Abstract. Junctions formed by skeletal muscles where they adhere to tendons, called myotendinous junctions, are sites of tight adhesion and where forces generated by the cell are placed on the substratum. In this regard, myotendinous junctions and focal contacts of fibroblasts in vitro are analogues. Talin is a protein located at focal contacts that may be involved in force transmission from actin filaments to the plasma membrane. This study investigates whether talin is also found at myotendinous junctions. Protein separations on SDS polyacrylamide gels and immunolabeling procedures show that talin is present in skeletal muscle. Immunofluorescence microscopy using anti-talin indicates that talin is found concentrated at myotendinous junctions and in lesser amounts in periodic bands over nonjunctional regions. Electron microscopic immunolabeling shows talin is a component of the digitlike processes of muscle cells that extend into tendons at myotendinous junctions. These findings indicate that there may be similarities in the molecular composition of focal contacts and myotendinous junctions in addition to functional analogies.

The molecular composition of sites at which cultured cells adhere to extracellular surfaces has been investigated with the goal, in part, of identifying how cytoskeletal structures join the plasma membrane (e.g., Mangeat and Burridge, 1984a, for review). The picture that has emerged from these studies of focal contacts of cultured cells is that several proteins including α-actinin, talin, and vinculin are located at these sites and some of these proteins display biochemical behavior consistent with that expected of a link in an actin-to-membrane chain (see Mangeat and Burridge, 1984a, for review).

A caveat for interpreting these studies is that stress fibers may be uncommon structures in vivo (Burridge, 1981; Herman et al., 1981; Byers et al., 1984). Focal contacts would also be expected to occur infrequently in vivo. Indeed, fibroblasts, which are frequently used in studies of focal contacts, do not commonly display these cellular specializations during normal, rapid, locomotor behavior (Couchman and Rees, 1979). The appearance of the contacts in vitro correlates with cell adhesion to the substratum, loss of motility, and stress fiber formation (Willingham et al., 1977; Couchman and Rees, 1979). These observations were among those that led to a hypothesis suggesting that stress fiber and focal contact formation may be structural correlates of tight adhesion of a cell to the substratum and tension exerted by the cell on the substratum (Burridge, 1981).

A natural site of actin–membrane interaction that has been little investigated in studies of the molecular composition of the membrane–cytoskeleton is the junction between skeletal muscle cells and tendon collagen fibers called the myotendinous junction. Skeletal muscle adheres strongly to the substratum and places tension on the substratum at the myotendinous junction, and in this regard myotendinous junctions are analogues of focal contacts. Furthermore, electron microscopic observations have shown that thin filaments extend into a dense, subsarcomemmal meshwork at these junctions (e.g., Tidball, 1983, 1984; Tidball and Daniel, 1986; Trotter et al., 1981, 1983), which suggests that the myotendinous junction may be an important site of actin–membrane interaction.

This investigation examines the possibility that talin, a protein that may be a component of actin–membrane interactions at focal contacts (Burridge and Connell, 1983a,b), is a component of myotendinous junctions. The possibility that talin is found subjacent to nonjunctional membrane is also investigated with the goal of contrasting junctional and nonjunctional sites of muscle membranes.

Materials and Methods

Electrophoresis and Immunoblotting

Extracts of adult chicken skeletal muscle were prepared by homogenizing the muscle in 10 vol of water plus the protease inhibitors: 10 μg/ml α-p-tosyl-L-arginine methyl ester–HCl, 10 μg/ml benzamidine, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 100 μM phenylmethylsulfonyl fluoride, 1 mM EGTA, and 10 μg/ml leupeptin. The extract was boiled with SDS-containing sample buffer, centrifuged to remove large fragments that had not dissolved, and electrophoresed on SDS-polyacrylamide gels (Laemmli, 1970). Gels contained 10% acrylamide and 0.13% bisacrylamide. Some gels were stained with Coomassie Blue while others, loaded identically, were transferred to nitrocellulose by electrophoresis (Burnette, 1981). The nitrocellulose sheets were then washed 1 h in 50 mM Tris buffer at pH 7.5 containing 150 mM NaCl and 0.1% NaN₃ (buffer A) to which 3% BSA, 0.2% gelatin, and 0.05% Tween-20 were added. The nitrocellulose sheets were then incubated 90 min at room temperature in anti-talin serum diluted 1:2,000 in...
0.2% gelatin, 0.05% Tween-20 and 5% vol/vol inactivated horse serum in buffer A. After extensive washing in 0.2% gelatin and 0.05% Tween in buffer A, the nitrocellulose sheets were incubated for 1 h at room temperature in 125I-affinity purified goat anti-rabbit IgG. This antibody was iodinated with iodine-125 using the chloramine T method (Hunter and Greenwood, 1982). Nitrocellulose sheets were incubated with a solution containing 1 × 10⁶ cpm/ml and 2% hemoglobin, 0.2% gelatin, and 0.05% Tween in buffer A, washed, air-dried, and exposed for autoradiography.

**Antisera**

Rabbit antisera prepared against either talin or its 190-kD proteolytic fragment (O'Halloran and Burridge, 1986) were used in the study. Affinity-purified antibodies were prepared by adsorption of the antisera on a column of talin immobilized on agarose and eluted with 0.1 M glycine-HCl (pH 2.3) to release bound immunoglobulin. This was neutralized and dialyzed against buffer A.

Affinity-purified antibodies against α-actinin were prepared by coupling chicken gizzard α-actinin (Fernamisco and Burridge, 1980) to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) activated with cyanogen bromide (Cuatrecasas et al., 1968). A rabbit antisera raised against chicken gizzard α-actinin was passed over the column, which was washed extensively with 150 mM NaCl, 0.1% NaN₃, and 50 mM Tris-Cl (pH 7.6) until the absorbance of the eluate returned to baseline. The antibody was then eluted with 0.1 M glycine-HCl (pH 2.3). This was rapidly neutralized with 1 M Tris-Cl (pH 9.0). After addition of BSA to 1 mg/ml, the antibody was dialyzed against 150 mM NaCl, 0.1% NaN₃, and 50 mM Tris-Cl (pH 7.6) and stored at 4°C until used.

**Preparation of Cells for Immunolabeling**

The plantaris muscles of adult chickens were exposed by dissection and while in situ were tied to an applicator stick while the muscle was at physiological length. The muscles were then removed and immersed for 10 min in 2.7% formalin in 15 mM sodium phosphate containing 10% sucrose, 2 mM CaCl₂, and 0.01 mM sodium azide at pH 7.1. The muscles were immersed next in the phosphate buffer alone and single cells were dissected out using needles and fine forceps. The myotendinous junctions at the insertion end of the cells were kept intact.

The cells were then washed for 10 min in buffer A, permeabilized in 0.5% Triton-X in buffer A for 15 min, and rinsed for 30 min in buffer A. The cells were then incubated at room temperature in one of the following solutions for 45 min if prepared for light microscopy or 5 h if for electron microscopy: affinity-purified, anti-talin (1:200 dilution); buffer A; preabsorbed talin; preimmune serum drawn from the animal before it was used for antibody production; or affinity-purified-anti-α-actinin. After incubation, the cells were rinsed in buffer A for 15 min and then buffer A containing 0.05% Tween, 0.2% gelatin, and 0.3% BSA for 1 h. Ferritin-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) was diluted 1:200 with buffer A containing 2.5% BSA and 5% horse serum that had been inactivated by heating to 56°C for 30 min. This second antibody had been pretreated with minced chicken tendon for 15 min with agitation and the tendon pieces were removed by centrifugation in a microcentrifuge for 45 s.

Cells for electron microscopic study were then incubated in this second antibody for 12 h at 4°C. Cells for light microscopic study were incubated in fluorescein isothiocyanate (FITC)—conjugated goat anti-rabbit IgG diluted 1:200 in buffer A for 1 h at room temperature.

The cells were washed after incubation in the second antibody with buffer A for 5 h (electron microscopy) or 1 h (light microscopy) at 4°C and then briefly rinsed in water. Cells to be studied by fluorescence microscopy were then mounted in water miscible mounting medium (Aquamount, Lerner Laboratories, New Haven, CT) and viewed on a Leitz Ortholux microscope equipped with epifluorescent optics.

After staining by the above method, cells for electron microscopy were fixed for 15 min in 1.4% glutaraldehyde in 0.1 M sodium cacodylate with 4 mM CaCl₂ at pH 7.20, rinsed in the cacodylate buffer, fixed for 10 min in 1% OsO₄, and then rinsed in buffer. These cells were dehydrated using a graded series of ethanols and embedded in epoxy resin. The cells were longitudinally sectioned at 0.5-μm thickness and viewed in a Siemens 101 electron microscope.

Other plantaris muscle samples were dissected into small bundles of cells and then dehydrated and embedded in paraffin. Longitudinal sections of the junctional regions of cells were cut at ~12-μm thickness, dehydrated, and hydrated. The sections were then immunolabeled by the procedure described above for single cell labeling for light microscopy.

**Grain Counting at Myotendinous Junctions**

Electron micrographs were made of the junctional region of treated cells at a magnification of 20,000. Fields to be photographed were identified at a magnification of 8,000 so that ferritin grains could not be distinguished before field selection. The first field viewed at the end of each cell that displayed digitilike processes characteristic of myotendinous junctions and free from dissection and sectioning artifacts was photographed.

Micrographs were printed at a magnification of 76,000. The number of grains overlaying digitilike extensions of the cell were counted for each micrograph. The area of each process was measured on a digitizing tablet interfaced with an IBM-PC equipped with a digitizing program (Bioquant, Nashville, TN). Grain counts are expressed as grains/area of process. Four to six sections were analyzed for each cell evaluated and the average grain density for each cell calculated. Multiple sections were analyzed to minimize variability in grain counts attributable to variability in section thickness.

**Grain Counting at Non-Junctional Regions**

Electron micrographs of nonjunctional regions of the plasma membrane were also prepared at a magnification of 76,000. Regions photographed were selected by criteria identical to that used to select junctional regions. The number of grains lying between the most peripheral myofibril and the surface of the cells were counted, the length of membrane in each analyzed micrograph, and sarcomere length were measured on the digitizing tablet. Data are expressed in grains/sarcomere length of membrane. Four to six sections from each cell were analyzed.

*Abbreviation used in this paper: FITC, fluorescein isothiocyanate.*

**Figures 1-5.** (Fig. 1) Coomassie Blue-stained SDS-polyacrylamide gel is shown with standard proteins (lane 1) adjacent to an extract of skeletal muscle (lane 2). The autoradiograph of a gel slice equivalent to lane 2. transferred to nitrocellulose and incubated with anti-talin and 125I-goat anti-rabbit IgG is shown in lane 2'. Anti-talin sera specifically binds two proteins with molecular masses corresponding to sarcomeric myosin (200 kD), β-galactosidase (130 kD), phosphorylase (95 kD), BSA (68 kD), ovalbumin (45 kD), and carbonic anhydrase (30 kD). (Fig. 2) Whole mount of single skeletal muscle cell from chicken plantaris. The tapering end of the cell is where it forms a junction with tendon collagen fibers. (a) A phase micrograph reveals the interface (arrows) between the cell on the left at connective tissue on the right. (b) Immunofluorescence of the same, anti-talin treated cell as shown in Fig. 4 a shows talin located at the junction. Bar, 40 μm.
Results

Immunoblots of chicken skeletal muscle probed with anti-talin sera reveal bands of apparent molecular masses of 225,000 and 190,000 D (Fig. 1). Chicken gizzard smooth muscle talin also has an apparent molecular mass of 225,000 D (Maloney et al., 1986) and has a proteolytic fragment of 190,000 D (O'Halloran and Burridge, 1986). The molecular masses of proteins in the immunoblots were confirmed in similar immunoblots in which purified chicken gizzard talin and the 190,000-D proteolytic fragment of talin were electrophoresed adjacent to experimental samples (data not shown). Chicken gizzard talin and its 190,000-D proteolytic fragment migrated identically with the two immunoreactive proteins of chicken skeletal muscle.

Single skeletal muscle cells dissected from chicken plantaris muscle show bright fluorescence at the myotendinous junction in permeabilized cells treated with affinity-purified, anti-talin (Fig. 2). These same cells show slight periodic bands of fluorescence at nonjunctional sites at the cell's surface when treated with anti-talin (Figs. 2 and 3). This periodic labeling was prominent at the cell margin but absent from deep regions of the cell when focused at that level. Control cells treated with preimmune serum or with anti-talin that had first been absorbed with purified talin and then with the second antibody displayed a slight fluorescence over the entire cell surface but showed no bright fluorescence of the junction and no periodic bands of fluorescence along the nonjunctional regions of the cell (Fig. 4). Cells prepared by the same procedure but treated with only the fluoresceinated second antibody displayed minimal fluorescence (data not shown).

Paraffin sections of small bundles of plantaris cells also show fluorescence at myotendinous junctions in cells treated with affinity-purified anti-talin (Fig. 5). Faint periodic bands of fluorescence were again observed in these preparations where the plane of section passed through nonjunctional sarcolemma (Fig. 6). Both whole cells and paraffin sections of muscle treated with antibodies to the 190-kD proteolytic fragment of talin produced identical labeling. Control sections displayed negligible fluorescence (Fig. 7).

Electron microscopic observations of single skeletal muscle cells dissected free from surrounding connective tissue show that the normal morphology of the myotendinous junction remains intact in these preparations (Fig. 8). Cells prepared by this technique and treated with affinity-purified, anti-talin and ferritin-conjugated goat anti-rabbit immunoglobulin show ferritin grains over the digitlike extensions of the cell at the junction (Fig. 9) and in lesser numbers over nonjunctional regions of the cell (Fig. 10). Grain counts over these regions were performed on unstained sections where cell boundaries could be distinguished and ferritin grains appeared in sharp contrast. Immunolabeling procedures using affinity-purified anti-α-actinin as a positive control for antibody penetration and labeling of internal structures show labeling of Z-discs and very little, nonspecific labeling at the cell surface (Fig. 11). This observation and the absence of intracellular labeling in paraffin sections indicate that the localization of talin near the cell surface in whole cell preparations is not an artifact of poor penetration by reagents.

Grain counts over the digitlike extensions of the cell at the myotendinous junction show significant labeling for anti-talin treated cells (778 grains/μm²; SD, 298; n = 10) (Fig. 12) when compared with cells treated with buffer (182 grains/μm²; SD, 156; n = 5), preimmune serum (226 grains/μm²; SD, 87; n = 4) or preabsorbed antitalin (357 grains/μm²; SD, 150; n = 5) in place of the antibody (Figs. 13 and 14).

Grain counts over nonjunctional regions of the cell surface show no significant increase in the number of grains/unit sarcomere length in anti-talin treated (153 grains/μm; SD, 47; n = 5) cells than in any of the three types of control preparations (buffer only: 131 grains/μm; SD, 74; n = 5; preimmune serum: 199 grains/μm; SD, 207; n = 5; preabsorbed antitalin: 199 grains/μm; SD, 142; n = 5) (Fig. 15).

Discussion

The molecular composition of sites for actin-membrane interaction has been most extensively studied in erythrocyte membrane cytoskeleton (see Branton et al., 1981; Bennett, 1982; and Goodman and Shiffer, 1983, for reviews), intestinal brush border (e.g., Bretsch, 1983; Moosker et al., 1984) and focal contacts of cultured cells (see Mangeat and Burridge, 1984a, for review). The protein composition of each of these sites of actin-membrane interaction is apparently unique. Associations between thin filaments and plasma membrane are mediated by ankyrin and spectrin in erythrocytes (e.g., Branton et al., 1981; Goodman and Shiffer, 1983) and by a 110-kD protein in microvilli of the brush border (Matsudaira and Burgess, 1979). Focal contacts do not appear to contain more spectrin or spectrin-like proteins than do nonjunctional regions of cultured cells (Mangeat and Burridge, 1984a). Furthermore, spectrin precipitation in cells possessing focal contacts does not affect stress fiber appearance or cell shape and movement (Mangeat and Burridge, 1984b). Spectrin, however, is essential in determining normal red cell shape and mechanical behavior (e.g., Greenquist et al., 1978; Lux, 1979; Agre et al., 1982; Waugh, 1983).

Proteins that are prominently localized at focal contacts include talin (Burridge and Connell, 1983b), α-actinin (Larabides and Burridge, 1975), and vinculin (Geiger, 1979; Geiger et al., 1980). All three proteins have also been identified in neuromuscular junctions (Bloch and Hall, 1983; Sealock et al., 1986). The present investigation shows that talin is also a component of myotendinous junctions, whereas previous studies have suggested that α-actinin (Trotter et al., 1983) and vinculin (Bloch and Bloch, 1985) may also be constituents of myotendinous junctions. Focal contacts and myotendinous junctions are thereby analogous not only in being sites where cells adhere tightly to and exert force upon extracellular structures, but they are analogous in their protein composition, at least to some degree. The localization of talin and vinculin at myotendinous junctions is consistent with the correlation noted by Geiger et al. (1985) that talin and vinculin occur at sites of cell-substratum adhesions, whereas vinculin occurs without talin at cell-cell adhesions.

Previous studies have shown that vinculin (Pardo et al., 1983) and spectrin (Repasky et al., 1982; Menold and Repasky, 1984) occur in periodic structures, called costameres, along the length of skeletal muscle. The immunofluorescence studies reported here show that talin also has a periodic distribution although whether its distribution coincides
Figures 6-9. (Fig. 6) Paraffin section of skeletal muscle fibers. (a) Phase micrograph shows sarcomere spacing at nonjunctional region of the cell. (b) Fluorescence micrograph of same section treated with anti-talin and FITC-conjugated second antibody displays periodic bands of fluorescence that correspond to sarcomere spacing at sites where the plane of section passes through the sarcolemma. Bar, 35 μm. (Fig. 7) Paraffin section of a single skeletal muscle cell with tendon connective tissue still attached. (a) Phase micrograph reveals interface between muscle cell and tendon at myotendinous junction (arrows). (b) Fluorescence micrograph of same section treated with anti-talin pre-absorbed with purified talin and FITC-conjugated second antibody. Film, f/stop, exposure time, film development, and printing condition of Figs. 5 b and 7 b were identical. Bar, 40 μm. (Fig. 8) Electron micrograph of a longitudinal section through a myotendinous junction. This cell was dissected free from connective tissue and surrounding cells. Several of the digitlike processes characteristic of the junction are indicated by arrows. Bar, 2.1 μm. (Fig. 9) Electron micrograph of two digitlike processes at a myotendinous junction. The cell was treated with anti-talin and a ferritin-conjugated second antibody and stained with uranyl acetate and lead citrate. Many ferritin grains overlie the two processes that are outlined by arrows. Bar, 250 nm.

with that of vinculin and spectrin has not been demonstrated. However, the anti-talin–treated fibers prepared for electron microscopy showed no more ferritin labeling at nonjunctional sites than buffer, preimmune sera, or preabsorbed antibody treated cells. This indicates that although specific binding to talin may occur in our immunoelectron microscopic preparations of nonjunctional membrane, levels of specific labeling cannot be distinguished from background levels of nonspecific binding. Thus, at the present time, electron microscopic labeling provides no information on the na-
Figures 10–13. (Fig. 10) Electron micrograph of nonjunctional region of muscle cell. The cell was treated with antititin and a ferritin-conjugated second antibody. This cell showed the mean number of ferritin grains ± 1 SD. Note that nearly all grains lie at the cell surface (between arrows). The intracellular region lies to the left in the micrograph. Statistical analysis of all preparations showed no significant increase in the number of ferritin grains at the nonjunctional, cell surface in experimental vs. control preparations. Thus, the sites of specific and nonspecific labeling cannot be distinguished at nonjunctional regions by our electron microscopic immunolabeling. Bar, 600 nm. (Fig. 11) Electron micrograph of a section from a cell treated with anti-α-actinin and ferritin-conjugated second antibody and stained with uranyl acetate and lead citrate. Note that ferritin grains overlie Z-discs (arrows) and that few grains are found at the cell surface. Bar, 750 nm. (Fig. 12) Electron micrograph of myotendinous junction digitlike processes. This cell was treated with anti-titin and ferritin-conjugated second antibody but otherwise unstained. This sort of micrograph enlarged to a total magnification of 76,000 was used for grain counts. This cell has a density of ferritin grains averaged over five sections of 556 grains/μm². This value is lower than the mean for all anti-titin-treated cells (778/μm²; SD, 298). Bar, 600 nm. (Fig. 13) Electron micrographs of a digitlike process of a myotendinous junction. This control cell was treated with buffer and then ferritin-conjugated second antibody. This control has a density of ferritin grains averaged over five sections of 289 grains/μm². This value is higher, but within 1 SD of the mean for all buffer-only treated controls (82 grains/μm²; SD = 156). Bar, 600 nm.
ture of the periodic distribution of talin observed by immunofluorescence. We interpret this to mean that talin occurs at these sites at levels too low to be resolved by our electron microscopic immunolabeling technique and conclude that the myotendinous junction is the site at which talin is most concentrated in the cell.

The functions and relationships of α-actinin, vinculin, and talin to other proteins of focal contacts, neuromuscular junctions and myotendinous junctions are not understood completely. In vitro studies have demonstrated the ability of muscle or nonmuscle α-actinin to crosslink actin filaments to neighboring actin filaments (Podlubnaya et al., 1975; Jockusch and Isenberg, 1981; Burridge and Feramisco, 1981). Electron microscopic observations have shown thin filaments extending into a dense, apparently crossbrided matrix subjacent to the myotendinous junction sarcolemma (Tidball, 1983, 1984; Tidball and Daniel, 1986; Trotter et al., 1981, 1983). Trotter and co-workers have proposed (1983) that α-actinin could be a component of that subsarcolemmal matrix.

Biochemical studies using purified proteins have recently identified binding between talin and two other proteins previously localized to focal contacts. Vinculin and talin have been shown to interact with relatively high affinity (Burridge and Mangeat, 1984). Binding between talin and a 140-kD fibronectin receptor has also been demonstrated (Horwitz et al., 1986). This receptor, a complex of glycoproteins with subunits of ~140-kD, has been localized to focal contacts (Chen et al., 1985a, b; Damsky et al., 1985) in smooth muscle (Chen et al., 1985b) and at the ends of myotubes in vitro (Damsky et al., 1985). The complex also serves as a receptor for laminin (Horwitz et al., 1985). The identification of talin in the myotendinous junction may suggest that it is interacting with an equivalent transmembrane component at this location. How the vinculin–talin complex interacts with actin filaments remains elusive. Originally, evidence was presented for a direct interaction between vinculin and actin but more recent work has shown that this apparent binding is due to a contaminant in vinculin preparations (Evans et al., 1984). If further work confirms that vinculin and talin are components of a chain of attachment between bundles of actin filaments and specialized regions of the plasma membrane, then an additional component(s) may remain to be identified.

Data on the mechanical loading of myotendinous junctions and focal contacts permit a comparison of the mechanical environment of these sites enriched in talin, vinculin, and α-actinin. The stress placed on myotendinous junctions during maximum isometric tension is ~2 × 10^4 N/m2 (Tidball, 1984; 1985; Tidball and Daniel, 1986). Essentially all of this loading is seen as shear stress at the junction. Fibroblasts in vitro exert shear stresses that exceed 10^-2 N/m of advancing cellular margin (Harris et al., 1980). Adhesion of any locomoting fibroblast occurs along a band 0.5 – 25 μm behind the advancing margin of the cell (Harris et al., 1980). A typical fibroblast ~15 μm wide adhering to the substratum along a 15-μm × 1-μm band (see micrographs in Harris et al., 1980; and Vasiliev and Gelfand, 1973) therefore loads its adhering membrane with shear stresses on the order of 10^4 N/m2. Fibroblasts therefore load their adhesive membrane with stresses similar in magnitude and type to those occurring at myotendinous junctions. However, the measurements of force placed on the substratum by fibroblasts used locomoting cells rather than cells with focal contacts (Harris et al., 1980). The same study showed that adherent cells possessing focal contacts also exert force on the substratum although its magnitude was not measured.

The similarities in molecular composition and mechanical behavior of myotendinous junctions and focal contacts suggest that this correlation may have functional significance. This correlation supports an earlier hypothesis (Burridge and Mangeat, 1984) that talin may be a force transmitting protein.

We thank Dr. Mary Beckerle for generous gifts of anti-sera to talin and Ms. Michelle Gadus for skilled technical assistance.

This investigation was supported by grants from the Academic Senate of the University of California and from the National Science Foundation (DCB-841785) to J. G. Tidball and from the National Institutes of Health (GM-29860) and Muscular Dystrophy Association to K. Burridge.
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