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The interaction between actin filaments and the cytoskeleton-membrane attachment site protein talin

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The interaction between actin filaments and the cytoskeleton-membrane attachment

site protein talin

by

Jinwen Zhang

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Talin, a protein localized primarily in cell-matrix type adherens junctions, is involved in linking the cytoskeleton to the cell membrane. The major objectives of my study were to (1) determine the structural basis for the effect of pH and ionic strength on the direct talin-actin interaction, (2) examine the effects of other factors, such as specific phospholipids, on the talin-actin interaction, and (3) compare abilities of human platelet and avian smooth muscle talins to interact with actin. Negative staining results showed that at pH 6.4 and low ionic strength, talin extensively crosslinked actin filaments into both tight networks and bundles. Some of the bundles consisted of parallel actin filaments with an interfilament spacing of ~13 nm, and talin crossbridges spaced at ~36 nm intervals along the actin bundles. As pH and/or ionic strength was increased, talin's actin bundling activity was decreased first, then its networking activity. Chemical crosslinking indicated that talin was present primarily as a dimer of two ~225 kDa subunits under all ionic conditions tested. Talin crosslinked actin filaments into networks and bundles after only five minutes of actin polymerization, with no evidence of shorter actin filaments formed in comparison to actin controls. Talin also crosslinked preformed actin filaments as well as it did actin filaments polymerized in its presence. The ~190 kDa talin fragment was able to crosslink actin filaments into networks and bundles, but required a slightly lower pH than did intact talin.

The phosphoinositides, PIP₂, PIP, or PI, but not IP₃ or the phosphoglycerides, PS or PC, greatly inhibited the ability of talin to crosslink actin filaments. The phosphoinositides bound talin to form talin/phosphoinositide complexes, which prevented talin from effectively
interacting with actin filaments. The detergent Triton X-100 or divalent cations, such as Ca\(^{2+}\), decreased the effect of the phosphoinositides on the talin-actin interaction.

Cosedimentation assays, low shear viscometry, and negative staining showed that platelet talin interacted with actin in a fashion similar to that of smooth muscle talin. However, some differences were noted between the two talins, with platelet talin exhibiting lower actin crosslinking activity.
INTRODUCTION

Dissertation Organization

The body of this dissertation consists of three papers that have been or will be submitted for publication, and one additional chapter. The additional chapter, which follows the first paper, contains my results that will be included in two papers being prepared for submission to The Journal of Biological Chemistry by Dr. Jean Schmidt, and on which I am a coauthor. I was responsible for nearly all of the experiments described in this dissertation. Jean Schmidt prepared some of the talin and actin used in the research of the first two chapters and she prepared the 190 kDa talin fragment that I used in some experiments. The three papers and the extra chapter are preceded by this Introduction, which includes a Literature Review, and will be followed by an Overall Summary chapter. The references cited in the Introduction and Overall Summary are listed in a Literature Cited chapter at the end of the dissertation.

Literature Review

Actin-membrane attachment sites

The interaction between the actin-based cytoskeleton and the plasma membrane is important in controlling cell shape, stabilizing attachments to other cells and to the substrate, regulating cell migration, and responding to growth factors and other external stimuli (Niggli and Burger, 1987; Luna and Hitt, 1992; Clark and Brugge, 1995). This diversity of cellular functions is regulated by several different biochemical mechanisms that mediate the
interactions between the plasma membrane and the cytoskeleton (for reviews see Luna and Hitt, 1992; Simon and Burridge, 1994). There are four major families of receptors on the cell membrane that mediate cell adhesion to other cells or to extracellular matrix: (1) cadherins; (2) members of the Ig superfamily; (3) selectins; and (4) integrins. The first three families primarily mediate cell-cell interactions, whereas the integrins are primarily involved in attachment of the cell to the substratum (Luna and Hitt, 1992; Hynes, 1994; Rosales et al., 1995). The cytoplasmic domains of these transmembrane receptors also may be involved in linking actin filaments to the cell membrane. The attachment of the cytoskeleton to the cell membrane has been fairly well-described in the erythrocyte, but much less is known about the proteins involved in this linkage in most other cells (Carraway and Carraway, 1989; Luna and Hitt, 1992). In the erythrocyte, the cytoskeleton consists of a meshwork of short actin filaments that are connected to the membrane via a multi-protein link involving actin, spectrin, protein 4.1, ankyrin, band 3 (the anion exchanger), and glycophorin C (Luna and Hitt, 1992).

In resting platelets, ABP-280 (filamin) is believed to play a major role in forming a cytoskeletal meshwork, similar to the one found in erythrocytes, by binding directly to GP Ib-IX, a transmembrane glycoprotein complex, and to actin filaments, even though spectrin and associated proteins also are present (Luna and Hitt, 1992).

In skeletal muscle cells, dystrophin, a 427 kDa member of the spectrin superfamily, has been shown to link F-actin to a specific set of glycoproteins present at or in the plasma membrane at myotendinous and neuromuscular junctions and at cos iameric sites (Luna and Hitt, 1992; Campbell, 1995). There also is a set of proteins, which includes talin, vinculin, α-
actinin, and filamin, which is thought to link actin filaments to the cell membrane via integrins at or near these sites, and components of the spectrin-based system are present as well (Luna and Hitt, 1992; Simon and Burridge, 1994; McDonald et al., 1995).

In cultured cells, the cell-matrix attachment sites are known as focal adhesions, focal contacts, or adhesion plaques (Burridge et al., 1988). There are several structures found in living tissues that appear to be homologous with focal adhesions, including the membrane-associated dense bodies of smooth muscle, the myotendinous junctions of skeletal muscle, and the adhesions made by activated platelets (Simon et al., 1991). In these areas, actin filaments are anchored to the cytoplasmic face of the plasma membrane at the sites where the cell interacts directly with the extracellular matrix, primarily through the integrins. Integrins are transmembrane, heterodimeric proteins composed of α and β subunits (Clark and Brugge, 1995). The β subunits are highly conserved while the α subunits are more varied. Cells express specific integrins with specific αβ combinations on their surface, which enable them to bind selectively to different extracellular matrix components (Albelda and Buck, 1990; Juliano and Haskill, 1993; Hynes, 1994). The cytoplasmic domain of integrin can bind to talin (Horwitz et al., 1986) and to α-actinin (Otey et al., 1990) each of which, in turn, may bind to actin filaments and thereby link them directly and/or indirectly to the plasma membrane (Luna and Hitt, 1992; Simon and Burridge, 1994). The redundancy of the membrane-cytoskeleton interactions at the cell-matrix attachment sites might enable cells to respond to different external stimulation.
Considerable interest has been directed towards understanding the proteins involved in linking actin filaments to the plasma membrane at adhesion plaques. As a result, a number of the molecular components, including talin and vinculin, have been identified (Geiger, 1983; Niggli and Burger, 1987; Lo and Chen, 1994). In cultured fibroblasts, talin has been localized by immunofluorescence in adhesion plaques, in the ruffling membranes and leading lamellae of the cell periphery, and in fibrillar patterns that align with microfilament bundles and/or with surface fibronectin (Burridge and Connell 1983a). Since then, talin has been found to be present primarily at the cytoplasmic face of a variety of cell-matrix junctions such as membrane-associated dense bodies in smooth muscle (Volberg et al., 1986), myotendinous junctions (Tidball et al., 1986), neuromuscular junctions (Sealock et al., 1986), and costameres in striated muscle (Belkin et al., 1986; Niggli and Burger, 1987). In general, talin is found where integrins are found, which is typically at regions of cell-matrix association (Beckerle and Yeh, 1990). Talin is absent from cell-cell junctions, such as zonulae adherens in epithelial cells (Geiger et al., 1985), even though other proteins that colocalize with talin at cell-matrix attachment sites, such as vinculin, α-actinin, and actin, can also be found at cell-cell attachment sites. These cellular locations suggest that talin could function either in the attachment of microfilaments to the plasma membrane or in the organization of microfilaments close to membrane attachment sites (Burridge and Connell, 1983a; Simon and Burridge, 1994).

Talin was discovered in 1982 (Burridge et al., 1982), was first purified from chicken gizzard by Burridge and Connell (1983b), and characterized later by Molony et al. (1987).
Talin is a high molecular weight protein that migrates with an apparent molecular mass of 225 kDa by SDS-PAGE. Electron microscope observations of rotary shadowed preparations showed that talin is a spherical protein at low ionic strength, but unfolds to become an elongated molecule with an average length of 60 nm at approximately physiological conditions or at higher ionic strength (Molony et al., 1987). Molony et al. (1987) also reported that talin is monomeric at concentrations less than 0.72 mg/ml, but exhibits the ability to self-associate into dimers at higher protein concentrations. More recent studies, however, have suggested that smooth muscle talin also is present as a dimer at lower concentration (0.5 mg/ml) (Muguruma et al., 1992). It has been speculated that the ability of talin to form dimers (i.e., to self-aggregate) is relevant to its function, such as cross-linking two structures (Molony et al., 1987).

At about the time talin was discovered, a protein called P235 (molecular mass of ~ 235 kDa by SDS-PAGE) that accounts for 3-8 % of total platelet protein was isolated from human platelets by Collier and Wang (1982). It is now well established that P235 corresponds to the platelet form of talin as shown by immunological crossreactivity, similar biophysical characteristics and one-dimensional peptide maps, and an ability to interact with vinculin (O'Halloran et al., 1985; Beckerle et al., 1986). Talin is now the commonly used name for the protein. The molecular structure of platelet talin has recently been studied using a variety of methods including rotary shadowing, analytical ultracentrifugation, and chemical crosslinking (Goldmann, et al., 1994). The results of that study showed that native platelet talin can exist as a dumbbell-shaped antiparallel homodimer with an average length of ~ 51 nm even at relatively low concentrations (0.1-0.4 mg/ml).
More information on the molecular structure of talin became known when murine talin was cloned and sequenced (Rees et al., 1990). The cDNA derived primary structure of talin can be considered as two domains. The first N-terminal 600 residues are highly polar (28% charged residues) and show no internal homologies, whereas the last 1,900 residues are highly enriched in alanine (18%), poor in aromatic residues (2%), and show apparent internal homologies (McLachlan et al., 1994). The C-terminal 60 residues are highly charged (>30%) (Rees et al., 1990). Secondary protein structure predictions suggest a high content of α-helix (McLachlan et al., 1994), consistent with the high α-helix content measured for talin (~80%) (Molony et al., 1987). The molecular mass of murine talin estimated from the cDNA sequence is 269,854 Da, which is larger than its apparent molecular weight determined by SDS-PAGE. This discrepancy is likely to be a consequence of the unusual amino acid composition, secondary structure of talin (Rees et al., 1990), and/or post-translational modifications of talin, such as glycosylation (Hagmann et al., 1992). The amino acid sequence of the large ~190-200 kDa C-terminal tail region, which contains the binding sites for vinculin, integrin and actin (Simon and Burridge, 1994), shows a strong but irregular pattern of repeats as analyzed by Fourier methods (McLachlan, et al., 1994). There are between 50 and 65 copies of this motif in the C-terminal tail region, which are likely to be short α-helical segments (McLachlan, et al., 1994). Proline residues often occur between the motifs. Even though talin does not share sequence homology with other proteins in this region, the series of short helical segments are somewhat analogous to those seen in proteins such as spectrin, α-actinin and dystrophin (McLachlan, et al., 1994), which belong to a group of actin-crosslinking proteins. A 200-220 amino acid segment from the N-terminal
region of talin shares homology with an N-terminal segment of proteins from the band 4.1 family (Rees et al., 1990), including band 4.1, which helps link the spectrin-actin cytoskeleton of erythrocytes to the plasma membrane (Luna and Hitt, 1992), ezrin, which is clearly localized in a submembranous region in the brush border of intestinal epithelial cells, moesin (Lankes and Furthmayr, 1991), and a protein-tyrosine phosphatase-related protein termed PTPH1 (Yang and Tonks, 1991). It has been suggested that the homologous regions in these proteins are involved in their binding to the cell membrane (Rees et al., 1990; Yang and Tonks 1991).

To study the in vivo function of talin in cultured cells, talin was inactivated by microinjection of antibodies against talin into fibroblasts (Nuckolls et al., 1992). The results of that study showed that the effect of the injected anti-talin antibodies on cell spreading was dependent upon how recently the fibroblasts had been plated. Cells that were in the progress of spreading on a fibronectin substratum, and which had newly developed focal adhesions, were induced to round up and to disassemble many of the adhesions. However, in cells that had been allowed to spread completely before they were microinjected with anti-talin antibody, focal adhesions remained intact, and the morphology of the cells was not affected. These results indicate that talin is essential for the spreading and migration of fibroblasts on fibronectin, as well as for the development and initial maintenance of focal adhesions on this substratum (Nuckolls et al., 1992). Recent studies, in which talin was down regulated by an antisense RNA strategy in Hela cells, demonstrated that talin plays a central role in establishment of cell-matrix contacts (Albiges-Rizo et al., 1995).

Post-translational modifications of talin, such as proteolysis, may have physiological
significance relative to structural changes that a cell undergoes (Beckerle and Yeh, 1990).
Talin can be proteolyzed by a variety of proteases, including calpain, into two prominent
proteolytic fragments, one of 47 kDa consisting of residues 1-434 (the N-terminus of talin)
and one of ~ 190-200 kDa containing residues 435-2541 (the C-terminus of talin)
(O'Halloran et al., 1985; Beckerle et al., 1986; Rees et al., 1990). Calcium-dependent
proteolytic cleavage of talin occurs in vivo when activated platelets aggregate with each
other (Fox et al., 1985). Interestingly, the calcium-dependent protease type II (CDP-II or m-
calpain) has been shown to colocalize with talin in adhesion plaques of cultured cells
(Beckerle et al., 1987). This implicates involvement of proteolysis in platelet activation and
clot formation. However, there has been no clear evidence demonstrating a relationship
between proteolysis of talin and platelet function (Wencel-Drake et al., 1991). The function
of talin's two proteolytic fragments was studied by microinjection and indirect
immunofluorescence (Nuckolls et al., 1990). It was shown that the large talin fragment
(>190 kDa) was incorporated quickly into focal contacts and zonulae adherentes, where it
remained stable for at least 14 hours. The small fragment (~ 47 kDa) associated with focal
adhesions of the fibroblasts, but was also found distributed in the cytoplasm and the nucleus.
In the same set of experiments, intact talin was localized to focal contacts, but was excluded
from the zonulae adherentes, which are cell-cell type adherens junctions (Nuckolls et al.,
1990). These experiments suggest that talin has at least two sites that contribute to its
location in focal adhesions, one in the N-terminal domain and one in the C-terminal domain.
In resting platelets, talin is distributed throughout the cytoplasm. However, upon activation
by thrombin, talin becomes translocated to the submembrane areas. This translocation
appeared to be accompanied by the proteolytic cleavage by calpain (Beckerle et al., 1989). One of the objectives of my Ph.D. research was to understand the effect of proteolysis of talin on its ability to interact with actin filaments.

Talin can be phosphorylated at multiple sites on serine, threonine, and tyrosine residues. It is an in vitro substrate for purified protein kinase C at serine and threonine residues (Litchfield and Ball, 1986). When chicken embryo fibroblasts were exposed to the tumor-promoting phorbol ester, 12-myristate 13 acetate, which augments protein kinase C activity, phosphorylation on serine and threonine residues of talin increased three fold (Beckerle, 1990). The phosphorylation sites are located mainly on the 47 kDa talin fragment (Beckerle, 1990). A recent study showed that the 47 kDa fragment of talin also is a substrate for protein kinase P, which is a protein kinase that exhibits phospholipid-stimulation in the presence of Mn$^{2+}$ (Simons and Elias, 1993). The biological significance of talin phosphorylation remains unknown. In BS-C-1 cells, a tumor promoter-induced increase in talin phosphorylation was correlated with a dramatic loss in the integrity of focal contacts and organized actin filament arrays (Turner et al., 1989). In chicken embryo fibroblasts, a similar increase in talin phosphorylation did not result in a detectable loss of established adhesion plaques or stress fibers; however, in this case, the tumor promoter did induce a loss of talin-rich focal contact precursors (Beckerle, 1990).

Viral transformation can also induce dramatic changes in the organization of the cytoskeleton and adhesion plaques. Certain oncogene tyrosine kinases, such as pp60$^{v-src}$, are localized at adhesion plaques (reviewed in Kellie, 1988). A number of adhesion plaque proteins, including talin and integrin, are phosphorylated on tyrosine residues by pp60$^{v-src}$ in
Rous Sarcoma Virus (RSV) infected cells (Pasquale et al., 1986; DoClue and Martin, 1987), but talin phosphorylation is not strictly correlated with the development of the transformed morphology in RSV infected cells (DoClue and Martin, 1987). Interestingly, phosphorylated integrin isolated from RSV-transformed cells fails to bind talin in equilibrium gel filtration assays (Buck and Horwitz, 1987). This suggests that modulation of the phosphorylation state of integrin can affect its ability to interact with talin and, thus, may be in part responsible for the transformed phenotype (Beckerle and Yeh, 1990).

The interaction of interleukin 1β and its receptor, which is primarily localized at focal adhesions, has been shown to induce rapid phosphorylation and redistribution of talin, which was accompanied by a simultaneous disorganization of the cytoskeleton (Quarnstorm et al., 1991). This suggests that the action of interleukin on fibroblasts may be partially mediated by direct phosphorylation of talin via activation of a protein serine/threonine kinase, which leads to changes in its interactions with transmembrane linkage proteins and the cytoskeleton (Quarnstorm et al., 1991). An interaction between talin and actin filaments has been identified (Muguruma et al., 1990, Kaufmann et al., 1991, Schmidt et al., 1993) and, therefore, post-translational modifications of talin may also have an effect on this important interaction.

As discussed earlier, in general, cell-matrix adhesion plaques in different tissues all appear to contain the same set of cytoskeletal proteins, e.g., actin, talin, vinculin, α-actinin, filamin, integrin (reviewed in Geiger 1983; Niggli and Burger, 1987; Rosales et al., 1995), which may be due to the similarity of cytoplasmic domains of the various integrins that are located at these sites (Simon et al., 1991). It is likely, therefore, that the interactions between
talin and other actin-membrane attachment site proteins play an important role in events that occur at these sites. Talin has so far been shown to interact in vitro with vinculin, metavinculin, integrin, and actin (reviewed in Simon et al., 1991). Recently, talin has also been shown to interact in vitro with dystrophin (Senter et al., 1993) and focal adhesion kinase (FAK) (Chen et al., 1995).

Vinculin has a calculated molecular mass of 117 kDa by cDNA sequence analysis (Coutu and Craig, 1988; Price et al., 1989). The sequence of vinculin shows a remarkable division of the molecule into an acidic (pI ~ 5.4) N-terminal domain of about 90 kDa separated from an extremely basic (pI ~ 9.7) C-terminal domain of about 25 kDa by a proline-rich stretch of about 50 amino acids. It is another protein localized mainly at adhesion plaques and has been shown to interact with talin in vitro with a moderately high affinity (Kd of ~10^-8 M) by a number of techniques, including sucrose density gradient centrifugation, gel filtration, co-immunoprecipitation, and gel overlay methods (Otto, 1983; Burridge and Mangeat, 1984; Beckerle and Yeh, 1990). Vinculin can be digested with V8 protease between residues 837-879 to generate two major fragments of approximately 90 kDa (N-terminus) and 30 kDa (C-terminus). The talin binding sites were localized to the 90 kDa vinculin fragment (Groesch and Otto, 1989; Jones et al., 1989), and the affinity of the 90 kDa domain for talin is about 3-fold higher than that of intact vinculin (Groesch and Otto, 1989). The latter result recently has been attributed to the intramolecular interaction of the 90 kDa and the 30 kDa fragments of vinculin molecules (Johnson and Craig, 1994). Other studies have shown that the N-terminal 258 amino acids of the vinculin molecule are required to form a functional talin binding domain (Gilmore et al., 1992). The vinculin binding site on talin has been localized
to the large 190 kDa talin fragment, which contains the C-terminal 435-2541 residues (Rees et al., 1990) and which can be generated by a variety of proteases including calcium-dependent protease (calpain) (O'Halloran and Burridge, 1986). Lee et al. (1992) later showed that there are two vinculin binding sites on talin, both located in the large 190 kDa portion of the molecule. One was located between residues 1653-1848. A second was located between residues 483 and 1652. An interaction between talin and metavinculin has also been shown by the gel overlay method (Burridge and Mangeat, 1984). Vinculin and metavinculin, a highly homologous 152 kDa protein, are derived from a single gene by alternative splicing of a 207-base pair exon unique to metavinculin (Byrne et al., 1992). Thus, the interaction between talin and metavinculin would be expected.

Integrins are a family of transmembrane glycoproteins, which are major cell surface receptors for various extracellular matrix proteins. They are heterodimeric proteins composed of $\alpha$ and $\beta$ subunits (Albelda and Buck, 1990; Simon and Burridge, 1994; Rosales et al., 1995). Integrins can be divided into groups based on their $\beta$ subunit. The $\beta$ subunits of various cell surface receptors are highly homologous with each other and so, too, are the $\alpha$ subunits; however, the $\alpha$ and $\beta$ subunits of a given integrin are not closely related. The various combinations of $\alpha$ and $\beta$ subunits allow integrins to recognize a wide variety of ligands (Hynes, 1994; Clark and Brugge, 1995). The large extracellular domains of the integrins are responsible for ligand binding, and the relatively short cytoplasmic domain of the $\beta$ subunit often interacts with cytoskeletal proteins (Hynes 1992; Simon and Burridge, 1994). The association of talin with integrins of the $\beta_1$ class exhibits a low affinity interaction with a $K_d$ of $\sim 10^{-6}$ M (Horwitz et al., 1986). These investigators also reported that the talin-
integrin complex did not appear to affect either the ability of talin to bind vinculin or the ability of integrin to bind fibronectin. The native integrin heterodimer was required for talin binding (Buck et al., 1986). So far, the interaction in vitro between talin and integrin has only been shown by one method, namely equilibrium gel filtration (Horwitz et al., 1986), and the direct association between talin and integrin within cells has not been proven (Simon and Burridge, 1994).

Early studies (Burridge and Mangeat, 1984) failed to detect any direct interaction between talin and actin. Based on in vitro binding assays of purified proteins, a potential multi-protein bridge connecting the membrane to actin filament bundles was proposed (Burridge et al., 1988). Because an interaction between vinculin and α-actinin, another actin-binding protein, had been reported (Wachsstock et al., 1987), actin filaments might be linked to the cytoplasmic face of the plasma membrane sequentially via integrin, talin, vinculin, and α-actinin (Burridge et al., 1988). Alternative connections between the cytoskeleton and plasma membrane almost certainly exist because an interaction between α-actinin and integrin has also been identified (Otey et al., 1990), which would provide a more direct linkage between the cytoskeleton and plasma membrane via these two proteins (Burridge et al., 1990; Simon and Burridge, 1994). Recent studies have shown that vinculin has an actin-binding site on its 30 kDa domain whose activity is regulated by the intramolecular interaction between it and the 90 kDa N-terminal head domain (Johnson and Craig, 1995). Intact vinculin shows little if any actin binding activity (Evans et al., 1984), but the isolated 30 kDa fragment crosslinks and bundles actin filaments strongly (Johnson and Craig, 1995). The physiological significance of the intramolecular interaction of vinculin is
not yet clear, and the \textit{in vivo} agents that may regulate the intramolecular interaction that controls vinculin's actin binding properties have not been identified. The identification of a vinculin-actin interaction \textit{in vitro} provides an alternative linkage between the actin-based cytoskeleton and the plasma membrane. The interactions among the proteins at focal adhesions appear to be very complex as more and more proteins, as well as interactions between and among them, have been and presumably will continue to be identified.

Although direct binding between talin and actin was identified by Muguruma et al. (1990), there is little agreement on the nature of the interaction. This interaction supposedly involves the binding of talin to both monomeric actin (G-actin) and polymerized actin (F-actin) as detected by cosedimentation, gel filtration, and chemical crosslinking (Muguruma et al., 1990, 1992). Muguruma et al. (1990) used talin purified from avian smooth muscle and showed that it binds to actin filaments and slightly increases the viscosity of F-actin solutions. However, no clear evidence of actin crosslinking activity was detected. Discovery of the direct talin-actin interaction generated yet another alternative direct linkage between actin filaments and the plasma membrane, i.e., talin might link actin to the transmembrane integrins (Simon et al., 1991). The actin binding site(s) in talin has been localized to the 190 kDa fragment, the major fragment generated by calpain (Muguruma et al., 1990, 1995). However, the specific binding sequence site(s) has not been identified. Human platelet talin has been reported to increase the nucleation process of actin filament formation and to decrease the viscosity of F-actin solutions (Kaufmann et al., 1991). The reported binding constant is 0.25 \( \mu \text{M} \), with a maximal binding molar ratio of 3 G-actins to 1 talin. Isenberg and Goldmann (1992) suggest this implicates talin as a key protein in mediating actin
filament assembly at the cell membrane. Schmidt et al. (1993) showed that talin bound to actin filaments and increases viscosity of the F-actin solutions. The interaction is much stronger at low pH and low ionic strength, and weakens as pH and/or ionic strength increases. Gelating of the F-actin solution by talin suggested the possibility that talin possessed actin crosslinking activity (Schmidt et al., 1993).

The apparent conflicts in the results reported on talin from different laboratories may be due to (1) the differing degree of protein homogeneity, (2) the different species and tissues used to prepare the talin, (3) the different types and/or degrees of post-translational modifications, and (4) the different ionic conditions employed in the experiments. Although a direct interaction between talin and actin has been shown, the regulation and exact nature of the interaction have yet to be clarified. A major objective of my Ph.D. research was to improve our understanding of the nature of the talin-actin interaction.

**Actin**

The actin molecule has been highly conserved during evolution by showing minimal differences in the actin sequences of different tissues and species. Multiple actin genes have been identified and found to be expressed in different tissues, but they all share a high degree of homology in sequence (Bamburg and Berstein, 1991). The structure of the actin molecule has been indirectly resolved by subjecting crystals formed from 1:1 complexes of actin with DNase I to X-ray diffraction analysis. The structure of the DNase I was then subtracted from the density map because it has been determined independently (Kabsch et al., 1990). The G-actin molecule consists of a single polypeptide chain of 375 amino acids (molecular weight
of 42,000) with a pI of ~ 5.4, a tightly complexed nucleotide (ATP or ADP) and divalent cation, and has overall dimensions of ~5.5 x 5.5 x 3.5 nm (Kabsch et al., 1990). The actin monomer is composed of a large and a small domain (actually they are nearly the same size), separated by a cleft in which the nucleotide (ATP or ADP) and an associated calcium ion are bound (Kabsch et al., 1990). There is one high affinity and several low affinity cation binding sites in the actin molecule. The small domain contains both the N- and C-termini, and can be further divided into subdomains 1 and 2, whereas the large domain can be further divided into subdomains 3 and 4. Subdomains 1 and 3 are structurally similar, suggesting that they may have arisen from gene duplication (Kabsch et al., 1990).

Monomeric actin (G-actin) of molecular weight 42,000 assembles in the presence of MgCl₂ or KCl to form linear polymers consisting of two staggered parallel rows twisted in a right-handed helix (F-actin), with a crossover occurring every 13 actin molecules or 36 nm along the actin filament (Kabsch et al., 1990). The actin monomer is arranged in the actin filament so that its large domain is located in the center of the filament, while its small domain is exposed (Kabsch et al., 1990). The actin polymerization reaction is reversible, and the extent of polymerization is determined by the temperature, ionic conditions, and the presence of actin binding proteins in the solution (for reviews see Stossel et al., 1985; Pollard and Cooper, 1986). Both hydrophobic and electrostatic forces are believed to contribute to the noncovalent bonds holding the subunits together within the polymer. As mentioned previously, actin monomers bind the adenine nucleotides ATP and ADP, and actin polymerization is associated with the hydrolysis of actin-bound ATP to ADP. Actin polymerization can be initiated by addition of monovalent and/or divalent cations (Stossel et
Actin filaments are polar structures, and have "barbed" (+) and "pointed" (-) ends, which are often determined by labelling the filaments with heavy meromyosin (Pollard, 1986). The concentration of free monomers required to maintain the steady state, known as the critical monomer concentration, is lower at the barbed end than at the pointed end. At approximately physiological conditions, these actin concentrations are 0.1 μM and 1.5 μM, respectively. Therefore, actin monomers add more rapidly to the barbed end than to the pointed end of an actin filament (Stossel et al., 1985; Pollard and Cooper, 1986).

Actin polymerization (for reviews see Stossel et al., 1985; Pollard and Cooper, 1986) has at least three major steps: (1) the activation step, which is solely dependent on the salt concentration, and leads to a conformational change in the monomeric actin molecules; (2) the nucleation step, which is the formation of dimeric and trimeric actin nuclei and is generally the rate-limiting step of actin polymerization; and (3) the elongation step, which is the process of adding actin monomers to the nuclei and growing actin filaments. Elongation of actin filaments proceeds at a high rate as soon as stable nuclei have been created.

Actin binding proteins can be classified into five groups according to their effects on different steps of actin polymerization and on F-actin organization (for reviews see Stossel et al., 1985; Pollard and Cooper, 1986; Hartwig and Kwiatkowski, 1991) as follows: (1) monomer sequestering proteins, including profilin, thymosin β-4, DNase I, vitamin D-binding proteins, etc., which predominantly bind to actin monomers and prevent actin from polymerizing; (2) filament-severing proteins, including gelsolin, fragmin, villin, severin, etc., which sever actin filaments and also nucleate and cap actin filaments in a Ca^{2+}-dependent
way; (3) filament end-blocking (capping) proteins, including CapZ, CapG, etc., nearly all of which bind to the barbed end of actin filaments and inhibit filament elongation in a calcium independent way. For many of these proteins, a nucleating activity also has been reported, but they normally do not have severing activities; (4) F-actin side-binding proteins, including tropomyosin, caldesmon, etc., which bind laterally to the sides of actin filaments; and (5) F-actin crosslinking proteins, including α-actinin, filamin, spectrin, fimbrin, ABP-50, etc., which bind to actin filaments and crosslink actin filaments into networks and/or bundles.

About 20 kinds of different actin binding domains have been identified, which don't display sequence homology with each other or with other non-actin binding proteins. Quite often, however, there is a sequence homology, or secondary structure similarities, among the proteins within the same group (e.g., the spectrin super family) (Hartwig and Kwiatkowski, 1991). One actin-binding protein may have multi-actin binding activities due to multiple actin binding domains. The activities of a number of actin binding proteins have been shown to be regulated by phosphoinositides (Isenberg, 1991; Janmey, 1994).

Actin-binding proteins and phosphoinositides

Inositol-containing phospholipids are ubiquitous components of eukaryotic cells (for reviews see Berridge, 1987; Majerus, 1992). They constitute 2-8% of total phospholipids. Compared to all the other phospholipids in the membrane, phosphatidylinositol (PI) is unique in that it can be further phosphorylated. A PI kinase transfers a phosphate from ATP onto the 4-position of the inositol head group to give phosphatidylinositol 4-phosphate (PIP). A separate PIP kinase adds another phosphate to the 5-position to give phosphatidylinositol
4,5-bisphosphate (PIP<sub>2</sub>). Among the three phosphoinositides, PI accounts for more than 80% of the total.

Increased hydrolysis of PIP<sub>2</sub> to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>) has been observed in many different cell types in response to a variety of external stimuli including neurotransmitters, hormones, growth factors, etc., and has led to the proposition of a bifurcating signal transduction pathway (Berridge, 1987; Berridge and Irvine, 1989). The stimulation of cell surface receptors initiates hydrolysis of the membrane-bound PIP<sub>2</sub> by phosphoinositide specific phospholipase C (PLC), and provides at least two second messengers, DAG, which remains in the membrane, and IP<sub>3</sub>, which is released into the cytosol (for reviews see Berridge, 1987; Berridge and Irvine, 1989). The DAG stimulates protein kinase C activity, whereas IP<sub>3</sub> binds to its receptor in the endoplasmic reticulum and mobilizes calcium. These signal transduction pathways are now known to regulate a large array of cellular processes, including metabolism, secretion, contraction, neural activity and cell proliferation (Berridge and Irvine, 1984, 1989; Berridge, 1987; Majerus, 1992). The PIP<sub>2</sub> is then regenerated through a series of phosphorylation steps, and thus completes the so-called PI (phosphoinositol) cycle. However, only a small proportion (10-20%) of the entire phosphoinositide pool is involved in signalling, and only ~ 60% of total PIP<sub>2</sub> in the plasma membrane is hormone-sensitive and available for signalling (Berridge, 1987). Recently, a non-signalling role for the phosphoinositides in vesicular traffic in yeast and mammalian cells has been described (Burgoyne, 1994). A phosphoinositide transfer protein (PITP) was found to transfer phosphoinositides and, to a lesser extent, phosphatidylcholine (PC), between lipid bilayers. The PITP may be involved in exocytosis
and in the maintenance of the PI levels required to provide the substrate PIP$_2$ for phospholipase C (Burgoyne, 1994). The presence of phosphoinositides in the lipid bilayer of organelles might be important in allowing fusion of transport vesicles with their target membranes, such as by inserting newly synthesized PI into membranes and/or in enabling binding of cytosolic and/or cytoskeletal proteins to transport vesicles (Burgoyne, 1994), especially since a number of cytoskeletal proteins have been shown to interact with phosphoinositides (for reviews see Isenberg, 1991; Janmey, 1994).

The following part of my review will emphasize the interaction between phospholipids, especially the phosphoinositides, and actin-binding proteins, and the effects of phosphoinositides on the activities of these actin-binding proteins.

Actin itself has been shown to interact with lipids. At a given surface pressure, increasing the density of positive charges in synthetic lipid monolayers causes a significant increase in the intercalation of the actin within the lipid molecules, thereby indicating that the adsorption of actin is facilitated by electrostatic interactions (Grimard et al., 1993).

*Actin monomer sequestering proteins* - Profilins are small (molecular mass of 12-15 kDa dependent on the source), soluble, basic proteins that are present in fairly high concentrations (30-40 $\mu$M) throughout the cytoplasm. Profilin inhibits actin polymerization by binding to actin monomer at its C-terminus to form a 1:1 complex (profilactin) (Lassing and Lindberg, 1985; Katakami et al., 1992; McLaughlin, 1995), and to a lesser extent to actin filament ends (Stossel et al., 1985; Pollard and Cooper, 1986).

The binding between profilin and actin is regulated by anionic phospholipids (Lassing and Lindberg, 1985; 1988a). Anionic phospholipids, at low ionic strength and relatively high
calcium concentration, interact with isolated profilin and with profiactin causing the dissociation of profiactin, and in turn permitting the released actin to polymerize. Among the anionic phospholipids, PIP$_2$ was found to be the most effective, followed by PIP, PI, phosphatidic acid (PA), and phosphatidylserine (PS), whereas cationic phospholipids (phosphatidylcholine and phosphatidylethanolamine), DAG, and IP$_3$ were inactive (Lassing and Lindberg, 1985; 1988a). As ionic strength was increased to near-physiological levels of KCl and Ca$^{2+}$, the phosphoinositides were the only phospholipids that remained highly active in binding to profilin and in dissociating the profiactin complex (Lassing and Lindberg, 1988a). On the other hand, a decrease in the concentration of calcium increases phosphoinositides' activity (Lassing and Lindberg, 1988a). The binding stoichiometry is about 10 molecules of PIP$_2$ needed to dissociate one molecule of profiactin. The PIP$_2$ acts on profilin or profiactin by binding to profilin, presumably by electrostatic forces between the negatively charged polar head groups of the micelle and the basic side chains on profilin. The increase seen in activity of the phosphoinositides with increasing phosphorylation state of the inositol ring demonstrates the importance of the phosphate groups in the inositol/profilin interaction. The relatively strong interference with this interaction caused by addition of Ca$^{2+}$ also demonstrates the importance of the phosphate groups, because chelation of divalent cations by these phospholipids most likely involves the phosphate groups on the inositol ring. However, IP$_3$ by itself does not cause dissociation of profiactin (Lassing and Lindberg, 1988a). The latter result indicates that the interaction may involve both the inositol ring and the acyl chains.

Profilin, in turn, also regulates the PI cycle by binding to PIP$_2$, and competes efficiently
with platelet cytosolic phosphoinositide-specific phospholipase C (PLC) for interaction with its substrate, PIP$_2$ (Goldschmidt-Clermont et al., 1990). Each profilin obstructs the access of PLC to ~ 10 molecules of PIP$_2$. However, only the activity of the unphosphorylated PLC-$\gamma$ isoform on PIP$_2$ hydrolysis is inhibited by binding of profilin. The phosphorylation of PLC-$\gamma$ by the epidermal growth factor (EGF) or platelet derived growth factor (PDGF) receptors, upon stimulation of the cell, overcomes the inhibition. Profilin does not inhibit the action of another PLC isoform, PLC-$\beta$, on PIP$_2$ hydrolysis (Goldschmidt-Clermont et al., 1991).

The high concentration of profilin in cells and its ability to bind actin monomers, which is regulated by phospholipids, have led to the suggestion that profilin together with phosphoinositides may help buffer the actin monomer concentration in cells. This role is now attributed in part to profilin and mainly to thymosin $\beta$-4 (Safer and Nachmias, 1994), and suggests a close relationship between the PI cycle and microfilament based cell motility (Lassing and Lindberg, 1988b).

The pancreatic secretory protein DNase-I (31 kDa), is another actin monomer sequestering protein, whose enzyme activity can be inhibited by the binding of PIP$_2$ (Yonezawa et al., 1990). DNase-I binds to actin at its N-terminus, which is different from most actin-binding proteins, and depolymerizes actin filaments and binds to the barbed end of actin filaments. Its actin-depolymerizing activity is also inhibited by PIP$_2$ and PIP, but not by PI, PS, PC, IP$_3$, and DAG (Yonezawa et al., 1990).

**Actin filament-severing proteins** - Gelsolin (87 kDa) is a multifunctional actin-binding protein, which (1) binds to actin monomers and stimulates the formation of actin nuclei; (2) acts as a capping protein at the barbed end of actin filaments, which takes place to a small
extent in the presence of submicromolar Ca\(^{2+}\), but to a greater extent at higher Ca\(^{2+}\) concentration; and (3) severs actin filaments in a Ca\(^{2+}\) dependent manner, which does not occur at submicromolar Ca\(^{2+}\) concentration (for reviews see Stossel et al., 1985; McLaughlin, 1995). One gelsolin can complex with two actin monomers, which in turn serves as a nucleus for actin polymerization. Removing Ca\(^{2+}\) with EGTA only dissociates one of the two actin monomers. The resultant EGTA-resistant gelsolin-actin complex cannot sever actin filaments. Micelles of PIP\(_2\) dissociate EGTA-resistant gelsolin-actin complexes, and restore gelsolin's capacity to sever actin filaments *in vitro* (Janmey et al., 1987). The severing function of gelsolin can be almost completely eliminated by low concentrations of PIP\(_2\), even in the presence of 200-1000 \(\mu\)M Ca\(^{2+}\). The half maximal inhibition of its severing function requires 20 moles of PIP\(_2\) for 1 mole of gelsolin. In contrast to the effect on severing, the ability of gelsolin to nucleate filament assembly is much less inhibited by an equivalent amount of PIP\(_2\) (Janmey and Stossel 1987). Gelsolin has at least three actin binding domains (Bryan, 1990), and its actin modulating activities are due to one or a combination of the domains. The site affected by PIP\(_2\) seems to be in the N-terminal half of gelsolin, and two potential PIP\(_2\) binding sites on gelsolin have been located at residues 130-149 (Yu et al., 1992) and residues 150-169, which both contain numerous basic amino acid residues (Janmey et al., 1992). Residues 150-169 may also be its actin binding site (Bryan, 1990). The effect of the lipid is highly specific, as there is no inhibition by PI, PA, DAG, IP\(_3\), glycerophosphatidylinositol 4,5-bisphosphate, or PS. Treatment of PIP\(_2\) with phospholipase A\(_2\) or C, or with an excess of the non-ionic detergents Triton X-100 or Tween-20 before addition to gelsolin eliminates its inhibitory effect, which suggests that micelle structure is
important for PIP$_2$'s function. A similar inhibitory effect occurs only when PIP is extensively sonicated, indicating that the effects of PIP$_2$ or PIP on gelsolin depend on the physical state of the phosphoinositides and, thus, can be affected by a variety of chemical agents. Any molecule that decreases the exposure of the hydrophilic head groups of the phosphoinositides to the solvent decreases inhibition of gelsolin by the phosphoinositides (Janmey et al., 1987). This can be achieved by trapping phosphoinositides in the interior of multilaminar vesicles composed of other lipids, by formation of aggregates containing PIP$_2$ by divalent cations, or by masking of the head groups following binding to profilin (Janmey et al., 1987). Alternatively, an agent that mixes with phosphoinositides at sufficiently high concentrations may also decrease the effect of phosphoinositides on gelsolin, apparently by a dilution effect (Janmey and Stossel, 1989). However, the charged groups on the phosphoinositide head groups do not appear to play a direct role in gelsolin regulation because both PIP and PIP$_2$ inhibit gelsolin's actin binding activities in the presence of millimolar Mg$^{2+}$ concentrations, and the effects of PIP$_2$ are constant over a range of pH within which phosphate groups become protonated (Janmey and Stossel, 1989).

Like profilin, gelsolin also competes with phospholipase C for interaction with PIP$_2$ and, thus, inhibits PIP$_2$ hydrolysis by PLC in platelets. The 1:1 gelsolin/actin complex does not have the inhibitory effect on PIP$_2$ hydrolysis (Banno et al., 1992).

Cofilin (21 kDa) is a widely distributed actin modulating protein that has the ability to bind along the side of F-actin and to depolymerize F-actin in a pH dependent manner (Yonezawa et al., 1990). It depolymerizes F-actin partially at approximately neutral pH and completely at more alkaline pH (>7.3). This pH-dependent change in its depolymerizing
activity is reversible (Yonezawa et al., 1990). It has a sequence homology to that of gelsolin, villin, fragmin, severin, and *Acanthamoeba* profilin in its C-terminal portion (Yonezawa et al., 1990). Under physiological conditions, it can bind both G-actin and F-actin in a 1:1 molar ratio. PI, PIP, and PIP$_2$ have an inhibitory effect on its actin binding activities, whereas PC, PS, and IP$_3$ have little or no effect (Yonezawa et al., 1990). Gel filtration analyses showed that PIP$_2$ bound to cofilin and thereby inhibited the binding of cofilin to G-actin (Yonezawa et al., 1990). The binding site for both actin and PIP$_2$ is located on residues 104-115 in cofilin, which indicates that PIP$_2$ may inhibit the interaction between cofilin and actin by competing with the binding of actin (Yonezawa et al., 1991). The binding of cofilin to PIP$_2$ inhibits PIP$_2$ hydrolysis by PLC-$\gamma$ (Yonezawa et al., 1990). Destrin (19 kDa) is a pH independent actin depolymerizing protein. Destrin's actin binding activity is also inhibited by PI, PIP, PIP$_2$, but not by PC, PS, and IP$_3$, and it is very similar in sequence to cofilin (Yonezawa et al., 1990).

*Actin filament end-blocking (capping) proteins* - CapZ is an $\alpha/\beta$ heterodimer that can bind to the barbed end of actin filaments and nucleate actin polymerization. It does not require Ca$^{2+}$ for these activities (Pollard and Cooper, 1986). Anionic phospholipids, such as PIP$_2$, completely inhibit the ability of CapZ to affect actin polymerization (Heiss and Cooper, 1991). The nucleation and capping activities of CapZ are both inhibited. PIP$_2$ inhibits the binding of actin to CapZ by binding to CapZ at a molar ratio of ~ 400 PIP$_2$ to 1 CapZ (Heiss and Cooper, 1991). All of the anionic phospholipids, including PIP$_2$, PIP, PI, PS, PG, and PA, inhibited CapZ in the order of PIP$_2$ = PIP > others, but the neutral phospholipids PC and PE do not inhibit CapZ (Heiss and Cooper, 1991).
Actin filament side-binding and crosslinking proteins - A recent study has shown that α-actinin from striated muscle is an endogenous PIP2-bound protein, containing a molar ratio of 20-30 moles of PIP2 to one mole of α-actinin (Fukami et al., 1992). The specific interaction between PIP2 and α-actinin regulates the F-actin-gelating activity of α-actinin (Fukami et al., 1992). Exogenously added PIP2 can enhance the actin gelating activity of smooth muscle α-actinin to the level seen for striated muscle α-actinin. However, a large amount of exogenous PIP2 inhibited the gelating activity of α-actinin on F-actin solutions (Fukami et al., 1992). The presence of 0.5% Triton X-100, even at the high concentration of PIP2, permitted the gelating activity of striated muscle α-actinin, suggesting that large PIP2 micelles induce aggregation of α-actinin-PIP2 complexes and prevent actin crosslinking (Fukami et al., 1992).

Both α-actinin and meta-vinculin have been shown to bind to phospholipid liposomes (Niggli and Gimona, 1993). Incubation of α-actinin with vinculin or metavinculin strongly inhibited the binding of α-actinin to phospholipid (Niggli and Gimona, 1993). These results suggest the formation of a ternary complex of vinculin, α-actinin and phospholipid (Niggli and Gimona, 1993). Vinculin and α-actinin were further shown to be present in PIP2-bound forms, and involved in signalling by tyrosine phosphorylation (Fukami et al., 1994).

Micelles of inositol phospholipids have been shown to bind to smooth muscle filamin and inhibit its ability to form a gel of F-actin (Furuhashi et al., 1992). The effectiveness of the phospholipids is in the order of PIP2 > PIP > PI. At a modest concentration of PIP2, activity of filamin is totally inhibited (Furuhashi et al., 1992).

The earliest studies on the interaction between talin and membrane components showed
that talin purified from detergent-lysed human platelets was reconstituted into neutral phospholipid PC bilayers with a low incorporation rate (Heise, et al., 1991). This interaction was greatly enhanced in the presence of negatively charged lipids. Platelet talin purified from detergent-lysed membrane fraction interacted electrostatically and hydrophobically with lipid membranes. The talin isolated from the cytoplasmic pool, however, failed to interact hydrophobically with lipid bilayers, and exhibited only electrostatic interactions with model membranes (Heise et al., 1991). A freeze-thaw technique was used to show a uniform orientation of platelet talin, with its large head group pointing to the exterior of the vesicles (right-side-out orientation) (Heise, et al., 1991). A film balance technique was used to show that fluorescently labelled talin, as well as native talin, interacts with negatively charged lipid monolayers (Dietrich et al., 1993). Further study showed that talin selectively reacts with the hydrophobic part of negatively charged liposome bilayers (Goldmann et al., 1992).

Thrombin proteolytic fragments of talin were purified, and their interactions with liposomes were studied (Niggli et al., 1994). The ~200 kDa fragment was shown to nucleate actin filament formation and reduce the viscosity of filamentous actin, which is comparable to these activities of intact talin. The 47 kDa fragment, however, did not have this activity. The 47 kDa fragment, but not the 200 kDa fragment, interacted specifically with large liposomes containing acidic phospholipids (Niggli et al., 1994). Similar results with acidic phospholipids have been shown with smooth muscle talin (Muguruma et al., 1995). These results indicate that the membrane binding ability of talin is in its N-terminal 47 kDa fragment.

One of the objectives of my dissertation research was to investigate the effect of
phospholipids, especially the phosphoinositides, on the talin-actin interaction.

The physiological significance of the interaction between phosphoinositides and actin-binding proteins remains to be resolved. In skeletal muscle, PIP and PI are found in transverse tubules and terminal cisternae of the sarcoplasmic reticulum, whereas PIP₂ is only found in the transverse tubules but not in terminal cisternae (Milting et al., 1994). In addition, a much greater pool of PIP₂ is located in the sarcolemma some distance away from the triadic junctions (Milting et al., 1994). By immunofluorescent staining, PIP₂ was found to be present in high density in the central areas around nuclei, microfilament bundles, and focal contacts (Fukami et al., 1994). The concentration of PIP₂ in cells such as platelets is around 300 μM when averaged over the entire cell volume (Rittenhouse and Sasson, 1985), which is about the same concentration of unpolymerized actin in platelets (Goldschmidt-Clermont et al., 1990). Therefore, the signal transduction pathway involving phosphoinositides may be directly linked to the cytoskeleton by regulating the activities of actin-binding proteins and, as a result, the organization/reorganization of the cytoskeleton and cell motility.
TALIN CAN CROSSLINK ACTIN FILAMENTS INTO BOTH NETWORKS AND BUNDLES

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Summary

The talin-actin interaction was examined by using negative staining and cosedimentation assays. At pH 6.4 and low ionic strength, talin extensively crosslinked actin filaments into both networks and bundles. The bundles consist of parallel actin filaments with a center-to-center distance of 13 nm, and talin crossbridges spaced at 36 nm intervals along the bundles. As pH was increased stepwise from 6.4 to 7.3, talin's bundling activity was decreased first, then its networking activity. Qualitatively similar results were obtained at pH 6.4 by increasing ionic strength. Chemical crosslinking indicated talin was present as a dimer from pH 6.4 to 7.3, with or without added KCl. The results show that talin can interact directly with actin filaments by formation of actin filament networks and bundles, with the bundles more sensitive to dissolution by increase in pH or ionic strength.

Introduction

Talin is a component of cell-matrix type adherens junctions where actin filaments are linked in some manner to the cell membrane (1,2). Talin has been shown to bind in vitro to vinculin (3-6), the integral membrane protein integrin (7), dystrophin (8), and focal adhesion kinase (9). Early reports, however, found no direct talin-actin interaction (1,4,10). Thus,
talin initially was proposed to be one component in a multi-protein bridge linking actin filaments to the cytoplasmic face of the cell membrane (1). Subsequently, direct interaction \textit{in vitro} between talin and actin was shown (11,12), indicating that talin also might directly link actin filaments to integrin of the cell membrane (13). The reported nature of the talin-actin interaction from different labs varies considerably, however, with talin shown to increase (11,14,15) or to decrease (12,16) viscosity of F-actin solutions. We have demonstrated that the direct talin-actin interaction is very sensitive to ionic conditions such as pH (15) that may explain, in part, variability in reports on talin from different labs. The purpose of this study is to provide a structural basis for the effects of ionic conditions on the talin-actin interaction. We show for the first time that talin can crosslink F-actin into both networks and bundles, and that formation of these macromolecular assemblies is reduced with increasing pH or ionic strength.

\textbf{Materials and Methods}

\textit{Actin and talin preparation} - Porcine skeletal muscle actin and highly homogeneous turkey (gizzard) smooth muscle talin were prepared as described (15). Purity of both proteins is shown in Fig. 1. The modified Lowry method (Sigma) was used to determine protein concentrations.

\textit{Electron microscopy} - Talin (0.10 mg/ml final concentration) and G-actin (0.25 mg/ml final concentration) were mixed in buffer A (10 mM imidazole-HCl, 1 mM ATP, 1 mM \textsuperscript{1}EGTA) at selected pH values, with pH adjustment of all buffers done at 25\degree C. Actin polymerization was induced by either MgCl\textsubscript{2} (2 mM final concentration) or MgCl\textsubscript{2} plus KCl.
Fig. 1. SDS-PAGE of purified talin and actin. Lane 1, talin (5 μg); lane 2, actin (5 μg). T = talin, A = actin.
(2 mM and 200 mM final concentrations, respectively). After addition of MgCl₂ or of MgCl₂ plus KCl, final pH values tested were 6.4, 6.9, or 7.3. After incubation for 1 hr at 25°C, a drop of the actin control or actin-talin mixture was applied to a 400-mesh carbon-coated copper grid, negatively stained with 2% aqueous uranyl acetate, and observed with a JEOL-100 CX II electron microscope operated at 80 kV. Representative areas were photographed. The distance between actin filaments in actin bundles and the spacing between crossbridges along actin bundles were determined from five different preparations of talin and actin. The average distance and spacing were each determined from more than 100 measurements (data reported as means ± S.D.) of randomly selected images.

**Cosedimentation assay** - Talin (0.20 mg/ml final concentration) and G-actin (0.50 mg/ml final concentration) were mixed in buffer A at 25°C. Actin polymerization was initiated by adding MgCl₂ (2 mM final concentration) or MgCl₂ plus KCl (2 mM and 200 mM final concentrations, respectively). The final pH was 6.4. The solution was mixed, incubated at 25°C for 1 hr, and centrifuged at 110,000 x g in a Beckman airfuge for 20 min. Comparable amounts of pellets and supernatants were analyzed by 10% SDS-PAGE (17).

**Chemical Crosslinking** - Talin (0.20 mg/ml) in buffer A containing 2 mM MgCl₂ at pH 6.9 and vinculin (0.20 mg/ml) in 10 mM imidazole-HCl, pH 6.9, were each crosslinked with EDC (20 mM final concentration). Aliquots were taken at specific time points and the reaction was quenched with 20 mM glycine, 20 mM Tris-HCl, pH 8.0. The samples were

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1The abbreviations used: EGTA, ethylene glycol bis (β-amino-ethyl ether) N,N,N',N'-tetraacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EF1α, elongation factor 1α; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
analyzed by SDS-PAGE. The vinculin, which served as a control, was obtained from a peak eluted during the DEAE-cellulose chromatography step used in the talin preparation, and then purified by sequential chromatography on hydroxyapatite (BioRad) and Sephacryl S-300 HR (Pharmacia) columns.

Results

We previously demonstrated a marked pH dependence in the talin-actin interaction, with talin's abilities to cosediment with actin filaments and to increase the low shear viscosity of F-actin solutions significantly increased as pH was lowered (15). The effect of pH on the talin-actin interaction as examined by negative staining is shown in Fig. 2. The actin filament controls formed in the absence of talin at pH 6.4 and 6.9 (Fig. 2A and 2C, respectively) are similar in appearance, with the filaments randomly dispersed. However, as shown in Fig. 2B, talin (at a molar ratio of ~ one 225 kDa talin to thirteen 42 kDa actin monomers) extensively crosslinked actin filaments at pH 6.4 into both networks and bundles. The networks (NW) consist of actin filaments brought into a close or tight meshwork. The bundles varied in appearance. Some appeared as thin sheet-like bundles (TSB), in which several actin filaments were aligned equidistant from each other, with periodic crosslinks (talin particles) giving them a distinct, ordered two-dimensional appearance. Others appeared as tight bundles (TB), in which the adjacent actin filaments were roughly parallel, but not equidistant from each other (Fig. 2B). As shown in Fig 2D, talin still had the ability to crosslink actin filaments at pH 6.9 into networks (NW) and bundles, but the activity was considerably decreased. At pH 6.9 thin sheet-like bundles were absent and the actin bundles often had
Fig. 2. The pH dependence of the talin-actin interaction as shown by electron microscopy. Actin (0.25 mg/ml) in the absence or presence of talin (0.10 mg/ml) was polymerized in buffer A by the addition of MgCl₂ (2 mM final concentration). (A) Actin control polymerized in the absence of talin at pH 6.4; (B) actin polymerized in the presence of talin at pH 6.4. NW = network, TSB = thin sheet-like bundle, TB = tight bundle; (C) actin control polymerized in the absence of talin at pH 6.9; (D) actin polymerized in the presence of talin at pH 6.9. Globular particles often were observed at points of filament intersection in networks (NW). TRA = talin rich actin filaments, BA = bare actin filaments. Bar = 100 nm for A-D.
alternating regions of talin-rich actin filaments (TRA) and bare actin filaments (BA) (Fig. 2D). At pH 7.3, talin's actin filament crosslinking activity was further reduced, with no evidence of actin bundles, and only slight evidence of actin networks (results not shown).

Examples of three actin bundle structures typically observed in the presence of talin at pH 6.4 and low ionic strength are shown at higher magnification in Fig. 3. The thin sheet-like bundle shown in Fig. 3A consists of four parallel actin filaments, with a consistent longitudinal periodicity of crossbridges along the bundle (shown by brackets). Each crossbridge, as defined herein, consists of two closely spaced talin particles (shown as pairs of arrows) perpendicular to the long axis of the bundle. The average center to center distance between adjacent actin filaments in the thin sheet-like bundles, and the spacing of crossbridges along the longitudinal axis of the actin bundles, were 12.9 ± 0.6 nm and 36.2 ± 1.7 nm, respectively. Other actin bundles were observed that exhibited a similar longitudinal periodicity (~36 nm) of talin crossbridges (Fig. 3B), but they were not as highly ordered in appearance as the bundle shown in Fig. 3A, and some actin bundles crosslinked by talin present at pH 6.4 exhibited no obvious longitudinal periodicity (Fig. 3C). We observed throughout our study that actin bundles exhibiting the ~36 nm longitudinal periodicity (e.g., Fig. 3A and 3B) were only observed at low pH (~6.4) and low ionic strength.

The effect of ionic strength on the interaction of talin and actin at pH 6.4 was examined by negative staining and cosedimentation (Fig. 4). Actin filaments, in the absence of talin, formed in the presence of 2 mM MgCl₂ and 200 mM KCl at pH 6.4 (Fig. 4A) were randomly dispersed and similar to the actin filament controls formed in the presence of 2 mM MgCl₂ at pH 6.4 (Fig. 2A). However, in the presence of the 200 mM KCl, talin lost its ability to
Fig. 3. Examples of talin's ability to bundle actin filaments at pH 6.4 as shown by electron microscopy. Actin (0.25 mg/ml) in the presence of talin (0.10 mg/ml) was polymerized in buffer A by the addition of MgCl₂ (2 mM final concentration) at pH 6.4. (A) Some actin filaments were crosslinked by talin (shown by arrows) into thin sheet-like bundles with a longitudinal periodicity (shown by brackets); (B) some actin filaments were crosslinked by talin (shown by arrows) into small bundles with a longitudinal periodicity (shown by bracket); (C) some actin filaments were crosslinked by talin (shown by arrows) into tight bundles with a less distinct longitudinal periodicity. Bar = 100 nm for A-C.
Fig. 4. The effect of ionic strength on the talin-actin interaction as shown by electron microscopy and cosedimentation. (A and B) Electron microscopy. (A) Micrograph of actin (0.25 mg/ml) polymerized in the absence (A) or presence (B) of talin (0.10 mg/ml) by the addition of MgCl₂ and KCl (2 mM and 200 mM final concentrations, respectively). Arrows in B indicate talin particles observed at points of filament intersection in networks. Bar = 100 nm for A and B.

(C) Cosedimentation assay. (a) Talin control in buffer A containing 2 mM MgCl₂; (b) talin/actin mixture polymerized in buffer A containing 2 mM MgCl₂; (c) actin control polymerized in buffer A containing 2 mM MgCl₂; (d) talin control in buffer A containing 2 mM MgCl₂ and 200 mM KCl; (e) talin/actin mixture polymerized in buffer A containing 2 mM MgCl₂ and 200 mM KCl; (f) actin control polymerized in buffer A containing 2 mM MgCl₂ and 200 mM KCl. s = supernatant, p = pellet, T = talin, A = actin.
crosslink actin filaments at pH 6.4 into bundles (cf., Fig. 4B with Fig. 2B and Fig. 3), and its ability to crosslink actin filaments into networks also was significantly reduced (cf., Fig. 4B with Fig. 2B). The results obtained by cosedimentation assays on the effect of ionic strength on the talin-actin interaction at pH 6.4 (Fig. 4C) were in concert with the microscopy results. At low ionic strength (only 2 mM MgCl$_2$ used in actin polymerization), about 90% of the talin cosedimented with the F-actin pellet (Fig 4C, panel b). In contrast, in the presence of 2 mM MgCl$_2$ and 200 mM KCl, approximately 15% of the talin cosedimented with the F-actin pellet (Fig. 4C, panel e), which is still more talin than was sedimented in the pellet of the talin control (panel d) under the same ionic conditions.

Recent crosslinking studies have indicated that talin probably functions as a dimeric molecule (two 225 kDa subunits) or possibly a higher oligomer (14,16,18). Our crosslinking results obtained with the zero-length crosslinker EDC at pH 6.9 and low ionic strength also showed that talin was present primarily as a dimer (Fig. 5A). With increasing crosslinking time, the amount of monomer band decreased concomitantly with an increase in amount of a band corresponding to an ~ 400 kDa protein (Fig. 5A). Vinculin, another adhesion plaque protein (19), remained as a monomer (Fig. 5B), as did G-actin when crosslinked in nonpolymerizing solutions (results not shown). We also observed crosslinking results on talin, similar to those shown in Fig. 5A, when the crosslinking was done at pH 6.9 in the presence of 100 mM KCl, as well as at pH 6.4 or 7.3, with or without 100 mM KCl (results not shown). Thus, talin was present primarily as a dimer from pH 6.4 to 7.3 at low and higher ionic strength. Overall, our crosslinking results on talin were in agreement with those of others (14,16,18).
Fig. 5. Chemical crosslinking of talin with EDC. Aliquots of the talin (A) and of the vinculin control (B) reaction mixtures were taken at specific time points, quenched, and analyzed by SDS-PAGE. Lane 1, proteins before addition of EDC; lanes 2, 3, 4, 5, 6, and 7, are after addition of EDC and represent 0, 10, 30, 60, 120, and 240 min of crosslinking, respectively. Numbers on the left of A correspond to the molecular mass (kDa) of protein standards. d = talin dimer corresponding to an ~ 400 kDa protein.
Discussion

We show for the first time that when actin is polymerized into filaments at pH 6.4 and low ionic strength in the presence of talin, both actin networks and bundles are formed. Talin's actin filament crosslinking activity decreases with increase in pH and/or ionic strength, with its bundling activity lost first. These results provide a structural explanation for the marked pH dependence of the direct talin-actin interaction observed by low shear viscosity and cosedimentation assays (15). The sensitivity of the talin-actin interaction to ionic conditions may explain some of the reported differences in talin's effect on actin among some labs that have used different ionic strength and/or higher pH (~ 7.5-8.0) (4,11,14,15). It does not, however, seemingly account for reports of a talin-induced decrease in viscosity of actin solutions (12,16), in which it was suggested the decrease in actin viscosity, especially at short polymerization times, was due to an increase in number of actin filaments but decrease in filament length. We observed, however, in samples of talin-actin mixtures negatively stained after only five minutes of polymerization, that talin had crosslinked actin filaments into networks and bundles and, in comparison to control F-actin, there was no evidence of shorter filaments (results not shown). An alternative possibility for talin's actin viscosity reducing activity may be the purity of the talin used, which is especially critical at the rather high molar ratio of talin to actin used (~ one talin to two actins) (12,16). Others have reported (13,20), that an actin severing contaminant(s) can be removed from platelet talin prepared by procedures similar to those described (12,16).

The talin crossbridge spacing of ~ 36 nm observed in the well-ordered lattice of the thin sheet-like bundles (Fig. 2B; Fig. 3A and 3B) matches the ~ 36 nm actin filament crossover
periodicity (21) and approximates the longitudinal crossbridge spacing in actin bundles formed by some other actin crosslinking proteins, such as EF1α (ABP-50) [33 nm (22)] and muscle α-actinin [36 nm (23)]. The filament to filament distance of ~13 nm is slightly larger than that in actin bundles formed by EF1α [5 or 8 nm (22)] and fimbrin [9 nm (24)], similar to that of villin [12-13 nm (24)] and much less than muscle α-actinin [36 nm (23)]. The exact arrangement of the talin molecule(s) within the crossbridges will require more detailed analysis. This, too, is complicated because the reported size and shape of talin varies considerably (15,16,25-27), and is evidently dependent upon factors such as protein concentration and ionic strength.

For an actin binding protein to crosslink actin filaments, it is expected to have two or more actin binding domains or one actin binding domain and the ability to self-associate (28). Although the ~190 kDa tail domain generated by calpain cleavage contains talin's actin-binding activity (11,18), the actin binding domain(s) on talin has not yet been determined. Several reports suggest that native talin can (27) or does (14,16,18,25,26) exist as dimers and, thus, talin can be expected to have two (or more) actin binding sites. We have shown talin exists primarily as a dimer from pH 6.4 to 7.3, in the absence or presence of KCl. Thus, the effect of pH and ionic strength on talin's actin crosslinking activity observed herein appears due to an effect on the talin-actin interaction rather than on the ability of talin to self-associate. Several other proteins also have pH-dependent actin binding activities, such as ADF (29), gelsolin (30), τ peptide (31), hisactophilin (32), and EF1α (33). Interestingly, Edmonds et al. (33) reported that increasing pH in the physiological range of 6.6 to 7.0, or increasing the ionic strength, significantly decreased EF1α-mediated F-actin bundling.
activity, a result analogous to what we observed with talin. Several studies (33-35) have shown that intracellular pH is altered in response to different cell signals. It is plausible the marked sensitivity to ionic conditions of the talin-actin interaction is important in cytoskeletal alterations occurring near the cell membrane \textit{in vivo}.

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\textbf{References}


Summary

Electron microscopy of negatively stained preparations was used to examine the effects of selected factors on talin's ability to interact with actin filaments. The results showed that increasing pH from 6.4 to 7.3 progressively decreased talin's ability to crosslink actin filaments into bundles and networks, and eventually eliminated talin's bundling activity. Increasing ionic strength at pH 6.4 by adding as little as 25 mM KCl considerably decreased talin's ability to crosslink actin filaments. Chemical crosslinking of talin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl showed that talin was present primarily as a dimer of two ~ 225 kDa polypeptides from pH 6.4 to 7.3, at both low and higher ionic strength, indicating that talin functions as a dimeric molecule when it crosslinks actin filaments. Thus, the effect of pH and/or ionic strength on talin's actin crosslinking ability appears due to the effect on the interaction between actin and talin, and not on talin's ability to self-associate. Results of electron microscopy demonstrated that even with very short polymerization times talin crosslinked actin filaments into both networks and bundles, without restricting the length of actin filaments in comparison to control F-actin. They also showed that talin crosslinked preformed actin filaments as well as it did actin filaments polymerized in its presence. The 190 kDa calpain generated fragment of talin had the ability to crosslink actin filaments into both networks and bundles. However, it required a slightly lower pH than did intact talin to effectively crosslink actin filaments into both networks and bundles.
Introduction

Talin is a protein localized primarily at cell-matrix attachment sites where actin filaments are attached to the cell membrane. Because early studies indicated no direct talin-actin interaction (Burridge and Mangeat, 1984), it was suggested, based primarily on *in vitro* protein binding studies, that talin was one component involved in a multi-protein bridge that included integrin, talin, vinculin, α-actinin, and actin that links the actin filament-based cytoskeleton to the cytoplasmic face of the cell membrane (Burridge et al., 1988). After a direct interaction *in vitro* between talin and actin was shown (Muguruma et al., 1990), a more direct role for talin in linking the cytoskeleton to the cell membrane was proposed (Simon et al., 1991). However, the nature of the talin-actin interaction reported by different labs has varied considerably. Kaufmann et al. (1991), for instance, reported that talin promoted the nucleation of actin filaments by binding to G-actin, and that talin decreased the viscosity of F-actin solutions. Muguruma et al. (1990, 1992), however, reported that talin increased the viscosity of F-actin solutions. Schmidt et al. (1993) showed that talin has the ability to cosediment with actin filaments and to increase the viscosity of F-actin solutions, and that this ability is enhanced by lowering the pH and ionic strength. As discussed in the previous chapter of this dissertation, my results obtained by electron microscopy have offered an explanation for the direct interaction between talin and actin filaments measured by the cosedimentation and viscometry assays (Schmidt et al., 1993) by showing that talin crosslinks actin filaments into both networks and bundles at pH 6.4 and low ionic strength.

Talin can be catalytically cleaved by a variety of proteases, including the calpains (Beckerle et al., 1986), into two prominent proteolytic fragments of 47 kDa [the N-terminal
head domain containing residues 1-434 (Rees et al., 1990) and ~190 kDa [the C-terminal tail domain containing residues 435-2541 (Rees et al., 1990)]. The ~190 kDa fragment has been shown to interact with actin filaments, whereas the 47 kDa fragment does not (Muguruma et al., 1990, 1995).

The objectives of this study were to examine the effects of selected factors, such as pH, ionic strength, actin polymerization time, and presence of intact vs. the 190 kDa talin fragment, on the interaction between talin and actin filaments.

**Experimental Procedures**

*Protein Preparation* - Talin was purified from turkey gizzard smooth muscle by the method of Schmidt et al. (1993) and Schmidt (1994), and was in a buffer containing 10 mM imidazole, pH 7.0, 0.1 mM PMSF1, 1 μg/ml E-64, and 0.5 mM MCE. Actin was purified from porcine skeletal muscle as described in Schmidt et al. (1993), and was in a buffer containing 2 mM Tris-Cl, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl2, and 0.5 mM MCE. Protein concentrations were determined by the modified Lowry method (Sigma). The purified 190 kDa fragment of talin was prepared as described in Schmidt (1994).

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1The abbreviations used: EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EGTA, ethylene glycol bis (β-amino-ethyl ether) N,N,N',N', tetraacetic acid; MCE, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Electron Microscopy - Talin (0.10 mg/ml final concentration) and G-actin (0.25 mg/ml final concentration) were mixed in buffer A (10 mM imidazole-HCl, 1 mM ATP, 1 mM EGTA) at selected pH values at 25°C. Actin polymerization was then induced by addition of either MgCl₂ (2 mM final concentration) or MgCl₂ (2 mM final concentration) plus selected concentrations of KCl. After incubation for 1 h at 25°C, samples of actin control and of the talin-actin mixture were negatively stained with 2% uranyl acetate, and were observed with a JEOL-100 CX II electron microscope operated at 80 kV as described in Schmidt et al. (1993), with representative areas photographed.

Chemical Crosslinking - Talin (0.20 mg/ml) samples in buffer A containing 2 mM MgCl₂, or 2 mM MgCl₂ and 100 mM KCl, at pH values of 6.4, 6.9, or 7.3 at 25°C were each crosslinked with EDC (40 mM final concentration). Aliquots were taken at specific time points and the reaction was quenched with 40 mM glycine, 40 mM Tris-HCl, pH 8.0 (final concentration). The samples were analyzed by SDS-PAGE (Laemmli, 1970).

Results

The Effect of pH and Ionic strength on the Interaction between Talin and Actin Filaments - Highly homogeneous talin and actin (Fig. 1) were used to examine the ability of talin to crosslink actin filaments at different pH values by using negative staining (Fig. 2). The actin filament controls at pH 6.4, 6.9, and 7.3 (Fig. 2A, 2C, and 2E, respectively) appeared similar, with long single filaments randomly dispersed. At pH 6.4, talin crosslinked actin filaments into both networks (NW) and bundles, including thin sheet-like bundles
Fig. 1. Analysis of purified talin and actin by SDS-PAGE. Lane 1, 5 µg actin; lane 2, 5 µg talin. T = talin, A = actin.
Fig. 2. Effect of pH on the ability of talin to crosslink actin filaments as shown by electron microscopy. Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of talin (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ (final concentration) at pH 6.4, 6.9, or 7.3. All samples were incubated for 1 h at 25°C and negatively stained. (A) Actin control polymerized at pH 6.4; (B) actin polymerized in the presence of talin at pH 6.4. TSB = thin-sheet like bundle, TB = tight bundle, NW = network; (C) actin control polymerized at pH 6.9; (D) actin polymerized in the presence of talin at pH 6.9. TRA = talin-rich actin filaments, BA = bare actin filaments; (E) actin control polymerized at pH 7.3; (F) actin polymerized in the presence of talin at pH 7.3. Bar = 100 nm in A-F.
(TSB) and tight bundles (TB) (Fig. 2B). The thin sheet-like bundles have an ordered two-dimensional appearance (Fig. 2B), which has been discussed in the previous chapter of this dissertation. At pH 6.9, the ability of talin to crosslink actin filaments decreased, as shown by the presence of fewer bundles and less tight networks (Fig. 2D). The appearance of the bundles formed at pH 6.9 also was different from the bundles formed at pH 6.4 by having alternating talin-rich actin filament areas (TRA) and bare actin filament areas (BA), as was also discussed in the previous chapter of the dissertation. At pH 7.3, talin's crosslinking activity is nearly absent (Fig. 2F), with no bundles present, and only slightly crosslinked networks evident. These results showed that talin's ability to crosslink actin filaments into both networks and bundles was very pH dependent, being stronger at low pH and weaker as pH was increased.

To examine the effect of ionic strength on the ability of talin to crosslink actin filaments, different concentrations of KCl were present when actin polymerization was initiated (Fig. 3). The actin filament controls polymerized at pH 6.4 in the presence of 25 mM KCl (Fig. 3A) or 75 mM KCl (Fig. 3C) appeared similar to those formed in the absence of KCl (Fig. 2A). As was shown in Fig. 2B, at pH 6.4 and low ionic strength, talin crosslinked actin filaments extensively into networks and bundles. However, at pH 6.4 with 25 mM KCl present, talin's ability to crosslink actin filaments was decreased considerably as shown by the presence of fewer bundles and less highly crosslinked networks (Fig. 3B). The thin sheet-like bundles seen at pH 6.4 with no KCl present (Fig. 2B) were absent, and those bundles present (Fig. 3B) appeared similar to the tight bundles formed with no KCl present (Fig.
Fig. 3. **Effect of ionic strength on the ability of talin to crosslink actin filaments at pH 6.4 as shown by electron microscopy.** Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of talin (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ (final concentration) and selected concentrations of KCl at pH 6.4. All samples were incubated for 1 h at 25°C and negatively stained. (A) actin control polymerized with 2 mM MgCl₂ and 25 mM KCl; (B) actin in the presence of talin polymerized with 2 mM MgCl₂ and 25 mM KCl. TB = tight bundle, NW = network; (C) actin control polymerized with 2 mM MgCl₂ and 75 mM KCl; (D) actin in the presence of talin polymerized with 2 mM MgCl₂ and 75 mM KCl. Bar = 100 nm in A-D.
At pH 6.9 with 75 mM KCl present, no bundles were present, but talin retained some of its ability to crosslink actin filaments into networks (Fig. 3D).

**Chemical Crosslinking of Talin** - To determine whether talin was present as a monomer or dimer under the conditions used to examine the talin-actin interaction herein, the zero-length chemical crosslinker, EDC, was used to crosslink talin (Fig. 4). At pH 6.4, after 10 min of crosslinking in the absence or presence of 100 mM KCl, a band corresponding to an ~400 kDa protein (D) was present (Fig. 4, lanes b). With increased crosslinking time, the amount of this band increased concomitantly with a decrease in the amount of the talin monomer band (M) (~225 kDa) in the absence or presence of 100 mM KCl (Fig. 4, lanes c-e). Similar qualitative results also were observed at both pH 6.9 and 7.3, with or without 100 mM KCl present (Fig. 4). The delay in the disappearance of talin monomers and simultaneous increase in dimers with increasing pH (especially noted at pH 7.3) is believed due to the reduced efficiency of EDC at higher pH (Hoare and Koshland, 1967). I observed much more conversion of monomer to dimer when I extended the crosslinking time to 4 h, or increased the EDC concentration (results not shown). Taken *in toto*, the results indicated that talin was present primarily as a dimer and, thus, the effect of pH and ionic strength on talin's ability to crosslink actin filaments appears due to an effect on the talin-actin interaction instead of an effect on talin's self-association properties. Similar conclusions indicating that native talin consists of two subunits were found by Muguruma et al. (1992) and Goldmann et al. (1994).
**Fig. 4. Chemical crosslinking of talin by EDC.** Aliquots of talin reaction mixtures (pH 6.4, 6.9, or 7.3, without or with 100 mM KCl present) were taken at specific time points, quenched, and analyzed by SDS-PAGE. Lane a, talin before addition of EDC; lanes b, c, d, and e, are after addition of EDC and represent 10, 30, 60, and 120 min of crosslinking, respectively. M = talin monomer; D = talin dimer corresponding to an ~400 kDa protein.
The Effect of Polymerization Time on the Talin-Actin Interaction - To examine the ability of talin to crosslink actin filaments after very short polymerization time, actin was polymerized in the presence of talin at pH 6.4 and 25°C for only 5 min. As shown in Fig. 5A, long randomly dispersed actin filaments were already formed in the absence of talin. And talin extensively crosslinked actin filaments into both networks and bundles (Fig. 5B). The bundles formed at 5 min were similar in appearance to the bundles examined after polymerization for 1 h (Fig. 2B). There also was no significant increase in shorter actin filaments found in talin-actin mixtures (Fig. 5B) in comparison to the actin filament control (Fig. 5A). Overall, these results indicated that talin's ability to crosslink actin filaments into both networks and bundles was not significantly affected by the actin polymerization time.

The Ability of Talin to Crosslink Preformed Actin Filaments - The ability of talin to crosslink preformed actin filaments was examined by first polymerizing actin filaments in the absence of talin at pH 6.4 for 1 h, before talin was added to the actin filament solution and incubated for another 1 h. Talin crosslinked the preformed actin filaments into both networks and bundles (Fig. 6C) to an extent similar to that observed when actin was polymerized in the presence of talin for 1 h (Fig. 6B). Furthermore, the bundles shown in Fig. 6C are generally similar in appearance to those formed when actin was polymerized in the presence of talin for 1 h (Fig. 6B). These results suggest that talin clearly interacts with F-actin, and crosslinks existing actin filaments into networks and bundles.

The Interaction Between the ~190 kDa Talin Fragment and Actin Filaments - The ability of the 190 kDa fragment of talin to crosslink actin filaments was examined by using negative staining (Fig. 7). At pH 6.4 and low ionic strength, intact talin extensively
Fig. 5. Effect of short polymerization time on the ability of talin to crosslink and bundle actin filaments at pH 6.4 as shown by electron microscopy. Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of talin (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ (final concentration) for 5 minutes at 25°C and immediately negatively stained. (A) Actin control; (B) actin polymerized in the presence of talin. B = bundle, NW = network. Bar = 100 nm for A and B.
Fig. 6. Ability of talin to crosslink preformed actin filaments at pH 6.4 as shown by electron microscopy. (A) Preformed actin (0.25 mg/ml final concentration) control polymerized by the addition of 2 mM MgCl₂ (final concentration); (B) Actin (0.25 mg/ml final concentration) was polymerized in the presence of talin (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ (final concentration); (C) Talin (0.10 mg/ml final concentration) was added to preformed actin filaments (0.25 mg/ml final concentration) that had been polymerized by the addition of 2 mM MgCl₂ (final concentration) for 1 h. B = bundle, NW = network. Bar = 100 nm for A-C.
Fig. 7. *Effect of pH on the ability of the 190 kDa talin fragment to crosslink actin filaments as shown by electron microscopy.* Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of talin (0.10 mg/ml final concentration) or of the 190 kDa talin fragment (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ (final concentration) at pH 6.4 or 6.0. All samples were incubated for 1 h at 25°C and negatively stained. (A) Actin control at pH 6.4; (B) actin polymerized in the presence of intact talin at pH 6.4; (C) actin polymerized in the presence of 190 kDa talin fragment at pH 6.4; (D) actin control at pH 6.0; (E) actin polymerized in the presence of 190 kDa talin fragment at pH 6.0. B = bundle, NW = network. Bar = 100 nm for A-E.
crosslinked actin filaments into both networks and bundles (Fig. 7B; also see Figs. 2B and 6B). As shown in Fig. 7C, the 190 kDa talin fragment had the ability to form actin networks, but did not exhibit any actin bundling activity at pH 6.4 and low ionic strength. When the actin crosslinking ability of the 190 kDa talin fragment was tested at pH 6.0, it crosslinked actin filaments into both networks and bundles (Fig. 7E) to a similar extent as did intact talin at pH 6.4 (Fig. 7B). However, the bundles formed in the presence of the 190 kDa talin fragment at pH 6.0 did not exhibit any obvious longitudinal periodicity in comparison to some of the bundles formed at pH 6.4 with intact talin (Fig. 7B; also see Figs. 2B and 6B). These results suggest that the 190 kDa talin fragment possesses the actin binding activity responsible for its actin filament crosslinking activity.

Discussion

Although independent research groups have reported a direct interaction in vitro between talin and actin filaments (Muguruma, et al., 1990, 1992, 1995; Kaufmann et al., 1991; Schmidt et al., 1993; Goldmann et al., 1994), the mode of the talin-actin interaction varies considerably among the labs. For instance, talin reportedly nucleates actin polymerization, and decreases the viscosity of F-actin solutions (Kaufmann et al., 1991; Goldmann et al., 1994), or it increases the viscosity of F-actin solutions and cosediments with F-actin (Muguruma et al., 1990, 1992; Schmidt et al., 1993). And, although the increase in the viscosity of F-actin solutions and the cosedimentation assays indicate an actin crosslinking activity for talin (Muguruma et al., 1990, 1992, 1995), no actin filament
crosslinking by talin was observed by electron microscopy by that laboratory (Muguruma et al., 1990). In our laboratory, talin has been identified as an actin filament crosslinking protein, especially at pH 6.4 and low ionic strength, and talin's crosslinking activity as measured by cosedimentation and viscometry assays is pH and ionic strength dependent, being decreased with increasing pH and/or ionic strength (Schmidt et al., 1993). As I demonstrated herein (and see previous chapter in this dissertation) talin crosslinks actin filaments into both networks and bundles, and these crosslinking abilities are pH and ionic strength dependent. Thus, I have provided a structural basis for results of the talin-actin cosedimentation and viscometry assays we reported earlier (Schmidt et al., 1993).

The results shown herein that talin crosslinked actin filaments into both networks and bundles after only five minutes of polymerization, without showing a significant number of shorter actin filaments compared to the F-actin controls, disagree with the reports of Kaufmann et al. (1991) and Goldmann et al. (1994) in which they suggested that talin decreases viscosity of actin filaments because it nucleates actin polymerization and increases the number of filaments but decreases their length. As shown herein, talin also crosslinked preformed actin filaments in a fashion similar to what it did when actin filaments were formed in its presence. These results clearly show that talin interacts with F-actin, which disagrees with the suggestion in Kaufmann et al. (1991) that talin has no effect on preformed actin filaments. The reason(s) for the variable observations on talin's ability to interact with actin reported by different research groups may be due, in part, to the different preparation procedures used to prepare talin, which, in turn, may result in different degrees of purity (Muguruma et al., 1990, 1992; Kaufmann et al., 1991; Schmidt et al., 1993; Goldmann et al.,
Also, the ionic conditions used in the experiments conducted in different labs may be responsible for some of the different results reported, especially since we have found the talin-actin interaction to be very sensitive to both pH and ionic strength.

To crosslink actin filaments, it appears that talin has either two or more actin binding domains, or one actin binding domain and the ability to self-associate. The actin binding activity of talin is contained in the 190 kDa fragment generated by calpain or other proteases (Muguruma et al., 1990, 1995; Niggli et al., 1994). However, neither the number nor the location of the specific actin binding domain(s) in the 190 kDa talin fragment has been identified. It was recently reported that platelet talin exists as an anti-parallel homodimer (Goldmann et al., 1994), although the reported size and shape of native talin varies considerably (e.g., Collier and Wang, 1982; Molony et al., 1987; Schmidt et al., 1993). The results of the chemical crosslinking experiments herein indicated that talin exists primarily as a dimer under the conditions of pH and ionic strength tested, which suggests the major effect of pH and ionic strength on talin's actin filament crosslinking activity is due to the direct talin-actin interaction rather than on talin's ability to self-associate.

The 190 kDa talin fragment was shown to have the ability to crosslink actin filaments into both networks and bundles. However, it required a slightly lower pH than did intact talin. This indicates that the actin binding site(s) in talin that are responsible for its crosslinking activity is located on the 190 kDa fragment. Muguruma et al. (1990, 1995) have reported that the 47 kDa head fragment of talin does not cosediment with actin filaments. As shown herein, the 190 kDa talin tail fragment did not form as highly ordered actin bundles as did intact talin. Perhaps the 47 kDa N-terminal fragment in some way regulates the actin
binding site(s) on the 190 kDa fragment.

References


EFFECT OF PHOSPHOINOSITIDES ON THE TALIN-ACTIN INTERACTION

A paper to be submitted to the Journal of Biological Chemistry

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Summary

Talin is a membrane-cytoskeleton linking protein primarily located in cell-matrix type adherens junctions. Talin has been shown to cosediment with F-actin, increase low shear viscosity of F-actin solutions, and crosslink actin filaments into networks and bundles at pH 6.4 and low ionic strength. In this study, the effects of phospholipids on the interaction between talin and actin filaments were examined by using cosedimentation and viscometry assays, and electron microscopy. The results showed that of the phospholipids tested, the phosphoinositides, including phosphatidylinositol 4,5-bisphosphate (PIP₂), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol (PI) bind talin in the form of talin/phosphoinositide complexes and thereby prevent talin from effectively interacting with actin filaments at pH 6.4 and low ionic strength. Under the same ionic conditions, phosphatidylserine (PS), phosphatidylcholine (PC), and inositol 1,4,5-trisphosphate (IP₃) did not.

The abbreviations used are: PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; IP₃, inositol 1,4,5-trisphosphate; MCE, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
not significantly affect the talin-actin interaction. Talin retained its ability to crosslink actin filaments when the phosphoinositide vesicle structures were disrupted with the detergent Triton X-100. Divalent cations such as Ca\(^{2+}\) significantly decreased the efficiency of the phosphoinositides in inhibiting the talin-actin interaction.

**Introduction**

Talin, a protein associated with the membrane regions specialized for cell-substrate contacts, has been shown to interact with actin *in vitro* (Muguruma et al., 1990, 1992; Kaufmann et al., 1991; Schmidt et al., 1993). Talin cosediments with actin filaments, increases the viscosity of F-actin solutions (Muguruma et al., 1990, 1992; Schmidt et al., 1993), and crosslinks actin filaments into both networks and bundles (Zhang et al., 1995). The predicted primary structure of talin from cDNA sequence analysis (Rees et al., 1990) showed that talin shares an ~ 200 amino acid sequence homologous to several other proteins, such as protein 4.1, ezrin, moesin (Lankes and Furthmayr, 1991), and a human protein-tyrosine phosphatase (PTPH1) (Yang and Tonks, 1991) at their N-terminal regions. The homologous N-terminal portion of protein 4.1 has been assigned an important role in linking the cytoskeleton to the cell membrane (reviewed in Luna and Hitt, 1992), and the homologous region of the N-terminal portion of talin may likewise be important in membrane binding (Rees et al., 1990). The interactions identified between talin and the transmembrane glycoprotein, integrin (Horwitz et al., 1986), and between talin and actin (Muguruma et al., 1990, 1992; Kaufmann et al., 1991) led to the suggestion that talin is directly involved in linking the actin-based cytoskeleton and the cell membrane (Simon et al.,
Talin's actin binding activity is associated with the large (~190 kDa) C-terminal tail fragment of talin (Muguruma et al., 1990, 1995, Niggli et al., 1994).

Previous studies have indicated that talin binds to acidic phospholipids (Heise et al., 1991; Goldmann et al., 1992, Dietrich et al., 1993). By using proteolytic fragments of talin, the 47 kDa N-terminal fragment, and not the ~ 190-200 kDa C-terminal fragment, of talin has been shown to possess these phospholipid binding properties (Niggli et al., 1994; Muguruma et al., 1995).

The effect of phosphoinositides, components of the PI signal transduction pathway (Berridge and Irvine, 1989), on several actin-binding proteins has been shown (Isenberg, 1991; Janmey, 1994). Phosphoinositides inhibit the interactions between actin and a number of actin-binding proteins such as profilin (Lassing and Lindberg, 1985, 1988), gelsolin (Janmey and Stossel, 1987, 1989; Janmey et al., 1992), CapZ (Heiss and Cooper, 1991), and filamin (Furuhashi et al., 1992) by interacting with the actin-binding proteins rather than the actin (Isenberg, 1991; Janmey, 1994). α-Actinin is an exception, in that it has been shown that the phosphoinositides PIP₂, PIP, and PI increased its actin gelating activity at low concentrations, but inhibited this activity at higher concentrations (Fukami et al., 1992). The effect, if any, of the phosphoinositides on the talin-actin interaction has not been shown. The objective of this study was to investigate the effect of specific phospholipids, especially the phosphoinositides, on the talin-actin interaction. The results indicate that phosphoinositides bind to talin and thereby inhibit the interaction between talin and actin.
Experimental Procedures

Protein Preparation - Smooth muscle talin was prepared from turkey gizzards as described (Schmidt et al., 1993; Schmidt, 1994). Purified talin was in a buffer containing 10 mM imidazole, pH 7.0, 0.1 mM PMSF, 1 µg/ml E-64, and 0.5 mM MCE. Actin was purified from porcine skeletal muscle as described in Schmidt et al. (1993), and in a buffer containing 2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl₂, and 0.5 mM MCE. Protein concentrations were determined by using the modified Lowry method (Sigma).

Lipids - Phospholipids including PIP₂, PIP, PI, PS, and PC, and another related compound, IP₃, were purchased from Sigma and used without further purification. All were dissolved (1 mM final concentration) in double-distilled, deionized water, sonicated until the solution was clear or homogenous, and aliquots were then stored at -70°C. The phospholipids were thawed at room temperature and sonicated for 1 min on ice immediately before use.

Cosedimentation Assay - Talin (0.20 mg/ml final concentration) was incubated with specific phospholipids at selected molar ratios at 25°C for 30 min in buffer A (10 mM imidazole, pH 6.6, 1 mM ATP). G-actin (0.50 mg/ml final concentration) was then added and polymerization induced by the addition of MgCl₂ (2 mM final concentration). The final pH was 6.4. After incubating for 1 h at 25°C, the samples were centrifuged at 110,000 x g for 20 min in a Beckman airfuge. Comparable amounts of the supernatants and pellets were analyzed by SDS-PAGE (Laemmli, 1970). Specific incubation conditions were tested and will be given in the Figure Legends. The figures shown are representative of results (with each experiment run at least in duplicate) obtained from a minimum of three different talin
and actin preparations.

*Low Shear Viscometry* - Talin (0.10 mg/ml final concentration) was incubated with specific phospholipids at selected molar ratios at 25°C for 30 min in buffer A. G-actin (0.25 mg/ml final concentration) was then added and polymerization induced by addition of MgCl₂ (2 mM final concentration). The final pH was 6.4. The low shear viscosity of the samples was measured as described (Schmidt et al., 1993). Results were expressed as normalized viscosities, obtained by dividing all actin/talin viscosity values by actin control viscosity values, and actin/talin/phospholipid viscosity values by actin/phospholipid control viscosity values. A normalized value of one indicates no change in viscosity from the control. Specific incubation conditions were tested and will be given in the Figure Legends. The viscosity experiments were done at least in duplicate, and the values reported represent the means obtained from three different talin and actin preparations.

*Electron Microscopy* - Talin (0.10 mg/ml final concentration) was first incubated with specific phospholipids at 25°C for 30 min in buffer A. G-actin (0.25 mg/ml final concentration) was then added and polymerization was induced by the addition of MgCl₂ (2 mM final concentration). The final pH was 6.4. After 1 h of polymerization at 25°C, the samples were negatively stained and examined as described (Schmidt et al., 1993; Zhang et al., 1995). Specific incubation conditions were tested and will be given in the Figure Legends.
Results

The Effect of Phospholipids on the Interaction Between Talin and Actin - The effect of phospholipids on the talin-actin interaction at pH 6.4 and low ionic strength was examined by cosedimentation assays (Figs. 1 and 2). At pH 6.4 and low ionic strength, talin bound and cosedimented with actin filaments by centrifugation (Fig. 1, panel c) as shown previously (Schmidt et al., 1993), while talin in the absence of actin remained in the supernatant (Fig. 1, panel b). As shown in Fig. 1 (panels d, f, and h), however, a significant proportion (> one-half) of talin in the absence of actin sedimented in the presence of PIP$_2$, PIP, or PI, indicating formation of large talin/phosphoinositide complexes. As a result, the talin observed in the pellets of the actin/talin/phosphoinositide mixtures (Fig. 1, panels e, g, and i) can be due to a combination of formation of the complexes of phosphoinositides and talin, and to the binding between talin and actin filaments. It is evident in Fig. 1, in the presence of the phosphoinositides, that more talin is sedimented with the actin present (panels e, g, and i) than in its absence (panels d, f, and h). The PIP$_2$, PIP, and PI used at this concentration (molar ratio of 20 phosphoinositides to one 225 kDa talin) did not affect the amount of control actin filaments sedimented (results not shown). As shown previously, the interaction between talin and actin is markedly pH and ionic strength dependent (Schmidt et al., 1993; Zhang et al., 1995), with increase of pH and/or ionic strength effectively decreasing the talin-actin interaction. The addition of the phosphoinositides at the concentrations used herein did not change the pH of the interaction solutions (results not shown), and the concentration of the phosphoinositides (< 1 mM) was not high enough to significantly alter ionic strength and
Fig. 1. Effect of phosphoinositides on the ability of talin to cosediment with actin filaments at pH 6.4. Talin (0.20 mg/ml final concentration) was first incubated with PIP$_2$, PIP, or PI, when present, at a molar ratio of 20 phosphoinositides to one talin in buffer A for 30 min at 25°C. G-actin (0.50 mg/ml final concentration) was then added, and polymerization initiated by the addition of 2 mM MgCl$_2$ (final concentration). All samples were incubated, centrifuged, and assayed as described in Experimental Procedures. (a) Actin control; (b) talin control; (c) actin/talin; (d) talin/PIP$_2$ control; (e) actin/talin/PIP$_2$; (f) talin/PIP control; (g) actin/talin/PIP; (h) talin/PI control; (i) actin/talin/PI. PL = phospholipid, T = talin, A = actin, s = supernatant, p = pellet.
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A-
Fig. 2. Effect of phosphatidyserine (PS), phosphatidylcholine (PC), and inositol 1,4,5-trisphosphate (IP$_3$) on the ability of talin to cosediment with actin filaments at pH 6.4.

Talin (0.20 mg/ml final concentration) was first incubated with PS, PC, or IP$_3$ at a molar ratio of 40 phospholipids or IP$_3$ to one talin in buffer A for 30 min at 25°C. G-actin (0.50 mg/ml final concentration) was then added, and polymerization initiated by the addition of 2 mM MgCl$_2$ (final concentration). All samples were incubated, centrifuged, and assayed as described in Experimental Procedures. (a) Actin/PS control; (b) talin/PS control; (c) actin/talin/PS; (d) actin/PC control; (e) talin/PC control; (f) actin/talin/PC; (g) actin/IP$_3$ control; (h) talin/IP$_3$ control; (i) actin/talin/IP$_3$. T = talin, A = actin, s = supernatant, p = pellet.
the talin-actin interaction (Schmidt, 1994). Addition of PS, an acidic phospholipid, PC, a neutral phospholipid, or IP₃, one of the hydrolysis products of PIP₂, did not affect the talin-actin interaction under the same ionic condition, even at higher concentrations (molar ratio of 40 to 1 talin) (Fig. 2), with the gel patterns appearing essentially the same as in the absence of phospholipids for the control actin, control talin, and control actin/talin mixture in Fig. 1 (panels a, b, and c, respectively). The results shown in Figs. 1 and 2 showed that of the phospholipids tested, only the phosphoinositides had an ability to affect the sedimentation behavior of talin.

The effect of phosphoinositides on the talin-actin interaction was also examined by low shear viscometry. We have previously shown that at pH 6.4 and low ionic strength, talin dramatically increases the viscosity of F-actin solutions (Schmidt et al., 1993). The increase in viscosity was sufficiently high in the present study (molar ratio of one 225 kDa talin to 13 42 kDa actins) that the ball would not travel through the solution inside the capillary tube, and is defined herein as "gel". As shown in Fig. 3, the ability of talin to increase the viscosity of F-actin solutions was inhibited when talin was preincubated with phosphoinositides for 30 min before actin was added and polymerization initiated by addition of MgCl₂. In the absence of phosphoinositides, talin gelated the F-actin solutions. As the molar ratio of the phosphoinositides (PIP₂, PIP, or PI) to talin was increased, the ability of talin to increase the viscosity of F-actin solutions was decreased. Among the three phosphoinositides, PI and PIP₂ were somewhat more effective than PIP in inhibiting talin's ability to increase the viscosity of F-actin solutions. However, at a molar ratio of 30:1 (phosphoinositide to talin),
Fig. 3. Effect of phosphoinositides on the ability of talin to increase the low shear viscosity of F-actin solutions at pH 6.4. Talin (0.10 mg/ml final concentration) was first incubated with PIP$_2$, PIP, or PI, when present, in buffer A for 30 min at 25°C. G-actin (0.25 mg/ml final concentration) was then added, and polymerization initiated by the addition of 2 mM MgCl$_2$ (final concentration). The viscosities of all samples were measured and expressed as normalized viscosities as described in Experimental Procedures.
Molar Ratios of Phosphoinositides to Talin
all three phosphoinositides totally inhibited talin's ability to increase the viscosity of F-actin solutions. None of the phosphoinositides affected the viscosity of F-actin controls (results not shown), indicating that phosphoinositides affect the talin-actin interaction by interacting with talin but not actin. In contrast to the phosphoinositides, the presence of PS, PC, or IP₃ did not inhibit the ability of talin to increase the viscosity of F-actin solutions (Fig. 4), even at higher concentrations than those of the phosphoinositides used in the previous assays (Fig. 3). The viscosity results shown in Figs. 3 and 4 are consistent with the results obtained from the cosedimentation assays (Figs. 1 and 2).

The results shown in Figs. 1 and 3 were from experiments that included preincubation of talin and the phosphoinositides before addition of G-actin and polymerization. The effect of the presence of G-actin during the preincubation period, before polymerization, on the interaction between talin and phosphoinositides was examined by incubating talin, G-actin, and phospholipids at pH 6.4 and low ionic strength for 30 min at 25°C, before polymerization was initiated by adding MgCl₂. The results of low shear viscometry assays shown in Fig. 5 are similar to those shown in Fig. 3, indicating that PIP₂, PIP, or PI inhibited ability of talin to increase the viscosity of F-actin solutions, regardless of the presence of G-actin during the preincubation period.

As shown in Fig. 6, and as we have shown previously (Zhang et al., 1995), talin crosslinks actin filaments into both networks and bundles (B) at pH 6.4 and low ionic strength. As shown in Fig. 7, preincubation of the talin with PIP₂, PIP, or PI for 30 min at 25°C before G-actin was added and polymerization initiated by the addition of MgCl₂, under
**Fig. 4.** Effect of phosphatidylserine (PS), phosphatidylcholine (PC), and inositol 1,4,5-trisphosphate (IP$_3$) on the ability of talin to increase the low shear viscosity of F-actin solutions at pH 6.4. Talin (0.10 mg/ml final concentration) was first incubated with PS, PC, or IP$_3$, when present, in buffer A for 30 min at 25°C before G-actin (0.25 mg/ml final concentration) was added, and polymerization initiated by the addition of 2 mM MgCl$_2$ (final concentration). The viscosities of all samples were measured and expressed as normalized viscosities as described in Experimental Procedures.
Molar Ratios of Phospholipids or IP-3 to Talin
Fig. 5. Effect of presence of G-actin on the interaction between talin and phosphoinositides during preincubation period prior to actin polymerization as measured by low shear viscometry of F-actin solutions at pH 6.4. Talin (0.10 mg/ml final concentration), phosphoinositides, when present, and G-actin (0.25 mg/ml final concentration) were mixed together for 30 min at 25°C before MgCl₂ (2 mM final concentration) was added to initiate actin polymerization. The viscosities of all samples were measured and expressed as normalized viscosities as described in the Experimental Procedures.
Molar Ratios of Phosphoinositides to Talin
Fig. 6. The ability of talin to crosslink actin filaments at pH 6.4 as shown by electron microscopy. Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of talin (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ (final concentration) at 25°C. All samples were incubated for 1 h and negatively stained as described in Experimental Procedures. (A) Actin control; (B) actin polymerized in the presence of talin. Globular particles often were observed at the points of filament intersection (small arrows) in networks. B = bundle. Bar = 100 nm for A and B.
Fig. 7. Effect of phosphoinositides on the ability of talin to crosslink actin filaments at pH 6.4 as shown by electron microscopy. Talin (0.10 mg/ml final concentration) was first incubated with PIP₂, PIP, or PI for 30 min at 25°C. G-actin (0.25 mg/ml final concentration) was then added, and polymerization initiated by the addition of 2 mM MgCl₂ (final concentration). Samples were negatively stained as described in Experimental Procedures. (A) PIP₂/actin (20:13) control; (B) PIP/actin (20:13) control; (C) PI/actin (20:13) control; (D) PIP₂/talin/actin (20:1:13); (E) PIP/talin/actin (20:1:13). B = bundle; (F) PI/talin/actin (20:1:13); (G) PIP₂/talin/actin (40:1:13); (H) PIP/talin/actin (40:1:13); (I) PI/talin/actin (40:1:13). Numbers within the parentheses are molar ratios. Bar = 100 nm for A-I.
the same ionic conditions as used for Fig. 6, inhibited the ability of talin to crosslink actin filaments at low concentrations of phosphoinositides and eliminated it at higher molar ratios of phosphoinositides to talin. Actin filament controls in the presence of PIP$_2$, PIP, or PI (Fig. 7A, 7B, and 7C, respectively) appeared randomly dispersed and similar to actin filament controls formed in the absence of the phosphoinositides (Fig. 6A), except for the presence of the phosphoinositide vesicles (Fig. 7A-C, arrows). The vesicles in the actin controls (Fig. 7A-C) were similar in structure to those observed for each specific phosphoinositide in the absence of actin (results not shown). At a molar ratio of 20:1 (phosphoinositides to talin), no bundles were observed in talin/PIP$_2$/F-actin (Fig. 7D) or in talin/PI/F-actin mixtures (Fig. 7F). Some shorter, thinner bundles, in comparison to the bundles formed in the absence of phosphoinositides, were found in talin/PIP/F-actin mixture (cf., Fig. 7E with Fig. 6B). These results were in concert with the results obtained from the viscometry assays (Fig. 3), in that PIP$_2$ and PI were somewhat more effective than PIP in inhibiting the interaction between talin and actin filaments. As the bundles disappeared, a number of talin/phosphoinositide complexes were present (Fig. 7D and 7F, arrows). The complexes, such as those seen in Fig. 7D and 7F, appeared similar to those observed in talin/phosphoinositide mixtures examined in the absence of actin (results not shown). As the concentration of phosphoinositides was elevated (40 phosphoinositides to 1 talin), no bundles or networks were observed in the talin/PIP$_2$/F-actin, talin/PIP/F-actin, or talin/PI/F-actin mixtures (Fig. 7G, 7H, and 7I, respectively), and large numbers of talin-phosphoinositide complexes were present (arrows).

The presence of PS, PC, or IP$_3$ did not affect either the polymerization of actin filaments
(cf., Fig. 8A, 8C, and 8E with Fig. 6A) or the ability of talin (molar ratio of 40 to 1 talin) to crosslink actin filaments, in that bundles and networks were observed (Fig. 8B, 8D, and 8F) as seen in the F-actin/talin mixture in the absence of phosphoinositides (cf., Fig. 8B, 8D, and 8F with Fig. 6B).

Taken together, the results of the cosedimentation assays, viscometry assays, and electron microscopy indicate that phosphoinositides interfere with the talin-actin interaction by binding to talin to form talin/phosphoinositide complexes, and thereby prevent talin from interacting with actin filaments.

The Effect of Triton X-100 on the Activity of Phosphoinositides - To gain more evidence as to whether inhibition of the talin-actin interaction by phosphoinositides was due to the formation of talin/phosphoinositide complexes, PI, PIP, or PIP₂ was preincubated with 0.5% Triton X-100 before talin was added. Even high concentrations of phosphoinositides no longer inhibited the talin-actin interaction as shown by both cosedimentation assays (Fig. 9a) and viscometry assays (Fig. 9b). As shown in Fig. 9a, the presence of Triton X-100 had no effect on the sedimentation behavior of actin, talin, or talin/actin mixture (cf., Fig. 9a, panels a, b, and c with Fig. 1, panels a, b, and c). As shown in Fig. 9a, after preincubation with Triton X-100, the phosphoinositides (PI is shown) no longer had the ability to bind talin to form talin/phosphoinositide complexes and sediment as they did without the Triton X-100 (cf., Fig. 9a, panel e with Fig. 1, panel h). These results indicate that phosphoinositide vesicles are necessary for forming complexes with talin, and thereby preventing talin from
Fig. 8. Effect of phosphatidylserine (PS), phosphatidylcholine (PC), and inositol 1,4,5-trisphosphate (IP₃) on the ability of talin to crosslink actin filaments at pH 6.4 as shown by electron microscopy. Talin (0.10 mg/ml final concentration) was first incubated with PS, PC, or IP₃ for 30 min at 25°C before G-actin (0.25 mg/ml final concentration) was added, and polymerization initiated by the addition of 2 mM MgCl₂ (final concentration). Samples were negatively stained as described in Experimental Procedures. (A) PS/actin (40:13); (B) PS/talin/actin (40:1:13); (C) PC/actin (40:13); (D) PC/talin/actin (40:1:13); (E) IP₃/actin (40:13); (F) IP₃/talin/actin (40:1:13). Numbers within the parentheses are molar ratios. B = bundle, NW = network. Bar = 100 nm for A-F.
Fig. 9. Effect of Triton X-100 on the ability of phosphoinositides to inhibit the talin-actin interaction at pH 6.4. When present, specific phosphoinositides were first incubated with 0.5% Triton X-100 (v/v) for 5 min before talin (0.10 mg/ml final concentration) was added, and incubated for another 30 min. G-actin (0.25 mg/ml final concentration) was then added and polymerization initiated by the addition of 2 mM MgCl₂ (final concentration).

a. Cosedimentation assay. All samples were incubated for 1 h at 25°C, centrifuged, and assayed as described in Experimental Procedures.

(a) Triton X-100/actin control; (b) Triton X-100/talin control; (c) Triton X-100/talin/actin control; (d) PI/Triton X-100/actin (30:13); (e) PI/Triton X-100/talin (30:1); (f) PI/Triton X-100/talin/actin (30:1:13). Numbers within the parentheses are molar ratios of PI to talin and/or actin in the order listed. T = talin, A = actin, s = supernatant, p = pellet.
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Fig. 9 (continued)

b. Viscometry assay. After incubation for 10 min at 25°C, the viscosities of all samples were measured and expressed as normalized viscosities as described in Experimental Procedures.
Molar Ratios of Phosphoinositides to Talin
binding to actin filaments.

The Effect of Ca$^{2+}$ on Activities of Phospholipids - That only the phosphoinositides among the five amphipathic phospholipids tested (PIP$_2$, PIP, PI, PS, and PC) affected the talin-actin interaction suggests that the inositol ring and/or the phosphate groups on the inositol ring may be important for their inhibitory action. However, that the hydrophilic IP$_3$ alone did not show any inhibitory effect suggests that the formation of vesicles by amphipathic phospholipids is important. To examine the importance of the phosphate groups in the phosphoinositides, the phosphoinositides were preincubated with 1 mM Ca$^{2+}$ before talin was added. The results showed that the presence of 1 mM Ca$^{2+}$ effectively suppressed the inhibitory effects of PI on the talin-actin interaction as shown by cosedimentation (Fig. 10a). The presence of 1 mM Ca$^{2+}$ did not affect the sedimentation of actin, talin, or talin/actin mixture controls (cf., Fig 10a, panels a, b, and c with Fig. 1, panels a, b, and c). However, the PI was no longer able to bind talin and sediment in the form of talin/PI complexes when the PI was preincubated with Ca$^{2+}$ (cf., Fig. 10a, panel e with Fig. 1, panel h). As shown in Fig. 10b, the preincubation of the phosphoinositides with 1 mM Ca$^{2+}$ decreased their inhibitory effect on the talin-actin interaction as shown by low shear viscometry assays. Results of negative staining also showed that preincubation of phosphoinositides with 1 mM Ca$^{2+}$ prevented the phosphoinositides from blocking the formation of actin networks and bundles (cf., Fig. 10c, B with C). Although the Ca$^{2+}$ concentration (1 mM) used was sufficiently high to complex all phosphate groups in the phosphoinositides (< 0.1 mM), it did not completely suppress the inhibitory effect of
Fig. 10. Effect of Ca\(^{2+}\) on the ability of phosphoinositides to inhibit the talin-actin interaction at pH 6.4. When present, specific phosphoinositides were first incubated with 1 mM Ca\(^{2+}\) (final concentration) for 5 min before talin (0.10 mg/ml final concentration) was added, and incubated for another 30 min. G-actin (0.25 mg/ml final concentration) was then added, and polymerization initiated with 2 mM MgCl\(_2\) (final concentration).

**a. Cosedimentation assay.** All samples were incubated for 1 h at 25°C, centrifuged, and assayed as described in Experimental Procedures.

(a) Ca\(^{2+}\)/actin control; (b) Ca\(^{2+}\)/talin control; (c) Ca\(^{2+}\)/talin/actin control; (d) Ca\(^{2+}\)/PI/actin (30:13); (e) Ca\(^{2+}\)/PI/talin (30:1); (f) Ca\(^{2+}\)/PI/talin/actin (30:1:13). Numbers within the parentheses are molar ratios of PI to talin and/or actin in the order listed. T = talin, A = actin, s = supernatant, p = pellet.
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Fig. 10 (continued).

b. Viscometry assay. After polymerization for 10 min at 25°C, the viscosities of all samples were measured and expressed as normalized viscosities as described in Experimental Procedures.
Molar Ratios of Phosphoinositides to talin

Normalized Viscosity

0 10 20 30 40 50

- PIP-2
- PIP
- PI

gel
Fig. 10 (continued).

c. Electron microscopy. All samples were incubated for 1 hr at 25°C and negatively stained as described in Experimental Procedures. (A) Pl/actin (30:13) (arrow points to PI vesicle); (B) PI/talin/actin (30:1:13) (arrows point to talin/PI complexes); (C) Ca$^{2+}$/PI/talin/actin (30:1:13) (B = bundle). Numbers within the parentheses are molar ratios of PI to talin and/or actin in the order listed. Bar = 200 nm for A and B.
phosphoinositides (see Fig. 10b). This suggests that phosphate groups are important for the talin-phosphoinositide interaction, but that other factors are involved as well.

Discussion

Based largely upon the in vitro talin-integrin (Horwitz et al., 1986) and talin-actin interactions (Muguruma et al., 1990), together with talin's primary location at cell-matrix type adherens junctions (Beckerle and Yeh, 1990), Simon et al. (1991) proposed a direct role for talin in linking actin filaments to the cell membrane. Talin recently has been identified as an actin crosslinking protein (Schmidt et al., 1993), having the ability to crosslink actin filaments into both networks and bundles at pH 6.4 and low ionic strength (Zhang et al., 1995). The marked pH and ionic strength dependence of the talin-actin interaction in vitro (Schmidt et al., 1993; Zhang et al., 1995) suggests that talin may be able to regulate actin filament organization in vivo at the cell-matrix attachment sites in response to specific cell signals.

Talin previously has been shown to bind selectively to acidic phospholipids, such as PS, but not to neutral phospholipids such as PC (Heise et al., 1991). The phospholipid binding site(s) on talin has been located on its N-terminal 47 kDa fragment (Niggli et al., 1994; Muguruma et al., 1995), rather than on the C-terminal 190-200 kDa fragment containing talin's actin binding activity (Muguruma et al., 1990, 1995, Niggli et al., 1994). No effect of phospholipids on the talin-actin interaction has been reported. The phosphoinositides, components of eukaryotic cell membranes, have been shown to inhibit the interaction of a number of actin binding proteins such as profilin (Lassing and Lindberg, 1985, 1988),
gelsolin (Janmey and Stossel, 1987, 1989; Janmey et al., 1992), CapZ (Heiss and Cooper, 1991), and filamin (Furuhashi et al., 1992) with actin (reviewed in Isenberg, 1991; Janmey, 1994). The effects of the phosphoinositides, if any, on the talin-actin interaction have not been previously reported.

We have used cosedimentation, viscometry, and electron microscopy analyses, and show for the first time that phosphoinositides including PIP$_2$, PIP, and PI have a strongly inhibitory effect on the talin-actin interaction. They inhibited talin's ability to increase the viscosity of F-actin solutions and to crosslink actin filaments into networks and bundles at pH 6.4 and low ionic strength. Our results indicate the phosphoinositides interact with talin by forming talin/phosphoinositide complexes, and thereby prevent talin from effectively interacting with actin filaments. Complete inhibition of talin's ability to crosslink actin filaments was obtained at a molar ratio of ~30 phosphoinositides to 1 talin. Of the phospholipids tested herein, only the phosphoinositides inhibited the talin-actin interaction, showing that the interaction between talin and phosphoinositides is specific. The PIP$_2$ and PI were somewhat more effective than PIP, but all three were capable of completely inhibiting the talin-actin interaction. All three phosphoinositides also have been shown to inhibit interaction of cofilin and destrin (Yonezawa et al., 1990, 1991), filamin (Furuhashi, 1992), and severin (Eichinger and Schleicher, 1992) with actin. The phosphoinositide binding sites on some actin binding proteins have been identified, with the sites consisting of 10 to 20 amino acids, but showing no obvious sequence homology (Janmey et al., 1992; Yonezawa et al., 1991). These sites do generally contain numerous basic residues, such as lysine and arginine, suggesting electrostatic interaction between the head groups of the phosphoinositides and actin binding.
proteins such as gelsolin (Janmey et al., 1992; McLaughlin, 1995), villin (Janmey et al., 1992), and coflin (Yonezawa et al., 1990, 1991). Further investigation will be required to identify the binding site(s) for phosphoinositides on talin.

The vesicle structures of the phosphoinositides have been shown to be essential for their action on some actin binding proteins such as gelsolin (Janmey and Stossel, 1989), α-actinin (Fukami et al., 1992), and CapZ (Heiss and Cooper, 1991). We also showed herein that the vesicle structure was involved in the inhibitory effect of the phosphoinositides on the talin-actin interaction. Preincubation of the phosphoinositides with the detergent Triton X-100 eliminated the inhibitory effect of the phosphoinositides on talin's ability to interact with actin. Preincubation of the phosphoinositides with divalent cations such as Ca$^{2+}$, which reportedly results in the aggregation of the phosphoinositides (Janmey et al., 1989), also decreased ability of the phosphoinositides to inhibit the talin-actin interaction.

The signal transduction pathway of the well described PI cycle is known to regulate a large array of cellular processes (Berridge, 1987; Berridge and Irvine, 1989). The concentration of the phosphoinositides near the cell membrane is sufficiently high (Rittenhouse and Sasson, 1985) to make their in vivo involvement in actin filament organization, via interaction with specific actin binding proteins such as talin, plausible. Thus, talin may function as an actin crosslinking protein at cell-matrix adherens junctions, and facilitate the actin filament organization/reorganization required in many cellular functions.
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References


FUNCTIONAL COMPARISON OF PLATELET TALIN (P235) WITH SMOOTH MUSCLE TALIN

A paper to be submitted to Biochemical Journal

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Summary

The abilities of purified human platelet talin (P235) and purified avian smooth muscle talin to interact with actin filaments were compared by using cosedimentation assays, viscometry assays, and electron microscopy. The results showed that platelet talin interacted directly with actin filaments in a fashion similar to that known for smooth muscle talin as follows: (1) platelet talin bound and cosedimented with actin filaments, gelated actin filament solutions, and crosslinked actin filaments into both networks and bundles at pH 6.4 and low ionic strength, (2) the interaction between platelet talin and actin was very sensitive to pH and ionic strength, (3) the interaction between platelet talin and actin was inhibited by phosphatidylinositol 4,5-bisphosphate, and (4) platelet talin was present primarily as a dimeric molecule under in vitro conditions in which it interacts with actin filaments. Some differences were noted between human platelet talin and avian smooth muscle talin, however, with platelet talin exhibiting lower actin crosslinking activity as measured by viscometry assays, and a different appearance in the actin bundles formed as observed by negative staining.
Introduction

Talin, a protein primarily concentrated at cell-matrix attachment sites, is believed to be involved in linking the cytoskeleton to the cell membrane (Beckerle and Yeh, 1990). Based upon in vitro protein binding studies showing that talin interacts with the transmembrane glycoprotein integrin (Horwitz et al., 1986), with another cytoskeletal protein vinculin (Burridge and Mangeat, 1984), but not with actin (Burridge and Mangeat, 1984), it was suggested that talin is one component in a multi-protein bridge including integrin, talin, vinculin, α-actinin, and actin that links actin filaments to the cell membrane (Burridge et al., 1988). More recently, a direct talin-actin interaction in vitro was found (Muguruma et al., 1990), which led to the proposition that talin may also link actin filaments directly to the cytoplasmic domain of integrin at the cell membrane (Simon et al., 1991).

At essentially the same time avian smooth muscle talin was discovered (Burridge et al., 1982; Burridge and Connell, 1983a, b), a protein called P235 that accounts for 3-8% of total platelet protein was isolated from human platelets (Collier and Wang, 1982a, b). It was subsequently shown that talin and P235 have similar biophysical properties, one-dimensional peptide maps, and that they are immunologically related (O'Halloran et al., 1985; Beckerle et al., 1986). Both talin and P235 have the ability to interact with vinculin in vitro (O'Halloran et al., 1985), and both talin and P235 are cleaved in vitro by the calcium-dependent proteases (calpains) to yield similar fragments (Collier and Wang, 1982a; Fox et al., 1985; O'Halloran et al., 1985; Beckerle et al., 1986, 1987). Thus, P235 is believed to be the human platelet form of talin. Recent studies also have shown that the N-terminal 47 kDa fragment, but not the C-terminal ~190-200 kDa fragment of both avian smooth muscle talin
(Muguruma et al., 1995) and human platelet talin (Niggli et al., 1994), contains the phospholipid binding site(s). Nearly all studies reported on isolated talin have been done with avian smooth muscle (gizzard) and/or human platelet talin. Some differences between human platelet talin and avian smooth muscle talin were reported. Human platelet talin has a slightly higher molecular mass (~235 kDa) by SDS-PAGE (Collier and Wang, 1982a; O'Halloran et al., 1985; Beckerle et al., 1986) than does smooth muscle talin (~225 kDa) (Molony et al., 1987), suggesting different primary structures and/or post-translational modifications, such as glycosylation (Hagmann et al., 1992). The predicted primary structure of murine talin from cDNA sequence analysis revealed an estimated molecular mass of 269,854 Da, and that the calpain cleavage site in talin is between residues 433 and 434 (Rees et al., 1990). However, the cDNA sequences corresponding to human platelet talin and avian smooth muscle talin are not yet available for comparison. The size and shape of human platelet talin reported by different labs vary (Collier and Wang, 1982a, b; Goldmann et al., 1994). Likewise, the shape and size of avian smooth muscle talin from different labs also vary (Molony et al., 1987; Muguruma et al., 1992; Schmidt et al., 1993), and may be dependent upon factors such as ionic strength and talin concentration (Molony et al., 1987).

Reports also have differed considerably with regard to the nature of the talin-actin interaction. Early reports on gizzard talin indicated no interaction with actin (Burridge and Mangeat, 1984; Beckerle and Yeh, 1990). More recently, gizzard talin has been shown to interact directly with actin in vitro (Muguruma et al., 1990, 1992, 1995; Goldmann and Isenberg, 1991; Schmidt et al., 1993; Zhang et al., 1995), although the mode of the direct talin-actin interaction reported varies considerably. Early reports on platelet talin indicated it
restricted the length of polymerizing actin filaments (Collier and Wang, 1982b). It was later suggested that the severing activity present in some platelet talin preparations might be due to the presence of severing contaminants (Burridge et al., 1990; Simon et al., 1991).

Kaufmann et al. (1991) and Goldmann et al. (1994) have reported that platelet talin interacts directly with actin in vitro and nucleates actin polymerization, which results in a decrease in F-actin viscosity. Unless human platelet talin and smooth muscle talin are markedly different, it is difficult to reconcile the latter findings with those of Muguruma et al. (1990, 1992) and Schmidt et al. (1993), who demonstrated that smooth muscle talin increased the viscosity of F-actin solutions. We recently have shown that highly purified avian smooth muscle talin is an actin filament crosslinking protein (Schmidt et al., 1993), crosslinking actin filaments into both networks and bundles at pH 6.4 and low ionic strength (Zhang et al., 1995). Talin's actin crosslinking activity is extremely sensitive to pH and ionic strength, being enhanced by decrease in pH and/or ionic strength (Schmidt et al., 1993; Zhang et al., 1995), which also may account for some of the differences in results on talin among several labs.

The reasons that account for the differences in talin's properties, and especially for those differences between human platelet talin and avian smooth muscle talin, are not clear. The purpose of this study was to directly compare the actin binding properties of purified human platelet talin and avian smooth muscle talin under identical experimental conditions.

**Experimental**

*Protein Preparation* - Smooth muscle talin was purified from turkey gizzards as described (Schmidt et al., 1993; Schmidt, 1994). Briefly, talin was extracted with a high
ionic strength buffer containing 0.5 M NaCl, and was subsequently purified sequentially on
DEAE-cellulose, hydroxyapatite, and phosphocellulose columns. Platelet talin was extracted
essentially following the procedures described in Collier and Wang (1982a). Briefly,
outdated platelets (generously provided by the Blood Bank, Mary Greeley Medical Center,
Ames, Iowa) were pelleted, washed, and extracted with a Triton X-100-containing buffer
and, then purified sequentially on DEAE-cellulose, hydroxyapatite, and phosphocellulose
columns as described for the smooth muscle talin. Purified smooth muscle talin and platelet
talin were each dialyzed into the same buffer containing 10 mM imidazole, pH 7.0, 0.1 mM
PMSF\(^1\), 1 \(\mu\)g/ml E-64, and 0.5 mM MCE immediately before use. Actin was purified from
porcine skeletal muscle as described (Schmidt et al., 1993; Schmidt, 1994), and was in a
buffer containing 2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl\(_2\), and 0.5 mM MCE.
Protein concentrations were determined by the modified Lowry method (Sigma).

**Cosedimentation Assays** - G-actin (0.50 mg/ml final concentration) was polymerized in
the absence or presence of platelet or smooth muscle talin (0.20 mg/ml final concentration)
in buffer A (10 mm imidazole, 1 mM ATP) at selected pH values, with pH adjustment of all
buffers done at 25\(^\circ\)C, by the addition of 2 mM MgCl\(_2\) (final concentration), or 2 mM MgCl\(_2\)
and 100 mM KCl (final concentrations). Final pH values were 6.4 or 6.9. All samples were
incubated, centrifuged, and then analyzed by SDS-PAGE (Laemmli, 1970) as described

\(^1\)The abbreviations used are: EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; MCE,
2-mercaptoethanol; PIP\(_2\), phosphatidylinositol 4,5-bisphosphate; PMSF,
phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel
electrophoresis.
(Schmidt et al., 1993; Zhang et al., 1995).

Low Shear Viscometry - Platelet or smooth muscle talin (0.10 mg/ml final concentration) was mixed with G-actin (0.25 mg/ml final concentration) in buffer A at specific pH values. Polymerization was then started by addition of 2 mM MgCl₂ (final concentration). The viscosities of all samples were measured after 10 min of polymerization as described (Schmidt et al., 1993). Results are expressed as normalized viscosity, obtained by dividing all talin-actin viscosity values by actin control viscosity values, and talin-actin-phospholipid viscosity values by actin-phospholipid control viscosity values. A value of one indicates no difference from controls. The phosphoinositide, PIP₂, was obtained from Sigma and used in some experiments. The PIP₂ was dissolved in water, sonicated, and stored at -70°C. It was thawed at room temperature and sonicated for 1 min on ice immediately before use. Specific conditions were tested and will be stated in the Figure Legends.

Electron Microscopy - Platelet or smooth muscle talin (0.10 mg/ml final concentration) was mixed with G-actin (0.25 mg/ml final concentration) in buffer A at specific pH values, and polymerization was induced by addition of 2 mM MgCl₂ (final concentration). Samples were negatively stained and observed as described (Schmidt et al., 1993; Zhang et al., 1995).

Chemical Crosslinking - Platelet talin (0.1 mg/ml) in buffer A containing 2 mM MgCl₂ at pH 6.9 was crosslinked with EDC (20 mM final concentration). Aliquots were taken at specific time points, and the reaction was stopped by the addition of 20 mM glycine, 20 mM Tris-HCl, pH 8.0 (final concentrations). Samples were analyzed by SDS-PAGE (Laemmli, 1970).
Results

*Talin Preparation* - Because of the composition of the starting material (muscle vs. platelets), the two types of talin were extracted by different methods, with smooth muscle talin being extracted with a high ionic strength buffer (Schmidt et al., 1993; Schmidt, 1994), and platelet talin with a low ionic strength buffer containing Triton X-100 (Collier and Wang, 1982a). After their extraction, however, they were subjected to the same series of chromatographic columns. The purified talins were consistently highly homogeneous (Fig. 1), with the only difference in purity detected being a tendency for the platelet talin to show more of a trace of the ~200 kDa C-terminal tail fragment that results from proteolysis (O'Halloran et al., 1985; Beckerle et al., 1986). Although not obvious in the gel shown in Fig. 1, platelet talin migrates slightly less distance than does gizzard talin, in agreement with previous studies (O'Halloran et al., 1985; Beckerle et al., 1986).

*Effects of pH and Ionic Strength on the Abilities of Platelet Talin and Smooth Muscle Talin to Interact with Actin Filaments* - The effect of pH on the abilities of platelet and smooth muscle talins to cosediment with actin filaments was examined by cosedimentation assays (Fig. 2). At pH 6.4 and low ionic strength, a majority of the platelet talin cosedimented with the actin filaments (Fig. 2, panel c). At pH 6.9, however, much less platelet talin cosedimented with the actin filaments (Fig. 2, panel f). Qualitatively similar results to those obtained with platelet talin were obtained with smooth muscle talin at pH 6.4 and 6.9 (Fig. 2, panels i and l, respectively). Similar results with smooth muscle talin have been shown previously (Schmidt et al., 1993). There was slightly more platelet talin than smooth muscle talin left in the supernatants (s) of the talin/actin mixtures at pH 6.4 (cf., Fig.
Fig. 1. Purified smooth muscle talin and platelet talin analyzed by SDS-PAGE. Lane 1, avian smooth muscle talin; lane 2, human platelet talin (P235).
Fig. 2. Effect of pH on the abilities of platelet and smooth muscle talins to cosediment with F-actin. Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of platelet or smooth muscle talin (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ (final concentration) at pH 6.4 or 6.9. Samples were incubated, centrifuged, and analyzed as described in Experimental. (a) actin control at pH 6.4; (b) platelet talin control at pH 6.4; (c) actin/platelet talin at pH 6.4; (d) actin control at pH 6.9; (e) platelet talin control at pH 6.9; (f) actin/platelet talin at pH 6.9; (g) actin control at pH 6.4; (h) smooth muscle talin control at pH 6.4; (i) actin/smooth muscle talin at pH 6.4; (j) actin control at pH 6.9; (k) smooth muscle talin control at pH 6.9; (l) actin/smooth muscle talin at pH 6.9. s = supernatant, p = pellet, T = talin, A = actin.
platelet talin

\[
\begin{array}{cccc}
pH 6.4 & pH 6.9 \\
\hline
a & b & c \\
sp & sp & sp \\
\end{array}
\]

smooth muscle talin

\[
\begin{array}{cccc}
pH 6.4 & pH 6.9 \\
\hline
g & h & i & k \\
sp & sp & sp & sp \\
\end{array}
\]
2, panels c with i), and slightly less platelet talin than smooth muscle talin cosedimented with F-actin in the pellets (p) of talin/actin mixtures at pH 6.9 (Fig. 2, cf., panels f with l), which indicate that, under the same ionic conditions, smooth muscle talin bound to F-actin slightly stronger than did platelet talin.

The effect of pH on the abilities of platelet and smooth muscle talins to increase the viscosity of F-actin solutions was examined by low shear viscometry assay (Fig. 3). At pH 6.4 and low ionic strength, both platelet and smooth muscle talins dramatically increased the viscosity of F-actin solutions and effectively "gelated" the F-actin solutions (i.e., the ball would not roll through the viscous solutions) (Fig. 3). As pH was increased to 6.9, both of the talins' abilities to increase the viscosity of F-actin solutions decreased as expected (Schmidt et al., 1993), with smooth muscle talin retaining stronger crosslinking activity than did platelet talin. At pH 7.3, the abilities of both smooth muscle and platelet talins to increase the viscosity of F-actin solutions decreased further, with values approaching those of the control actin (results not shown). Neither platelet nor smooth muscle talin exhibited any ability, under any conditions tested, to decrease the viscosity of actin solutions in our studies.

The nature of the interaction between platelet and smooth muscle talins and actin filaments was examined by electron microscopy (Fig. 4). At pH 6.4 or pH 6.9, and low ionic strength, the control actin filaments were randomly dispersed (Fig. 4A and 4D, respectively). At pH 6.4 and low ionic strength, smooth muscle talin crosslinked actin filaments into both networks (NW) and bundles (B) (Fig. 4B) as we have shown previously (Zhang et al., 1995). As shown in Fig. 4C, platelet talin also crosslinked actin filaments into networks
Fig. 3. Effect of pH on the abilities of platelet and smooth muscle talins to increase the viscosity of F-actin solutions. Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of platelet or smooth muscle talin (0.10 mg/ml final concentration) by addition of 2 mM MgCl₂ (final concentration) at pH 6.4 or 6.9. The viscosities of all samples were measured as described in Experimental. gel = gelated actin (i.e., ball stopped).
pH of Interaction Solutions

- Smooth muscle talin
- Platelet talin (P235)
Fig. 4. Effect of pH on the abilities of platelet and smooth muscle talins to crosslink actin filaments as shown by electron microscopy. Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of platelet or smooth muscle talin (0.10 mg/ml final concentration) by addition of 2 mM MgCl$_2$ (final concentration) at pH 6.4 or 6.9. Samples were then negatively stained as described in Experimental. (A) actin filament control at pH 6.4; (B) actin/smooth muscle talin at pH 6.4; (C) actin/platelet talin at pH 6.4; (D) actin filament control at pH 6.9; (E) actin/smooth muscle talin at pH 6.9; (F) actin/platelet talin at pH 6.9. Single arrows in C and F point to structures described in the text. NW = network, B = bundle; Bar = 100 nm for A-F.
(NW), but bundles similar to those formed by smooth muscle talin (Fig. 4B) were not seen. Instead, the platelet talin-rich actin bundles observed have aggregates of talin associated with the actin filaments in a discontinuous pattern (Fig. 4C), rather than uniformly as along the more ordered bundles observed with smooth muscle talin (Fig. 4B). At pH 6.9, smooth muscle talin's ability to crosslink actin filaments decreased, as shown by the presence of fewer and smaller bundles than at pH 6.4 (cf. Fig. 4E with Fig. 4B). At pH 6.9, platelet talin retained ability to crosslink actin filaments into networks (NW), but fewer talin aggregates were associated with actin filaments to form talin-rich actin bundle-like structures than at pH 6.4 (cf., Fig. 4F with Fig. 4C). Smooth muscle talin retained more pronounced bundling activity than did platelet talin at pH 6.9 (cf., Fig. 4E with 4F).

The effect of ionic strength on the interaction between platelet talin and actin was examined by cosedimentation assays (Fig. 5). In the presence of 100 mM KCl, platelet talin lost its ability to cosediment with actin filaments at pH 6.4 (cf., Fig. 5, panel c with Fig. 2, panel c). Schmidt (1994) showed that smooth muscle talin's ability to cosediment with F-actin at pH 6.4 in the presence of 100 mM KCl also was considerably decreased in comparison to pH 6.4 and no KCl, but a small amount (~ 20%) of the smooth muscle talin still was found in the F-actin pellet.

The results of viscometry assays (Fig. 6) are in concert with the cosedimentation assays (Fig. 5). As shown in Fig. 6, with increasing concentration of KCl, both smooth muscle and platelet talins' abilities to increase the viscosity of F-actin solutions decreased, with smooth muscle talin retaining stronger ability than platelet talin to increase the viscosity of F-actin solutions. Neither of the talins decreased the low shear viscosity of F-actin below that of F-
Fig. 5. Effect of ionic strength on the ability of platelet talin to cosediment with F-actin at pH 6.4. Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of platelet talin (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ and 100 mM KCl (final concentrations) at pH 6.4. Samples were incubated, centrifuged, and analyzed as described in Experimental. (a) actin control; (b) platelet talin control; (c) actin/platelet talin. s = supernatant, p = pellet, A = actin, P235 = platelet talin.
100 mM KCl

\[
\begin{array}{ccc}
a & b & c \\
\text{s} & \text{p} & \text{p} & \text{p} & \text{p}
\end{array}
\]

P235- 

A-
Fig. 6. Effect of ionic strength on the abilities of platelet and smooth muscle talins to increase the viscosity of F-actin solutions at pH 6.4. Actin (0.25 mg/ml final concentration) was polymerized in the absence or presence of platelet or smooth muscle talin (0.10 mg/ml final concentration) by the addition of 2 mM MgCl₂ (final concentration) and selected concentrations of KCl at pH 6.4. Samples were incubated and the viscosities of all samples were measured and expressed as normalized viscosities as described in Experimental.
Smooth muscle talin
Platelet talin (P235)
actin controls, regardless of the ionic strength tested (results not shown).

**The Effect of Phosphatidylinositol 4,5-bisphosphate on the Talin-Actin Interaction** -
The effects of PIP$_2$ on the interaction of platelet and smooth muscle talins with actin filaments were compared by viscometry assays. As shown in Fig. 7, the interaction between each type of talin and actin was inhibited by PIP$_2$. However, smooth muscle talin appeared to be more sensitive to inhibition of its ability to increase the low shear viscosity of F-actin by PIP$_2$ than was platelet talin. Both talins lost their ability to increase the viscosity of F-actin solutions at a molar ratio of ~ 30 PIP$_2$ to one talin.

**Chemical Crosslinking of Platelet Talin** - Recent studies have suggested that smooth muscle talin functions as a dimeric molecule (i.e., two subunits in the native molecule) (Muguruma et al., 1992, 1995; Zhang et al., 1995) as also suggested for platelet talin (Goldmann et al., 1994). Our crosslinking results obtained with the zero-length chemical crosslinking reagent, EDC are shown in Fig. 8. With increasing crosslinking time at pH 6.9 and low ionic strength, the amount of a band corresponding to an approximately 400 kDa protein increased, concomitantly with a decrease in the amount of the monomer band of platelet talin. Thus, platelet talin was present as a dimer, a result in agreement with that obtained with platelet talin by Goldmann et al. (1994), and similar to what we obtained with smooth muscle talin (Zhang et al., 1995).

**Discussion**

We used the same series of chromatographic columns to purify both the platelet and smooth muscle talins, and obtained highly homogenous samples of proteins. We then
Fig. 7. Effect of the phosphoinositide PIP$_2$ on the abilities of platelet and smooth muscle talins to increase the viscosity of F-actin solutions at pH 6.4. Platelet or smooth muscle talin (0.10 mg/ml final concentration) was incubated with phosphatidylinositol 4,5-bisphosphate (PIP$_2$) for 30 min at 25°C before G-actin (0.25 mg/ml final concentration) was added, and polymerization started by the addition of 2 mM MgCl$_2$ (final concentration). The viscosities of all samples were measured and expressed as normalized viscosities as described in Experimental.
Molar Ratio of PIP-2 to Talin

- smooth muscle talin
- platelet talin(P235)

Normalized Viscosity vs. Molar Ratio of PIP-2 to Talin
Fig. 8. Chemical crosslinking of platelet talin by EDC. Platelet talin (0.1 mg/ml final concentration) in buffer A containing 2 mM MgCl₂ at pH 6.9 was crosslinked with EDC (20 mM final concentration). Aliquots of the reaction mixture were taken at specific time points, quenched, and analyzed by SDS-PAGE. Lane 1, talin before addition of EDC; lanes b, c, d, e, and f are after addition of EDC and represent 10, 30, 60, 120, and 240 min of crosslinking, respectively. Numbers to the right of the gel correspond to the molecular mass (kDa) of protein standards in lane g. M = talin monomer, D = talin dimer corresponding an ~ 400 kDa protein.
compared, under the same sets of experimental conditions, the abilities of human platelet and smooth muscle talins to interact with actin by using cosedimentation and viscometry assays, and electron microscopy. In general, the results showed that platelet talin interacted with actin in a fashion similar to that obtained with smooth muscle talin. At pH 6.4 and low ionic strength, both proteins cosedimented with actin filaments, markedly increased the viscosity of F-actin solutions, and crosslinked actin filaments as shown by negative staining. Furthermore, the abilities of both proteins to interact with actin were very sensitive to pH and/or ionic strength, being decreased with increase in pH and/or ionic strength. The abilities of both types of talin to interact with actin were effectively eliminated by their interaction with PIP$_2$.

The results of the cosedimentation and viscometry assays obtained herein, with both talins, are similar to those obtained with avian smooth muscle talin by Muguruma and associates (Muguruma et al., 1990, 1992, 1995) and by Schmidt et al. (1993) and Zhang et al. (1995) in our lab, but are different from those obtained with human platelet talin by Isenberg and associates (Kaufmann et al., 1991; Goldmann et al., 1994). Kaufmann et al. (1991) and Goldmann et al. (1994) reported a platelet talin-induced decrease in the viscosity of F-actin solutions, and suggested that the decrease in actin viscosity, especially at short polymerization times, resulted from an increase in number of actin filaments but decrease in their length. We did not observe any decrease in F-actin viscosity as a result of addition of either platelet or smooth muscle talin under any experimental conditions. And, we measured the low shear viscosity after only 10 minutes of polymerization. Thus, these differing results obtained by different labs seem unlikely to be due primarily to functional differences between
human platelet talin and avian smooth muscle talin. The reason for platelet talin's actin viscosity-reducing activity (Kaufmann et al., 1991; Goldmann et al., 1994) may be the purity of the talin used. Those investigators used a rather high molar ratio of talin to actin (~ one talin to two actins), which can increase the problem with even a small amount of contaminants having either potent nucleating, capping, or severing activity. Our lab showed, for instance, that actin binding contaminants in conventionally purified preparations of vinculin significantly decreased the low shear viscosity of F-actin solutions (Evans et al., 1984). Interestingly, others have reported (Burridge et al., 1990; Simon et al., 1991) that an actin severing contaminant(s) can be removed from human platelet talin prepared by procedures similar to those described in Kaufmann et al. (1991) and Goldmann et al. (1994).

The differences observed herein that avian smooth muscle talin possesses somewhat stronger actin crosslinking activity than platelet talin under the same ionic conditions, and the different appearance of the actin bundle-like structures formed in the presence of platelet talin in comparison to the more highly ordered actin bundles formed in the presence of smooth muscle talin, may be related to different molecular properties of the two talins. Human platelet talin migrates slightly slower than avian smooth muscle talin by SDS-PAGE (O'Halloran et al., 1985; Beckerle et al., 1986), suggesting different primary structure/size and/or post-translational modifications. No sequence information for the two talins is yet available for comparison. One report (Hagmann et al., 1992) indicated that a small proportion of smooth muscle talin, but none of the platelet talin, is glycosylated. Unfortunately, most primary structure data are derived from analysis of cDNA sequence, and the type and degree of post-translational modification in the two talins may not become
available for some time. In cells grown in culture, talin is found together with vinculin in focal adhesions (Burridge and Connell 1983a; Burridge et al., 1988). In adult smooth muscle cells, talin is primarily localized at the analogous membrane-associated dense bodies (Volberg et al., 1986). In resting platelets, however, talin is absent from the vinculin-rich areas at the ends of stress fibers near the cell membrane (Nachmiyas and Golla, 1991) and instead has a uniform, cytoplasmic distribution (Beckerle et al., 1989). The distribution of talin in platelets changes dramatically when platelets are activated to become adhesive, as the talin is translocated to a peripheral submembrane location (Beckerle et al., 1989). These functional differences between smooth muscle talin and platelet talin suggest the possibility of some differing molecular properties of the two proteins.

Heise et al. (1991) have shown that talin binds acidic phospholipids such as phosphatidylserine, and the phospholipid binding sites have been located on talin's N-terminal ~ 47 kDa fragment (Niggli et al., 1994; Muguruma et al., 1995). No evidence, however, for an effect of any of the phospholipids on the talin-actin interaction has been reported. The phosphoinositides have been shown to inhibit the interaction of a number of actin binding proteins with actin (Isenberg, 1991; Janmey, 1994). We have shown in this study, by using low shear viscometry assays, that the phosphoinositide PIP₂ inhibits the abilities of both platelet and smooth muscle talins to increase the viscosity of F-actin solutions at pH 6.4 and low ionic strength. The results suggest that the actin binding activities of both platelet and smooth muscle talins may be regulated in a similar fashion by phosphoinositides.

Overall, the results of this study indicate that human platelet talin and avian smooth
muscle talin share many similarities in their abilities to interact with actin, but that some differences remain.

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References


OVERALL SUMMARY

The major objectives of my dissertation studies were to (1) determine the structural basis for the effect of pH and ionic strength on the talin-actin interaction, (2) examine the effects of other factors, including specific phospholipids, on the talin-actin interaction, and (3) compare abilities of human platelet and smooth muscle talins to interact with actin. In the first and second chapters included herein, I used cosedimentation assays, electron microscopy, and chemical crosslinking experiments to examine the structural basis for the pH and ionic strength dependence of the talin-actin interaction. The major findings from the results of these experiments are as follows:

(1) At pH 6.4 and low ionic strength, talin extensively crosslinked actin filaments into both networks of intermeshed filaments and bundles. The bundles consist of parallel actin filaments with a center-to-center filament spacing of ~ 13 nm, and talin crossbridges positioned at ~ 36 nm intervals along the actin bundles. As the pH was increased from 6.4 to 7.3, talin's bundling activity was first decreased, then its networking activity, with both activities nearly absent at pH 7.3. The talin-actin interaction also was very sensitive to ionic strength. Talin's actin bundling activity at pH 6.4 was dramatically decreased when the ionic strength was increased by adding as little as 25 mM KCl to the protein solution, and disappeared with 75 mM KCl present, with only its networking activity remaining. The results show that talin can interact directly with actin filaments by formation of actin filament networks and bundles, with the bundles more sensitive to dissolution by increase in pH and/or ionic strength.
(2) Talin was present primarily as a dimeric molecule at pH 6.4 to 7.3, with or without 100 mM KCl present. These results show that talin's ability to self-associate is not noticeably pH or ionic strength dependent, indicating that the effect of pH or ionic strength on the talin-actin interaction appears due to the direct talin-actin interaction rather than the self-association of talin.

(3) Talin's ability to crosslink actin filaments was not noticeably affected by the length of polymerization time. Even with a short, five minute polymerization time, talin crosslinked actin filaments into both networks and bundles, without noticeably restricting the length of actin filaments in comparison to control F-actin.

(4) Talin was able to crosslink preformed actin filaments into both networks and bundles as well as it did when actin was polymerized in its presence.

(5) Talin's ~ 190kDa C-terminal tail fragment had the ability to crosslink actin filaments into both networks and bundles. However, it required a slightly lower pH than did intact talin.

The major objective of the third chapter of my dissertation was to examine the effect of specific phospholipids, especially phosphoinositides, on the talin-actin interaction by using cosedimentation assays, low shear viscometry assays, and electron microscopy. The major findings obtained from the results in this chapter are as follows:

(1) The three phosphoinositides, phosphatidylinositol 4.5-bisphosphate (PIP$_2$), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol (PI), when incubated with talin (molar ratio of ~ 30 phosphoinositides to one talin) prior to its addition to actin and polymerization, effectively inhibited the ability of talin to crosslink actin filaments into both
networks and bundles at pH 6.4 and low ionic strength. Phosphoinositides affected the talin-actin interaction by interacting with talin, but not actin, by forming talin/phosphoinositide complexes, which in turn prevented talin from effectively interacting with actin filaments.

(2) The presence of phosphatidylserine (PS), phosphatidylcholine (PC), and inositol 1,4,5-trisphosphate (IP₃) under the same ionic conditions did not affect the talin-actin interaction.

(3) The presence of G-actin during the talin-phosphoinositide preincubation period did not affect the interaction between talin and phosphoinositides.

(4) The inhibitory effect of phosphoinositides on the talin-actin interaction was eliminated by preincubating phosphoinositides with the detergent Triton X-100, suggesting that phosphoinositide vesicles are necessary for their inhibitory effects. The inhibitory effect of phosphoinositides on the talin-actin interaction also was decreased by preincubation with divalent cations such as Ca²⁺.

The major objective of the fourth chapter in my dissertation was to compare the abilities of purified human platelet and avian smooth muscle talins to interact with actin filaments, under the same experimental conditions, by using cosedimentation assays, low shear viscometry assays, and electron microscopy. The major findings obtained from the results in this chapter are as follows:

(1) Highly homogeneous human platelet talin and avian smooth muscle talin were obtained by following similar preparation procedures.

(2) Platelet talin (P23S) interacted with actin filaments in a manner similar to that of smooth muscle talin. At pH 6.4 and low ionic strength, both talins bound and cosedimented
with actin filaments, dramatically increased the viscosity of F-actin solutions, and crosslinked actin filaments into networks and bundles. As pH and/or ionic strength was increased, platelet talin's ability to crosslink actin filaments decreased as also was observed for smooth muscle talin.

(4) The interaction of platelet or smooth muscle talin with actin was inhibited by PIP2.

(5) Some differences were detected between platelet and smooth muscle talins, with smooth muscle talin exhibiting a stronger actin crosslinking activity, and platelet talin crosslinking actin filaments into bundle-like structures that appeared different than the more highly ordered actin bundles obtained with smooth muscle talin.

Overall, the results presented in my dissertation provide a structural explanation for the reported talin-actin interaction, in which talin directly binds to actin filaments and increases the viscosity of F-actin solutions (Muguruma, et al., 1990; 1992; Schmidt et al., 1993). I have shown that talin crosslinks actin filaments into both networks and bundles at pH 6.4 and low ionic strength. These results suggest that talin may function as an actin crosslinking protein at cell-matrix adherens junctions, and thereby facilitate the actin filament organization/reorganization required in many cellular functions.

My results do not support the reports that platelet talin decreases the viscosity of actin solutions, especially at short polymerization times, by increasing the number of actin filaments and decreasing their length (Kaufmann et al., 1991; Goldmann et al., 1994). The results presented herein showed that after only a five minute-polymerization talin crosslinked actin filaments into networks and bundles, without showing a significant number of shorter actin filaments in comparison to the actin controls. I also compared the abilities of human
platelet and avian smooth muscle talins to interact with actin under the same experimental conditions. These results showed that human platelet talin behaved in an overall fashion similar to that of smooth muscle talin, but with some differences noted. One possibility for some of the differences in the talin-actin interaction reported by different labs is the degree of purity of the talin used. Burridge et al. (1990) and Simon and Burridge (1994) have noted that actin severing contaminants are present in some platelet talin preparations. Another possibility for some of the differences in the talin-actin interaction reported from different labs is the marked sensitivity of the talin-actin interaction that I observed. Even rather small differences in experimental conditions can significantly influence the results.

My studies also showed for the first time that talin interacts with phosphoinositides. These results open up an entirely new avenue with regard to how talin may interact with actin or other talin binding proteins in cells.
LITERATURE CITED


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