.2.3 14-3-3

The 14-3-3 proteins are small (~30 kDa) acidic cytoplasmic proteins that are expressed in all eukaryotic organisms (Aitken 2006). The 14-3-3 proteins are among the most abundant proteins in cell. In mammals, these proteins are widely expressed, especially in the nervous system. The seven mammalian 14-3-3 isoforms are named as Greek letters (β, γ, ε, ζ, η, τ/θ and σ), two of which (β and ζ) also have phosphorylated forms (α and δ, respectively) (Aitken 2002, Aitken 2006). The 14-3-3 proteins are highly conserved in their sequence and structure, with deviations mainly in their dimerization site (Gardino et al. 2006).

The crystal structures of 14-3-3 isoforms reveal a highly helical fold unrelated to other protein families (Liu et al. 1995, Xiao et al. 1995, Aitken 2002). The 14-3-3ζ monomer consists of a bundle of nine antiparallel α-helices (αA-αI) that are connected by short loops (Fig. 6). Four N-terminal α-helices form a dimerization site where the A- and B-helices face the C- and D-helices of the adjacent monomer. Dimerization interactions include three salt bridges, and hydrophobic and polar interactions. Consequently, the 14-3-3ζ proteins form horse-shoe-like concave dimers with a large central canal. Helices αC, αE, αG and αI enclose an amphipatic groove. The αG and αI helices form a hydrophobic surface on one side of the groove, whereas the side chains of the αC and αE helices form a polar and charged surface on the other side. Residues Lys49, Arg56 and Arg127 form the basic ligand-binding pocket (Liu et al. 1995).

The last αI helix is followed by a diverse region that is not visible in crystal structures. This unconserved segment has certain Asp and Glu residues that bind the basic ligand-binding site of the amphipatic groove (Obsilova et al. 2004, Silhan et al. 2004). This C-terminal stretch is assumed to have an autoinhibitory role, as the 14-3-3ζ with truncated C-terminus binds ligands more readily (Truong et al. 2002).

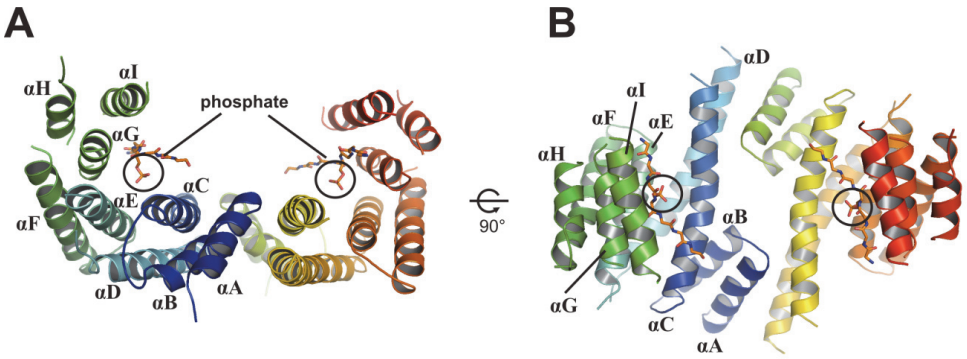

FIGURE 6 Structure of 14-3-3ζ in complex with a phosphopeptide. The crystal structure is shown from the front (A) and above (B). Nine helices are indicated, as well as the phosphorylated residue of the extended mode I peptide (PDB code: 1A37) (Petosa et al. 1998). All the loop regions connecting the helices were not visible in the crystal structure.

The 14-3-3 proteins are reported to interact with more than 200 partners, including DNA (Aitken 2006). The 14-3-3 proteins mainly bind to motifs that are phosphorylated on serine or threonine (Muslin et al. 1996). Several optimal binding motifs are defined, including mode I (RSxpSxP), mode II (RxYxpSxP) and mode III motifs (Yaffe et al. 1997, Rittinger et al. 1999, Ganguly et al. 2005). Most of the interactions resemble modes I and II, but the 14-3-3 proteins have also been found to bind unphosphorylated sequences (Petosa et al. 1998, Aitken 2006). The actual biological interactions with 14-3-3 usually deviate from the optimal mode interactions and have lower binding affinities, and the overall binding can also be affected by sites outside the binding pocket (Obsil et al. 2001, Gardino et al. 2006).

The structures of 14-3-3 in complex with ligand peptides show that 14-3-3 conformation is unaffected by a ligand (Yaffe et al. 1997, Petosa et al. 1998, Rittinger et al. 1999). An extended ligand peptide is aligned in the amphipatic groove and interacts with the side chains of helices αE, αG and αI. The positively charged basic pocket binds to the negative phosphoserine or -threonine of the ligand. In addition, the interaction is further strengthened by a hydrogen bond between phosphate and Tyr128 of 14-3-3ζ. Only a few solved structures of 14-3-3 proteins are in complex with entire proteins as with serotonin N-acyltransferase (Obsil et al. 2001), and no structures are directly related to integrins.

2.3 Regulation of integrin function by binding proteins

Integrin outside-in and inside-out signaling involves numerous cellular components that assemble and disassemble in a regulated way. Short integrin cytoplasmic tails do not show any enzymatic activity or ability to bind actin, micro-

tubules or intermediate filaments. However, integrins work as signaling hubs. Recently, over 40 proteins were reported to interact directly with integrin β tails (Legate & Fässler 2009) (Table 1). These interacting proteins form an adhesion complex, integrin adhesome. Integrin adhesion complexes consist of an extensive network of proteins that interact with each other (Zaidel-Bar et al. 2007). Spatially, the proteins of the adhesion complex seem to organize into distinct horizontal layers with a specific function. Three layers have been proposed: integrin signaling layer, force transduction layer and actin regulatory layer (Kanchanawong et al. 2010).

The integrin-associated proteins can be divided into several categories. One way to group these proteins is to 1) cytoskeletal or structural proteins, 2) scaffolding adapters and 3) catalytic and signaling proteins. Although different integrin-binding proteins can be divided into three classes, their function is remarkably redundant in cells (Legate & Fässler 2009). The proteins that are reported to interact with β integrin tails are tabulated in Table 1.

TABLE 1 Proteins that interact with integrin β cytoplasmic tails. The table is modified from (Legate & Fässler 2009).

Protein	Integrin tail	Reference
Cytoskeletal or structural proteins		
α-actinin	β1, β2, β3	(Otey et al. 1993, Pavalko & LaRoche 1993)
Bullous pemphigoid antigen 180 (BP180)	β4	(Schaapveld et al. 1998, Koster et al. 2003)
Filamin	β1, β2, β3, β7	(Sharma et al. 1995, Pfaff et al. 1998, Calderwood et al. 2001, Travis et al. 2004)
Myosin	β1, β3, β5	(Jenkins et al. 1998, Sajid et al. 2000, Zhang et al. 2004)
Plectin	β4	(Geerts et al. 1999)
Skelemin	β1, β3	(Reddy et al. 1998)
Talin	β1, β2, β3, β5, β7	(Horwitz et al. 1986, Pfaff et al. 1998, Sampath et al. 1998, Calderwood et al. 1999, Calderwood et al. 2001, Calderwood et al. 2003)
Tensin	β1, β3, β5, β7	(Calderwood et al. 2003, McCleverty et al. 2007)
Scaffolding adapters		
14-3-3	β1, β2, β3, β4	(Han et al. 2001, Santoro et al. 2003, Fagerholm et al. 2005)
β3-endonexin	β3	(Shattil et al. 1995, Eigenthaler et al. 1997)
Disabled 1 (Dab1)	β1, β2, β3, β5, β7	(Calderwood et al. 2003)
Disabled homolog 2 (Dab2)	β3, β5	(Calderwood et al. 2003)
Docking protein 1 (Dok1)	β2, β3, β5, β7	(Calderwood et al. 2003)
Four and a half LIM domains protein 2 (Fhl2)	β1, β2, β3, β6	(Wixler et al. 2000)

Table 1 continues

Table 1 continues

Four and a half LIM domains protein 3 (Fhl3)	β1	(Samson et al. 2004)
Growth factor receptor-bound protein 2 (Grb2)	β3	(Blystone et al. 1996, Law et al. 1996)
Integrin-associated protein (IAP)	β3	(Brown et al. 1990)
Integrin cytoplasmic domain-associated protein-1α (ICAP-1α)	β1	(Chang et al. 1997, Zhang & Hemler 1999)
Integrin-linked kinase (ILK)	β1, β3	(Hannigan et al. 1996, Pasquet et al. 2002)
Jun activation domain-binding protein 1 (JAB1)	β2	(Bianchi et al. 2000)
Kindlin 1	β1, β3	(Harburger et al. 2009)
Kindlin 2	β1, β3	(Ma et al. 2008, Montanez et al. 2008)
Kindlin 3	β1, β2, β3	(Moser et al. 2008, Moser et al. 2009a)
Melusin	β1	(Brancaccio et al. 1999)
Numb	β3, β5	(Calderwood et al. 2003)
Paxillin	β1, β3	(Schaller et al. 1995, Chen et al. 2000)
Receptor for activated protein kinase 1 (Rack1)	β1, β2, β5	(Liliental & Chang 1998)
Shc	β3, β4	(Law et al. 1996, Dans et al. 2001)
Theta-associated protein 20 (TAP20)	β5	(Tang et al. 1999)
WAIT1	β7	(Rietzler et al. 1998)
Catalytic and signaling proteins		
Cytohesin 1	β2	(Kolanus et al. 1996)
EGF receptor pathway substrate 8 (Eps8)	β1, β3, β5	(Calderwood et al. 2003)
Extracellular signal-regulated kinase 2 (ERK2)	β6	(Ahmed et al. 2002)
Focal adhesion kinase (FAK)	β1, β2, β3, β5	(Schaller et al. 1995, Chen et al. 2000, Eliceiri et al. 2002)
Fyn	β3	(Arias-Salgado et al. 2005)
Lyn	β1, β2, β3	(Arias-Salgado et al. 2005)
Protein kinase D1 (PKD1)	β1, β3	(Woods et al. 2004, Medeiros et al. 2005)
Protein phosphatase 2A (PP2A)	β1	(Kim et al. 2004)
Shp2	β4	(Bertotti et al. 2006)
Src	β3	(Arias-Salgado et al. 2003, Arias-Salgado et al. 2005)
Yes	β1, β2, β3	(Arias-Salgado et al. 2005)
Other proteins		
Cluster of differentiation 98 (CD98)	β1, β3	(Zent et al. 2000)
Muscle-specific beta1 integrin binding protein (MIBP)	β1	(Li et al. 1999)

There is prominent overlap between protein binding sites on the integrin β tails, which leads to competition between interacting proteins (Fig. 7). There are three conserved regions on β tails, so-called hot spots that are the preferred binding sites of several proteins. According to Legate and Fässler, these spots include a membrane-proximal HDR(R/K)E motif, a membrane-proximal NPxY motif and a membrane-distal NxxY motif (Legate & Fässler 2009). Additionally, an intervening Ser/Thr-rich sequence located between the NPxY/NxxY motifs has an important role in several protein interactions (Ma et al. 2006).

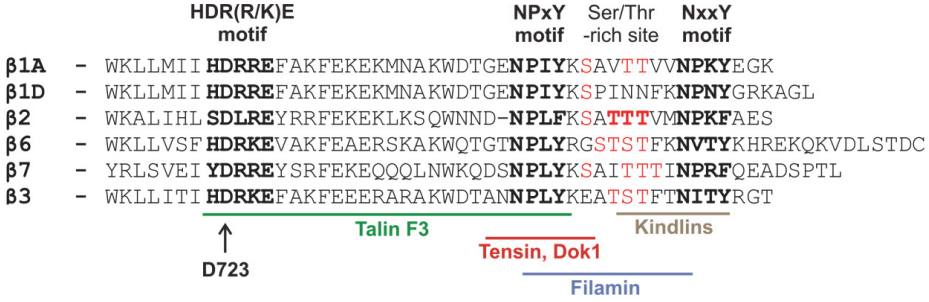

FIGURE 7 Alignment of integrin β cytoplasmic tails with some key interactions (Legate & Fässler 2009, Anthis & Campbell 2011). Manual alignment of the integrins shows the three conserved sites that are highlighted. The possible phosphorylation sites of the Ser/Thr-rich sequence are colored in red, and the β2 threonine triplet is highlighted. D723 that is important in integrin activation is marked. The binding sites of the discussed proteins are indicated.

Integrin function has to be carefully coordinated in order to enable proper cellular function. Therefore, the activation of integrins is regulated spatially and temporally (Gahmberg et al. 2009). There are several ways of regulating integrin cytoplasmic interactions and integrin function (Fig. 8):

1) Protein autoinhibition

Several integrin-binding proteins can exist in an auto-inhibited form (Lad et al. 2007, Goksoy et al. 2008). This autoinhibition can be unraveled by several ways, as discussed in the context of the proteins talin and filamin.

2) Integrin β tail phosphorylation

The main locations for integrin phosphorylation are the hot spots regions. Several PTB domains are shown to bind NxxY motifs in integrins (Calderwood et al. 2003), but the specificity for the motif phosphorylation varies; the phosphorylation status can either increase or abrogate this binding (Garcia-Alvarez et al. 2003, McCleverty et al. 2007, Oxley et al. 2008). In addition, β integrin cytoplasmic serine and threonine residues can be phosphorylated (Fagerholm et al. 2004).

3) Competitive or co-operative adapter binding to integrin tails

The overlap of the integrin-binding sites causes the interacting proteins to compete for integrin binding. Other proteins can either compete or cooperate with

talin, hence modulating integrin activation (Kiema et al. 2006, Moser et al. 2008). In addition, the phosphorylation of integrin can sometimes be considered a molecular switch that displaces one protein in favor of another (Oxley et al. 2008).

4) Recruitment by other proteins (co-adapters)
The intracellular localization and recruitment of integrin-binding proteins is one way to regulate the integrin cytoplasmic interactions (Anthis & Campbell 2011). Talin recruitment to the plasma membrane is discussed later.

2.3.1 Regulation of integrin-talin interaction

Talin plays a vital role in integrin-mediated events such as cell adhesion, migration and survival. Depletion of talin can cause phenotypes similar to those generated by defective integrins. Talin1 is important for animal development since its absence in mice is embryonically lethal (Monkley et al. 2000). In T-cells, talin is required for T-cell receptor (TCR)-mediated ICAM-1-adhesion, regulation of affinity, migration, polarization and clustering of the αLβ2 integrin. Talin accumulates F-actin and integrin at the immunological synapse. Talin is also required, *e.g.*, for platelet adhesion and macrophage phagocytosis (Critchley & Gingras 2008).

Talin acts as a mechanical link between integrins and the actin cytoskeleton (Roberts & Critchley 2009). Therefore, it is essential for coupling the cytoskeleton to adhesion structures and ECM, and to related signaling events (Zhang et al. 2008). Talin acts as a scaffold for other proteins, and it recruits these proteins, like vinculin, to FAs. Talin also promotes integrin clustering (Cluzel et al. 2005). With multiple integrin-binding sites, it can cross-link or cluster integrins into adhesive structures. Therefore, talin is required for FA assembly, signaling and stabilization (Roberts & Critchley 2009). As already discussed, talin is essential for integrin activation (Calderwood 2004, Campbell & Ginsberg 2004). Because of this crucial role, integrins can be regulated by controlling the talin-integrin interaction itself. This regulation is achieved by various elaborate mechanisms that include post-translational modifications, other proteins and interacting molecules (Fig. 8).

Talin autoinhibition
Intact talin can exist in autoinhibited form in which the integrin-binding site of the head domain can be masked by the rod region. Consequently, full-length talin is a relatively poor integrin activator whereas the talin head only is a notably more potent one (Goksoy et al. 2008). Talin belongs to the band 4.1 superfamily that also includes the ezrin/radixin/moesin (ERM) protein family. In ERM proteins, the interaction sites are masked by intramolecular masking that resembles talin autoinhibition. The unmasking of the ERM ligand-binding site is achieved by several ways that include protein cleavage, binding to phosphoinositides and the phosphorylation of the tail region (Hamada et al. 2000, Pearson et al. 2000). In addition, talin autoinhibition seems to be released in similar ways.

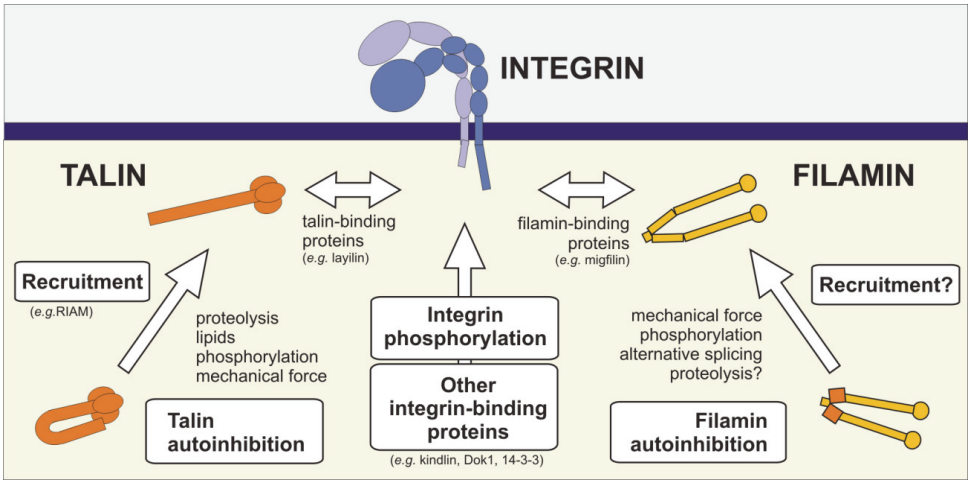

FIGURE 8 Regulation of integrin-talin and integrin-filamin interaction. The interactions are affected by the ways presented in the boxes. Talin and filamin can exist as an autoinhibited form (bottom corners). This autoinhibition is unraveled by several means also discussed in the text. The interaction with integrins can be further regulated by integrin phosphorylation. Other proteins can compete with talin and/or filamin for binding to the integrin tail; or other proteins can compete with the integrin tail for binding to talin or filamin. In addition, the proteins can be recruited to the integrins by other proteins.

The **calpain** family of proteases is implicated in multiple cellular processes, including cell migration and spreading. Calpains regulate the dynamics of integrin-mediated adhesions, and their substrates include numerous adhesion complex proteins (Franco & Huttenlocher 2005). Calpain is shown to cleave talin resulting in a talin head and a talin rod fragment. Because the talin head has six times higher binding affinity for the β3 tail than full-length talin has, the cleavage can facilitate talin binding and integrin activation (Yan et al. 2001). Calpain contributes to the activation of αVβ3 and αIIbβ3 integrins. However, calpain also acts downstream of integrin activation and cleaves integrin cytoplasmic tails, which makes the role of calpain in integrin activation more complicated (Calderwood 2004). Although calpains promote talin-mediated integrin activation, they do not appear for the assembly of adhesion complexes. Instead, calpain-mediated proteolysis of talin is shown to have a major role in FA disassembly (Franco et al. 2004, Franco & Huttenlocher 2005).

Talin binding to **PIP2** causes conformational changes that disrupt talin autoinhibition and facilitate the binding of talin to integrin (Martel et al. 2001, Goksoy et al. 2008, Saltel et al. 2009). Talin binds to the C-terminal part of PIPKIγ-90, and this interaction has been shown to activate the enzyme (Di Paolo et al. 2002, Ling et al. 2002). Active PIPKIγ-90 induces localized synthesis of PIP2 and recruitment of talin to focal adhesions. Thus, PIPKIγ-90 is a potential positive regulator of integrin activation and FA assembly (Di Paolo et al. 2002, Ratnikov et al. 2005b). On the other hand, there is a possibility of counteracting factors such as competitive binding of integrin and PIPKIγ-90 on the talin head. Src kinase, a major player in integrin outside-in signaling, is shown to phos-

phorylate PIPKIγ-90. This phosphorylation greatly facilitates PIPKIγ-90 binding to talin and thus outcompetes the talin-integrin interaction. Therefore, Src phosphorylation could act as a switch from integrin binding to PIPKIγ-90 binding by talin (Barsukov et al. 2003, Ling et al. 2003, de Pereda et al. 2005).

As in other FERM family proteins, **phosphorylation** can be important in talin function. Phosphorylation could alter the tendency of talin for calpain cleavage or change talin interactions. In agonist-induced platelets, talin is shown to be phosphorylated mainly on T144, T159 and S446 (Ratnikov et al. 2005a). These sites reside in the F1 loop of talin FERM and in a hinge near the calpain cleavage site, respectively. Indeed, phosphorylation of residues T144 and T150 is shown to inhibit talin membrane association, thereby modulating integrin activation (Goult et al. 2010).

Scientists have also proposed that talin has **mechanosensory** characteristics. When talin forms a link between integrins and actin cytoskeleton, it is a subject of external mechanical force. Force exerted by actomyosin contraction may alter the conformation of the rod helical bundles and exposes the buried VBSs (Lee et al. 2007). The greater the force, the larger the number of cryptic VBSs exposed. This then enables the vinculin binding to increase progressively, which would gradually strengthen the interface between cytoskeleton and integrins (Ziegler et al. 2006, Humphries et al. 2007). However, whether the exerted force had an effect on the unmasking of the integrin-binding site is not known.

Integrin phosphorylation

The interactions between talin and integrin cytoplasmic tails are controlled by integrin **phosphorylation.** The integrin outside-in signaling is accompanied by tyrosine phosphorylation of the β cytoplasmic tails. For example, αIIbβ2 integrin-dependent platelet aggregation causes increased tyrosine phosphorylation of signaling proteins (Law et al. 1996). The tyrosine phosphorylation of the β integrin tail is shown to be important in cell transformation (Sakai et al. 2001, Datta et al. 2002), cell migration (Sakai et al. 1998) and hemostasis (Law et al. 1999). The integrin membrane-proximal NPxY motif is phosphorylated by Src-family kinases (Law et al. 1996, Sakai et al. 2001), which is shown to lead to impaired cell adhesion (Wennerberg et al. 2000, Datta et al. 2002). The NPxY phosphorylation by Src abrogates the binding of talin (Garcia-Alvarez et al. 2003, Ling et al. 2003). Thus, the tyrosine phosphorylation of integrins can be regarded as a negative regulator of integrin activation.

Other PTB proteins

Integrin cytoplasmic tails have two recognition sites for PTB domains: a membrane-proximal NPxY motif and a membrane-distal NxxY motif (Calderwood et al. 2003). In addition to talin, several other proteins with a PTB domain can bind to these motifs. These proteins include tensin, Numb, docking protein 1 (Dok1), integrin cytoplasmic domain-associated protein-1α (ICAP-1α) and kindlins. Depending on the type of the PTB domain, the protein can show a preference for either phosphorylated or unphosphorylated tyrosine, or can be independent

of the phosphorylation state. In addition, the integrin sequence upstream of the NPxY or NxxY motifs accounts for the specificity of interactions. Naturally, the binding of these proteins to integrin tails has to be regulated spatially and temporally. As with talin, this regulation can be achieved by lipids and integrin tyrosine phosphorylation (Calderwood 2004, Legate & Fässler 2009).

The recruitment of **tensin** is temporally distinct to talin: talin is recruited to early adhesion sites whereas tensin resides at mature FAs and fibrillar adhesions (Zamir et al. 1999, Zaidel-Bar et al. 2007). The relatively weak binding of tensin to integrins is insensitive to NPxY phosphorylation, which is caused by the flexibility of the PTB domain binding pocket (McCleverty et al. 2007). While this same phosphorylation abrogates talin biding (Garcia-Alvarez et al. 2003, Ling et al. 2003), it can be considered a talin-tensin phosphotyrosine switch. This switch can have a role in the conversion from a structural focal adhesion to a fibrillar adhesion with signaling functions (Geiger et al. 2001, Legate & Fässler 2009). Indeed, FA maturation is characterized by tyrosine phosphorylation of integrins by the Src kinase (Ling et al. 2003).

Dok1 negatively regulates integrin activation and interacts exclusively with the canonical membrane-proximal NPxY motif. While talin also binds to this motif, the proteins compete in integrin binding (Wegener et al. 2007, Oxley et al. 2008). The Dok1 binding to unphosphorylated NPxY is relatively weak but is greatly increased by the integrin tyrosine phosphorylation. This suggests that integrin phosphorylation can act as a switch between talin and Dok1 binding (Oxley et al. 2008). **ICAP-1α** is shown to bind the membrane-distal NxxY motif in a β1A integrin-specific way. This binding requires an unphosphorylated ^{792}NxxY^{795} motif and an upstream valine (V787) (Chang et al. 2002). Consequently, ICAP-1α binding competes with the neighboring talin-integrin interaction and facilitates FA disassembly in cells (Bouvard et al. 2003).

The **kindlins** (Karaköse et al. 2010) are found to assist talin in integrin activation, hence acting as integrin co-activators. Three kindlins are expressed in mammals, kindlin-1, kindlin-2 and kindlin-3, and each one localizes to integrin adhesion sites. Kindlins are important components in integrin adhesion since failure of their function leads to integrin activation-deficient phenotypes. Diseases related to kindlins include Kindler syndrome, leukocyte adhesion deficiency type III (LAD-III) and some bleeding disorders. The mechanism underlying kindlin function is still unknown, but it requires direct contact with integrin β tails. Additionally, kindlins are known to bind migfilin and integrin-linked kinase (ILK), and to orchestrate protein recruitment to FAs (Mackinnon et al. 2002, Tu et al. 2003).

Like talin, kindlins consist of a FERM domain with an acidic binding pocket and a preceding F0 subdomain (Goult et al. 2009). In addition, kindlins have a PH domain inserted into the F2 subdomain. The F3 subdomain of kindlins resembles that of talin but binds to the membrane-distal NxxY of integrin β subunits. Indeed, mutation of this motif abrogates cell spreading (Ylänne et al. 1995). Additionally, the Ser/Thr-rich site between the NxxY motifs is important for kindlin, but not talin, binding (Garcia-Alvarez et al. 2003, Ma et al.

2008). Because talin and kindlins bind to different NxxY motifs, the simultaneous integrin binding might in theory be possible, thus enabling cooperative roles in integrin activation. This hypothesis is supported by the results where integrin activation requires simultaneous expression of talin and kindlins (Ma et al. 2008, Moser et al. 2008, Meves et al. 2009, Moser et al. 2009b, Karaköse et al. 2010).

2.3.2 Integrins, Rap1 and RIAM

Several different signaling pathways can regulate integrin activation, and often these pathways converge to the talin-integrin interaction. Proteins that have membrane-targeting motifs can associate directly or indirectly with talin. Consequently, these proteins have an ability to target talin to the plasma membrane and to integrins. For example, platelet agonist stimulation increases αIIbβ3 affinity that is accompanied by talin redistribution from cytoplasm to the plasma membrane (Beckerle et al. 1989). One of these recruiting pathways includes WASP-family verprolin homologue 2 (WAVE2) that recruits vinculin and talin to the immunological synapse (Nolz et al. 2007). Another pathway includes PIPKIγ and the second messenger PIP2 that, in concert with protease calpain, facilitate talin recruitment and integrin activation (Anthis & Campbell 2011). One major group of signaling intermediates that regulate integrin activation is the Ras superfamily of small guanosine triphosphatases (GTPases).

More than 21 members of the Ras GTPase exist, and they act as molecular switches that cycle between active GTP-bound and inactive GDP-bound forms. The GTPase-activating proteins (GAPs) facilitate the inactive 'off' state while guanine nucleotide exchange factors (GEFs) induce the active 'on' state. The activity state of small GTPase determines its ability to bind effectors. The switch regions (switch I, switch II and P-loop) that border the nucleotide-binding site are flexible and stabilized in the GTP-bound state so that effectors are allowed to interact. Ras-family proteins are structurally conserved and share some activators and effectors, but these proteins have distinct biological functions (Bos et al. 2001, Kinbara et al. 2003, Raaijmakers & Bos 2009).

Small GTPases have major roles in cell growth and proliferation, and they are also involved in cell adhesion and migration. Integrin function can be regulated by several small GTPases, including H-Ras, R-Ras and Rap. The proteins share overlapping pathways; therefore, crosstalk between them in integrin regulation is possible. H-Ras can suppress or promote integrin-mediated adhesion, depending on the cell type. H-Ras prevents integrin activation in fibroblasts but activates integrins in hematopoietic cell lines. The effectors mediate the effects of the H-Ras onwards. For example, Raf1 suppresses integrin activation, but phosphatidylinositol 3-kinase (PI3K) enhances integrin activation; the effector chosen by H-ras depends on the cellular context. R-Ras promotes integrin activation and consequent behavior change in Chinese hamster ovary (CHO) cells, breast epithelial cells and macrophages. R-Ras also counters the suppressive effect of H-Ras. The membrane association of R-ras is important in integrin activation, as well as effector phosphatidylinositol 3-kinase that propagates sig-

nals to Rac and protein kinase Cε. These proteins then could further regulate integrin-dependent cells spreading (Kinbara et al. 2003).

Rap1 GTPase

The Rap GTPases are the closest homologs of the Ras GTPases. Mammals have four Rap isoforms, Rap1A, Rap1B, Rap2A and Rap2B. The Rap1A and Rap1B proteins are 97 % identical and functionally equivalent, often referred to commonly as Rap1. Rap1 is activated by several transmembrane receptors that include cytokine receptors, growth factors receptors, receptor tyrosine kinases, G-protein-coupled receptors and cell adhesion molecules. Therefore, it can be regarded as a ubiquitous mediator between integrins and other surface receptors (Bos et al. 2001, Kinbara et al. 2003). The signals from these receptors are mediated by second messengers such as AMP, Ca^{2+} and diacyl glycerol (DAG). GEFs that activate Rap1 include Ca^{2+}- and DAG-regulated guanine nucleotide exchange factor 1 (CalDAG-GEF1), C3G, members of Epac and PDZ-GEF subfamilies. GAPs regulating Rap1 include at least the Rap1GAP and Spa1 family of GAPs (Boettner & Van Aelst 2009, Raaijmakers & Bos 2009).

Whereas Ras mainly plays a role in cell proliferation and survival, Rap1 is involved in integrin- and cadherin-dependent cell adhesion and cell spreading (Boettner & Van Aelst 2009). Rap1 regulates β1, β2 and β3 integrins, therefore affecting integrin activity (affinity) and clustering (avidity) (Bos 2005). The first evidence of the role of Rap1 in integrin function came from leukocytes where Rap1 regulates αLβ2-dependent adhesion upon agonist TCR ligation (Katagiri et al. 2000, Reedquist et al. 2000). Rap1A enhances integrin-mediated T-cell adhesion through avidity modulation *in vivo* (Sebzda et al. 2002). Rap1 also has a role in chemokine-induced integrin activation and transmigration of T-cells (Shimonaka et al. 2003). In mouse platelets, the lack of Rap1B leads to impaired αIIbβ3-dependent platelet aggregation. This causes bleeding defects and reduced thrombus formation, which indicates that Rap1 is required for normal hemostasis (Chrzanowska-Wodnicka et al. 2005).

Rap1 effectors

Many Rap1 effectors have been found. Some are involved in integrin signaling; some are involved in other cellular events (Bos et al. 2001, Bos 2005, Raaijmakers & Bos 2009). Among the effectors, afadin (AF6), a regulator for cell adhesion and polarization enriched in lymphoid tissues (RAPL), and RIAM are associated with Rap1-mediated integrin activation (Bos 2005). The unifying characteristic of these effectors is the Ras-association (RalGDS/AF6; RA) domain or structurally similar Ras-binding domain (RBD). These domains form a ubiquitin-like fold that interacts with small GTPases in a conserved way. This binding depends on the activation state of the GTPase, whereas other secondary interactions define the specificity of Rap1-effector interaction (Nassar et al. 1995, Herrmann 2003).

Effector AF6 contains two RA domains, and it binds Ras and Rap GTPases. In T-cells, AF6 has been shown to act as a negative regulator of Rap1-induced integrin adhesion (Zhang et al. 2005). RAPL regulates Rap1-induced integrin

activation and adhesion in lymphocytes. RAPL is probably recruited by active Rap1 to the plasma membrane where RAPL associates with the α cytoplasmic tail of αLβ2, which results in integrin activation (Katagiri et al. 2003). Still, RAPL has been shown not to mediate the Rap1-induced recruitment of talin in platelets integrin αIIbβ3 (Han et al. 2006).

RIAM

RIAM (or PREL1) is a Rap1 effector that has been shown to induce integrin activation and to regulate actin cytoskeleton dynamics (Lafuente et al. 2004, Jenzora et al. 2005). RIAM is enriched in hematopoietic cells, such as platelets and lymphocytes, whereas lamellipodin, a RIAM paralogue, is present in fibroblasts and other cells (Krause et al. 2004). In T-cells, overexpression of RIAM promotes β1 and β2 integrin-mediated adhesion, and the existence of RIAM is required in Rap1-induced T-cell adhesion. RIAM also has a role in recruiting Rap1 at the plasma membrane. In cells, RIAM resides at the locations where integrin activation is expected to take place: leading edge, lamellipodia and filopodia (Lafuente et al. 2004, Watanabe et al. 2008).

Active Rap1 stimulates the membrane localization of talin and its association with αIIbβ3 integrin (Han et al. 2006). This recruitment is mediated by RIAM (Watanabe et al. 2008, Lee et al. 2009). By binding both Rap1 and talin, RIAM forms a ternary integrin activation complex that facilitates integrin activation in platelets and fibroblasts (Fig. 9A) (Han et al. 2006). The most N-terminal part of RIAM constitutes an amphipatic α-helix that binds talin. This, in addition to the RA domain, is sufficient for talin-mediated integrin activation (Lee et al. 2009). Activated Rap1 is targeted to the lipid membrane due to its membrane-targeting CAAX motif that is prenylated in cells. Membrane targeting of the talin-binding site of RIAM via CAAX prenylation can bypass Rap1 for talin-mediated integrin activation. The RIAM/Rap1 complex thus recruits talin to the plasma membrane where talin can induce integrin activation (Lee et al. 2009).

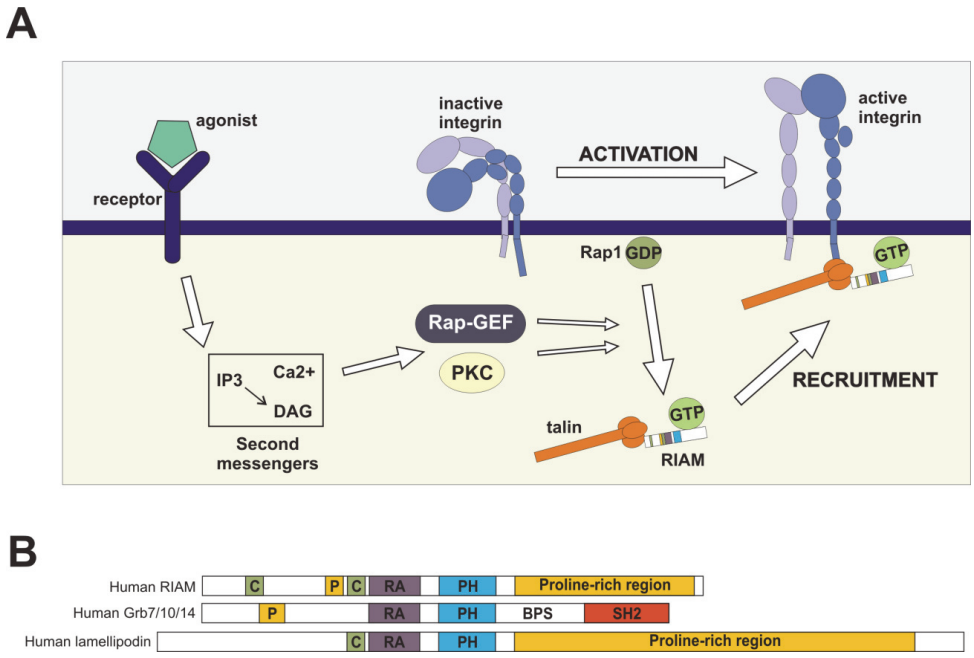

FIGURE 9 RIAM signaling and structure. A. Signaling events preceding talin recruitment, adapted from (Shattil et al. 2010). Agonist (e.g., thrombin) ligation of the receptor (e.g., thrombin receptor) causes the release of second messengers. These include inositol triphosphate (IP3), calcium (Ca2+) and diacyl glycerol (DAG). The second messengers in turn activate GEFs and protein kinase C (PKC) that activate Rap1 GTPase (Shattil et al. 2010). Rap1 then forms a complex with RIAM that recruits talin to integrins. B. Domain structure of selected Mig-10/RIAM/Lamellipodin (MRL) and related Grb7 family proteins as in (Lafuente et al. 2004, Jenzora et al. 2005). Abbreviations: C, coiled-coil; P: Proline-rich region; SH2: Src-homology region 2; BPS: Between PH and SH2.

RIAM binds to profilin and Enabled/protein vasodilator-stimulated phosphoprotein (Ena/VASP) and therefore regulates actin cytoskeleton remodeling (Lafuente et al. 2004, Jenzora et al. 2005). Interestingly, RIAM interaction with VASP and talin has also been shown to have a role in the β3 integrin-dependent regulation of adhesion dynamics and β1 integrin activation. β3 integrin promotes VASP phosphorylation that abrogates RIAM-VASP interaction, but the loss of β3 leads to enhanced association of the VASP/RIAM/talin complex and β1 activation (Worth et al. 2010). Additionally, RIAM has been shown to bind a complex of adhesion and degranulation-promoting adapter protein (ADAP) and 55-kDa src kinase-associated phosphoprotein (SKAP-55) with its RA-PH domain region. This ADAP/SKAP-55 module then recruits RIAM and Rap1 to the plasma membrane, thereby facilitating integrin activation (Ménasché et al. 2007).

In addition to recruiting effects, RIAM may function in relieving talin autoinhibition. An activated talin head fragment that consists of F2 and F3 subdomains can bypass the need for Rap1 activity, whereas full-length talin cannot (Han et al. 2006). Therefore, RIAM binding to talin could expose the masked

integrin binding site (Calderwood 2004, Goksoy et al. 2008). Still, the RIAM-binding site in talin is yet unknown as well as the possible conformational changes accompanied by this interaction.

RIAM belongs to the Mig-10/RIAM/Lamellipodin (MRL) family of adapter proteins (Lafuente et al. 2004). The MRL proteins include RIAM and lamellipodin in human and related orthologs Mig-10 in nematode and pico in the common fruit fly. MRL family members consist of a conserved RA-PH domain pair that is preceded by coiled-coil regions and flanked by several proline-rich regions (Fig. 9B). Another family of proteins, the Grb7-related proteins, shares a similar RA-PH domain pattern with MRL proteins. RIAM interaction with Rap1 has been shown to require an intact RA-PH domain pair (Lafuente et al. 2004). Although in contrast the PH domain was shown not to be necessary in integrin activation in CHO cells (Lee et al. 2009). The exact role of the PH domain in the RIAM-Rap1 interaction remains to be solved.

2.3.3 Regulation of integrin function by filamins

FLNa expression is essential for the development of mammals, and the vast number of FLNa's divergent roles is reflected by related diseases. In mice, the loss of FLNa is embryonically lethal with defects in bone development and cardiac and vascular development. In humans, mutations of the FLNa gene cause X-linked periventricular heterotopia that is caused by defective neuronal migration. Disorders related to filamin mutations include many congenital, skeletal and cardiac anomalies. FLNa mutations also cause familiar cardiac valvular dystrophy (Robertson 2005, Nakamura et al. 2011).

FLNa has been shown to orthogonally cross-link F-actin into a three-dimensional gel representing a characteristic structure of the cortical actin network (Flanagan et al. 2001). These networks are responsible for the mechanical integrity and movement of cells. Cells lacking FLNa exhibit unstable surfaces (blebbing phenotype), locomotion defects and impaired mechanical resistance (Cunningham et al. 1992, Flanagan et al. 2001, Kainulainen et al. 2002). At the same time, filamins can accommodate sufficient flexibility to account for elasticity and binding to other ligands (Nakamura et al. 2007). Filamins anchor transmembrane receptors to the actin cytoskeleton and act as scaffolds for numerous proteins, such as receptors, adapters and signaling proteins. This provides membrane stability, integration of signaling as well as mechanoprotection (Stossel et al. 2001, Nakamura et al. 2011).

Filamin A binds to integrin β cytoplasmic tails, thus linking them to the actin cytoskeleton (Calderwood et al. 2001). One role of FLNa interaction with integrin is to regulate cell spreading and survival (Kim et al. 2008). Filamin also seems to act as a negative regulator of integrin activation, since FLNa knockdown increases integrin activation (Kiema et al. 2006). The binding site of filamin on integrin β tails overlaps that of talin (Fig. 7), which results in competitive binding in integrin β cytoplasmic tails. Increased filamin-integrin association causes decreased integrin-mediated cell migration with impaired cell polarization and membrane protrusion (Pfaff et al. 1998). In support of that, FLNa

suppresses the invasive behavior of breast cancer cells and regulates calpain-mediated FA disassembly in (Xu et al. 2010).

The filamin binding site on integrins overlaps with several other integrin-binding proteins. These proteins include β3 endonexin, ICAP-1α and kindlins (Legate & Fässler 2009). Still, the competitive effects of these proteins are poorly known. As with talin, the interaction between filamin and integrins is regulated by several means (Nakamura et al. 2011). These include mechanical force, alternative splicing, phosphorylation, proteolysis and competition with binding partners (Fig. 8).

Filamin autoinhibition

As discussed earlier in this thesis, the main integrin binding site is masked in resting filamin (Lad et al. 2007). Computational analyses have shown that the cryptic binding sites can be exposed by a physiologically relevant **mechanical force** (Chen et al. 2009, Pentikäinen & Ylänne 2009). Similar to the case of talin and vinculin (Lee et al. 2007), the force applied by the actomyosin contraction could lead to the displacement of filamin A-strands and increased integrin binding. The mechanosensory characteristic of FLNa has also been implicated in several phenomena *in vivo* (Nakamura et al. 2011). For example, filamin has been shown to have mechanoprotective effects by preventing cell death induced by mechanical force and mediated by integrins (Kainulainen et al. 2002). Filamin has also been shown to have a mechanosensory role as the FLNa/β1 integrin complex regulates cell response to matrix tension. The FLNa-β1 interaction is important for contracting and remodeling collagen in response to matrix stiffness (Gehler et al. 2009).

FLNa is **phosphorylated** by several kinases, which can lead to increased resistance to calpain cleavage or altered binding to actin (van der Flier & Sonnenberg 2001, Nakamura et al. 2011). Many of the kinases are reported to phosphorylate FLNa at Ser2152 that resides near the A-strand of FLNa20. This phosphorylation is believed not to affect integrin binding and autoinhibition, but currently there are conflicting results whether it might affect force-induced relieving of autoinhibition by decreasing the required mechanical force (Travis et al. 2004, Chen et al. 2009, Pentikäinen & Ylänne 2009).

The third way to regulate FLNa interaction with integrins is **alternative splicing** of the filamin (van der Flier & Sonnenberg 2001). Several filamin variants have been found (van der Flier & Sonnenberg 2001). Two heart-specific FLNb variants (var-2 and var-3) lack their last four C-terminal domains, but their role is unknown (van der Flier et al. 2002). Some found splice variants of FLNb and FLNc lack the hinge1 region between FLNa15 and FLNa16. This deletion has an effect on myogenesis. The FLNb double variant (var-1 with hinge1 deletion) induces a change in filamin localization and accelerated muscle differentiation (van der Flier et al. 2002).

FLNa and FLNb have a naturally occurring splice variant (var-1) that is widely distributed in human tissues. The splice variant lacks a segment of 41 amino acids that corresponds the C-terminal part of FLNa19 and the N-terminal part of FLNa20 (van der Flier et al. 2002). The missing fragment also includes

the autoinhibitory A-strand of domain 20 (Fig. 5A) (Lad et al. 2007). Therefore, the var-1 binds different integrin isoforms more strongly (van der Flier et al. 2002), as it is also shown for FLNa with β1 and β7 integrins in cells and *in vitro* (Travis et al. 2004, Lad et al. 2007). The same interactions were not necessarily detectable in the non-variant filamin constructs, probably due to the present autoinhibition (van der Flier et al. 2002, Lad et al. 2007). Still, the structural and functional details of FLNa var-1 remain to be elucidated.

The two hinge regions of filamin A are cleaved by calpain, which generates the rod1, rod2 and self-association domain fragments. This **proteolysis** is followed by rod2 translocation into the nucleus with the interacting transcription factors (Loy et al. 2003). Ankyrin repeat-containing protein with a suppressor of cytokine signaling box 2 (ASB2) targets filamins to proteosomal degradation. Proteolysis by these proteins inhibits cell spreading but not migration. This is probably due to the altered FA dynamics (Nakamura et al. 2011).

Proteins can **compete** with integrin cytoplasmic tails for FLNa binding. One of them, migfilin, is thought to provide a connection between the ECM and the actin cytoskeleton (Wu 2005). In cultured cell lines, migfilin is required for efficient integrin-mediated cell spreading and possibly achieves this in concert with kindlin-2 (Tu et al. 2003). Migfilin localizes with cell-matrix adhesion structures, where it is recruited by kindlin-2. Migfilin is also associated with actin filaments via filamin. Migfilin consists of an N-terminal portion, a central proline-rich region and three C-terminal LIM domains. The LIM domains bind to kindlin-2, and the largely disordered N-terminal region binds filamins (Tu et al. 2003, Lad et al. 2008). There are multiple migfilin binding sites in the rod1 and rod2 regions of filamins (Takafuta et al. 2003, Tu et al. 2003). In the rod2 segment, the preferential interaction is with FLNa21, with weaker binding at domains 19 and 22 (Lad et al. 2008). The migfilin binds to the CD-face of FLNa21 in a very similar way to integrins but with a higher affinity; hence, migfilin can readily compete for filamin binding. Consequently, migfilin acts as a molecular switch in integrin activation via its binding to filamin. Migfilin competitively inhibits binding of filamin to β-integrin tails, and enhances integrin activation in cells (Lad et al. 2008, Ithychanda et al. 2009a).

Integrin phosphorylation

The filamin binding region of β tails contains several potential phosphorylation sites (Fagerholm et al. 2004). Filamin binding to integrins is abrogated by Tyr to Ala substitution of the membrane-proximal NPxY motif (Pfaff et al. 1998, Kiema et al. 2006). Talin binding depends on this Tyr residue, but the impact of the phosphorylation on filamin binding is not known. The serine and threonine phosphorylation of the Thr/Ser-rich region has been predicted to have an impact on filamin interaction (Calderwood et al. 2001). Indeed, filamin binding is obstructed by phospho-mimicking residues in this triplet in β7 integrin, whereas talin binding is unchanged (Kiema et al. 2006). Therefore, the threonine phosphorylation of this site can modulate the competition status of the filamin and talin in integrin tails.

2.3.4 Regulation of integrin function by 14-3-3

The 14-3-3 proteins participate in many cellular processes. These include cell signaling, cell cycle progression, stress responses, transcription, apoptosis, cytoskeletal structure, as well as adhesion and migration (van Hemert et al. 2001, Aitken 2006). Therefore, the changes in the normal function of especially 14-3-3σ isoform relate to cancer (Tzivion et al. 2006). The 14-3-3 proteins are also associated with several neuronal disorders, such as Creuzfeld-Jakob, Alzheimer's and Parkinson's disease (Wilker & Yaffe 2004, Tzivion et al. 2006). Animal models lacking one 14-3-3 appear normal, probably due to the redundant function of the different isoforms (Aitken 2006).

It has been proposed that 14-3-3 proteins have various functions at the molecular level. The 14-3-3 binding can alter the target protein's conformation by acting as a rigid molecular anvil (Yaffe 2002), or by changing the target's activity (Obsil et al. 2001), cellular localization or degradation. As dimeric molecules, 14-3-3 proteins could also function as adapters (van Hemert et al. 2001). The interaction between a 14-3-3 protein and a target protein is regulated in several ways. These include phosphorylation or dephosphorylation of the 14-3-3 or target protein, regulation of 14-3-3 dimerization, protein localization and isoform-specific expression (Aitken 2006).

Whereas integrin tyrosine phosphorylation is related to integrin outside-in signaling, threonine/serine phosphorylation (Fig. 7) may be important in inside-out activation events (Fagerholm et al. 2004, Gahmberg et al. 2009). Indeed, the adhesion events of β1A, β2, β3 and β7 integrins involve phosphorylation of threonines (Peter & O'Toole 1995, Valmu & Gahmberg 1995, Wennerberg et al. 1998, Valmu et al. 1999, Hilden et al. 2003). In platelets, the β3 cytoplasmic domain is phosphorylated on threonines in response to thrombin stimulation (Parise et al. 1990, Lerea et al. 1999). The β1A tail can be phosphorylated at Ser785, Thr788 and Thr789, and the β3 can be phosphorylated at Thr751 and Thr753. These threonine residues are important for integrin activation, cell adhesion and spreading, and phosphorylation can affect these events (Fagerholm et al. 2004).

The leukocyte-specific β2 integrins are phosphorylated after cell stimulation, either by TCR ligation or by phorbol esters. The β2 Ser756 can be phosphorylated in some circumstances (Hibbs et al. 1991, Fagerholm et al. 2004). The β2 threonine triplet (^{758}TTT^{760}) has a role in the cytoskeletal interactions and in modulation of cell spreading and adhesion (Valmu et al. 1999, Fagerholm et al. 2004). This triplet is also vital for β2-mediated adhesion to ICAM-1 (Hibbs et al. 1991). Protein kinase C phosphorylates the threonine triplet as well as Ser745 of the β2 tail (Fagerholm et al. 2002). Moreover, cell activation by receptor ligation induces Thr758 phosphorylation of β2 integrin. In addition, the corresponding threonine triplet of β7 is phosphorylated after agonist stimulation, which leads to increased cell adhesion (Valmu et al. 1999, Hilden et al. 2003).

The 14-3-3 proteins have been shown to bind Thr758-phosphorylated β2 integrins in response to TCR-induced inside-out activation (Fagerholm et al.

2002, Fagerholm et al. 2005, Nurmi et al. 2006). This binding has a role in cytoskeletal rearrangements, cell spreading, adhesion to ICAM-1 and downstream signaling events (Fagerholm et al. 2005, Nurmi et al. 2007). However, the role of 14-3-3 binding in integrin activation remains unknown.

In addition to β2 integrin, 14-3-3 has been shown to bind to β1, β3 and β4 integrin cytoplasmic tails (Han et al. 2001, Santoro et al. 2003). In contrast to 14-3-3-β2 integrin interaction, the interaction between 14-3-3β and β1 and β3 integrin has been proposed to be phosphorylation-independent. The 14-3-3 overexpression induced integrin-mediated cell spreading and migration, but via an unidentified mechanism (Han et al. 2001). The interaction of 14-3-3 with β4 involves integrin phosphorylation at serine in hemidesmosomes (Santoro et al. 2003).

3 AIMS OF THE STUDY

Cytoplasmic interactions are important in regulating talin-mediated integrin activation. We examined with structural and biochemical methods three proteins that affect these events. The structural characteristics of RIAM and its interaction with Rap1 are closely associated with talin recruitment to the plasma membrane. Filamin A competes with talin in integrin binding, which can be affected in splice variant-1 of FLNa. Phosphorylation of the β2 integrin has an effect on the binding of proteins 14-3-3ζ, talin and FLNa. The detailed aims of this thesis were as follows:

I. To characterize the interaction between RIAM and the Rap1 GTPase, and to study the structural properties of the central part of RIAM. How does the PH domain contribute to the RIAM-Rap1 interaction?

II. To investigate the interaction between filamin splice variant-1 domains 18-21 with its ligand peptide. Does domain 19 of var-1 bind the ligand, and are the binding properties of the filamin C-terminus unaffected by the lacking 41 residues in var-1?

III. To determine the structure of 14-3-3ζ in complex with the Thr758-phosphorylated integrin β2 cytoplasmic tail, and, additionally, to characterize the effects of the phosphorylation on talin, filamin and 14-3-3ζ binding with integrin.

4 SUMMARY OF THE METHODS

Table 2 summarizes the methods used in this thesis. More detailed descriptions can be found in the original publications indicated by Roman numerals. The 14-3-3ζ binding assay with integrin peptides is not included in the publications.

TABLE 2 Methods

Method	Publication
DNA cloning and sequencing	I, II
QuikChange mutagenesis	I, II
Protein expression and purification	I, II, III
Crystallization	III
X-ray diffraction data processing	III
Structure definition and refinement	III
Pull-down binding assay	I, II, III
Thermal unfolding assay	I
Limited proteolysis	I, II
Cell culture and transfection	I
Western analysis with antibody detection	I

4.1 Integrin phosphopeptide binding to 14-3-3ζ

The 14-3-3ζ was expressed and purified as in (III). The integrin peptides (Table 3) were coupled to vinyl sulphon-activated agarose (Sigma) according to the manufacturer's instructions. The interaction assay was conducted as in (III).

TABLE 3 Integrin peptides used in 14-3-3ζ interaction assays

Peptide name	Sequence*	Source
Control†	CRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES	Helsinki‡
β1A integrin	CKFEKEKMNAKWDTGENPIYKSAV(pT)TVVNPKYEGK	EZBiolab
β2 integrin	CRRFEKEKLKSQWNNDNPLFKSA(pT)TTVMNPKFAES	Helsinki‡
β3 integrin	CKFEEERARAKWDTANNPLYKEA(pT)STFTNITYRGT	EZBiolab
β6 integrin	CKFEAERSKAKWQTGTNPLYRGS(pT)STFKNVTYKHR	EZBiolab
β7 integrin	CQLNWKQDSNPLYKSAI(pT)TTINPRFQE	EZBiolab

* pT is phosphothreonine

† Unphosphorylated β2 integrin peptide

‡ Faculty of Biosciences, Division of Biochemistry, University of Helsinki, Helsinki, Finland

5 RESULTS AND DISCUSSION

5.1 The properties of RIAM and its interaction with Rap1B (I)

RIAM has been shown to be an important link between Rap1 signaling and talin-mediated integrin activation (Lee et al. 2009). By binding talin and Rap1, RIAM forms an integrin activation complex that is recruited to integrins (Han et al. 2006). The structural and binding properties of RIAM RA and PH domains are largely unknown. Here, we studied the RIAM interaction with Rap1 and the structural properties of the RIAM fragments.

5.1.1 The Ras-association domain of RIAM is sufficient for Rap1 interaction

In order to characterize the interaction between RIAM and Rap1 GTPase, various GST-RIAM fragments were tested using a pull-down assay (Table 4). The constructs with an RA domain were able to bind the GTPase, whereas the controls or constructs without an RA domain were not. The RA domain of RIAM seemed to be both necessary and sufficient for the interaction with Rap1B.

TABLE 4 Interactions of RIAM constructs with Rap1B

Construct	Including domains	Binding to Rap1B
RIAM1-176	N*	-
RIAM1-261	N-RA	+
RIAM1-502	N-RA-PH	+
RIAM150-261	RA	+
RIAM150-502	RA-PH	+
GST or agarose	-	-

*N, N-terminal region of RIAM (amino acids 1-176 or 1-150)

The affinities of the RA domain construct (RIAM150-261) and the RA-PH domain pair construct (RIAM150-502) were further quantified with pull-down affinity measurements (Table 5). RIAM150-261 bound active Rap1B with a dissociation constant that is comparable to other Ras-effector interactions (Wohlgemuth et al. 2005). This interaction was specific for the GTPase activity state because Rap1B-GDP was unable to bind the construct (I, Fig. 2). In addition, the RA-PH fragment (RIAM150-502) showed a similar binding affinity to the RA domain only (RIAM150-261), which suggests that the PH domain does not participate directly in the RIAM-Rap1B interaction *in vitro*.

TABLE 5 Dissociation constants of RIAM-Rap1B interactions

Sample	K_d (μM)	Std. Error
RIAM150-261 + Rap1B-GTP	0.3	0.1
RIAM150-502 + Rap1B-GTP	0.7	0.4
RIAM150-261 + Rap1B-GDP	*n.d.* †	*n.d.*

† *n.d.*, could not be defined

The results are consistent with the hypothesis that the interaction between RIAM and Rap1B is a canonical Rap1 GTPase-effector interaction. This interaction would take place between the switch regions of the GTP-bound Rap1 and an ubiquitin-like RA domain of RIAM (Nassar et al. 1995, Herrmann 2003). Experiments with a yeast two-hybrid assay have previously implied that the RIAM-Rap1 interaction would require an intact RA-PH domain pair (Lafuente et al. 2004). Our results however argue that the PH domain is not required for this interaction. The finding that the RA domain is sufficient in Rap1-mediated integrin activation events in CHO cells (Lee et al. 2009) supports our results.

The PH domain can have an indirect effect on the RIAM-Rap1 interaction. The PH domain has been shown to have an important role in Rap1 localization in cells (Lafuente et al. 2004, Ménasché et al. 2007). The PH domain of RIAM can bind to membrane phospholipids in a canonical way, or it can bind to other membrane-targeting proteins. For example, RIAM interaction with the ADAP/SKAP-55 protein complex depends on the intact RA-PH domain pair. The ADAP/SKAP-55 then facilitates the recruitment of the RIAM/Rap1 complex to the plasma membrane (Ménasché et al. 2007). However, the lipid anchor of Rap1 can compensate for the lost PH domain (Lee et al. 2009), at least to some extent. In addition to localizing effects, the PH domain of RIAM was proposed to influence the conformational state of the protein. This was studied next.

5.1.2 The pleckstrin homology domain stabilizes the fold of the Ras-association domain in RIAM

The stability of the RIAM constructs was analyzed with limited proteolysis by using α-chymotrypsin (I, Fig. 3A-D). The RIAM constructs yielded smaller fragments that imply folded domains flanked by unstructured regions. The resulting fragments included the predicted domains (Lafuente et al. 2004).

RIAM1-261 yielded a fragment that contained an RA domain, and the construct consisting of an RA domain only (RIAM150-261) did not result smaller fragments. RIAM150-502 was trimmed to consist of the core RA-PH domain pair. The rate of proteolysis was considerably faster in RIAM150-261 when compared to glutathione *S*-transferase (GST), a stable control protein, which suggests the unstable nature of the RIAM construct.

The stabilities of the proteins were further analyzed with a thermal stability assay. We utilized the ThermoFluor assay (Pantoliano et al. 2001) where the fluorescent dye binds only to hydrophobic areas of proteins exposed upon denaturation. ThermoFluor shows the thermal unfolding of the protein as a function of temperature. Each protein has its characteristic melting profile, and therefore melting temperature (T_m). A high melting temperature indicates a stable and folded protein. The high fluorescent signal even in the ambient temperatures from the RA domain only (RIAM150-261), suggests that this construct is largely unfolded or unstable. The N-terminal part of RIAM or included Rap1B did not seem to stabilize the fragment (I, Fig. 3E-F). In contrast, the constructs consisting of a complete RA-PH domain pair yielded profiles of a stable protein with T_m around 50 °C. The turnover of RIAM constructs was further tested in CHO cells. After transfection, the protein synthesis was abolished with cycloheximide. The analysis of resultant cell lysate showed that the RA domain was readily degraded within two hours, whereas the RA-PH pair was relatively stable (I, Fig. 3G-H). Therefore, the RA domain is stabilized by the PH domain *in vitro* and in cells.

The MRL proteins and Grb7-related proteins share a conserved RA-PH domain pattern (Fig. 9B) (Lafuente et al. 2004). These domains are separated by only a short linker, which could suggest close interdomain interactions. The crystal structure of Grb10, a member of the Grb7-related protein family, shows extensive interdomain interactions (Depetris et al. 2009). As the MRL proteins share a similar domain pattern and sequence homology with Grb10, these families probably share similar RA-PH domain structure. In several other proteins, the RA domain forms an independent folded entity that is stable *per se*. On the contrary, the RA domain of RIAM cannot fold properly in the absence of the PH domain. These results therefore imply that the PH domain has a stabilizing role in MRL proteins. Here we showed that the RA domain binds Rap1 regardless of the PH domain, but the PH domain is needed to stabilize the RA domain.

5.2 Filamin variant-1 folding and binding characteristics (II)

Filamin A has a splice variant (var-1) that lacks 41 residues (residue numbers 2127-2167) (van der Flier & Sonnenberg 2001, van der Flier et al. 2002). These residues include the last β-strand of domain 19 and the first strand of domain 20. FLNa var-1 binds integrins more strongly than the wild-type counterpart (Travis et al. 2004, Lad et al. 2007), but the structural and functional details had

remained obscure. Here, these properties were studied with several biochemical and structural methods.

5.2.1 Filamin A variant-1 is partially unstructured

NMR and ThermoFluor data analysis suggested that the truncated domain 19 of FLNa18-21 var-1 was a flexible structure, but the corresponding wild-type construct (FLNa18-21) behaved as a folded protein (II, Fig. 2 and Fig. 3). The stabilities of the constructs were further tested with limited proteolysis (Fig. 10). In conditions where the wild type FLNa18-21 was largely resistant to chymotrypsin for 120 min, the FLNa18-21 var-1 segment readily degraded in 30 min to fragments ranging between 22 kDa and 12 kDa. N-terminal sequencing revealed that the emerged fragments spanned from the unstructured domain 19 to the ends of the construct. Therefore, domain 19 in var-1 seemed to be more readily accessible to proteolysis than the wild-type counterpart. This together with NMR and ThermoFluor data is consistent with the var-1 deletions unstabilizing the structure of FLNa domain 19.

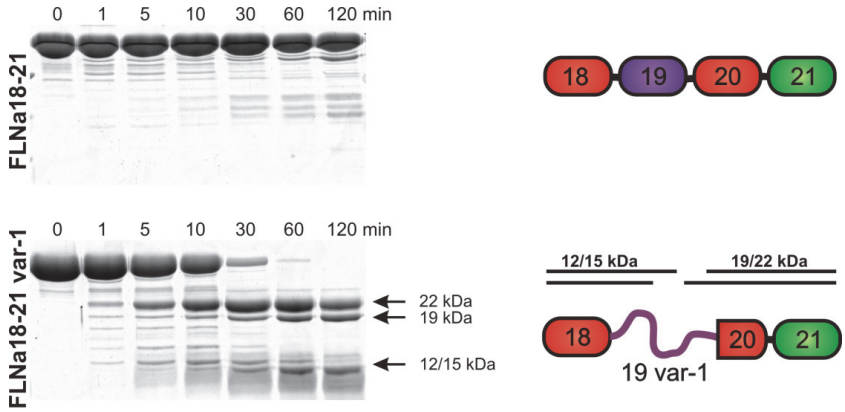

FIGURE 10 Limited proteolysis of FLNa18-21 and FLNa18-21 var-1. The var-1 construct was more readily digested and from the site of the unfolded 19th domain. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels stained for protein in the left, domain structure of the constructs in the right. The figure is based on the Figure 3 of the original publication (II).

The small angle X-ray scattering (SAXS) measurements showed that the FLNa18-21 forms a rigid L-shaped structure where the arms are formed by domain pairs 18-19 and 20-21 (II, Fig. 5). On the contrary, SAXS data and ensemble-optimized model fitting to the data revealed that var-1 has a flexible structure with multiple possible conformations (II, Fig. 7). This is also consistent with the unstructured nature of domain 19 in FLNa var-1.

5.2.2 Filamin A variant-1 binding to migfilin peptide

Integrins and migfilin have several binding sites in the FLNa rod2 region. Within the fragment studied here, the primary binding site resides in FLNa21, and a secondary site is in FLNa19 (Kiema et al. 2006, Lad et al. 2008, Ithychanda et al. 2009a, Ithychanda et al. 2009b). To study if these sites are functional in FLNa var-1, we measured migfilin binding to FLNa18-21 var-1 constructs containing mutations that abrogate the binding site on either FLNa19 (I2092C) or FLNa21 (I2283C) (Kiema et al. 2006, Lad et al. 2008, Ithychanda et al. 2009a, Ithychanda et al. 2009b). The affinities were quantified with a pull-down assay.

The approximate dissociation constant between migfilin and FLNa18-21 var-1 I2092C (the FLNa19 mutation) was similar to that of migfilin and FLNa21 alone (Table 6). The K_d values between migfilin and canonical FLNa18-21 seem to differ from those with FLNa21. This is consistent with the results obtained with integrin binding (Lad et al. 2007), suggesting that in var-1 the masking of FLNa21 binding site is released. When migfilin binding to the FLNa18-21 var-1 I2283C construct (the FLNa21 mutation) was tested, no specific interaction could be observed (II, Fig. 9A). This construct showed weaker interaction than FLNa19 alone, although the K_d values could not be accurately determined even with FLNa19. These results are consistent with the unfolded nature of domain 19 in FLNa var-1.

TABLE 6 Dissociation constants of FLNa fragments with a migfilin peptide

Sample	K_d (μM)	Std. Error
FLNa18-21	62	16
FLNa18-21 I2092C	41	21
FLNa18-21 var1 I 2092C	13	4
FLNa18-21 var-1 I2283C	*n.d.*†	*n.d.*
FLNa19	~150	~70
FLNa21	20	7

† *n.d.*, could not be defined

Our results suggest that, when compared to the canonical FLNA, the binding site for migfilin in var-1 domain 19 is lost, and at the same time the masking of domain 21 binding site is relieved. As the contribution of domain 19 binding is rather small, the var-1 splice variants result in overall higher affinity interaction. Considering the unfolded nature of domain 19 in var-1, other ligands cannot be expected to interact with this domain in var-1, either. Our results regarding the unmasking of the FLNa21 binding site of var-1 are in agreement with its increased binding to various integrins as well (van der Flier et al. 2002, Travis et al. 2004, Lad et al. 2007).

5.3 The structure of 14-3-3ζ/β2 complex and competitive binding (III)

5.3.1 Crystal structure of the 14-3-3ζ in complex with β2 integrin peptide

Following T-cell stimulation, β2 integrin becomes phosphorylated on Thr758 (Valmu & Gahmberg 1995, Hilden et al. 2003, Nurmi et al. 2007), and this phosphorylation is followed by 14-3-3 binding (Fagerholm et al. 2002, Fagerholm et al. 2005). To reveal the atomic details of the 14-3-3ζ interaction with β2 integrin tail, we co-crystallized the complex. The 14-3-3ζ crystals formed in conditions reported earlier (Liu et al. 1995), and the phosphopeptide was applied to the crystals by soaking. The crystals belonged to space group $P6_5$ and diffracted to 2.5 Å resolution. The structure was solved using molecular replacement by using the PDB entry 1A4O (Liu et al. 1995) as the search model. Cumulative intensity distribution of the diffraction data revealed that the crystals were hemihedrally twinned. Accordingly, the structure was built and refined by taking this twinning into account.

The structure (Fig. 11) reveals that the 14-3-3ζ interaction involves β2 integrin residues ^{755}KSApTTTVM^{762} (pT, key phosphothreonine). The structure resembles the canonical mode I interaction where the peptide is extended along the 14-3-3 binding groove and the phosphate is the key determinant for interaction (Yaffe et al. 1997, Petosa et al. 1998, Rittinger et al. 1999). The phosphate group of Thr758 interacted with the key residues of the basic ligand-binding pocket (Liu et al. 1995). The majority of the electrostatic interactions were with the phosphate group whereas the side chains of the β2 peptide had only a few polar interactions (Fig. 11B). The side chain of Ser756 forms hydrogen bonds with 14-3-3ζ residues Glu180 and Trp228. In addition, the main chain atoms flanking the pThr758 interact with the 14-3-3ζ.

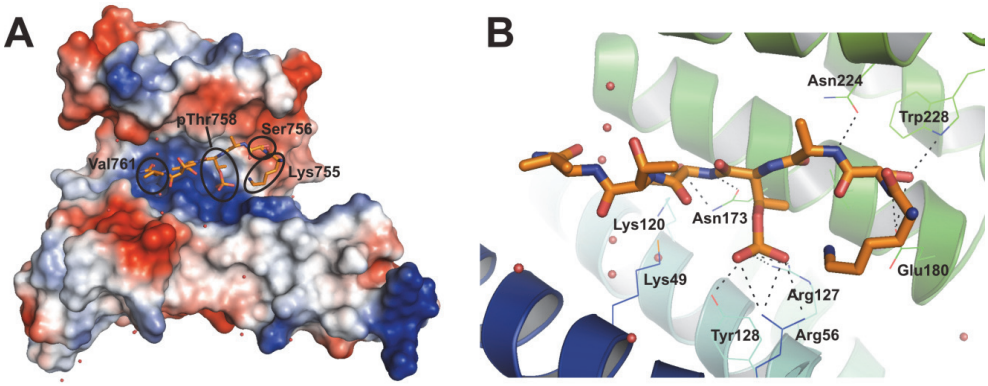

FIGURE 11 Structure of the 14-3-3ζ/β2 complex. A. 14-3-3ζ monomer shown as surface presentation and bound peptide as sticks. Some integrin residues are marked. The basic ligand-binding pocket can be seen as the blue area below the pThr758 residue. B. Interactions between the peptide and 14-3-3ζ. Hydrogen bonds are shown as dashed lines, and interacting 14-3-3 residues are indicated.

Protein 14-3-3ζ was the third type of protein crystallized in complex with an integrin β cytoplasmic tail. The interaction resembles canonical 14-3-3 interaction with a phosphorylated peptide. The bound integrin β tail adopts an extended conformation that differs from the conformation in complex with FLNa21 (Kiema et al. 2006). When not bound to proteins, this intervening region of the β integrin tail seems to adopt a helical conformation (Li et al. 2002, Vinogradova et al. 2004, Ma et al. 2006). The structures imply that integrin β cytoplasmic tails seem to be rather flexible regions that adopt a conformation depending on the interacting protein. This integrin disorder allows weak but specific protein interactions, controlled by the entropic cost of an interaction (Anthis & Campbell 2011).

5.3.2 Effects of the Thr758 phosphorylation and 14-3-3ζ binding on filamin and talin interactions

In cells, 14-3-3 is shown to bind only the Thr758-phosphorylated β2 integrin peptide, whereas the same phosphorylation inhibited filamin binding (III, Fig. 1A, B). The surface plasmon resonance (Biacore) experiments also verified these results *in vitro*. The 14-3-3ζ bound the Thr758 phosphorylated β2 integrin peptide with an affinity (K_d ~260 nM) comparable to other similar 14-3-3 interactions (III, Fig. 2A). In contrast, FLNa21 bound only to the unphosphorylated peptide with relatively weak affinity (K_d ~0.5 mM) (III, Fig. 2C). The strong binding of the talin head fragment (F2-F3) was unaffected by Thr758 phosphorylation (III, Fig. 2B).

The crystal structures of both FLNa21 and 14-3-3ζ in complex with the β2 integrin peptide explain at the atomic level how Thr758 phosphorylation affects the interactions. As discussed above, 14-3-3ζ interaction largely depends on the phosphate moiety. The crystal structure of the FLNa21/β2 integrin complex (III, Fig. 3) is very similar to the complex FLNa21 with β7 (Kiema et al. 2006). The Thr758 of β2 points toward the hydrophobic interior of the CD face. The insertion of the phosphate group should make this interaction highly unfavorable. Therefore, β2 integrin phosphorylation on Thr758 acts as a molecular switch for 14-3-3ζ and FLNa interactions.

The effects of Thr758 phosphorylation and 14-3-3 on talin binding were further analyzed with a competition assay (III, Fig. 1C). In accordance with the Biacore results, talin binding was unchanged by Thr758 phosphorylation or Thr758Ala mutation. This is also supported by previous results where this residue did not participate in the binding of talin (Fagerholm et al. 2005), and the structures of talin that do not overlap with the residue corresponding Thr758 (Garcia-Alvarez et al. 2003, Anthis et al. 2010). Surprisingly, the assay showed that talin binding to the phosphopeptide is completely outcompeted by the 14-3-3ζ (III, Fig. 1C). In addition, the crystal structure of the 14-3-3ζ/β2 complex implies that the concomitant binding of these proteins is sterically unfavorable and the binding sites overlap (III, Fig. 7D).

Talin is an important regulator of αLβ2 integrins in TCR-induced T-cell adhesion (Simonson et al. 2006). The interaction of talin with the β2 tail is im-

portant in integrin activation, and this is not affected by Thr758 phosphorylation. On the contrary, the subsequent binding of 14-3-3ζ abrogates the β2-talin interaction. The influence that these events have on integrin-mediated adhesion can be explained with a sequential model (Fig. 12). In this model, filamins inhibit integrin activation by competing with talin (Kiema et al. 2006). This would happen in early stages after TCR stimulation. The subsequent Thr758 phosphorylation inhibits FLNa binding, thus favoring talin interaction. However, the phosphorylation indirectly inhibits talin interaction via 14-3-3ζ binding.

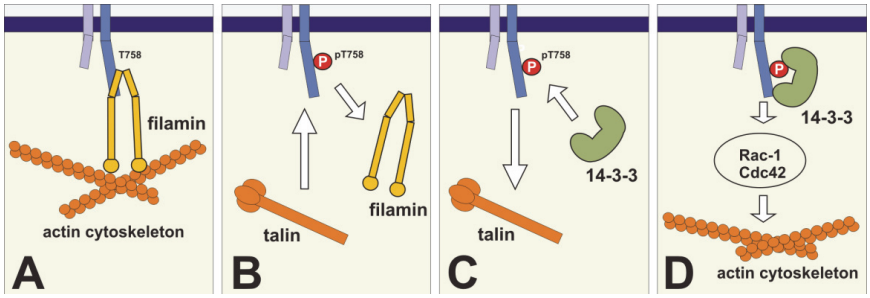

Figure 12 Schematic presentations of the events that relate to β2 Thr758 phosphorylation. This figure summarizes the conclusions based on our observations. Filamin binds to the unphosphorylated β2 integrin tail, therefore connecting integrin to the cytoskeleton and other proteins (A). When Thr758 is phosphorylated, filamin cannot bind to integrin, which favors talin interaction (B). 14-3-3 binds to the phosphorylated β2 tail, which outcompetes talin binding (C) and leads to downstream signaling effects such as actin cytoskeleton reorganization (D).

The bound 14-3-3 then mediates further downstream signaling events, such as small GTPase Rac-1 and Cdc42 activation and actin cytoskeleton reorganization (Nurmi et al. 2007). These events further affect cell spreading and adhesion that no longer depend on talin. The 14-3-3 proteins can affect these signaling events by several mechanisms that include acting as a dimeric scaffold protein or as an allosteric modulator (van Hemert et al. 2001, Yaffe 2002). However, the signaling events that immediately follow 14-3-3 interaction with integrins are not known.

5.3.3 Binding of 14-3-3 to other integrin β tails

The possibility that 14-3-3 also binds other integrins was also speculated. The phosphorylated β2 sequence that binds 14-3-3ζ resembles closely the mode I optimal binding motif (Yaffe et al. 1997). When other integrin cytoplasmic tail sequences with mode I and mode II consensus motifs are compared, some similarities can be seen (Fig. 13A). These include a positive Arg or Lys side chain that follows the membrane-proximal NPxY motif. Several integrins have a Ser/Thr residue at a site that correlates with the first threonine (Thr758) of the β2 triplet. These include Thr788 in β1 that is phosphorylated and is important for integrin function (Kim et al. 2004, Nilsson et al. 2006). In addition, Thr777 in

β3 integrin can be phosphorylated in cells (Parise et al. 1990, van Willigen et al. 1996, Lerea et al. 1999), as well as the threonine triplet of β7 integrins (Hilden et al. 2003). The muscle-specific integrin β1D variant does not have a Ser/Thr residue in the corresponding site (not shown), which indicates that it is regulated in a different way.

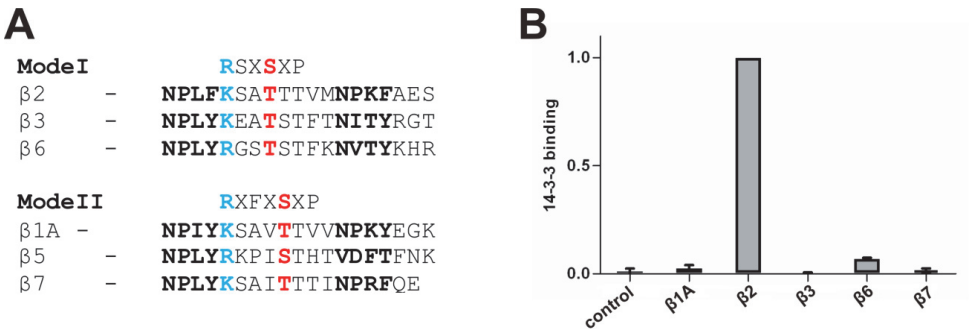

Figure 13 Integrins as canonical mode I ligands. A. Comparison of integrin tails with mode I and mode II optimal binding motifs. Ser/Thr residues are in red, and shared positive side chains (Arg/Lys) are in blue. B. Phosphorylated forms of other integrins were tested for 14-3-3ζ binding (mean columns ± standard deviation, n=4). The protocol and the peptides used were described in section 4.1 above.

However, when the binding of 14-3-3ζ to other integrin phosphopeptides was tested, no significant interaction was detected (Fig. 13B). Only the phosphorylated β6 integrin, which resembles β2 most, showed weak binding. Therefore, the interactions between 14-3-3 and other β integrins, if present, might follow a different kind of binding mode. For example, 14-3-3 has been shown to interact with unphosphorylated β1 (and probably with β3) at a site upstream from the Thr/Ser-rich site (Han et al. 2001). The 14-3-3 interaction studied here may therefore be specific for leukocyte β2 integrins, and the phosphorylation of the Thr/Ser-rich region could have other regulatory roles in other integrins.

These results add one piece to the elaborate puzzle of integrin cytoplasmic interactions. Integrins are regulated by several means, and here we described one scenario where threonine phosphorylation changes the binding of some key protein interactions. Still, several other integrin β tail interactions, their mutual effects and the role of phosphorylation remain to be elucidated. For example, kindlin-3 has been shown to associate with β2 integrins (Moser et al. 2009a), and the binding requires the Thr/Ser-rich site between the NxxY motifs. Whereas both 14-3-3 and filamin A compete with talin for integrin binding, their effect on kindlin function is not yet known.

6 CONCLUDING REMARKS

The main conclusions of this thesis are as follows:

I RIAM binds small GTPase Rap1 with its RA domain. The RA domain is both sufficient and necessary for this interaction, and the PH domain does not participate in the binding *in vitro*. On the other hand, the PH domain stabilizes the RA domain structure and forms a stable functional RA-PH entity *in vitro* and in cells.

II The domain 19 of FLNa var-1 is unfolded and does not bind its ligands. FLNa18-21 var-1 binds its ligands with an affinity that is comparable to isolated domain 21. However, the lack of an autoinhibitory A-strand does not seem to significantly facilitate the binding of migfilin.

III The crystal structure of 14-3-3ζ in complex with the Thr758-phosphorylated β2 integrin peptide reveals the details of the interaction at the atomic level. The 14-3-3ζ binds the extended peptide with an interaction that is similar to the phosphorylation-dependent mode I binding motif. The Thr758 phosphorylation abrogates filamin binding but does not affect talin interaction. However, the binding of 14-3-3ζ completely outcompetes talin in the phosphorylated β2 integrin.

In this thesis several mechanisms affecting integrin function were investigated. However, these mechanisms may not be generalized to all systems. For example, the recruitment of talin by RIAM is yet poorly studied and *e.g.* animal models with specific mutations would be informative. The expression of FLNa var-1 can be restricted to certain tissues, so the exact role of the variant remains a mystery. Although other integrins are also phosphorylated on threonines, my preliminary experiments reported in this thesis suggest that the subsequent binding of 14-3-3 may be specific to β2 integrins.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Kolme integriinin toimintaa säätelevää proteiinia – filamiini, 14-3-3 ja RIAM

Integriinit ovat solukalvon lävistäviä proteiineja, jotka ovat tärkeitä solujen tarttumisessa, liikkumisessa sekä eloonjäännissä. Integriinit välittävät informaatiota solun ulko- ja sisäpuolen välillä, ja niiden avulla solut kiinnittyvät solunulkoisiin rakenteisiin tai toisiin soluihin. Integriinit ovat dimeerisiä proteiineja, jotka koostuvat α- ja β-alayksiköstä. Kukin alayksikkö koostuu suuresta solunulkoisesta osasta, solukalvon lävistävästä osasta sekä lyhyestä solunsisäisestä hännästä. Integriinit voivat olla solukalvolla joko inaktiivisessa tai aktiivisessa tilassa. Muutosta inaktiivisesta muodosta aktiiviseen muotoon sanotaan integriinin aktivaatioksi. Integriinin sisältäpäin tapahtuvassa aktivaatiossa solunsisäiset signaalit aiheuttavat muutoksia integriinin konformaatiossa, mikä lisää proteiinin kykyä sitoa solunulkoisia ligandeja. Tätä aktivaatiota säädellään integriinien solunsisäisiin häntiin sitoutuvien proteiinien avulla. Parhaiten tunnettu solunsisäinen aktivoiva tekijä on proteiini nimeltään taliini. Monet muut proteiinit säätelevät taliinin toimintaa joko edistävästi tai inhiboivasti. Tässä väitöskirjassa tutkittiin taliinin toimintaa säätteleviä proteiineja RIAM, filamiini ja 14-3-3.

Adapteriproteiini RIAM yhdessä Rap1-proteiinin kanssa rekrytoivat taliinin solukalvolle, missä syntynyt kompleksi pystyy aktivoimaan integriinejä. Ensimmäisessä osatyössä tutkimme RIAM:n rakenteellisia ominaisuuksia sekä sitä, miten RIAM sitoo Rap1:ä. Selvitimme, että vain RIAM:n RA-domeeni sitoo Rap1:ä, eikä tämä interaktio vaadi muita proteiinin osia. Proteiinin stabiilisuuskokeet kuitenkin osoittivat, että RA-domeeni vaatii viereisen PH-domeenin pysyäkseen stabiilina kokonaisuutena. Näin ollen PH-domeeni stabiloi RA-domeenia, muttei osallistu suoranaisesti interaktioon Rap1-proteiinin kanssa.

Myös filamiinit sitoutuvat integriinin solunsisäiseen osaan. Tämä sitoutuminen kilpailee taliinin sitoutumisen kanssa, minkä takia filamiinia voi pitää integriinin aktivaatiota estävänä proteiinina. Myös itse filamiinin sitoutumista integriiniin säädellään monin tavoin, joista yksi on lähetti-RNA:n vaihtoehtoinen silmikoituminen. Filamiini koostuu 24:stä immunoglobuliinin kaltaisesta domeenista, joista domeenit 19 ja 21 sitovat integriiniä tai migfiliiniä. Silmikoinnin seurauksena syntyvästä filamiinin variantti-1:stä puuttuu alue, joka osallistuu proteiinin sitoutumisen itsesäätelyyn. Toisessa osatyössä tutkimme, kuinka variantin rakenteelliset ja toiminnalliset ominaisuudet eroavat normaalista proteiinista. Variantin puuttuvan osan seurauksena domeeni 19 oli laskostumaton, minkä takia tämä ei pystynyt sitomaan migfiliiniä. Sen sijaan migfiliinin sitoutuminen variantin 21. domeeniin oli jonkin verran tehostunut.

Integriinin kykyä sitoa solunsisäisiä proteiineja säädellään myös fosforylaation avulla. Integriinien solunsisäiset osat fosforyloituvat soluadheesioon liittyvissä tapahtumissa, kuten valkosolujen kiinnittyessä toisten solujen ICAM-proteiineihin. Valkosolujen β2-integriinin solunsisäisen hännän aminohappo-

tähde, treoniini 758 (Thr758), fosforyloituu integriinin aktivoituessa, ja tätä fosforylaatiota seuraa 14-3-3-proteiinin sitoutuminen integriiniin. Väitöskirjani kolmannessa osatyössä ratkaisimme rakenteen, jossa proteiini 14-3-3ζ on sitoutuneena Thr758-fosforyloituun β2-integriiniin. Rakenne paljastaa atomitasolla, kuinka proteiinit sitoutuvat toisiinsa fosforylaatiosta riippuvalla tavalla. Osoitimme, että integriinin fosforylaatio estää filamiinin sitoutumisen, mutta on edellytys 14-3-3:n sitoutumiselle. Fosforylaatio sinällään ei vaikuttanut taliinin sitoutumiseen, mutta siitä riippuva 14-3-3:n sitoutuminen syrjäytti taliinin integriiniltä. Tämä tutkimus paljasti odottamattomia molekyylitason mekanismeja valkosolujen integriinien säätelystä, joilla on merkitystä immuniteetissä.

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