# INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

**Molecular interactions of talin and actin: A study on the specific domains of talin involved in the interactions**

by

**Ho-Sup Lee**

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Neuroscience; Molecular, Cellular and Developmental Biology

Program of Study Committee:
Richard M. Robson, Co-major Professor
Ted W. Huiatt, Co-major Professor
Jorgen Johansen
Steven M. Lonergan
Marvin H. Stromer

Iowa State University

Ames, Iowa

2002

UMI Number: 3073463

# UMI®

Graduate College
Iowa State University


This is to certify that the doctoral dissertation of

Ho-Sup Lee

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

**Co-major Professor**

Signature was redacted for privacy.

**Co-major Professor**

Signature was redacted for privacy.

**For the Co-major Program**

Signature was redacted for privacy.

**For the Co-major Program**

# TABLE OF CONTENTS

# ABSTRACT

Talin is an essential cytoskeletal protein present in cell-matrix type adherens junctions where it helps in attachment of the actin cytoskeleton to the plasma membrane. The nature of the interaction between talin and actin was examined in this dissertation by investigating three actin binding sites (ABS1-3), with ABS1 located in the 47 kDa N-terminal head and ABS2 and ABS3 present in the 190 kDa C-terminal tail of the talin molecule. Cosedimentation of expressed GST-fusion deletion constructs from the talin head with F-actin showed that actin binding activity of the talin head is contained within residues 271-320. Talin constructs containing the narrowed ABS1 exhibited less pH dependence compared to those containing ABS3. Both ABS1 and ABS3 showed high dependence on ionic strength in ability to bind actin. Nearly all of the N-terminal head fragment purified from calpain-cleaved talin cosedimented with F-actin under conditions (pH 6.4 and low ionic strength) found to be optimal for the talin-actin interaction. EGFP-fusion talin constructs containing ABS1 were primarily colocalized with actin stress fibers when transfected into COS cells, whereas EGFP-ABS3 was predominantly colocalized with actin-rich membrane ruffles.

The ABS2 and ABS3 within the C-terminal tail were further defined to residues 1051-1250 (ABS2) and 2331-2530 (ABS3), respectively. ABS2 exhibited high dependence on both pH and ionic strength in interacting with actin, which is similar to that found with ABS3. Individual transfections of EFGP-ABS2 and EGFP-ABS3 into COS cells showed that ABS2 was primarily colocalized with actin stress fibers, whereas ABS3 was colocalized with actin filaments at both membrane ruffles and microspike-like cell extensions. Site-directed mutagenesis studies on ABS1 showed that substitution of alanine for the five positively charged residues $K^{295}$, $R^{297}$, $K^{300}$, $R^{303}$ and $K^{306}$, or for all six residues within 295-300 resulted

in significant reduction in actin binding. Taken together, these results suggest that the ABS1 located within the head domain, and the ABS2 and ABS3 present in the C-terminal tail domain, of talin each may play distinct roles in the interaction of talin with actin. These differences may be important in regulating integrin-mediated cell adhesion and migration.

# GENERAL INTRODUCTION

## Dissertation Organization

My dissertation is composed of one manuscript and one additional chapter within the main body. The manuscript is being prepared for submission for publication to the Biochemical Journal. The preliminary results in the additional chapter will be useful for more comprehensive studies conducted by future graduate students or postdoctoral associates in Dr. Richard M. Robson's lab. I have performed essentially all of the experiments described in the dissertation. Chicken talin cDNA used in this study was obtained from Dr. David R. Critchley (University of Leicester, U.K.). This General Introduction, which contains a Literature Review and References cited, precedes the main body. An Overall Summary and Comprehensive Bibliography follows the main body at the end of the dissertation. All of the references cited in the Literature Review, the main body and the Comprehensive Bibliography were formatted to the requirements of the Biochemical Journal, which includes the titles of the articles cited.

## Literature Review

Molecular interactions between cells and their extracellular matrix (ECM) are critical for many biological events such as cell proliferation, motility, differentiation, survival, embryonic morphogenesis, and regulation of gene expression, which are regulated by multi-molecular protein complexes localized at the cell-matrix junctions (Burridge and Chrzanowska-Wodnicka, 1996; Critchley, 2000; Geiger et al., 2001; Jockusch et al., 1995). Multiple protein-protein interactions within these protein complexes provide the necessary molecular linkages for mechano- and signal-transduction between the extracellular and

intracellular environments (Burridge and Chrzanowska-Wodnicka, 1996; Geiger et al., 2001). The high degree of complexity and redundancy of molecular interactions in the cell-matrix junctions may permit cells to respond to a variety of signals and provide more adaptive capability (Burridge and Chrzanowska-Wodnicka, 1996; Geiger et al., 2001). These protein complexes are usually composed of intracellular domains of (1) transmembrane proteins that are adhesion receptors for the ECM, and (2) cellular proteins including both signaling and cytoskeletal proteins, some of which bind to the cytoplasmic domain of the integral membrane proteins (Burridge and Chrzanowska-Wodnicka, 1996; Geiger et al., 2001).

The cell-matrix type adherens junctions in muscle cells include the myotendinous and neuromuscular junctions of skeletal muscle, costameres of striated muscles, and membrane-associated dense bodies of smooth muscle, all of which play roles in mediating bi-directional signals between cells and the ECM, and in transmitting the force generated by the actomyosin contractile apparatus to the ECM via transmembrane adhesion receptors (Small et al., 1992).

Myotendinous junctions are sites of tight adhesions of the terminal ends of skeletal muscle cells to the adjacent tendons, thereby mediating tensional forces from muscle contraction to the surrounding, extracellular tendon collagen fibers (Small et al., 1992). The thin filaments emanating from the terminal Z-line of the muscle fibers are firmly attached to the sarcolemma at these sites. The highly convoluted structure of the myotendinous junctions helps the ends of the muscle cell to adhere to the tendon. The intracellular site in this region contains junctional protein components such as vinculin and talin on the cytoplasmic side, the transmembrane protein integrins, and proteins in the ECM such as laminin and heparin sulfate proteoglycans (Small et al., 1992).

Neuromuscular junctions are specialized sites of adhesion in skeletal muscle where the post-synaptic muscle cell membrane has distinct invaginations folded or protruding into the muscle cell (Small et al., 1992). This adhesion is similar to other adherens junctions in that the plasma membrane of the cell associates with extracellular matrix, such as the basal lamina in the synaptic cleft (Small et al., 1992). Dense, thick submembranous coats are localized to the crests of the invaginations where cytoskeletal proteins such as talin, vinculin, α-actinin and filamin are enriched. It is not clear whether the cytoskeletal proteins play an important role in accumulating acetylcholine receptors, although the cytoskeletal proteins are believed to be involved in the formation and establishment of the neuromuscular junctions (Small et al., 1992).

Costameres in striated muscle cells are sites of transverse connections between the Z-lines of peripheral myofibrils and the nearby sarcolemma (Pardo et al., 1983; Small et al., 1992). Thus, the costameres are localized periodically along the longitudinal, cytoplasmic face of the muscle cell membrane. The costameric structures were first recognized in an immunofluorescence localization study of vinculin in skeletal muscle (Pardo et al., 1983). Vinculin staining exhibited dense patches of rib-like lattice that ran perpendicular to, and repeated, along the longitudinal axis of the cell. Costameres are believed to help transmit the forces generated from contraction across the sarcolemma to the extracellular matrix by coupling/linking the Z-lines of peripheral myofibrils to γ-actin present within the costameres along the sarcolemma.

Membrane-associated dense bodies of smooth muscle are adherens-type junctions located between caveolae-rich membrane domains (Small, 1995; Stromer, 1998). The membrane-associated dense bodies are ovoid shaped, electron dense plaques located along

the cytoplasmic face of the plasma membrane (Small, 1995; Stromer, 1998). The membrane-associated dense bodies are sites where actin filaments from the intracellular contractile machinery are attached to the ECM via binding to integral membrane proteins (Small, 1995; Stromer, 1998). The molecular composition of the membrane-associated dense bodies is somewhat similar to that of focal adhesions in cultured cells. The membrane-associated dense bodies also appear to function as anchoring sites for intermediate filaments (Chou et al., 1994).

Membrane-attachment sites for the actin cytoskeleton in adherent cells, which correlate to cell-matrix junctions *in vivo*, include focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Geiger et al., 2001; Jockusch et al., 1995). Focal adhesions are structures specialized for the tight adhesion of cultured cells to the ECM, playing both structural and signal transduction roles important in cell adhesion, growth, and differentiation (Burridge and Chrzanowska-Wodnicka, 1996; Geiger et al., 2001; Jockusch et al., 1995). Focal adhesions are very heterogeneous in their size, morphology and molecular composition, although integrins and actin are always included (Zamir et al., 1999). The most common types of integrins observed in focal adhesions are $\alpha_5\beta_1$ for adhesion to fibronectin, and $\alpha_v\beta_3$ for adhesion to vitronectin (Burridge and Chrzanowska-Wodnicka, 1996). Focal adhesions range from less than a square micrometer to several square micrometers in size, and usually take on a flat, elongated shape. Focal adhesions contain electron-dense, multi-cytoskeletal protein complexes at the cytoplasmic face, which anchor bundles of actin filaments to transmembrane adhesion receptors (Geiger et al., 2001).

Immunofluorescence microscope observation and morphometric analysis of the focal adhesions of fibroblasts have recently shown (Zamir et al., 1999) that the regions of focal

adhesion can be grouped into the following three categories: (1) classical focal adhesions that contain large amounts of vinculin, paxillin and phosphotyrosine; (2) fibrillar adhesions that contain high concentrations of tensin, but retain low amounts of classical focal adhesion proteins; and (3) mosaic adhesions that contain proteins of both classical focal- and fibrillar adhesions. A vast amount of research has been conducted to investigate the molecular mechanisms of cell adhesion to the ECM, which has resulted in identification of several transmembrane proteins such as integrins, syndecan IV and dystroglycan, and of many cytoskeletal proteins located at the cytoplasmic face of the cell membrane, including α-actinin, talin, filamin, vinculin, tensin, etc. (Burridge and Chrzanowska-Wodnicka, 1996; Geiger et al., 2001; Jockusch et al., 1995).

Based upon functional and biochemical characteristics, the cytoskeletal proteins have been classified into four groups (Geiger et al., 2001): (1) cytoskeletal proteins such as tensin, α-actinin, talin, and filamin that play a scaffolding role by directly interacting with both integrin and actin filaments; (2) integrin-bound molecules, including paxillin, focal adhesion kinase, integrin-linked kinase (ILK), and 14-3-3β, which interact indirectly with actin via binding to other protein members of the focal adhesions; (3) actin binding proteins such as vasodilator-stimulated phosphoprotein (VASP), fimbrin, ERM proteins, and vinculin, which interact indirectly with integrins; and (4) adaptor proteins such as zyxin, cysteine-rich protein, PINCH, vinexin, ponsin, and Grb-7, which can serve to bind and thereby connect/link integrin- and actin-associated proteins. Many of the focal adhesion proteins have multiple interaction partners, which further increases the complexity of the molecular interactions occurring at/in the focal adhesions (Geiger et al., 2001). The small GTPase, Rho, plays a major role in regulating the assembly of focal adhesions and actin stress fibers

(Ridley, et al., 1992). Activation of Rho induces the enhancement of cell contractility and isometric tension, which leads to integrin clustering. This integrin activation stimulates the growth of focal adhesions by recruiting cytoskeletal proteins (Burridge and Chrzanowska-Wodnicka, 1996).

Talin is a cytoskeletal protein localized primarily at cell-matrix type adherens junctions, such as the myotendinous junctions (Tidball et al., 1986) and costameres of skeletal and cardiac muscle cells (Belkin et al., 1986), membrane-associated dense bodies of smooth muscle cells (Geiger et al., 1985), and neuromuscular junctions of skeletal muscle cells (Sealock et al., 1986). Talin is also located in focal adhesions and membrane ruffles of many cultured cells (Burridge and Connell, 1983), where it is believed to play key roles in linking the actin cytoskeleton to the plasma membrane via binding to integral transmembrane proteins including the integrins (Calderwood et al., 2002; Calderwood et al., 1999; Xing et al., 2001) and to layilin at membrane ruffles (Borowsky and Hynes, 1998), and also in mediating signals between the extracellular matrix and cell cytoskeleton, which are important for cell growth, motility and differentiation (Critchley, 2000).

Talin was discovered independently by two research groups in 1982 (Burridge and Connell, 1982; Collier and Wang, 1982) using two different cell types, namely chicken embryonic fibroblasts (Burridge and Connell, 1982) and human platelets (Collier and Wang, 1982). Talin was found to be localized at focal adhesions and membrane ruffles in the cultured fibroblasts by using immunofluorescence microscopy (Burridge and Connell, 1983). Platelets contain a large amount of talin (more than 3% of total protein) (Collier and Wang, 1982). Talin is distributed diffusely throughout the cytoplasm of resting platelets, and is then rapidly redistributed to the submembranous region upon thrombin activation, during which it

is phosphorylated and becomes involved in linking the actin cytoskeleton to the platelet membrane (Bertagnolli et al., 1993). Talin is not found in cell-cell adhesions such as the zonula adherens junctions in epithelial cells (Geiger et al., 1985). However, talin has been identified in the cell-cell junction between cytotoxic lymphocytes and target cells (e.g., the junction between helper T cells and antigen-presenting B cells) where integrins are enriched (Kupfer et al., 1986).

Various lines of evidence recognize and support important roles for talin in cell migration. When monoclonal antibodies raised against the N-terminal and against the C-terminal regions of platelet talin were microinjected into human fibroblast cells, the actin stress fibers and focal adhesions were disrupted, and cell motility was decreased (Bolton et al., 1997) These results, in turn, are consistent with results obtained previously by microinjection of fluorescently labeled talin fragments into fibroblasts (Nuckolls et al., 1992). Down-regulation of talin synthesis by using antisense RNA resulted in the inhibition of cell spreading, reduction in the size of focal contacts, and a dramatic decrease in the number of actin stress fibers in HeLa cells (Albiges-Rizo et al., 1995). The down-regulation of talin also generated β1 integrin having lower molecular mass, suggesting that processing of β1 integrin is also affected. Inactivation of talin in the neuronal growth cones of chick dorsal root ganglia obtained by a microscale, chromophore-assisted laser treatment caused the extension and retraction of filopodia to stall temporarily (Sydor et al., 1996). Stimulation of Dictyostelium discoideum with cAMP was shown to induce talin accumulation at the leading edge of aggregating cells, which suggests that talin is also involved in mediating signals in response to this chemoattractant (Kreitmeier et al., 1995). All of these results, taken

together, indicate that talin plays important roles in organizing the actin cytoskeleton and in regulating cell motility.

Studies of talin gene disruption in Dictyostelium (Niewohner et al., 1997) and in embryonic stem (ES) cells (Priddle et al., 1998) showed that talin is essential for cell adhesion to the ECM. Dictyostelium mutants in which a talin homologue (filopodin) had been eliminated (Niewohner et al., 1997) exhibited strong defects in their adhesion to substrates and in phagocytosis due to the diminished adhesion of the cells to yeast particles, whereas cytokinesis was slightly impaired. The attachment of the talin mutants to BSA-coated glass was almost lost. However, the talin-deficient cells were not defective in responding to stimulation with a chemoattractant such as cAMP, although the spreading and the fusion of the cells were reduced (Niewohner et al., 1997).

Cultured ES cells, in which both copies of the talin gene had been disrupted (Priddle et al., 1998), showed that: (1) expression of the $\beta 1$ integrin subunit was markedly decreased; (2) spreading and adhesion to gelatin and laminin, but not fibronectin, were reduced, and the cells continued to grow in a round morphology having many surface protrusions; (3) ability to form focal adhesions and actin stress fibers on fibronectin was lost in undifferentiated talin ES cell mutants, whereas differentiated talin ES cell mutants were able to spread and to assemble vinculin- and paxillin-containing focal adhesions, and to express the $\beta 1$ integrin subunit to as high a level as the differentiated wild-type ES cells (Priddle et al., 1998).

In recent studies in which disrupted talin alleles were introduced into mice, the homozygous talin (-/-) mutant mice did not develop past the gastrulation stage (6.5-7.5 days post coitum) (Monkley et al., 2000). These mutant mice exhibited defects in the organization of several tissues in the embryos, indicating that talin is required for embryogenesis.

Embryonic death resulted from developmental arrest at the gastrulation stage, primarily due to the lack of cell migration because trophoblast cells from the blastocysts of the mutant embryos demonstrated reduced ability to spread on, and adhere to, fibronectin and laminin. These results suggest that the role talin plays in cell motility is essential for the morphogenetic events occurring at the gastrulation stage.

The biochemical/molecular characteristics of talin are in accord with its functional role *in vivo*. Talin has a high molecular mass (~270 kDa from both mouse and chicken cDNA) and migrates as an ~230 kDa protein by SDS-PAGE (Rees et al., 1990). The talin molecule contains a high content (~80%) of α-helix, which is primarily contained in the long C-terminal tail (Molony et al., 1987). The C-terminal tail (domain) is predicted to have 50 to 60 copies of irregular repeats that are approximately 34 residues long, and rich in alanine and leucine.

The conformation of talin appears to be very flexible, depending on the ionic strength (Molony et al., 1987). Talin adopts a compact, globular shape in low ionic strength buffers, whereas it appears elongated (~50-60 nm long) at high ionic strength (Molony et al., 1987). Energy-filtered, electron microscope observations of negatively stained chicken talin showed that the talin molecule is composed of globular masses (3.8 nm in diameter) appearing in a "beads on a string-like structure" at physiological ionic strength (Winkler et al., 1997). Glycerol-spray and rotary-shadow electron microscope analysis of the molecular domain structure of platelet talin indicated that talin likely exists as a homodimer arranged in an antiparallel dumbbell shape of ~51 nm length (Goldmann et al., 1994). Only the 190 kDa C-terminal tail of talin can be covalently crosslinked, suggesting that it is the properties of the tail that result in dimerization of the two talin polypeptides (Goldmann et al., 1994). That the

two subunits in talin are arranged in an antiparallel fashion was later confirmed by using polyclonal antibodies specific to a phospholipid binding site located within the globular 47 kDa N-terminal talin head (Isenberg and Goldmann, 1998). The antiparallel arrangement of subunits within the talin dimer enables talin to cross-link actin filaments (Zhang et al., 1996; Schmidt et al., 1999).

Talin is highly susceptible to proteolytic cleavage by various proteases. Calcium-activated m-calpain readily cleaves talin between residues 433 and 434, thereby generating the 47 kDa N-terminal head and 190 kDa C-terminal tail fragments (Beckerle et al., 1986). Analysis of the cDNA sequence of mouse talin (Rees et al., 1990) shows that the N-terminal head fragment/domain shares homology with the ERM protein family containing the FERM domain (band 4.1-ezrin-radixin-moesin). The FERM domain reportedly serves in the attachment of cytoskeletal proteins to the cell membrane by binding to the intracellular domains of transmembrane proteins (Bretscher et al., 2000; Chishti et al., 1998). The FERM domain was first identified in purified band 4.1 protein obtained from human erythrocytes (Leto and Marchesi, 1984). The FERM domain is located in the N-terminal region of most FERM-containing proteins. However, myosin VIIA, a membrane-associated motor protein that is responsible for Usher syndrome type III disease (Chen et al., 1996), has a FERM domain in its C-terminal region, and FAP-1, a protein tyrosine phosphatase that interacts with the cytoplasmic domain of the Fas receptor (Sato et al., 1995), has a FERM domain located close to the middle of the protein.

The crystal structures of the FERM domain in radixin (Hamada et al., 2000), moesin (Pearson et al., 2000) and band 4.1 protein (Han et al., 2000) show that the FERM domain is composed of three subdomains, termed A, B, and C, arranged in a cloverleaf-like structure. It

has been reported (Hamada et al., 2000) that inositol-(1,4,5)-trisphosphate (IP3) binds to a basic cleft between subdomains A and C. The basic, juxta-membrane regions of adhesion proteins, including CD44 and CD 43, were proposed to bind to an acidic groove located between subdomains B and C of the FERM domain in radixin (Hamada et al., 2000). Subdomains A and B of band 4.1 protein have binding sites for integral membrane proteins, such as band 3 and glycophorin C, whereas subdomain C has a binding region for p55 (Han et al., 2000).

The globular-like N-terminal head of the talin molecule interacts with the cytoplasmic domains of integral membrane proteins such as some of the integrins (Calderwood et al., 2002; Calderwood et al., 1999) and with layilin (Borowsky and Hynes, 1998). The talin head also has binding sites for focal adhesion kinase (FAK) (Borowsky and Hynes, 1998; Chen et al., 1995) and phospholipids (Seelig et al., 2000). Based upon secondary protein structure predictions (Tempel et al., 1995), it has been proposed that the talin head contains three potential phospholipid binding sites (amino acid residues 21-39, 287-304 and 385-406). One of these three short amino acid stretches (residues 385-406) was recently reported to have the ability to insert into neutral and negatively charged membranes by an entropy-driven process, which suggests that the isolated peptide retains binding energy for interaction with phospholipids, and also may act as an intrinsic membrane anchor (Seelig et al., 2000). The large C-terminal tail of talin has been shown to interact with vinculin (Bass et al., 2002; Bass et al., 1999; Gilmore et al., 1993), actin (Hemmings et al., 1992) and specific integrins (Xing et al., 2001). Based upon the domain structure of talin, which is similar to that of members of the ERM protein family, it has been suggested (Critchley, 1999) that talin associates with the

cell membrane via the N-terminal head, and interacts with actin primarily via the C-terminal tail.

The integrins are a large family of transmembrane receptor proteins, located primarily at sites of cell adhesion to the extracellular matrix, which, in turn, is essential for several biological events such as cell growth, motility, differentiation and survival (Burridge and Chrzanowska-Wodnicka, 1996; Hynes, 1992; van der Flier and Sonnenberg, 2001). Integrin molecules are heterodimers composed of noncovalently-associated α- and β-subunits, which integrate/link the extracellular matrix via their large, globular head domains to the intracellular cytoskeleton via short (30-50 amino acids long) cytoplasmic tails. In humans, 24 heterodimers of the integrin family, resulting from the combinations of 18 α- and 8 β-subunits, have been identified (van der Flier and Sonnenberg, 2001). Alternative splicing and posttranslational modification, such as glycosylation, of some subunits also increases the complexity of the integrin family. Recent sequence analyses (Venter et al., 2001) on data from the human genome reveals that the integrin family in the human contains 24 α- and 9 β-subunits, although the presence of the newly identified six α- and 1 β subunits requires further investigation.

Activation of integrins induced by agonist, or upon ligand binding, involves conformational changes and clustering of the integrins, which increases affinity for their ligands (van der Flier and Sonnenberg, 2001). The clustering of integrins also recruits signaling proteins such as tensin and FAK, and cytoskeletal proteins including talin, vinculin and α-actinin (Burridge and Chrzanowska-Wodnicka, 1996). Tyrosine phophorylation events involving the signaling proteins, which are induced upon ligand binding at the focal

adhesions, lead to activation of downstream signaling cascades (Burridge and Chrzanowska-Wodnicka, 1996).

The cytoplasmic tail domains of integrins play critical roles in targeting integrins to focal adhesions, and in transmitting signals from ligand binding to the cytoplasm via interactions with cytoskeletal proteins (Burridge and Chrzanowska-Wodnicka, 1996). Talin binds to the cytoplasmic tails of integrin $\beta1A$, $\beta1D$ (Pfaff et al., 1998), $\beta2$ (Sampath et al., 1998) and $\beta3$ (Knezevic et al., 1996). The FERM domain within the talin head contains the binding site (186-435 residues) for the integrins. Overexpression of a peptide containing the integrin binding site of the talin head in CHO cells induced the activation of integrin $\alpha_{IIb}\beta_3$ (Calderwood et al., 1999). This result suggests that talin may regulate the binding affinity of the integrins to their ligands. When this talin peptide was transfected into cells expressing a mutant $\beta3$ integrin subunit lacking the C-terminal 85 amino acid residues, the activation of integrin was not observed, thereby indicating that physical interaction between the talin head and the integrin cytoplasmic tail is required for the integrin activation.

Another integrin binding site located within the C-terminal tail of talin was recently localized to the end (residues 1984-2541) of the C-terminal tail (Xing et al., 2001). The integrin binding site located within the talin head has much higher affinity than the one located within the tail of talin (Yan et al., 2001).

The two integrin binding sites may be positioned in close proximity within the antiparallel homodimeric talin molecule, which may help facilitate the interaction between talin and integrins. Indeed, calpain-digested talin containing the 47 kDa head and 190 kDa tail showed a dramatic increase in affinity to $\beta3$ integrin, which resulted from cooperative interactions of the head and tail domains of talin with the integrin (Yan et al., 2001). These

latter results also suggest that the interaction of the talin head with integrin is masked in native talin, as is the case for members of the FERM protein family in which the binding ability of the FERM domain to integral membrane proteins is masked by the C-terminal tail, and then unmasked by phosphorylation of the C-terminal tail or by addition of phosphoinositides (Bretscher et al., 2000). In fact, the interaction of talin with phosphoinositides was recently reported to induce a conformational change in the talin molecule, which enhanced the affinity of talin for $\beta 1$ integrin (Martel et al., 2001). It has also been shown that phosphoinositides, such as $PI4,5P_2$, are important for regulating the localization of talin in/to focal adhesions (Martel et al., 2001). When $PI4,5P_2$ was sequestered by overexpressing a pleckstrin homology (PH) domain, which has specific affinity for $PI4,5P_2$, the majority of talin in NIH3T3 cells was excluded from the focal adhesions.

In recent studies, subdomain F3 (residues 309-405) of the talin FERM domain has been predicted to contain a phosphotyrosine binding (PTB)-like domain based on its sequence similarity to ERM proteins and a three dimensional homology model (Calderwood et al., 2002). Overexpression of the F3 domain in CHO cells induced the activation of integrin $\alpha_{IIb}\beta_3$. The PTB-binding peptides have been known to form $\beta$ turns when bound to a PTP domain (Forman-Kay and Pawson, 1999). The highly conserved NPxY motif of the $\beta$ integrin tail tends to form a $\beta$-turn conformation, which suggests that the $\beta$ turn in the NPxY motif is required for the activation of $\beta$ integrins (Calderwood et al., 2002).

Layilin is a novel transmembrane protein, which shares homology with C-type lectins, and is colocalized with talin at membrane ruffles of cultured cells (Borowsky and Hynes, 1998). The interaction with layilin is via a region containing residues 280-435 in the talin head that overlaps with the FAK binding site (residues 225-327). Layilin is a

glycoprotein that is expressed widely in various cells and tissues. Layilin was recently identified as a cell surface receptor specific for hyaluronan, a ubiquitous extracellular matrix protein (Bono et al., 2001). The cellular localization of layilin suggests this protein serves as a membrane-attachment site for talin at the membrane ruffles of cultured cells, which may be important for cell adhesion and migration at those sites. In contrast, the integrins appear to play a role in docking talin at focal adhesion sites. The precise functional significance of the interaction between layilin and talin remains to be shown.

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase localized at focal adhesions of adherent cells (Burridge and Chrzanowska-Wodnicka, 1996; Schaller, 2001). FAK plays an essential role in regulating integrin-mediated signal transduction processes involved in cell adhesion to the extracellular matrix, motility and survival. The generation of transgenic mice containing a disrupted FAK gene showed a reduction in cell motility and an increase in the number of focal adhesions (Ilic et al., 1995). Inhibition of FAK activation in fibroblast cells resulting from microinjection of anti-FAK antibody or a peptide containing the FAK binding site of the $\beta 1$ integrin cytoplasmic tail, caused the cells to quickly apoptose (Hungerford et al., 1996). On the other hand, increased FAK activation in CHO cells obtained by overexpressing exogenous FAK enhanced cell migration on fibronectin (Cary et al., 1996).

The molecular domain structure of FAK is composed of three domains including large N- and C-terminal domains, and a kinase domain located between these two domains (Schaller, 2001). The N-terminal domain has homology with the FERM domain, and mediates interaction with the cytoplasmic tail of transmembrane proteins such as specific integrins and growth factor receptors.

A primary site of autophosphorylation in the FAK molecule is tyrosine residue 397, which is one of the six tyrosine residues within the FAK molecule (Schaller, 2001). The autophosphorylation site serves as a docking site for the proteins containing a phosphotyrosine-binding SH2 domain such as Src tyrosine kinases and PI3-kinase (Schaller, 2001). The C-terminal domain of FAK contains a focal adhesion targeting (FAT) sequence that is essential for correct localization of FAK to the focal adhesions (Schaller, 2001). Both paxillin and talin, two components of focal adhesions, interact with the FAT sequence, and both have been proposed to target the FAK to the focal adhesions (Schaller, 2001). However, recent evidence does not support the idea. Some FAK mutants defective for binding to paxillin are still targeted to focal adhesions (Cooley et al., 2000), and a deletion mutant of FAK, which was not localized to the focal adhesions, retains ability to associate with talin (Chen et al., 1995). The binding of talin to FAK appeared to mediate signals from FAK activation by integrins (Chen et al., 1995). However, the functional significance and molecular mechanism of the talin-FAK interaction in transmitting signals at the focal adhesions remain unclear.

Vinculin is a cytoskeletal protein localized at the cytoplasmic face of both cell-matrix and cell-cell type adherens junctions (Rudiger, 1998; Yamada and Geiger, 1997). Vinculin interacts with the plasma membrane via the indirect interaction with the integrin family at cell-matrix junctions such as focal adhesions in cultured cells and membrane-associated dense plaques of smooth muscle cells. Vinculin also interacts with members of the classical cadherin protein family at cell-cell junctions, including the zonula adherens (Rudiger, 1998; Yamada and Geiger, 1997). Vinculin is one of most abundant structural protein components at both these junctions. Vinculin is believed to anchor actin filaments to the plasma

membrane at these two types of cell contacts (Rudiger, 1998; Yamada and Geiger, 1997). Disruption of both alleles of the vinculin gene in F9 embryonal carcinoma and embryonic stem cells was shown to decrease cell spreading and adhesion to fibronectin, whereas the motility of the vinculin mutants was increased 2.4-times (Coll et al., 1995). The vinculin-deficient cells had a round shape and exhibited reduced lamellipodial extensions. The vinculin-deficient F9 cells were, however, still able to organize focal adhesions that contained classical constitutive proteins such as integrin, α-actinin, talin and paxillin (Volberg et al., 1995). Alteration of the vinculin gene in *C. elegans* resulted in developmental arrest at the larval stage, and the mutants showed defects in muscle organization suggesting that vinculin is required for muscle function (Barstead and Waterston, 1991). A homozygous vinculin (-/-) mutant of the mouse embryo also exhibited embryonic lethality due to defects in the development of the heart and brain (Xu et al., 1998). Conversely, results obtained with *Drosophila* showed that mutation of the vinculin homologue due to *X* chromosome inversion showed no obvious defects in viability and fertility, indicating that the vinculin gene is not essential in *Drosophila*, and that other alternative cytoskeletal components may compensate for the vinculin deficiency (Alatortsev et al., 1997).

Vinculin is highly conserved across species, and migrates by SDS-PAGE with an apparent molecular mass of 117 kDa (Geiger et al., 1980). The V-8 protease readily cleaves within a proline-rich region in the vinculin molecule that connects two prominent fragments, namely a 90-kDa globular N-terminal head and a 30-kDa elongated C-terminal tail (Otto, 1990). The N-terminal head has been shown to contain binding sites for talin and α-actinin, whereas the C-terminal tail binds to F-actin and paxillin (Jockusch et al., 1995). These multiple protein-protein interactions are modulated by an intramolecular association between

the globular head domain and elongated tail domain of vinculin (Johnson and Craig, 1994). Interaction of the tail domain with acidic phospholipids, including $PIP_2$, interrupts the head-tail interaction, exposing binding sites for talin, $\alpha$-actinin, VASP (vasodilator-stimulated phosphoprotein) and a protein kinase C phosphorylation site (Critchley, 2000). The addition of $PIP_2$, however, was shown to inhibit the interaction between F-actin and the vinculin tail domain, which contains the F-actin binding site (Steimle et al., 1999). The intramolecular head-tail association was also disrupted by a vinculin peptide, isolated from a phage-displayed random peptide library, which contains or is located next to the talin-binding site.

The structure of the vinculin tail (residues 879-1066), which was recently determined (Bakolitsa et al., 1999), showed that the vinculin tail contains five amphipathic helices (H1-H5) arranged as an antiparallel bundle 60 Å in length and 20-30 Å in width (Bakolitsa et al., 1999). The H2 and H3 helices have a highly amphipathic nature, interact with acidic phospholipids, and also are involved in actin binding. A hydrophobic hairpin located at the end of the C-terminus of the five helices (H1-H5) is thought to insert into the lipid bilayer. The conformational changes induced by the interactions of the vinculin tail with the acidic phospholipids and with actin may, in turn, cause the amphipathic helix to insert its hydrophobic face into the lipid bilayer, while allowing the hydrophilic face to interact with cytoplasmic ligands such as actin.

Metavinculin is a vinculin variant resulting from alternative splicing, which is expressed only in muscle cells (Siliciano and Craig, 1982). Metavinculin has an insertion of 68 and 69 amino acids, respectively in the tails of the porcine (Gimona et al., 1988) and chicken (Byrne et al., 1992) molecules. Similarities in cellular localization and domain structure of metavinculin and vinculin suggest that the functions of metavinculin may be

similar to those of vinculin. However, metavinculin has been reported to be involved in dilated cardiomyopathy, suggesting that metavinculin may have a distinct role in cardiac muscle cells (Maeda et al., 1997).

The large, ~190 kDa C-terminal tail of talin has been shown to contain three vinculin binding sites (residues 498-656, 852-950 and 1929-2029) by using solid-phase binding assays (Gilmore et al., 1993). These three sites were subsequently further narrowed to smaller peptides containing residues 607-636, 852-876 and 1944-1969 by using yeast two-hybrid assays (Bass et al., 1999). The latter set of results suggests that the vinculin binding sites within talin are rather short linear stretches of the polypeptide chain. The sequences of the three sites are homologous (59% similarity) to each other. The talin binding site on vinculin originally was localized to the globular vinculin head (residues 1-258), (Gilmore et al., 1992), and more recently has been further defined to residues 1-167 (Bass et al., 1999). The three vinculin binding sites within the talin molecule were shown to bind to the same, or overlapping, sites in the vinculin head (Bass et al., 2002). Interaction between a talin peptide containing one of the vinculin binding sites (1943-2157) and vinculin was shown to be dependent on temperature, with maximal interaction at 37°C (Bass et al., 2002). This interaction was not dependent on ionic strength, and point mutations of hydrophobic and basic residues in the three vinculin binding sites did not affect the binding to vinculin, which suggested that not just hydrophobic or electrostatic interactions are responsible for the talin-vinculin interaction (Bass et al., 2002).

A muscle specific protein, N-RAP (nebulin-related protein), was recently discovered, and shown to be located in the myotendinous junctions in skeletal muscle and in the intercalated disks in cardiac muscle (Luo et al., 1999). The N-RAP was found to interact with

talin through the LIM domain located at the N-terminus of N-RAP. Localization of N-RAP at these cell-matrix type junctions suggests that N-RAP and talin play a role in transmitting tension resulting from contraction to the extracellular matrix by anchoring the terminal actin filaments to the cell membrane.

Actin is one of the most highly conserved and abundant proteins in eukaryotic cells. Actin filaments represent one of the three major cytoskeletal systems in cells of the animal kingdom (Pollard et al., 2000; Pollard and Cooper, 1986; Weeds et al., 1991). The actin cytoskeleton plays critical roles in many biological events, including establishment and maintenance of cytoplasmic structure, generation of force for cell locomotion, cell division, and intracellular vesicle transport. The actin monomer (G-actin) is globular and consists of a single polypeptide chain (molecular mass ~42 kDa). The actin molecule is the smallest (5.5 × 5.5 × 3.5 nm) in size of the major proteins present in the three cytoskeletal, filamentous systems. The actin monomer is composed of two domains called the large and small domains (Kabsch et al., 1990). The small domain consists of subdomains one and two, and the larger domain consists of subdomains three and four. The cleft between the large and small domains has binding sites for the nucleotide ATP or ADP, and for $Ca^{2+}$. Actin is present *in vitro* as a monomer at very low ionic strength. An increase in ionic strength to physiological levels induces a conformational change in the actin monomer, and polymerization into long, two-stranded helical filaments (F-actin) (Pollard et al., 2000; Pollard and Cooper, 1986).

The assembly and disassembly of actin, which are very dynamic processes and important for many biological events, are primarily regulated by many actin binding proteins. Based largely upon regulatory mechanisms, actin binding proteins are often divided (Pollard et al., 2000; Pollard and Cooper, 1986; Weeds et al., 1991) into the following five

representative groups: (1) proteins that bind to the sides of actin filaments and cross-link them to form bundles or networks, such as fimbrin, α-actinin, and filamin; (2) proteins that bind and sequester actin monomers, such as profilin, deoxyribonuclease I, and vitamin D-binding protein; (3) proteins that sever actin filaments and cap the ends of the filaments, such as gelsolin; (4) proteins that link actin filaments to plasma membranes, such as talin and vinculin; and (5) proteins that generate contractile forces such as myosin.

For many years, investigators (reviewed in Burridge et al., 1988) failed to identify a direct interaction between talin and actin, concluding incorrectly that talin could only function with actin via a multi-protein bridge containing vinculin and α-actinin. The direct talin-actin interaction was first shown by Muguruma et al. (1990) who demonstrated that talin is able to bind to both G- and F-actin by using gel filtration chromatography, actin cosedimentation and cross-linking assays. Actin binding activity of talin was shown in studies from our lab (Schmidt et al., 1993; Schmidt et al., 1999) to be highly sensitive to pH, ionic strength and temperature, with maximal binding found at low pH and ionic strength, and physiological temperature (37°C). Those conditions suggest that the talin-actin interaction includes those of an electrostatic nature.

Talin had not previously been known for possessing an ability to cross-link actin filaments because platelet talin was shown to decrease the viscosity of F-actin solutions (Kaufmann et al., 1991). Smooth muscle talin, however, was reported to increase the viscosity and to cross-link actin filaments into bundles and networks in studies from our lab (Schmidt et al., 1999; Zhang et al., 1996). The 190 kDa C-terminal tail of talin showed less actin binding and cross-linking activity than did intact talin under assay conditions favorable for intact talin (Schmidt et al., 1999). However, the cross-linking activity of the talin tail was

increased at a lower pH of 6.0. Intact talin retains significant actin filament cross-linking activity at pH 6.9, 100 mM KCl and 37°C, conditions similar to the physiological environment.

Talin has been reported to contain three actin binding sites, one (ABS1, residues 102-497) in the N-terminal head, and the other two (ABS2, residues 951-1327; ABS3, residues 2269-2541) in the C-terminal tail (Hemmings et al., 1996). The ABS3 was found to contain a new actin binding module that is conserved across species (McCann and Craig, 1999). Four blocks of I/LWEQ module in the ABS3 were identified from an analysis of talin sequences of mouse, *Caenorhabditis elegans*, *Dictyostelium* filopodin, *C. elegans* Sla2 homologue, and *Saccharomyces cerevisiae* Sla2 (McCann et al., 1997). This actin binding module competed with intact talin for F-actin binding activity. Additional sequence analysis on 23 available I/LWEQ modules in proteins among eukaryotes suggested that the proteins containing this module can be grouped into four distinct families. The high conservation of this module across species suggests an important function for the module as an adaptor, coupling proteins involved in several biological events such as cell adhesion, migration and cortical actin organization to actin cytoskeleton (McCann and Craig, 1999).

Biochemical properties and functional roles of ABS1 and ABS2 have not heretofore been characterized further than what was originally reported from the Critchley lab (Bolton et al., 1997; Hemmings et al., 1996). The presence of ABS1 in the N-terminal head has been controversial. Cosedimentation at high pH and ionic strength of calpain-digested talin containing both the N-terminal head and C-terminal tail fragments with F-actin showed that only the tail cosedimented with F-actin (Muguruma et al., 1995). Biophysical analysis using quasi-elastic light scattering (Goldmann et al., 1999) confirmed the high dependence of the

intact talin/actin interaction on pH and ionic strength (Schmidt et al., 1993; Schmidt et al., 1999), and showed that the interaction of the C-terminal talin tail with F-actin affected the dynamics of actin filaments, whereas the N-terminal head had no effect. In contrast, an expressed construct containing the talin head domain was shown to have F-actin binding activity (Bolton et al., 1997; Hemmings et al., 1996). As will be shown in this dissertation, I have characterized the three actin binding sites of talin, concentrating primarily upon ABS1. My studies strongly indicate that the talin head possesses actin binding activity.

At least one gene encoding talin has been identified in various eukaryotes, from *Dictyostelium* to mammals. A talin cDNA (GenBank Accession: X56123) was first cloned from mouse by antibody screening of an expression library (Rees et al., 1990). Two homologues of talin protein (TALA, GenPept Accession: A57036 and TALB, GenPept Accession: BAA75511) were identified in *Dictyostelium discoideum*, both of which share higher homology with the mouse talin sequence in their N-terminal 400 amino acids and C-terminal 200 amino acids, respectively, than in the rest of the molecules (Kreitmeier et al., 1995; Tsujioka et al., 1999). TALB contains an additional C-terminal domain, which contains about 60 amino acid residues that are homologous to the C-terminal domain of villin. The talin protein homologue of *C. elegans* (GenBank Accession: L46861) shows approximately 39% homology with mouse talin in protein sequence (Moulder et al., 1996). The protein sequence of chicken talin shows very high homology (89% identity) with that of the mouse talin. A homology search of the *Drosophila melanogaster* database resulted in the identification of a predicted protein (GenPept Accession: AAF50399) that shares homology with the FERM domain within the N-terminal head and with the actin binding domain (ABS3) near the end of the C-terminal tail of talin.

The human talin gene (GenPept Accession: Q9Y490) was found to contain 57 exons, and its sequence exhibited high homology with those of mouse (98% identity) and chicken (88% identity) talins (Ben-Yosef and Francomano, 1999). The human talin gene (*TLN*) was localized to human chromosome 9p13 (Ben-Yosef and Francomano, 1999). The sequence of a second talin gene (*TLN2*) has recently been assembled from the genomic and the expressed sequence tag (EST) databases (Monkley et al., 2001). The human *TLN2* gene codes for a protein (talin2) containing 2,532 amino acids, which shows high homology (74% identity and 86% similarity) with human talin1. The FERM domain in the human talin2 head is very similar to that of talin1 and, therefore, may be expected to bind to similar ligands as talin1. The talin2 protein has the same calpain cleavage site between the head and tail domains, and the alanine-rich repeats in the C-terminal tail, suggesting that talin1 and 2 have very similar domain structures. Expression of the talin2 gene was detected in a variety of tissues, with the highest level of expression in heart. More than one talin2 transcript was observed in heart, brain, lung, skeletal muscle, liver and spleen, which suggests the presence of various isoforms of talin2. The biological functions of the multiple transcripts in various tissues remain to be established.

# References

Alatortsev, V. E., Kramerova, I. A., Frolov, M. V., Lavrov, S. A., and Westphal, E. D. (1997). Vinculin gene is non-essential in Drosophila melanogaster. FEBS Lett *413*, 197-201.

Albiges-Rizo, C., Frachet, P., and Block, M. R. (1995). Down regulation of talin alters cell adhesion and the processing of the alpha 5 beta 1 integrin. J Cell Sci *108*, 3317-3329.

Bakolitsa, C., de Pereda, J. M., Bagshaw, C. R., Critchley, D. R., and Liddington, R. C. (1999). Crystal structure of the vinculin tail suggests a pathway for activation. Cell *99*, 603-613.

Barstead, R. J., and Waterston, R. H. (1991). Vinculin is essential for muscle function in the nematode. J Cell Biol *114*, 715-724.

Bass, M. D., Patel, B., Barsukov, I. G., Fillingham, I. J., Mason, R., Smith, B. J., Bagshaw, C. R., and Critchley, D. R. (2002). Further characterization of the interaction between the cytoskeletal proteins talin and vinculin. Biochem J *362*, 761-768.

Bass, M. D., Smith, B. J., Prigent, S. A., and Critchley, D. R. (1999). Talin contains three similar vinculin-binding sites predicted to form an amphipathic helix. Biochem J *341*, 257-263.

Beckerle, M. C., O'Halloran, T., and Burridge, K. (1986). Demonstration of a relationship between talin and P235, a major substrate of the calcium-dependent protease in platelets. J Cell Biochem *30*, 259-270.

Belkin, A. M., Zhidkova, N. I., and Koteliansky, V. E. (1986). Localization of talin in skeletal and cardiac muscles. FEBS Lett *200*, 32-36.

Ben-Yosef, T., and Francomano, C. A. (1999). Characterization of the human talin (TLN) gene: genomic structure, chromosomal localization, and expression pattern. Genomics *62*, 316-319.

Bertagnolli, M. E., Locke, S. J., Hensler, M. E., Bray, P. F., and Beckerle, M. C. (1993). Talin distribution and phosphorylation in thrombin-activated platelets. J Cell Sci *106*, 1189-1199.

Bolton, S. J., Barry, S. T., Mosley, H., Patel, B., Jockusch, B. M., Wilkinson, J. M., and Critchley, D. R. (1997). Monoclonal antibodies recognizing the N- and C-terminal regions of talin disrupt actin stress fibers when microinjected into human fibroblasts. Cell Motil Cytoskeleton *36*, 363-376.

Bono, P., Rubin, K., Higgins, J. M., and Hynes, R. O. (2001). Layilin, a novel integral membrane protein, is a hyaluronan receptor. Mol Biol Cell *12*, 891-900.

Borowsky, M. L., and Hynes, R. O. (1998). Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. J Cell Biol *143*, 429-442.

Bretscher, A., Chambers, D., Nguyen, R., and Reczek, D. (2000). ERM-Merlin and EBP50 protein families in plasma membrane organization and function. Annu Rev Cell Dev Biol *16*, 113-143.

Burridge, K., and Chrzanowska-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. Annu Rev Cell Dev Biol *12*, 463-518.

Burridge, K., and Connell, L. (1983). A new protein of adhesion plaques and ruffling membranes. J Cell Biol *97*, 359-367.

Burridge, K. Fath, K. Kelly, T. Nuckolls, G., and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu Rev Cell Biol *4*, 487-525.

Burridge, K., Kelly, T., and Connell, L. (1982). Proteins involved in the attachment of actin to the plasma membrane. Phil Trans R Soc Lond B *299*, 291-299.

Byrne, B. J., Kaczorowski, Y. J., Coutu, M. D., and Craig, S. W. (1992). Chicken vinculin and meta-vinculin are derived from a single gene by alternative splicing of a 207-base pair exon unique to meta-vinculin. J Biol Chem *267*, 12845-12850.

Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y., Liddington, R. C., and Ginsberg, M. H. (2002). The phosphotyrosine binding-like domain of talin activates integrins. J Biol Chem *277*, 21749-21758.

Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O., and Ginsberg, M. H. (1999). The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. J Biol Chem *274*, 28071-28074.

Cary, L. A., Chang, J. F., and Guan, J. L. (1996). Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J Cell Sci *109*, 1787-1794.

Chen, H. C., Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D., and Guan, J. L. (1995). Interaction of focal adhesion kinase with cytoskeletal protein talin. J Biol Chem *270*, 16995-16999.

Chen, Z. Y., Hasson, T., Kelley, P. M., Schwender, B. J., Schwartz, M. F., Ramakrishnan, M., Kimberling, W. J., Mooseker, M. S., and Corey, D. P. (1996). Molecular cloning and domain structure of human myosin-VIIa, the gene product defective in Usher syndrome 1B. Genomics *36*, 440-448.

Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N., Chasis, J. A., Conboy, J. G., Gascard, P., Takakuwa, Y., Huang, S. C., Benz, E. J., Jr., Bretscher, A., Fehon, R. G., Gusella, J. F., Ramesh, V., Solomon, F., Marchesi, V. T., Tsukita, S., Hoover, K. B., and et al. (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. Trends Biochem Sci *23*, 281-282.

Chou, R. G. Stromer, M. H. Robson, R. M., and Huiatt, T. W. (1994). Substructure of cytoplasmic dense bodies and changes in distribution of desmin and alpha-actinin in developing smooth muscle cells. Cell Motil Cytoskeleton *29*, 204-214.

Coll, J. L., Ben-Ze'ev, A., Ezzell, R. M., Rodriguez Fernandez, J. L., Baribault, H., Oshima, R. G., and Adamson, E. D. (1995). Targeted disruption of vinculin genes in F9 and embryonic stem cells changes cell morphology, adhesion, and locomotion. Proc Natl Acad Sci U S A *92*, 9161-9165.

Collier, N. C., and Wang, K. (1982). Purification and properties of human platelet P235. A high molecular weight protein substrate of endogenous calcium-activated protease(s). J Biol Chem *257*, 6937-6943.

Cooley, M. A., Broome, J. M., Ohngemach, C., Romer, L. H., and Schaller, M. D. (2000). Paxillin binding is not the sole determinant of focal adhesion localization or dominant-negative activity of focal adhesion kinase/focal adhesion kinase-related nonkinase. Mol Biol Cell *11*, 3247-3263.

Critchley, D. R. Holt, M. R. Barry, S. T. Priddle, H. Hemmings, L., and Norman, J. (1999). Integrin-mediated cell adhesion: the cytoskeletal connection. Biochem Soc Symp *65*, 79-99.

Critchley, D. R. (2000). Focal adhesions - the cytoskeletal connection. Curr Opin Cell Biol *12*, 133-139.

Forman-Kay, J. D., and Pawson, T. (1999). Diversity in protein recognition by PTB domains. Curr Opin Struct Biol *9*, 690-695.

Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001). Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. Nat Rev Mol Cell Biol *2*, 793-805.

Geiger, B., Tokuyasu, K. T., Dutton, A. H., and Singer, S. J. (1980). Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. Proc Natl Acad Sci U S A *77*, 4127-4131.

Geiger, B., Volk, T., and Volberg, T. (1985). Molecular heterogeneity of adherens junctions. J Cell Biol *101*, 1523-1531.

Gilmore, A. P., Jackson, P., Waites, G. T., and Critchley, D. R. (1992). Further characterisation of the talin-binding site in the cytoskeletal protein vinculin. J Cell Sci *103*, 719-731.

Gilmore, A. P., Wood, C., Ohanian, V., Jackson, P., Patel, B., Rees, D. J., Hynes, R. O., and Critchley, D. R. (1993). The cytoskeletal protein talin contains at least two distinct vinculin binding domains. J Cell Biol *122*, 337-347.

Gimona, M., Small, J. V., Moeremans, M., Van Damme, J., Puype, M., and Vandekerckhove, J. (1988). Porcine vinculin and metavinculin differ by a 68-residue insert located close to the carboxy-terminal part of the molecule. Embo J *7*, 2329-2334.

Goldmann, W. H., Bremer, A., Haner, M., Aebi, U., and Isenberg, G. (1994). Native talin is a dumbbell-shaped homodimer when it interacts with actin. J Struct Biol *112*, 3-10.

Goldmann, W. H., Hess, D., and Isenberg, G. (1999). The effect of intact talin and talin tail fragment on actin filament dynamics and structure depends on pH and ionic strength. Eur J Biochem *260*, 439-445.

Hamada, K., Shimizu, T., Matsui, T., Tsukita, S., and Hakoshima, T. (2000). Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. EMBO J *19*, 4449-4462.

Han, B. G., Nunomura, W., Takakuwa, Y., Mohandas, N., and Jap, B. K. (2000). Protein 4.1R core domain structure and insights into regulation of cytoskeletal organization. Nat Struct Biol *7*, 871-875.

Hemmings, L., Kuhlman, P. A., and Critchley, D. R. (1992). Analysis of the actin-binding domain of alpha-actinin by mutagenesis and demonstration that dystrophin contains a functionally homologous domain. J Cell Biol *116*, 1369-1380.

Hemmings, L., Rees, D. J., Ohanian, V., Bolton, S. J., Gilmore, A. P., Patel, B., Priddle, H., Trevithick, J. E., Hynes, R. O., and Critchley, D. R. (1996). Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. J Cell Sci *109*, 2715-2726.

Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G., and Otey, C. A. (1996). Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. J Cell Biol *135*, 1383-1390.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11-25.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539-544.

Isenberg, G., and Goldmann, W. H. (1998). Peptide-specific antibodies localize the major lipid binding sites of talin dimers to oppositely arranged N-terminal 47 kDa subdomains. FEBS Lett *426*, 165-170.

Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. (1995). The molecular architecture of focal adhesions. Annu Rev Cell Dev Biol *11*, 379-416.

Johnson, R. P., and Craig, S. W. (1994). An intramolecular association between the head and tail domains of vinculin modulates talin binding. J Biol Chem *269*, 12611-12619.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., and Holmes, K. C. (1990). Atomic structure of the actin:DNase I complex. Nature *347*, 37-44.

Kaufmann, S., Piekenbrock, T., Goldmann, W. H., Barmann, M., and Isenberg, G. (1991). Talin binds to actin and promotes filament nucleation. FEBS Lett *284*, 187-191.

Knezevic, I., Leisner, T. M., and Lam, S. C. (1996). Direct binding of the platelet integrin alphaIIbbeta3 (GPIIb-IIIa) to talin. Evidence that interaction is mediated through the cytoplasmic domains of both alphaIIb and beta3. J Biol Chem *271*, 16416-16421.

Kreitmeier, M., Gerisch, G., Heizer, C., and Muller-Taubenberger, A. (1995). A talin homologue of Dictyostelium rapidly assembles at the leading edge of cells in response to chemoattractant. J Cell Biol *129*, 179-188.

Kupfer, A., Swain, S. L., Janeway, C. A., Jr., and Singer, S. J. (1986). The specific direct interaction of helper T cells and antigen-presenting B cells. Proc Natl Acad Sci U S A *83*, 6080-6083.

Leto, T. L., and Marchesi, V. T. (1984). A structural model of human erythrocyte protein 4.1. J Biol Chem *259*, 4603-4608.

Luo, G., Herrera, A. H., and Horowits, R. (1999). Molecular interactions of N-RAP, a nebulin-related protein of striated muscle myotendon junctions and intercalated disks. Biochemistry *38*, 6135-6143.

Maeda, M., Holder, E., Lowes, B., Valent, S., and Bies, R. D. (1997). Dilated cardiomyopathy associated with deficiency of the cytoskeletal protein metavinculin. Circulation *95*, 17-20.

Martel, V., Racaud-Sultan, C., Dupe, S., Marie, C., Paulhe, F., Galmiche, A., Block, M. R., and Albiges-Rizo, C. (2001). Conformation, localization, and integrin binding of talin depend on its interaction with phosphoinositides. J Biol Chem *276*, 21217-21227.

McCann, R. O., and Craig, S. W. (1997). The I/LWEQ module: a conserved sequence that signifies F-actin binding in functionally diverse proteins from yeast to mammals. Proc Natl Acad Sci U S A *94*, 5679-5684.

McCann, R. O., and Craig, S. W. (1999). Functional genomic analysis reveals the utility of the I/LWEQ module as a predictor of protein:actin interaction. Biochem Biophys Res Commun *266*, 135-140.

Molony, L., McCaslin, D., Abernethy, J., Paschal, B., and Burridge, K. (1987). Properties of talin from chicken gizzard smooth muscle. J Biol Chem *262*, 7790-7795.

Monkley, S. J., Pritchard, C. A., and Critchley, D. R. (2001). Analysis of the mammalian talin2 gene tln2. Biochem Biophys Res Commun *286*, 880-885.

Monkley, S. J., Zhou, X. H., Kinston, S. J., Giblett, S. M., Hemmings, L., Priddle, H., Brown, J. E., Pritchard, C. A., Critchley, D. R., and Fassler, R. (2000). Disruption of the talin gene arrests mouse development at the gastrulation stage. Dev Dyn *219*, 560-574.

Moulder, G. L., Huang, M. M., Waterston, R. H., and Barstead, R. J. (1996). Talin requires beta-integrin, but not vinculin, for its assembly into focal adhesion-like structures in the nematode Caenorhabditis elegans. Mol Biol Cell *7*, 1181-1193.

Muguruma, M., Nishimuta, S., Tomisaka, Y., Ito, T., and Matsumura, S. (1995). Organization of the functional domains in membrane cytoskeletal protein talin. J Biochem (Tokyo) *117*, 1036-1042.

Niewohner, J., Weber, I., Maniak, M., Muller-Taubenberger, A., and Gerisch, G. (1997). Talin-null cells of Dictyostelium are strongly defective in adhesion to particle and substrate surfaces and slightly impaired in cytokinesis. J Cell Biol *138*, 349-361.

Nuckolls, G. H., Romer, L. H., and Burridge, K. (1992). Microinjection of antibodies against talin inhibits the spreading and migration of fibroblasts. J Cell Sci *102*, 753-762.

Otto, J. J. (1990). Vinculin. Cell Motil Cytoskeleton *16*, 1-6.

Pardo, J. V., Siliciano, J. D., and Craig, S. W. (1983). A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements (&quot;costameres&quot;) mark sites of attachment between myofibrils and sarcolemma. Proc Natl Acad Sci U S A *80*, 1008-1012.

Pearson, M. A., Reczek, D., Bretscher, A., and Karplus, P. A. (2000). Structure of the ERM protein moesin reveals the FERM domain fold masked by an extended actin binding tail domain. Cell *101*, 259-270.

Pfaff, M., Liu, S., Erle, D. J., and Ginsberg, M. H. (1998). Integrin beta cytoplasmic domains differentially bind to cytoskeletal proteins. J Biol Chem *273*, 6104-6109.

Pollard, T. D., Blanchoin, L., and Mullins, R. D. (2000). Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu Rev Biophys Biomol Struct *29*, 545-576.

Pollard, T. D., and Cooper, J. A. (1986). Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. Annu Rev Biochem 55, 987-1035.

Priddle, H., Hemmings, L., Monkley, S., Woods, A., Patel, B., Sutton, D., Dunn, G. A., Zicha, D., and Critchley, D. R. (1998). Disruption of the talin gene compromises focal adhesion assembly in undifferentiated but not differentiated embryonic stem cells. J Cell Biol 142, 1121-1133.

Rees, D. J., Ades, S. E., Singer, S. J., and Hynes, R. O. (1990). Sequence and domain structure of talin. Nature 347, 685-689.

Ridley, A. J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70, 389-399.

Rudiger, M. (1998). Vinculin and alpha-catenin: shared and unique functions in adherens junctions. Bioessays 20, 733-740.

Sampath, R., Gallagher, P. J., and Pavalko, F. M. (1998). Cytoskeletal interactions with the leukocyte integrin beta2 cytoplasmic tail. Activation-dependent regulation of associations with talin and alpha-actinin. J Biol Chem 273, 33588-33594.

Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995). FAP-1: a protein tyrosine phosphatase that associates with Fas. Science 268, 411-415.

Schaller, M. D. (2001). Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophys Acta 1540, 1-21.

Schmidt, J. M., Robson, R. M., Zhang, J., and Stromer, M. H. (1993). The marked pH dependence of the talin-actin interaction. Biochem Biophys Res Commun 197, 660-666.

Schmidt, J. M., Zhang, J., Lee, H. S., Stromer, M. H., and Robson, R. M. (1999). Interaction of talin with actin: sensitive modulation of filament crosslinking activity. Arch Biochem Biophys 366, 139-150.

Sealock, R., Paschal, B., Beckerle, M., and Burridge, K. (1986). Talin is a post-synaptic component of the rat neuromuscular junction. Exp Cell Res 163, 143-150.

Seelig, A., Blatter, X. L., Frentzel, A., and Isenberg, G. (2000). Phospholipid binding of synthetic talin peptides provides evidence for an intrinsic membrane anchor of talin. J Biol Chem 275, 17954-17961.

Siliciano, J. D., and Craig, S. W. (1982). Meta-vinculin—a vinculin-related protein with solubility properties of a membrane protein. Nature 300, 533-535.

Small, J. V., Furst, D. O., and Thornell, L. E. (1992). The cytoskeletal lattice of muscle cells. Eur J Biochem *208*, 559-572.

Small, J. V. (1995). Structure-function relationships in smooth muscle: the missing links. Bioessays *17*, 785-792.

Steimle, P. A., Hoffert, J. D., Adey, N. B., and Craig, S. W. (1999). Polyphosphoinositides inhibit the interaction of vinculin with actin filaments. J Biol Chem *274*, 18414-18420.

Stromer, M. H. (1998). The cytoskeleton in skeletal, cardiac and smooth muscle cells. Histol Histopathol *13*, 283-291.

Sydor, A. M., Su, A. L., Wang, F. S., Xu, A., and Jay, D. G. (1996). Talin and vinculin play distinct roles in filopodial motility in the neuronal growth cone. J Cell Biol *134*, 1197-1207.

Tempel, M., Goldmann, W. H., Isenberg, G., and Sackmann, E. (1995). Interaction of the 47-kDa talin fragment and the 32-kDa vinculin fragment with acidic phospholipids: a computer analysis. Biophys J *69*, 228-241.

Tidball, J. G., O'Halloran, T., and Burridge, K. (1986). Talin at myotendinous junctions. J Cell Biol *103*, 1465-1472.

Tsujioka, M., Machesky, L. M., Cole, S. L., Yahata, K., and Inouye, K. (1999). A unique talin homologue with a villin headpiece-like domain is required for multicellular morphogenesis in Dictyostelium. Curr Biol *9*, 389-392.

van der Flier, A., and Sonnenberg, A. (2001). Function and interactions of integrins. Cell Tissue Res *305*, 285-298.

Venter, J. C. Adams, M. D. Myers, E. W. Li, P. W. Mural, R. J., and et al. (2001). The sequence of the human genome. Science *291*, 1304-1351.

Volberg, T., Geiger, B., Kam, Z., Pankov, R., Simcha, I., Sabanay, H., Coll, J. L., Adamson, E., and Ben-Ze'ev, A. (1995). Focal adhesion formation by F9 embryonal carcinoma cells after vinculin gene disruption. J Cell Sci *108*, 2253-2260.

Weeds, A. G. Gooch, J. Hawkins, M. Pope, B., and Way, M. (1991). Role of actin-binding proteins in cytoskeletal dynamics. Biochem Soc Trans *19*, 1016-1020.

Winkler, J., Lunsdorf, H., and Jockusch, B. M. (1997). Energy-filtered electron microscopy reveals that talin is a highly flexible protein composed of a series of globular domains. Eur J Biochem *243*, 430-436.

Xing, B., Jedsadayanmata, A., and Lam, S. C. (2001). Localization of an integrin binding site to the C terminus of talin. J Biol Chem *276*, 44373-44378.

Xu, W., Baribault, H., and Adamson, E. D. (1998). Vinculin knockout results in heart and brain defects during embryonic development. Development *125*, 327-337.

Yamada, K. M., and Geiger, B. (1997). Molecular interactions in cell adhesion complexes. Curr Opin Cell Biol *9*, 76-85.

Yan, B., Calderwood, D. A., Yaspan, B., and Ginsberg, M. H. (2001). Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. J Biol Chem *276*, 28164-28170.

Zamir, E., Katz, B. Z., Aota, S., Yamada, K. M., Geiger, B., and Kam, Z. (1999). Molecular diversity of cell-matrix adhesions. J Cell Sci *112*, 1655-1669.

Zhang, J., Robson, R. M., Schmidt, J. M., and Stromer, M. H. (1996). Talin can crosslink actin filaments into both networks and bundles. Biochem Biophys Res Commun *218*, 530-537.

# CHARACTERIZATION AND FUNCTIONAL IMPLICATIONS OF AN ACTIN-BINDING SITE WITHIN THE TALIN HEAD DOMAIN

Ho-Sup Lee, Robert M. Bellin, Diana L. Walker, Marvin H. Stromer, David R. Critchley*,

and Richard M. Robson[1]

## Summary

Talin is a large cytoskeletal protein that links integrins to F-actin. Talin reportedly contains three actin binding sites (ABS1-3),[1] one in the N-terminal head domain, and two in the C-terminal rod domain (Hemmings et al., 1996, J. Cell Sci. 109, 2715-2726). Although the C-terminal ABS3 has been well characterized, the properties of ABS1 within the talin head have not been defined. We show herein that the N-terminal head fragment purified from calpain-digested talin cosediments with F-actin, and that a recombinant talin polypeptide spanning residues 102-497 has similar activity. Using deletion analysis, we show that residues (271-320) within the talin FERM (band 4.1-ezrin-radixin-moesin) domain are essential for actin binding, and are highly conserved across species. Binding of both ABS1 and ABS3 to actin was highly dependent on ionic strength (as for native talin), but binding of ABS1 was much less pH dependent than was ABS3 or native talin. EGFP-tagged ABS1

Muscle Biology Group, Departments of Biochemistry, Biophysics, and Molecular Biology and of Animal Science, Iowa State University, Ames, Iowa, 50011-3260, and *Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom. [1]To whom correspondence should be addressed (rmrobson@iastate.edu).

expressed in COS cells colocalized with actin-rich structures, especially stress fibers, whereas EGFP-ABS3 was found in membrane ruffles. Overall, these studies provide strong evidence for an actin binding site in the talin FERM domain. This region also contains an integrin-binding site suggesting that it may link integrins to F-actin independent of the C-terminal actin binding site.

## Introduction

Talin is a large (270 kDa, 2,541 amino acids) cytoskeletal protein that is localized primarily in cell-extracellular matrix adherens-type junctions, although it is also found in membrane ruffles of migrating cells [1], human platelets [2], and the junctions between T-cells and antigen presenting cells [3]. Thus, it is localized in the focal adhesions of adherent cells in culture [1], the myotendinous [4] and neuromuscular junctions [5] of skeletal muscle cells, the costameres of skeletal and cardiac muscle cells [6], and the membrane-associated dense bodies of smooth muscle cells [7]. Talin is believed to play a key role at these sites in linking the actin cytoskeleton to the cytoplasmic domain of the integrin family of cell adhesion molecules [8, 9]. Evidence for such a role has come from a variety of sources. When antibodies against talin were microinjected into fibroblasts, migration of the cells was inhibited, and focal adhesions and actin stress fibers were disassembled [10, 11]. Inactivation of talin in neuronal growth cones by using chromophore-assisted laser inactivation resulted in neurite retraction [12], and downregulation of talin in HeLa cells by using antisense RNA technology caused decreased cell spreading and reduced the size of focal adhesions [13]. A similar phenotype was observed in mouse embryonic stem cells carrying mutations in both talin alleles [14]. Finally, talin (-/-) mouse embryos were arrested at gastrulation, apparently

due to a failure of mesoderm migration [15], which may reflect defects in coupling integrins to the actomyosin contractile apparatus within the cell.

The biochemical properties of talin are also consistent with a role in linking integrins to F-actin. The N-terminal head domain of talin contains a FERM (band 4.1-ezrin-radixin-moesin) domain with binding sites for the cytoplasmic domains of $\beta 1$ and $\beta 3$ integrins [16] and for layilin [17], a C-type lectin that colocalizes with talin in membrane ruffles. It also contains a binding site for FAK [18, 19], which is implicated in coupling cell adhesion to cell proliferation and suppression of apoptosis and cell migration [20]. The large C-terminal rod domain of talin contains a second integrin binding site [21], three vinculin binding sites [22, 23], and is able to bind F-actin [24-26]. An as yet undefined domain of talin also has recently been shown to bind to an N-terminal LIM domain within a striated muscle-specific, nebulin related protein, N-RAP [27], suggesting that N-RAP and talin may together play a significant role in anchoring actin filaments of the terminal myofibrillar sarcomeres to the sarcolemma at myotendinous junctions in skeletal muscle cells and the intercalated disks in cardiac muscle cells. The talin molecule is comprised of two subunits that are likely arranged in an antiparallel manner [28, 29], which probably explains why it can cross-link actin into networks and bundles [26, 30-33]. The ability of talin to crosslink F-actin is highly dependent on pH, ionic strength and temperature, and talin exhibits optimal actin binding activity *in vitro* at pH 6.4, low ionic strength and 37°C [26].

The best characterized actin binding site in talin (ABS3) is located close to the C-terminal region of the protein [10, 34], and is highly conserved across species. It contains a so-called I/LWEQ module that is also found in a number of other actin binding proteins such as Sla2p and Hip-1 [35, 36]. A recombinant polypeptide containing this module was shown to inhibit

binding of intact talin to F-actin suggesting that it is a major determinant of actin binding *in vitro* [35]. However, preliminary evidence for two other actin binding sites in talin has been presented [34], one of which (ABS1) is located within the N-terminal head domain. The presence of an actin binding site in the talin head is controversial because the purified head, which can be liberated from talin by calpain II cleavage [37], did not bind actin as detected by biophysical analysis [31] or by actin cosedimentation analysis of a calpain-digest of talin containing both the head and tail domains [38]. In the present study, we have used a series of GST talin head fusion proteins in actin cosedimentation assays, and provide evidence that residues 271-320 within the talin FERM domain contains an actin binding site. Moreover, we also show that the purified talin head isolated from talin after calpain cleavage does indeed bind F-actin at low ionic strength and at pH 6.4. Finally, when EGFP fusion proteins containing this region were expressed in COS cells, they colocalized with actin-rich structures. Together, these data provide strong evidence for the presence of an actin binding site in the talin head domain.

## Materials and Methods

### GST talin fusion constructs

DNA fragments encoding various regions of the talin head (Fig. 1) and the C-terminal ABS3 of talin (residues 2269-2541) were amplified by PCR from chicken talin cDNAs [34] using oligonucleotides containing a *Bam*HI restriction enzyme cleavage site at the 5' end and an *Eco*RI at the 3' end. The amplified PCR products were gel purified, digested with *Bam*HI and *Eco*RI, and ligated into the glutathione *S*-transferase (GST) fusion vector pGEX4T1 (Amersham Biosciences, Piscataway, NJ). The reading frames of all fusion constructs were

confirmed by DNA sequencing. Expression of the recombinant plasmids in *Escherichia coli*

(BL21-Codon⁻-RIL, Stratagene, La Jolla, CA) and the purification of the fusion proteins

using glutathione-crosslinked agarose (Amersham Biosciences) were performed as described

by Carroll et al. [39]. GST fusion proteins were further purified using DEAE-cellulose

(DE52, Whatman, Fairfield, NJ) anion exchange column (1.0 x 10 cm) chromatography.

Protein concentrations were measured by using a modified Lowry method (Sigma, St. Louis,

MO).

## Purification of talin and the talin head domain

All steps were carried out at 0-4 °C unless stated otherwise, and the pH of buffers was

adjusted at the temperature at which they were used. Highly purified talin was prepared from

turkey gizzard smooth muscle as described [26]. The ~47 kDa N-terminal head fragment of

talin was prepared from purified talin by proteolytic digestion with m-calpain prepared by

procedures modified from Edmunds et al. [40]. Purified talin was cleaved by m-calpain in 20

mM Tris-HCl, 15 mM β-mercaptoethanol, pH 7.0, with a talin to m-calpain molar ratio of

300:1. Proteolysis was initiated by addition of $CaCl_2$ (2.5 mM final concentration), and after

1 h at 25 °C, the reaction was stopped by adding an excess of E-64 (10 µg/ml, Peptides

International, Louisville, KY). The talin head was then purified by chromatography on a 1.0

x 10 cm DEAE-cellulose (DE52, Whatman) column.

## Actin cosedimentation assays

G-actin was purified from actin powder preparations of porcine skeletal muscle as described

[41], followed by gel filtration column chromatography (2.5 x 70 cm, Sephacryl S-300 HR,

Amersham Biosciences). Actin cosedimentation assays, all done in duplicate, were performed essentially as described by Schmidt et al. [26]. Each sample (GST fusion protein, talin, calpain-digested talin, or purified talin head fragment (0.2 mg/ml)) was individually mixed with actin (0.5 mg/ml) in cosedimentation buffer (10 mM imidazole-HCl, 1 mM ATP, 1 mM β-mercaptoethanol, 1 mM EGTA, 0.1 mM $CaCl_2$) at the designated pH, and actin polymerization was initiated by adding $MgCl_2$ (2 mM final concentration). The mixture (final volume 150 μl) was incubated at 25 °C for 1 h and centrifuged at 100,000 × g for 20 min in a Beckman airfuge. The resulting pellets were resuspended in 100 μl of the cosedimentation buffer including 1% (w/v) SDS, and comparable amounts of supernatants and pellets (supernatants 21 μl; pellets 14 μl) were analyzed by SDS-PAGE. The gels were stained with Coomassie brilliant blue, scanned, and the integrated density of the bands was measured by using the Scion Image program (Scion Corporation, Frederick, MD).

## EGFP fusion constructs and transfections into COS cells

The EcoRI and BamHI restriction enzyme cleavage sites of the green fluorescent protein (GFP) fusion vector pEGFPC2 (Clontech, Palo Alto, CA) were altered, creating a BamHI site at the 5′ end and EcoRI at the 3′ end in order to ligate the PCR products generated for cloning in the proper orientation. The oligonucleotides for reconstructing the restriction cleavage sites of pEGFPC2 were as follows: 5′-AGCTTCGGATCCTGCAGTCGACGGTACCGCGGGC-CCGGGGGAATTCA-3′ and 5′-GATCTGAATTCCCCCGGGCCCGCGGTACCGTCGAC-TGCAGGATCCGA-3′. The two oligonucleotides were mixed in buffer (10 mM Tris-HCl, pH 7.4), heated for 30 sec at 100 °C, and the mixture cooled to room temperature in order to

anneal the two oligonucleotides. These were then ligated into *Hind*III-*Xba*I digested pEGFPC2 vector and transformed into *E. coli* (XL1 Blue). Amplified talin PCR products were gel purified, digested with *Bam*HI/*Eco*RI and ligated into the modified pEGFPC2 vector digested with the same enzymes. A DNA fragment encoding the ABS3 (residues 2269-2541) from the C-terminal end of talin was also amplified by PCR and cloned into the modified pEGFPC2 vector. The actin binding site of α-actinin (residues 1-269) [42] was prepared by PCR amplification of a chicken α-actinin cDNA (GeneBank™ accession number J03486) as template, and cloned into the *Sal*I and *Sac*II restriction enzyme sites of pEGFPC3. Plasmid DNA for transfection experiments was purified by using the EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA). All constructs were sequenced to confirm the reading frame and authenticity of the constructs.

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco/BRL, Rockville, MD) supplemented with 10% fetal calf serum. Transfections were carried out by using the FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) as follows. Cells were grown on fibronectin (10 μg/ml)-coated sterile coverslips in 60 mm culture dishes until they reached 50-70% confluence. Serum free Dulbecco's modified Eagle's medium (200 μl) was mixed with 6 μl of FuGENE 6, and incubated at 25 °C for 5 min. Two μg of GFP fusion plasmid were added to the mixture that was incubated for an additional 15 min at 25 °C, and then added to the culture dishes. The cells were incubated at 37 °C for 12 to 20 h, then washed three times with PBS (80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4 \cdot H_2O$, 100 mM NaCl, pH 7.4), and fixed with 4% (w/v) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS for 20 min at 25 °C. The cells were

permeabilized with 0.1% Triton X-100 in fixative for 5 min, and then washed three times in

PBS. Cells were stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR), which

had been diluted 1:40 in PBS, for 1 h at 37 °C to visualize F-actin. After washing with PBS

two times and once with $H_2O$, the cells were mounted on slides with Gelvatol (Air Products

and Chemicals, Inc., Allentown, PA) and observed with a Zeiss Photomicroscope III

equipped with phase contrast and epifluorescence optics. The images of the cells were

obtained with a SPOT RT camera (Diagnostic Instruments, Inc.).

## Results

{SUGGESTED PLACEMENT FOR FIG. 1}

**Defining an actin binding site within the N-terminal head of talin**

Talin reportedly contains three actin binding sites, one in the N-terminal head domain (ABS1

residues 102-497) and two in the C-terminal rod domain (ABS2 residues 951-1327; ABS3

residues 2269-2541) [34]. To confirm that the talin head does indeed contain an actin binding

site and to further define the residues involved, various GST-talin head constructs were tested

in actin cosedimentation assays for their abilities to bind F-actin at pH 6.4 and low ionic

strength, conditions that are optimal for the talin-actin interaction [26]. Most of the fusion

proteins starting at residue 102 were insoluble. In contrast, those starting at residue 201 were

soluble, and moreover a construct spanning residues 201-497 bound to F-actin as well as the

102-497 construct. Therefore, ABS1 was mapped by making N- and C-terminal deletions of

the 201-497 construct (Fig. 1).

{SUGGESTED PLACEMENT FOR FIG. 2}

Progressive C-terminal deletions up to residue 320 had little or no effect on binding to F-actin, and ~92% of a GST fusion protein containing talin residues 201-320 was able to co-sediment with F-actin (Fig. 2, panels c and d). GST alone did not bind to F-actin (data not shown). However, further deletions led to substantial loss of binding activity (Fig. 1), and a GST 201-270 construct showed only low levels of specific F-actin binding, with ~96% of the protein remaining in the supernatant (Fig. 2, panels a and b). Progressive deletion from the N-terminus up to residue 270 resulted in some reduction in protein solubility and stability and a noticeable fraction of the GST 271-497 construct sedimented in the absence of F-actin (Fig. 2, panel g). However, virtually all (~99%) of the protein was recovered in the pellet when centrifuged in the presence of F-actin (Fig. 2, panel h) indicating that residues 271-497 still retained binding activity. A construct containing talin residues 295-497 also co-sedimented (over 90%) with F-actin (Fig. 1), whereas one containing residues 321-497 failed to do so (Fig. 2, panels e and f). These results suggest that residues 271-320 within the talin head region are responsible for binding to F-actin, with residues 295-320 containing the major determinants of F-actin binding.

{SUGGESTED PLACEMENT FOR FIG. 3}

**Effect of pH on the interaction between ABS1 and F-actin**

We have previously shown that the interaction of talin with F-actin is highly dependent on pH and that the ability of talin to cosediment with F-actin increased dramatically as the pH was decreased to pH 6.4 [26]. We therefore analyzed the effect of pH on the interaction between ABS1 and F-actin in cosedimentation buffers at pH 6.4, 6.9 and 7.3, respectively. Two GST fusion proteins were analyzed, one containing residues 201-320 (Fig. 3A), and the

other residues 271-497 (Fig. 3B), both of which encompass the narrowed ABS1. The results

in Fig. 3 show that the ability of ABS1 to bind F-actin is not highly dependent on pH, and

that decreasing the pH from 7.3 to 6.4 only slightly increased the ability of the fusion

proteins to cosediment with actin. Approximately 98% of GST 201-320 cosedimented with

F-actin at pH 6.4, whereas about 91% and 80% of the fusion protein was cosedimented at pH

6.9 and pH 7.3, respectively (Fig. 3A, compare panels b, d, and f). Binding of the 271-497

construct (Fig. 3B) was even less pH sensitive, with ~99% of the fusion protein cosedimented

with F-actin at pH 6.4, 96% at pH 6.9, and 93% at pH 7.3, respectively (Fig. 3B, compare

panels b, d, and f). Thus, near maximal binding of ABS1 to F-actin is retained at a higher pH

than was observed with native talin.

{SUGGESTED PLACEMENT FOR FIG. 4}


## Effect of pH on the interaction of ABS3 with F-actin

Sequence alignments show that the C-terminal ABS3 contains a new actin binding module

(I/LWEQ), which is also found in a number of other actin binding proteins [35, 36]. We,

therefore, analyzed the effect of pH on the ability of ABS3 to bind F-actin in order to

compare with that obtained with ABS1. The results (Fig. 4) showed that decreasing the pH

from 7.3 to 6.4 significantly increased the amount of ABS3 that cosedimented with F-actin.

Approximately 76% of the ABS3 was sedimented at pH 6.4, compared to 30% at pH 6.9, and

18% at pH 7.3 (Fig. 4, compare panels b, d and f). These results are in marked contrast to

those obtained with ABS1 (Fig. 3), and indicate that the ability of ABS3 to cosediment with

actin shows a similar pH dependence to that seen with native talin. These differences suggest

that the electrostatic properties of ABS1 may be different from those of ABS3.

{SUGGESTED PLACEMENT FOR FIG. 5}

## Effect of ionic strength on the interaction of ABS1 and ABS3 with F-actin

The talin-actin interaction has been shown to be markedly reduced at high ionic strength [26]. Since the minimal ABS1 (residues 271-320) is highly basic (pI ~10.15), the interaction between ABS1 and F-actin may be primarily electrostatic in nature. To test this hypothesis, we investigated the effect of ionic strength on the interaction by using two fusion proteins containing residues 201-320 and 271-497. The results showed that increasing the KCl concentration progressively decreased the ability of both fusion proteins to cosediment with F-actin (Fig. 5), although even at 100 mM KCl, there was still significant binding. In contrast, the ability of native talin to bind F-actin was almost completely inhibited in 100 mM KCl at 25 °C [26].

{SUGGESTED PLACEMENT FOR FIG. 6}

We next examined the effect of ionic strength on binding of ABS3 to F-actin. Concentrations as low as 25 mM KCl markedly decreased the ability of ABS3 to bind F-actin (~48% inhibition), and adding 50 mM and 100 mM KCl further inhibited binding. In comparison to ABS1, which showed most significant inhibition at KCl concentrations between 50 mM and 100 mM, the decrease in the ability of ABS3 to bind to F-actin was most marked between 0 mM and 25 mM. These results indicate that ABS3 is even more sensitive to ionic strength than is ABS1, and that electrostatic interactions may also be involved in the interaction of ABS3 and F-actin.

{SUGGESTED PLACEMENT FOR FIG. 7}

## The purified talin head fragment binds to F-actin

The $Ca^{--}$-dependent protease type II (m-calpain) readily cleaves talin between residues 433 and 434 [43], generating an ~47 kDa N-terminal head and an ~190 kDa C-terminal rod domain [37]. Previous reports have indicated that the ~190 kDa rod domain, but not the ~47 kDa head, binds to F-actin [31, 38]. Because recombinant talin head polypeptides bind to F-actin (studies herein and see Ref. 34), we re-examined the ability of the purified N-terminal head fragment liberated from intact talin by m-calpain to cosediment with F-actin at pH 6.4 and low ionic strength. Intact talin almost quantitatively cosedimented with F-actin under these conditions (Fig. 7, panel b). Interestingly, when calpain-cleaved talin that contained both the talin head and rod domains was similarly analyzed, a considerable portion of both polypeptides was found to cosediment with F-actin (Fig. 7, panel c), and quantitative analysis showed that ~54% of the rod domain and 47% of the N-terminal head cosedimented with F-actin. To verify and extend these findings, the talin head fragment was purified, and the results showed that it almost quantitatively cosedimented with F-actin (Fig. 7, panels d and e). This confirms the data obtained using recombinant talin polypeptides and establishes that there is indeed an actin binding site in the talin head.

{SUGGESTED PLACEMENT FOR FIG. 8}

## Colocalization of EGFP-tagged ABS1 with F-actin in COS cells

To investigate whether the talin head has actin binding activity *in vivo*, we generated EGFP fusion constructs that included ABS1 residues 271-320, and transfected them into COS cells. COS cells transiently transfected with EGFP constructs encoding talin residues 201-320 (Fig. 8A) and 271-497 (Fig. 8C), both of which contain ABS1, colocalized with phalloidin-labeled

actin stress fibers. The constructs did not localize to focal adhesions. The EGFP fluorescence was also distributed diffusely in the cytoplasm, presumably due to the high level of expression. COS cells expressing EGFP 201-270 (Fig. 8B) and 321-497 (Fig. 8D) (corresponding to the control constructs that do not include residues 271-320 and that do not bind F-actin *in vitro*) showed diffuse EGFP fluorescence distributed throughout the cytoplasm with no distinct localization at actin-rich structures. This strongly suggests that the colocalization of EGFP constructs 201-320 and 271-497 with F-actin is indeed specific.

We also transfected COS cells with an EGFP 2269-2541 (Fig. 8E) construct that contains ABS3 [34] and an EGFP construct containing the N-terminal ABS of $\alpha$-actinin (residues 1-269) (Fig. 8F) as positive controls. As shown in Fig. 8E, the EGFP fluorescence with ABS3 was predominantly colocalized with actin-rich membrane ruffles and more weakly with cortical actin filaments and actin stress fibers. As shown in Fig. 8F, the $\alpha$-actinin construct showed extensive colocalization of EGFP fluorescence with actin stress fibers visualized with rhodamine-phalloidin, as well as strong fluorescence concentrated in focal adhesion-like structures at the ends of stress fibers. The $\alpha$-actinin construct also colocalized with membrane-associated actin filaments. The results shown in Fig. 8 support the conclusion that ABS1 is able to bind to F-actin *in vivo*, and also suggest that ABS1 and ABS3 may play distinctive roles in interacting with cellular actin.

## Discussion

We have used a series of recombinant talin polypeptides to provide strong evidence that the talin head contains an actin binding site (ABS1) that is defined by residues 271-320. We have also shown that the N-terminal head purified from calpain-digested, tissue-purified talin has

the ability to interact with actin. The interaction between F-actin and ABS1 was relatively insensitive to pH, which is in contrast to the properties of ABS3 (and of intact talin), which showed a marked reduction in binding at pH 7.3. However, binding of ABS1 to actin was inhibited by KCl concentrations above 50 mM and was only marginally less salt sensitive than intact talin. The above data initially appear to conflict with previous studies that failed to detect any interaction between the talin head and F-actin. Quasi-elastic light scattering studies showed that the purified ~190 kDa C-terminal rod domain, but not the purified ~47 kDa N-terminal head of talin, affected actin dynamics [31]. However, the rod domain reportedly contains two well-separated, distinct actin binding sites [34], which may affect actin dynamics differently than would one actin binding site in the head domain in their assays [31]. Furthermore, those studies [31] included assays conducted below pH 6.4, where we have found the talin head region becomes insoluble[2]. We also have shown that talin is much more effective than is the ~190 kDa talin rod domain in formation of actin filament networks and bundles [26], again supportive of an actin binding function for the talin head. It was reported in one study [38] that only the C-terminal rod domain in a calpain-digested talin sample was observed to cosediment with F-actin. However, the "purified" N-terminal talin head has not heretofore been tested biochemically for its ability to bind actin. The fact that the previous study failed to detect binding of the talin head, when present within a talin digest, to F-actin may be due at least in part to several factors; (i), Muguruma et al. [38] carried out their studies at high pH (7.5) and ionic strength (100 mM KCl), conditions under which binding of even intact talin to actin is drastically reduced [26]; (ii), competition may exist between the talin head and rod domains for binding sites on F-actin; and (iii), the C-terminal rod domain has been reported to contain two actin binding sites [34], and might

therefore bind F-actin more tightly than the N-terminal head that contains a single actin

binding site. That the C-terminal rod domain in the talin digest cosedimented with F-actin

less efficiently than did intact talin (Fig 7), coupled with the observation that the purified C-

terminal rod domain has less actin binding and crosslinking activity than does intact talin

[26] are consistent with the concept that the talin head contributes significantly to the overall

actin binding activity of intact talin. In additional support of this conclusion, the GST talin

201-320 construct competes with intact talin for binding to actin, and a 10:1 molar ratio

inhibited talin binding by ~67%$^2$.

{SUGGESTED PLACEMENT FOR FIG. 9}

The talin head contains a region of homology to the FERM proteins band 4.1, ezrin, radixin

and moesin, which act as linkers between the actin cytoskeleton and type 1 integral

membrane proteins such as CD44 and ICAM 1-3 [44]. The crystal structure of the radixin

[45], moesin [46] and band 4.1 [47] FERM domains have recently been reported revealing

three subdomains (A-C; using the nomenclature of Ref. 45) connected by short linkers.

Although the original sequence alignments suggested that the homology between talin and

ERM proteins was restricted to residues 165-373 [43], it is now apparent that the homology

extends from approximately residue 86 to 405 [45]. This suggests that the talin FERM

domain will have a similar three lobed structure, and the predicted boundaries of subdomains

A-C in talin are approximately 86-195, 208-303 and 310-405, respectively. However, the

talin FERM domain is distinct from that of the ERM proteins in terms of sequence identity

(25% as opposed to ~74% within the ERM family), and the presence of an ~33 residue

insertion between subdomains A and B. Our deletion analysis indicates that putative

subdomain A of the talin FERM domain (residues 86-195) is not required for actin binding

because fusion proteins lacking this region (e.g., GST 201-497) bind F-actin as well as GST 102-497. Similarly, subdomain C (residues 310-405) is unlikely to bind F-actin since GST 321-497, which contains most of subdomain C, does not bind actin. Instead, the talin polypeptide GST 201-320, which contains the whole of subdomain B (residues 208-303), binds strongly to F-actin. Further deletion analysis has allowed us to map ABS1 to residues 271-320, which is predicted to correspond to the C-terminal half of subdomain B, the linker region between subdomain B and C, and the start of subdomain C. In radixin, the equivalent region (residues 164-212) is negatively charged (pI ~5.26), and the linker region forms an acidic groove in the middle of the negatively charged surface that has been suggested as a possible binding site for the basic juxta-membrane domains of integral membrane proteins [45]. However, talin ABS1 (residues 271-320) has a pI of ~10.15, and thirteen of the fifty residues are positively charged. Nine of eleven negatively charged residues in the acidic groove of radixin are correspondingly substituted with positively charged or uncharged residues in the talin ABS1 (Fig. 9, panel A). These positively charged residues may be part of the site involved in binding F-actin. The FERM domain of ezrin has also been reported [48] to possess an actin binding site (residues 288-310) located at the end of subdomain C, a region that contains the highly conserved basic sequence RRRK. Although the equivalent region of talin (residues 396-410) contains a similar conserved sequence (KKKK), a GST 321-497 talin fusion protein containing this sequence did not bind actin.

Protein sequence alignments of ABS1 (residues 271-320) with talin sequences from other species show that this region is highly conserved (Fig. 9, panel B). Moreover, the sequence identity of ABS1 between chicken and mouse (94%), is somewhat greater than that of the whole talin sequence (89%) [34], which is consistent with the conclusion that this region

contains a binding site for actin that is itself highly conserved. Another talin gene (*TLN 2*) previously identified from mouse and human EST databases has been assembled using human genomic sequences [49]. Talin ABS1 shows a higher degree of relatedness to the equivalent region in talin-2 (82% identity, 91% similarity) than that shown by the sequences as a whole (76% identity, 86% similarity). The fact that GST constructs spanning talin residues 295-497 and 201-320 both bind strongly to F-actin focuses attention on the role of residues 295-320 in actin binding, and we refer to this region as the "core ABS1." Interestingly, this region has more conserved residues than the rest of the ABS1, and is identical in human, mouse and chicken.

The ability of EGFP ABS1 fusion proteins to colocalize with actin-rich structures in COS cells is consistent with the conclusion that ABS1 has actin binding activity. The weak colocalization with actin stress fibers is similar to that observed by Hemmings et al. [34] when a GST talin fusion protein (residues 102-497) containing the ABS1 was microinjected into chick embryo fibroblasts. This colocalization was abolished when cells were extracted with Triton X-100 before fixation suggesting that the interaction between ABS1 and the actin cytoskeleton is labile [34]. In contrast, the EGFP construct (residues 2269-2541) containing ABS3 localized strongly to membrane ruffles and membrane-associated actin filaments with weaker colocalization to actin stress fibers in the cytoplasm. Again, this localization is reminiscent of that observed with the equivalent GST fusion protein that was resistant to detergent extraction [34].

The differences between ABS1 and ABS3 in resistance to Triton X-100 extraction [34], plus the differences shown herein between ABS1 and ABS3 in cellular localization and the fact that actin binding by ABS3 (but not ABS1) is markedly pH dependent, indicate that the

two sites have quite distinct properties. However, the structural basis of this difference is unclear. Charged groups and their distribution on the surface of a protein will clearly influence the pH dependence of protein-protein interactions. For instance, binding of *Dictyostelium* hisactophilin to F-actin was shown to be markedly pH dependent (within pH 5.7–6.7) [50], and the high content (26%) of histidine residues (p$K$ 6.04) is believed to contribute to this effect. ABS1 (pI ~10.15) contains a high content (20%) of lysine residues (p$K$ 10.54) and this may explain the lack of pH dependence of actin binding to ABS1 in the physiological range. The presence of a large number (26%) of positively charged residues in ABS1 may also account for the marked inhibitory effects of increasing ionic strength on binding to actin. In contrast, ABS3 has a pI of ~5.80 which is close to the p$K$ of histidine, and the three histidine residues within ABS3 may account for the pH dependence of actin binding in the pH range of 6.4 to 7.3. It is also possible that the change in pH may induce a change in the conformation of ABS3 that is more favorable for the interaction with actin. The finding that talin has at least two actin binding sites with different pH sensitivities suggests that the ability of talin to interact with actin may be finely modulated in response to local changes in pH within the cell, and that each actin binding site may have a distinctive role in interacting with actin.

In addition to ABS1, the talin FERM domain also contains binding sites for integral membrane proteins including integrins (residues 186-435) [16] and layilin (residues 280-435) [17], as well as for FAK (residues 225-357) [17], a cytoplasmic protein tyrosine kinase implicated in the regulation of focal adhesion dynamics [20]. Recently, a second integrin binding site in talin has been identified [21] towards the C-terminal region of the protein (residues 1984-2541). The investigators [21] speculated that these two integrin binding sites,

one in the N-terminal and the other in the C-terminal region of talin, may lie in close apposition within the talin antiparallel dimeric molecule [28, 29], and that this may serve to regulate the strength of the interaction between integrins and talin. It may be significant that both integrin binding sites are within the same vicinity as actin binding sites, thus raising the possibility that the talin head and C-terminal rod might both participate in coupling integrins to the actin cytoskeleton. Furthermore, the expressed N-terminal domain of talin (residues 1-435) recently was shown to have higher affinity for the cytoplasmic domain of the $\beta$ integrins than does intact talin [51]. Interestingly, calpain digestion of talin resulted in cooperative and increased binding of talin head and rod domains to the cytoplasmic domain of $\beta_3$ integrin, suggesting that the talin head may even function as an independent entity in some cellular events [51, 52]. Furthermore, it recently has been reported [53] that subdomain F3[3] of the talin FERM domain is predicted to contain a phosphotyrosine binding (PTB)-like domain and expression of this domain in cells fostered activation of integrins. Those results indicate that the talin FERM domain plays an important role in signal tranduction. The presence of the PTB-like domain adjacent to, and slightly overlapped with, ABS1 (271-320) further supports important functional roles for the talin head.

In conclusion, we have demonstrated the presence of an actin binding site in the N-terminal talin head, and have narrowed down the region involved to residues 271-320. We have shown that this site has different characteristics than those of ABS3 in the C-terminal rod domain, and suggest that ABS1 might provide a means of coupling the high affinity integrin binding site in the talin FERM domain to the actin cytoskeleton.

## References

1    Burridge, K. and Connell, L. (1983) Talin: a cytoskeletal component concentrated in adhesion plaques and other sites of actin-membrane interaction. Cell Motil. **3**, 405-417

2    Collier, N. C. and Wang, K. (1982) Purification and properties of human platelet P235. A high molecular weight protein substrate of endogenous calcium-activated protease(s). J. Biol. Chem. **257**, 6937-6943

3    Kupfer, A., Swain, S. L., Janeway, C. A., Jr. and Singer, S. J. (1986) The specific direct interaction of helper T cells and antigen-presenting B cells. Proc. Natl. Acad. Sci. U S A **83**, 6080-6083

4    Tidball, J. G., O'Halloran, T. and Burridge, K. (1986) Talin at myotendinous junctions. J. Cell Biol. **103**, 1465-1472

5    Sealock, R., Paschal, B., Beckerle, M. and Burridge, K. (1986) Talin is a post-synaptic component of the rat neuromuscular junction. Exp. Cell Res. **163**, 143-150

6    Belkin, A. M., Zhidkova, N. I. and Koteliansky, V. E. (1986) Localization of talin in skeletal and cardiac muscles. FEBS Lett. **200**, 32-36

7       Geiger, B., Volk, T. and Volberg, T. (1985) Molecular heterogeneity of adherens junctions. J. Cell Biol. **101**, 1523-1531

8       Critchley, D. R. (2000) Focal adhesions - the cytoskeletal connection. Curr. Opin. Cell Biol. **12**, 133-139

9       Lee, H.-S., Bellin, R. M., and Robson, R. M. (2001) in Wiley Encyclopedia of Molecular Medicine, Vol. **5**, 3087-3089, John Wiley & Sons, Inc., New York

10      Bolton, S. J., Barry, S. T., Mosley, H., Patel, B., Jockusch, B. M., Wilkinson, J. M. and Critchley, D. R. (1997) Monoclonal antibodies recognizing the N- and C-terminal regions of talin disrupt actin stress fibers when microinjected into human fibroblasts. Cell Motil. Cytoskeleton **36**, 363-376

11      Nuckolls, G. H., Romer, L. H. and Burridge, K. (1992) Microinjection of antibodies against talin inhibits the spreading and migration of fibroblasts. J. Cell Sci. **102**, 753-762

12      Sydor, A. M., Su, A. L., Wang, F. S., Xu, A. and Jay, D. G. (1996) Talin and vinculin play distinct roles in filopodial motility in the neuronal growth cone. J. Cell Biol. **134**, 1197-1207

13      Albiges-Rizo, C., Frachet, P. and Block, M. R. (1995) Down regulation of talin alters cell adhesion and the processing of the alpha 5 beta 1 integrin. J. Cell Sci. **108**, 3317-3329

14      Priddle, H., Hemmings, L., Monkley, S., Woods, A., Patel, B., Sutton, D., Dunn, G. A., Zicha, D. and Critchley, D. R. (1998) Disruption of the talin gene compromises focal adhesion assembly in undifferentiated but not differentiated embryonic stem cells. J. Cell Biol. **142**, 1121-1133

15      Monkley, S. J., Zhou, X. H., Kinston, S. J., Giblett, S. M., Hemmings, L., Priddle, H., Brown, J. E., Pritchard, C. A., Critchley, D. R. and Fassler, R. (2000) Disruption of the talin gene arrests mouse development at the gastrulation stage. Dev. Dyn. **219**, 560-574

16      Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O. and Ginsberg, M. H. (1999) The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. J. Biol Chem. **274**, 28071-28074

17      Borowsky, M. L. and Hynes, R. O. (1998) Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. J. Cell Biol. **143**, 429-442

18      Chen, H. C., Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D. and Guan, J. L. (1995) Interaction of focal adhesion kinase with cytoskeletal protein talin. J. Biol Chem. **270**, 16995-16999

19      Hayashi, I., Vuori, K. and Liddington, R. C. (2002) The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. Nat. Struct. Biol. **9**, 101-106

20      Ilic, D., Damsky, C. H. and Yamamoto, T. (1997) Focal adhesion kinase: at the crossroads of signal transduction. J. Cell Sci. **110**, 401-407

21      Xing, B., Jedsadayanmata, A. and Lam, S. C. Localization of an integrin binding site to the C terminus of talin. (2001) J. Biol. Chem. **276**, 44373-44378

22      Bass, M. D., Smith, B. J., Prigent, S. A. and Critchley, D. R. (1999) Talin contains three similar vinculin-binding sites predicted to form an amphipathic helix. Biochem J. **341**, 257-263

23    Bass, M. D., Patel, B., Barsukov, I. G., Fillingham, I. J., Mason, R., Smith, B. J.,

Bagshaw, C. R. and Critchley, D. R. (2002) Further characterization of the interaction

between the cytoskeletal proteins talin and vinculin. Biochem J. **362**, 761-768

24    Muguruma, M., Matsumura, S. and Fukazawa, T. (1990) Direct interactions between

talin and actin. Biochem. Biophys. Res. Commun. **171**, 1217-1223

25    Niggli, V., Kaufmann, S., Goldmann, W. H., Weber, T. and Isenberg, G. (1994)

Identification of functional domains in the cytoskeletal protein talin. Eur. J. Biochem.

**224**, 951-957

26    Schmidt, J. M., Zhang, J., Lee, H. S., Stromer, M. H. and Robson, R. M. (1999)

Interaction of talin with actin: sensitive modulation of filament crosslinking activity.

Arch. Biochem. Biophys. **366**, 139-150

27    Luo, G., Herrera, A. H. and Horowits, R. (1999) Molecular interactions of N-RAP, a

nebulin-related protein of striated muscle myotendon junctions and intercalated disks.

Biochemistry **38**, 6135-6143

28    Goldmann, W. H., Bremer, A., Haner, M., Aebi, U. and Isenberg, G. (1994) Native

talin is a dumbbell-shaped homodimer when it interacts with actin. J. Struct. Biol.

**112**, 3-10

29    Isenberg, G. and Goldmann, W. H. (1998) Peptide-specific antibodies localize the

major lipid binding sites of talin dimers to oppositely arranged N-terminal 47 kDa

subdomains. FEBS Lett. **426**, 165-170

30    Muguruma, M., Matsumura, S. and Fukazawa, T. (1992) Augmentation of alpha-

actinin-induced gelation of actin by talin. J. Biol. Chem. **267**, 5621-5624

31    Goldmann, W. H., Hess, D. and Isenberg, G. (1999) The effect of intact talin and talin tail fragment on actin filament dynamics and structure depends on pH and ionic strength. Eur. J. Biochem. **260**, 439-445

32    Schmidt, J. M., Robson, R. M., Zhang, J. and Stromer, M. H. (1993) The marked pH dependence of the talin-actin interaction. Biochem. Biophys. Res. Commun. **197**, 660-666

33    Zhang, J., Robson, R. M., Schmidt, J. M. and Stromer, M. H. (1996) Talin can crosslink actin filaments into both networks and bundles. Biochem. Biophys. Res. Commun. **218**, 530-537

34    Hemmings, L., Rees, D. J., Ohanian, V., Bolton, S. J., Gilmore, A. P., Patel, B., Priddle, H., Trevithick, J. E., Hynes, R. O. and Critchley, D. R. (1996) Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. J. Cell Sci. **109**, 2715-2726

35    McCann, R. O. and Craig, S. W. (1997) The I/LWEQ module: a conserved sequence that signifies F-actin binding in functionally diverse proteins from yeast to mammals. Proc. Natl. Acad. Sci. U S A **94**, 5679-5684

36    McCann, R. O. and Craig, S. W. (1999) Functional genomic analysis reveals the utility of the I/LWEQ module as a predictor of protein:actin interaction. Biochem. Biophys. Res. Commun. **266**, 135-140

37    Beckerle, M. C., O'Halloran, T. and Burridge, K. (1986) Demonstration of a relationship between talin and P235, a major substrate of the calcium-dependent protease in platelets. J. Cell Biochem. **30**, 259-270

38    Muguruma, M., Nishimuta, S., Tomisaka, Y., Ito, T. and Matsumura, S. (1995)

Organization of the functional domains in membrane cytoskeletal protein talin. J.

Biochem. (Tokyo) 117, 1036-1042

39    Carroll, C. W., Altman, R., Schieltz, D., Yates, J. R. and Kellogg, D. (1998) The

septins are required for the mitosis-specific activation of the Gin4 kinase. J. Cell Biol.

143, 709-717

40    Edmunds, T., Nagainis, P. A., Sathe, S. K., Thompson, V. F., and Doll, D. E. (1991)

Comparison of the autolyzed and unautolyzed forms of mu- and m-calpain from

bovine skeletal muscle. Biochim. Biophys. Acta .1077, 197-208

41    Spudich, J. A. and Watt, S. (1971) The regulation of rabbit skeletal muscle

contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin

complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. 246,

4866-4871

42    Hemmings, L., Kuhlman, P. A. and Critchley, D. R. (1992) Analysis of the actin-

binding domain of alpha-actinin by mutagenesis and demonstration that dystrophin

contains a functionally homologous domain. J. Cell Biol. 116, 1369-1380

43    Rees, D. J., Ades, S. E., Singer, S. J. and Hynes, R. O. (1990) Sequence and domain

structure of talin. Nature 347, 685-689

44    Bretscher, A., Chambers, D., Nguyen, R. and Reczek, D. (2000) ERM-Merlin and

EBP50 protein families in plasma membrane organization and function. Annu. Rev.

Cell Dev. Biol. 16, 113-143

45    Hamada, K., Shimizu, T., Matsui, T., Tsukita, S. and Hakoshima, T. (2000) Structural

basis of the membrane-targeting and unmasking mechanisms of the radixin FERM

domain. EMBO J. **19**, 4449-4462

46    Pearson, M. A., Reczek, D., Bretscher, A. and Karplus, P. A. (2000) Structure of the

ERM protein moesin reveals the FERM domain fold masked by an extended actin

binding tail domain. Cell **101**, 259-270

47    Han, B. G., Nunomura, W., Takakuwa, Y., Mohandas, N. and Jap, B. K. (2000)

Protein 4.1R core domain structure and insights into regulation of cytoskeletal

organization. Nat. Struct. Biol. **7**, 871-875

48    Roy, C., Martin, M. and Mangeat, P. (1997) A dual involvement of the amino-

terminal domain of ezrin in F- and G-actin binding. J. Biol. Chem. **272**, 20088-20095

49    Monkley, S. J., Pritchard, C. A. and Critchley, D. R. (2001) Analysis of the

mammalian talin2 gene tln2. Biochem. Biophys. Res. Commun. **286**, 880-885

50    Habazettl, J., Gondol, D., Wiltscheck, R., Otlewski, J., Schleicher, M. and Holak, T.

A. (1992) Structure of hisactophilin is similar to interleukin-1 beta and fibroblast

growth factor. Nature **359**, 855-858

51    Yan, B., Calderwood, D. A., Yaspan, B. and Ginsberg, M. H. (2001) Calpain

cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. J. Biol.

Chem. **276**, 28164-28170

52    Glading, A., Lauffenburger, D. A. and Wells, A. (2002) Cutting to the chase: calpain

proteases in cell motility. Trends Cell Biol. **12**, 46-54

53      Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y.,

        Liddington, R. C. and Ginsberg, M. H. (2002) The phosphotyrosine binding-like

        domain of talin activates integrins. J. Biol. Chem. 277, 21749-21758

54      Sato, N., Funayama, N., Nagafuchi, A., Yonemura, S. and Tsukita, S. (1992) A gene

        family consisting of ezrin, radixin and moesin. Its specific localization at actin

        filament/plasma membrane association sites. J. Cell Sci. 103, 131-143

55      Funayama, N., Nagafuchi, A., Sato, N. and Tsukita, S. (1991) Radixin is a novel

        member of the band 4.1 family. J. Cell Biol. 115, 1039-1048

56      Tsujioka, M., Machesky, L. M., Cole, S. L., Yahata, K. and Inouye, K. (1999) A

        unique talin homologue with a villin headpiece-like domain is required for

        multicellular morphogenesis in Dictyostelium. Curr. Biol. 9, 389-392

57      Moulder, G. L., Huang, M. M., Waterston, R. H. and Barstead, R. J. (1996) Talin

        requires beta-integrin, but not vinculin, for its assembly into focal adhesion-like

        structures in the nematode Caenorhabditis elegans. Mol. Biol. Cell 7, 1181-1193

## Footnotes

Abbreviations used: ABS, actin binding site; EGFP, enhanced green fluorescent protein; ERM, ezrin-radixin-moesin; FERM, band 4.1-ezrin-radixin-moesin; GST, glutathione $S$-transferase; PAGE, polyacrylamide gel electrophoresis; PTB, phosphotyrosine binding.

[2]H.-S. Lee and R. M. Robson, unpublished observations.

[3]Subdomain F3 described in Pearson et al. [46] is essentially equivalent to subdomain C described in Hamada et al. [45]. We have used the designation subdomain C throughout the remainder of this report to refer to this subdomain within the FERM domain of the talin head.

**Figure 1  GST-talin constructs used to identify the actin-binding site in the talin head**

The $NH_2$- and COOH-terminal amino acid residues of the talin polypeptides expressed as GST-fusion proteins are indicated. Each deletion mutant (0.2 mg/ml final concentration) was mixed with G-actin (0.5 mg/ml final concentration) in cosedimentation buffer, and actin polymerization was induced by addition of $MgCl_2$ (2 mM final concentration). Samples were incubated for 1 h at 25 °C, and centrifuged at 100,000 × g for 20 min. The actin binding ability of each mutant was analyzed by running supernatants and pellets on SDS-PAGE and quantified by densitometry of Coomasie blue stained bands. An indication of the amount of a given talin polypeptide that cosedimented with F-actin is indicated as follows: ++++ = over 90%; +++ = ~80%; ++ = ~50%; + = ~35%; --- = ~5%.

**Actin Binding**

| | | |
|---|---|---|
| 102 — 497 | ++++ |
| 201 — 497 | ++++ |
| 271 — 497 | ++++ |
| 295 — 497 | ++++ |
| 321 — 497 | - - - |
| 201 — 400 | ++++ |
| 201 — 365 | ++++ |
| 201 — 330 | ++++ |
| 201 — 320 | ++++ |
| 201 — 310 | +++ |
| 201 — 294 | + |
| 201 — 285 | ++ |
| 201 — 270 | - - - |

**Figure 2  Analysis of the ability of GST-talin head fusion proteins to cosediment with F-actin**

GST-talin fusion proteins (0.2 mg/ml) containing residues 201-270 (a, b), 201-320 (c, d), 321-497 (e, f) and 271-497 (g, h) were

mixed with G-actin (0.5 mg/ml) in cosedimentation buffer, and 2 mM $MgCl_2$ was added to polymerize G-actin. Samples were

incubated for 1 h at 25 °C, and centrifuged at 100,000 × g for 20 min. Supernatants (S) and pellets (P) resulting were analyzed by

SDS-PAGE (b, d, f ,h). Fusion proteins centrifuged in the absence of F-actin (a, c, e, g) as controls.

201-270    201-320        321-497    271-497

a   b    c   d       e   f    g   h

S P S P  S P S P     S P S P  S P S P

← Actin

← 271-497

← 321-497 →
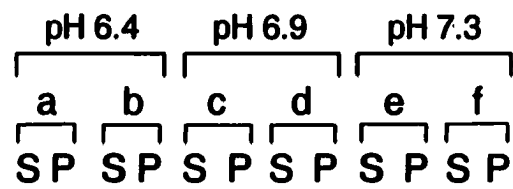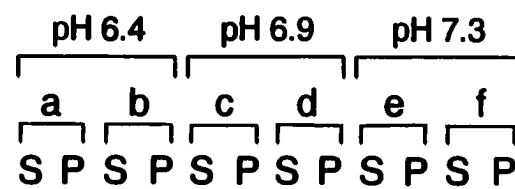
201-320

201-270 →

← Actin

**Figure 3  Effect of pH on the ability of GST-talin 201-320 and GST-talin 271-497 to cosediment with F-actin**

The ability of GST-fusion proteins containing talin residues 201-320 (A) and talin residues 271-497 (B) to cosediment with F-actin was assayed at the following final pH values: pH 6.4 (a, b), pH 6.9 (c, d) and pH 7.3 (e, f). Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. Fusion proteins centrifuged in the presence of F-actin (b, d, f). Fusion proteins centrifuged in the absence of F-actin as controls (a, c, e).

(A)                                              (B)

| pH 6.4 | pH 6.9 | pH 7.3 |
|---|---|---|
| a b | c d | e f |
| S P S P | S P S P | S P S P |

201-320→

271-497→

←Actin

←Actin

**Figure 4  Effect of pH on the ability of GST-talin 2269-2541 (ABS3) to cosediment with F-actin**

The ability of a GST-fusion protein containing talin residues 2269-2541 to cosediment with F-actin was assayed at the following final pH values: pH 6.4 (a, b), pH 6.9 (c, d) and pH 7.3 (e, f). Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. Fusion proteins centrifuged in the presence of F-actin (b, d, f). Fusion proteins centrifuged in the absence of F-actin as controls (a, c, e).

pH 6.4 | pH 6.9 | pH 7.3

| a | b | c | d | e | f |
| S P | S P | S P | S P | S P | S P |

ABS3 →
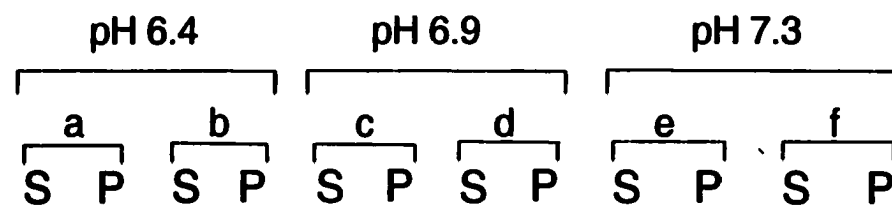
← Actin

**Figure 5  Effect of ionic strength on the ability of GST-talin 201-320 and GST-talin 271-497 to cosediment with F-actin**

The ability of GST-fusion proteins containing talin residues 201-320 (A) and 271-497 (B) to cosediment with F-actin at pH 6.4 in the presence of increasing concentrations of KCl was analyzed by SDS-PAGE. Supernatant (S) and pellet (P) of the fusion proteins centrifuged in the absence of F-actin, and without the addition of KCl (a); pellets (P) only of the GST-talin fusion protein/F-actin mixture in the presence of increasing concentrations of KCl (b). The addition of 25 mM KCl did not significantly decrease the ability of GST-talin 201-320 to cosediment with F-actin, whereas there was a 36% reduction in the amount of binding of GST-talin 271-497. The addition of 50 mM KCl decreased the ability of both fusion proteins to bind actin by approximately 50%. At 100 mM KCl, binding of GST-talin 201-320 and GST-talin 271-497 was reduced by 62% and 70%, respectively.
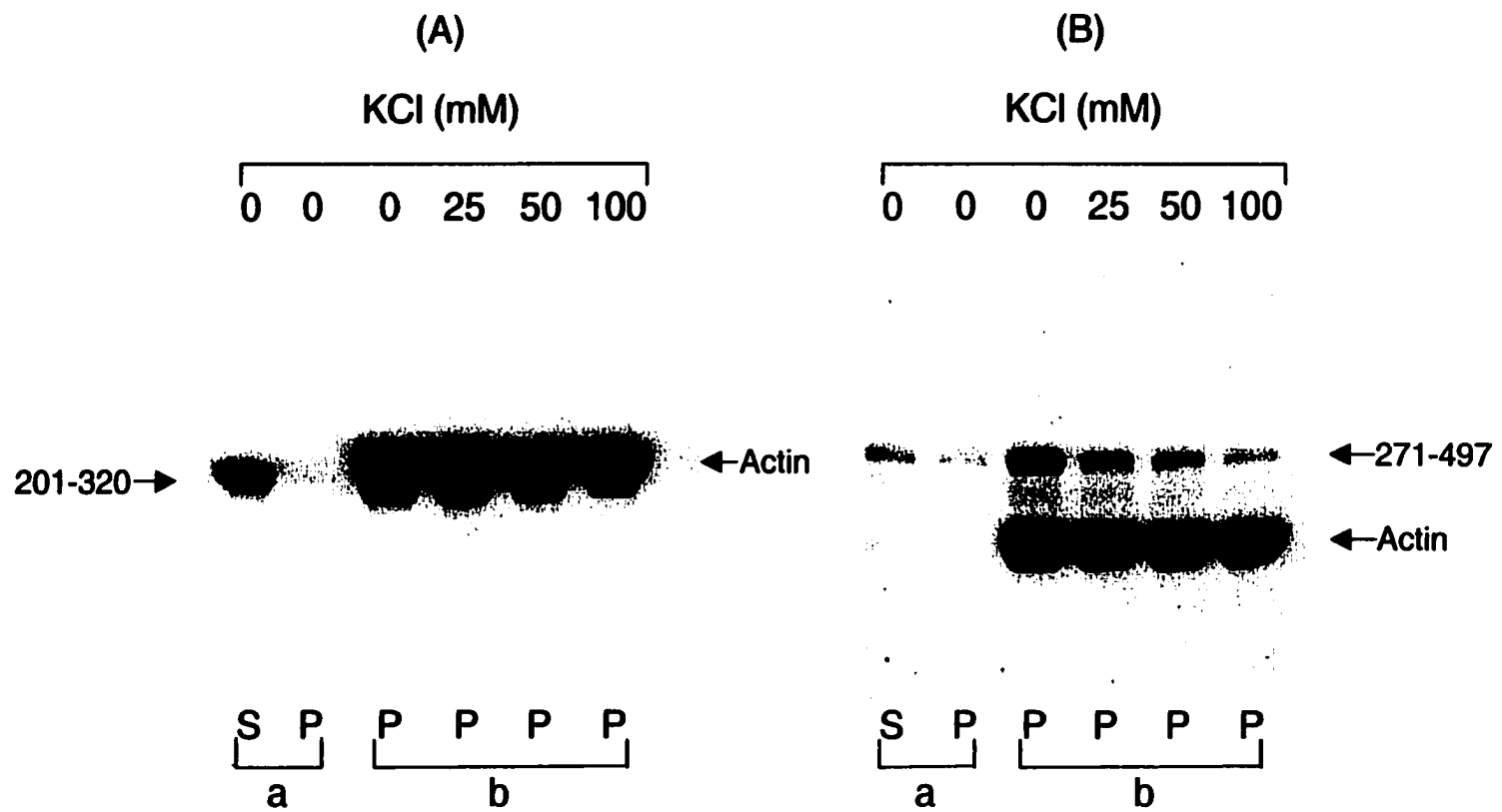
**Figure 6 Effect of ionic strength on the ability of GST-talin 2269-2541 (ABS3) to cosediment with F-actin**

The ability of a GST-fusion protein containing talin residues 2269-2541 to cosediment with F-actin at pH 6.4 in the presence of increasing concentrations of KCl was analyzed by SDS-PAGE. Supernatant (S) and pellet (P) of the fusion protein centrifuged in the absence of F-actin, and without the addition of KCl (a); pellets (P) only of the GST-talin fusion protein/F-actin mixture in the presence of increasing concentrations of KCl (b). The addition of 25 mM KCl significantly decreased the ability of GST-talin 2269-2541 to cosediment with F-actin by approximately 48 %. The addition of 50 mM and 100 mM KCl further decreased the ability of the fusion protein to bind actin by approximately 75%, and 87%, respectively.

KCl (mM)

| 0 | 0 | 0 | 25 | 50 | 100 |

ABS3 →

← Actin

| S | P | P | P | P | P |
| a | | b | | | |
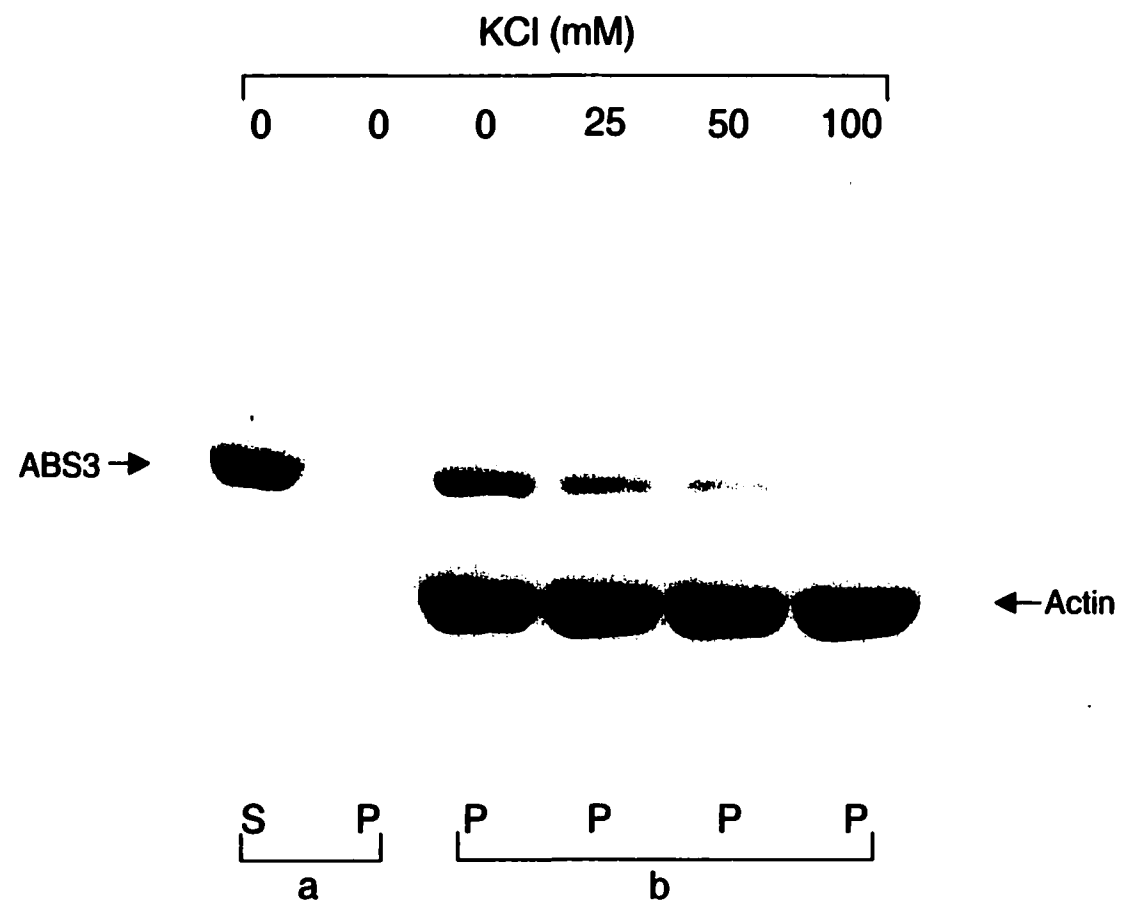
**Figure 7 Cosedimentation of the calpain-generated talin head fragment with F-actin**

The ability of talin, and of talin fragments generated by calpain cleavage, to cosediment with F-actin was assayed at pH 6.4.

Actin alone (a), 0.2 mg/ml of intact talin (b), calpain-digested talin (c), and purified N-terminal talin head fragment in the

absence (d) and presence (e) of F-actin. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE.

Talin→ ←—190 kDa fragment

Actin→ ←—47 kDa fragment
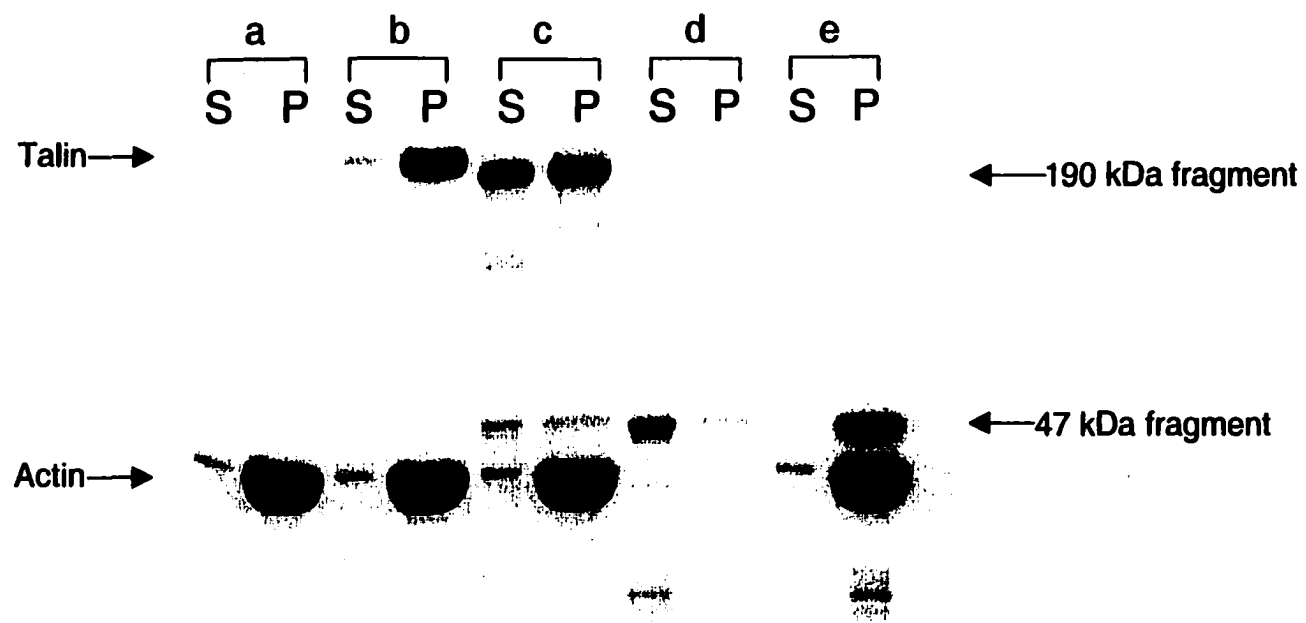
**Figure 8  Transient expression of EGFP-talin and EGFP-α-actinin fusion constructs in COS-7 cells**

EGFP-talin head constructs containing ABS1 talin residues 201-320 (A) and EGFP-talin head constructs lacking ABS1 talin residues 201-270 (B); EGFP-talin head constructs containing ABS1 talin residues 271-497 (C) and EGFP-talin head constructs lacking ABS1 talin residues 321-497 (D); EGFP-talin rod construct (residues 2269-2541) containing ABS3 (E); actin binding site (residues 1-269) of α-actinin (F). Cells were counter stained with rhodamine-phalloidin to visualize F-actin. Bar = 20 μm.

| EGFP | Phalloidin | | EGFP | Phalloidin |
|------|------------|---|------|------------|

A

B

C

D

E

F
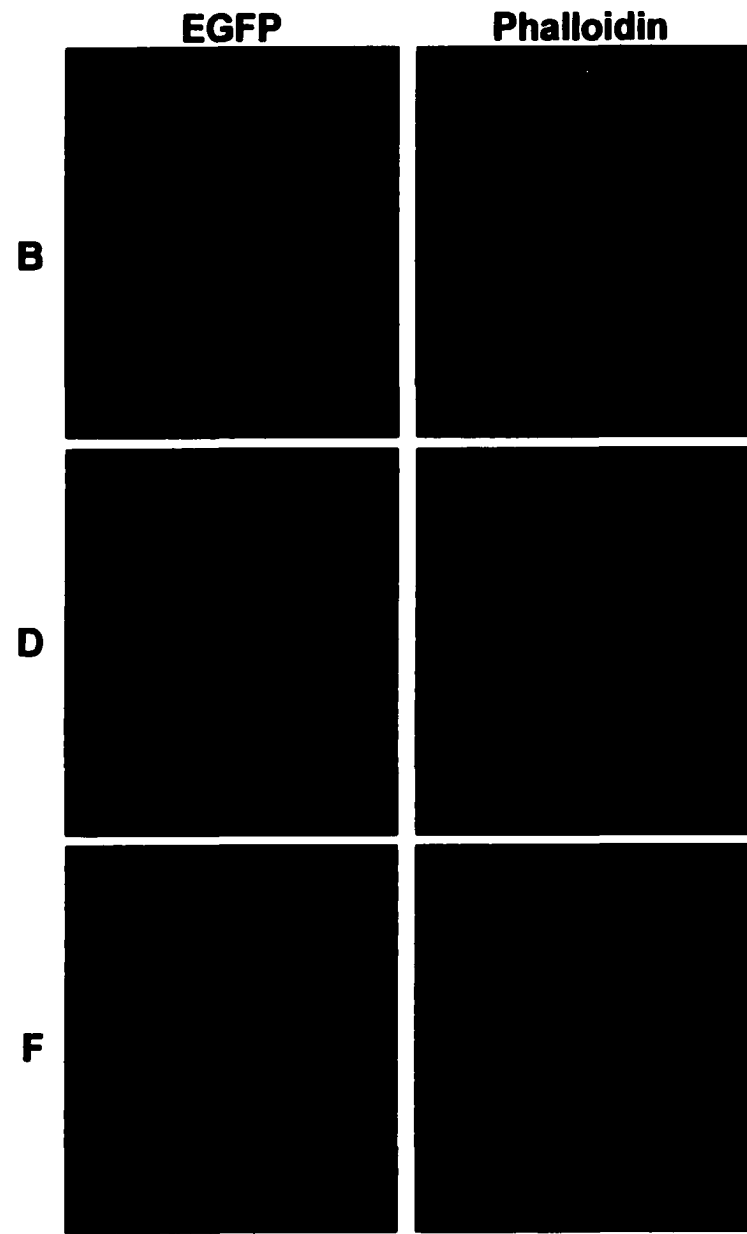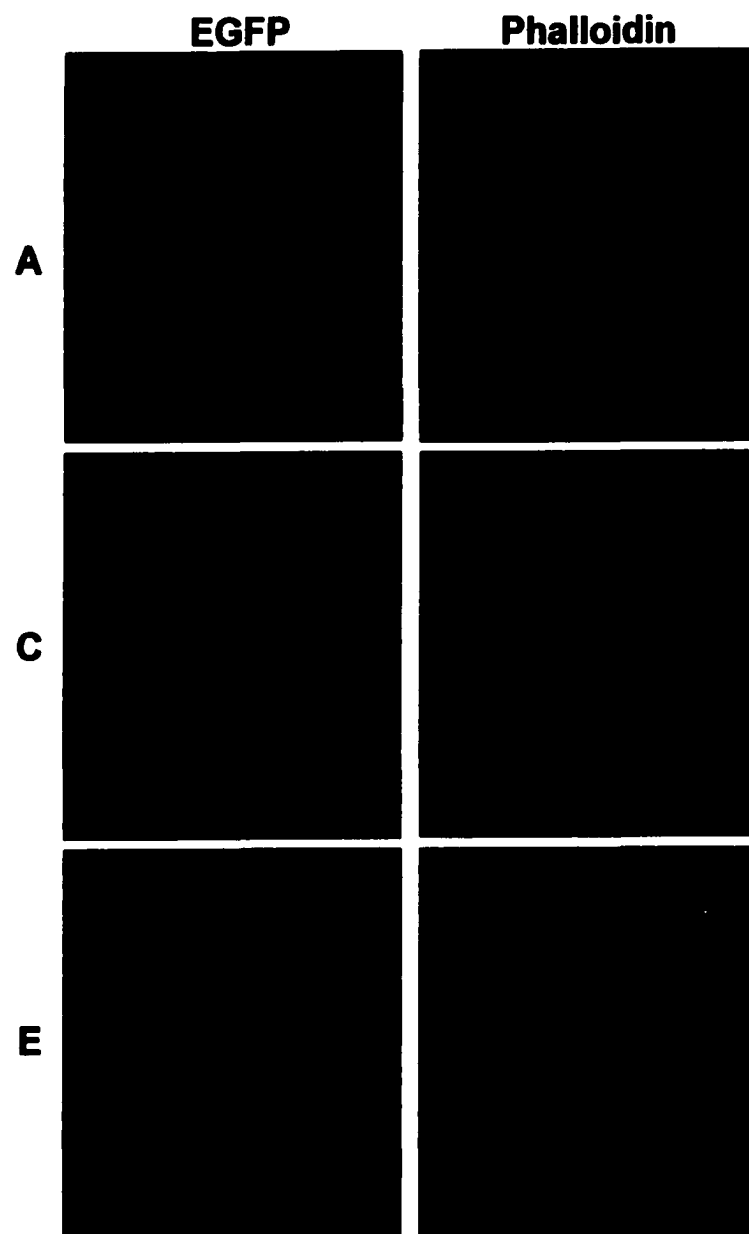
**Figure 9 Sequence alignment of talin ABS1 with ERM proteins and of talin across species** (A) The ABS1 sequence of chicken talin is compared with those of mouse ERM proteins including moesin [54], radixin [55] and ezrin [55]. The conserved sequences are aligned and identified by using the CLUSTALW and BOXSHADE program. (*) indicates identical residues in all sequences, (:) represents conserved substitutions, and (.) shows semi-conserved substitutions. (B) Talin sequences are from human (GenPept accession: Q9Y490), mouse [43], chicken [34], Drosophila (GenPept accession: AAF50399), *D. discoideum* [56] and *C. elegans* [57].

(A)

Core ABS 1

| | | | |
|---|---|---|---|
| TALIN | 271 | I QKG R F A KNCGN SEI K R RS KT S L E MK | 320 |
| MOESIN | 164 | N S | 213 |
| RADIXIN | 164 | N | 213 |
| EZRIN | 164 | A S | 213 |

::: * :* * : .* . .: :.*:*:*:.*: **:.:* :*:*

(B)

Core ABS 1

| | | | |
|---|---|---|---|
| HUMAN | 271 | - Q | 320 |
| MOUSE | 271 | - Q | 320 |
| CHICKEN | 271 | - M | 320 |
| DROSOPHILA | 279 | T I SE KHVD L I T E P N | 329 |
| DICTYOSTELIUM | 236 | H K DA D KEF KLTG SN F Q C T | 286 |
| C.ELEGANS | 301 | A EN VAMY ELSGT S C G P | 348 |

: * *:.: .:: ** ::* *.: .* * ***::**:.**::

# FURTHER CHARACTERIZATION OF TWO COOH-TERMINAL ACTIN-BINDING SITES AND AN ADDITIONAL MUTAGENESIS STUDY ON THE NH₂-TERMINAL ACTIN-BINDING SITE OF TALIN

An additional study to provide background for future research

Ho-Sup Lee

## Summary

Talin is one of the major components in cell-matrix type adherens junctions where it presumably interacts with actin via its three actin binding sites (ABS)[1], with one located in the 47 kDa N-terminal head (ABS1) and the other two (ABS2 and ABS3) in the 190 kDa C-terminal tail domain. Thus, talin is believed to help link the actin cytoskeleton to the plasma membrane. In the studies described herein, the characteristics of ABS2 and ABS3 were examined by using deletion analyses, actin cosedimentation, and cell transfection assays. The ABS2 and ABS3 were defined to residues 1051-1250 and residues 2331-2530, respectively. The ABS2 exhibits high dependence on pH and ionic strength, with much stronger binding to F-actin at lower pH and ionic strength, which is similar to the results previously obtained with ABS3 (Lee, H.-S., Bellin, R. M., Walker, D. L., Stromer, M. H., Critchley, D. R., and Robson, R. M. 2002, manuscript in preparation). Results of COS cell transfections with EGFP-fusion constructs showed that ABS2 is primarily colocalized with actin stress fibers

---

[1]The abbreviations used are: ABS, actin binding site; ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N, N',N'-tetraacetic acid; GST, glutathione S-transferase; MCE, β-mercaptoethanol.

whereas ABS3 is predominantly colocalized with actin-rich membrane ruffles.

As shown earlier in this dissertation, the actin binding activity of the N-terminal head of talin is contained within residues 271-320 (ABS1). To identify determinants involved in the actin binding, I performed *in vitro* site-directed mutagenesis on specific amino acid residues within ABS1. I have found that substitution of alanine for the amino acid residues 295-306 within the ABS1 core resulted in marked reduction in actin binding, suggesting that these residues are potentially major determinants for interaction of ABS1 with actin.

## Introduction

Cell-matrix type adherens junctions are sites of tight adhesion of cells to their surrounding extracellular matrix (ECM), and are important for many biological events such as cell growth, motility, differentiation and survival [1-3]. Talin is one of the major structural proteins primarily localized in these cell-matrix type adherens junctions, and is believed to play a critical role in linking the actin cytoskeleton to the plasma membrane by interacting with actin and with the cytoplasmic domains of transmembrane proteins [4,5] such as the integrins [6] and layilin [7, 8]. Several lines of study, including the downregulation of talin by anti-sense RNA technology [9] and microinjection of talin antibodies into fibroblasts [10], have shown that talin is one of the key links between the ECM and the actin cytoskeleton. Talin also has been shown to be important for the formation and extension of filopodia and lamellipodia [11, 12], and it is essential for embryonic tissue morphogenesis [13].

Talin is a high molecular weight (~ 230 kDa by SDS-PAGE) cytoskeletal protein and exists as an antiparallel homodimer [14, 15] with a flexible-like conformation [16, 17]. Talin is composed of a globular ~47 kDa N-terminal head and an elongated ~190 kDa C-terminal

tail, which are obtained by m-calpain proteolytic cleavage [18]. The N-terminal head domain

shares homology with the FERM domain (band 4.1-ezrin-radixin-moesin) of members of the

ERM protein family, which have been shown to function as cytoskeleton-membrane cross-

linkers [19, 20]. The FERM domain of talin contains the binding sites for most of the

proteins that bind to the talin head, including integrins [6, 21], layilin [7, 8], focal adhesion

kinase (FAK) [22], and actin [23]. It also contains the binding sites for phospholipids [24,

25]. The C-terminal tail domain of talin has binding sites for vinculin [26-28], actin and

integrin [21]. The talin molecule contains three actin-binding sites, with one (ABS1) in the

N-terminal head, and two (ABS2 and 3) located in the C-terminal tail [10, 23]. A new actin

binding module (I/LWEQ), which is highly conserved across species, was recently found in

the ABS3 [29, 30]. A biophysical analysis of the talin-actin interaction from another lab [31]

identified actin binding activity in the tail domain, but not in the head domain of talin. We,

however, have shown that the purified N-terminal head from calpain-digested talin is able to

bind actin, and that 50 amino acid residues (271-320) within the talin head are responsible for

the binding [32].

In an effort to examine the roles played by the C-terminal tail of talin in interaction

with actin at cell-matrix junctions, I have further defined ABS2 and ABS3, and have

compared the characteristics of ABS2 with those of ABS3 by using actin cosedimentation

and cell transfection assays. I have also employed *in vitro* site-directed mutagenesis to

identify some of the determinants of ABS1 within the talin head that may be critical for the

interaction of ABS1 with actin.

## Materials and Methods

*GST talin fusion constructs and expression in E. coli* - GST talin constructs were made by amplifying the corresponding chicken talin cDNA using PCR, and cloning the PCR products into glutathione S-transferase (GST) fusion vector, pEGX4T1 (Amersham Biosciences, Piscataway, NJ). The talin constructs were expressed in *E. coli* (BL21-codon⁻-RIL, Stratagene, La Jolla, CA), and purified as described [33] with affinity column chromatography on glutathione-agarose beads.

*Actin Cosedimentation Assay* - Cosedimentation of fusion proteins with F-actin was performed in cosedimentation buffer (10 mM imidazole-HCl, pH 6.4, 1 mM ATP, 1 mM MCE, 1 mM EGTA, 0.1 mM $CaCl_2$) as described [34]. G-actin was prepared from porcine skeletal muscle as described [35]. Each fusion protein (0.2 mg/ml) and G-actin (0.5 mg/ml) were mixed in the cosedimentation buffer at the selected pH. Actin polymerization was induced by the addition of 2 mM $MgCl_2$ (final concentration). The reaction mixtures were incubated for 1 hr at 25°C, and centrifuged at 100,000 × g for 20 min in a Beckman airfuge. The resulting supernatants and pellets were analyzed by SDS-PAGE. The density of the band in Coomassie brilliant blue stained gels was determined by using the Scion Image program (Scion Corporation, Frederick, MD).

*EGFP talin fusion constructs and transient expression in COS Cells* – The designated regions of talin were amplified by PCR using a chicken talin cDNA as a template, and the PCR products were subcloned into the green fluorescent protein fusion vector, pEGFPC2 (Clontech, Palo Alto, CA), which has been modified in its restriction cleavage sites in order to clone the PCR products in proper orientation. The plasmid DNAs for transfection were prepared by using Endofree Plasmid Maxi Kit (Qiagen, Valencia, CA). The transfections into

COS cells were performed by using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN) as described [32]. The COS cells were fixed with 4% paraformaldehyde for 20 min at 25°C, and permeabilized with 0.1 % Triton X-100 in fixative for 5 min. F-actin in the cells was visualized by staining with rhodamine-phalloidin for 1 hr at 37°C. Samples were then washed with PBS three times, and then mounted with Gelvatol (Air Products and Chemicals, Inc., Allentown, PA). The cells were viewed with a Zeiss phase/epifluorescence Photomicroscope III, and photographed with a SPOT RT camera and software (Diagnostic Instruments, Inc.).

*In vitro site-directed mutagenesis* – The recombinant plasmid containing chicken talin cDNA corresponding to residues 196-400 cloned within the histidine-tag fusion vector, pET-15b (Novagen, Madison, WI) was obtained for use as a mutagenesis template from Dr. Robert C. Liddington (Burnham Institute, CA). The mutations of selected amino acid residues of ABS1 (within residues 271-320) to alanine were introduced by using the PCR-based QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The DNA template was amplified with the mutagenic primers by PCR, and the PCR products were then treated for 1 hr at 37°C with DpnI in order to digest non-mutated parental DNA template. The digest was then mixed with competent cells to transform *E. coli* (XL-1 Blue, Stratagene, La Jolla, CA). The DNA sequences and the reading frames of all mutant plasmids were confirmed by DNA sequencing. The mutated plasmids were then expressed in *E. coli* (BL21-Codon$^+$-RIL, Stratagene, La Jolla, CA), and the his-tagged proteins were purified by chromatography on $Ni^{2+}$ matrix (Qiagen, Valencia, CA).

## Results

*Definition of ABS2 and ABS3 within the C-terminal tail domain of talin* – An initial study including the presence of ABS2 and ABS3 within the C-terminal tail of talin was reported from the lab of Dr. David R. Critchley [23]. To further define and characterize ABS2 and ABS3, I generated a deletion series of GST- talin tail fusion proteins, and tested their ability to bind actin by using an actin cosedimentation assay. The optimal conditions (pH 6.4 and low ionic strength) found for the intact talin-actin interaction [34] were used.

The deletion of ABS2 (residues 951-1327) from the N-terminus to residue 986 resulted in the loss of ~30% of the actin binding activity (Fig. 1). Removal of residues 986 to 1050 showed no further effect. A little over 30% of the GST-fusion protein containing residues 1051-1327 cosedimented with F-actin. Further deletion of only 10 residues resulted in a dramatic loss of actin binding (Fig. 1), with ~95% of the talin polypeptide containing residues 1061-1327 remaining in the supernatant. Deletion from the C-terminus to residue 1250 showed some reduction (~30%) in actin binding activity, and further deletion of 20 amino acid residues (i.e., construct containing 951-1230) almost abolished the ability to bind actin. About 94% of the fusion protein containing residues 951-1230 remained in the supernatant. These preliminary results indicate that much of the actin binding activity of ABS2 is located within residues 1051-1250, although some appears to reside in residues 951-986 and 1251-1327, respectively. Approximately ~39% of the fusion protein containing residues 1051-1250 cosedimented with F-actin (Fig. 2). Two short regions of sequence (residues 1051-1060 and residues 1231-1251) were found to be quite important in actin binding, and the regions containing residues 951-985 and 1251-1327 also appeared to be important.

Progressive deletion from the N-terminus of ABS3 (residues 2269-2541) up to residue 2331 showed little decrease in actin binding (Fig. 3). Further deletions resulted in a dramatic reduction in protein stability (i.e., these expressed proteins were insoluble), which made it difficult to examine the effects of the deletions. Expressed construct 2464-2541 was soluble and exhibited some actin binding ability. The deletion from the C-terminus of ABS3 to residue 2530 did not greatly affect the ability of the fusion protein to bind actin. Further deletion of 50 amino acid residues resulted in a major decrease in ability of the fusion protein (residues 2306-2480) to bind to actin. These preliminary results indicate that residues within 2331-2530 are important for the actin binding activity of ABS3, with more than 90% of the fusion protein containing residues 2331-2530 cosedimented with F-actin (Fig. 4).

*Effect of pH and ionic strength on the interaction between ABS2 and F-actin* – Talin has been shown to be highly sensitive to pH and ionic strength in interaction with actin, with optimal binding at low pH (~6.4) and ionic strength [34, 36]. We have previously shown that both ABS1 and ABS3 are very dependent on ionic strength, and that ABS1 is much less dependent on pH than is ABS3, with the latter exhibiting much stronger binding to actin at low pH 6.4 [32]. Therefore, I investigated the effect of pH and ionic strength on the ability of ABS2 to bind actin. The results in Fig. 5 show that the progressive decrease in pH from 7.3 to 6.4 significantly enhanced the ability of ABS2 to bind F-actin. Approximately 80% of ABS2 (residues 951-1327) cosedimented with F-actin at pH 6.4, whereas ~62% and ~47 % of ABS2 cosedimented, respectively, at pH 6.9 and 7.3.

The results in Fig. 6 show that the progressive increase in the concentration of KCl to 100 mM significantly decreased the ability of ABS2 to cosediment with F-actin. The addition of 25 mM KCl decreased by ~41% the amount of ABS2 that cosedimented with F-actin.

Approximately 32% and 19% of the amount of ABS2 that cosedimented (in comparison to no KCl added) was reduced at the KCl concentrations of 50 mM and 100 mM, respectively. These results suggest that the electrostatic/ionic interactions are involved in the ABS2/actin interaction, which is similar to the characteristics of ABS3.

*Localization of EGFP-tagged ABS2 and ABS3 with F-actin in COS cells* – I transfected and transiently expressed the EGFP-fusion constructs of ABS2 (residues 951-1327) and ABS3 (residues 2269-2541) individually into COS cells to investigate the interactions of the two ABSs with F-actin in cells. Fluorescence microscope observations of COS cells transfected with EGFP-fusion ABS2 showed that ABS2 was primarily colocalized with actin stress fibers, with some localization of the cortical actin (Fig. 7A). Diffuse EGFP fluorescence was also observed in the cytoplasm because of high expression of the fusion protein. The COS cells transfected with EGFP-fusion ABS3 construct exhibited strong EGFP fluorescence colocalized primarily with actin-rich membrane ruffles (Fig. 7B), and much weaker colocalization with actin stress fibers. These results indicate different localizations between the ABS2 and the ABS3 in the actin-rich structures of COS cells.

*In vitro site-directed mutagenesis of ABS1* – We have previously shown that the actin binding activity of the talin head is contained within talin residues 271-320 [32]. To begin to determine the amino acid residues essential for actin binding, I systematically mutated specific amino acid residues located within ABS1 to alanine by using PCR-based *in vitro* mutagenesis. Alanine, a small amino acid, is located in both buried (interior) and exposed (exterior) positions in proteins, although it is more often found exposed at the surface of proteins. Protein folding is generally believed to be only minimally affected by the alanine substitution [37, 38, 45]. I focused on regions containing the charged residues because ABS1

is highly basic in nature (pI ~10.15), which may be important in the interaction with actin. I generally mutated from two to five residues at a time to increase the likelihood of identifying important determinants in binding to actin. Some hydrophobic residues in one of the mutants ($K^{295}V^{296}R^{297}Y^{298}V^{299}K^{300}$) in Table 1 were also substituted with alanine. The preliminary results presented in Table 1 show that mutations of a segment containing residues within 295-306 resulted in a significant reduction of actin binding ability. Two of the eight mutants showed a marked decrease in binding to F-actin, although a portion of the two mutants precipitated by themselves in the cosedimentation buffer. Mutation of positively charged residues between 295 and 306 significantly decreased the ability of the mutant to bind actin. Mutation of all of the residues between 295-300 to alanine also significantly reduced actin binding. Residues $R^{297}$ and $K^{300}$, however, were found not to be absolutely essential for the actin binding because the mutant containing alanine substituted for $K^{278}M^{281}N^{285}E^{293}R^{297}K^{300}$ cosedimented with F-actin. These very preliminary results suggest that the charged residues, such as $K^{295}$, $R^{303}$ and $K^{306}$, may be potential determinants involved in interaction of ABS1 with actin.

**Discussion**

We have previously demonstrated that ABS1 within the N-terminal head of talin can be defined to residues 271-320, and that the biochemical and cellular characteristics of ABS1 are distinct from those of ABS3, which is located near the end of the C-terminus [32]. Talin possesses yet another ABS (ABS2) within the C-terminal tail [23], which has not previously been further characterized with regard to its interaction with actin. To initiate studies examining properties of the C-terminal talin tail/actin interaction at cell-matrix type

junctions, I have further defined the amino acid residues in ABS2 and ABS3, and have compared the actin binding properties of the ABS2 with those of the ABS3 by actin cosedimentation assays and by transient cell transfection.

My deletion analysis of ABS2 showed that the region containing residues 1051-1250 is minimal for significant binding to actin. Deletion of internal residues 1091-1097 in ABS2 also resulted in the loss of actin binding activity (data not shown). These results suggest that the actin binding domain in ABS2, which contains 200 amino acid residues, is not comprised of a simple linear peptide. The higher order protein conformation (i.e., secondary and tertiary) of ABS2 may consist of a native folded structure that is important for interaction with F-actin.

The ABS3 was further defined to residues 2331-2530. The ABS3 has been reported to contain four blocks (residues 2344-2368, 2388-2410, 2421-2444 and 2500-2528) of a novel actin binding module (I/LWEQ) [29]. The construct I made containing residues 2464-2541, which includes only the fourth block of the module, showed some weak actin binding activity. In contrast, the construct containing residues 2306-2480, which contains the first three of the four actin binding modules exhibited very low actin binding activity, suggesting the fourth block, which has higher affinity for actin than the other three blocks, might play a major role in binding to actin.

Sequence alignment of ABS2 and of ABS3 with talin proteins across species shows that ABS2 and ABS3 are highly conserved among chicken, mouse, and human to a similar degree (~75% identity for ABS2 and ~78% identity for ABS3) (data not shown). However, ABS3 is much more conserved in lower organisms such as *Drosophila, C. elegans* and

*Dictyostelium* than is ABS2. Both ABS2 and ABS3 are, overall, less conserved than ABS1 in talin molecules across species.

The study of COS cell transfections with EGFP-fusion constructs containing the two actin binding sites showed that ABS2 is primarily colocalized with actin stress fibers within the cytoplasm, whereas ABS3 is primarily colocalized with actin filaments at the membrane ruffles. The localization of ABS3 at the membrane ruffles, together with the fact that the ABS3 shares homology with the yeast protein Sla2p that is involved in assembly of cortical actin cytoskeleton [39], suggest that ABS3 may be important in the assembly of cortical actin filaments and focal adhesions.

We have previously found that ABS3, in contrast to ABS1, is highly sensitive to pH and ionic strength in interacting with actin [32]. The ability of native talin to crosslink actin filaments has been shown to be highly dependent on both pH and ionic strength [34, 40]. The purified C-terminal tail of talin was demonstrated to crosslink actin filaments, but at a pH lower than was optimal for intact talin [34]. The high dependence on pH and ionic strength of both ABS2 and ABS3 suggest that these two actin binding sites in the large C-terminal talin tail domain may be involved in crosslinking actin filaments. The EGFP fluorescence of ABS3 in COS cells indicated it more strongly colocalized with actin filaments than did ABS2, suggesting that ABS3 within the antiparallel talin dimer may play a larger role in crosslinking actin filaments. The localized pH in the leading edge of moving cells is increased due to the activation of the $Na^+/H^-$ exchanger, which is involved in cell migration and the assembly of focal adhesions [41]. Based upon my findings that increased pH significantly decreases ability of ABS2 and ABS3 to crosslink actin filaments, whereas the transmembrane proteins such as the integrins and layilin may still bind to actin via the more

pH-independent ABS1 within the talin FERM domain, raises the possibility that ABS1 may function to permit cells to remodel cortical actin filaments during cell migration.

In the previous chapter herein, we have shown that residues 271-320 within the N-terminal head of talin are responsible for ability of the head domain to bind to actin [32]. To more precisely identify the determinants involved in binding to actin, I have initiated experiments in which I have employed in vitro site-directed mutagenesis of specific amino acid residues within ABS1. Results of my preliminary study show that mutation within the region containing amino acid residues 295-306, which are located within the core ABS1 region, resulted in significant reduction in binding to F-actin. These results suggest that those residues are possible determinants important in the interaction of ABS1 with actin. Sequence comparisons to the FERM domains of the ERM protein family, for which X-ray crystal protein structures have been published [42-44], show that the residues 295-306 are located primarily in the fourth $\alpha$-helix within subdomain B (nomenclature in [42]) and in the first half of the linker region between subdomains B and C. This region contains five positively charged residues and four hydrophobic residues. The residues $R^{297}$ and $K^{300}$ do not appear to be essential for the binding to actin because alanine substitution of residues $K^{278}M^{281}N^{285}E^{293}R^{297}K^{300}$ resulted in little effect on the ability of the mutant to bind actin (Table 1). Residues $K^{295}$, $Y^{298}$, $R^{303}$ and $L^{305}$ are identical within the talin molecules from *C. elegans* to human. Further mutations of those four residues may provide us better insight into the critical determinants of ABS1 involved in the interaction with actin. Because protein-protein interactions are known to often include more than one determinant (amino acid residue) in contributing to the interactions [37, 38], a combination of charged and hydrophobic amino acid residues within the region may be required for binding to actin.

Small amounts of these two mutants, which had reduced ability to bind to F-actin, precipitated in the cosedimentation buffer suggesting that the protein folding of these two mutants was altered. However, the expression level of these mutants in *E. coli* was similar to that of wild type proteins, which suggests that structural integrity of the two mutants may not have been affected by alanine substitution because misfolded proteins are usually poorly expressed [45]. Clearly, a much more detailed and comprehensive study involving site-directed mutagenesis will be required to elucidate the nature of the ABS1/actin interaction.

## References

1    Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. Annu. Rev. Cell Dev. Biol. **12**, 463-518

2    Geiger, B., Bershadsky, A., Pankov, R. and Yamada, K. M. (2001) Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. Nat. Rev. Mol. Cell Biol. **2**, 793-805

3    Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G. and Winkler, J. (1995) The molecular architecture of focal adhesions. Annu. Rev. Cell Dev. Biol. **11**, 379-416

4    Critchley, D. R. (2000) Focal adhesions - the cytoskeletal connection. Curr. Opin. Cell Biol. **12**, 133-139

5    Lee, H.-S., Bellin, R. M., and Robson, R. M. (2001) in Wiley Encyclopedia of Molecular Medicine, Vol. **5**, 3087-3089, John Wiley & Sons, Inc., New York

6    Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O. and Ginsberg, M. H. (1999) The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. J. Biol. Chem. **274**, 28071-28074

7    Bono, P., Rubin, K., Higgins, J. M. and Hynes, R. O. (2001) Layilin, a novel integral membrane protein, is a hyaluronan receptor. Mol. Biol. Cell **12**, 891-900

8    Borowsky, M. L. and Hynes, R. O. (1998) Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. J. Cell Biol. **143**, 429-442

9       Albiges-Rizo, C., Frachet, P. and Block, M. R. (1995) Down regulation of talin alters cell adhesion and the processing of the alpha 5 beta 1 integrin. J. Cell Sci. **108**, 3317-3329

10      Bolton, S. J., Barry, S. T., Mosley, H., Patel, B., Jockusch, B. M., Wilkinson, J. M. and Critchley, D. R. (1997) Monoclonal antibodies recognizing the N- and C-terminal regions of talin disrupt actin stress fibers when microinjected into human fibroblasts. Cell Motil. Cytoskeleton **36**, 363-376

11      Niewohner, J., Weber, I., Maniak, M., Muller-Taubenberger, A. and Gerisch, G. (1997) Talin-null cells of Dictyostelium are strongly defective in adhesion to particle and substrate surfaces and slightly impaired in cytokinesis. J. Cell Biol. **138**, 349-361

12      Sydor, A. M., Su, A. L., Wang, F. S., Xu, A. and Jay, D. G. (1996) Talin and vinculin play distinct roles in filopodial motility in the neuronal growth cone. J. Cell Biol. **134**, 1197-1207

13      Monkley, S. J., Zhou, X. H., Kinston, S. J., Giblett, S. M., Hemmings, L., Priddle, H., Brown, J. E., Pritchard, C. A., Critchley, D. R. and Fassler, R. (2000) Disruption of the talin gene arrests mouse development at the gastrulation stage. Dev. Dyn. **219**, 560-574

14      Isenberg, G. and Goldmann, W. H. (1998) Peptide-specific antibodies localize the major lipid binding sites of talin dimers to oppositely arranged N-terminal 47 kDa subdomains. FEBS Lett. **426**, 165-170

15      Goldmann, W. H., Bremer, A., Haner, M., Aebi, U. and Isenberg, G. (1994) Native talin is a dumbbell-shaped homodimer when it interacts with actin. J. Struct. Biol. **112**, 3-10

16      Winkler, J., Lunsdorf, H. and Jockusch, B. M. (1997) Energy-filtered electron microscopy reveals that talin is a highly flexible protein composed of a series of globular domains. Eur. J. Biochem. **243**, 430-436

17      Molony, L., McCaslin, D., Abernethy, J., Paschal, B. and Burridge, K. (1987) Properties of talin from chicken gizzard smooth muscle. J. Biol. Chem. **262**, 7790-7795

18      Beckerle, M. C., O'Halloran, T. and Burridge, K. (1986) Demonstration of a relationship between talin and P235, a major substrate of the calcium-dependent protease in platelets. J. Cell Biochem. **30**, 259-270

19      Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N., Chasis, J. A., Conboy, J. G.,

Gascard, P., Takakuwa, Y., Huang, S. C., Benz, E. J., Jr., Bretscher, A., Fehon, R. G., Gusella, J. F., Ramesh, V., Solomon, F., Marchesi, V. T., Tsukita, S., Hoover, K. B. and et al. (1998) The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. Trends Biochem. Sci. 23, 281-282

20    Bretscher, A., Chambers, D., Nguyen, R. and Reczek, D. (2000) ERM-Merlin and EBP50 protein families in plasma membrane organization and function. Annu. Rev. Cell Dev. Biol. 16, 113-143

21    Xing, B., Jedsadayanmata, A. and Lam, S. C. (2001) Localization of an integrin binding site to the C terminus of talin. J. Biol. Chem. 276, 44373-44378

22    Chen, H. C., Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D. and Guan, J. L. (1995) Interaction of focal adhesion kinase with cytoskeletal protein talin. J. Biol. Chem. 270, 16995-16999

23    Hemmings, L., Rees, D. J., Ohanian, V., Bolton, S. J., Gilmore, A. P., Patel, B., Priddle, H., Trevithick, J. E., Hynes, R. O. and Critchley, D. R. (1996) Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. J. Cell Sci. 109, 2715-2726

24    Tempel, M., Goldmann, W. H., Isenberg, G. and Sackmann, E. (1995) Interaction of the 47-kDa talin fragment and the 32-kDa vinculin fragment with acidic phospholipids: a computer analysis. Biophys. J. 69, 228-241

25    Seelig, A., Blatter, X. L., Frentzel, A. and Isenberg, G. (2000) Phospholipid binding of synthetic talin peptides provides evidence for an intrinsic membrane anchor of talin. J. Biol. Chem. 275, 17954-17961

26    Gilmore, A. P., Wood, C., Ohanian, V., Jackson, P., Patel, B., Rees, D. J., Hynes, R. O. and Critchley, D. R. (1993) The cytoskeletal protein talin contains at least two distinct vinculin binding domains. J. Cell Biol. 122, 337-347

27    Bass, M. D., Patel, B., Barsukov, I. G., Fillingham, I. J., Mason, R., Smith, B. J., Bagshaw, C. R. and Critchley, D. R. (2002) Further characterization of the interaction between the cytoskeletal proteins talin and vinculin. Biochem. J. 362, 761-768

28    Bass, M. D., Smith, B. J., Prigent, S. A. and Critchley, D. R. (1999) Talin contains three similar vinculin-binding sites predicted to form an amphipathic helix. Biochem. J. 341, 257-263

29    McCann, R. O. and Craig, S. W. (1997) The I/LWEQ module: a conserved sequence that signifies F-actin binding in functionally diverse proteins from yeast to mammals. Proc. Natl. Acad. Sci. U S A 94, 5679-5684

30    McCann, R. O. and Craig, S. W. (1999) Functional genomic analysis reveals the utility of the I/LWEQ module as a predictor of protein:actin interaction. Biochem. Biophys. Res. Commun. **266**, 135-140

31    Goldmann, W. H., Hess, D. and Isenberg, G. (1999) The effect of intact talin and talin tail fragment on actin filament dynamics and structure depends on pH and ionic strength. Eur. J. Biochem. **260**, 439-445

32.   Lee, H.-S., Bellin, R. M., Walker, D. L., Stromer, M. H., Critchley, D. R., and Robson, R. M. (2002). Characterization and Functional Implications of an Actin-Binding Site within the Talin Head Domain. manuscript in preparation.

33    Carroll, C. W., Altman, R., Schieltz, D., Yates, J. R. and Kellogg, D. (1998) The septins are required for the mitosis-specific activation of the Gin4 kinase. J. Cell Biol. **143**, 709-717

34    Schmidt, J. M., Zhang, J., Lee, H. S., Stromer, M. H. and Robson, R. M. (1999) Interaction of talin with actin: sensitive modulation of filament crosslinking activity. Arch. Biochem. Biophys. **366**, 139-150

35    Spudich, J. A. and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. **246**, 4866-4871

36    Schmidt, J. M., Robson, R. M., Zhang, J. and Stromer, M. H. (1993) The marked pH dependence of the talin-actin interaction. Biochem. Biophys. Res. Commun. **197**, 660-666

37    Argos, P. (1988) An investigation of protein subunit and domain interfaces. Protein Eng. **2**, 101-113

38    Janin, J., Miller, S. and Chothia, C. (1988) Surface, subunit interfaces and interior of oligomeric proteins. J. Mol. Biol. **204**, 155-164

39    Holtzman, D. A., Yang, S. and Drubin, D. G. (1993) Synthetic-lethal interactions identify two novel genes, SLA1 and SLA2, that control membrane cytoskeleton assembly in Saccharomyces cerevisiae. J. Cell Biol. **122**, 635-644

40    Zhang, J., Robson, R. M., Schmidt, J. M. and Stromer, M. H. (1996) Talin can crosslink actin filaments into both networks and bundles. Biochem. Biophys. Res. Commun. **218**, 530-537

41    Putney, L. K., Denker, S. P. and Barber, D. L. (2002) The changing face of the Na+/H+ exchanger, NHE1: structure, regulation, and cellular actions. Annu. Rev.

Pharmacol. Toxicol. **42**, 527-552

42    Hamada, K., Shimizu, T., Matsui, T., Tsukita, S. and Hakoshima, T. (2000) Structural
      basis of the membrane-targeting and unmasking mechanisms of the radixin FERM
      domain. EMBO J. **19**, 4449-4462

43    Han, B. G., Nunomura, W., Takakuwa, Y., Mohandas, N. and Jap, B. K. (2000)
      Protein 4.1R core domain structure and insights into regulation of cytoskeletal
      organization. Nat. Struct. Biol. **7**, 871-875

44    Pearson, M. A., Reczek, D., Bretscher, A. and Karplus, P. A. (2000) Structure of the
      ERM protein moesin reveals the FERM domain fold masked by an extended actin
      binding tail domain. Cell **101**, 259-270

45    Bass, S. H., Mulkerrin, M. G. and Wells, J. A. (1991) A systematic mutational
      analysis of hormone-binding determinants in the human growth hormone receptor.
      Proc. Natl. Acad. Sci. U S A **88**, 4498-4502

Fig. 1. **Deletion scheme to define ABS2 within the C-terminal tail of talin.** The deletion constructs of talin fused to GST were generated and expressed in *E. coli*, and the NH2- and COOH-terminal amino acid residues of the fusion proteins are indicated. Each talin mutant (0.2 mg/ml final concentration) was mixed with G-actin (0.5 mg/ml final concentration) in the cosedimentation buffer at pH 6.4, and G-actin was polymerized to F-actin by the addition of $MgCl_2$ (2 mM final concentration). The mixtures were incubated for 1 hr at 25°C, and centrifuged at 100,000 × g for 20 min. The resulting supernatants and pellets were subjected to SDS-PAGE analysis, and the actin binding ability was determined by measuring the density of Coomasie blue stained bands. The amount of talin fusion protein cosedimented with F-actin is indicated as follows: +++ = ~65%; ++ = ~30-40%; --- = ~5%.

# ABS 2

Actin Binding

| | |
|---|---|
| 951 ————————————— 1327 | +++ |
| 986 ————————— 1327 | ++ |
| 1051 ——————— 1327 | ++ |
| 1061 ——————— 1327 | --- |
| 951 ——————— 1250 | ++ |
| 951 ——————— 1230 | --- |
| 1051 ————— 1250 | ++ |

Fig. 2. **Cosedimentation of GST-talin ABS2 with F-actin.** The narrowed talin ABS2 (0.2 mg/ml) containing residues 1051-1250, fused to GST, was mixed with G-actin (0.5 mg/ml) in the cosedimentation buffer at pH 6.4, and 2 mM $MgCl_2$ was added to polymerize the G-actin. The sample was incubated for 1 hr at 25°C and centrifuged at 100,000 × g for 20 min. The resulting supernatants (S) and pellets (P) were analyzed by SDS-PAGE. *Panel* a, ABS2 alone; *Panel* b, mixture of ABS2 and actin.

1051-1250 →

← Actin
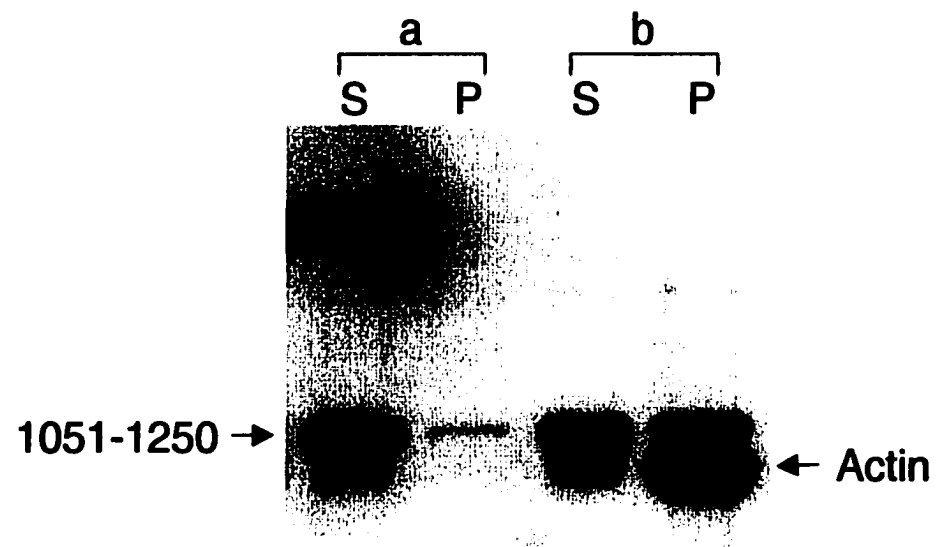
Fig. 3. **Deletion scheme to define ABS3 within the C-terminal tail of talin.** The deletion constructs of talin fused to GST were generated and expressed in *E. coli*, and the NH2- and COOH-terminal amino acid residues of the fusion proteins are indicated. Each talin mutant (0.2 mg/ml final concentration) was mixed with G-actin (0.5 mg/ml final concentration) in the cosedimentation buffer at pH 6.4, and G-actin was polymerized to F-actin by the addition of $MgCl_2$ (2 mM final concentration). The mixtures were incubated for 1 hr at 25°C, and centrifuged at 100,000 × g for 20 min. The resulting supernatants and pellets were subjected to SDS-PAGE analysis, and the actin binding ability was determined by measuring the density of Coomasie blue stained bands. The amount of talin fusion protein cosedimented with F-actin is indicated as follows: ++++ = over 90%; +++ = ~80%; + = ~ 25%; --- = ~5%.

# ABS 3

Actin Binding

2269 ——————————————————— 2541    +++

2306 ——————————————————— 2541    ++++

2331 ——————————————————— 2541    ++++

2464 ————————— 2541    +

2306 ——————————————— 2530    +++

2306 ————————— 2480    ---

2331 ——————————————— 2530    ++++

Fig. 4. **Cosedimentation of GST-talin ABS3 with F-actin.** The narrowed talin ABS3 (0.2 mg/ml) containing residues 2331-2530, fused to GST, was mixed with G-actin (0.5 mg/ml) in the cosedimentation buffer at pH 6.4, and 2 mM MgCl₂ was added to polymerize the G-actin. The sample was incubated for 1 hr at 25°C and centrifuged at 100,000 × g for 20 min. The resulting supernatants (S) and pellets (P) were analyzed by SDS-PAGE. *Panel* a, ABS3 alone; *Panel* b, mixture of ABS3 and actin.

2331-2530 →

← Actin

Fig. 5. **Effect of pH on the ability of GST-talin ABS2 to cosediment with F-actin.** Cosedimentation of GST-talin ABS2 containing residues 951-1327 with F-actin was performed in cosedimentation buffer at the following pH values: pH 6.4 (a,b), pH 6.9 (c,d) and pH 7.3 (e,f). Supernatants (S) and pellets (P) from the cosedimentation assays with actin (b,d,f) and without actin (a,c,e) were analyzed by SDS-PAGE.
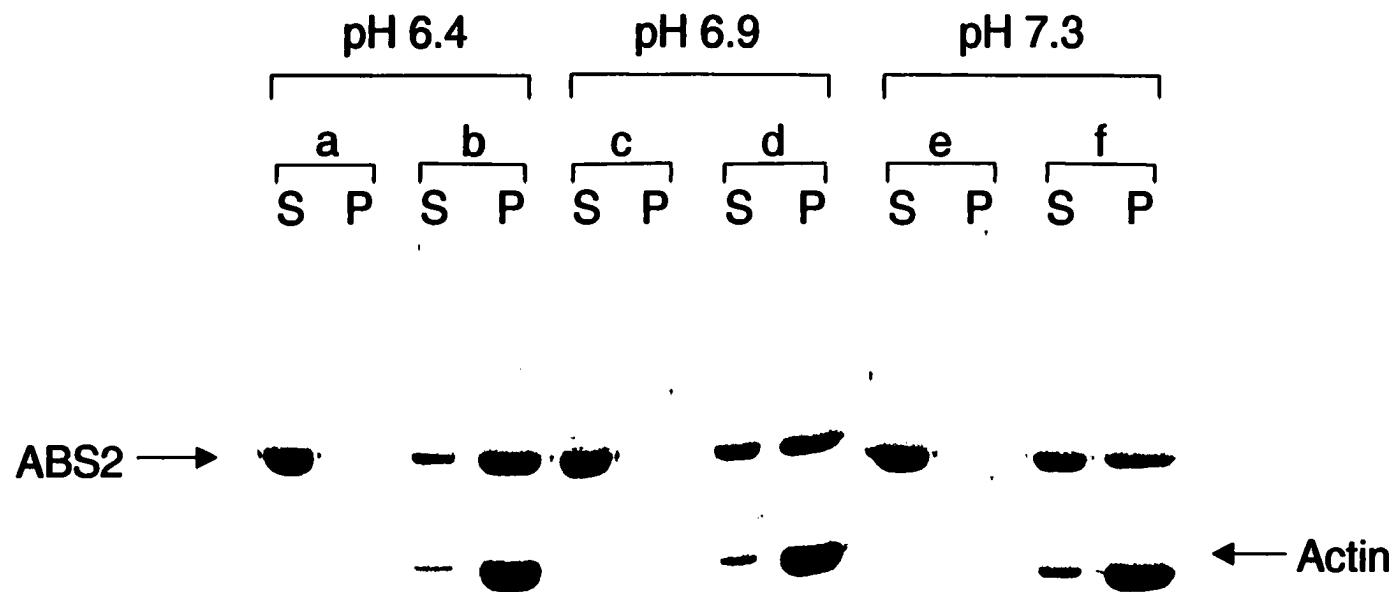
Fig. 6. **Effect of ionic strength on the ability of GST-talin ABS2 to cosediment with F-actin.** Cosedimentation of GST-talin ABS2 containing residues 951-1327 with F-actin was performed at pH 6.4 in cosedimentation buffer containing increasing concentrations of KCl. Supernatants (S) and pellets (P) resulting from the cosedimentation assays without actin at 0 mM KCl (a); pellets (P) resulting from the cosedimentation assays of ABS2 with actin in the presence of increasing concentrations of KCl (b).

KCl (mM)

0    0    0    25   50   100

ABS 2 →

← Actin

S    P    P    P    P    P

a         b
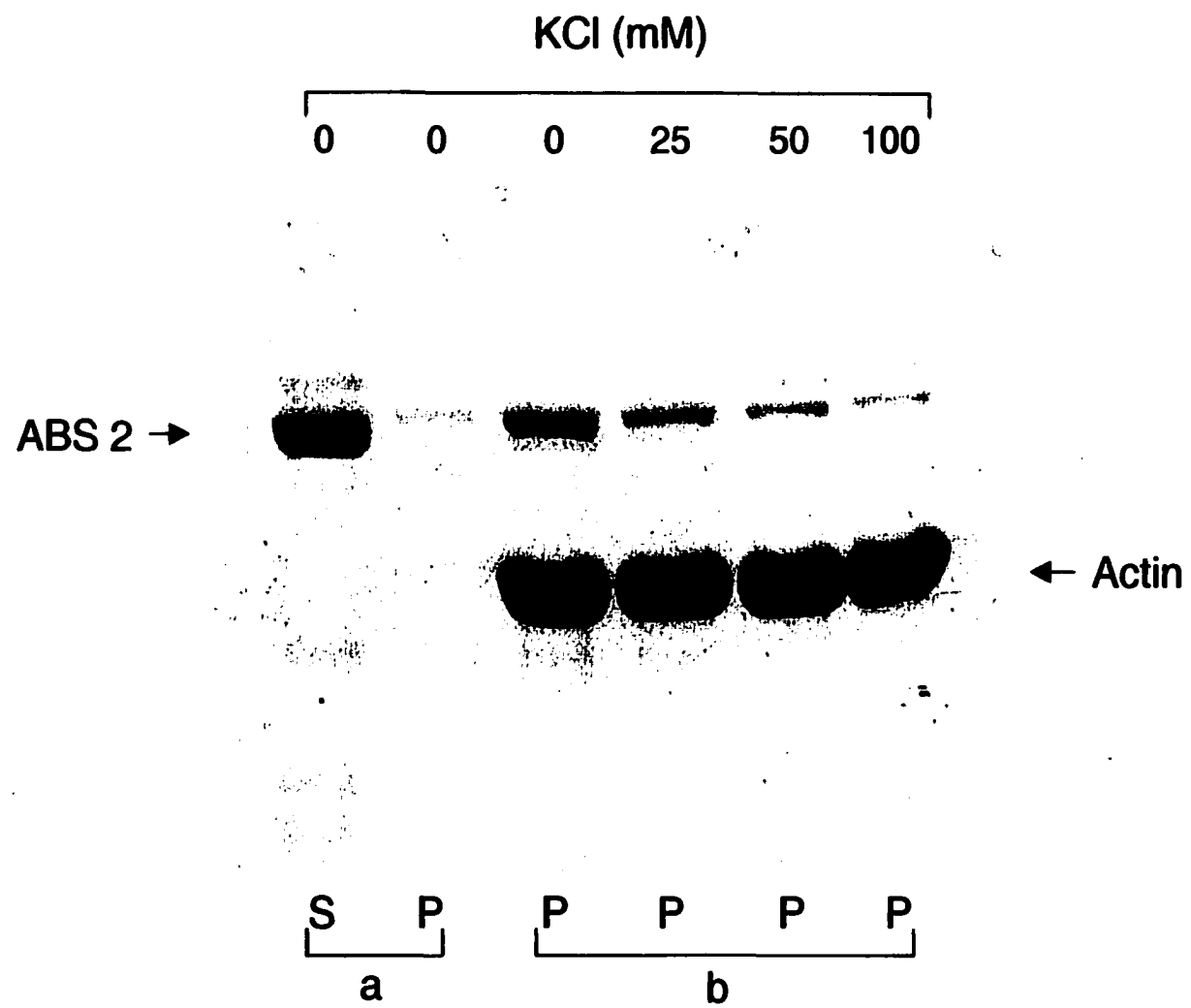
Fig. 7. **Transient expression of EGFP-talin fusion constructs in COS-7 cells.** (A) EGFP-talin ABS2 construct containing residues 951-1327; (B) EGFP-talin ABS3 construct containing residues 2269-2541; (C) EGFP-fusion control construct that does not contain the three actin binding sites of talin. F-actin was visualized by staining with rhodamine-phalloidin.
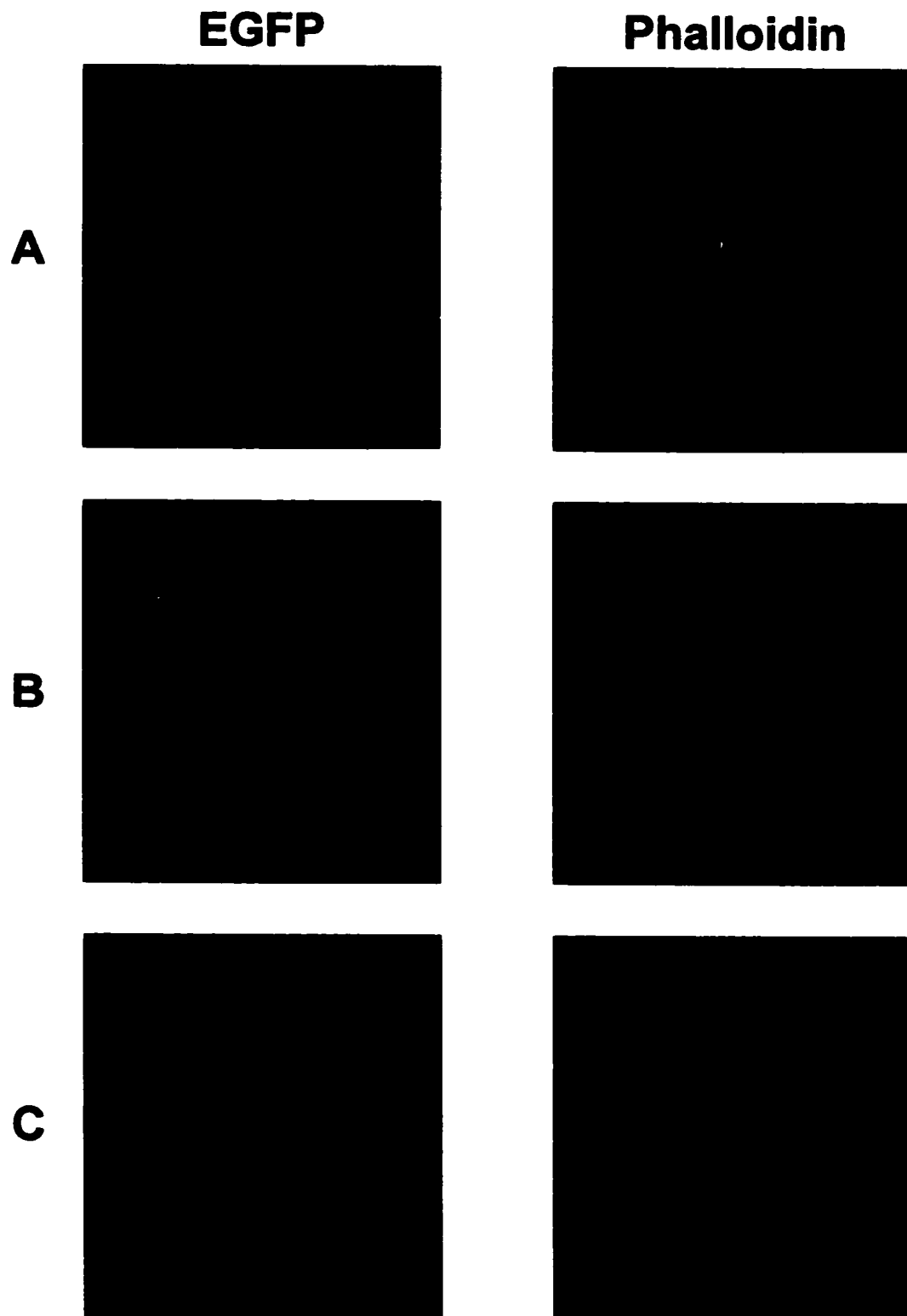
**EGFP**

**Phalloidin**

A

B

C

Table 1. *In vitro* site-directed mutagenesis of ABS1

| ABS1 mutants with alanine substitution | Actin binding ability |
|---|---|
| $R^{297}K^{300}$ | Precipitated |
| $M^{281}N^{285}$ | Very little effect |
| $D^{233}K^{234}$ | Very little effect |
| $K^{278}M^{281}N^{285}E^{293}R^{297}K^{300}$ | Very little effect |
| $D^{233}K^{234}E^{293}R^{297}K^{300}$ | Very little effect ** |
| $D^{233}K^{234}K^{278}M^{281}N^{285}E^{293}R^{297}K^{300}$ | Precipitated |
| $K^{295}R^{297}K^{300}R^{303}K^{306}$ | Large change in binding to actin** |
| $K^{295}V^{296}R^{297}Y^{298}V^{299}K^{300}$ | Large change in binding to actin * |

Actin binding ability of mutants was measured by densitometry of Coomassie brilliant blue-stained bands resulting from actin cosedimentation assays, and is indicated as follows: Very little effect = ~90% of the mutant cosedimented with F-actin; Large change in binding to actin = only ~5% of the mutant cosedimented with F-actin; Precipitated = ~90% of the mutant precipitated by itself in the cosedimentation buffer; ** = ~50% of the mutant precipitated by itself in the cosedimentation buffer; * = ~30% of the mutant precipitated by itself in the cosedimentation buffer. For reference, approximately 95% of a control construct containing ABS1 (residues 196-400) cosedimented with F-actin.

## OVERALL SUMMARY

The primary objective of my dissertation research was to examine the characteristics of the interaction between talin and actin by investigating specific domains within talin involved in the interaction. The manuscript included within the main body of the dissertation was primarily focused on the actin binding site (ABS1) located within the 47 kDa N-terminal head. The second part of the main body described preliminary studies of the ABS2 and ABS3 within the 190 kDa C-terminal tail of talin, plus preliminary results of a mutagenesis study on ABS1. The conclusions obtained from the results of the manuscript include the following:

(1) The ABS1 in the N-terminal head domain of talin was defined to residues 271-320, which are within the FERM (band 4.1-ezrin-radixin-moesin) domain of talin. GST-talin fusion proteins containing residues 201-320 and 271-497 (i.e., included ABS1 residues 271-320) cosedimented with F-actin, whereas GST-talin fusion proteins containing residues 201-270 and 321-497 (i.e., did not include residues 271-320) did not cosediment with F-actin.

(2) The interaction of ABS1 with actin is only weakly pH dependent. The GST-talin fusion proteins containing ABS1 did not show a significant increase in the amount cosedimented with F-actin as pH of the cosedimentation buffer was decreased to 6.4. A high level of the interaction of ABS1 with actin was maintained from pH 6.4 to 7.3.

(3) The interaction of ABS3, which is located near the end of the C-terminal tail of talin, with F-actin is highly dependent on pH. Progressive decrease in the pH from 7.3 to 6.4 markedly increased the amount of GST-talin fusion protein containing ABS3 that cosedimented with F-actin, which is very similar to the characteristics of native tissue-purified talin.

(4) The interaction of ABS1 with actin is highly sensitive to ionic strength. A progressive increase in the KCl concentration of the cosedimentation buffer up to 100 mM significantly decreased the ability of GST-talin fusion proteins containing ABS1 to cosediment with F-actin.

(5) The interaction of ABS3 with actin is highly sensitive to ionic strength. The presence of 25 mM KCl inhibited approximately 48% of the ability of GST-talin fusion protein containing ABS3 to cosediment with F-actin. Increasing the KCl concentration to 50 mM and 100 mM further decreased actin binding.

(6) The purified 47 kDa N-terminal head of talin was able to bind actin. This N-terminal fragment, purified from calpain-digested talin, almost completely cosedimented with actin at optimal *in vitro* conditions of talin-actin interaction. These findings demonstrate the presence of ABS1 in the N-terminal head, and support/confirm the results obtained with the recombinant talin constructs.

(7) EGFP-fusion ABS1 colocalized with F-actin in COS cells. The EGFP-fusion constructs containing ABS1 colocalized primarily with actin stress fibers when transfected into COS cells. In contrast, transfected EGFP-fusion ABS3 colocalized primarily with actin filaments at membrane ruffles, thereby showing that the interaction of ABS1 with actin is specific, and that the localization pattern of ABS1 is distinct from that of ABS3 in COS cells.

(8) Talin ABS1 showed relatively lower conservation (~25% identity) in amino acid sequences among the ERM proteins, in comparison to higher conservation (~48% identity with *C. elegans* to ~94% identity with human and mouse) among talin proteins across species. The highly positive nature of side chains of amino acid residues (271-320) in ABS1 (pI ~10.15) is quite different from the negative nature of side chains of corresponding

residues (e.g., pI ~5.26 in radixin) in the FERM domain of ERM proteins. The ABS1 was somewhat more conserved than was the entire talin sequence in comparisons of the chicken and mouse. The core ABS1 residues (295-320) were identical in the amino acid sequences among human, mouse and chicken.

The major objectives of the second part of the main body of my dissertation were to initiate studies in which I have defined and characterized the ABS2 and ABS3 in the C-terminal tail of talin, and to conduct preliminary *in vitro* mutagenesis studies on ABS1 in the N-terminal head. The conclusions obtained from the results of this second part were as follows:

(1) ABS2 was further defined to residues 1051-1250. Deletion from the N-terminus to residue 986 and from the C-terminus to residue 1250 resulted in some loss of actin binding. Additional deletion from the N-terminus to residue 1050 did not affect the binding to actin. Further deletions of 10 to 20 residues from either the $NH_2$-terminus of GST-talin fusion protein containing residues 1051-1327, or from the COOH-terminus of GST-talin fusion protein containing residues 951-1250 significantly decreased the ability of these fusion proteins to cosediment with F-actin, respectively.

(2) ABS3 was further defined to residues 2331-2530. Deletion from the N-terminus to residue 2330 and from the C-terminus to residue 2531 did not highly affect the ability of ABS3 to bind to actin. Further deletions from the COOH-terminus (e.g., GST-talin 2306-2480) almost abolished actin binding, and deletions from the $NH_2$-terminus greatly reduced the solubility of the fusion proteins, which made it difficult to test the effect of these deletions. A GST-talin fusion protein containing residues 2464-2541 that contains the fourth block of the I/LWEQ module retains some actin binding ability, whereas a GST-talin fusion

protein containing residues 2306-2480 that contains the other three blocks of this module did not retain actin binding ability, thereby suggesting the fourth block may be very important in binding to actin.

(3) ABS2 was highly dependent on pH in binding to actin. Cosedimentation of GST-talin fusion protein containing ABS2 (residues 951-1327) with F-actin at low ionic strength showed that the ability of ABS2 to bind actin was progressively increased as pH was decreased from 7.3 to 6.4.

(4) ABS2 was highly sensitive to ionic strength in ability to interact with actin. Progressive increase in the concentration of KCl to 100 mM significantly reduced the amount of GST-talin fusion protein containing ABS2 to cosediment with F-actin. Approximately 81% of the ability of GST-ABS2 to cosediment with F-actin at low pH (6.4) was lost in the presence of 100 mM KCl.

(5) Transfection of EGFP-tagged ABS2 (residues 951-1327) and EGFP-tagged ABS3 (2269-2541) into COS cells, respectively, showed that ABS2 was primarily colocalized with actin stress fibers, whereas ABS3 was predominantly colocalized with actin filaments at membrane ruffles.

(6) Alanine substitution of amino acid residues between 295-306 of ABS1 resulted in a significant reduction in binding to actin. The preliminary *in vitro* site-directed mutagenesis study on ABS1 showed that the ability of two mutants, one containing mutation of residues $K^{295}R^{297}K^{300}R^{303} K^{306}$ and the other containing mutation of all residues within 295-300, to cosediment with F-actin was almost abolished. Taken together with the result that residues $R^{297}K^{300}$ were not found to be essential for ABS1 to bind actin, these preliminary mutagenesis

studies suggest that $K^{295}$, $R^{303}$ and $K^{306}$ within the ABS1 core region may be potentially important/critical for interaction of ABS1 with actin.

The results in this dissertation provide strong evidence for the presence of an actin binding site (ABS1) within the N-terminal head domain of talin, which was defined to residues 271-320. The biochemical and cell biological characteristics of ABS1 were different from those of ABS3, which suggest that these two ABS may play distinctive roles in ability of talin to interact with actin. The ABS1 is located within the major integrin binding site of the FERM domain, suggesting it may serve to directly link the actin cytoskeleton to the integrins. The subdomain F3 (the nomenclature of Pearson et al., 2000; or subdomain C in the nomenclature of Hamada et al., 2000) within the talin FERM domain has recently been predicted to contain a phosphotyrosine binding (PTB)-like domain, and to have the ability to induce integrin activation when expressed in cultured cells. The localization of ABS1 close to the PTB-like domain further suggests the possibility of a functional role for ABS1 in signal transduction. The actin filament crosslinking ability of native talin has been shown to be significantly dependent on pH and ionic strength. The fact that ABS2 and ABS3 both showed high pH and ionic strength dependence in interacting with actin suggests both ABS2 and ABS3, which are located in the C-terminal tail, may play a role in crosslinking actin filaments in response to local changes in cellular pH, whereas ABS1 in the N-terminal head may directly link integrins to the actin cytoskeleton in a pH-independent manner. The ability of talin to modulate actin binding activity via its pH-insensitive (ABS1) and pH–sensitive ABS (ABS2 and ABS3) may be important for attachment of actin filaments to the plasma membrane and for remodeling of the actin cytoskeleton necessary during cell adhesion and migration. A localized pH increase within microenvironments of the cell, such as at the

leading edge, may reduce/inactivate actin crosslinking activity of ABS2 and ABS3, thereby facilitating remodeling of the actin cytoskeleton necessary for cell migration, while talin remains attached to the plasma membrane via ABS1.

These results, taken *in toto*, suggest distinctive functional roles for ABS1 located within the N-terminal head, and for ABS2 and ABS3 located within the long C-terminal tail domain of talin, respectively. The ABS1 may serve in part to attach actin filaments to the integrins in a pH-independent manner, whereas ABS2 and ABS3 may serve to crosslink actin filaments in a pH-dependent manner.

# COMPREHENSIVE BIBLIOGRAPHY

Alatortsev, V. E., Kramerova, I. A., Frolov, M. V., Lavrov, S. A. and Westphal, E. D. (1997) Vinculin gene is non-essential in Drosophila melanogaster. FEBS Lett. **413**, 197-201.

Albiges-Rizo, C., Frachet, P. and Block, M. R. (1995) Down regulation of talin alters cell adhesion and the processing of the alpha 5 beta 1 integrin. J. Cell Sci. **108**, 3317-3329

Argos, P. (1988) An investigation of protein subunit and domain interfaces. Protein Eng. **2**, 101-113

Bakolitsa, C., de Pereda, J. M., Bagshaw, C. R., Critchley, D. R. and Liddington, R. C. (1999) Crystal structure of the vinculin tail suggests a pathway for activation. Cell **99**, 603-613

Barstead, R. J. and Waterston, R. H. (1991) Vinculin is essential for muscle function in the nematode. J. Cell Biol. **114**, 715-724

Bass, S. H., Mulkerrin, M. G. and Wells, J. A. (1991) A systematic mutational analysis of hormone-binding determinants in the human growth hormone receptor. Proc. Natl. Acad. Sci. U S A **88**, 4498-4502

Bass, M. D., Smith, B. J., Prigent, S. A. and Critchley, D. R. (1999) Talin contains three similar vinculin-binding sites predicted to form an amphipathic helix. Biochem. J. **341**, 257-263

Bass, M. D., Patel, B., Barsukov, I. G., Fillingham, I. J., Mason, R., Smith, B. J., Bagshaw, C. R. and Critchley, D. R. (2002) Further characterization of the interaction between the cytoskeletal proteins talin and vinculin. Biochem. J. **362**, 761-768

Beckerle, M. C., O'Halloran, T. and Burridge, K. (1986) Demonstration of a relationship between talin and P235, a major substrate of the calcium-dependent protease in platelets. J. Cell Biochem. **30**, 259-270

Belkin, A. M., Zhidkova, N. I. and Koteliansky, V. E. (1986) Localization of talin in skeletal and cardiac muscles. FEBS Lett. **200**, 32-36

Ben-Yosef, T. and Francomano, C. A. (1999) Characterization of the human talin (TLN) gene: genomic structure, chromosomal localization, and expression pattern. Genomics **62**, 316-319

Bertagnolli, M. E., Locke, S. J., Hensler, M. E., Bray, P. F. and Beckerle, M. C. (1993) Talin distribution and phosphorylation in thrombin-activated platelets. J. Cell Sci. **106**, 1189-1199

Bolton, S. J., Barry, S. T., Mosley, H., Patel, B., Jockusch, B. M., Wilkinson, J. M. and Critchley, D. R. (1997) Monoclonal antibodies recognizing the N- and C-terminal regions of talin disrupt actin stress fibers when microinjected into human fibroblasts. Cell Motil. Cytoskeleton 36, 363-376

Bono, P., Rubin, K., Higgins, J. M. and Hynes, R. O. (2001) Layilin, a novel integral membrane protein, is a hyaluronan receptor. Mol. Biol. Cell 12, 891-900

Borowsky, M. L. and Hynes, R. O. (1998) Layilin, a novel talin-binding transmembrane protein homologous with C-type lectins, is localized in membrane ruffles. J. Cell Biol. 143, 429-442

Bretscher, A., Chambers, D., Nguyen, R. and Reczek, D. (2000) ERM-Merlin and EBP50 protein families in plasma membrane organization and function. Annu. Rev. Cell Dev. Biol. 16, 113-143

Burridge, K., Kelly, T., and Connell, L. (1982). Proteins involved in the attachment of actin to the plasma membrane. Phil. Trans. R. Soc. Lond. B 299, 291-299

Burridge, K. and Connell, L. (1983) A new protein of adhesion plaques and ruffling membranes. J. Cell Biol. 97, 359-367

Burridge, K. and Connell, L. (1983) Talin: a cytoskeletal component concentrated in adhesion plaques and other sites of actin-membrane interaction. Cell Motil. 3, 405-417

Burridge, K. Fath, K. Kelly, T. Nuckolls, G. and Turner, C. (1988) Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu. Rev. Cell. Biol. 4, 487-525

Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. Annu. Rev. Cell Dev. Biol. 12, 463-518

Byrne, B. J., Kaczorowski, Y. J., Coutu, M. D. and Craig, S. W. (1992) Chicken vinculin and meta-vinculin are derived from a single gene by alternative splicing of a 207-base pair exon unique to meta-vinculin. J. Biol. Chem. 267, 12845-12850

Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O. and Ginsberg, M. H. (1999) The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. J. Biol. Chem. 274, 28071-28074

Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y., Liddington, R. C. and Ginsberg, M. H. (2002) The phosphotyrosine binding-like domain of talin activates integrins. J. Biol. Chem. 277, 21749-21758

Carroll, C. W., Altman, R., Schieltz, D., Yates, J. R. and Kellogg, D. (1998) The septins are required for the mitosis-specific activation of the Gin4 kinase. J. Cell Biol. **143**, 709-717

Cary, L. A., Chang, J. F. and Guan, J. L. (1996) Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J. Cell Sci. **109**, 1787-1794

Chen, H. C., Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D. and Guan, J. L. (1995) Interaction of focal adhesion kinase with cytoskeletal protein talin. J. Biol. Chem. **270**, 16995-16999

Chen, Z. Y., Hasson, T., Kelley, P. M., Schwender, B. J., Schwartz, M. F., Ramakrishnan, M., Kimberling, W. J., Mooseker, M. S. and Corey, D. P. (1996) Molecular cloning and domain structure of human myosin-VIIa, the gene product defective in Usher syndrome 1B. Genomics **36**, 440-448

Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N., Chasis, J. A., Conboy, J. G., Gascard, P., Takakuwa, Y., Huang, S. C., Benz, E. J., Jr., Bretscher, A., Fehon, R. G., Gusella, J. F., Ramesh, V., Solomon, F., Marchesi, V. T., Tsukita, S., Hoover, K. B. and et al. (1998) The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. Trends Biochem. Sci. **23**, 281-282

Chou, R. G. Stromer, M. H. Robson, R. M. and Huiatt, T. W. (1994) Substructure of cytoplasmic dense bodies and changes in distribution of desmin and alpha-actinin in developing smooth muscle cells. Cell Motil. Cytoskeleton **29**, 204-214

Coll, J. L., Ben-Ze'ev, A., Ezzell, R. M., Rodriguez Fernandez, J. L., Baribault, H., Oshima, R. G. and Adamson, E. D. (1995) Targeted disruption of vinculin genes in F9 and embryonic stem cells changes cell morphology, adhesion, and locomotion. Proc. Natl. Acad. Sci. U S A **92**, 9161-9165

Collier, N. C. and Wang, K. (1982) Purification and properties of human platelet P235. A high molecular weight protein substrate of endogenous calcium-activated protease(s). J. Biol. Chem. **257**, 6937-6943

Cooley, M. A., Broome, J. M., Ohngemach, C., Romer, L. H. and Schaller, M. D. (2000) Paxillin binding is not the sole determinant of focal adhesion localization or dominant-negative activity of focal adhesion kinase/focal adhesion kinase-related nonkinase. Mol. Biol. Cell **11**, 3247-3263

Critchley, D. R. Holt, M. R. Barry, S. T. Priddle, H. Hemmings, L. and Norman, J. (1999) Integrin-mediated cell adhesion: the cytoskeletal connection. Biochem. Soc. Symp. **65**, 79-99

Critchley, D. R. (2000) Focal adhesions - the cytoskeletal connection. Curr. Opin. Cell Biol.

**12**, 133-139

Edmunds, T., Nagainis, P. A., Sathe, S. K., Thompson, V. F. and Doll, D. E. (1991) Comparison of the autolyzed and unautolyzed forms of mu- and m-calpain from bovine skeletal muscle. Biochim. Biophys. Acta .**1077**, 197-208

Forman-Kay, J. D. and Pawson, T. (1999) Diversity in protein recognition by PTB domains. Curr. Opin. Struct. Biol. **9**, 690-695

Funayama, N., Nagafuchi, A., Sato, N. and Tsukita, S. (1991) Radixin is a novel member of the band 4.1 family. J. Cell Biol. **115**, 1039-1048

Geiger, B., Tokuyasu, K. T., Dutton, A. H. and Singer, S. J. (1980) Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. Proc. Natl. Acad. Sci. U S A **77**, 4127-4131

Geiger, B., Volk, T. and Volberg, T. (1985) Molecular heterogeneity of adherens junctions. J. Cell Biol. **101**, 1523-1531

Geiger, B., Bershadsky, A., Pankov, R. and Yamada, K. M. (2001) Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. Nat. Rev. Mol. Cell Biol. **2**, 793-805

Gilmore, A. P., Jackson, P., Waites, G. T. and Critchley, D. R. (1992) Further characterisation of the talin-binding site in the cytoskeletal protein vinculin. J. Cell Sci. **103**, 719-731

Gilmore, A. P., Wood, C., Ohanian, V., Jackson, P., Patel, B., Rees, D. J., Hynes, R. O. and Critchley, D. R. (1993) The cytoskeletal protein talin contains at least two distinct vinculin binding domains. J. Cell Biol. **122**, 337-347

Gimona, M., Small, J. V., Moeremans, M., Van Damme, J., Puype, M. and Vandekerckhove, J. (1988) Porcine vinculin and metavinculin differ by a 68-residue insert located close to the carboxy-terminal part of the molecule. EMBO J. **7**, 2329-2334

Glading, A., Lauffenburger, D. A. and Wells, A. (2002) Cutting to the chase: calpain proteases in cell motility. Trends Cell Biol. **12**, 46-54

Goldmann, W. H., Bremer, A., Haner, M., Aebi, U. and Isenberg, G. (1994) Native talin is a dumbbell-shaped homodimer when it interacts with actin. J. Struct. Biol. **112**, 3-10

Goldmann, W. H., Hess, D. and Isenberg, G. (1999) The effect of intact talin and talin tail fragment on actin filament dynamics and structure depends on pH and ionic strength. Eur. J. Biochem. **260**, 439-445

Habazettl, J., Gondol, D., Wiltscheck, R., Otlewski, J., Schleicher, M. and Holak, T. A. (1992) Structure of hisactophilin is similar to interleukin-1 beta and fibroblast growth factor. Nature **359**, 855-858

Hamada, K., Shimizu, T., Matsui, T., Tsukita, S. and Hakoshima, T. (2000) Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. Embo J. **19**, 4449-4462

Han, B. G., Nunomura, W., Takakuwa, Y., Mohandas, N. and Jap, B. K. (2000) Protein 4.1R core domain structure and insights into regulation of cytoskeletal organization. Nat. Struct. Biol. **7**, 871-875

Hayashi, I., Vuori, K. and Liddington, R. C. (2002) The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. Nat. Struct. Biol. **9**, 101-106

Hemmings, L., Kuhlman, P. A. and Critchley, D. R. (1992) Analysis of the actin-binding domain of alpha-actinin by mutagenesis and demonstration that dystrophin contains a functionally homologous domain. J. Cell Biol. **116**, 1369-1380

Hemmings, L., Rees, D. J., Ohanian, V., Bolton, S. J., Gilmore, A. P., Patel, B., Priddle, H., Trevithick, J. E., Hynes, R. O. and Critchley, D. R. (1996) Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. J. Cell Sci. **109**, 2715-2726

Holtzman, D. A., Yang, S. and Drubin, D. G. (1993) Synthetic-lethal interactions identify two novel genes, SLA1 and SLA2, that control membrane cytoskeleton assembly in Saccharomyces cerevisiae. J. Cell Biol. **122**, 635-644

Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G. and Otey, C. A. (1996) Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. J. Cell Biol. **135**, 1383-1390

Hynes, R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell **69**, 11-25

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature **377**, 539-544

Ilic, D., Damsky, C. H. and Yamamoto, T. (1997) Focal adhesion kinase: at the crossroads of signal transduction. J. Cell Sci. **110**, 401-407

Isenberg, G. and Goldmann, W. H. (1998) Peptide-specific antibodies localize the major lipid binding sites of talin dimers to oppositely arranged N-terminal 47 kDa subdomains. FEBS Lett. **426**, 165-170

Janin, J., Miller, S. and Chothia, C. (1988) Surface, subunit interfaces and interior of oligomeric proteins. J. Mol. Biol. **204**, 155-164

Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G. and Winkler, J. (1995) The molecular architecture of focal adhesions. Annu. Rev. Cell Dev. Biol. **11**, 379-416

Johnson, R. P. and Craig, S. W. (1994) An intramolecular association between the head and tail domains of vinculin modulates talin binding. J. Biol. Chem. **269**, 12611-12619

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F. and Holmes, K. C. (1990) Atomic structure of the actin:DNase I complex. Nature **347**, 37-44

Kaufmann, S., Piekenbrock, T., Goldmann, W. H., Barmann, M. and Isenberg, G. (1991) Talin binds to actin and promotes filament nucleation. FEBS Lett. **284**, 187-191

Knezevic, I., Leisner, T. M. and Lam, S. C. (1996) Direct binding of the platelet integrin alphaIIbbeta3 (GPIIb-IIIa) to talin. Evidence that interaction is mediated through the cytoplasmic domains of both alphaIIb and beta3. J. Biol. Chem. **271**, 16416-16421

Kreitmeier, M., Gerisch, G., Heizer, C. and Muller-Taubenberger, A. (1995) A talin homologue of Dictyostelium rapidly assembles at the leading edge of cells in response to chemoattractant. J. Cell Biol .**129**, 179-188

Kupfer, A., Swain, S. L., Janeway, C. A., Jr. and Singer, S. J. (1986) The specific direct interaction of helper T cells and antigen-presenting B cells. Proc. Natl. Acad. Sci. U S A **83**, 6080-6083

Lee, H.-S., Bellin, R. M., and Robson, R. M. (2001) in Wiley Encyclopedia of Molecular Medicine, Vol. **5**, 3087-3089, John Wiley & Sons, Inc., New York

Lee, H.-S., Bellin, R. M., Walker, D. L., Stromer, M. H., Critchley, D. R., and Robson, R. M. (2002). Characterization and Functional Implications of an Actin-Binding Site within the Talin Head Domain. manuscript in preparation.

Leto, T. L. and Marchesi, V. T. (1984) A structural model of human erythrocyte protein 4.1. J. Biol. Chem. **259**, 4603-4608

Luo, G., Herrera, A. H. and Horowits, R. (1999) Molecular interactions of N-RAP, a nebulin-related protein of striated muscle myotendon junctions and intercalated disks. Biochemistry **38**, 6135-6143

Maeda, M., Holder, E., Lowes, B., Valent, S. and Bies, R. D. (1997) Dilated cardiomyopathy associated with deficiency of the cytoskeletal protein metavinculin. Circulation **95**, 17-20

Martel, V., Racaud-Sultan, C., Dupe, S., Marie, C., Paulhe, F., Galmiche, A., Block, M. R. and Albiges-Rizo, C. (2001) Conformation, localization, and integrin binding of talin depend on its interaction with phosphoinositides. J. Biol. Chem. **276**, 21217-21227

McCann, R. O. and Craig, S. W. (1997) The I/LWEQ module: a conserved sequence that signifies F-actin binding in functionally diverse proteins from yeast to mammals. Proc. Natl. Acad. Sci. U S A **94**, 5679-5684

McCann, R. O. and Craig, S. W. (1999) Functional genomic analysis reveals the utility of the I/LWEQ module as a predictor of protein:actin interaction. Biochem. Biophys. Res. Commun. **266**, 135-140

Molony, L., McCaslin, D., Abernethy, J., Paschal, B. and Burridge, K. (1987) Properties of talin from chicken gizzard smooth muscle. J. Biol. Chem. **262**, 7790-7795

Monkley, S. J., Zhou, X. H., Kinston, S. J., Giblett, S. M., Hemmings, L., Priddle, H., Brown, J. E., Pritchard, C. A., Critchley, D. R. and Fassler, R. (2000) Disruption of the talin gene arrests mouse development at the gastrulation stage. Dev. Dyn. **219**, 560-574

Monkley, S. J., Pritchard, C. A. and Critchley, D. R. (2001) Analysis of the mammalian talin2 gene tln2. Biochem. Biophys. Res. Commun. **286**, 880-885

Moulder, G. L., Huang, M. M., Waterston, R. H. and Barstead, R. J. (1996) Talin requires beta-integrin, but not vinculin, for its assembly into focal adhesion-like structures in the nematode Caenorhabditis elegans. Mol. Biol. Cell **7**, 1181-1193

Muguruma, M., Matsumura, S. and Fukazawa, T. (1990) Direct interactions between talin and actin. Biochem. Biophys. Res. Commun. **171**, 1217-1223

Muguruma, M., Matsumura, S. and Fukazawa, T. (1992) Augmentation of alpha-actinin-induced gelation of actin by talin. J. Biol. Chem. **267**, 5621-5624

Muguruma, M., Nishimuta, S., Tomisaka, Y., Ito, T. and Matsumura, S. (1995) Organization of the functional domains in membrane cytoskeletal protein talin. J. Biochem. (Tokyo) **117**, 1036-1042

Niewohner, J., Weber, I., Maniak, M., Muller-Taubenberger, A. and Gerisch, G. (1997) Talin-null cells of Dictyostelium are strongly defective in adhesion to particle and substrate surfaces and slightly impaired in cytokinesis. J. Cell Biol. **138**, 349-361

Niggli, V., Kaufmann, S., Goldmann, W. H., Weber, T. and Isenberg, G. (1994) Identification of functional domains in the cytoskeletal protein talin. Eur. J. Biochem. **224**, 951-957

Nuckolls, G. H., Romer, L. H. and Burridge, K. (1992) Microinjection of antibodies against talin inhibits the spreading and migration of fibroblasts. J. Cell Sci. **102**, 753-762

Otto, J. J. (1990) Vinculin. Cell Motil. Cytoskeleton **16**, 1-6

Pardo, J. V., Siliciano, J. D. and Craig, S. W. (1983) A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. Proc. Natl. Acad. Sci. U S A **80**, 1008-1012

Pearson, M. A., Reczek, D., Bretscher, A. and Karplus, P. A. (2000) Structure of the ERM protein moesin reveals the FERM domain fold masked by an extended actin binding tail domain. Cell **101**, 259-270

Pfaff, M., Liu, S., Erle, D. J. and Ginsberg, M. H. (1998) Integrin beta cytoplasmic domains differentially bind to cytoskeletal proteins. J. Biol. Chem. **273**, 6104-6109

Pollard, T. D. and Cooper, J. A. (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. Annu. Rev. Biochem. **55**, 987-1035

Pollard, T. D., Blanchoin, L. and Mullins, R. D. (2000) Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. Annu. Rev. Biophys. Biomol. Struct. **29**, 545-576

Priddle, H., Hemmings, L., Monkley, S., Woods, A., Patel, B., Sutton, D., Dunn, G. A., Zicha, D. and Critchley, D. R. (1998) Disruption of the talin gene compromises focal adhesion assembly in undifferentiated but not differentiated embryonic stem cells. J. Cell Biol. **142**, 1121-1133

Putney, L. K., Denker, S. P. and Barber, D. L. (2002) The changing face of the Na+/H+ exchanger, NHE1: structure, regulation, and cellular actions. Annu. Rev. Pharmacol. Toxicol. **42**, 527-552

Rees, D. J., Ades, S. E., Singer, S. J. and Hynes, R. O. (1990) Sequence and domain structure of talin. Nature **347**, 685-689

Ridley, A. J. and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70**, 389-399

Roy, C., Martin, M. and Mangeat, P. (1997) A dual involvement of the amino-terminal domain of ezrin in F- and G-actin binding. J. Biol. Chem. **272**, 20088-20095

Rudiger, M. (1998) Vinculin and alpha-catenin: shared and unique functions in adherens junctions. Bioessays **20**, 733-740

Sampath, R., Gallagher, P. J. and Pavalko, F. M. (1998) Cytoskeletal interactions with the leukocyte integrin beta2 cytoplasmic tail. Activation-dependent regulation of associations with talin and alpha-actinin. J. Biol. Chem. **273**, 33588-33594

Sato, N., Funayama, N., Nagafuchi, A., Yonemura, S. and Tsukita, S. (1992) A gene family consisting of ezrin, radixin and moesin. Its specific localization at actin filament/plasma membrane association sites. J. Cell Sci. **103**, 131-143

Sato, T., Irie, S., Kitada, S. and Reed, J. C. (1995) FAP-1: a protein tyrosine phosphatase that associates with Fas. Science **268**, 411-415

Schaller, M. D. (2001) Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim. Biophys. Acta **1540**, 1-21

Schmidt, J. M., Robson, R. M., Zhang, J. and Stromer, M. H. (1993) The marked pH dependence of the talin-actin interaction. Biochem. Biophys. Res. Commun. **197**, 660-666

Schmidt, J. M., Zhang, J., Lee, H. S., Stromer, M. H. and Robson, R. M. (1999) Interaction of talin with actin: sensitive modulation of filament crosslinking activity. Arch. Biochem. Biophys. **366**, 139-150

Sealock, R., Paschal, B., Beckerle, M. and Burridge, K. (1986) Talin is a post-synaptic component of the rat neuromuscular junction. Exp. Cell Res. **163**, 143-150

Seelig, A., Blatter, X. L., Frentzel, A. and Isenberg, G. (2000) Phospholipid binding of synthetic talin peptides provides evidence for an intrinsic membrane anchor of talin. J. Biol. Chem. **275**, 17954-17961

Siliciano, J. D. and Craig, S. W. (1982) Meta-vinculin--a vinculin-related protein with solubility properties of a membrane protein. Nature **300**, 533-535

Small, J. V., Furst, D. O. and Thornell, L. E. (1992) The cytoskeletal lattice of muscle cells. Eur. J. Biochem. **208**, 559-572

Small, J. V. (1995) Structure-function relationships in smooth muscle: the missing links. Bioessays **17**, 785-792

Spudich, J. A. and Watt, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. J. Biol. Chem. **246**, 4866-4871

Steimle, P. A., Hoffert, J. D., Adey, N. B. and Craig, S. W. (1999) Polyphosphoinositides inhibit the interaction of vinculin with actin filaments. J. Biol. Chem. **274**, 18414-18420

Stossel, T. P., Chaponnier, C., Ezzell, R. M., Hartwig, J. H., Janmey, P. A., Kwiatkowski, D. J., Lind, S. E., Smith, D. B., Southwick, F. S., Yin, H. L. and et al. (1985) Nonmuscle actin-binding proteins. Annu. Rev. Cell Biol. **1**, 353-402

Stromer, M. H. (1998) The cytoskeleton in skeletal, cardiac and smooth muscle cells. Histol. Histopathol. **13**, 283-291

Sydor, A. M., Su, A. L., Wang, F. S., Xu, A. and Jay, D. G. (1996) Talin and vinculin play distinct roles in filopodial motility in the neuronal growth cone. J. Cell Biol. **134**, 1197-1207

Tempel, M., Goldmann, W. H., Isenberg, G. and Sackmann, E. (1995) Interaction of the 47kDa talin fragment and the 32-kDa vinculin fragment with acidic phospholipids: a computer analysis. Biophys. J. **69**, 228-241

Tidball, J. G., O'Halloran, T. and Burridge, K. (1986) Talin at myotendinous junctions. J. Cell Biol. **103**, 1465-1472

Tsujioka, M., Machesky, L. M., Cole, S. L., Yahata, K. and Inouye, K. (1999) A unique talin homologue with a villin headpiece-like domain is required for multicellular morphogenesis in Dictyostelium. Curr. Biol. **9**, 389-392

van der Flier, A. and Sonnenberg, A. (2001) Function and interactions of integrins. Cell Tissue Res. **305**, 285-298

Venter, J. C. Adams, M. D. Myers, E. W. Li, P. W. Mural, R. J. and et al. (2001) The sequence of the human genome. Science **291**, 1304-1351

Volberg, T., Geiger, B., Kam, Z., Pankov, R., Simcha, I., Sabanay, H., Coll, J. L., Adamson, E. and Ben-Ze'ev, A. (1995) Focal adhesion formation by F9 embryonal carcinoma cells after vinculin gene disruption. J. Cell Sci. **108**, 2253-2260

Weeds, A. G. Gooch, J. Hawkins, M. Pope, B. and Way, M. (1991) Role of actin-binding proteins in cytoskeletal dynamics. Biochem. Soc. Trans. **19**, 1016-1020

Winkler, J., Lunsdorf, H. and Jockusch, B. M. (1997) Energy-filtered electron microscopy reveals that talin is a highly flexible protein composed of a series of globular domains. Eur. J. Biochem. **243**, 430-436

Xing, B., Jedsadayanmata, A. and Lam, S. C. (2001) Localization of an integrin binding site to the C terminus of talin. J. Biol. Chem. **276**, 44373-44378

Xu, W., Baribault, H. and Adamson, E. D. (1998) Vinculin knockout results in heart and brain defects during embryonic development. Development **125**, 327-337

Yamada, K. M. and Geiger, B. (1997) Molecular interactions in cell adhesion complexes. Curr. Opin. Cell Biol. **9**, 76-85

Yan, B., Calderwood, D. A., Yaspan, B. and Ginsberg, M. H. (2001) Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. J. Biol. Chem. **276**, 28164-28170

Zamir, E., Katz, B. Z., Aota, S., Yamada, K. M., Geiger, B. and Kam, Z. (1999) Molecular diversity of cell-matrix adhesions. J. Cell Sci. **112**, 1655-1669

Zhang, J., Robson, R. M., Schmidt, J. M. and Stromer, M. H. (1996) Talin can crosslink actin filaments into both networks and bundles. Biochem. Biophys. Res. Commun. **218**, 530-537

# ACKNOWLEDGMENTS

I thank my major professor, Dr. Richard Robson for his encouragement, advice and support throughout my Ph.D. studies. I also thank Dr. Ted Huiatt, for serving as a co-major professor for me, and express my gratitude to the other members of my graduate committee, Dr. Jorgen Johansen, Dr. Steven Lonergan and Dr. Marvin Stromer, for their useful suggestions in my research program.

I am indebted to Dr. David R. Critchley for sending our lab the talin constructs used in my research and for some excellent ideas during conduct of my research.

I am grateful to the other members of the Muscle Biology Group for their friendship and research suggestions. I am quite thankful to Dr. Robert Bellin for his suggestions, especially for his advice in molecular biology techniques, and to Suzy Sernett for helping to teach me cytoskeletal protein purification skills.

I especially thank my wife, Jungmi Kim, and my young son, Paul Saeyon Lee, for their support and love. I express my sincere appreciation to my mother, Jinsil Ghang and my father, Eungmo Lee, who passed away during the time of my graduate work. And I am very thankful to my heavenly Father for His guidance throughout my Ph.D. studies and life.