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Examination of the effects of selected factors on the talin-actin interaction

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Examination of the effects of selected factors on the talin-actin interaction

by

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INTRODUCTION

Dissertation Organization

My dissertation is arranged following an alternate format with the inclusion of three papers. The main body consists of one short paper, which has been published in Biochemical and Biophysical Research Communications, followed by two longer papers that will be submitted for publication to the Journal of Biological Chemistry. I was responsible for essentially all of the experimentation described, with the exception of the electron microscopy that was done by Jinwen Zhang. The protease calpain that I used in experiments in the third paper was purified by Elisabeth Lonergan, a fellow graduate student in our lab. The second and third papers in this dissertation differ from the final forms that will be submitted for publication, which will each contain electron micrographs done by Jinwen Zhang. They are not included herein because they will be included with other electron micrographs in a section of Jinwen Zhang's Ph.D dissertation. The three papers are preceded by this Introduction, which includes a literature review, and will be followed by an Overall Summary. The references cited specifically in the Introduction and Overall Summary are included in the Literature Cited section at the end of this dissertation.

General Overview

Adhesion plaques are specialized areas where the attachment of actin filaments to the cytoplasmic side of the cell membrane coincides with attachment of the cell to either extracellular matrix components (cell-matrix junctions) or to other cells (cell-cell junctions) [for review, see Burridge et al., 1988]. These sites are very complex and are believed to be involved in a variety of important cellular processes such as cell adhesion, cell motility, muscle cell contraction, growth, and differentiation. Adhesion plaques contain a wide range of proteins including several

different actin binding proteins, kinases, and at least one protease, all of which are thought to help regulate interactions at these sites [for review, see Lo and Chen; 1994]. These sites also can be affected by the local lipid environment at the membrane and by potentially significant changes in various ion concentrations due to their proximity to ion channels within the membrane. This high degree of complexity and regulation is essential in these sites, which must be able to rapidly assemble or disassemble in response to a variety of extracellular signals [Lo and Chen, 1994]. Talin has been implicated as having a key role in the attachment of actin filaments to the cell membrane at cell-matrix adhesion plaques based upon its location and ability to bind to both the integral membrane protein integrin [Horwitz et al., 1986] and to actin filaments [Muguruma et al., 1990; Kaufmann et al., 1991; Schmidt et al., 1993] *in vitro*. The focus of the studies that follow is on selected factors that influence the interaction between smooth muscle talin and actin including pH, ionic strength, protein molar ratio, temperature, and the presence of other adhesion plaque proteins. The results that will be described in my dissertation are supportive of a role for talin in the organization and attachment of actin filaments to the cell membrane at adhesion plaques.

Literature Review

In the first part of this review, I will briefly discuss three systems in which adhesion plaques play a key role, namely skeletal muscle, smooth muscle, and platelets. I then will focus on the following individual adhesion plaque proteins: talin, actin, integrin, vinculin, filamin, α-actinin, tropomyosin, and calpain. These proteins are all thought to be important in adhesion plaque function and, with the exception of integrin, each is used in specific experiments described in this dissertation. The main body of my work involves the talin-actin interaction and, therefore, these

two proteins will be reviewed in more detail than the others.

Skeletal Muscle

Skeletal muscle cells (muscle fibers) are long cylindrical cells with tapering ends, which have varying lengths (20-30 mm average) and diameters (10-100 μm) depending on the muscle type [for reviews, see Goll et al., 1984; Alberts et al., 1994; Craig, 1994]. The ends of each muscle fiber are connected to other muscle fibers or to bone via tendons at myotendinous junctions. Each fiber is surrounded by a thin sheet of connective tissue called the endomysium, which is in close contact with the muscle cell sarcolemma. The sarcolemma consists of an outer basement membrane, which contains extracellular matrix components, that can be difficult to distinguish from the continuing endomysium. The inner part of the sarcolemma is similar to the plasmalemma (cell membrane) of non-muscle cells and consists primarily of membrane proteins, phospholipids, and cholesterol. Motor neurons contact muscle fibers at specialized areas on the sarcolemma called neuromuscular junctions [for review, see Small et al., 1992]. The sarcolemma differs from most other non-muscle cells in that it can propagate an action potential in a manner similar to nerve cells. The sarcolemma also has a series of thin tubular invaginations called transverse or T tubules, which run perpendicular to the long cellular axis, and are located at regular intervals along the long axis of the cell. The T tubules run into the interior of the muscle cell and allow the signals from the motorneurons to be carried quickly throughout the cell.

Within the cell, T tubules are usually in close contact with the terminal cisternae that are part of the extensive membranous network called the sarcoplasmic reticulum, which is analogous to the endoplasmic reticulum of non-muscle cells [Goll et al., 1984; Alberts et al., 1994]. The sarcoplasmic reticulum functions as the primary storage site of calcium inside the muscle fiber

until it is released during contraction. The nuclei of skeletal muscle cells are located just beneath the sarcolemma. Mature skeletal muscle fibers are multinucleated because they are formed in the embryo from the fusion of multiple (~ 100-200) myoblasts. The number of mitochondria in a muscle cell depends on the cell type, with cells that require prolonged or sustained contractions containing more mitochondria. Muscle cells of the latter type also contain myoglobin in relatively larger amounts. The interior of the long skeletal muscle cell is nearly packed with myofibrils, which make up the skeletal muscle contractile apparatus. Myofibrils are protein threads (~ 1-3 μm in diameter) that are aligned parallel to the long axis of the muscle fiber and that extend from one end of the cell to the other [Goll et al., 1984; Alberts et al., 1994; Craig, 1994]. The myofibrils consist of alternating dark (A-bands) and light (I-bands) bands, which are bisected by an M-line and a Z-line, respectively. The contractile unit of the myofibril that extends from one Z-line to the next Z-line is called a sarcomere. All of the myofibrils are in register and give skeletal muscle its characteristic cross-striated appearance.

The contractile apparatus (myofibril) of skeletal muscle cells is highly organized with regular arrays of interdigitating thick and thin filaments held in register at the level of the transverse M-lines and Z-lines, respectively [Small et al., 1992]. The thick filaments (~ 15 nm in diameter by 1.5 μm long) are composed primarily of myosin. Myosin is a hexamer consisting of two large or "heavy" subunits (~ 220 kDa each) that each have a globular head, a long tail that forms the long myosin rod in conjunction with the tail of the other large subunit, and two pairs of light subunits (~ 20 kDa each) [Goll et al., 1984; Alberts et al., 1994; Craig, 1994]. The complete myosin molecule consists of a long α-helical coiled-coil rod with the two globular heads located on the same end. One of each pair of light chains or subunits is associated with each globular head. Myosin molecules can aggregate to form the bipolar thick filaments in skeletal muscle, with the

rod portions of the molecules forming the backbone of the filament, and the globular heads extending from the filament surface and free to associate with actin. The thin filaments (~ 6-8 nm in diameter by 1 μm long) are composed primarily of F-actin, tropomyosin, and the troponin complex. The very high molecular weight protein nebulin also is associated with the thin filament and may help regulate thin filament length [Small et al., 1992]. The actin-containing thin filaments are oriented in the sarcomere such that the pointed end of each filament interdigitates with the myosin-containing thick filaments, and the barbed end is anchored at the Z-line, presumably by α-actinin [Yamaguchi et al., 1985; Vigoreaux, 1994]. The thick filaments all seem to be held in register in cross section at the level of the M-line, which contains M-protein, myomesin, and creatine kinase [Small et al., 1992]. The giant protein titin is a very long extensible molecule that extends from the Z-line to the M-line, and may play a role in maintaining the structural integrity of the sarcomere. Adjacent myofibrils appear to be held in register by desmin intermediate filaments, which encircle the myofibrils at the level of the Z-line and tie adjacent myofibrils together, and by skelemin, which encircles myofibrils at the level of the M-line and also may tie adjacent myofibrils together [Small et al., 1992].

Contraction is thought to occur by a sliding of the thick and thin filaments with respect to each other. The actin-activated ATPase of myosin provides the energy needed for the force of the contraction. Regulation of contraction resides primarily on the thin filament, and may function via the steric blocking model [for reviews, see Zott and Potter, 1987; Payne and Rudnick, 1989]. In this model, the tropomyosin-troponin complex binds along the actin filament, and in the absence of Ca^{2+}, tropomyosin is positioned such that it inhibits the interaction between actin and myosin by sterically blocking the myosin binding sites on the actin filament [Zott and Potter, 1987; Payne and Rudnick, 1989]. When the signal to contract is received, the T tubules carry the message to

the sarcoplasmic reticulum, which releases stored Ca^{2+} [Goll et al., 1984]. Troponin C then binds the calcium, which causes a change in the conformation of the entire tropomyosin-troponin complex. This causes a shift in the position of tropomyosin on the actin filament, thereby opening up the myosin binding sites on actin and allowing contraction to proceed [Zott and Potter, 1987].

The force of contraction is transmitted across the muscle cell membrane to the extracellular matrix at the myotendinous junctions at the ends of the cell, and laterally at sites called costameres. Myotendinous junctions occur at each end of the muscle fiber where the muscle is attached to tendons. The terminal thin filaments of each myofibril are attached to the sarcolemma via their barbed ends at these sites [Small et al., 1992] and they, therefore, fall under the category of cell-matrix adhesion plaques. Costameres are structures that are located at the cytoplasmic face of the sarcolemma and run perpendicular to the long axis of the cell on either side of each myofibrillar Z-line [Pardo et al., 1983; Small et al., 1992]. Costameres are thought to be responsible for linking the peripheral layer of myofibrils, perhaps through actin, to the membrane [Small et al., 1992]. The name costamere comes from the ribbed-like appearance (from Latin *costa,* meaning rib and Greek *meros,* meaning part) that these structures exhibit when fluorescently labelled by antibodies to the adhesion plaque protein vinculin [Pardo et al., 1983]. Costameres also appear to be areas of the muscle cell that help link the extracellular matrix components and internal cytoskeletal elements. This has been shown to be likely because several adhesion plaque proteins are located in the costameres, and contracting cells show a pleated pattern, with the sarcolemma appearing to be anchored near the Z-lines but free to balloon out in the middle of the sarcomere as the costameres move closer to each other [Small et al., 1992]. Neuromuscular junctions are also sites that contain several adhesion plaque proteins such as integrin, talin, vinculin, α-actinin, and filamin [Small et al., 1992]. Talin has been localized at

each of the adhesion plaque structures (costameres, neuromuscular junctions, and myotendinous junctions) present in skeletal muscle [Belkin et al. 1986; Sealock et al., 1986; Tidball et al. 1986] and, thus, may play a key role in (a) transmitting the force of muscle cell contraction across the cell membrane to the extracellular matrix at these sites, and (b) assisting in the maintenance of the highly ordered internal structure of the skeletal muscle cell.

Smooth Muscle

Smooth muscle cells are often spindle shaped and ~ 3-6 μm in diameter by ~ 100-200 μm long in appearance [for review, see Somlyo and Somlyo, 1992]. The cells are surrounded by connective tissue that contributes to the distribution of force within the smooth muscle. Smooth muscle cells have a high surface area to volume ratio that is increased by the presence of flask-shaped invaginations called caveolae. Unlike the multinucleated skeletal muscle cell, there is only one, centrally located nucleus per smooth muscle cell. The contractile apparatus of smooth muscle lacks the striated appearance and high degree of organization found in the skeletal muscle system. Thick and thin filaments are arranged in parallel arrays that are not in register and that give the cells their smooth appearance. The thin filaments are more numerous and longer (~ 1-2 μm) than in skeletal muscle cells, and contain actin and tropomyosin but not troponin [Somlyo and Somlyo, 1992]. The thin filaments are anchored at their barbed ends by α-actinin-containing cytoplasmic dense bodies that are thought to be analogous to the skeletal muscle Z-lines [Small et al., 1992]. The thick filaments are also longer (~ 2.2 μm) in smooth muscle than in skeletal muscle and contain a smooth muscle isoform of myosin [Somlyo and Somlyo, 1992]. Smooth muscle cells contain large numbers of intermediate filaments, which are thought to be important in maintaining the cytoskeletal stability of the cell by binding to the cytoplasmic and membrane-

associated dense bodies [Small et al., 1992; Somlyo and Somlyo, 1992]. According to Small et al. [1992], the interior of the cell can be separated into two basic domains, with one containing the actin-myosin based contractile apparatus, and the other one containing the actin-intermediate filament based cytoskeleton, although there is some overlap between these two general domains.

Smooth muscle contraction is not voluntary, and is under the control of both the autonomous nervous system and hormonal signals. Gap junctions are areas of close contact between adjacent smooth muscle cells, and permit the rapid spread of the electrical signal for contraction throughout the tissue and possibly also the exchange of metabolites between cells [Somlyo and Somlyo, 1992]. Smooth muscle cells lack the T tubule system found in skeletal muscle, but do have a sarcoplasmic reticulum that acts as a store for intracellular calcium. Calcium release from the sarcoplasmic reticulum is triggered by the depolarization of the sarcolemma and/or by the action of inositol 1,4,5-trisphosphate (IP_3), which is formed as a second messenger in response to hormonal stimulation [Somlyo and Somlyo, 1992].

Smooth muscle contraction occurs by the sliding of thin and thick filaments with respect to each other, in a manner roughly similar to that of skeletal muscle. It has been suggested that there are groupings of three to five thick filaments, which could form a kind of "mini sarcomere" analogous to the well defined sarcomeric structure of skeletal muscle [Somlyo and Somlyo, 1992]. The energy required for contraction is supplied by the actin-activated smooth muscle myosin ATPase, as is the case in skeletal muscle. However, the regulation of contraction by calcium is thought to reside primarily with the thick filaments in smooth muscle as opposed to the thin filament system in skeletal muscle. Thick filament regulation of contraction in smooth muscle occurs because the smooth muscle myosin isoform is sensitive to phosphorylation of one class of the myosin light chains by a Ca^{2+}-calmodulin-dependent myosin light chain kinase [for review, see

Trybus, 1991].

Smooth muscle myosin is very similar to skeletal muscle myosin in overall structure, consisting of two heavy chains that combine to form a long rod tail domain with two globular heads at one end of the molecule. Each of the heads is associated with two light chains of ~ 20 kDa and 17 kDa mass. Dephosphorylated smooth muscle myosin adopts a folded conformation and cannot form filaments *in vitro* [Trybus, 1991]. Phosphorylation of the 20 kDa light chain *in vitro* by myosin light chain kinase (MLCK) results in a change in the conformation of the myosin molecule allowing it to form filaments, bind to actin, and increase the actin-activated ATPase to ~ 500 times that of the dephosphorylated form [Trybus, 1991]. This activation by phosphorylation of myosin is the basis of the major form of contractile regulation in smooth muscle [Trybus, 1991; Somlyo and Somlyo, 1992]. When the cell is stimulated to contract, the sarcoplasmic reticulum releases its stores of calcium, and some extracellular Ca^{2+} enters the cell as well. The Ca^{2+} binds to calmodulin, which in turn interacts with and activates MLCK. The activated MLCK then phosphorylates the regulatory class of myosin light chains and allows contraction to occur. As calcium levels subsequently decrease, the MLCK activity decreases and phosphatase activity increases, resulting in deactivation of myosin and relaxation of the muscle cell. Thick filament regulation accounts for the majority of regulation of smooth muscle contraction. However, there also may be some regulation via the thin filament [Somlyo and Somlyo, 1992].

Some degree of thin filament regulation of smooth muscle cell contraction is possible through the interactions among several actin binding proteins. Smooth muscle thin filaments contain tropomyosin, which can activate the actin-activated ATPase of smooth muscle myosin [Lees-Miller and Helfman, 1991]. However, another actin binding protein in smooth muscle called caldesmon

can bind to tropomyosin and actin when Ca^{2+} levels are low and, thus, inhibit the actin-activated ATPase [Sobue and Sellers, 1991]. When Ca^{2+} binds to calmodulin, the calmodulin can bind to caldesmon and release caldesmon from the thin filament. This, in turn, releases the inhibition of the actin-myosin interaction and permits contraction to occur [Sobue and Sellers, 1991]. There also is another possible form or model of thin filament regulation in smooth muscle. The troponin-T like protein called calponin [for review, see Winder and Walsh, 1993] can also bind to actin filaments and to tropomyosin, and can inhibit the actomyosin ATPase in a Ca^{2+}- and phosphorylation-dependent manner. Like caldesmon, the inhibitory action of calponin is released by binding of Ca^{2+}-calmodulin. Calponin can be phosphorylated by protein kinase C, which also interferes with the ability of calponin to inhibit the actomyosin ATPase activity [Winder and Walsh, 1993]. In both of these latter thin filament-based models, Ca^{2+} triggers a release of inhibition of actin-myosin interaction, thus allowing contraction to occur in a manner similar to that described for the steric blocking model proposed to explain the thin filament-based regulation in skeletal muscle. It remains to be seen whether either or both of these models can explain thin filament regulation in smooth muscle, and how important either is in comparison to the thick filament-based regulatory system.

The adhesion plaques in smooth muscle are the membrane-associated dense bodies [Small et al., 1992]. These structures are located in areas along the cytoplasmic face of the cell membrane that are structurally distinct from the caveolae and from the areas of the membrane associated with the sarcoplasmic reticulum [Somlyo and Somlyo, 1992]. The membrane-associated dense bodies are areas of thin filament attachment to the inside face of the cell membrane, and also are sites where the membrane is attached to extracellular matrix components. The membrane-associated dense bodies contain many of the proteins commonly associated with adhesion plaques such as

talin, vinculin, filamin, and integrin [Small et al., 1992]. Presumably these areas are important in linking the cell cytoskeleton to the extracellular matrix and, thus, in transmitting the force of smooth muscle cell contraction to the rest of the tissue.

Platelets

Platelets are involved in the clot formation that is important in the repair of injured blood vessels. Platelets are small cell fragments, ~ 2-4 μm in diameter, that are generated from the rupture of megakaryocytes [for review, see Marieb, 1991]. They lack a nucleus and will degenerate in ~ 10 days in the bloodstream if not activated for clot formation. The resting platelet is non-adhesive and, therefore, does not contain any recognizable separate adhesion plaque structures. The cytoskeleton of the resting platelet is thought to be made up primarily of filamin (actin binding protein) that binds to the cytoplasmic side of the transmembrane GPIb-IX complex and to actin to form a membrane skeletal network similar to the spectrin-based cytoskeleton of the red blood cell [Fox et al., 1988; Fox, 1993]. The platelet can be activated by binding to the collagen exposed during blood vessel injury, by factors that are released by cells from the injured tissue, or by factors released by other activated platelets [Marieb, 1991]. When the platelet is activated, extensive cytoskeletal reorganization takes place and several adhesion plaque components become preferentially located at sites of platelet cell adhesion [for review, see Fox, 1993]. Activated platelets adhere to each other and to the insoluble fibrin strands, which make up the structural foundation of the clot. The platelets induce clot retraction through an actin-myosin based contraction, which exerts force on the surrounding network of fibrin strands and, in turn, results in a compaction of the clot [Marieb, 1991]. This causes the injured walls of the blood vessel to move closer together, and aids in tissue repair. Activated platelets also release factors

that stimulate tissue repair in the surrounding cells, such as platelet derived growth factor [Marieb, 1991]. Although these events have been studied extensively, the details of the reactions involved in the activation process are still unclear.

Activation causes a dramatic change in the polymerization state of platelet actin. In the resting platelet, ~ 30-40% of the actin exists as filaments, with the remaining ~ 60% existing as G-actin that is presumably complexed with actin sequestering proteins such as profilin and/or thymosin β_4 [Fox, 1993]. However, upon activation as much as 70% of the total actin in platelets is in the filamentous form. The activation of actin polymerization could be triggered by a release of monomers from monomer sequestering proteins, by the uncapping of filament ends, and/or by the activation of actin filament severing proteins that would create more filament ends for polymerization [Fox, 1993]. The activities of several actin binding proteins seemingly must be coordinated to permit such a large shift in the polymerization state of actin and the extensive cytoskeletal reorganization that accompanies platelet activation.

Talin is very abundant in platelets and makes up from ~ 3 to 8% of the total platelet protein [Collier and Wang, 1982a]. In resting platelets talin has a diffuse cytoplasmic location. However, upon activation talin becomes rapidly localized to the membrane [Beckerle et al, 1989]. This redistribution of talin may be due to a phosphorylation event, and does not appear to be directly related to the activation of the platelet integrin or to increased calpain proteolytic activity [Bertagnolli et al., 1993]. It is thought that the talin present in activated platelets may have a role in linking actin filaments and, therefore, the actin-myosin contractile apparatus to the platelet membrane, as is generally proposed for talin at adhesion plaques in other cells [Fox, 1993].

Talin

Talin was discovered in 1982 by two independent research groups working with different cell systems. Burridge and associates [Burridge et al., 1982; Burridge and Connell, 1983a] described an ~ 215 kDa protein that could be purified from avian smooth muscle and localized by immunofluorescence microscopy at focal contacts and ruffling membranes in cultured fibroblasts. They named the protein "talin" from the Latin word *talus*, meaning ankle, because of its proposed function in linking actin filaments to the membrane. At approximately the same time, Collier and Wang [1982a; 1982b] isolated a protein that they called P235 from platelets, which was ~ 235 kDa in molecular mass, could bind to actin, and could easily be proteolytically degraded by calpain. These two forms of talin subsequently have been shown to be related by immunological crossreactivity, similar biophysical characteristics, similar one-dimensional peptide maps, and an ability to bind to vinculin [Beckerle et al., 1986]. Both proteins are now generally referred to as talin. There are, however, some differences between the two types of talin, which may be due to either species-specific or tissue-specific isoforms. One notable difference is found in the molecular mobility as analyzed by SDS-PAGE, where the platelet (235 kDa) [Collier and Wang, 1982] form migrates slower than the gizzard smooth muscle form (225 kDa) [Molony et al., 1987]. Unfortunately, an extensive comparison between talin from the same species, but different cell origin, has not yet been reported, and the only complete sequence information comes from mouse cDNA studies [Rees et al., 1990]. This makes it difficult to directly compare results on talin from various laboratories. Talin has been shown to be a complex molecule that can adopt different conformations under different conditions *in vitro* [Molony et al., 1987]. Thus, discrepancies among results from various laboratories could be related to the use of different sources of talin, the use of different *in vitro* conditions to study the molecule, and the possibility

that protein preparations may contain small amounts of contaminants that may exert a large effect on the properties assigned to talin. When comparing results involving talin from different laboratories, I will endeavor to point out in this review the different sources and conditions used in the specific studies.

Talin has been localized primarily to cell-matrix type adhesion plaques in a variety of cell types including focal contacts and ruffling membranes in cultured cells [Burridge and Connell, 1983a; 1983b], membrane-associated dense plaques in smooth muscle [Geiger et al., 1985; Volberg et al., 1986], and myotendinous junctions [Tidball et al., 1986], neuromuscular junctions [Sealock et al., 1986], and costameres [Belkin et al., 1986] in skeletal muscle. Talin does not appear to be associated with cell-cell type adhesion plaques, such as zonula adherens in epithelial cells [Geiger et al., 1985], even though other proteins that colocalize with talin at cell-matrix type junctions can also be found at cell-cell junctions (e.g., vinculin, α-actinin, and actin). It is also interesting that talin is often present at these sites in the earliest stages of adhesion plaque formation. For example, talin is associated with the ends of small bundles of actin filaments in the leading lamellae of motile cells before the adhesion plaque is actually formed [DePasquale and Izzard, 1991]. Talin also is found at developing membrane-associated dense bodies in embryonic chicken gizzard muscle as early as 16 days, whereas vinculin only appears at these sites at ~ 1 to 3 days post-hatching [Volberg et al., 1986]. Talin is relatively abundant in platelets, where it makes up ~ 3-8 % of the total platelet protein [Collier and Wang, 1982a]. In resting (nonadherent) platelets talin is uniformly distributed throughout the cytoplasm; however, upon platelet activation, talin becomes concentrated at the cytoplasmic face of the plasma membrane [Beckerle et al., 1989]. This dramatic, activation-dependent redistribution of platelet talin to the membrane suggests that talin may play a role in platelet adhesion, which could be analogous to its

role at adhesion plaques in other cells.

Several of the biophysical characteristics of avian smooth muscle talin have been reported by Molony et al. [1987]. Their experiments were done in a 20 mM Tris-buffer at pH 7.6, containing 20 mM NaCl, 1 mM EDTA, 0.1% 2-mercaptoethanol, and different amounts of NaCl or of ammonium formate. Circular dichroism experiments showed that the secondary structure of talin consists primarily of α-helix (~ 80%) with the remainder being random coil. It also was shown that talin existed as a monomer at low protein concentrations and high salt (220 mM NaCl), but that at higher protein concentrations (above 0.72 mg/ml) a monomer-dimer equilibrium existed. The apparent molecular mass of talin was 225 kDa as determined by SDS-PAGE, which was similar to the relative molecular weight of 213,000 ±15,000 obtained by sedimentation equilibrium experiments (high salt, concentration < 0.72 mg/ml). The Stokes radius and sedimentation coefficient of talin varied with the ionic strength of the experiment, thus, suggesting an ionic strength-dependent conformational change in the talin molecule. At low ionic strength, the Stokes radius was 6.5 nm and the sedimentation coefficient was 9.4 S (uncorrected), indicating an asymmetric globular molecule. At high ionic strength (200 mM NaCl), the Stokes radius was 7.8 nm and the sedimentation coefficient was 8.8 S (uncorrected), indicating a more elongated molecule. These results were confirmed by using electron microscopy, which indicated that at low ionic strengths talin was a roughly globular molecule with varying diameters, and that as the ionic strength was increased, the molecule seemed to gradually unwind. In the presence of 200 mM ammonium formate, talin appeared as an elongated, flexible molecule, ~ 60 nm in length [Molony et al., 1987].

Human platelet talin migrates slightly slower than gizzard talin when analyzed by SDS-PAGE, exhibiting an apparent molecular mass of 235 kDa [Collier and Wang, 1982a]. Collier and Wang

[1982a] also reported several other biophysical characteristics of platelet talin. The platelet talin had a Stokes radius of 6.7 nm and a sedimentation coefficient of 9.8 S (uncorrected) at low ionic strength (pH 8.0), which were similar to the values reported for gizzard talin under these conditions [Molony et al., 1987]. It was concluded that, under these conditions, platelet talin existed as a roughly globular dimer with an approximate molecular weight of 440,000 ±20,000 [Collier and Wang, 1982a]. Because the concentrations of platelet talin used in these latter experiments were greater than 1 mg/ml, these results are not in direct conflict with the results from Molony et al. [1987], who reported that gizzard talin could form dimers at protein concentrations greater than 0.72 mg/ml. Goldmann et al. [1994] have recently shown by electron microscopy of rotary shadowed preparations (pH 8.0, low ionic strength) that platelet talin could form dumbbell shaped homodimers with an average length of 51 nm. Their talin dimers seemed to be made up of two large globular heads joined by a flexible rod-like region, with some indication of a hinge or knob in the middle of the rod. In this same report, sedimentation experiments yielded a molecular weight of 412,000 ±28,000 and a corrected sedimentation coefficient S_{20w} of 11.2. Because of the very low protein concentrations (0.1-0.4 mg/ml) used in these experiments, the results of Goldmann et al. [1994] disagree with the suggestion of Molony et al. [1987] that dimer formation only occurs at higher talin concentrations (≥ 0.72 mg/ml). The equilibrium sedimentation experiments done by Molony et al. [1987] differed from those used by Goldmann et al. [1994] in the ionic strength used, which may have contributed to the differing results.

Talin also is very susceptible to proteolytic cleavage by a variety of proteases, including the calpains. Calpains are calcium-activated proteases and can catalytically cleave talin into two major fragments/domains with apparent molecular masses of 190 kDa and 47 kDa [Beckerle et al.,

1986]. The cDNA sequence of mouse talin shows that the cleavage site is at residue 434, and that the 47 kDa fragment (predicted molecular mass from sequence of ~ 50 kDa) contains talin's N-terminus and the 190 kDa fragment (predicted molecular mass from sequence of ~ 220 kDa) contains talin's C-terminus [Rees et al., 1990]. The overall experimentally determined isoelectric point of gizzard talin was reported as 6.7-6.8 [Molony et al., 1987]. The isoelectric points of the 47 kDa and the 190 kDa fragments are 8.4 and 5.4, respectively (predicted from the cDNA sequence). Thus, the talin molecule consists of a relatively basic N-terminus and an acidic C-terminus. The 190 kDa talin fragment has a Stokes radius of 7.5 nm and a sedimentation coefficient of 8.0 S that is independent of ionic strength, which indicates that the 190 kDa fragment has an elongated conformation in both high and low salt conditions [O'Halloran and Burridge, 1986]. This suggests that the 47 kDa fragment may be important in regulating the conformation of the overall talin molecule.

Results of cDNA studies show that the 47 kDa fragment contains a small region of sequence homology with members of the band 4.1-ezrin-radixin family of proteins [Rees et al., 1990]. Like talin, this family of proteins is thought to associate with the membrane through interactions with cytoplasmic domains of integral membrane proteins and also to interact with the cytoskeleton [for review, see Aprin et al., 1994]. The area of sequence homology coincides with the membrane-associated portion of band 4.1, and it has, therefore, been suggested that the 47 kDa talin fragment/domain may be important in targeting talin to the membrane. Although there is some evidence of a regulatory function for the 47 kDa fragment in determining the location of talin, it does not seem to be required for actual membrane attachment. When Nuckolls et al. [1990] microinjected fluorescently labelled 47 kDa fragments into cultured cells, a portion of the protein localized to focal contacts, but a significant portion remained diffusely located in the cytoplasm.

When the 190 kDa fragment was microinjected, most of it localized to focal contacts but, interestingly, some of this fragment also localized to cell-cell contacts that usually do not contain intact talin [Nuckolls et al., 1990]. These studies suggest that the 47 kDa fragment/domain may interfere with the ability of intact talin to interact with proteins at cell-cell contact sites and indicate that this fragment is not required for talin localization at cell-matrix adhesion plaques [Nuckolls et al., 1990]. In fact, all of the interactions that have been described between talin and other specific proteins including vinculin [Gilmore et al.,1993], integrin [Horwitz et al., 1986], and actin [Muguruma et al., 1990] appear to involve the 190 kDa fragment/domain of talin.

Vinculin is an ~ 117 kDa protein that can be found at both cell-matrix adhesion plaques and at cell-cell adhesion plaques [for review, see Otto, 1990]. Talin has been shown to interact with vinculin by a variety of *in vitro* binding assays including cosedimentation on sucrose density gradients [Burridge and Mangeat, 1984], gel and blot overlays [Drenckhahn et al., 1988; Groesch and Otto, 1990; Lee et al., 1992], immunoprecipitation assays [Groesch and Otto, 1990], and microtiter assays [Gilmore et al., 1992, 1993]. The vinculin-talin interaction is of moderately high affinity with a reported K_d of ~ 10^{-8} M [Burridge and Mangeat, 1984]. The binding site for talin on the vinculin molecule has been narrowed down to residues 1-258 by using a series of vinculin polypeptides, which were expressed as fusion proteins in *Escherichia coli*, in binding assays with intact talin [Gilmore et al., 1992]. There are at least two binding sites for vinculin on talin within residues 498-950 in the 190 kDa talin fragment as shown by using ^{125}I-vinculin in overlays and microtiter assays with a series of talin polypeptides, which were expressed as fusion proteins in *Escherichia coli* [Gilmore et al., 1993]. Talin [Beckerle and Yeh, 1990] and vinculin [Otto, 1990] are both located at cell-matrix adhesion plaques and, therefore, an *in vivo* interaction between the two proteins seems likely. However, a functional significance for the talin-vinculin

interaction remains to be clearly defined.

Integrins are a large family of heterodimeric integral membrane proteins that bind to extracellular matrix proteins through their extracellular domains and to the actin filament-based cytoskeleton through their cytoplasmic domains [for review, see Hynes, 1992]. Talin has been shown, in equilibrium gel filtration experiments, to interact *in vitro* with the β_1 class of integrins with a relatively weak affinity (K_d of ~ 10^{-6} M) [Buck et al., 1986; Horwitz et al. 1986]. Talin will only bind to native heterodimeric integrin in this type of study [Buck et al., 1986]. This suggests that both the α and β subunits are necessary to generate the talin binding site on integrin, even though the binding of talin to the native integrin could be eliminated by competition with a ten-amino acid polypeptide sequence from the cytoplasmic domain of the β_1 subunit of integrin [Tapley et al., 1989]. This may explain why talin did not bind to affinity columns containing only the cytoplasmic domain of the β_1 subunit of integrin in more recent studies [Otey et al., 1990]. It also is interesting that integrins isolated from Rous sarcoma virus (RSV)-transformed cells are phosphorylated on tyrosine residues in the cytoplasmic domain of the β_1 subunit, and that these phosphorylated forms of integrin were unable to bind to talin [Tapley et al., 1989]. This latter finding correlates nicely with the *in vivo* effects of cytoskeletal disruption that are observed when cells are transformed by the Rous sarcoma virus [Pasquale et al., 1986], and suggests that the talin-integrin interaction may be important in maintaining cytoskeletal integrity within the cell [Tapley et al., 1989]. Although the gel filtration equilibrium experiments are the only way in which a direct interaction between talin and integrin have been shown to date, there is evidence for extensive colocalization of talin and integrin in a variety of cells [Beckerle and Yeh, 1990], and it is likely that this interaction is important *in vivo*.

Dystrophin is the protein that was found to be missing in patients with Duchenne muscular

dystrophy. Dystrophin has been localized to areas that also contain talin, and is thought to be important in binding actin to the cell membrane via association with a large glycoprotein complex [for review, see Ervasti and Campbell, 1993]. Senter et al. [1993] have recently described an interaction between talin and dystrophin by using microtiter techniques. The interaction between talin and dystrophin could be inhibited by the addition of vinculin, indicating that the interaction was specific. Although the possibility of an interaction between talin and dystrophin is intriguing, more studies will need to be done to confirm and better define this interaction.

Platelet talin was shown to be an actin binding protein when it was first described by Collier and Wang [1982b]. They showed the platelet talin decreased the viscosity of F-actin solutions, apparently by decreasing the average length of filaments formed in its presence. Addition of platelet talin, however, did not affect the length of actin filaments that were first preformed in its absence, suggesting that talin did not act to sever filaments [Collier and Wang, 1982b]. The interaction between platelet talin and actin was investigated again by Kaufmann et al. [1991]. Once more, platelet talin was described as a protein that could reduce the viscosity of F-actin by increasing the number of shorter actin filaments. However, Kaufmann et al. [1991] also presented evidence that platelet talin could increase the polymerization rate of actin. This increase in polymerization rate resulted in an increase in the number of short actin filaments rather than an increase in the length of filaments, thus suggesting that platelet talin could nucleate actin filament assembly [Kaufmann et al., 1991; Isenberg and Goldmann, 1992].

Smooth muscle talin has not been shown to reduce the length of actin filaments formed in its presence [Burridge et al., 1990; Muguruma et al., 1990; Schmidt et al., 1993]. In fact, no evidence for a direct interaction between smooth muscle talin and actin was observed in early cosedimentation experiments [Burridge and Mangeat, 1984]. It was not until 1990 that Muguruma

et al. [1990] reported that talin could cosediment with actin filaments, could slightly increase the rate of polymerization and the final viscosity of F-actin, and also could bind to G-actin. In this same report it was shown that the extent of the talin-actin interaction (at pH 7.5) was strongest when 2 mM $MgCl_2$ was used to induce actin polymerization and was decreased when 100 mM KCl was used to induce polymerization. Muguruma et al. [1990] also stated (based upon unpublished observations) that talin did not appear to crosslink actin filaments when talin-actin mixtures were observed by electron microscopy. Schmidt et al. [1993; paper included in this dissertation] also reported that smooth muscle talin could interact directly with actin, but that the talin-actin interaction was very dependent on pH. At low pH and low ionic strength (1 mM ATP, 1 mM EGTA, 10 mM imidazole-HCl, pH 6.6, with 2 mM $MgCl_2$ added to induce actin polymerization), talin almost completely cosedimented with F-actin and markedly increased the low shear viscosity of F-actin solutions. However, as the pH was increased in the range of 6.6 to 7.4, the ability of talin to interact with actin decreased significantly in both cosedimentation and low shear viscometry assays. Electron microscope observations showed that talin could crosslink actin filaments at pH 7.0 and that at lower pH the crosslinking was more extensive. At a pH above 7.4 the interaction was difficult to detect [Schmidt et al., 1993]. These studies agree overall with those of Muguruma et al. [1990] except that the extent of the cosedimentation between talin and F-actin under similar conditions in the latter report was much higher than that reported by Schmidt et al. [1993]. The results of Schmidt et al. [1993] may explain in part why some of the earlier studies failed to detect any talin-actin interaction, because the conditions commonly employed in actin binding assays (pH 7.5-8.0, 100 mM KCl) do not favor the talin-actin interaction.

Burridge et al. [1990] reported, as unpublished observations, that they also were able to

detect evidence for a direct interaction between smooth muscle talin and actin, and that they did not detect any decrease in the length of actin filaments by viscometry studies using highly purified gizzard or platelet talin. They suggested that the filament shortening activity obtained by others was due to contaminants in the platelet preparations, and that they were able to remove these contaminants by further purification [Burridge et al., 1990]. Unfortunately, none of the actual results backing up these observations have yet been published. Goldmann et al. [1994] recently have reported that platelet talin has its greatest effect on actin filament length at early times (~ five minutes) in the polymerization process. They suggested that studies performed after longer actin polymerization times may not detect evidence for short filaments due to the annealing of these shorter filaments into longer filaments with increased time [Goldmann et al., 1994]. The talin-actin interaction appears to be very complex, and it remains difficult to sort out the various factors that may be responsible for the conflicting reports from different laboratories because of the (a) use of talin from different sources, (b) use of different conditions in the interaction assays, and/or (c) effects of contaminants in the talin preparations.

Muguruma et al. [1992] reported that talin can augment the ability of α-actinin to gelate F-actin. Their cosedimentation results, using both talin and α-actinin with actin, showed that talin and α-actinin apparently did not compete for binding sites on actin. It also was stated that there was no evidence for an interaction between α-actinin and talin. Viscosity results showed that, although talin did not form a gel with actin filaments by itself, talin could decrease the amount of α-actinin required for gel formation. It was concluded that talin could crosslink actin at limited regions. Chemical crosslinking studies also suggested that talin formed dimers and higher oligomers when crosslinking actin. The conditions for these experiments were 20 mM Tris-HCl, pH 7.5, containing 0.5 mM ATP, 0.5 mM $CaCl_2$, 2 mM dithiotheitol, and 1 mM NaN_3, with 100

mM KCl and 2 mM $MgCl_2$ added to initiate polymerization [Muguruma et al., 1992].

Goldmann et al. [1992] examined the effect of vinculin on the ability of talin to bind to actin and to liposomes. The results obtained from their kinetic and thermodynamic studies suggested that, although talin, vinculin, and actin can form a ternary complex, vinculin did not affect the interaction of talin with actin or the ability of talin to insert into lipid bilayers.

Talin can be phosphorylated on serine and threonine residues by protein kinase C [Litchfield and Ball, 1986; Beckerle, 1990], and on tyrosine residues by $pp60^{v\text{-}src}$ [Pasquale et al., 1986; DeClue and Martin, 1987]. Although both the 190 kDa talin fragment and the 47 kDa talin fragment can be phosphorylated by protein kinase C, the 47 kDa fragment is phosphorylated to a higher degree than the 190 kDa fragment [Beckerle, 1990]. The 47 kDa fragment of talin was also found to be a substrate for protein kinase P, which is a protein kinase found in human hematopoietic cells and characterized by its stimulation by phospholipids in the presence of Mn^{2+} ions [Simons and Elias, 1993]. Protein kinase P also phosphorylated intact talin, but at a decreased rate (~ 1/20) compared to the 47 kDa fragment [Simons and Elias, 1993]. Tidball and Spencer [1993] have reported that talin is phosphorylated on tyrosine residues in response to platelet derived growth factor (PDGF) stimulation of myoblasts. Talin is not phosphorylated by c-AMP dependent kinase [Beckerle, 1990] or by the recently discovered tyrosine kinase $pp125^{FAK}$, which is activated in response to cell adhesion at focal contacts [Bockholt and Burridge, 1993].

There is some indirect evidence that the phosphorylation state of talin may affect its ability to interact with other proteins within the cell. Phorbol esters are a group of tumor promoters known to cause an increase in the activity of protein kinase C [for review, see Nishizuka, 1986] and often to cause a disruption of the intracellular cytoskeleton [Beckerle and Yeh, 1990]. Several studies have been done to attempt to correlate the extent of talin phosphorylation with the disruption of

the cytoskeleton after cells have been treated with phorbol esters. Although the response seems to vary among different cell lines, increased phosphorylation of talin was observed in response to phorbol ester treatment [Beckerle and Yeh, 1990]. In BSC-1 cells treated with the tumor promotor 12-*o*-tetradeconoyl phorbol-13-acetate, the increase in talin phosphorylation was accompanied by a dramatic decrease in stress fibers and a disappearance of focal contacts [Turner et al., 1989]. In chick embryo fibroblasts treated with phorbol 12-myristate 13-acetate, an increase in talin phosphorylation was correlated with a loss in talin-rich focal contact precursor structures, but established focal contacts seemed unaffected [Beckerle, 1990]. Interleukin 1β activation of fibroblasts also resulted in an increase in talin phosphorylation that appeared to be related to the change in cell shape and actin filament distribution [Qwarnström et al., 1991]. Talin is phosphorylated in response to PDGF stimulation on tyrosine residues, and PDGF also affects cytoskeletal organization [Tidball and Spencer, 1993]. Interestingly, PDGF activation resulted in the release of vinculin and actin from focal contacts, but integrin and talin localization remained unchanged and the shape of the cells was largely unaffected. This suggested that the increase in phosphorylation of talin may have decreased its ability to interact with vinculin and/or actin [Tidball and Spencer, 1993]. Another interesting finding was that platelet talin showed a four-fold increase in phosphorylation in response to platelet activation by thrombin, which appeared to correlate with a dramatic redistribution of talin from the cytoplasm to the membrane [Bertagnolli et al., 1993]. This change in talin's subcellular distribution was found not to be related to its cleavage by calpain or to the presence of the platelet integrin, GPIIb-IIIa [Bertagnolli et al., 1993]. Although the results of the previous studies taken *in toto* point to a physiological link between phosphorylation of talin and alterations in cytoskeletal organization, it is important to note that other factors also are likely to contribute to the changes observed in these various cells

and that, to date, there have not been any studies describing the effects of phosphorylation of talin on its ability to interact directly with proteins at adhesion plaques.

Platelet talin has been reported to interact preferentially with negatively charged phospholipids [Heise et al., 1991]. Talin that was isolated from the membrane fraction of platelets appeared able to interact with mixed lipid bilayers both electrostatically and hydrophobically, whereas talin isolated from the cytoplasm appeared able to interact only electrostatically. These results could indicate some kind of secondary modification to talin, such as acylation of the talin located at the membrane [Heise et al., 1991]. The ability of talin to interact with negatively charged phospholipids did not seem to be altered by the presence of vinculin, and talin also appeared to promote attachment of actin filaments to talin-containing lipid vesicles [Goldmann et al., 1992]. An association between talin and membrane lipids may aid in stabilizing the interactions of talin at the platelet membrane.

Hagmann et al. [1992] reported that talin was O-glycoslyated to a slight extent in smooth muscle. Smooth muscle talin has two sequences within its 190 kDa C-terminal fragment that can be glycoslyated with an O-linked N-acetylglucosamine (GlcNAc). Less than 6% of isolated chicken gizzard talin and 3% of porcine stomach talin contained GlcNAc moieties, and platelet talin did not appear to be glycosylated at all [Hagmann et al., 1992]. A functional significance for the glycosylation, if any, remains unknown.

Actin

Actin is one of the most abundant proteins in eucaryotic cells [for reviews, see Pollard and Cooper, 1986; Bamburg and Bernstein, 1991; Kabsch and Vandekerckhove, 1992]. It can exist as a monomer with a roughly globular shape (known as G-actin) under low ionic strength conditions,

and can spontaneously polymerize into long filaments (F-actin) when the ionic strength is raised to physiological levels. The G-actin molecule is a single polypeptide chain, with a molecular mass of ~ 42 kDa, and a primary sequence that has been highly conserved throughout evolution. The diversity among actins within the animal kingdom is usually less than 5% and the maximum diversity found, when comparing sequences from higher plants, protozoans, and vertebrates, is only ~ 10-12% [Bamburg and Bernstein, 1991]. Actin is relatively acidic with an isoelectric point of ~ 5.4, and three closely-spaced isoelectric variants (α, β, γ) can be obtained depending on the source of the actin. Six isoforms are expressed in warm-blooded vertebrates, namely two striated muscle forms (cardiac and skeletal, both α), two smooth muscle forms (vascular α, and visceral γ), and two non-muscle or cytoplasmic forms (β and γ) [Kabsch and Vandekerckhove, 1992]. Actin can bind one molecule of ATP or ADP and exhibits a slow rate of ATPase activity. There is one high affinity and several low affinity divalent cation binding sites within the actin molecule. The crystal structure of G-actin complexed with DNase I has been analyzed by X-ray diffraction at a resolution of 2.8 Å [Kabsch et al., 1990]. Actin consists of what is called a "small" domain, which contains amino acid residues from both the N-terminus (residues 1-144) and C-terminus (residues 338-375), and a "large" domain, which contains residues 145-337, although the two domains are not actually very different in size [Kabsch et al., 1990]. Each of these two domains can be further divided into two subdomains, with the small domain containing subdomains 1 and 2, and the large domain containing subdomains 3 and 4. The overall dimensions of the actin monomer are ~ 55 × 55 × 35 Å, and the two domains create a bilobular appearance with the nucleotide binding site and high affinity divalent cation binding site located in the cleft present between the two domains [Kabsch et al., 1990]. The nucleotide and high affinity divalent cation interact at several sites within both domains of actin, which contributes to the stability of the

molecule. Their removal causes actin to denature rapidly [Kabsch and Vandekerckhove, 1992].

The actin filament (F-actin) can be characterized as a left-handed (one start or genetic) helix with a rotation angle of approximately -166°, which gives it the appearance of two long-pitch (two start) right-handed helices (strands) wound around each other [Holmes et al., 1990; Kabsch and Vandekerckhove, 1992]. The maximum diameter of the filament is ~ 9 nm, and each monomer is arranged so that the "large" domain is located near the center of the filament axis and the "small" domain is exposed at the periphery of the actin filament. There are 13 actin molecules per turn of the double-stranded (two start) helix, and a repeat distance of ~ 36 nm along the actin filament. The actin filament exhibits a polarity that can easily be observed when actin is allowed to interact with myosin S1 heads. This results in filaments that are decorated with an arrowhead pattern, with one end of each filament appearing pointed and the other end barbed.

The polymerization of G-actin into F-actin occurs when the lower affinity cation binding sites are filled [Kabsch and Vandekerckhove, 1992]. This induces a conformational change in the actin monomer, allowing for self-association into filaments. *In vitro*, F-actin is usually dialysed into a very low ionic strength solution containing calcium and ATP in order to obtain G-actin, and then $MgCl_2$ (1 to 3 mM) and/or KCl (100 to 150 mM) can be added to induce polymerization into F-actin [Bamburg and Bernstein, 1991]. The polymerization reaction can be thought of as occurring in four steps [for review, see Pollard and Craig, 1982], namely (1) activation, where the cation sites are filled and a conformational change takes place in the monomer; (2) nucleation, a slow step where the number of monomers that self-associate favors polymerization over disassociation; (3) elongation, where the filaments grow by the addition of monomers to either end; and (4) annealing, where two shorter filaments can anneal or join to form one longer filament. The time course of this interaction proceeds with an initial lag phase followed by rapid polymerization

(elongation) until an equilibrium is reached. The rate limiting step appears to be the nucleation step, which requires that at least three actin monomers self-associate before polymerization is favored [Pollard and Craig, 1982]. Hydrolysis of the bound ATP also occurs during actin polymerization. However, the rate of ATP hydrolysis lags behind the rate of actin polymerization. Thus, the ends of the polymerizing actin filament are thought to be capped with ATP-containing actin molecules, while the actin monomers in the middle of the filament contain ADP [Bamburg and Bernstein, 1991]. It also has been shown that the polymerization rate for actin filaments is 10 to 40 times faster at the barbed end than at the pointed end. This can result in a treadmilling effect where there is a net polymerization at the barbed end and a net depolymerization at the pointed end [Bamburg and Bernstein, 1991].

The polymerization of actin can be affected by several factors *in vitro*, including the type and concentration of cation used, the pH, and the temperature. The rate of actin polymerization is faster when $MgCl_2$ (1-2 mM) is used in place of KCl (100-150 mM) to induce actin filament assembly, and the combination of $MgCl_2$ and KCl results in a larger increase in the rate than with either used alone [Wang et al., 1989]. The rate of polymerization of actin filaments is also increased as pH is lowered in the range of 8.0 to 6.6, regardless of the cation used [Wang et al., 1989]. The lag time is decreased and the polymerization rate is increased with increasing temperature in the range of 10 to 30°C [Zimmerle and Frieden, 1986]. ATP-actin can polymerize faster than ADP-actin.

It is unlikely that the concentrations of magnesium and potassium are ever low enough to favor the G-form of actin *in vivo*, but actin interacts with a wide range of proteins that are believed to control actin assembly and disassembly within most cells. These actin binding proteins are often separated into different classes based upon their overall effect on actin organization [for

reviews, see Gaertner et al., 1989; Vandekerckhove, 1989; Dubreuil, 1991; Hartwig and Kwiatkowski, 1991; Vandekerckhove and Vancompernolle, 1992]. The types of actin binding proteins include: (a) actin monomer sequestering proteins, which bind monomeric actin and control the pool of unpolymerized actin in the cell; (b) capping proteins, which can bind to one end of the actin filament and sometimes inhibit polymerization at that end; (c) severing proteins, which can disrupt the interactions between adjacent actin molecules within the filament and produce shorter filaments; (d) nucleating proteins, which can increase the polymerization rate of actin by increasing the rate of nucleation; (e) crosslinking proteins, which can bind laterally to actin filaments and link neighboring filaments to one another; and (f) proteins that bind along actin filaments, but do not crosslink neighboring filaments, and which can stabilize the actin filament and regulate its ability to interact with other proteins. Some actin binding proteins exhibit more than one type of actin-binding activity, and there often are several proteins with apparently similar actin binding activities within the same cell [Hartwig and Kwiatkowski, 1991]. This apparent redundancy would seemingly permit different control mechanisms to operate under different conditions. Actin binding activities also often are regulated by factors such as phosphorylation, calcium concentration, phospholipid environment, and pH.

Although physiological conditions would seemingly favor the formation of F-actin *in vivo*, there is a relatively large pool of unpolymerized actin (~ 50%) in many cell types, which contain proteins that bind to G-actin and prevent its incorporation into actin filaments [for review, see Safer and Nachmias, 1994]. These monomer sequestering proteins include profilin [for review, see Sohn and Goldschmidt-Clermont, 1994], actobindin [Bubb et al., 1991], DNase I [Weber et al., 1994], actin depolymerizing factor (ADF) [Hayden et al., 1993] and thymosin β_4 [Safer and Nachmias, 1994]. Profilin (~ 17 kDa) can bind to G-actin *in vitro* and inhibit its incorporation

into F-actin, indicating that profilin could act as an actin monomer sequestering protein within cells [Sohn and Goldschmidt-Clermont, 1994]. The binding of profilin to G-actin can be decreased by phosphoinositides, providing a possible mechanism for regulating the amount of G-actin available for polymerization in cells [Sohn and Goldschmidt-Clermont, 1994]. Recent studies suggest that the amounts of profilin present and actually bound to G-actin do not approach the levels of G-actin in cells and, thus, that actin monomer sequestering activity may not be the primary function of profilin [Sohn and Goldschmidt-Clermont, 1994]. Thymosin β_4 and its homologs appear to be good candidates for G-actin sequestering activity [Safer and Nachmias, 1994]. Thymosin β_4 is an ~ 5 kDa peptide that binds to G-actin at a 1:1 molar ratio, is present at relatively high concentrations within cells, and, thus, it may function as the primary G-actin sequestering protein [Safer and Nachmias, 1994]. Monomer sequestering proteins can help to regulate actin polymerization by decreasing the amount of G-actin available for polymerization when they are complexed with the monomers, and by increasing polymerization when they release the bound monomers.

Actin filament capping proteins are proteins that can bind to one end of the actin filament and sometimes restrict actin polymerization at that end [Gaertner et al., 1989]. Most capping proteins bind to actin at the barbed end and include gelsolin [Lamb et al., 1993], tensin [Lo and Chen, 1994], Cap $Z_{(36/32)}$ [Cassella and Torres, 1994], gCap39 [Yu et al., 1990], and Cap 100 [Hoffmann et al., 1992]. Some actin capping proteins also can nucleate filament assembly by allowing actin monomers to add on or bind to them and thereby decrease the lag phase of polymerization. Some actin capping proteins also can sever actin filaments, presumably by intercalating between two adjacent actin monomers within the filament. Gelsolin is an ~ 87 kDa actin binding protein that can cap and sever actin filaments, and nucleate actin filament assembly in a Ca^{2+}-dependent

manner [Lamb et al., 1993]. Gelsolin does not bind to actin at neutral pH in the absence of Ca^{2+}. However, when the Ca^{2+} concentration is raised (10 -100 μM) gelsolin can sever actin filaments and cap the newly formed barbed ends [Lamb et al., 1993]. Gelsolin also can nucleate actin filament assembly by binding to actin monomers in the presence of Ca^{2+} [Gaertner et al., 1989; Vandekerckhove, 1989]. The overall effects of activated gelsolin would be to cleave existing actin filaments and to provide new nuclei for the growth of filaments [Gaertner et al., 1989]. Capping proteins that do not sever or nucleate assembly also may be important in regulating the length of actin filaments and in conserving ATP by preventing additional growth of actin filaments and the ATP hydrolysis that follows polymerization [Pollard and Cooper, 1986].

Actin filament crosslinking proteins can bind laterally to actin filaments and link neighboring filaments to each other [for reviews, see Dubreuil, 1991; Otto, 1994]. This class of proteins can form networks and/or bundles of actin filaments. In order to crosslink actin filaments, these proteins must contain at least two actin binding sites or contain one actin binding site and be able to function as dimers [Otto, 1994]. Fimbrin has two actin binding domains on the same polypeptide chain, and can crosslink actin into tight bundles with the same polarity [Bamburg and Bernstein, 1991]. Fimbrin has been located in a variety of cells including the intestinal brush border and may be important in maintaining the organization and polarity of actin filaments at these sites [Bamburg and Bernstein, 1991; Otto, 1994]. Actin crosslinking proteins also are important in maintaining cytoskeletal stability at the level of the cell membrane, as can be illustrated by the spectrin-actin based cytoskeleton in the red blood cell [Bennett, 1990] or the filamin-actin cytoskeleton in resting platelets [Fox, 1993]. In these examples, actin is part of an extensive network of proteins that bind to the membrane via transmembrane proteins and thereby help to maintain cell shape. Actin crosslinking proteins also are assumed to play a role in linking

actin filaments to the membrane at adhesion plaques that contain both filamin [Pavalko et al., 1989] and α-actinin [Blanchard et al., 1989]. In striated muscle cells, α-actinin is believed to crosslink actin filaments at the Z-line [Yamaguchi et al., 1985; Vigoreaux, 1994], which is important in maintaining the structural integrity of the myofibrils and the muscle cell. Overall, crosslinking proteins are important in the organization and attachment of actin filaments to each other and to other proteins within cells.

Tropomyosin is a rod-like protein that binds all along the actin filament and increases its stability, but does not crosslink actin filaments [for review, see Lees-Miller and Helfmann, 1991]. Because of its location along the actin filament, tropomyosin is in a position to possibly regulate the interactions between several different actin binding proteins and actin. Actin filaments coated with tropomyosin, for instance, are not severed by gelsolin or ADF [Bamburg and Bernstein, 1991]. Tropomyosin also competes with filamin for binding to actin filaments *in vitro* [Zeece et al., 1979]. Tropomyosin in combination with caldesmon or the troponin complex also regulates the interaction between actin and myosin during muscle cell contraction [Payne and Rudnick, 1989; Sobue and Sellers, 1991].

The state of polymerization and the organization of actin are controlled by its interactions with the various types of actin binding proteins [Gaertner et al., 1989; Vandekerckhove, 1989; Dubreuil, 1991; Hartwig and Kwiatkowski, 1991; Vandekerckhove and Vancompernolle, 1992]. The interactions between these proteins and actin can be affected by a variety of factors including Ca^{2+} concentration, phosphoinositides [Janmey et al.,1994], pH [Lamb et al., 1993], and covalent modification [Lo and Chen, 1994]. These interactions presumably must be carefully regulated to allow actin filaments to respond to various cellular signals in a rapid and sensitive manner [Lo and Chen, 1994]. These signals also may be restricted to very specific areas of a cell. For example,

motile cells would be required to respond to signals in a directed manner to allow for movement of the cell. *In vitro* studies have been useful in determining how each of these factors affect the ability of isolated actin binding proteins to interact with actin. An understanding of how individual factors affect actin *in vitro* should lead to a better understanding of how the complex events involving actin within the cell may occur.

Calcium can affect the ability of several proteins to bind to actin [for review, see Janmey et al., 1994]. Gelsolin, villin, severin, and fragmin have calcium dependent severing, capping, and nucleating activities as mentioned earlier. The protein gCap39 binds actin monomers and caps barbed filament ends in the presence of μM concentrations of Ca^{2+}, but not in the absence of Ca^{2+}. Some non-muscle isoforms of α-actinin can bind Ca^{2+} ions, which then decreases the ability of the α-actinin to interact with and crosslink actin filaments. The ubiquitous protein calmodulin can bind to Ca^{2+}, and this complex can affect the interactions of several actin binding proteins with actin including caldesmon and spectrin/band 4.1, which show decreased binding to actin in the presence of Ca^{2+}-calmodulin. Actin binding proteins also can be affected indirectly by the Ca^{2+}-induced activation of the calpain proteases [for review, see Goll et al., 1992], which can cleave filamin and spectrin and thereby disrupt their ability to crosslink actin filaments. In general, Ca^{2+} ions appear to encourage reorganization of the actin-based cytoskeleton [Janmey et al., 1994]. However, Ca^{2+} also is the primary regulator of actin-myosin based contraction and can activate protein kinase C. It is difficult, therefore, to assign one general effect or role to such a broad based second messenger.

It is known that phosphoinositides, especially phosphatidylinositol 4,5-bisphosphate (PIP_2), affect the interactions between several proteins and actin [for review, see Janmey, 1994]. The PIP_2 permits the release of sequestered actin monomers by profilin, inhibits the severing and

capping activities of gelsolin, severin, and villin, releases several other barbed end capping proteins from filaments including CapZ, Cap100, and gCap39, and affects the ability of crosslinking proteins to interact with actin filaments. The ability of α-actinin to crosslink actin filaments is enhanced in the presence of PIP_2 [Fukami et al., 1992], whereas the ability of filamin to crosslink actin filaments is decreased by PIP_2 [Furuhashi et al., 1992]. Although no specific effect of PIP_2 has been shown for talin or vinculin, both proteins preferentially bind to negatively charged lipid bilayers [Niggli et al., 1986; Heise et al., 1991], and it has recently been reported that vinculin binds to PIP_2 [Fukami et al., 1994]. Therefore, it is possible that PIP_2 also may affect interactions with these latter two proteins. In general, phosphoinositides appear to favor the polymerization and organization of actin filaments in the cell [Janmey, 1994].

Several proteins recently have been shown to have pH-dependent actin binding activities. These include gelsolin [Lamb et al., 1993], ADF [Hayden et al., 1993], τ protein [Moraga et al., 1993], talin [Schmidt et al., 1993], and ABP50 (EF-1α) [Edmonds and Condeelis, 1993]. Gelsolin demonstrated a decreased requirement for Ca^{2+} for its severing, capping, and nucleating activities as the pH was lowered between 7.4 and 6.5, and at pH below 6.0 the Ca^{2+}requirement was eliminated [Lamb et al., 1993]. At pH values between 6.5 and 7.1 ADF showed only weak depolymerization activity and could cosediment with actin filaments [Hayden et al., 1993]. As the pH was increased between 7.1 and 7.7, ADF showed increasing depolymerization activity and a decreased ability to cosediment with F-actin. At pH 8.0 ADF primarily depolymerized actin filaments [Hayden et al., 1993]. Tau protein can bundle actin filaments to a greater extent as the pH is decreased from 7.6 to 4.7 [Moraga et al., 1993]. We have shown that talin can crosslink actin filaments in a pH-dependent manner, and that the crosslinking activity increased with decreasing pH in the range of 7.4 to 6.4 [Schmidt et al., 1993]. It was shown that ABP50

bundled actin filaments at pH 6.4 and low ionic strength and that the bundling activity was decreased with increasing pH in the range of 6.6 to 6.8 [Edmonds and Condeelis, 1993]. Thus, three of these proteins show increased actin crosslinking activities at lower pH, and a decrease in crosslinking activities as the pH increased toward neutral. ADF switched from having F-actin binding activity to depolymerizing activity in the same range that the three crosslinking proteins showed reduced activities [Hayden et al., 1993]. With the exception of gelsolin [Lamb et al., 1993], these effects in general seem to point to an increase in actin filament organization with decreasing pH and a decrease in the organization at higher pH. There have been reports that the intracellular pH of cells can be increased by certain growth factors including PDGF [Moolenaar, 1986], and that intracellular pH can decrease in contracting smooth muscle cells [Taggart and Wray, 1993]. It is possible that changes in the intracellular pH, perhaps even within microenvironments within cells, are important regulators of actin binding proteins *in vivo*.

Phosphorylation is likely to affect the interactions between actin binding proteins and actin. Several actin binding proteins are substrates for one or more protein kinase including talin [Beckerle and Yeh, 1990], myosin [Trybus, 1991], filamin [Weihing, 1985], spectrin [Bennett, 1990], and tensin [Lo and Chen, 1994]. In the case of smooth muscle myosin, phosphorylation appears to be the primary regulator of the interaction between actin and myosin [Trybus, 1991]. When myosin light chain kinase is activated by calcium-calmodulin, it, in turn, phosphorylates myosin and allows the interaction between actin and myosin to take place. Although the spectrin-actin interaction does not appear to be directly affected by the phosphorylation state of spectrin, phosphorylation of band 4.1 decreases the latter protein's ability to promote the spectrin-actin association [Bennett, 1990]. Activation of kinases is a common messenger in signal transduction and, because several actin binding proteins are substrates for a variety of kinases, it is probable

that phosphorylation is an important regulator of many actin-protein interactions within cells.

It seems likely that several factors combine to influence the complex events that involve actin within the cell. Many of these factors are linked to each other. For example, PIP_2 is formed from the phosphorylation of phosphatidylinositol located in the cell membrane [for review, see Berridge, 1987]. Several hormones can cause an activation of phospholipase C, which then cleaves PIP_2 into two products, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) [Berridge, 1987]. The IP_3 can then act to release intracellular stores of Ca^{2+}, and the other product from the cleavage of PIP_2, DG, can act with Ca^{2+} to activate protein kinase C [Berridge, 1987]. Therefore, the signal triggering the phosphatidylinositol cascade would affect actin through a change in PIP_2 levels, in Ca^{2+} concentration, and in the phosphorylation state of certain actin binding proteins. Each of these effects would be transitory in nature, and also could be subject to modulation by yet other intracellular signals. Because actin is involved in so many important cellular processes such as cell adhesion, cell motility, maintenance of cell shape, growth, and differentiation, it makes sense that actin requires a high degree of regulation. Indeed, the carefully controlled interactions between actin and several types of actin binding proteins are presumed to be necessary for actin to carry out its diverse cellular functions.

Integrin

Integrins are a family of transmembrane proteins that integrate the interactions between extracellular components and the intracellular cytoskeleton [for reviews, see Hynes, 1992; 1994]. This area of research is expanding rapidly and new information on different properties of integrins is continually coming out [Hynes, 1994]. These proteins are not only involved in cell adhesion, but also have been found to be important in transmitting information from both sides of the

membrane [for reviews, see Ginsberg et al., 1992; Hynes, 1992; Juliano and Haskill, 1993]. The interaction of integrins with other proteins has proven to be quite complex, and can be regulated by many factors including lipid environment, phosphorylation, and divalent cations [Hynes, 1992].

Integrins are a family of α/β heterodimeric transmembrane proteins that can bind to extracellular proteins through their extracellular domains and to the actin-based cytoskeleton through their cytoplasmic domains [Hynes, 1992; 1994]. The α and β subunits both have a large extracellular domain, a single membrane spanning region, and a relatively short cytoplasmic domain. There are ≥ 8 different types of β subunits (~ 90 to 110 kDa) and ≥ 15 α subunits (~ 120 to 180 kDa) [Hynes, 1994]. Although theoretically any α subunit should be able to bind with any β subunit, not all combinations appear to be expressed *in vivo* [Hynes, 1992; 1994]. Most, but not all (e.g., α_v), α subunits combine with a specific class of β subunit, resulting in >20 known α/β combinations [Hynes, 1994]. The different β subunits are fairly homologous, especially in their cytoplasmic domains, but there is considerable diversity among the various α subunits. The α and β subunits combine on the outside of the cell to form a large head domain, which contains the extracellular ligand binding site and cation binding sites [Hynes, 1992]. Each α/β pair is a receptor for one or more extracellular ligand, with the affinity for any given ligand being largely affected by the conformational state of the receptor. Some different α/β pairs can have similar ligand binding specificities [Hynes, 1992]. Although integrins are primarily considered as receptors for extracellular matrix proteins, some integrins also interact directly with cell surface receptors from other cells. For example, $\alpha_1\beta_2$ (LFA-1) on lymphocytes can bind ICAM on target cells [Hynes, 1992].

The interactions of integrins with cytoskeletal proteins via their cytoplasmic domains are still poorly understood. Two cytoskeletal proteins, talin [Horwitz et al., 1986] and α-actinin [Otey et

al., 1990], have been shown to interact directly with integrin *in vitro*. The interaction of α-actinin and integrin occurs through the β subunit's cytoplasmic domain, as demonstrated by using both affinity column binding and solid phase binding studies [Otey et al., 1990]. The interaction between talin and integrin requires both the α and β subunits of integrin, but the interaction can be eliminated by competition with a 10 amino acid sequence from the β subunit's cytoplasmic domain [Buck et al., 1986; Tapley et al., 1989]. It is likely that *in vivo* interactions are affected by the conformational state of the integrin molecule, which can be altered by binding to its extracellular ligand and/or by activation by signals within the cell [Hynes, 1992]. One interesting example of an activation dependent talin-integrin interaction was shown when it was found that talin did not associate with integrin in rounded cells that had been transformed by Rous sarcoma virus, but that the talin was diffusely located in the cytoplasm while the integrins were randomly distributed in the membrane [Mueller et al., 1989]. When these cells were allowed to bind to fibronectin-coated beads, however, integrin and talin were colocalized in aggregates at the site of interaction between the bead and the cell [Mueller et al., 1989]. These results suggested that the integrin-talin interaction was dependent upon the binding of integrin to the fibronectin-coated beads. This dependence could be due to a conformational change in the integrin receptor induced by the binding of integrin to fibronectin and/or by the aggregation of integrin receptors at these sites.

It has been shown that integrins transmit signals from the exterior to the interior of the cell in a number of ways. For instance, when integrins bind to their extracellular ligand they can activate a tyrosine kinase ($pp125^{FAK}$), which in turn can phosphorylate itself and at least two other proteins [Burridge et al., 1992; Bockholt and Burridge, 1993]. This phosphorylation event may then contribute to the overall events triggered by cell adhesion, including the formation of

adhesion plaque structures, but the mechanisms behind these events are still largely unknown [Hynes, 1992]. Integrin-mediated adhesion can also induce an alkalinization of the cytoplasm by activating the Na^+/H^+ antiporter [Schwartz et al., 1991], which may in turn affect interactions of various proteins within the cell. These types of outside-in signals are thought to be especially important in cell growth and differentiation.

Integrins also are important in transmitting inside-out signals [Ginsberg et al., 1992; Hynes, 1992]. One good example of this type of signalling occurs during platelet activation. The platelet integrin $\alpha_{IIb}\beta_3$ (GPIIIa/IIb) does not bind to its soluble extracellular ligand when the platelet is in its inactive or resting state. Activation of the platelet by thrombin or another stimulating factor causes a conformational change in the $\alpha_{IIb}\beta_3$ integrin, which then allows it to recognize and bind to its ligand(s), resulting in adhesion and clot formation [Hynes et al., 1992; Ginsberg et al., 1992]. In this case the activation of the platelet by thrombin caused activation of the integrin molecule through signals inside the platelet, possibly a phosphorylation event [Ginsberg et al., 1992], thereby transmitting a signal to $\alpha_{IIb}\beta_3$ to bind to its ligand from the inside-out. As mentioned above, the platelet also then responds to the outside-in signal of $\alpha_{IIb}\beta_3$ binding to its ligand by activating $pp125^{FAK}$, thus contributing to the changes that occur upon stimulation of platelets [Hynes, 1992].

Vinculin

Vinculin was first described as a 130 kDa protein that was localized to the termini of stress fibers in cultured cells by Geiger [1979]. It was given the name vinculin from the Greek word *vinculum*, meaning link, based upon its proposed role in linking actin filaments to the membrane [Geiger et al., 1980]. Burridge and Feramisco [1980] shortly thereafter also described a 130 kDa

protein that they isolated from gizzard. This protein had similar properties to those of vinculin, and was named "focin" for its location at focal contacts in cultured cells [Burridge and Feramisco, 1980]. The two proteins were found to be identical and the name vinculin was adopted. Vinculin has come to be used as a marker for adhesion plaques because of its location at both cell-matrix and cell-cell attachments [for review, see Otto, 1990], even though its role at these sites remains unclear.

The cDNA structure for vinculin is known and its predicted molecular mass is ~ 117 kDa [Couto and Craig, 1988]. The avian vinculin molecule appears to consist of a roughly globular, acidic (pI ~ 5.4), N-terminal head domain (~ 90 kDa), which is ~ 8 nm in diameter, and a basic (pI ~ 9.7), C-terminal, rod-like tail (~ 25 kDa), which is ~ 20 nm long [Otto, 1990]. Although the primary structures of avian and porcine vinculin are not very different, the porcine vinculin molecule does not have a discernable tail domain and instead appears to be roughly spherical with a diameter of ~ 10-12 nm [Otto, 1990]. A larger related form of vinculin also is present in some tissues, and has been termed meta-vinculin [Otto, 1990]. Meta-vinculin and vinculin arise from differential splicing of the same transcript, with meta-vinculin containing an extra 68 amino acid sequence inserted in the C-terminal region [Byrne et al., 1992]. It is not yet clear whether vinculin and meta-vinculin have different functions in the cell.

Early reports on vinculin demonstrated an actin binding activity that resulted in a dramatic decrease in the viscosity of actin filaments [Jockusch and Isenberg, 1981; Wilkins and Lin, 1982]. Evans et al. [1984], however, showed in our laboratory that the viscosity reducing activity was due to contaminants in vinculin preparations and that highly purified vinculin had little effect, or even slightly increased the viscosity of actin filaments. A re-examination of the vinculin-actin interaction in other laboratories confirmed that vinculin did not decrease the viscosity of actin

filaments, and found that vinculin did not appear to bind to actin at all under the conditions of their experiments [Otto, 1986; Wilkins and Lin, 1986]. Yamamoto et al. [1987] reported that vinculin could increase the viscosity of actin filaments in the presence of phosphatidylserine, but not in its absence. Belkin et al. [1987] found that ^{125}I-vinculin could bind to actin in a blot overlay type experiment. Ruhnau and Wegner [1988] reported that fluorescently labelled F-actin bound to vinculin in a gel overlay assay, and a later report from the same laboratory [Westmeyer et al., 1990] showed that an antibody against vinculin could specifically block the binding of fluorescently labelled F-actin to vinculin. At present, the ability of vinculin to interact with actin remains controversial.

Vinculin has been reported to interact with α-actinin with low affinity (K_d ~ 10^{-6} M) using a fluorescent transfer-type experiment [Wachsstock et al., 1987]. Pavalko and Burridge [1991] subsequently used ^{125}I-vinculin overlays and showed that vinculin binds to the 27 kDa actin binding fragment of α-actinin. The interaction between vinculin and α-actinin led to the suggestion [Burridge et al., 1988] that vinculin may be involved as one member of a multiprotein bridge in linking actin filaments to the membrane indirectly via its interactions with α-actinin, and with talin, which could, in turn, bind to integrin as described earlier in this review.

Vinculin can also bind to paxillin [for review, see Turner, 1994], a recently discovered protein that colocalizes with vinculin at cell-matrix adhesion plaques, but that is absent from cell-cell adhesion plaques. Paxillin is an ~ 68 kDa protein that migrates as a broad band by SDS-PAGE and exhibits a series of isoforms with isoelectric points ranging from 6.31 to 6.86 in isoelectric focusing studies [Turner, 1994]. Paxillin originally was discovered as one of two proteins that were localized to focal adhesions with a series of monoclonal antibodies that had been raised against phosphotyrosine-containing proteins from RSV-transformed cells [Glenney and

Zokas, 1989]. The K_d for the interaction between paxillin and intact vinculin is ~ 6×10^{-8} M, and paxillin binds specifically to the C-terminal tail domain (~ 25 kDa) of vinculin [Turner et al., 1990]. Paxillin can be phosphorylated on its tyrosine residues by $pp60^{v\text{-}src}$ (the RSV gene product) to a much higher degree (~ 30%) than either talin, integrin, or vinculin (~ 1%) [Turner, 1994]. Paxillin is also one of the few proteins that can be phosphorylated on tyrosine residues by $pp125^{FAK}$ [Burridge et al., 1992], which is a focal adhesion kinase that is activated in response to integrin-mediated cell adhesion [Hynes, 1992]. This points to a possible regulatory role for the paxillin-vinculin interaction at adhesion plaques. However, just how tyrosine phosphorylation may affect the interactions of proteins at adhesion plaques remains to be determined.

Vinculin itself is a phosphoprotein, and it can be phosphorylated by protein kinase C on its serine and threonine residues *in vitro* [Werth et al., 1983]. Vinculin can be phosphorylated by the RSV gene product $pp60^{v\text{-}src}$ on its tyrosine residues *in vivo* [Kellie, 1988], and there is a tyrosine phosphorylation consensus sequence within its C-terminal domain [Price et al., 1989]. There is some evidence that phosphorylation may affect the ability of vinculin to associate with adhesion plaques [Otto, 1990], but there have not been any studies which describe the effect of phosphorylation of vinculin on its ability to interact directly with other adhesion plaque proteins. Therefore, the significance of phosphorylation of vinculin is still unknown. Vinculin is not phosphorylated on tyrosine residues by $pp125^{FAK}$ [Bockholt and Burridge, 1993] or in response to PDGF activation [Tidball and Spencer, 1993].

It has been reported that vinculin can be acylated with both myristic acid [Kellie and Wigglesworth, 1987] and palmitic acid [Burn and Burger, 1987]. There are several cysteines in the vinculin sequence that could be palmitoylated, but vinculin does not contain an N-terminal glycine believed to be required for myristoylation [Couto and Craig, 1988; Price et al., 1989].

Although acylation could be one way to target vinculin to the membrane, the level of myristoylated vinculin was not shown to be enhanced in the cytoskeletal fraction isolated from chicken embryo fibroblast cells [Kellie and Wigglesworth, 1987]. Vinculin, like talin, has been shown to associate preferentially with negatively charged phospholipids [Niggli et al., 1986], and it recently has been reported to also bind to phosphatidylinositol 4,5-bisphosphate [Fukami et al., 1994]. This latter association and/or acylation may help to stabilize interactions of vinculin at adhesion plaques.

It is generally assumed that vinculin is involved in the linkage of actin filaments to the membranes at both cell-cell and cell-matrix adhesion plaques [Otto, 1990], but it is uncertain as to how it may be participating in this process. Vinculin was originally reported to bind directly to actin filaments *in vitro* [Jockusch and Isenberg, 1981; Wilkins and Lin, 1982], but this interaction was later shown to be due to contaminants rather than to the vinculin itself [Evans et al., 1984]. As a result, it subsequently was proposed that vinculin was one member of a multiprotein bridge that linked actin filaments to the membrane via α-actinin, vinculin, talin, and integrin [Burridge et al., 1988], and this model is still used in conjunction with more direct models that also show actin filament linkage via talin and integrin and/or α-actinin and integrin [Luna and Hitt, 1992]. Because vinculin (a) is often incorporated into the adhesion plaque at a later time than talin and actin [Volberg et al., 1986], and (b) does not appear to affect the binding of talin to either actin [Goldmann et al., 1992] or integrin [Horwitz et al., 1986], the role of vinculin in actin membrane attachment is still very unclear.

α-Actinin

α-Actinin is a dimeric molecule containing two identical ~ 100 kDa subunits that can crosslink

actin filaments [for review, see Blanchard et al., 1989]. α-Actinin is a rod-shaped molecule, ~ 30-40 nm long. The subunits of α-actinin are arranged in an antiparallel fashion. Each subunit has an actin binding domain at its N-terminus (i.e., one at each end of the molecule), a central rod domain containing four spectrin-like repeats, and two EF hands at the C-terminal domain [Blanchard et al., 1989]. The EF hands are responsible for Ca^{2+} binding in non-muscle α-actinins, but they are nonfunctional in muscle α-actinins [Blanchard et al., 1989]. α-Actinin is a member of the spectrin family of actin crosslinking proteins, and contains four quasi-repetitive spectrin-like repeats [for review, see Dubreuil, 1991]. The actin binding domain of α-actinin shows homology to the actin binding domains in several other actin crosslinking proteins including spectrin, dystrophin, filamin, and fimbrin [Dubreuil, 1991]. α-Actinin is located at both cell-cell and cell-matrix adhesion plaques, but also can be found at actin binding structures within the cell that are not associated with the cell membrane. For example, α-actinin is present in the cytoplasmic dense bodies of smooth muscle cells and the Z-lines of skeletal muscle cells [Blanchard et al., 1989].

The ability of α-actinin to bind to actin is strongly affected by temperature [Holmes et al., 1971; Goll et al., 1972], ionic strength [Kuroda et al., 1994], phospholipids [Fukami et al., 1992; Janmey, 1994], and, in at least some non-muscle cells, $[Ca^{2+}]$ [Janmey, 1994]. α-Actinin crosslinks actin filaments extensively at low temperatures (0-4°C), but the effect is decreased as temperature is increased to 37°C [Holmes et al., 1971; Goll et al., 1972]. Decreasing the ionic strength also appears to decrease the extent of the α-actinin-actin interaction [Kuroda et al., 1994]. α-Actinin can bind to phosphatidylinositol 4,5-bisphosphate (PIP_2), and PIP_2 binding increases the interaction between α-actinin and actin [Fukami et al., 1992; Janmey et al., 1994]. In at least some non-muscle α-actinins, Ca^{2+} can decrease the interaction between α-actinin and actin

[Blanchard et al., 1989; Janmey, 1994]. The Ca^{2+} binding domains of muscle α-actinins, however, are considered non-functional and they are not sensitive to $[Ca^{2+}]$. Tropomyosin also is known to affect the interaction between α-actinin and actin, especially at higher temperatures [Goll et al., 1972]. Tropomyosin binds all along the actin filament and can almost completely displace previously bound α-actinin from actin at 37°C [Goll et al., 1972]. It is plausible that under physiological conditions tropomyosin may help limit α-actinin binding along the filaments to discrete areas, such as their Z-line end in skeletal muscle cells.

α-Actinin also can bind directly to the integral membrane protein integrin *in vitro*, as shown by microtiter assays and affinity column binding studies [Otey et al., 1990]. Limited proteolysis of α-actinin molecules by thermolysine produces two monomeric 27 kDa N-terminal fragments containing the actin binding domains, and a dimer of 53 kDa fragments (simply referred to as the 53 kDa fragment), which can bind to the cytoplasmic domain of the β subunit of integrin [Otey et al., 1990]. When the 53 kDa fragment (containing the spectrin-like repeats) of α-actinin was microinjected into cells, it localized quickly to focal contacts, and after 30-60 minutes most of the stress fibers were disassembled even though the cells remained well spread [Pavalko and Burridge, 1991]. This suggests that the 53 kDa fragment of α-actinin may disrupt integrin-actin linkages by binding to integrin in place of intact α-actinin and, because there is no actin binding site on the 53 kDa fragment, actin filaments would be released from the focal contact [Pavalko and Burridge, 1991].

α-Actinin also has been shown to bind to vinculin [Pavalko and Burridge, 1991] and to zyxin [Crawford et al., 1992] through its 27 kDa fragment. The binding of α-actinin to vinculin was discussed earlier in the vinculin section of this literature review. The binding of α-actinin to zyxin was shown by using blot overlays, gel filtration, sedimentation, and microtiter assays

[Crawford et al., 1992]. Zyxin is a very low-abundance protein of ~ 82 kDa that colocalizes with α-actinin at adhesion plaques and is phosphorylated on several sites *in vivo* [Crawford et al., 1991]. Zyxin has been sequenced and found to contain two LIM domains, which can bind Zn^{2+} ions, and that also have been described in proteins involved in the control of gene expression and differentiation [Sadler et al., 1992]. It is possible that zyxin may have some kind of regulatory role at adhesion plaques, but no function is yet known for this protein.

It also has been reported that α-actinin can bind to nebulin in blot overlay experiments [Nave et al., 1990]. Nebulin is an insoluble, high molecular weight protein that is present only in skeletal muscle cells [Wang and Wright, 1988]. It has been suggested that nebulin may act as a scaffolding protein for thin filament assembly, help control thin filament length, and contribute to the high degree of order found in the skeletal muscle system [Wang and Wright, 1988; Small et al., 1992]. An interaction between α-actinin and nebulin at the Z-line would support these proposed roles for nebulin in skeletal muscle cells.

Although α-actinin has been studied extensively, its precise role in cells remains unclear. It is assumed that α-actinin is important in the organization of actin filaments at the Z-lines in striated muscle cells, but studies have shown that α-actinin can be released from the Z-line by the Ca^{2+}-activated protease calpain even though calpain does not degrade either actin or α-actinin [Goll et al., 1991]. This suggests that some other protein also may be involved in attaching actin to the Z-line. α-Actinin can bind to both integrin [Otey et al., 1990] and actin [Blanchard et al., 1989], which indicates that α-actinin could directly link actin filaments to the membrane through integrin [Otey et al., 1990]. The studies using microinjected α-actinin fragments described earlier herein suggest that α-actinin does have an important role in actin membrane attachment [Pavalko and Burridge, 1991]. However, α-actinin does not appear at the focal contact until after talin and

actin, which suggests that it is not essential to the early stages of this process. Thus, it has been suggested that talin may be involved in the early stages of actin-membrane attachment and that α-actinin may be more important in subsequently stabilizing and strengthening interactions between actin filaments and the membrane [Pavalko et al., 1991].

Filamin

Filamin, also known as ABP (actin binding protein), is a dimer of identical ~ 250 kDa subunits [for review, see Weihing, 1985]. It originally was isolated from avian smooth muscle [Wang et al., 1975; Shizuta et al., 1976], and has been identified in a number of different cell types as a family of isoforms that can crosslink actin filaments [Weihing, 1985]. The filamin molecule is composed primarily of β-sheet structure, and each subunit contains a self-association site in its C-terminus that allows it to form long (~ 160-190 nm) and flexible dimers [Gorlin et al., 1990]. The actin binding domain has been localized to the N-terminus of each subunit, and shows considerable homology to the actin binding domains of several other actin filament crosslinking proteins including, α-actinin, spectrin, dystrophin, and fimbrin [Gorlin et al., 1990]. The rest of the filamin sequence is not related to the spectrin family of proteins [for review, see Dubreuil, 1991]. Filamin is located at adhesion plaques, but also can be found at sites that are not associated with the cell membrane such as cytoplasmic dense bodies in smooth muscle cells [Small et al., 1986; Fujimoto and Ogawa, 1988] and along stress fibers in cultured cells [Langanger et al., 1984; Mittal et al., 1987].

Filamin can crosslink actin filaments into networks and bundles *in vitro* [Davis et al., 1978; Weihing, 1985]. The interaction between filamin and actin can be affected by ionic strength [Wang and Singer, 1977; Brotschi et al., 1978], phosphoinositides [Furuhashi et al., 1992;

Janmey, 1994], and the presence of tropomyosin [Maruyama and Ohashi, 1978; Zeece et al., 1979]. Filamin binds to actin best at low ionic strength, and the interaction is decreased as ionic strength is increased [Wang and Singer, 1977; Brotschi et al., 1978]. Phosphatidylinositol 4,5-bisphosphate (PIP_2) can decrease the ability of filamin to interact with actin [Furuhashi et al., 1992; Janmey, 1994]. Phosphatidylinositol 4-monophosphate and phosphatidylinositol also can decrease the extent of the filamin-actin interaction, but not as strongly as PIP_2 [Furuhashi et al., 1992]. Tropomyosin decreased the extent of the interaction between filamin and actin when the tropomyosin was allowed to interact with actin filaments before the filamin was added [Maruyama and Ohashi, 1978; Zeece et al., 1979]. However, when filamin was allowed to interact with actin before the addition of tropomyosin, the amount of tropomyosin that bound to actin was reduced [Maruyama and Ohashi, 1978; Zeece et al., 1979]. This indicates that filamin and tropomyosin compete for binding sites on actin filaments.

Filamin from platelets also can bind to the cytoplasmic domain of the transmembrane protein complex GPIb-IX [Fox et al., 1988; Fox, 1993]. GPIb-IX is a heterotrimer consisting of GP IX (22 kDa), $GPIb_\alpha$ (135 kDa), and $GPIb_\beta$ (25 kDa) that acts as a receptor for von Willebrand factor [Fox, 1993]. It is believed that filamin may bind to actin filaments and to the GPIb-IX complex to form a membrane skeleton in platelets [Fox, 1993], similar to the spectrin-based cytoskeleton in the red blood cell [Bennett, 1990] and, thus, may help the resting platelet maintain its shape [Fox, 1993].

Filamin is also a good substrate for calpain [Davies et al., 1978], a calcium activated protease. It has been reported that calpain cleaves avian smooth muscle filamin polypeptides into two major fragments of ~ 240 kDa and ~ 10 kDa [Davies et al., 1978]. Some mammalian filamins are cleaved into large fragments of 190 kDa and 100 kDa, the latter of which can be further degraded

into 90 kDa and 10 kDa fragments [Hock et al., 1990]. In each of these cases, calpain cleavage results in separation of the actin binding domain from the self-association site, thereby preventing filamin from crosslinking actin filaments, except under very low salt conditions [Davies et al., 1978; Hock et al., 1990]. Filamin can be phosphorylated by cAMP-dependent protein kinase [Wallach et al., 1978], and it has been reported that this phosphorylation can protect filamin from degradation by calpain [Zhang et al., 1988; Chen and Stracher, 1989]. Phosphorylation, thus, could be important in maintaining the integrity of the platelet membrane skeleton. Filamin also has been reported to be phosphorylated to a small extent by protein kinase C and by the Rous sarcoma virus transforming gene product $pp60^{v\text{-}src}$, but the effect of phosphorylation by these kinases on the interactions of filamin are unknown [Weihing, 1985].

Tropomyosin

Tropomyosin is a rod-shaped dimer of two parallel, in register, α-helical coiled-coil subunits of ~ 33 kDa each [for reviews, see Phillips et al., 1980; Lees-Miller and Helfman, 1991; Pittenger et al., 1994]. Tropomyosin can bind to F-actin and colocalizes with actin filaments throughout cells [Trombitas et al., 1990; Pittenger et al., 1994]. There are several homologous isoforms of tropomyosin ranging in size from ~ 30-45 kDa [Bamburg and Bernstein, 1991], which can have slightly different properties that may be related to their function in different cell types [Lees-Miller and Helfman, 1991; Pittenger et al., 1994]. In general, tropomyosin is a flexible rod-like protein, which can self-associate in a head to tail fashion to form a long strand that binds along actin filaments in a cooperative manner. A tropomyosin strand is present in each of the two grooves of the actin filament. Each muscle tropomyosin molecule can bind to seven contiguous actin monomers along the filament, corresponding to one tropomyosin/turn of the two start actin

helix/actin strand. This extensive association with actin filaments allows tropomyosin to stabilize actin filaments and to influence the ability of certain other proteins to bind to actin filaments.

The interaction between actin and tropomyosin depends on the ionic strength [Maruyama, 1964; Tanaka, 1972], temperature [Tanaka, 1972], and the presence of other proteins such as filamin [Zeece et al., 1979], troponin [Zott and Potter, 1987], and caldesmon [Sobue and Sellers, 1991]. Tropomyosin shows optimal binding to actin in the presence of 100 mM KCl, with either lower ionic strength conditions or very high ionic strength conditions decreasing the ability of tropomyosin to bind to actin [Maruyama, 1964; Tanaka, 1972]. Tropomyosin dissociates from actin filaments at high temperatures (35 - 50°C), depending upon other conditions of the experiment [Tanaka, 1972]. For example, as the pH is lowered in the range of 8.0 to 6.5, the dissociation temperature becomes higher (ranging from 35 - 50°C) [Tanaka, 1972]. Other proteins also can affect the interaction between actin and tropomyosin. Filamin and tropomyosin appear to compete for similar sites on the actin filament, and *in vitro* binding depends on the order in which the two proteins were added to F-actin [Zeece et al., 1979]. Troponin and caldesmon also can affect the position of tropomyosin on the actin filament [see earlier skeletal muscle and smooth muscle sections of this literature review], and are regarded as important in regulating the actin-myosin interaction.

It recently has been shown that tropomyosin also can bind to an ~ 40 kDa protein called tropomodulin [Fowler et al., 1993]. Tropomodulin can bind to tropomyosin and inhibit the head to tail self-association of tropomyosin molecules. It has been suggested that this association between tropomodulin and tropomyosin may be important in regulating actin filament length at the pointed end of the filament, which is where tropomodulin is located in skeletal muscle cells [Fowler et al., 1993].

Calpain

Calpains are calcium activated cysteine proteases that are found in all vertebrate cells [for reviews, see Goll et al., 1992; Suzuki et al., 1992]. There are two well characterized forms of calpains (μ- and m-) that differ in their requirement for Ca^{2+}. The μ-calpain form requires 3-50 μM Ca^{2+} for half maximal activity, and the m-calpain form requires 0.2-1.0 mM Ca^{2+} for half maximal activity [Goll et al., 1992]. Both calpains have an ~ 80 kDa catalytic subunit and an ~ 28 kDa regulatory subunit. The regulatory subunit is identical for both forms of calpain. The 80 kDa subunit for μ- and m-calpain are homologous (~ 50%), but are different gene products, and are responsible for the differences in the Ca^{2+} requirement between the two proteins [Goll et al., 1992]. Calpains rapidly autolyze both subunits when activated, resulting in a reduced requirement for Ca^{2+} to ~ 0.6-0.8 μM for μ-calpain and 50-150 μM for m-calpain [Goll et al., 1992]. Phospholipids can reduce the amount of Ca^{2+} required to activate autolysis of calpains, with phosphatidylinositol being the most effective [Goll et al., 1992; Suzuki et al., 1992].

The function(s) of the calpains in cells is still unclear. Several cytoskeletal proteins are good substrates for calpain (e.g., filamin, talin, and spectrin) and it has been suggested that calpains may be important in cytoskeletal reorganization [Fox et al., 1985; Goll et al., 1992]. Evidence for this is especially strong in the platelet system, where activation of platelets is associated with both increased calpain activity and extensive changes in the cytoskeleton [Fox et al., 1985; Goll et al., 1992]. Calpains also may be important in myofibrillar protein turnover in muscle [Goll et al., 1992]. Calpains degrade proteins that are important in signal transduction pathways such as protein kinase C and the transcription factors c-Jun and c-Fos [Suzuki et al., 1992].

The mechanism that regulates the function of calpains in cells remains uncertain. The concentration of Ca^{2+} required to activate m-calpain, even in the presence of phospholipids, is higher than what would be expected to be found *in vivo* [Goll et al., 1992]. In addition, cells

contain a potent inhibitor of calpains called calpastatin, which would seemingly inactivate calpain at the calcium concentrations needed for activation. This has led some to the suggestions that calpains may require an as yet unidentified activator that could allow calpain to be active under specific physiological conditions and that the calpastatin-calpain interaction also must be regulated in some way to permit calpain activity [Goll et al., 1992].

THE MARKED pH DEPENDENCE OF THE TALIN-ACTIN INTERACTION

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Summary: The effect of pH on the interaction between talin and actin was examined by using cosedimentation assays, low shear viscometry, and electron microscopy. When the pH at which the interaction was tested was lowered from 7.5 to 7.0 and below, the ability of talin to cosediment with F-actin was greatly enhanced, with maximal binding at pH 6.6. Talin's ability to increase the low shear viscosity of F-actin solutions was also significantly elevated as pH was decreased from 7.4 to 6.6. Electron microscope observations of negatively stained actin controls and talin-actin mixtures supported these results by demonstrating an increase in the degree of crosslinking of actin filaments in the presence of talin at pH values of 7 and lower.

Adherens junctions are areas where attachment of actin filaments to the cell membrane coincides with attachment of the cell to other cells (cell-cell junctions) or to proteins of the extracellular matrix (cell-matrix junctions). These sites are involved in a variety of important cellular processes including cell motility, cell adhesion, signal transduction, and muscle cell contraction (1). Talin is an ~225 kDa protein [by SDS-PAGE (2); 270 kDa from sequence (3)] believed to play a key role in the attachment of actin filaments to the cell membrane at cell-matrix junctions. Talin is concentrated at these sites in a variety of cell types and has been reported to bind *in vitro* with low affinity to the transmembrane receptor integrin (4,5), with higher affinity *in vitro* to vinculin, another cytoskeletal protein present in adherens junctions (6-8), and possibly to dystrophin (9), the protein missing in Duchenne muscular dystrophy (10). Because no direct

interaction between talin and actin was observed (5,7,11), early reports suggested that talin might help link actin filaments to the membrane in an indirect fashion, by composing one member of a multi-protein bridge containing vinculin and alpha-actinin. More recently, a direct interaction between talin and actin *in vitro* has been reported (12-15), suggesting that talin also may link actin filaments directly to an integral membrane protein such as integrin; however, reports vary considerably with respect to the nature of the talin-actin interaction. As part of our overall interest in understanding cytoskeletal proteins that may help anchor actin filaments to their attachment sites (16-21), and specifically to better understand the nature of the talin-actin interaction, we have used three different types of experimental approaches, cosedimentation, low shear viscometry and electron microscopy. We show that talin clearly interacts with actin filaments and that the interaction is remarkably pH dependent.

Materials and Methods

Protein purification. Actin was prepared from porcine skeletal muscle as described (22) with further purification by gel filtration on Sephacryl S-300 HR (Pharmacia). Smooth muscle talin was prepared from turkey gizzards essentially as described (12), with purification by sequential chromatagraphy on DEAE-cellulose (DE-52, Whatman), hydroxyapatite (HA Ultrogel, Sepracor) and phosphocellulose (P11, Whatman) used to obtain highly homogenous talin. Full details of the preparation will be presented elsewhere. Protein concentrations were determined by the modified Lowry method (Sigma).

Cosedimentation assays. Talin (0.2 mg/ml) and G-actin (0.5 mg/ml) were mixed in a solution containing 10 mM imidazole-HCl, 1 mM ATP, 1 mM EGTA at a pH of 7.4, 7.0, or 6.6, with pH adjustment of buffers done at 25°C. Actin polymerization was initiated by adding $MgCl_2$ to a

final concentration of 2 mM. The solution was mixed and then incubated at 25°C for 1 hr. The mixture was centrifuged at 110,000 x g in a Beckman airfuge for 20 min, and the pellets and supernatants were analyzed by 10% SDS-PAGE (23).

Low shear viscosity. Low shear viscosity was done by using the method of MacLean-Fletcher and Pollard (24). Talin (0.1 mg/ml) was mixed with G-actin (0.5 mg/ml) in a solution containing 10 mM imidazole-HCl, 1 mM ATP, 1 mM EGTA with pH values of 7.4, 7.0, or 6.6, and incubated for 5 min at 25°C. Polymerization was initiated by adding $MgCl_2$ to a final concentration of 2 mM. The solution was mixed and immediately drawn up into a capillary tube that was placed at a fixed angle of 20° from horizontal and incubated an additional 10 min before measuring the viscosity. Results are expressed as normalized viscosity, obtained by dividing all talin-actin values by the viscosity of actin controls at each respective pH condition, to minimize differences between actin preparations and polymerization conditions (17).

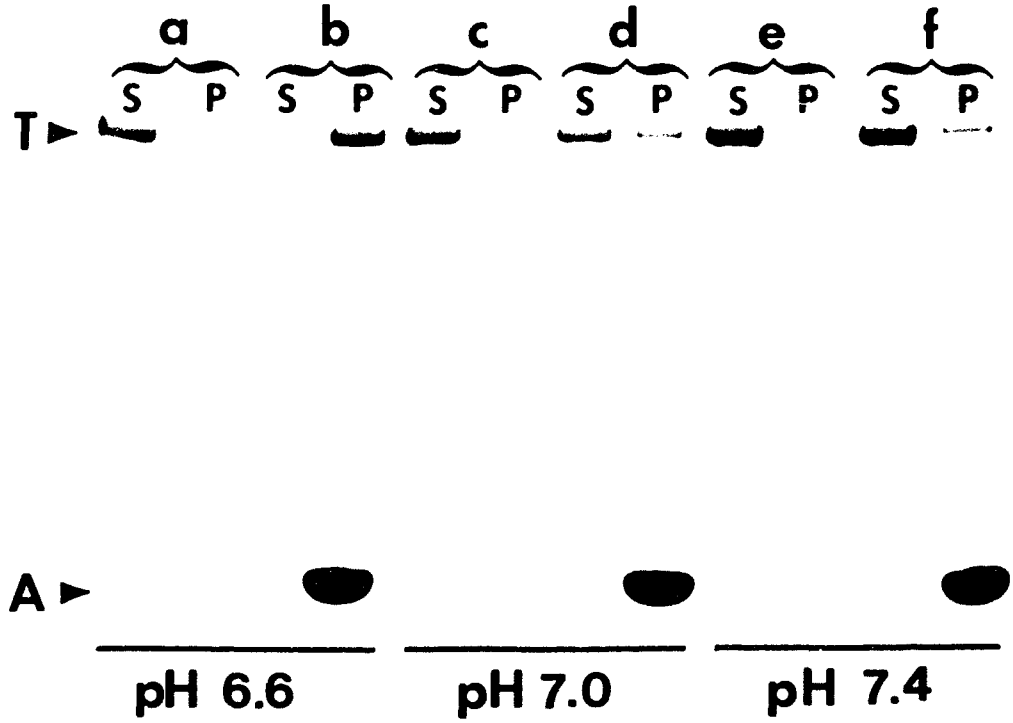
Electron microscopy. Negatively stained preparations of actin filaments in the presence or absence of talin were prepared by placing a drop of actin (0.25 mg/ml) control or actin (0.25 mg/ml)/talin (0.10 mg/ml) mixture, which had been polymerized under conditions similar to those used for cosedimentation assays, on a 400-mesh carbon coated grid. Samples were negatively stained with 2% aqueous uranyl acetate and were examined with a JEOL-100 CX II electron microscope operated at 80 kV with representative areas photographed.

Results

The effect of pH on the ability of talin to bind to and cosediment with actin filaments is shown in Fig. 1. Under the conditions of these assays, over 95% of the actin sedimented in the presence (Fig. 1, b,d,f) or absence (results not shown) of talin. Conversely, nearly all of the talin

Fig. 1. The effect of pH on the ability of talin to cosediment with actin filaments.

Experiments were performed at three different pH values, 6.6 (a,b), 7.0 (c,d) and 7.4 (e,f). Supernatants (S) and pellets (P) resulting from the sedimentation of talin controls with no actin (a,c,e) and of talin/actin mixtures (b,d,f) were analyzed by SDS-PAGE. T = talin, A = actin.


a
b
c
d
e
f
S P S P S P S P S P S P
T
A
pH 6.6
pH 7.0
pH 7.4

remained in the supernatant when centrifuged in the absence of actin, with only traces of talin in the pellet (compare S and P lanes in Fig. 1, a,c,e). When talin/actin mixtures (~ molar ratio of one 225 kDa talin to thirteen 42 kDa actins) were centrifuged, some talin cosedimented with actin filaments under all pH conditions tested (Fig. 1, b,d,f). However, a strong pH dependence was observed in the talin-actin interaction. As pH decreased, an increasing proportion of the talin cosedimented with actin (compare f, d, and b, respectively, in Fig. 1). At pH 6.6, almost all of the talin was in the pellet with actin (Fig. 1, b). The results shown in Fig. 1 were consistent and are representative of seven different talin preparations. Similar results were obtained when 0.2 mM $CaCl_2$ was substituted for EGTA in the incubation, and when other buffers were substituted for imidazole (results not shown).

As shown in Fig. 2, low shear viscometry experiments also demonstrated a direct interaction between talin and actin, as evidenced by an increase in the viscosity of F-actin solutions in the presence of talin (~1:26 molar ratio of talin to actin). Again, although talin consistently and significantly increased the viscosity of actin filaments at all pH values shown, a marked pH dependence was evident. At pH 6.6, the viscosity of the talin-actin mixture was much higher than the normalized viscosity obtained at pH 7.4. The viscosity of talin (only) control samples (results not shown) was negligible, regardless of pH; thus, viscosity increases shown in Fig. 2 involved talin/actin interaction.

To gain additional insight into the nature of the talin/actin interaction, negatively stained samples of actin (control), of talin/actin mixtures (~1:13 molar ratio of talin to actin) and of talin (control) were examined by electron microscopy (Fig. 3). Talin increases the crosslinked appearance of actin filaments at pH 7.0 (Fig. 3B) in comparison to the actin control (Fig. 3A). Because the total amount of actin is constant, talin appears to bring actin filaments into a closer,

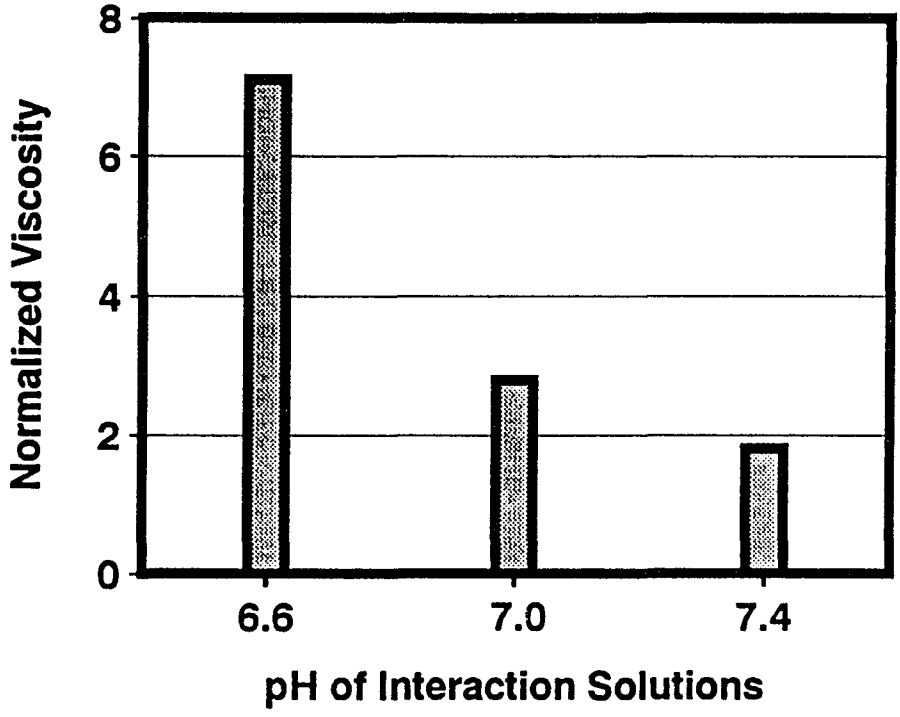
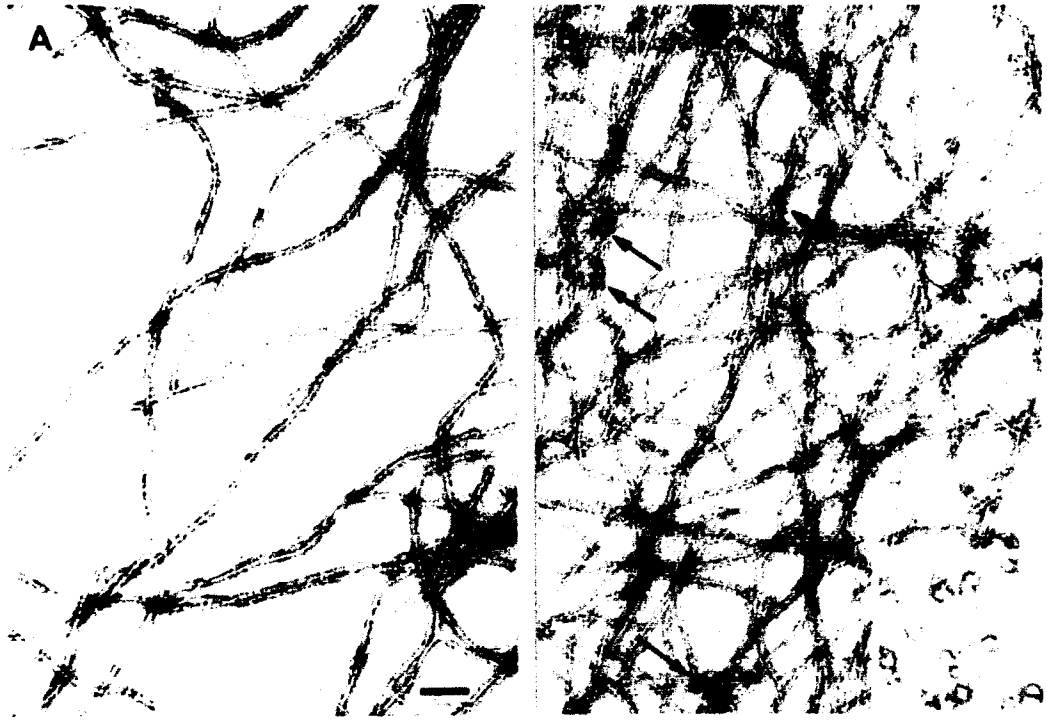


Fig. 2. The effect of pH on the ability of talin to increase the low shear viscosity of F-actin solutions. Assays were performed at three different pH values (6.6, 7.0, 7.4). Values for normalized viscosity were obtained by dividing the apparent viscosity of talin/actin mixtures by the apparent viscosity of actin controls (no talin) at each pH. For reference, a value of one for normalized viscosity would indicate no change in comparison to actin controls. Each viscosity assay was done in duplicate. Values are overall averages from four talin preparations.

Fig. 3. Electron microscopy of negatively stained actin filaments polymerized in the absence and the presence of talin at pH 7.0. Actin (0.25 mg/ml) was polymerized by the addition of $MgCl_2$ (2 mM final concentration) and incubated for 1 hr at 25°C, pH 7.0. (A) Actin control polymerized in the absence of talin, (B) actin polymerized in the presence of talin (0.10 mg/ml). Globular particles often were observed at points of filament intersection (arrows). Inset, negatively stained talin (in same polymerization buffer, pH 7.0) appears as globular particles with variable diameters (range 12-27 nm) that have some tendency to aggregate. Bar = 100 nm for A, B and inset.


A

tighter network. Globular particles also can be observed at several actin filament intersection points (Fig. 3B, arrows). These particles are not present in the actin filament control (Fig. 3A), and are similar in appearance to globular particles (minimum diameters of particles ranged from 12-27 nm) in the talin control (no actin) samples (Fig. 3B, inset). Molony et al. (2) also saw globular particles when talin was rotary shadowed in buffers of low ionic strength. When compared with results obtained at pH 7.0 (Fig. 3), talin is less able to crosslink actin filaments at pH 7.4, but much more extensively crosslinks actin filaments at pH 6.6 (results not shown).

Discussion

We have used three experimental approaches to study the interaction between talin and actin, under conditions in which actin is induced to polymerize into filaments (F-actin). Taken *in toto*, our results consistently demonstrate a direct interaction between talin and actin. Because most studies on talin published from 1983 to 1990 failed to demonstrate a direct talin-actin interaction *in vitro* (5,7,11), but did show both talin-integrin (4,5) and talin-vinculin (6-8) interactions *in vitro*, a working model or proposal for how talin might link actin filaments to cell-matrix junctions had talin as part of a multi-protein bridge (5,11). Recently, some reports (12-15) have indicated a direct talin-actin interaction *in vitro*, which presents the possibility that talin may directly link actin filaments to a transmembrane protein. The nature of the direct talin-actin interaction has, however, remained very unclear. Nevertheless, working models have been presented that illustrate talin involved in both indirect and direct linkages (25,26).

For the first time, we demonstrate that the talin-actin interaction is heavily dependent upon pH, and increases with decreasing pH (Figs. 1-3). Talin's abilities to cosediment with actin filaments and to increase the low shear viscosity of F-actin solutions were both significantly

enhanced as the pH was lowered. Electron microscope observations of talin/actin mixtures revealed a crosslinked appearance that became more extensive as pH decreased from 7.4 to 6.6. These observations were consistent with results from the viscometry and cosedimentation studies. The strong dependence on pH suggests that binding of talin to actin reflects, at least in part, electrostatic interactions between the two proteins. This suggestion is also supported by our results (not shown) of experiments that indicate reduced binding between actin and talin as the ionic strength of the solution is elevated, regardless of the pH tested.

Detailed comparison of our results to those of others is somewhat difficult. Nearly all reports have utilized talin prepared from gizzard smooth muscle (e.g., 2,7,12,14) or human platelets (e.g., 13,27,28). Of the several reports on gizzard talin, many indicated no interaction with actin (5,7,11) while others indicated a direct actin interaction (12,14,15,30). Of the latter studies, some indicated that talin interacts with G-actin, nucleates actin polymerization, but does not exhibit capping or severing properties (14,30). Platelet talin from that same laboratory also causes a decrease in the viscosity of actin filaments (13). Muguruma et al. (12,15), however, reported that gizzard talin increases the viscosity of actin filaments. In the earliest studies on platelet talin (sometimes called P235), it was reported that talin interacts with actin, causing a significant decrease in the viscosity of F-actin solutions (28). They also showed that actin filaments formed in the presence of talin were much shorter than controls, raising the possibility that talin may possess actin filament severing activity. Burridge et al. (25,31) have suggested that severing activity present in some preparations of platelet talin may be attributable to a contaminant(s), but those results were only referred to as unpublished observations (25,31).

We have made extensive, determined efforts to work with highly homogeneous talin preparations, and have found the effects of talin on actin to be highly reproducible. It is important

in studies of actin binding proteins that contaminants with ability to alter actin's properties be removed, as we demonstrated in earlier studies on vinculin (17). The results presented here indicate that the talin-actin interaction is complex, sensitive, and strongly dependent upon the ionic conditions employed *in vitro*. As many studies on talin/actin interactions have used relatively higher pH (7.5 to 8.0) and/or differing ionic strengths, our results may also explain, in part, why results among several labs are so variable.

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FACTORS AFFECTING THE INTERACTION BETWEEN TALIN AND ACTIN

A paper to be submitted to the Journal of Biological Chemistry

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Talin is an adhesion plaque protein believed to be involved in linking actin filaments to the cell membrane. The effects of selected factors including pH, ionic strength, temperature, and the molar ratio of talin to actin on the interaction between highly purified talin and actin were studied using low shear viscometry, cosedimentation assays, and electron microscopy. The ability of talin to bind to and increase the viscosity of actin filaments in the pH range from 6.4 to 7.4 and low ionic strength was increased with decreasing pH and increasing molar ratio of talin to actin. At pH 6.4 and low ionic strength, talin bound to actin at molar ratios as high as one talin to two actin monomers. Low shear viscometry and cosedimentation studies showed that increasing the ionic strength by as little as 0.025 decreased the ability of talin to bind to actin, regardless of pH. Results from low shear viscometry studies demonstrated a significant increase in the ability of talin to interact with actin with increasing temperature from 4° to 37°C. Cosedimentation assays showed that talin cosedimented to the same extent when mixed with preformed actin filaments as it did with G-actin that was then polymerized to F-actin. The effect of pH and ionic strength on the ability of talin to cosediment with F-actin was reversible. The results of this study consistently demonstrated a direct interaction between talin and actin, and that this interaction was highly sensitive to and dependent upon ionic conditions and temperature.

A role for talin in linking actin filaments to the cell membrane was proposed as early as 1982 (1), when it was first localized at focal contacts and ruffling membranes of cultured fibroblast cells. These early studies, however, did not detect any evidence for a direct interaction between

smooth muscle talin and actin (2). When talin was found to interact *in vitro* with vinculin (2) and subsequently the transmembrane receptor integrin (3), a model describing a multiprotein bridge between integrin and actin (via integrin-talin-vinculin-α-actinin-actin) was proposed (4) based upon the *in vitro* interactions between integrin and talin (3), talin and vinculin (2), vinculin and α-actinin (5), and α-actinin and actin (6). It was not until 1990 that a direct interaction between smooth muscle talin and actin was reported by Muguruma et al. (7). Their results showed that talin could cosediment with F-actin and increase the viscosity of F-actin solutions, thereby suggesting that smooth muscle talin also may act to directly crosslink actin filaments to integrin (8). We also recently have presented evidence for a direct talin-actin interaction, and have found that under conditions of low ionic strength the ability of talin to crosslink actin filaments was significantly increased as pH is decreased in the range of 7.4 to 6.6 (9). Those studies (9) suggested that one possible reason several earlier studies failed to detect a direct interaction between talin and actin was that conditions commonly employed in cosedimentation assays (pH 7.5-8.0 and 100 mM KCl) do not favor talin-actin interaction.

A platelet form of talin (originally referred to as P235) was also reported in 1982 (10, 11). It was described as an actin binding protein that could decrease the viscosity of F-actin by decreasing the length of filaments formed in its presence. Recent reports on platelet talin (12, 13) confirm these earlier viscosity results (11), and suggest that platelet talin acts to nucleate actin filament formation, thereby resulting in an increase in filament number over filament length and a concomitant decrease in actin viscosity. The differences reported in talin's properties (i.e., smooth muscle vs. platelet talin) remain unresolved. It seems plausible that the different conditions used in the studies reported (2, 7, 9, 11-13) may have contributed to at least some of the differences observed in the effect of the two talins on the viscosity of actin filaments. And,

Burridge and associates (14, 15) have suggested that an actin-severing activity contaminates platelet talin preparations, and that more highly purified platelet talin does not possess actin-severing or viscosity reducing properties. As one attempt to reconcile the varying results on talin's effect on actin, Goldmann et al. (16) have examined and reported that the effect of platelet talin on the length of actin filaments is more pronounced at very early times in the actin polymerization process (~ 5 min), and that experiments done with longer actin polymerization times may allow short filaments to anneal into longer filaments. It is evident that, although there is an increasing consensus over the last four years that talin can interact directly with actin, the mode of interaction remains unclear.

In an effort to better understand the talin-actin interaction, this study examined the effects of selected factors on the ability of purified smooth muscle talin to interact with actin including the effects of pH, ionic strength, protein molar ratio, and temperature on the talin-actin interaction. We demonstrate that smooth muscle talin acts to rapidly (within 5 min) crosslink actin filaments into networks and bundles at pH 6.4 and low ionic strength, that the talin-actin interaction is significantly decreased with increasing pH and ionic strength, and that these pH/ionic strength effects on the interaction are reversible. We also show for the first time that increasing the temperature from 4°C to 37°C markedly increases the ability of talin to increase the low shear viscosity of F-actin.

Experimental Procedures

Protein Purification - All steps were carried out at 0-4°C unless stated otherwise. The pH adjustment of buffers was done at the temperature at which the buffer was to be used. Actin was prepared from porcine skeletal muscle as described by Spudich and Watt (17) with further

purification by gel filtration on Sephacryl S-300 HR (Pharmacia). In our hands, other methods for the preparation of talin (7, 10, 18) resulted in protein of inconsistent purity and amount of degradation. Therefore, we have devised the following method for the preparation of smooth muscle talin from turkey gizzards, which is based in part on the methods of Muguruma et al. (7) and Molony et al. (18). Four hundred grams of trimmed, ground, frozen turkey gizzards were washed twice with 5 vol (v/w) H_2O containing 0.2 mM PMSF[1], and centrifuged at 17,700 x *g* for 30 min. The resulting pellet was extracted overnight with 10 vol (v/w) of Buffer A (500 mM NaCl, 3 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 0.1 mM trypsin inhibitor (Sigma, No. T-9253), 3 mM MCE, and 20 mM Tris-HCl, pH 8.6). The extract was centrifuged at 17,700 x *g* for 1 hr, the resulting supernatant (Fig. 1, *lane 3*) was slowly brought to 7% (w/v) ammonium sulfate, stirred for 1 hr, and centrifuged as above. The resulting supernatant was brought up to 28% (w/v) ammonium sulfate, stirred for 1 hr, and centrifuged as above. The pellet was resuspended in Buffer B (3 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 3 mM MCE, and 20 mM Tris-HCl, pH 8.0) and dialyzed extensively against Buffer B. The solution was clarified by centrifugation at 183,000 × *g* for 1 hr, and the supernatant (Fig. 1, *lane 4*) was loaded onto a DEAE-cellulose (DE-52, Whatman) column (2.5 x 70 cm) previously equilibrated in Buffer B. The column was washed with Buffer B until the absorbance at 280 nm attained a steady baseline value. The protein was then eluted from the column using a gradient of 0 to 300 mM NaCl in Buffer B (4 liters total volume). Talin-enriched fractions that did not include filamin were pooled, and the protease inhibitor E-64 (Peptides International) was added to a final concentration of 2.5 μM. The talin pool was dialyzed against Buffer C (0.2 mM PMSF, 3 mM MCE, 50 mM

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[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; MCE, 2-mercaptoethanol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

potassium phosphate, pH 7.0) (Fig. 1, lane 5), and loaded onto a hydroxylapatite (HA-Ultrogel, Sepracor) column (2.5 x 30 cm) previously equilibrated in Buffer C. Elution was obtained with a gradient of 50 to 400 mM potassium phosphate, pH 7.0, containing 0.2 mM PMSF, 2.5 μM E-64, and 3 mM MCE (800 ml total volume). Talin-enriched fractions were pooled and dialyzed against Buffer D (3 mM EGTA, 0.2 mM PMSF, 3 mM MCE, 25 mM potassium phosphate, pH 7.0) (Fig. 1, *lane 6*). Highly homogeneous talin was then routinely obtained by subsequent chromatography on a phosphocellulose (P-11, Whatman) column (1.6 x 50 cm) previously equilibrated in Buffer D, and eluted with a gradient of 0 to 500 mM NaCl in Buffer D (600 ml total volume). Talin fractions, free of an ~ 130 kDa contaminant, were pooled, E-64 (2.5 μM final concentration) and PMSF (0.2 mM final concentration) were added, and the purified talin was stored at 4°C (Fig. 1, *lane 7*). For all subsequent studies, the talin was dialyzed into the appropriate buffer immediately before use.

Other Analytical Methods - Protein concentrations were determined by using a modified (19) Lowry (20) assay (Sigma). Analysis by SDS-PAGE was done by the method of Laemmli (21). Molecular weight markers (see Fig. 1, *lane 1*) were obtained from Sigma, and contained myosin (~ 205 kDa), β-galactosidase (~ 116 kDa), phosphorylase b (~ 97 kDa), bovine albumin (~ 66 kDa), egg albumin (~ 45 kDa), and carbonic anhydrase (~ 29 kDa). Whole gizzard homogenate (Fig. 1, *lane 2*) was prepared by homogenization of gizzard smooth muscle in 10 vol (w/v) of 10% SDS, 0.1%MCE, 10 mM sodium phosphate, pH 7.0, followed by heating at 100°C for 5 min. After cooling, the samples were centrifuged at 2,000 x *g* for 30 min, filtered, and aliquots of the supernatant were prepared for electrophoresis and used as an additional set of molecular weight markers.

Cosedimentation Assays - Specific conditions for each cosedimentation study are stated in the

figure legends. Briefly, talin and actin were mixed in Buffer E (1 mM ATP, 1 mM EGTA, 1 mM MCE, 10 mM imidazole-HCl) at an initial pH of 7.4, 7.0, or 6.6. Actin polymerization was initiated by the addition of $MgCl_2$ (2 mM final concentration, Figs. 3, 7-9) or by the addition of $MgCl_2$ (2 mM final concentration) with selected concentrations of KCl (Fig. 5). The solution (final volume of 150 μl) was mixed, and then incubated at 25°C for the stated time. The mixture was centrifuged at 100,000 x *g* in a Beckman airfuge for 5 min (Fig. 7) or for 20 min (Figs. 3, 5, 8, 9). One hundred μl of the supernatants were collected, the final pH of each supernatant was measured with a microelectrode (Radiometer), and then samples were prepared for SDS-PAGE. The pellets were resuspended in 100 μl of 1% SDS in Buffer E and prepared for SDS-PAGE. Samples were analyzed by 10% SDS-PAGE and, to compensate for the different volumes used, protein loads were adjusted (supernatants = 21 μl and pellets = 14 μl) to permit a direct comparison between supernatants and pellets. The pH of Buffer E decreased slightly, but consistently, upon the addition of $MgCl_2$ and resulted in final pH values of 7.3, 6.9 and 6.4 for initial values of 7.4, 7.0 and 6.6, respectively. Throughout this paper we will refer to the final pH in the discussion of our results. The figures for the cosedimentation experiments show gels that are representative of results obtained from a minimum of three different talin preparations and three different actin preparations.

Low Shear Viscometry - Low shear viscometry was done by using the method of MacLean-Fletcher and Pollard (22). Talin (at final concentrations varying from 50 μg/ml to 200 μg/ml) was mixed with G-actin (0.5 mg/ml final concentration) in a solution containing 1 mM ATP, 1 mM EGTA, 1 mM MCE, 10 mM imidazole-HCl (Buffer E) with initial pH values of 7.4, 7.0, or 6.6, and incubated for 5 min at 25°C (Figs. 2 and 4) or for 10 min at 4°C, 25°C or 37°C (Fig. 6). Polymerization of G-actin was initiated by adding $MgCl_2$ to a final concentration of 2 mM

(Figs. 2 and 6) or by $MgCl_2$ (2 mM final concentration) with selected concentrations of KCl (Fig. 4). The solution was mixed, immediately drawn up into a capillary tube that was placed at a fixed angle of 20° from horizontal, incubated an additional 10 min, and the viscosity was measured as a function of the time it took a ball to travel a given distance through the sample. Results are expressed as normalized viscosity, obtained by dividing the apparent viscosity of the talin/actin mixtures by the apparent viscosity of the actin only controls for each different set of conditions, in order to minimize differences between actin preparations and in polymerization conditions. The viscosity results represent the means obtained from four different protein preparations tested in duplicate.

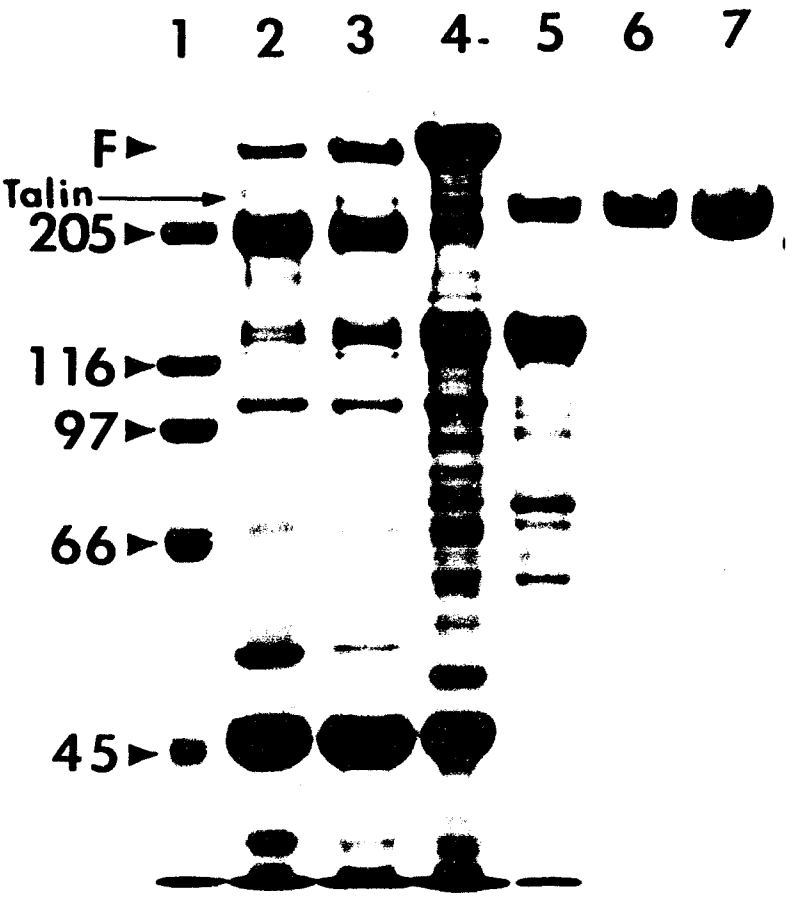
Results

Purification of Talin - Because the results reported on talin and its interactions with actin have been highly inconsistent and variable (2, 7, 11-14), and may have reflected small amounts of contaminants that could alter actin's properties (14, 15), we have made considerable effort to work with highly homogeneous talin. In our hands, previously published procedures for the purification of talin (1, 7, 10, 18) resulted in talin with variable degrees of purity, yield, and amount of proteolytic degradation. Our procedure for preparing smooth muscle talin as described in the Experimental Procedures is closest to that of Muguruma et al. (7) that in turn was a modification of Molony et al. (18), but differs in two significant ways. The first major difference is that other procedures, including the one described by Muguruma et al. (7), have used low ionic strength extraction buffers, which left almost half of the talin remaining in the pellet (unpublished observations). We have found that increasing the ionic strength of the extraction buffer resulted in an increased overall yield of talin. Samples from the major steps in the purification procedure are

shown in Fig. 1. A comparison of *lanes 2* and *3* shows that most of the major protein components in the whole gizzard homogenate were also extracted with the high ionic strength buffer used in this preparation. Ammonium sulfate fractionation, dialysis, and clarification of the extract resulted in a reduction in the amounts of several proteins, especially myosin and actin, while enriching for others, including filamin, talin and ~ 130 kDa protein(s) (*lane 4*). Column chromatography using DEAE-cellulose removed several contaminants such as filamin, myosin and actin, but not protein(s) migrating at ~ 130 kDa (*lane 5*). This contaminant(s) and others are removed by hydroxylapatite chromatography, which results in talin containing only traces of ~ 130 kDa protein and two very light bands around 66 kDa (*lane 6*). The second major difference between our procedure and that of Muguruma et al. (7) is that their final column was gel filtration, whereas the final column in this procedure was phosphocellulose, which, in our hands, more effectively removed the remaining contaminants and resulted in highly homogeneous talin (*lane 7*). Talin produced by this procedure has also been analyzed using 20% SDS-PAGE (results not shown), and no evidence for lower molecular weight contaminants was observed. Typical yields range from 5 to 8 mg of highly homogeneous talin/100 g starting material in comparison to the 3 to 6 mg talin/100 g starting material reported by Molony et al. (18). The talin produced is also substantially freer of proteolytic activity and can be stored for several months at 4°C without noticeable degradation. Overall, this procedure consistently resulted in talin of improved purity and yield, with little evidence of the well-known 190 kDa talin degradation product.

The Effect of pH and Molar Ratio of Talin to Actin on the Talin-Actin Interaction - We have recently shown that the talin-actin interaction is markedly dependent on pH, and that talin's ability to increase the viscosity of F-actin (at a molar ratio of ~ one 225 kDa talin to twenty-six 42 kDa

Fig. 1. Preparation of highly homogeneous talin from smooth muscle. Samples were taken at selected steps in the talin purification procedure as described in Experimental Procedures and analyzed by SDS-PAGE. *Lane 1*, molecular weight markers (Sigma); *lane 2*, whole gizzard homogenate; *lane 3*, high ionic strength extract; *lane 4*, crude talin-containing fraction obtained after ammonium sulfate fractionation, dialysis, and clarification; *lane 5*, talin-enriched pool obtained from DEAE-cellulose column; *lane 6*, talin-enriched pool obtained from hydroxylapatite column; *lane 7*, talin pool obtained from phosphocellulose column (purified talin). Numbers to the left of arrows represent approximate molecular mass in kDa. F = filamin.


1
2
3
4
5
6
7
F
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205
116
97
66
45

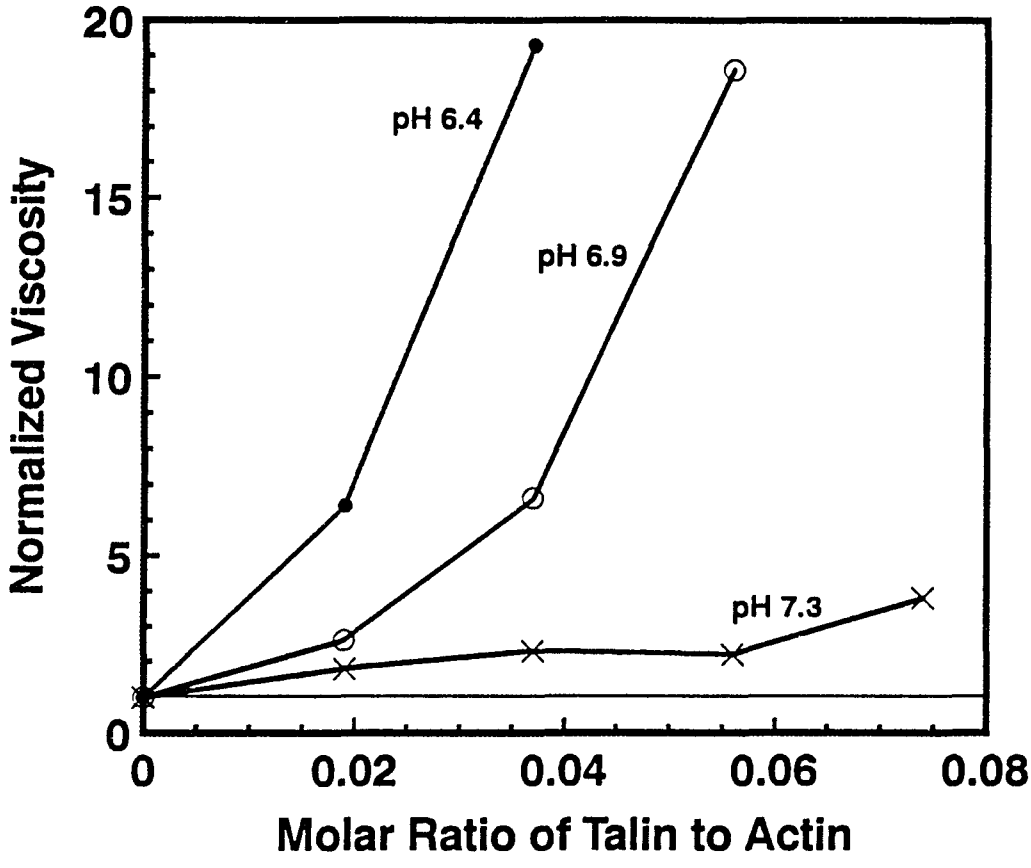
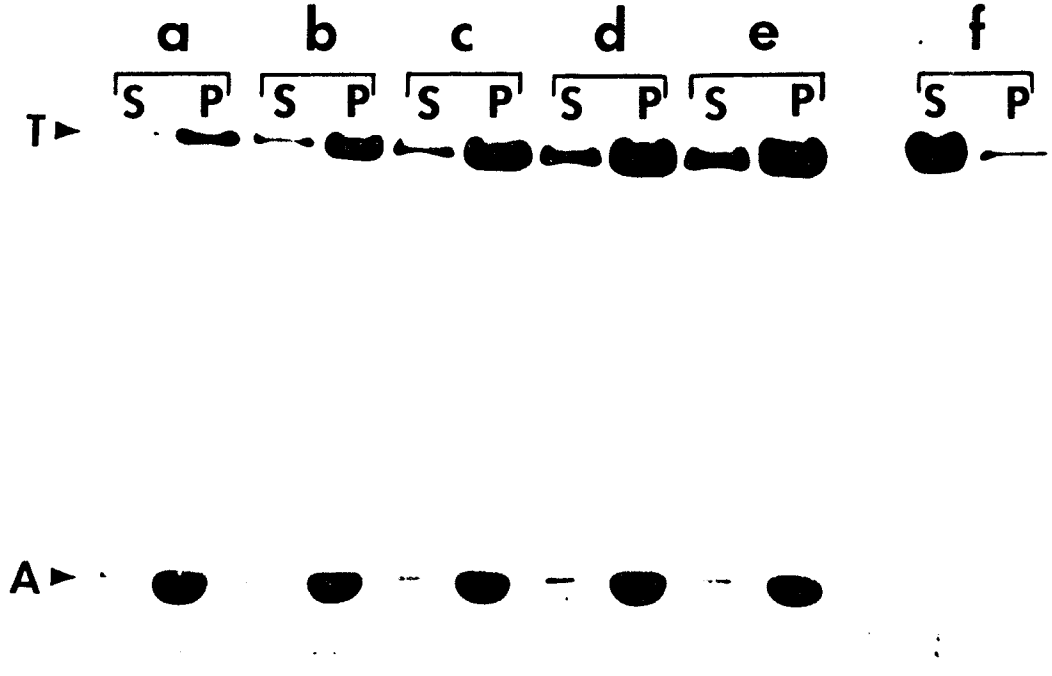


Fig. 2. Effect of pH on the ability of talin to increase the low shear viscosity of F-actin solutions. Assays were performed at three pH values (● = 6.4; O = 6.9; × = 7.3) at 25°C with talin at selected concentrations and actin at a final concentration of 0.5 mg/ml as described in Experimental Procedures. Results are expressed as normalized viscosity as described in Experimental Procedures. For reference, a value of one for normalized viscosity would indicate no change in comparison to actin controls, and is represented by a solid horizontal line on the graph.

actin monomers) was increased with decreasing pH in the range of 7.4 to 6.6 (9). We have also shown that almost all of the talin (at a molar ratio of ~ 1 talin to 13 actin monomers) could cosediment with F-actin in a low ionic strength buffer (Buffer E) at pH 6.6 (final pH after addition of $MgCl_2$ was 6.4) (9). The results in Fig. 2 show that talin's ability to increase the low shear viscosity of F-actin was dependent on both pH and the molar ratio of talin to actin. Talin increased the viscosity of F-actin under all conditions of pH and concentrations of talin tested. However, the effect at pH 6.4 was much more dramatic, even at lower concentrations of talin, than the effect at pH 7.3 at the highest concentration of talin tested (compare pH 6.4, molar ratio of ~ 0.02, with pH 7.3, molar ratio of ~ 0.08). At pH 6.4, the viscosity of talin/actin mixtures at molar ratios above 0.04 was high enough to completely stop the ball from moving through the solution. The SDS-PAGE results in Fig. 3 show a typical cosedimentation experiment in which we have progressively increased the molar ratio of talin to actin from 0.2 to 0.8 to saturate the talin binding sites on actin at pH 6.4 and low ionic strength. The relative proportion of talin in the supernatant compared to the pellet appeared to be constant in *panels a* (molar ratio ~ 0.2) and *b* (molar ratio ~ 0.4), but a detectable increase in the proportion of talin left in the supernatant was observed in *panels d* (molar ratio ~ 0.75) and *e* (molar ratio ~ 0.8). Very little talin sedimented in the absence of actin, even at the highest talin concentration used (*panel f*). A Scatchard plot of densitometry results obtained from similar experiments done under these conditions gave a binding ratio of ~ 1 talin to 2 actin monomers, which is close to the molar ratio of 0.56 in *panel c*, and an estimated K_d of ~ 7×10^{-8} M.

The Effect of Ionic Strength on the Talin-Actin Interaction - Potassium chloride at a concentration of 100 mM has been shown to decrease the ability of talin to cosediment with F-actin (7). The effect of increasing ionic strength on the ability of talin to increase the low shear

Fig. 3. Effect of increasing molar ratio of talin to actin on talin's ability to cosediment with F-actin at pH 6.4. Talin at final concentrations of 0.10 mg/ml (*panel a*), 0.20 mg/ml (*panel b*), 0.30 mg/ml (*panel c*), 0.40 mg/ml (*panel d*), and 0.43 mg/ml (*panel e*), was mixed with G-actin (fixed final concentration, 0.1 mg/ml) in Buffer E, and $MgCl_2$ was added to a final concentration of 2 mM. *Panel f* is a talin only control representing the highest concentration used (0.43 mg/ml). Samples were incubated for 1 hr and centrifuged at 100,000 x *g* for 20 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. T=talin; A=actin.


a
b
c
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e
f
S P S P S P S P S P S P
T►
A►

viscosity of F-actin solutions is shown in Fig. 4. As the concentration of KCl was increased, the ability of talin to increase the low shear viscosity of F-actin solutions was markedly decreased. The degree of the effect was most pronounced at KCl concentrations between 0 mM and 50 mM, with the talin-dependent increase in the viscosity of F-actin at pH 6.9 decreasing by ~ 70% in the presence of 50 mM KCl. Similar decreases were obtained at pH 6.4 (data not shown). Talin was still able to increase the viscosity of F-actin at pH 6.9 in the presence of 150 mM KCl (note that all values lie above a normalized viscosity of 1, Fig. 4), but the effect was small. A similar effect of increasing KCl concentration on the ability of talin to cosediment with F-actin is shown in Fig. 5. The presence of 25 mM KCl significantly decreased the amount of talin that cosedimented with F-actin at both pH 6.4 (Fig. 5*a*) and at pH 6.9 (Fig. 5*b*). Once again, the effect was most dramatic at salt concentrations between 0 mM and 50 mM, although a small portion of the talin still cosedimented with F-actin in the presence of 150 mM KCl at pH 6.4 and at pH 6.9. Similar results were obtained when NaCl was used in place of KCl (data not shown). Electron microscope observations of talin/actin mixtures in Buffer E at a final pH of 6.4 showed that talin crosslinked actin filaments into networks and bundles, but when 75 mM KCl was added to this buffer, talin no longer bundled actin filaments (data not shown).

The Effect of Temperature on the Talin-Actin Interaction - The effect of temperature on the ability of talin to increase the viscosity of F-actin at pH 6.9 is shown in Fig. 6. Decreasing the temperature from 25°C to 4°C resulted in a decrease in the ability of talin to increase the viscosity of F-actin, whereas increasing the temperature to 37°C resulted in a substantial increase in the normalized viscosity of talin/actin mixtures. The experiments shown in Fig. 6 were done using a low molar ratio of talin to actin of ~ 1:50, because higher ratios (1:25) often increased the viscosity of talin-actin solutions measured at 37°C to the point that the ball could no longer roll

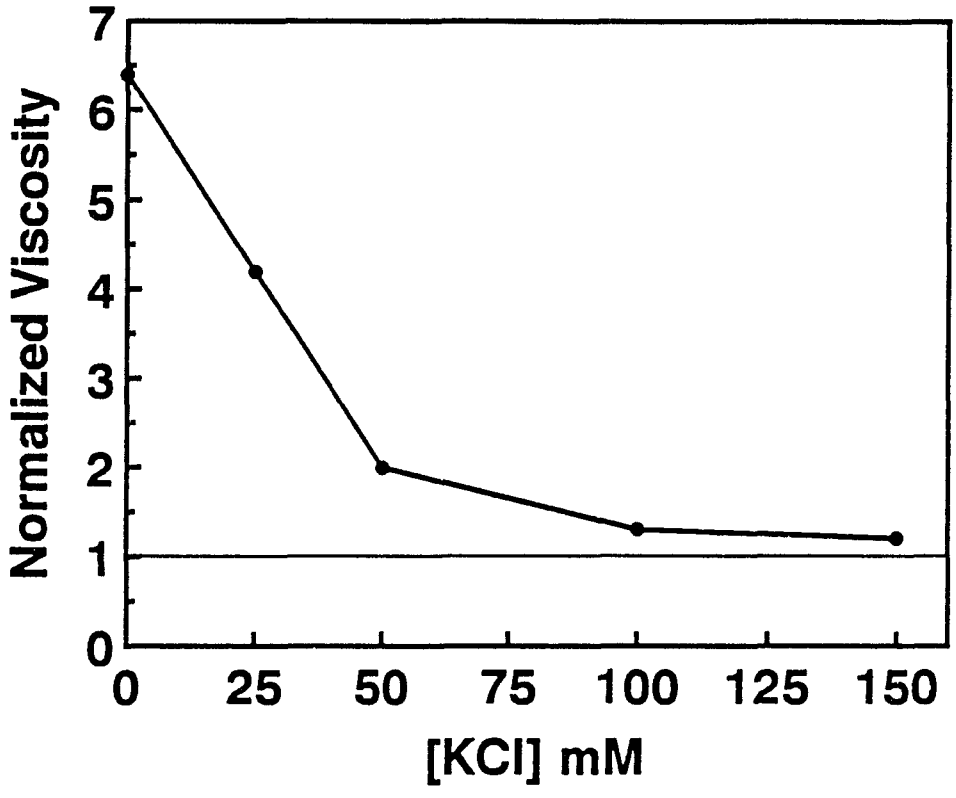
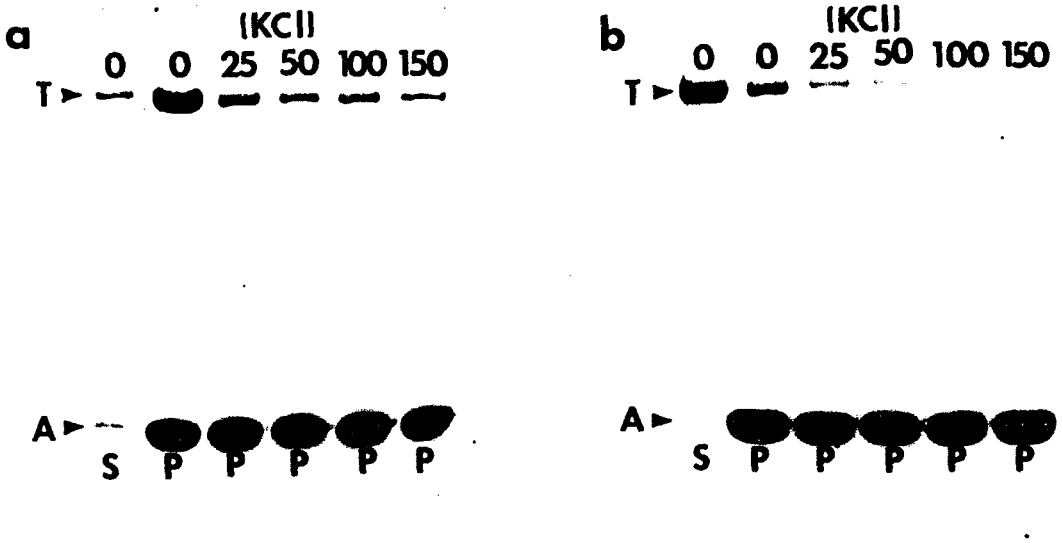


Fig. 4. Effect of ionic strength on the ability of talin to increase the low shear viscosity of F-actin solutions. Assays were performed at pH 6.9 at 25°C with fixed final concentrations of talin (0.1 mg/ml) and actin (0.5 mg/ml). Polymerization was induced by the addition of $MgCl_2$ (2 mM final concentration), with selected concentrations of KCl. The results are expressed as normalized viscosity as described in Experimental Procedures. A value of one for normalized viscosity would indicate no change in comparison to actin only controls, and is represented by a solid horizontal line on the graph.

Fig. 5. Effect of ionic strength on the ability of talin to cosediment with actin filaments. Experiments were performed at pH 6.4 (Fig. 5*a*) and pH 6.9 (Fig. 5*b*) at 25°C with fixed final concentrations of talin (0.2 mg/ml) and actin (0.5 mg/ml). Polymerization of actin was induced by the addition of $MgCl_2$ (2 mM final concentration), with selected final concentrations of KCl. Samples were incubated for 1 hr and centrifuged at 100,000 x *g* for 20 min. Pellets (P) resulting from sedimentation of the talin/actin mixtures were analyzed by SDS-PAGE. The final concentration of KCl (mM) in each sample is shown by the numbers above each lane. The supernatant (S), obtained from the talin/actin control mixture with no KCl, is shown in the first lane in both Fig. 5*a* (pH 6.4) and Fig. 5*b* (pH 6.9). T=talin; A=actin.


a
[KCl]
0 0 25 50 100 150
T
A
S P P P P P
b
[KCl]
0 0 25 50 100 150
T
A
S P P P P P

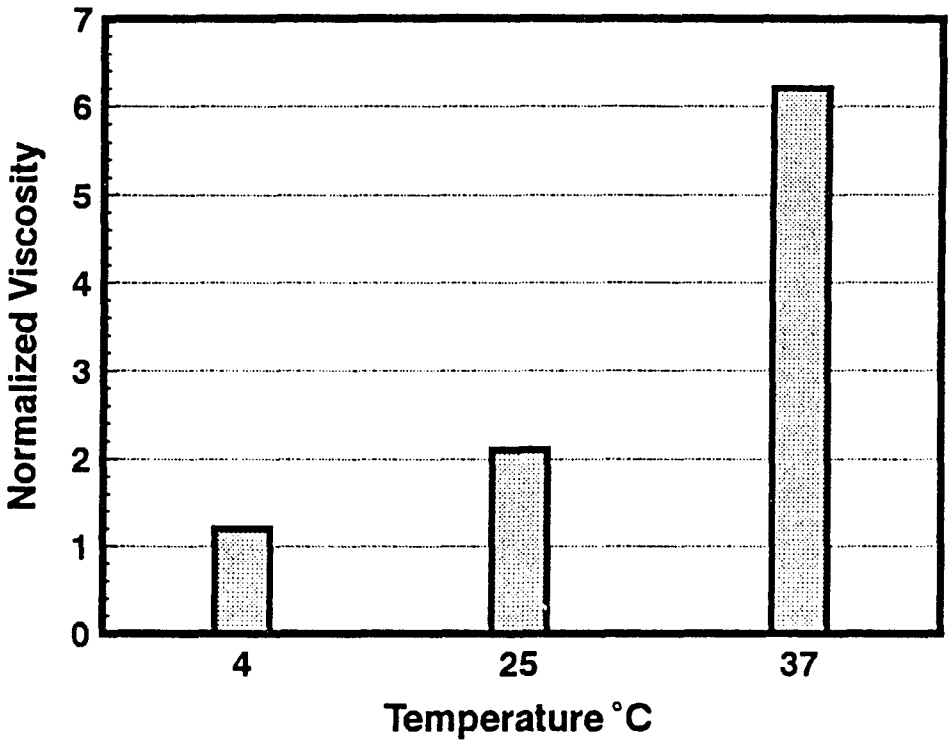
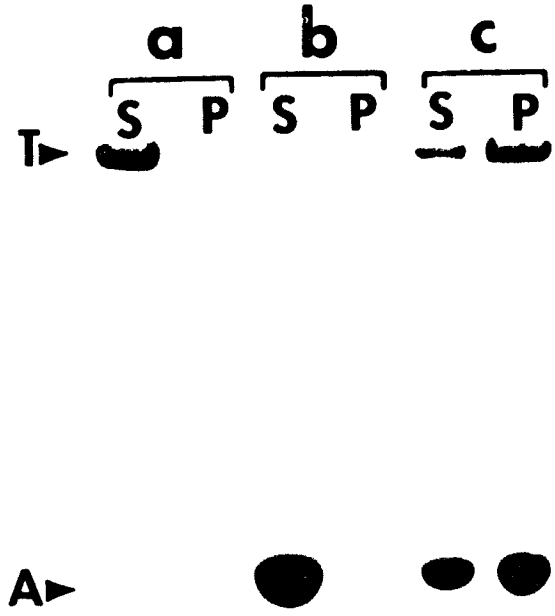


Fig. 6. Effect of temperature on the ability of talin to increase the low shear viscosity of F-actin solutions. Assays were performed at 4°C, 25°C, or 37°C at pH 6.9 with fixed final concentrations of talin (0.05 mg/ml) and actin (0.5 mg/ml). The pH adjustment of each buffer was done at the temperature of each experiment. Samples were incubated for 10 min at the selected temperature before polymerization was initiated by the addition of $MgCl_2$ to a final concentration of 2 mM. Viscosity was measured after an additional 10-min incubation, and results are expressed as normalized viscosity as described in Experimental Procedures.

through the mixture in the tube. For direct comparison between experiments done at the three different temperatures, the values shown in Fig. 6 were obtained 10 min after actin polymerization was initiated. Similar values for normalized viscosity were also obtained 20 min after actin polymerization was initiated, indicating that the small effect at 4°C was not simply due to lower rates of actin polymerization at this temperature.

The Effect of Short Polymerization Time on the Talin-Actin Interaction - In our earlier report (9) using smooth muscle talin, we showed that talin caused an increase in actin viscosity and that talin particles could be seen at the intersections of long actin filaments in talin/actin mixtures by electron microscopy. It has been reported, however, that platelet talin nucleated actin filament assembly (12, 13), resulting in a decrease in actin viscosity presumably due to an increase in the overall number of actin filaments and a decrease in filament length. It was later reported in studies from the same laboratory (16) that these effects were greater at very short times in the actin polymerization process (~ 5 min), and it was suggested that after longer polymerization times the short filaments might anneal to form longer filaments. In an effort to determine if the length of time the actin was polymerized affected our results obtained with smooth muscle talin, we decreased the polymerization times and examined the results by cosedimentation, low shear viscometry, and electron microscopy experiments. In Fig. 7 are shown the results from a representative cosedimentation experiment done in Buffer E at a final pH of 6.4 in which a talin only control (*panel a*), an actin only control (*panel b*), and a talin/actin mixture (*panel c*) were incubated for only 1 min after polymerization was initiated by the addition of $MgCl_2$ and then centrifuged for 5 min at 100,000 x *g*. As expected, the actin only control did not sediment well under these conditions (*panel b*); however, in the presence of talin, more than 50% of the actin was found in the pellet of the talin/actin mixture (*panel c*). These results indicated that talin was

Fig. 7. Effect of short polymerization time on the ability of talin to cosediment with actin filaments at pH 6.4. Magnesium chloride (2 mM final concentration) was added to a talin only control (0.2 mg/ml final concentration) (*panel a*), an actin only control (0.5 mg/ml final concentration) (*panel b*), and a talin (0.2 mg/ml final concentration)/actin (0.5 mg/ml final concentration) mixture (*panel c*) in Buffer E (final pH 6.4). The samples were incubated for 1 min at 25°C and then centrifuged at 100,000 x *g* for 5 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. T =talin; A =actin.


a
b
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S
P
S
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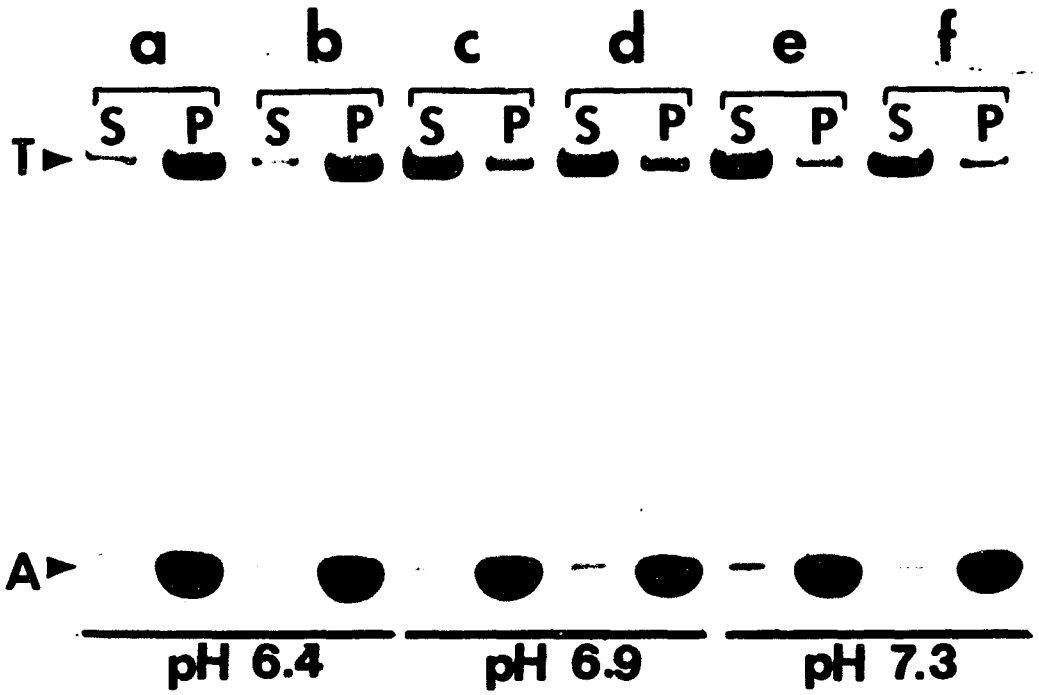
T►
A►

able to crosslink actin filaments under these conditions in a relatively short period of time. Talin/actin mixtures at pH 6.4 and low ionic strength that were incubated for 1 min after polymerization, but centrifuged for 10 min at 110,000 x *g*, appeared identical (data not shown) to talin/actin mixtures that had been incubated for 1 hr after polymerization, and then centrifuged at 100,000 x *g* for 20 min (e.g., Fig. 5*a*, 0 mM KCl). Low shear viscosity experiments done on samples incubated for times as short as 5 min after polymerization was initiated also yielded increases in normalized viscosity similar to those seen when measurements were taken 1 hr after polymerization was initiated (data not shown). Electron microscope observations of samples that had been fixed only 5 min after polymerization was initiated also showed that in Buffer E (final pH of 6.4) talin crosslinked actin filaments into networks and bundles (results not shown). In addition, no evidence was observed for the formation of short filaments in the presence of talin under these conditions. Taken *in toto*, we have found no evidence that smooth muscle talin's abilities to increase the viscosity of F-actin above actin only controls, or to crosslink actin filaments, were reversed by very short polymerization times. Therefore the length of time that actin was polymerized cannot explain the discrepancies between the effects of smooth muscle talin (7, 8) and platelet talin (10, 11, 14) on actin filaments.

The Ability of Talin to Interact with Preformed Actin Filaments versus Actin Filaments Formed in its Presence - It has been reported that platelet talin does not have an effect on preformed actin filaments (11), and that smooth muscle talin does not cosediment as well with preformed actin filaments as it does with actin filaments formed in its presence (23). Thus, we have compared the ability of talin to bind to preformed actin filaments with its ability to bind to filaments formed in its presence. The results in Fig. 8 show that talin cosedimented with preformed actin filaments (*panels a, b, c*), that the binding of talin to preformed filaments was also pH dependent (compare

Fig. 8. Ability of talin to cosediment with preformed actin filaments versus actin filaments formed in its presence, at pH 6.4, 6.9, or 7.3. Experiments were done at three pH values, 6.4 (*panels a, b*), 6.9 (*panels c, d*) and 7.3 (*panels e, f*) at 25°C as described in Experimental Procedures. Talin (0.2 mg/ml final concentration) was added to preformed actin filaments (0.5 mg/ml final concentration), which had been induced to polymerize with $MgCl_2$ and incubated for 1 hr at 25°C (*panels a, c, e*). For direct comparison, talin (0.2 mg/ml final concentration) was mixed with G-actin (0.5 mg/ml final concentration), which was then induced to polymerize by the addition of $MgCl_2$ (2 mM final concentration) (*panels b, d, f*), so that the final buffer conditions were identical for *panels a* and *b* (pH 6.4), for *panels c* and *d* (pH 6.9), and for *panels e* and *f* (pH 7.3). All samples were mixed by gentle inversion of the tube, allowed to incubate for 1 hr at 25°C after the addition of talin, and then centrifuged at 100,000 x *g* for 20 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE.


a
b
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d
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S
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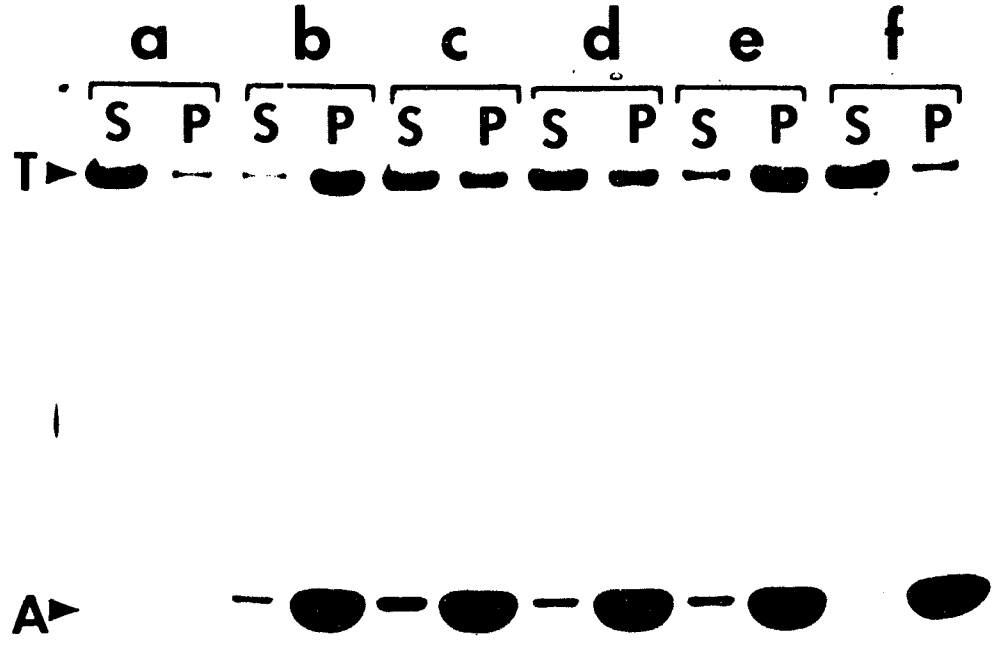
T
A
pH 6.4
pH 6.9
pH 7.3

panels a, c and *e*), and that the extent of talin binding to preformed filaments was equal to that of talin binding to filaments polymerized in its presence under the same conditions (compare *panel a* to *panel b, panel c* to *panel d,* and *panel e* to *panel f*). Likewise, electron microscope studies showed that talin crosslinked preformed actin filaments into networks and bundles in Buffer E at pH 6.4 and that the extent of the crosslinking was similar to that observed under the same conditions when actin was polymerized in the presence of talin (results not shown).

The Effects of pH and Ionic Strength on the Talin-Actin Interaction Are Reversible - The results shown in Fig. 9 demonstrated that the talin-actin interaction was responsive to subsequent changes in pH and ionic strength. Comparing the control sample at pH 6.4 in *panel b* to *panel c* shows that subsequently increasing the pH from 6.4 to 6.9 decreased the amount of talin that cosedimented with actin filaments. Comparing the results in *panel c* to *panel d* shows that the amount of talin that cosedimented with actin filaments after the pH was raised from 6.4 to 6.9 (*panel c*) was similar to that of the talin/actin control mixture at pH 6.9 (*panel d*). A decrease in pH from 6.9 to 6.4 (*panel e*) caused an increase in the ability of talin to cosediment with F-actin compared to the talin/actin control at pH 6.9 (*panel d*). Comparing the control sample at pH 6.4 and low ionic strength in *panel b* to *panel f* shows that subsequently increasing the ionic strength decreased the amount of talin that cosedimented with actin filaments. Talin responded rapidly to these changes as they could be visually seen as soon as the solutions were gently inverted for mixing. Samples in which the pH or ionic strength was subsequently increased immediately became noticeably less viscous, whereas the sample in which the pH was dropped immediately became noticeably more viscous (unpublished observations).

Fig. 9. Reversibility of the talin-actin interaction in response to changes in the pH or ionic strength. Talin (0.2 mg/ml final concentration) was mixed with G-actin (0.5 mg/ml final concentration) in Buffer E. Polymerization was initiated by the addition of $MgCl_2$ (2 mM final concentration), and samples were allowed to incubate for 30 min at 25°C before any changes were made in the pH or ionic strength of the test samples by the addition of NaOH, HCl or KCl. Control samples were treated in the same manner as the test samples, except that water was added in place of NaOH, HCl or KCl so that the final volumes for all samples were identical. All samples were then mixed by gentle inversion of the tubes, incubated for an additional 10 min, and then centrifuged at 100,000 x *g* for 20 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. *Panel a*, talin only control with no change in the pH of 6.4. *Panel b*, talin/actin control with no change in the pH of 6.4. *Panel c*, talin/actin mixture with the pH changed after 30 min from 6.4 to 6.9 by the addition of NaOH. *Panel d*, talin/actin control with no change in the pH of 6.9. *Panel e*, talin/actin mixture with the pH changed after 30 min from 6.9 to 6.4 by the addition of HCl. *Panel f*, talin/actin mixture with the ionic strength changed after 30 min by the addition of KCl to 100 mM final concentration with no change in the pH of 6.4.


a
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A

Discussion

Talin has been implicated in the attachment of actin filaments to the membrane because of its location at adhesion plaques and its ability to interact with the transmembrane receptor integrin (3). Because early reports did not detect any evidence for a direct interaction between smooth muscle talin and actin (2), it originally was proposed that talin might link actin to the membrane as one member of a multiprotein bridge including integrin, talin, vinculin, α-actinin, and actin (4). More recently, evidence for a direct interaction between talin and actin has been reported (7, 9, 12), suggesting that a more direct linkage among integrin, talin, and actin may also be possible (8). Unfortunately, the reported direct effects of talin on actin have been inconsistent, and the nature of the reported effects varies considerably among different laboratories (2, 7, 9, 11-16, 23). It has been suggested that at least some of these differences may be due to contaminants in the talin preparations (14, 15). As we have shown in earlier studies on the adhesion plaque protein vinculin (24), it is important to avoid contaminants that affect actin's properties in studies on actin binding proteins. Thus, we have made determined efforts to work with highly homogeneous talin and have devised the procedure described in the Experimental Procedures. In our hands, this procedure resulted in talin of consistently improved purity, yield, and decreased evidence of proteolytic activity upon storage. Different talin preparations using this method gave consistent, reproducible results in their effects on actin filaments. Overall, our results demonstrated a direct talin-actin interaction, but one that was very sensitive to experimental parameters. This sensitivity of the interaction may be another cause for the differences seen in the effects of talin on actin from different laboratories (2, 7, 9, 11-16, 23).

We have examined the effects of selected factors on the talin-actin interaction by using low shear viscosity, cosedimentation, and electron microscopy. *In toto*, our results show that talin

interacts directly with actin filaments and that the interaction is very dependent on pH, ionic strength, protein molar ratio, and temperature. The talin-actin interaction was significantly increased with decreasing pH from 7.4 to 6.4 and increasing molar ratio of talin to actin. The interaction was markedly decreased with increasing ionic strength. We also have shown clearly for the first time that the talin-actin interaction is very temperature dependent, with an increase in temperature from 4°C to the more physiological-like temperature of 37°C resulting in a stronger interaction. The sensitivity of the talin-actin interaction to changes in pH and ionic strength (Figs. 2-5, and 9) suggests that electrostatic interactions between talin and actin and/or talin and itself via self-association may affect the ability of talin to interact with actin. Our results have shown that talin can crosslink actin filaments, indicating that each talin molecule possesses at least two actin binding sites, or one actin binding site and the ability to self-associate (25). Others have presented evidence for dimeric or oligomeric formation for both smooth muscle (18, 23) and platelet (10, 16) talin under certain conditions, raising the possibility that self-association may be important in talin's crosslinking activity. Molony et al. (18) reported that talin exists as a compact globular molecule at low ionic strength (pH 7.6), but unwinds into a flexible, elongated molecule at higher ionic strengths. Thus, there also is evidence for an ionic strength-dependent conformational change in the talin molecule. It seems possible that talin may need to adopt a specific conformation in order to self-associate and/or to form the actin binding site(s) that may be enhanced at low pH, low ionic strength, and higher temperatures.

Kaufmann et al. (12) found that their platelet talin decreased the viscosity of actin filaments by decreasing the length of actin filaments, and proposed a nucleation activity for talin based upon evidence that talin could also increase the rate of actin polymerization. We, however, consistently have found that smooth muscle talin increased the viscosity of actin filaments (9, and this study) in

agreement with the results of Muguruma et al. (7, 23). In an effort to explain some of these differences (e.g., a decrease versus an increase in viscosity of actin filaments), Goldmann et al. (16) suggested that the decrease in actin filament length in the presence of their platelet talin was more pronounced at very early times in the actin polymerization process (~ 5 min). They suggested that these short filaments might then anneal to form longer filaments during the longer polymerization times used in the experiments from other laboratories (7, 9). We have shortened the actin polymerization times to ~ 5 min and examined the results by cosedimentation, low shear viscosity, and electron microscopy, and we have found no evidence for the very short actin filaments observed for platelet talin (11, 12, 16) in the presence of our smooth muscle talin. Our results showed that smooth muscle talin acted to rapidly (within ~ 5 min) increase the viscosity of actin filaments and crosslink long actin filaments into networks and bundles at low pH and low ionic strength. Therefore, the length of time actin was polymerized was not responsible for the differences reported on the effects of platelet (11, 12, 16) and smooth muscle (7, 9, 23) talin on actin. We also have shown that talin interacted equally well with actin filaments that had been polymerized in its presence or in its absence under the same conditions (Fig. 8). These results also argue in favor of crosslinking as the primary actin binding activity for smooth muscle talin. It is possible that the differences reported for human platelet talin and avian smooth muscle talin are due to species- or tissue-related differences between the two proteins, or that the platelet talin preparation is more prone to contamination by actin binding proteins as has been suggested by Burridge and associates (14, 15).

Overall, our results show that talin can interact directly to crosslink actin filaments, and that the interaction is very sensitive to changes in pH, ionic strength, protein molar ratio, and temperature. The ability of talin to respond to changes in these factors *in vitro* may be indicative

of a similar ability to respond to changes that occur *in vivo*. The talin-actin interaction is thought to occur primarily at the cell membrane where ion channels are located, and transient, localized changes in pH and ionic strength at these sites may be large enough to affect the talin-actin interaction. Several reports, for instance, have shown that the intracellular pH is altered by a variety of cell signals (26-28), and several other proteins recently have been reported to have pH-dependent actin binding activities (29-32). The latter findings suggest that changes in intracellular pH and/or ionic strength may serve as important messengers in signal transduction. Because talin can be phosphorylated by protein kinase C (33) and can associate with negatively charged lipids (34), it is probable that other factors such as covalent modification, lipid environment, and additional accessory proteins may also affect the ability of talin to interact with actin *in vivo*. The ability of the talin-actin interaction to respond to signals transmitted across the cell membrane by a variety of mechanisms is likely to be important in organization and function of the cell cytoskeleton. It seems plausible that talin must bind strongly to actin filaments under certain conditions such as when force is transduced across the membrane of muscle cells during contraction, and weakly under other conditions such as when cells require cytoskeletal reorganization such as for growth or cell repair. The results presented herein demonstrate a high degree of sensitivity in the talin-actin interaction and are consistent with a role for talin in the organization and attachment of actin filaments at adhesion plaques.

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THE EFFECT OF SELECTED ADHESION PLAQUE PROTEINS ON THE TALIN-ACTIN INTERACTION

A paper to be submitted to the Journal of Biological Chemistry

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These studies examined the effects of the adhesion plaque proteins filamin, vinculin, α-actinin, tropomyosin, and calpain (via its degradation of talin) on the ability of talin to interact with actin. Smooth muscle talin was cleaved by calpain into a 190 kDa fragment and a 47 kDa fragment. The ability of the 190 kDa proteolytic fragment of talin to bind to actin was markedly decreased when compared to intact talin under conditions favorable for the talin-actin interaction (pH 6.4 to 6.9). However, the 190 kDa fragment interacted strongly with actin filaments at a lower pH of 6.0. Filamin, at relatively high molar ratios to actin monomers (~ 1:10 and higher), but not at lower ratios (< 1:20), decreased the amount of talin that cosedimented with F-actin. Neither vinculin (molar ratio of ~ 1 vinculin to 12 actin monomers) or α-actinin (molar ratio of ~ 1 α-actinin to 5 actin monomers) affected the ability of talin to cosediment with actin. However, talin slightly increased the amount of vinculin that cosedimented in talin/vinculin/F-actin mixtures, and decreased the amount of α-actinin that cosedimented in talin/α-actinin/F-actin mixtures. Under conditions where neither talin or tropomyosin could completely cosediment with actin filaments, tropomyosin decreased the amount of talin that cosedimented with F-actin. None of the proteins used in these studies completely eliminated the talin-actin interaction, thus providing support for a direct role for talin in actin-membrane attachment.

Adhesion plaques are complex structures involved in a variety of important cellular processes

including cell motility, cell adhesion, muscle cell contraction, growth, and differentiation. Many proteins have been localized to these regions, including several actin binding proteins, protein kinases, and at least one protease (1), which are thought to be important in regulating the interactions occurring at these sites. Although it was originally suggested that talin linked actin filaments to the membrane as one member of a multiprotein bridge (via integrin, talin, vinculin, α-actinin, and actin), talin also may have a role in directly linking actin filaments to the membrane at these sites because it can bind to the transmembrane receptor integrin (2) and to actin filaments (3-5). We recently have reported that the direct binding of talin to actin is markedly dependent upon pH, ionic strength, protein molar ratio, and temperature (5, 6). The effects that other adhesion plaque proteins may have on the talin-actin interaction are largely unknown, but the influences of these proteins presumably must be coordinated with each other to respond to specific cellular needs. In this study we have investigated the *in vitro* effects of several proteins including filamin, vinculin, α-actinin, and tropomyosin on the ability of talin to cosediment with F-actin. These proteins were selected because they are found at adhesion plaques where the talin-actin interaction takes place, and they are known to bind to actin and/or to talin. Filamin (7, 8) and α-actinin (9, 10) are both actin binding proteins that also may interact directly with integral membrane proteins (11, 12). Therefore, they may be important in directly linking actin filaments to the membrane (11, 12) in a manner similar to that proposed for talin (13). Vinculin has been shown to bind to talin (14-17), and also may interact with actin (18, 19), although the latter interaction remains controversial (20-22). Tropomyosin is an important actin binding protein bound to actin filaments throughout the cell (23, 24), and is known to affect interactions between other actin binding proteins and actin (25-29). We will show herein that tropomyosin and filamin could decrease the amount of talin that cosedimented with F-actin, but

that the talin-actin interaction was not eliminated. Vinculin and α-actinin did not affect the amount of talin that cosedimented with F-actin. However, talin slightly increased the ability of vinculin to cosediment in talin/vinculin/actin mixtures and decreased the amount of α-actinin that cosedimented with F-actin.

The calpains are calcium activated proteases that also in part are located at adhesion plaques (30), and it has been suggested they might be important in cytoskeletal reorganization and in specific processes such as platelet activation (31, 32). Talin also is believed to be involved in these processes and is a very good substrate for calpain, which cleaves it into a 190 kDa fragment and a 47 kDa fragment (33). Although it has been reported that the 190 kDa fragment of talin contains the actin binding sites, we present results in this report that show the 190 kDa fragment of talin did not bind to actin as well as did intact talin under identical *in vitro* conditions.

Experimental Procedures

Protein Purification - All steps were carried out at 0-4°C unless stated otherwise. The pH adjustment of buffers was done at the temperature at which the buffer was to be used. Actin was prepared from porcine skeletal muscle as described (34) with further purification by gel filtration on Sephacryl S-300 HR (Pharmacia). Smooth muscle talin was prepared from turkey gizzards as described by Schmidt et al. (6). Highly purified m-calpain was purified according to Edmonds et al. (35). The 190 kDa fragment of talin was prepared by digestion of purified talin by m-calpain (~ 1: 300 molar ratio of m-calpain to talin) in 15 mM MCE[1], 20 mM Tris-HCl, pH 7.0, with 2.5 mM $CaCl_2$ (final concentration) added to initiate the digestion. The digestion was done for 1 hr at

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; MCE, 2-mercaptoethanol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

25°C and stopped by the addition of the calpain inhibitor, E-64 (Peptides International). The 190 kDa fragment was then purified by chromatography on a small (1.6 x 5 cm) hydroxylapatite (HA-Ultrogel, Sepracor) column. Tropomyosin was prepared from turkey gizzards (36) and further purified by hydroxylapatite column chromatography (37). Crude fractions of filamin, vinculin, and α-actinin were each obtained from the first DEAE-cellulose (DE-52, Whatman) column used during the talin preparation (6), in which the order of elution was vinculin, talin, filamin, and α-actinin. Filamin was further purified by hydroxylapatite (HA-Ultrogel, Sepracor) chromatography, and fractions containing primarily filamin and actin were collected. Highly purified filamin was then obtained by phosphocellulose column chromatography (P-11, Whatman) by using the same buffers and gradients described for purifying talin with these columns (6). Vinculin was further purified by sequential hydroxylapatite (HA-Ultrogel, Sepracor) and gel filtration (Sephacryl S-300 HR, Sigma) chromatography. The α-actinin was further purified by chromatography on DEAE-cellulose (DE-52, Whatman) and hydroxylapatite (HA-Ultrogel, Sepracor) columns (9), followed by additional gel filtration (Sephacryl S-300 HR, Sigma) chromatography when necessary. After addition of the protease inhibitor E-64 (Peptides International) to a final concentration of 2.5 μM and PMSF (Sigma) to a final concentration of 0.2 mM, purified filamin, vinculin, and α-actinin were stored at 4°C. All proteins were dialyzed into the appropriate buffer immediately before use. Protein concentrations were determined by the modified (38) Lowry (39) method (Sigma). Electrophoresis was done by the method of Laemmli (40).

Cosedimentation Assays - Specific conditions for each cosedimentation experiment are stated in the figure legends. Briefly, talin, or the 190 kDa talin fragment, and actin were mixed in Buffer A (1 mM ATP, 1 mM EGTA, 1 mM MCE, 10 mM imidazole-HCl, at selected pH

values). Actin polymerization was initiated by the addition of $MgCl_2$ (2 mM final concentration). The pH stated in the figure legends is the pH of Buffer A after $MgCl_2$ was added. The solution (final volume of 150 μl) was mixed, and then incubated at 25°C for 1 hr. The mixture was centrifuged at 100,000 x *g* in a Beckman airfuge for 20 min. One hundred μl of the supernatants were collected, the final pH of each supernatant was measured with a microelectrode (Radiometer), and samples were then prepared for SDS-PAGE. The pellets were resuspended in 100 μl of 1% SDS in Buffer A and prepared for SDS-PAGE. Samples were analyzed by 10% SDS-PAGE and, to compensate for the different volumes used, protein loads were adjusted (supernatants =21 μl and pellets = 14 μl) to permit a direct comparison between supernatants and pellets. The figures for the cosedimentation experiments show gels that are representative of results obtained from a minimum of three different protein preparations. The following molecular masses were used for the calculations of molar ratios: talin, 225 kDa; actin, 42 kDa; filamin, 500 kDa; vinculin, 117 kDa; α-actinin, 200 kDa; tropomyosin, 66 kDa.

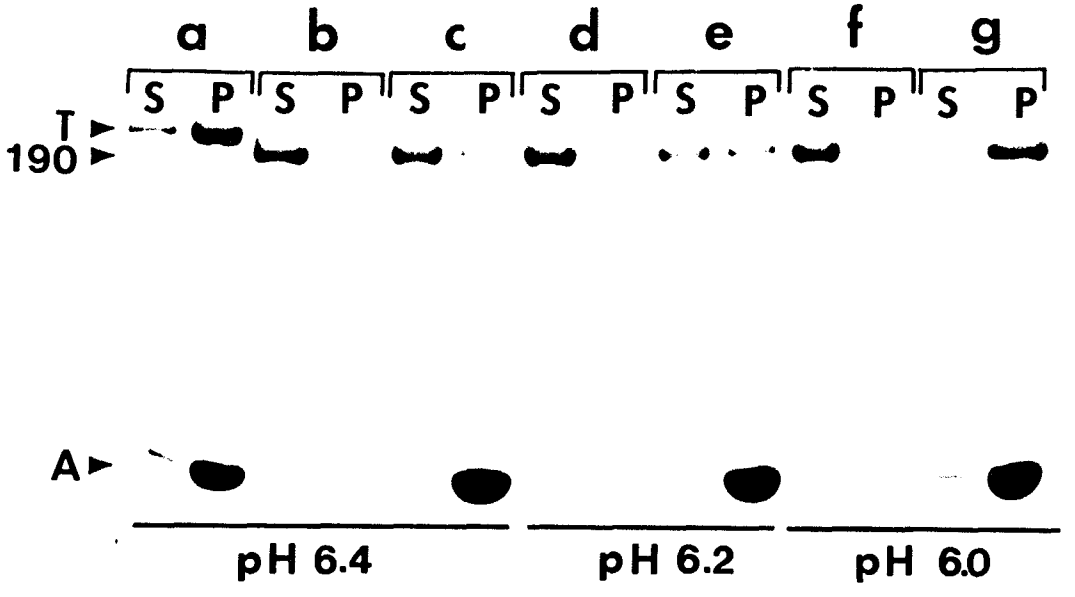
Low Shear Viscometry - Low shear viscometry was done by using the method of MacLean-Fletcher and Pollard (41). Talin (at final concentration of 100 μg/ml), or its 190 kDa proteolytic fragment (at final concentration of 100 μg/ml), was mixed with G-actin (0.5 mg/ml final concentration) in a solution containing 1 mM ATP, 1 mM EGTA, 1 mM MCE, 10 mM imidazole-HCl (Buffer A) with initial pH values of 7.0 or 6.6 (final pH of Buffer A with 2 mM $MgCl_2$ was 6.9 or 6.4, respectively), and incubated for 5 min at 25°C. Polymerization of G-actin was initiated by adding $MgCl_2$ to a final concentration of 2 mM. The solution was mixed and immediately drawn up into a capillary tube that was placed at a fixed angle of 20° from horizontal and incubated an additional 10 min before measuring the viscosity. Results are expressed as normalized viscosity, obtained by dividing the apparent viscosity of each talin/actin or 190 kDa

talin fragment/actin mixture by the apparent viscosity of actin controls (no talin or 190 kDa talin fragment) for each respective pH condition, to minimize differences between actin preparations and polymerization conditions. The viscosity results represent the means obtained from four different protein preparations tested in duplicate.

Results

The pH Dependence of the Interaction Between the 190 kDa Fragment of Talin and Actin - Talin is very susceptible to proteolytic cleavage by a variety of proteases including the calpains, which cleave talin into two fragments of 190 kDa and 47 kDa (33). It has been reported that the 190 kDa fragment contains the binding sites for vinculin (17), integrin (2), and actin (3). We had observed in preliminary studies that when talin preparations containing some contamination with the 190 kDa talin fragment were used in cosedimentation studies with actin, that the intact talin cosedimented with F-actin to a greater extent than did the 190 kDa fragment. The results from a cosedimentation study using the purified 190 kDa fragment of talin and actin at three different pH values are shown in Fig. 1. Because we had previously shown that intact talin cosediments almost completely at a pH of 6.4 and low ionic strength (5, 6, and see Fig.1, *panel a*), we compared the ability of the 190 kDa fragment to cosediment with actin under these identical conditions (*panel c*). The ability of the isolated 190 kDa fragment to cosediment with F-actin was greatly reduced in comparison to the intact talin (compare *panel c* to *panel a*), however a small amount of the 190 kDa fragment cosedimented with F-actin. Lowering the pH progressively increased the ability of the 190 kDa fragment to cosediment with F-actin (*panel e*), with maximal binding at a pH of 6.0 (*panel g*). The results of low shear viscometry studies comparing the relative abilities of the 190 kDa fragment and of intact talin to increase the viscosity of F-actin under identical conditions are

Fig. 1. Effect of pH on the ability of the 190 kDa talin fragment to cosediment with F-actin. Experiments were performed at final pH values of 6.4 (*panels a, b, c*), 6.2 (*panels d, e*), and 6.0 (*panels f, g*). Magnesium chloride was added to all samples (2 mM final concentration) in Buffer A, which were incubated for 1 hr and centrifuged at 100,000 x *g* for 20 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. *Panel a*, talin (0.15 mg/ml final concentration) /actin (0.5 mg/ml final concentration) mixture. *Panels b, d,* and *f,* 190 kDa talin fragment (0.12 mg/ml final concentration) only. *Panels c, e,* and *g,* 190 kDa talin fragment (0.12 mg/ml final concentration)/actin (0.5 mg/ml final concentration) mixtures. T = talin; A = actin; 190 = 190 kDa talin fragment.


a
b
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g
S P S P S P S P S P S P S P
T
190
A
pH 6.4
pH 6.2
pH 6.0

shown in Fig. 2. At a pH of 6.9, the 190 kDa fragment only slightly increased the viscosity of F-actin over the actin only controls (solid bar). Under these same conditions, however, intact talin significantly increased the viscosity of F-actin (open bar). At a pH of 6.4, there was an approximate two-fold increase in the ability of the 190 kDa fragment to increase the viscosity of F-actin (solid bar), but this increase was still much less than that found with intact talin at pH 6.4 (open bar). Experiments done at a pH of 6.0 showed that the 190 kDa fragment increased the viscosity of F-actin to the point where the ball would no longer roll though the solution (data not shown). Electron microscope observations of 190 kDa fragment/actin mixtures at pH 6.4 showed that the ability of the 190 kDa fragment to crosslink actin filaments at pH 6.4 was difficult to detect, although intact talin showed extensive crosslinking of actin filaments into both networks and bundles under these conditions. We observed, however, that when the pH was decreased to 6.0, the 190 kDa fragment was able to crosslink actin filaments into networks and bundles (results not shown). These results demonstrated that the 190 kDa fragment of talin contains the binding sites required to crosslink actin filaments, and that its ability to crosslink actin filaments was very dependent on pH. The optimal pH for crosslinking actin filaments was lowered from 6.4 for intact talin to 6.0 for the 190 kDa talin fragment.

The Effect of Filamin on the Ability of Talin to Cosediment with Actin Filaments - Results from a cosedimentation study including talin, filamin, and actin at a pH of 6.4 and low ionic strength are shown in Fig. 3. The molar ratio of talin to actin monomer was ~ 1:5 and the molar ratio of filamin to actin monomer was ~ 1:12. Under these experimental conditions, filamin sedimented to a small extent without actin present (*panel e*), and all of the filamin cosedimented when F-actin was present (*panel f*). Talin also sedimented to a very small extent in the absence of actin (*panel a*), and most of the talin cosedimented with F-actin (*panel b*). When both proteins

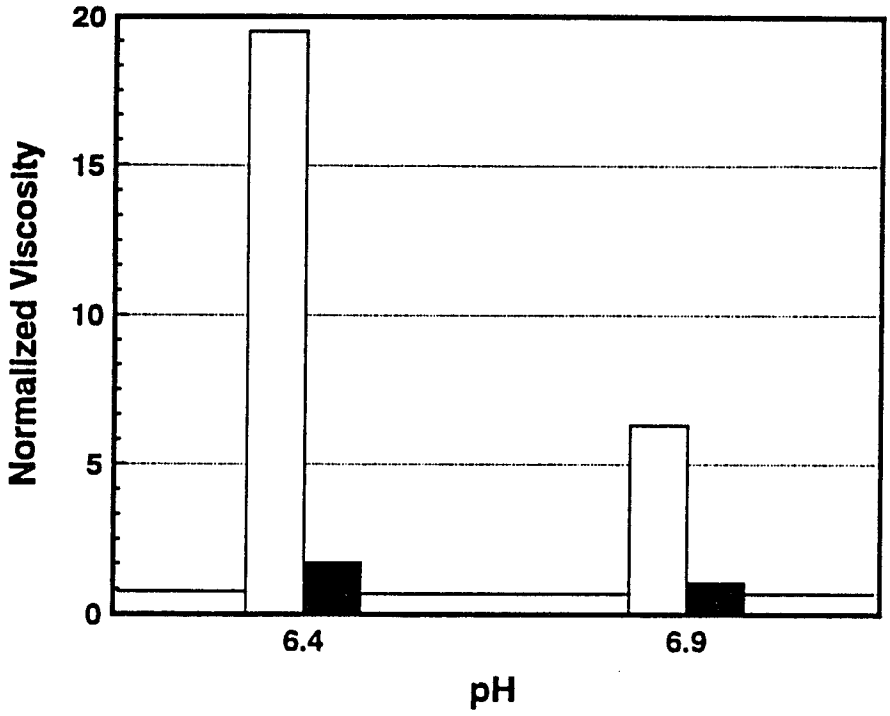
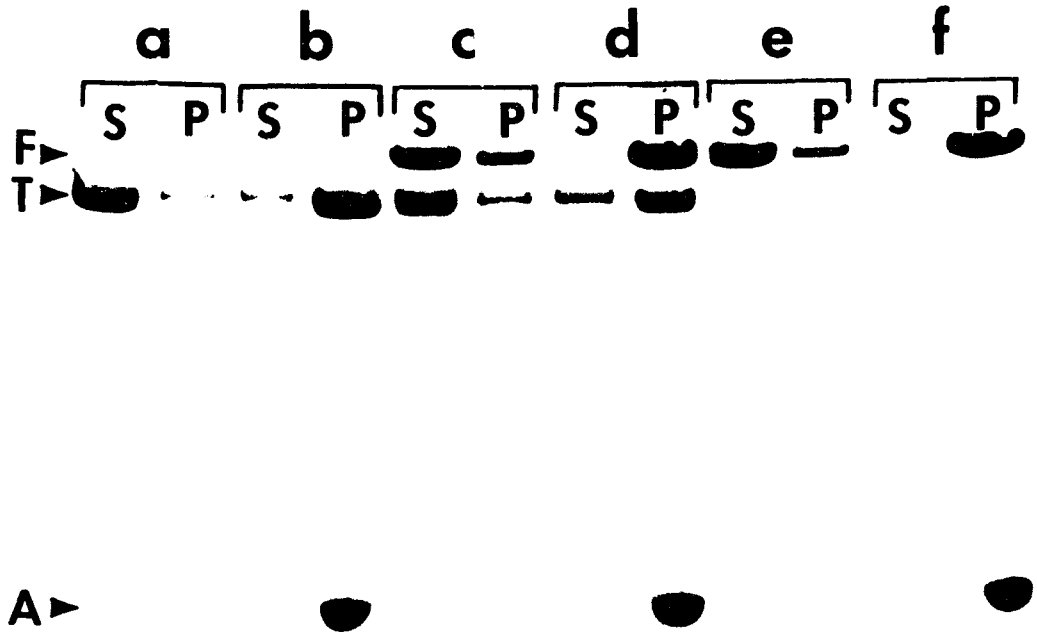


Fig. 2. Comparison of the ability of intact talin and of the 190 kDa talin fragment to increase the viscosity of F-actin solutions. Experiments were performed as described in Experimental Procedures at pH 6.4 and 6.9. Results are expressed as normalized viscosity. For reference, a value of one for normalized viscosity would indicate no change in comparison to actin controls, and is indicated by a solid horizontal line on the graph. Talin/actin = open vertical bars; 190 kDa talin fragment/actin = solid vertical bars.

Fig. 3. Effect of filamin on the ability of talin to cosediment with F-actin at pH 6.4. Talin (0.2 mg/ml final concentration) and/or filamin (0.2 mg/ml final concentration) were mixed with or without G-actin (0.2 mg/ml final concentration) in Buffer A. Magnesium chloride was added (2 mM final concentration) to all samples, which were incubated for 1 hr and centrifuged at 100,000 x *g* for 20 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. *Panel a*, talin alone; *panel b*, talin and actin; *panel c*, talin and filamin; *panel d*, talin, filamin, and actin; *panel e*, filamin alone; *panel f*, filamin and actin. T = talin; F = filamin; A = actin.


a
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d
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S P S P S P S P S P S P
F►
T►
A►

were allowed to interact with actin (*panel d*), all of the filamin still cosedimented; however, the amount of talin found in the supernatant compared to the pellet was slightly higher than found in talin/actin mixtures without filamin (compare *panels b* and *d*). This indicated that filamin, at these molar ratios, displaced some of the talin from actin. When the concentration of filamin was increased to the point where it just saturated actin (i.e., a molar ratio of ~ 1 filamin to 4 actin monomers, in which a small amount of filamin was present in the supernatant), the amount of talin that cosedimented with F-actin was decreased further, albeit approximately one third of the talin still cosedimented with F-actin. In contrast, at lower molar ratios (~ 1 filamin to 26 actin monomers), filamin did not affect the ability of talin to cosediment with F-actin (results not shown).

The Effect of Vinculin on the Ability of Talin to Cosediment with Actin Filaments - Vinculin is another well known adhesion plaque protein that has been shown to bind to talin with moderately high affinity (K_d ~ 10^{-8} M) (14). There have been conflicting reports as to whether vinculin can interact directly with actin (18-22). Results from cosedimentation studies including talin, vinculin, and actin are shown in Fig. 4. These experiments were performed at pH 6.4 and low ionic strength, conditions that favor the talin-actin interaction (*panel b*). Vinculin did not cosediment with F-actin under these conditions (*panel f*). The addition of vinculin did not significantly affect the amount of talin that cosedimented with F-actin (compare *panels b* and *d*); but, there was a small increase in the amount of vinculin that was found in the pellet in the talin/vinculin/actin mixture (*panel d*) in comparison to the vinculin/actin mixture (*panel f*).

The Effect of α-Actinin on the Ability of Talin to Cosediment with Actin Filaments - Alpha-actinin is an actin binding protein that is located at adhesion plaques and has been shown to bind directly to integrin (12). Based upon cosedimentation studies using conditions where neither

Fig. 4. Effect of vinculin on the ability of talin to cosediment with F-actin at pH 6.4. Talin (0.2 mg/ml final concentration) and/or vinculin (0.12 mg/ml final concentration) were mixed with or without G-actin (0.5 mg/ml final concentration) in Buffer A. Magnesium chloride was added (2 mM final concentration) to all samples, which were incubated for 1 hr and centrifuged at 100,000 x *g* for 20 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. *Panel a*, talin alone; *panel b*, talin and actin; *panel c*, talin and vinculin; *panel d*, talin, vinculin, and actin; *panel e*, vinculin alone; *panel f*, vinculin and actin. T =talin; V = vinculin; A = actin.

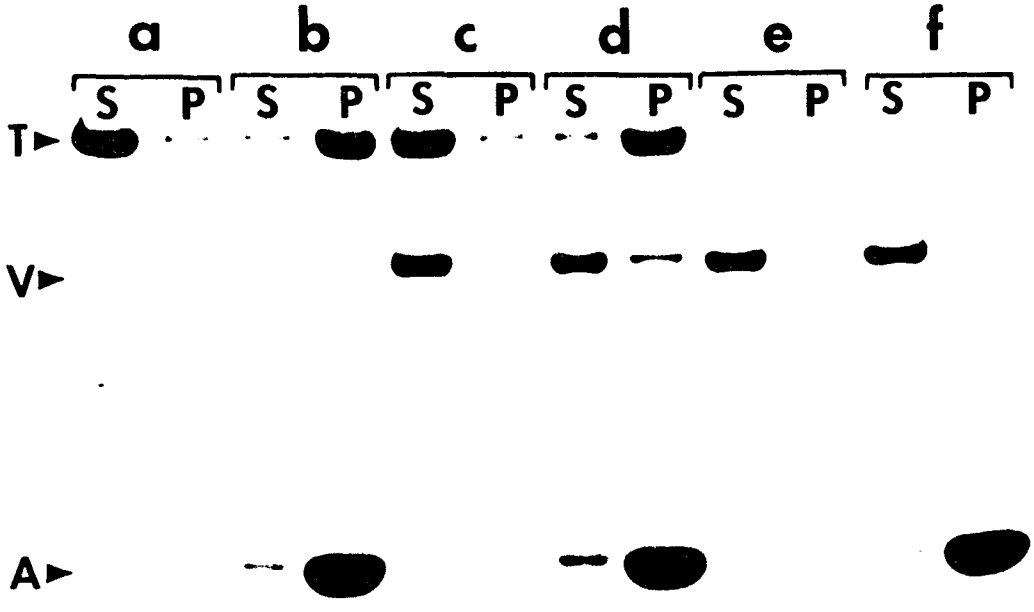
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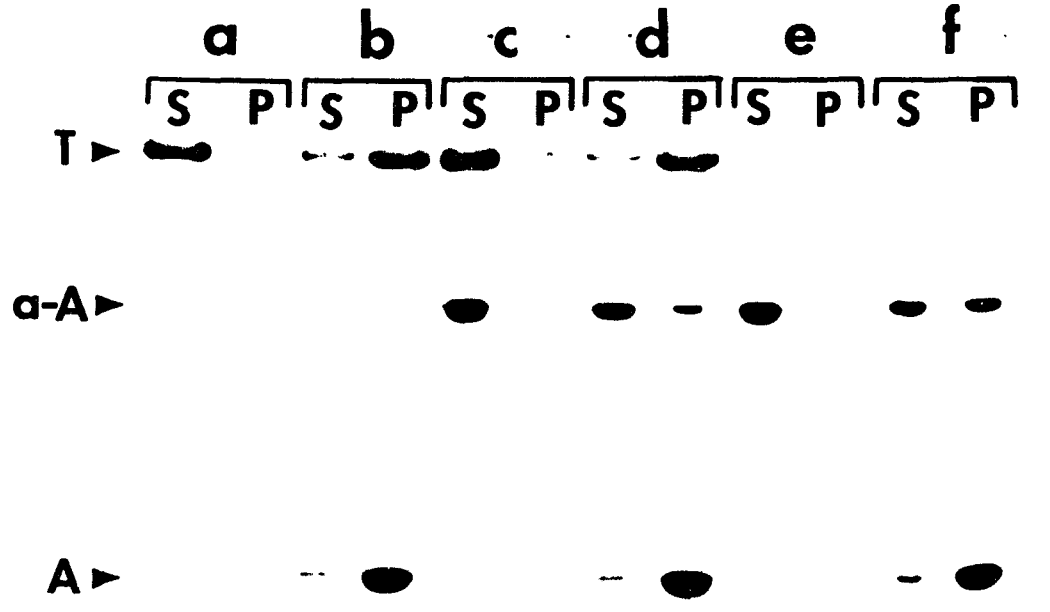
Fig. 5. Effect of α-actinin on the ability of talin to cosediment with F-actin at pH 6.4. Talin (0.2 mg/ml final concentration) and/or α-actinin (0.1 mg/ml final concentration) were mixed with or without G-actin (0.1 mg/ml final concentration) in Buffer A. Magnesium chloride was added (2 mM final concentration) to all samples, which were incubated for 1 hr and centrifuged at 100,000 x *g* for 20 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. *Panel a*, talin alone; *panel b*, talin and actin; *panel c*, talin and α-actinin; *panel d*, talin, α-actinin, and actin; *panel e*, α-actinin alone; *panel f*, α-actinin and actin. T =talin; a-A =α-actinin; A = actin.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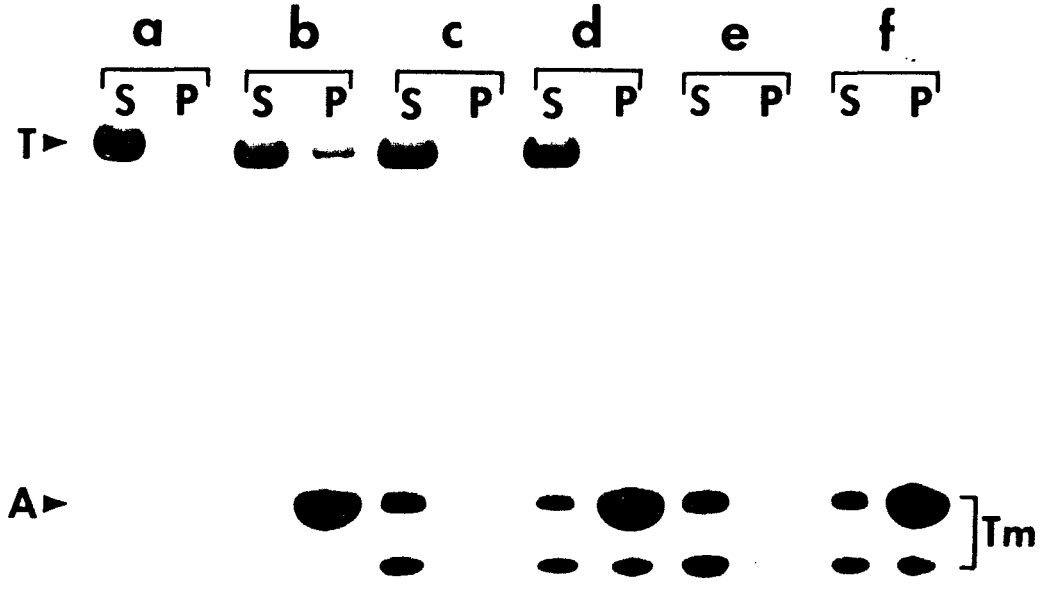
protein completely cosedimented with actin, it was suggested that talin and α-actinin may both be able to bind to actin filaments, but at different sites (42). Our results from cosedimentation studies with talin, α-actinin, and actin are shown in Fig. 5. Comparing *panel b* (talin/actin) with *panel d* (talin/α-actinin/actin) shows that under these conditions the amount of talin which cosedimented with F-actin was not affected by the presence of α-actinin. However, talin at a molar ratio of ~ 1 talin to 3 actin monomers noticeably decreased the amount of α-actinin that cosedimented with actin (*panel d*) compared to α-actinin/actin mixtures without talin (*panel f*).

The Effect of Tropomyosin on the Ability of Talin to Cosediment with Actin Filaments - Tropomyosin is generally believed to be associated with actin filaments throughout the cell (23, 24). It is, therefore, likely that an interaction between talin and actin filaments *in vivo* would involve tropomyosin-coated actin filaments. Unfortunately, at least for *in vitro* experiments, it was rather difficult to find ideal conditions to test the effect of tropomyosin on the talin-actin interaction because the talin-actin interaction is favored under conditions of low ionic strength (5, 6), but the tropomyosin-actin interaction is favored under conditions of higher ionic strength (43). Tropomyosin also becomes very viscous under conditions of low ionic strength, and it loses its ability to interact with actin (44). In order for both proteins to interact with actin we have chosen conditions of pH 6.4 and 25 mM NaCl. To help avoid extensive self-association, tropomyosin was dialyzed into Buffer A containing 125 mM NaCl so that when it was added to the experimental mixture the final NaCl concentration was 25 mM. An equal amount of Buffer A containing 125 mM NaCl was added to samples not containing tropomyosin. The results obtained from cosedimentation experiments with talin, tropomyosin (~ 1 tropomyosin to 6.5 actin monomers), and actin are shown in Fig. 6. Under these conditions, the ability of talin to cosediment with F-actin was greatly reduced compared to experiments done at pH 6.4 without

Fig. 6. Effect of tropomyosin on the ability of talin to cosediment with F-actin at pH 6.4. Talin (0.2 mg/ml final concentration) and/or tropomyosin (0.12 mg/ml final concentration) were mixed with or without G-actin (0.5 mg/ml final concentration) in Buffer A containing 25 mM NaCl. Magnesium chloride was added (2 mM final concentration) to all samples which were incubated for 1 hr and centrifuged at 100,000 x *g* for 20 min. Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. *Panel a,* talin alone; *panel b*, talin and actin; *panel c,* talin and tropomyosin; *panel d*, talin, tropomyosin, and actin; *panel e*, tropomyosin alone; *panel f,* tropomyosin and actin. T = talin; A = actin; TM = tropomyosin (Note that tropomyosin migrates as two subunits, with the slower migrating species comigrating with actin).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NaCl added (compare Fig. 1, *panel a*, with Fig. 6, *panel b*), and only ~ 50 % of the tropomyosin cosedimented with F-actin (Fig. 6, *panel f*). The amount of talin that cosedimented with actin was slightly reduced when tropomyosin was also included in the sample (compare *panels b* and *d*). As measured by cosedimentation, these results indicated that tropomyosin decreased the extent of the talin-actin interaction under these conditions.

Discussion

Talin is thought to be important in the organization and attachment of actin filaments to the cell membrane at adhesion plaques because it can bind to both the integral membrane receptor integrin (2) and to actin filaments (3-5). We recently have shown that a variety of factors can influence the ability of talin to bind to actin including pH, ionic strength, protein molar ratio, and temperature *in vitro* (5, 6). It is likely that additional factors such as covalent modification, proteolytic degradation, and interactions with other adhesion plaque proteins also affect the talin-actin interaction *in vivo*. The first part of this study was focused on the effect of proteolytic degradation of talin by calpain. Several adhesion plaque proteins such as talin (33) and filamin (7) are good substrates for proteolytic degradation by calpain. It has been suggested that calpains may play an important role in platelet activation and cytoskeletal reorganization (31, 32) and, because talin also may be important in these two processes (31), degradation of talin into its 190 kDa fragment and 47 kDa fragment by calpain may have physiological significance. We have shown that the isolated 190 kDa fragment of talin did not bind well to actin filaments under conditions that favor the crosslinking of actin filaments into both networks and bundles by intact talin (pH 6.4, low ionic strength), and at pH 6.9 it was difficult to see any evidence for a 190 kDa fragment-actin interaction. However, the 190 kDa fragment apparently contains sites required for

crosslinking actin filaments into networks and bundles (i.e., the 190 kDa fragment has at least two actin binding sites or one actin binding site and the ability to function as a dimer) as observed herein in experiments done at a lower pH of 6.0. It has been reported that the 190 kDa fragment is in an elongated conformation regardless of the ionic strength, whereas the intact talin molecule is elongated in high ionic strength conditions, but has a more globular shape in low ionic strength conditions (45). Interestingly, the isoelectric point for intact talin has been reported as 6.7-6.8 (45), and the isoelectric points calculated from the cDNA-derived sequence of the 190 kDa talin fragment and of the 47 kDa talin fragment are 5.4 and 8.4, respectively (46). Our results show that removal of the basic 47 kDa fragment decreased the optimal pH for actin binding from 6.4 for intact talin to 6.0 for the 190 kDa fragment, which is closer to the isoelectric point of the 190 kDa fragment. It is plausible that altering the electrostatic environment by lowering the pH to 6.0 allows the 190 kDa fragment to adopt a conformation better able to bind actin and/or itself via self-association. The 47 kDa fragment may be important in modulating the ability of talin to interact with actin, possibly by stabilizing conformational changes in talin that favor an interaction with actin at pH of 6.4 and above. These results also suggest the possibility that proteolytic cleavage by calpain *in vivo* could disrupt the talin-actin interaction, which could be important in cytoskeletal reorganization.

The second part of this study examined the effects of the adhesion plaque proteins filamin, vinculin, α-actinin, and tropomyosin on the ability of talin to cosediment with actin filaments. Filamin is known to crosslink actin filaments (7, 8) under conditions which also favor a talin-actin interaction (5, 6). Filamin may also be able to directly link actin filaments to the membrane in some instances by interacting with integral membrane proteins (11). We have shown that filamin can decrease the amount of talin that binds to actin when relatively high molar ratios of both

proteins to actin (≥ 1:12) are used. However, at lower molar ratios (≤ 1:20) of talin and filamin to actin, both proteins could bind equally well in the presence or absence of the other. The precise microenvironment and extent of colocalization of filamin and talin at adhesion plaques is unknown, but these results indicate that both proteins may be able to bind to actin filaments at these sites *in vivo*.

Vinculin can bind to talin with a moderately high affinity as has been shown by a variety of methods (14-17). It seemed likely, therefore, that the talin-vinculin interaction would affect the talin-actin interaction. However, at low pH and low ionic strength, vinculin did not affect the talin-actin interaction to a noticeable extent, and the amount of vinculin cosedimenting with actin in the presence of talin was only slightly greater than controls in the absence of talin. Although these results were somewhat surprising, they agree with those of Goldmann et al. (47), who also observed little effect of vinculin on the talin-actin interaction using kinetic and thermodynamic studies. It is possible that the talin-vinculin interaction is much weaker at low ionic strength and pH 6.4 and/or that, under these conditions, actin is able to compete with vinculin for binding to talin. We also noted that there was no significant interaction between vinculin and actin under these conditions, which is in general agreement with results from earlier studies (20-22).

Alpha-actinin, like talin, has been shown to bind to both actin filaments (10, 25, 28) and to integrin (12), and it has been suggested that α-actinin and talin may serve a similar function of directly linking actin filaments to the membrane at adhesion plaques (13). When α-actinin and talin were both allowed to cosediment with F-actin at lower molar ratios, both proteins bound to actin to the same extent as controls of the proteins added individually (42, and our unpublished observations). However, as shown herein at a pH of 6.4 and low ionic strength, talin decreased the amount of α-actinin that cosedimented with actin when the talin to actin molar ratio was high

(~ 1:3, Fig. 5). It is interesting that *in vitro* the α-actinin-actin interaction is favored at a higher ionic strength (48), and lower temperatures (25), whereas the talin-actin interaction is favored at very low ionic strength, higher temperatures, and lower pH (5, 6). It is plausible that talin and α-actinin serve a similar overall function in linking actin filaments to the membrane, but that their ability to do so may be complementary, rather than redundant. One interaction may be dominant under one set of conditions, but the other interaction may become more important as cellular conditions change. It has been suggested by others (49), for instance, that talin may be involved in the early stages of actin-membrane attachment, and that α-actinin may subsequently be more important in stabilizing and strengthening the interactions between actin filaments and the membrane.

Tropomyosin is an important actin binding protein thought to bind to actin filaments throughout the cell (23, 24) and known to affect the interactions between actin and several other proteins (25-29). We have shown, under conditions that are suboptimal for the binding of either tropomyosin or talin to actin, that tropomyosin decreased, but did not eliminate, the amount of talin that bound to actin. However, under conditions that favor talin-actin interaction (pH 6.4 and very low ionic strength), tropomyosin did not bind well to actin. On the other hand, the higher ionic strength required for optimal tropomyosin-actin interaction resulted in greatly reduced talin-actin interaction even in the absence of tropomyosin. It is possible that *in vivo*, tropomyosin may interfere with the binding of talin along actin filaments, and thereby restrict the binding of talin towards the ends of actin filaments at adhesion plaques. We cannot rule out the possibility that there may be some situations in cells, however, where the talin-actin interaction would be favored over the binding of tropomyosin to actin.

Although we have found that some of the proteins tested did influence the ability of talin to

interact with actin, under our conditions none of the proteins completely eliminated the talin-actin interaction. The interactions among the proteins located at the adhesion plaque are complex and under the control of several factors possibly including covalent modifications, proteolysis, the presence of lipids, and the microenvironment of pH and other ionic conditions. This complexity may permit the adhesion plaque to respond to a variety of extracellular signals important in the control of cellular processes such as cell adhesion, motility, growth, and differentiation. Elucidating how these proteins affect each other *in vitro* should provide a better understanding of these important events *in vivo*.

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OVERALL SUMMARY

The major objectives of the work presented here were to examine the effects of selected factors on the ability of talin to interact with actin. In the first and second papers included in my dissertation, cosedimentation, low shear viscometry, and electron microscopy were used to determine the effect of pH, ionic strength, increasing molar ratios of talin to actin, temperature, and length of actin polymerization time on the talin-actin interaction. The major conclusions that can be drawn from the results of these experiments include the following:

(1) The procedure that I developed for purifying talin results in 5-8 mg of highly homogeneous talin/100 g starting muscle. This method was developed because previously published procedures [Collier and Wang, 1982a; Molony et al., 1987; Muguruma et al., 1990] for the purification of talin that were used in my preliminary studies resulted in protein of inconsistent purity, yield, and amount of proteolytic degradation. I made two major changes to the procedure of Muguruma et al. [1990] including (a) the replacement of the low ionic strength extract with one that contained 0.5 M NaCl, which resulted in an increased overall yield, and (b) a different sequence of column chromatography, which increased the final purity and decreased the amount of proteolysis during purification and storage.

(2) The talin-actin interaction was markedly dependent upon pH and the molar ratio of talin to actin. At low ionic strength, decreasing the pH in the range from 7.4 to 6.4 resulted in increased talin-actin cosedimentation, increased low shear viscosity of talin-actin mixtures, and increased crosslinking of actin filaments in the presence of talin as observed by electron microscopy. Increasing the molar ratio of talin to actin resulted in an increase in the extent of the interaction, and at pH 6.4 and low ionic strength talin could bind to actin at molar ratios as high as one talin to two actin monomers.

(3) The talin-actin interaction was very sensitive to ionic strength. Cosedimentation and low shear viscosity studies showed that, when the ionic strength of the interaction solution was increased by adding as little as 25 mM KCl, the talin-actin interaction was dramatically decreased, regardless of pH. However, there still was evidence for a slight talin-actin interaction when as much as 150 mM KCl was present in the interaction solution.

(4) The talin-actin interaction was affected by temperature. Talin increased the viscosity of F-actin solutions to a much greater extent as the temperature was increased from 4° to the more physiological-like temperature of 37°C at pH 6.9 and low ionic strength.

(5) The mode of the talin-actin interaction was not affected by the length of time that actin was polymerized. Within approximately five minutes of polymerization time, talin could cosediment with F-actin, increase the low shear viscosity of F-actin, and crosslink actin filaments as observed by electron microscopy at pH from 6.4 to 6.9 and low ionic strength. These results showed that smooth muscle talin did not measurably decrease the length of actin filaments formed in its presence even at short polymerization times, which is unlike the results reported for platelet talin [Collier and Wang, 1982b; Kaufmann et al., 1991; Goldmann et al., 1994].

(6) Talin was able to bind to and crosslink actin filaments that had been preformed in its absence, and the extent of binding and crosslinking was similar to that seen when actin was polymerized in the presence of talin at pH between 6.4 and 7.3 and low ionic strength. These results disagree with those of others who reported a reduced ability of talin to bind to preformed actin filaments [Collier and Wang, 1982b; Muguruma et al., 1992].

(7) The effects of pH and ionic strength on the talin-actin interaction were reversible. The ability of talin to cosediment with actin filaments was very responsive to subsequent changes in pH and ionic strength, and talin that had been bound to actin was released by an increase in pH or

ionic strength.

The major objectives of the third paper in my dissertation were to determine the effects of the adhesion plaque proteins filamin, vinculin, α-actinin, tropomyosin, and calpain (via its proteolytic degradation of talin) on the talin-actin interaction. The major conclusions obtained from the results in this paper were as follows:

(1) The ability of the 190 kDa talin fragment (obtained by proteolytic cleavage of talin with calpain) to bind to actin was markedly decreased when compared to intact talin under conditions favorable to the talin-actin interaction (pH 6.4 and 6.9, and low ionic strength). However, the 190 kDa fragment interacted strongly with F-actin at a lower pH of 6.0.

(2) Filamin decreased the amount of talin that cosedimented with F-actin at relatively high molar ratios (~ one filamin to twelve actin monomers), but not at lower molar ratios (≤ one filamin to twenty actin monomers) at pH 6.4 and low ionic strength.

(3) Vinculin did not appear to affect the ability of talin to cosediment with F-actin, but talin did slightly increase the amount of vinculin that cosedimented in talin/vinculin/F-actin mixtures at pH 6.4 and low ionic strength. No evidence was observed for a direct vinculin-actin interaction under these conditions.

(4) α-Actinin did not affect the ability of talin to cosediment with F-actin, but when talin was present at a molar ratio of one talin to three actin monomers, talin decreased the amount of α-actinin that cosedimented in talin/α-actinin/F-actin mixtures at pH 6.4 and low ionic strength.

(5) Tropomyosin decreased the amount of talin that cosedimented in talin/tropomyosin/F-actin mixtures, but did not eliminate the talin-actin interaction under conditions that were suboptimal for the binding of either talin or tropomyosin to F-actin.

The results presented in this dissertation consistently showed evidence for a direct interaction

between talin and actin that was sensitive to and dependent upon the ionic conditions and temperature. It also was shown that the primary actin binding activity for smooth muscle talin is crosslinking actin filaments. Although an ability to also increase the rate of polymerization was not ruled out, smooth muscle talin did not measurably decrease the length of actin filaments under any of the conditions studied, which was unlike the results reported for platelet talin [Collier and Wang, 1982b; Kaufmann et al., 1991; Goldmann et al., 1994]. It is possible that the different results obtained for smooth muscle [Muguruma et al., 1990; Schmidt et al., 1993; and other results presented in this dissertation] and platelet talin [Collier and Wang, 1982b; Kaufmann et al., 1991; Goldmann et al., 1994] were due to species- or tissue-specific differences in the two proteins, or that the platelet preparation is more prone to contamination with other actin binding proteins as suggested by Burridge et al. [1990].

Overall, these results support a role for talin in the organization and attachment of actin filaments at adhesion plaques. Talin has been localized to the appropriate area of the cell, and was shown to be present at the earliest stages of the plaque formation [Volberg et al., 1986; DePasquale and Izzard, 1991]. The studies in this dissertation have shown that talin could rapidly crosslink actin filaments into networks and bundles at pH between 6.4 and 7.0 and low ionic strength, but demonstrated limited actin binding at higher pH and ionic strength. The interaction between talin and actin also was shown herein to be very responsive to subsequent changes in the ionic conditions *in vitro*. It is possible that the talin-actin interaction also may be responsive to cellular signals *in vivo*, such as changes in pH, phosphorylation state, and lipid environment. An ability to modulate its interaction with actin in response to cellular signals would allow talin to play a potentially key role in first organizing actin filaments at newly forming adhesion plaques, then attaching the actin filaments to the cell membrane by binding to integrins, and finally in

releasing actin filaments from these sites when triggered by specific cell signals. My results also suggest that talin can bind to actin filaments in the presence of filamin, vinculin, α-actinin, and tropomyosin, proteins that also are located at adhesion plaques and that might reasonably be expected to affect the talin-actin interaction *in vivo*. Calpain cleavage of talin at adhesion plaques also may provide a way to regulate the talin-actin interaction *in vivo* because the 190 kDa talin fragment did not bind to actin as well as intact talin under identical conditions *in vitro*. These studies taken *in toto* have been important in defining the conditions that affect the talin-actin interaction, and have shown that the conditions employed for actin binding studies *in vitro* can be very important in determining the extent and mode of the interaction.

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