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Talin Requires β-Integrin, but not Vinculin, for Its Assembly into Focal Adhesion-like Structures in the Nematode *Caenorhabditis elegans*

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In cultured cells, the 230-kDa protein talin is found at discrete plasma membrane foci known as focal adhesions, sites that anchor the intracellular actin cytoskeleton to the extracellular matrix. The regulated assembly of focal adhesions influences the direction of cell migrations or the reorientation of cell shapes. Biochemical studies of talin have shown that it binds to the proteins integrin, vinculin, and actin in vitro. To understand the function of talin in vivo and to correlate its in vitro and in vivo biochemical properties, various genetic approaches have been adopted. With the intention of using genetics in the study of talin, we identified a homologue to mouse talin in a genetic model system, the nematode *Caenorhabditis elegans*. *C. elegans* talin is 39% identical and 59% similar to mouse talin. In wild-type adult *C. elegans*, talin colocalizes with integrin, vinculin, and α-actinin in the focal adhesion-like structures found in the body-wall muscle. By examining the organization of talin in two different *C. elegans* mutant strains that do not make either β-integrin or vinculin, we were able to determine that talin does not require vinculin for its initial organization at the membrane, but that it depends critically on the presence of integrin for its initial assembly at membrane foci.

INTRODUCTION

The protein talin is found at sites in which the actin cytoskeleton is linked via integral membrane proteins to the extracellular matrix (ECM) (Burridge and Connell, 1983). In cultured cells such sites are known as focal adhesions. Several other proteins are found along with talin at focal adhesions, including vinculin (Geiger, 1979; Burridge and Feramisco, 1980) and the integral membrane protein integrin (for review, Buck and Horwitz, 1987; Hynes, 1987). As measured in vitro, each of the proteins in a focal adhesion has multiple, interdependent interactions, the nature of which may change as each is assembled into a functional structure (Niggli and Gimona, 1993). For example, the cytoplasmic side of β-integrin interacts with both talin (Horwitz *et al.*, 1986) and another focal adhesion protein α-actinin (Otey *et al.*, 1989). Talin interacts with integrin, vinculin (Burridge and Mangeat, 1984), actin (Muguruma *et al.*, 1990; Kaufmann *et al.*, 1991), and focal adhesion kinase (FAK; Chen *et al.*, 1995). Vinculin binds to talin, α-actinin (Wachsstock *et al.*, 1987), and actin (Menkel *et al.*, 1994; Johnson and Craig, 1995). Further, these interactions may be facilitated by allosteric changes in protein conformation; the interaction between talin and vinculin and between vinculin and actin may depend on a change in the structure of vinculin to unmask the appropriate binding sites (Johnson and Craig, 1994, 1995). With this biochemical complexity it is difficult to assess the relative importance of any given binary interaction in vivo.

The presence of talin at focal adhesions is strictly regulated. In nonadhesive-resting platelets, talin is found diffusely distributed in the cytoplasm. Upon activation and attachment of platelets to a substratum,

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Note: The assembled DNA sequence for the talin cDNA is available from GenBank under accession number L46861.

talin is seen to accumulate at the plasma membrane (Beckerle *et al.*, 1989). The recruitment of talin to such membrane sites in vivo may involve binding to integrin. For example, integrin and talin coaggregate at sites where cells form attachments to a fibronectin substrate (Mueller *et al.*, 1989). Integrin and talin also coaggregate at antibody-induced integrin caps, but only when integrin has been activated by phorbol esters (Burn *et al.*, 1988; Kupfer *et al.*, 1990). The linkage of talin to integrin may be regulated by phosphorylation of integrin and talin. Phosphorylation of integrin reduces its affinity for talin in vitro (Buck and Horwitz, 1987; Tapley *et al.*, 1989), and agents that increase phosphorylation of talin in cultured cells are generally associated with focal adhesion disassembly (Pasquale *et al.*, 1986; DeClue and Martin, 1987; Turner *et al.*, 1989; Beckerle, 1990; Qwarnström *et al.*, 1991), with the exception of one cell type in which decreased phosphorylation was associated with focal adhesion disassembly (Turner *et al.*, 1989).

Based on the observed biochemical interaction between talin and vinculin in vitro, one step in the assembly of a focal adhesion may be the recruitment of vinculin to the talin–integrin complex. This notion is supported by the appearance of talin before vinculin in the precursors of focal adhesions (DePasquale and Izzard, 1991). A similar sequence was also observed in nascent membrane-bound dense plaques within developing vertebrate smooth muscle; talin was detected several days before the appearance of vinculin (Volberg *et al.*, 1986). Interestingly, this sequence is reversed during focal adhesion disassembly, which can be induced by infecting cells with Rous sarcoma virus (Brands *et al.*, 1990). In such infected cells, vinculin disassociates rapidly from the cell membrane, well before any observed changes in the organization of talin and well before the disappearance of microfilament bundles.

The three studies cited above document apparently functional actin–membrane linkages that contain talin but not vinculin in either nascent structures or during the induced disassembly of mature structures. Further evidence suggesting a more complex functional relationship between talin and vinculin comes from the study of developing avian neural crest cells. Both the ratio and organization of vinculin and talin change differentially as these cells migrate to their embryonic targets (Duband and Thiery, 1990). The cumulative evidence suggests that 1) vinculin is not necessary for linkage of actin to the membrane in some situations, and 2) that talin can associate with the membrane in the absence of vinculin. A significant objection to these two conclusions, however, is that one cannot be certain that the structures observed are completely devoid of vinculin; functional levels of vinculin may exist below the level of detection of the protein. Nonetheless, these data challenge the simple model whereby talin and vinculin are associated in a one-to-one complex that functions as a chain of proteins linking actin to the plasma membrane.

Structures analogous to vertebrate focal adhesions are found in the body-wall muscle of the nematode *Caenorhabditis elegans* (Figure 1). These so-called dense-body structures, like the focal adhesion plaques of vertebrate nonmuscle cells and the dense plaques of smooth muscle, contain the proteins actin (Rosenbluth *et al.*, 1965), α-actinin (Francis and Waterston, 1985; Barstead *et al.*, 1991), vinculin (Barstead and Waterston, 1989), and β-integrin (Francis and Waterston, 1985; Gettner *et al.*, 1995). To exploit the strengths of the *C. elegans* model system, we have undertaken a genetic approach to the study of these proteins. Our genetic studies of vinculin in *C. elegans* have confirmed that vinculin is essential for the formation of the dense body (Barstead and Waterston, 1991). Mutations that eliminate vinculin from the *C. elegans* body-wall muscle cause complete muscle paralysis and prevent the formation of normal, functional actin–membrane linkages. Others have shown that β-integrin is essential for these same processes (Williams and Waterston, 1994; Gettner *et al.*, 1995). Further, when Coutu-Hresko *et al.* (1994) examined the distribution of normal vinculin in mutant animals that lacked integrin, they found that vinculin requires integrin for its membrane localization. Conversely, integrin seemed normally organized in mutant animals that lacked vinculin, suggesting that integrin does not require vinculin for its localization to membrane sites (Coutu-Hresko *et al.*, 1994).

Here we report the identification of a homologue to vertebrate talin in the nematode *C. elegans*. *C. elegans* talin is very similar to the known sequence of mouse talin (Rees *et al.*, 1990). Our localization of this homologue to the dense bodies in the body-wall muscle further emphasize the relationship of these *C. elegans* structures to vertebrate adherens junctions. We have examined the organization of this talin homologue in *C. elegans* mutants that lack either vinculin or β-integrin. We conclude from our data that vinculin is not necessary for the localization of talin to the plasma membrane in the body-wall muscle of *C. elegans*, whereas integrin is critical for this event.

MATERIALS AND METHODS

C. elegans Strains

General methods for the growth and maintenance of nematode strains were as described in Brenner (1974). The N2 strain was used for the analysis of the wild-type organization of talin. The *C. elegans* vinculin mutant, *deb-1(st555)*, was isolated as described in Barstead and Waterston, 1991. The mutation in the β-integrin gene *pat-3(rh54)* (Gettner *et al.*, 1995) was generously provided by Dr. Edward Hedgcock (The Johns Hopkins University, Baltimore, MD), in whose laboratory this mutation was isolated. Mutations in both the vinculin and β-integrin genes are lethal when homozygous, and so they were maintained in combination with appropriate chromosomal

duplications. Animals with the following genotypes were used for this study: for the vinculin mutant, *deb-1(st555)unc-44(e362)IV;nDp5*; for the integrin mutant, *pat-3(rh54)ncl-1(e1865)dpy-1(e1)III;sDp3*. The DNA sequence alteration in *deb-1 (st555)* changes a splice acceptor site and leads to the elimination of all detectable vinculin in the mutant as assayed by immunofluorescence microscopy with anti-vinculin antibodies (Barstead and Waterston, 1991). The sequence alteration in *pat-3(rh54)* introduces a premature termination codon in the message (Edward Hedgcock, personal communication). This mutation leads to the elimination of all detectable β-integrin in the affected animals (Edward Hedgcock, personal communication; our unpublished results).

Recovery and Sequencing of Talin cDNAs

Our initial talin cDNA came from a serendipitous discovery by Michael Hengartner and Robert Horvitz. In cloning cDNA for an unrelated gene, these investigators recovered one artifactual chimeric cDNA, a portion of which showed sequence homology to mouse talin. On the basis of our previous work, we had hypothesized that the *C. elegans* dense body was analogous to vertebrate focal adhesions (Barstead and Waterston, 1991), and we therefore recognized immediately the importance of their discovery to our work. Hengartner and Horvitz kindly sent to us the putative talin cDNA. This initial cDNA was used as a probe to recover proper talin cDNAs from the cDNA library λSZAP-RB1 (Barstead and Waterston, 1989). We recovered a 2.4-kb cDNA that encompassed 25% of the full-length talin cDNA. To recover cDNAs from the 5′ end of the message, we constructed another cDNA library by using random hexamers for the first-strand synthesis. The entire coding sequence was cloned in four steps. Both strands of each cDNA were sequenced via the method of Sanger (Sanger *et al.*, 1977). Sequence assembly and analysis were done with either the Wisconsin Package computer software (G.C.G., 1994), or MACAW (Multiple Alignment Construction and Analysis Workbench), version 2.0.5 (Schuler, 1995). The assembled DNA sequence is available from GenBank under accession number L46861.

Antibody Production

To make protein for use as an immunogen, we inserted a talin cDNA encoding the C-terminal 20% of the protein into the *E. coli* plasmid vector p-Mal2c (New England Biolabs, Beverly, MA) so as to generate a translational fusion between the bacterial maltose-binding protein and *C. elegans* talin. This fusion protein was expressed and purified as described (Riggs, 1992).

Two New Zealand white rabbits were injected subcutaneously with 1 mg of this purified fusion protein in Freund's complete adjuvant (Sigma Chemical, St. Louis, MO). This initial injection was followed by subcutaneous boosts at 4, 8, 12, and 14 wk with 0.100 mg of fusion protein in Freund's incomplete adjuvant (Harlow and Lane, 1988). Test bleeds were done at 0, 6, 8, 12, 14, and 16 wk. Antibodies from the preimmune and immune sera were partially purified by ammonium sulfate fractionation (Harlow and Lane, 1988). Antibodies specific for *C. elegans* talin were then purified from the serum in a two-step process. The serum was first passed over a Sepharose column to which had been coupled a soluble protein extract from an *E. coli* strain that was induced to produce maltose-binding protein. In this way, antibodies directed at either general *E. coli* proteins or at the maltose-binding portion of the fusion protein

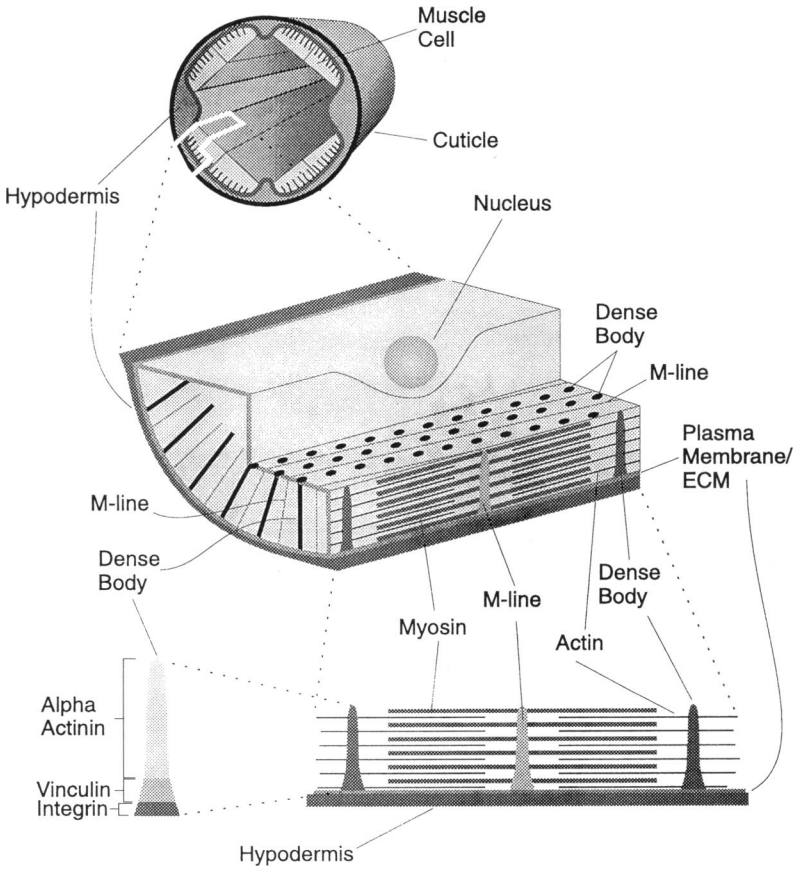

Figure 1. *C. elegans* muscle cell anatomy. A cross-section through the *C. elegans* body wall is shown at the top of the diagram. For clarity, the internal anatomy of the animal, including the gonad and gut, is not shown in this diagram. The body-wall muscle is located in four quadrants around the circumference of the animal. Within each quadrant are either 23 or 24 mononucleate muscle cells in two rows. In adult animals each muscle cell is ~100 84 μm long in the anterior to posterior dimension, or one-tenth the length of the animal. The muscle cells are associated, through a thin extracellular matrix, with an adjacent cell layer called the hypodermis. The hypodermis produces the flexible exoskeleton on the surface of the animal, known as the cuticle. The body-wall muscle cells are polarized such that all of the contractile filaments are located on the basal surface of the cell associated with the hypodermis. Within the muscle cells, dense bodies project from the plasma membrane into the cell. Actin filaments emanate from the dense bodies and interdigitate with myosin filaments. Myosin filaments are organized at their midpoint by a structure known as the M-line. As in vertebrate muscle, the interaction between the actin and myosin filaments generates a contractile force. This force is transmitted laterally to the plasma membrane through the dense bodies. Previous to this report, investigators had identified several dense-body proteins, including α-actinin (Francis and Waterston, 1985; Barstead *et al.*, 1991), vinculin (Barstead and Waterston, 1990), and integrin (Gettner *et al.*, 1995). The vertebrate homologues of these three proteins are found in structures known as focal adhesions.

were eliminated from the serum (our unpublished results). Anti-talin antibodies were then affinity purified from this adsorbed serum by passing it over a second column to which had been coupled the original purified talin-fusion protein. Antibodies that bound to this second column were eluted with a buffer containing 0.2 M glycine, pH 2.5. The pH of the eluate was quickly neutralized, and the purified antibody solution was then dialyzed against phosphate-buffered saline (PBS), pH 7.0. The purified antibody was stored at −20° in a 1:1 solution of PBS and glycerol.

Western Blots

SDS-soluble nematode protein was extracted as described in Moerman *et al.* (1988). Proteins were separated on SDS-polyacrylamide gels (Laemmli, 1970) and electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Keene, NH; Towbin *et al.*, 1979) for 1.5 h at 1 A constant current in a buffer containing 25 mM tris(hydroxymethyl)aminomethane (Tris), 192 mM glycine, 10% methanol, and 0.1% SDS. Transferred protein was detected by staining the membrane with a solution of 0.5% Ponceau S in 1% acetic acid and partially destaining in a 1% acetic acid solution. The membrane was then completely destained in TBS-T (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20). Before the addition of antibodies, the membrane was treated to block nonspecific antibody binding sites in a buffer containing the following: TBS-T, 10% normal goat serum, and 3% bovine serum albumin (BSA). Primary antibody incubations were done in this blocking buffer for 60 min at room temperature, followed by three 5-min washes in TBS-T. An alkaline phosphatase-conjugated secondary antibody (Tago, Burlingame, CA) was then added to the membrane in blocking solution. The bound alkaline-phosphatase secondary antibody was detected as described by Ey and Ashman (1986).

In Situ Localization

Staining of Embryonic Stages. Embryos were released from gravid adults by alkaline hypochlorite treatment (Sulston and Hodgkin, 1988) and prepared for immunofluorescence as described (Barstead and Waterston, 1991). The embryos were fixed for 10 min in 3% formaldehyde made from paraformaldehyde (Electron Microscopy Services, Ft. Washington, PA) and buffeted with 0.1 M sodium phosphate, pH 7.0, and 0.1 mM EDTA. After aldehyde fixation, embryos were collected by centrifugation and then post-fixed for 10 min in 100% methanol at −20°. The fixed embryos were then washed two times in PBS, pH 7.0, and once in PBS-T. Embryos were then resuspended in blocking buffer (PBS-T, 30% normal goat serum).

The following antibodies were used for this study: anti-β-integrin, MH25 (Francis and Waterston, 1985; Gettner *et al.*, 1995); anti-vinculin, MH24 (Francis and Waterston, 1985; Barstead and Waterston, 1989); and an antibody that recognizes the cell boundaries of the hypodermal cells, MH27− (Francis and Waterston, unpublished data). These three mouse monoclonal antibodies were detected in situ by using a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody (Chemicon, Temecula, CA). A rhodamine-labeled goat anti-rabbit antibody was use to detect the putative anti-talin (Chemicon).

Embryos were incubated with primary antibody for 60 min in blocking solution. The primary antibodies were removed by four washes in PBS-T and resuspended in blocking buffer for the addition of secondary antibody. The embryos were then incubated in secondary antibody for 60 min. After washing, the embryos were resuspended in Sigma's DABCO medium (20 mM Tris pH 8.0, 0.2 M 1, 4 diazabicyclo[2.2.2]octane in 90% glycerol).

Stained embryos were studied on a Zeiss Axioplan microscope equipped for epifluorescence and photographed with Kodak TMAX 400 film (Eastman Kodak, Rochester, NY). The TMAX film was processed according to the manufacturer's instructions.

Staining of Larval Stages

Larval-staged animals were collected by centrifugation and then floated in a solution of 30% sucrose (Sulston and Hodgkin, 1988). The animals were then washed three times in cold M9 buffer (Sulston and Hodgkin, 1988). The collected animals were prepared for immunofluorescence as described in Francis and Waterston (1985). This involved a limited French pressure shearing of the animals to allow for antibody penetration of the cuticle. The tissue was then processed with primary and secondary antibodies in the same manner as the embryos, except that incubations for primary and secondary antibodies were between 12 and 24 h.

RESULTS

C. elegans Talin Is 59% Similar to Mouse Talin

A cDNA from *C. elegans* with homology to mouse talin was cloned and sequenced as described in MATERIALS AND METHODS. As this initial cDNA did not contain all of the coding sequence for *C. elegans* talin, we screened a random primed cDNA library for the remainder of the talin-coding sequence. Upon completing the entire sequence of four overlapping cDNAs, we were able to deduce, provisionally, the amino acid sequence of *C. elegans* talin. A dot matrix comparison of the *C. elegans* and mouse talins is shown in Figure 2. This comparison shows homology throughout the length of both proteins, with no substantial shifts in the main diagonal of homology. Figure 3 shows the alignment of these two proteins. Overall, the sequence of *C. elegans* talin is 39% identical, 59% similar, to mouse talin. The similarity is greater in the amino- and carboxyl-terminal portions of the molecule, with 78% similarity between amino acids 1–450 and 52% similarity between amino acids 2000 and the C terminus. As with mouse talin, the sequence of *C. elegans* talin is similar also to the cytoskeletal proteins moesin, ezrin, radixin, and band 4.1 (our unpublished results).

Genetic Map Position of C. elegans Talin

To determine whether any of the muscle-affecting genes from *C. elegans* could be correlated with talin, we first determined the position of the talin gene with respect to genes on the *C. elegans* genetic map. This was done by hybridizing the initial talin cDNA to a set of YAC (yeast artificial chromosome) clones obtained from the *C. elegans* Genome Consortium (Coulson *et al.*, 1986, 1988). This set of clones encompassed most of the genome from *C. elegans*. Further, most of the clones in the set had already been mapped by the Genome Consortium to specific sites on the six *C. elegans* chromosomes (Coulson *et al.*, 1986, 1988). The cDNA hybridized to the YAC Y71G12 (Figure 4a), a clone on the left of *C. elegans* chromosome *I* (Figure 4b). There are no known dense-body or muscle-affecting genes at this location (Hodgkin *et al.*, 1993).

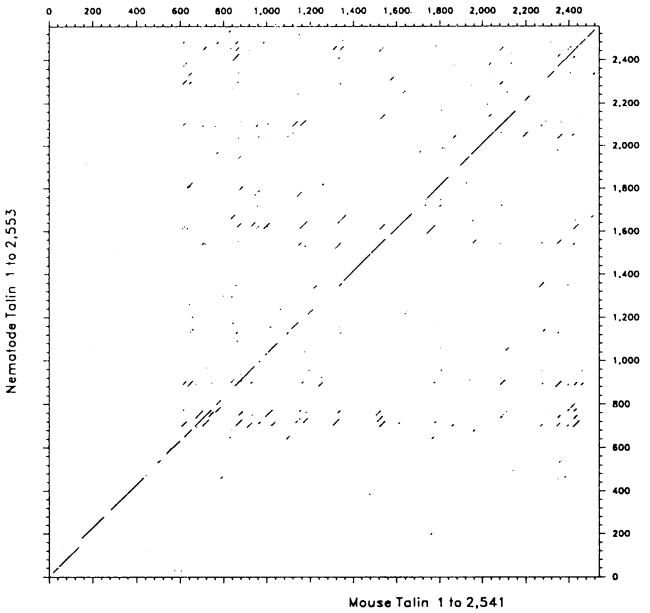

Figure 2. Dot matrix comparison of talin from mouse and *C. elegans*. The G.C.G. program Compare, with a window of 35 and a stringency of 20, was used to compare the amino acid sequences of these two proteins. The plot demonstrates that there are no large segments of either of these two proteins that are absent from the other. As observed by Rees *et al.* (1990) for mouse talin, the off-line homologies in the latter 60% of these proteins are due to the high frequency of alanine in this region of these molecules. The DNA sequence for the *C. elegans* talin cDNA is available from GenBank under accession number L46861.

Antibodies to C. elegans Talin Recognize a Protein of 230 kDa

To estimate the molecular weight of *C. elegans* talin, we examined its behavior on SDS-polyacrylamide gels by Western blot analysis. The affinity-purified antibodies, made to the portion of *C. elegans* talin corresponding to amino acid residues 2016–2553 (see MATERIALS AND METHODS), recognized a polypeptide of ~230 kDa, the size expected for a talin homologue (Figure 5). The very weak band at ~140 kDa is an artifact resulting from the very large amounts of a *C. elegans* yolk protein at this position, which leads to a discontinuity in the background staining. The preimmune serum, purified as described for the immune serum, did not stain any protein on the Western blots (our unpublished results). Further, the antibodies to *C. elegans* talin did not cross-react with talin from chick smooth muscle (our unpublished results).

C. elegans Talin Is Located in Focal Adhesion-like Structures

We used the affinity-purified antibodies described above to examine by indirect immunofluorescence the location of *C. elegans* talin in situ. We found that talin was a component of the body-wall muscle dense bodies (Figure 6a). Dense bodies are arrayed in a regular pattern on the basal surface of the body-wall muscle (Figures 1 and 6c; for review, Waterston, 1988). They function to link actin filaments in the muscle to the adjacent plasma membrane. Further, we found also that talin was positioned in a thin line between the dense bodies in the center of the muscle A-band, a position that corresponds to the location of the M-line (Figure 6a; see diagrams in Figures 1 and 6c). Like the dense bodies, the M-line structures are attached to the muscle cell membrane. As previously demonstrated, the protein β-integrin is found also at both the dense bodies and the M-line (Francis and Waterston, 1985; Gettner *et al.*, 1995) (Figure 6b). Whereas all adult animals showed anti-talin staining at the dense bodies, however, only 10% of the animals showed anti-talin staining at the M-line. Finally, in addition to the body-wall muscle, our anti-talin antibodies detected talin in all of the accessory muscles that were reported by Francis and Waterston (1985) to contain vinculin, with the exception of the pharyngeal muscle (our unpublished results). We did not detect talin in any nonmuscle cells.

Talin Behaves like other Dense Body Proteins in Developing Muscle

We examined the organization of talin in the muscle of wild-type *C. elegans* embryos. Figure 7 shows a developmental time line and illustrates those stages of *C. elegans* embryogenesis that are relevant to this work. A diagram showing the relevant embryonic muscle anatomy in a twofold-stage embryo is shown in Figure 8. Our results for talin are comparable to those of Epstein *et al.* (1993) and Coutu-Hresko *et al.* (1994), who examined the organization of several other muscle proteins in developing *C. elegans* muscle. In the early stages of wild-type muscle assembly, talin appears as a diffusely staining halo surrounding the muscle cell nuclei (Figure 9a). At ~300 min of development, it becomes asymmetrically distributed in the cell, with all of the staining in a single focal plane at the basal surface of the cell associated with the adjacent epidermal tissue, the hypodermis. It accumulates at the basal surface in a single, narrow longitudinal stripe (Figure 9d). This single stripe resolves into two separate parallel stripes over time (Figure 9g). Organization into discrete-dense, body-like structures is apparent at ~450 min of embryogenesis (Figure 9j), at the same time as integrin and vinculin.

β-Integrin, but not Vinculin, Is Required for the Organization of Talin at the Membrane

To determine the relationship of β-integrin and vinculin to talin function, we examined the organization of talin in embryos that lack either of these two proteins. As already described (Barstead and Water-

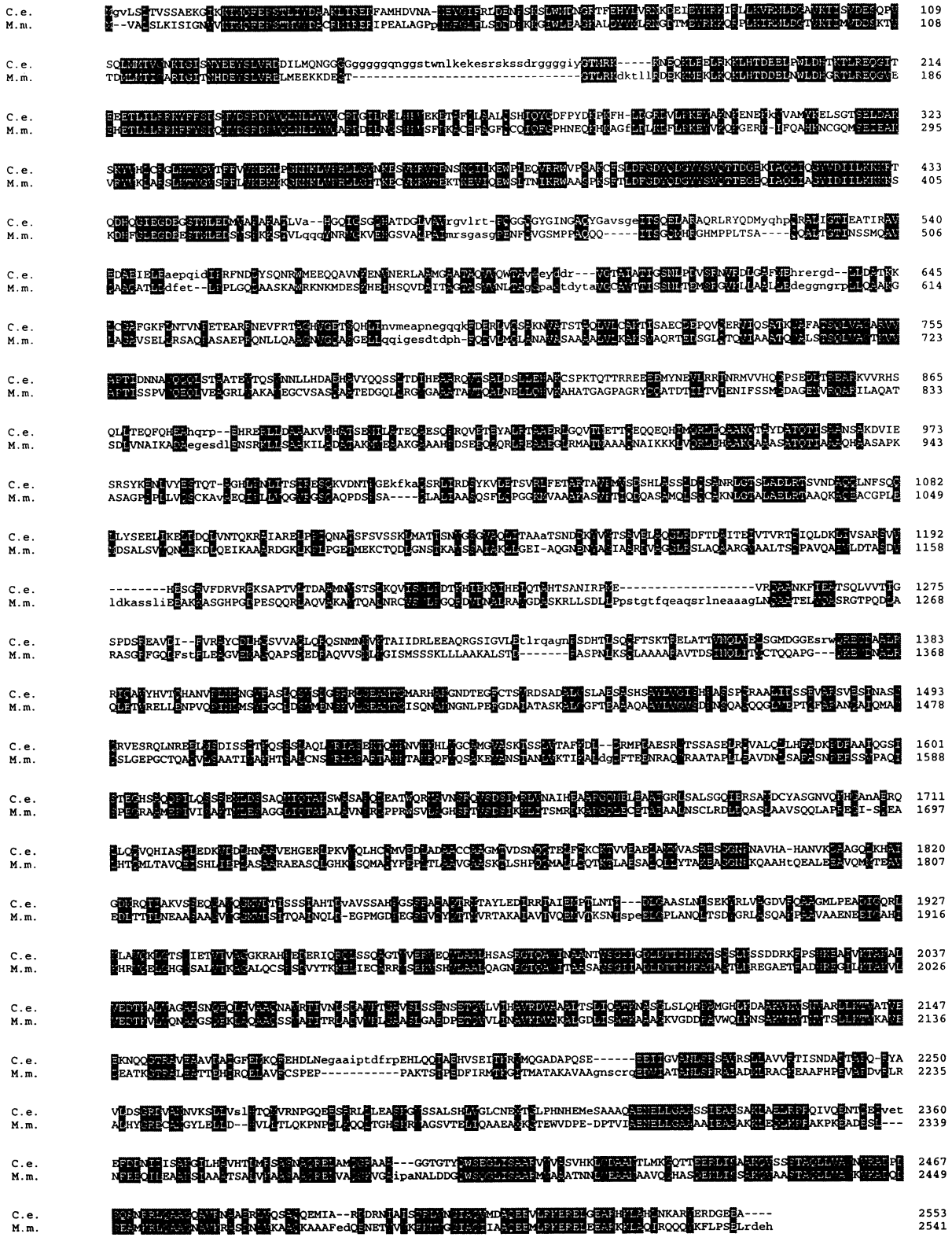

Figure 3. Sequence alignment of *C. elegans* and mouse talins. The program MACAW was used to align and highlight the similar residues between these two sequences, as shown. The highest homology is within the first 25% of these molecules. The terminal 25% also shows relatively high homology.

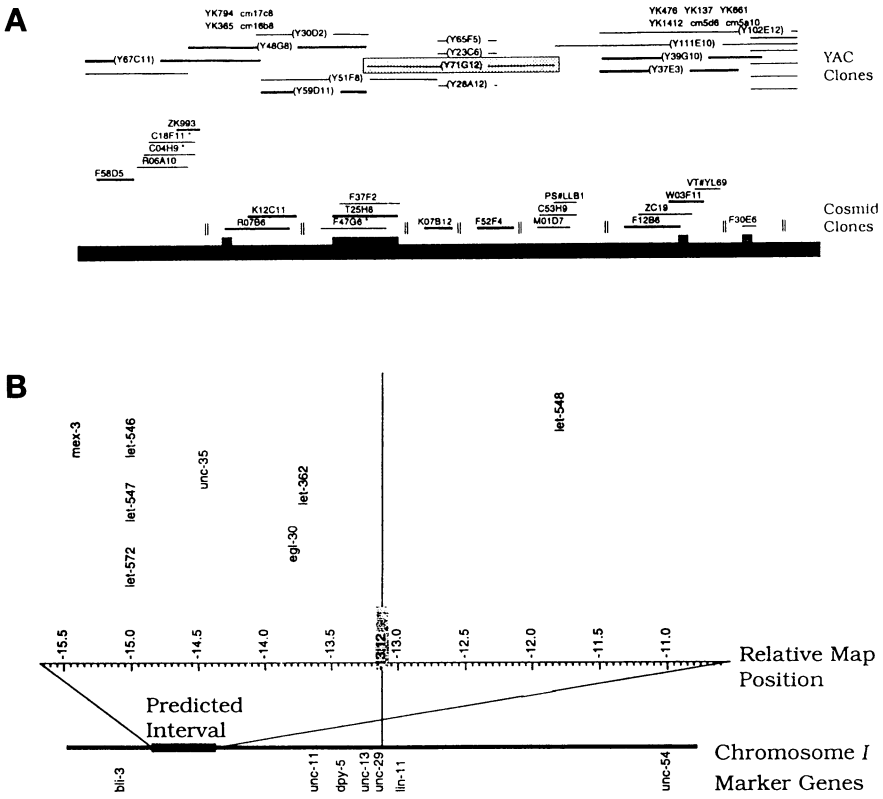

Figure 4. Physical and genetic location of the *C. elegans* talin gene. (A) The *C. elegans* talin cDNA was hybridized to clones from the physical map representing the entire *C. elegans* genome. Hybridization to the clone Y71G12 was observed. This clone is highlighted above and shown in the context of other physically mapped clones. (B) This physical contig set has been mapped to the left of linkage group I on the *C. elegans* genetic map (Coulson *et al.*, 1988). Genes that are predicted to be within this region are shown above the line. One of the well-mapped genetic markers in the region includes the gene *sup-34*, a tRNA non-sense translational suppressor (Kondo *et al.*, 1990). None of the known genes in this region is a strong candidate for the talin gene.

ston, 1991; Coutu-Hresko *et al.*, 1994), mutations that eliminate the function of either of these two genes have disorganized actin and are completely paralyzed. These mutants also show defects in embryonic morphogenesis, such that they fail to completely elongate to their normal wild-type lengths (see Figure 7). The mutants complete enough of their development, however, to hatch from the egg, but they arrest development as short, paralyzed L1 larvae (Barstead and Waterston, 1991; Williams and Waterston, 1994; E. Hedgcock, personal communication). Because such mutants are lethal, however, the mutant genes must be propagated in heterozygotes, animals that carry both a copy of the mutant gene and the wild-type gene. Twenty-five percent of the progeny of such heterozygotes will be homozygous for the mutation in question and therefore will not contain any detectable integrin or vinculin, respectively; the remaining progeny, however, produce functional protein from the wild-type copy of the gene.

To study mutant animals from such mixed populations, we needed to identify those animals in the population that were homozygous for the vinculin or integrin mutations. We did this by doubly staining mixed populations of animals with mouse monoclonal antibodies to either vinculin or integrin, respectively, in addition to the affinity-purified anti-talin antibodies described above. We photographed animals that were positive for talin but completely negative for the corresponding mutant protein. The results are shown in Figure 9. Talin was organized normally in both vinculin and integrin mutants at early stages of differentiation, appearing as a halo surrounding the cell nucleus (Figure 9, b and c). It did not organize further in animals that lacked integrin (Figure 9, f, i, and l). Note especially Figure 9l, in which talin is seen to surround the muscle cell nuclei in a later-stage integrin mutant, just as was seen in the early embryos. In an early

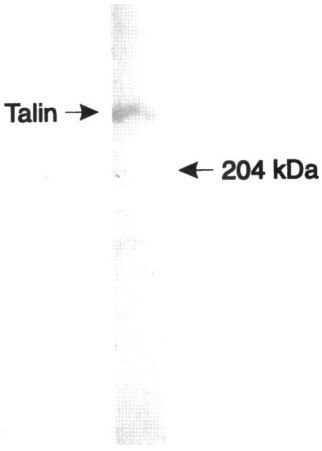

Figure 5. Western blot identification of *C. elegans* talin. Rabbit antibodies were generated against the C-terminal portion of *C. elegans* talin. The antibodies were affinity purified and used to detect *C. elegans* talin on a Western blot of a complete protein extract from a mixed staged population of animals. The position of the highest molecular weight marker, 204 kDa, is shown at the left. We calculated the approximate size of the *C. elegans* protein to be 230 kDa. The preimmune serum did not recognize any proteins on the Western blot (our unpublished results).

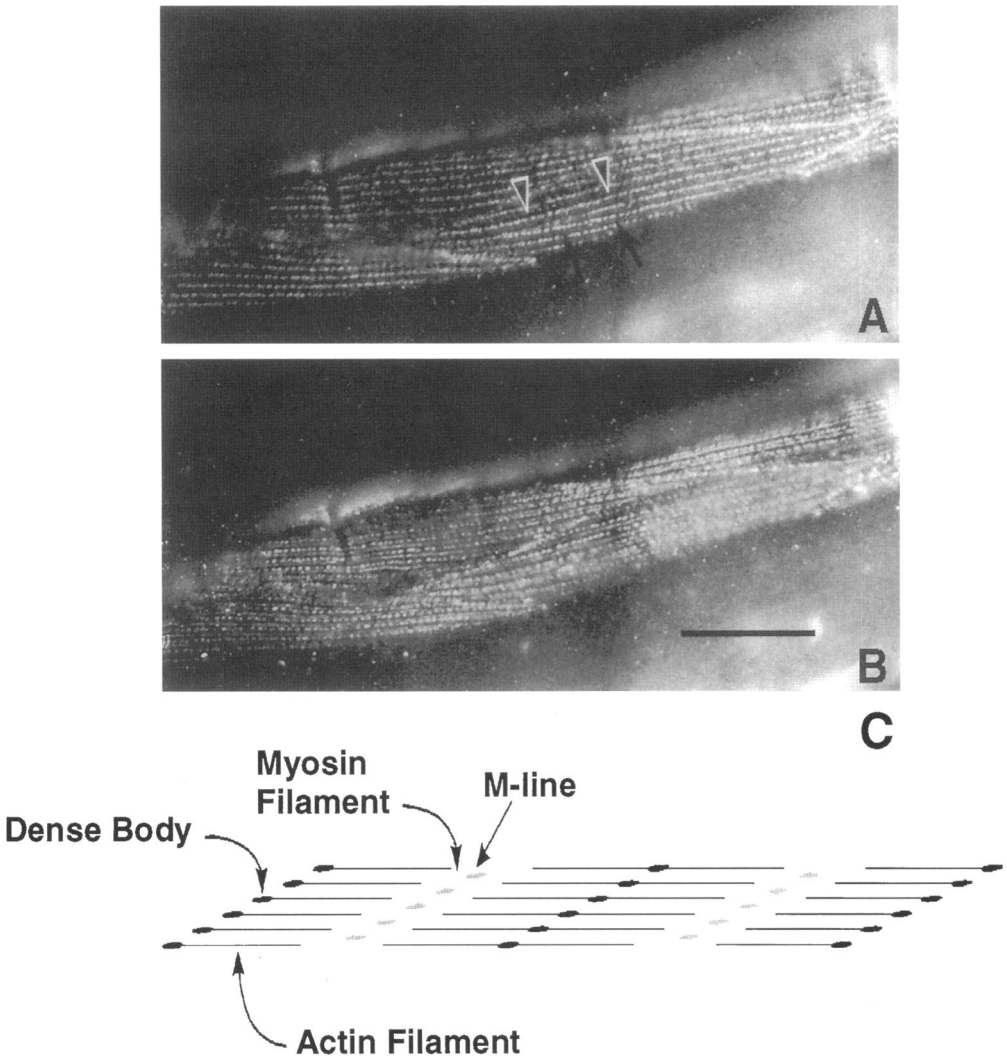

Figure 6. *C. elegans* talin is found in the body-wall muscle-dense bodies. (A) The anti-talin antibodies were used to locate the position of *C. elegans* talin in situ. Fragments of adult *C. elegans* were incubated with both rabbit anti-talin (A) and mouse anti β-integrin (B). As with β-integrin, talin is observed at dense bodies (arrows) and at the M-line (arrowheads). (C) A diagram illustrating the architecture of *C. elegans* muscle is shown. Bar, 20 μm.

vinculin mutant, however, talin seemed to undergo the same developmental progression as seen in wild-type animals at the same stage; after its initial appearance surrounding the cell nucleus, it was seen to accumulate at the basal surface of the cell in a single, narrow longitudinal stripe (Figure 9e). It was finally observed as two separate parallel stripes, in a single focal plane, within each muscle cell quadrant (Figure 9h), a pattern that was indistinguishable from the pattern in wild-type animals at this developmental stage (Figure 9g). In the vinculin mutants, however, further organizational changes did not take place, and we did not see the development of discrete-dense, body-like structures (Figure 9k).

DISCUSSION

The following data support our conclusion that we have cloned a *C. elegans* homologue to mouse talin: first, the sequence of four overlapping *C. elegans* cDNAs, when merged, encodes a polypeptide with a predicted size of 270 kDa, the same predicted size as mouse talin and with a sequence that is 38% identical and 59% similar to the mouse protein (Rees *et al.*, 1990). Although from our data we cannot determine whether the deduced amino acid sequence of the existing *C. elegans* cDNAs encompasses the true translational start for the *C. elegans* protein, the deduced sequence does match the sequence of mouse talin beginning at the known initiator methionine of the mouse mRNA. Second, antibodies to the *C. elegans* protein identify a polypeptide on Western blots that is the same approximate size as vertebrate talin, ~230 kDa. This is smaller than the 270-kDa size predicted from the sequence data, a discrepancy that is also seen for the vertebrate protein. Finally, as with mouse talin, the *C. elegans* protein is associated with focal adhesion-like structures in vivo.

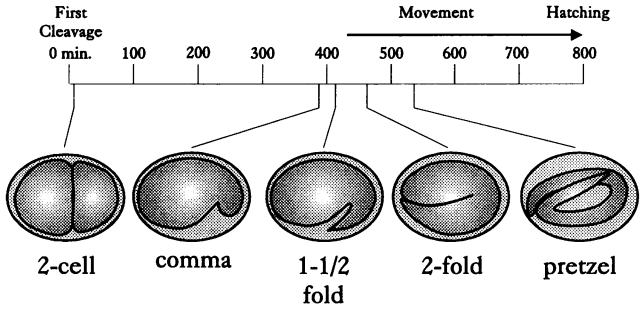

Figure 7. Morphological changes during *C. elegans* embryogenesis. *C. elegans* develops from first cleavage to hatching in 800 min at 20°C. The first half of embryogenesis is taken up with cell divisions and cell migrations. Tissue and organ morphogenesis takes place during the second half of development. The embryo begins to elongate (transform from a ball of cells to a worm) at ~380 min. Elongation begins with an indentation on the ventral surface of the embryo. The tail then pushes forward toward the head. Eventually, wild-type embryos elongate to what is known as the pretzel stage. Vinculin and integrin mutants fail to elongate past the twofold stage. The development of other tissues proceeds normally in these mutants, however, including the maturation of the hypodermis, pharynx, and gut. Muscle function is also defective in vinculin and integrin mutants. In wild-type embryos the body-wall muscle starts contracting at the 1.5-fold stage such that the embryo begins to twitch and then roll around inside the egg. Vinculin and integrin mutants, however, are paralyzed throughout embryogenesis.

As the similarity between the mouse and *C. elegans* proteins is not uniformly distributed across the sequence, it is useful to consider the sequence with respect to the functions of the protein. Note also that the sequence of a talin-like protein from *Dictyostelium* has been reported recently (Kreitmeier *et al.*, 1995), and although the overall similarity of this protein to vertebrate talin is lower than that seen for *C. elegans* talin, the *Dictyostelium* protein shows the same pattern of local similarities as are observed in comparisons of the mouse and nematode proteins. The greatest similarity between mouse and *C. elegans* talin falls within the first 450 amino acids. Although none of the biochemical interactions seen for talin in vitro map to this region, in vivo this domain, by itself, localizes to focal adhesions (Nuckolls *et al.*, 1990). The high level of sequence conservation seen for this part of the molecule across such a large evolutionary distance suggests that the function of this region is also conserved and that, therefore, genetic studies of the *C. elegans* protein will be instructive generally. Several vinculin binding sites have been mapped to residues 450–900 and 1600–2200 (Lee *et al.*, 1992; Gilmore *et al.*, 1993) of vertebrate talin. These segments show relatively low overall similarity. The study of those residues that are conserved may identify amino acids that are necessary for the talin–vinculin interaction. The actin binding site of talin has not been precisely mapped, but it is known to reside within the C-terminal 75% of the molecule. Because *C. elegans* actin is 90% identical to vertebrate actin, we expect that the actin binding site within talin should be relatively well conserved also. The most highly conserved C-terminal segment within these sequences falls between residues 2000 and the end of the molecule, and it would not be surprising if the actin binding site of talin falls within this region.

Our finding that talin is a component of the dense bodies in *C. elegans* body-wall muscle, together with previous findings that integrin (Francis and Waterston, 1985; Gettner *et al.*, 1995), vinculin (Barstead and Waterston, 1989), and α-actinin (Francis and Waterston, 1985; Barstead *et al.*, 1991) are also components of the dense bodies, further solidifies our assertion that this structure is analogous to the focal adhesions found in vertebrate cells. We also found that talin was located at the muscle M-line in a small fraction of adult animals. In all cases the antibody staining for talin was weak at the M-line, and so the absence of M-line staining in most animals may simply reflect variable accessibility for the antibody at this location. That talin may have a significant role at the M-line is supported by previous observations that β-integrin is found at relatively high levels at this location (Francis and Waterston, 1985; Gettner *et al.*, 1995). Vinculin and α-actinin, however, are not found at this site. At present we cannot speculate intelligently about the function of talin or integrin at the M-line of *C. elegans* body-wall muscle, but we anticipate that the recovery of mutations in the *C. elegans* talin gene will allow for a determination of its function at this site and may eventually lead to the identification of those sites within talin that regulate the apparently different protein–protein interactions required for its various functions in *C. elegans*.

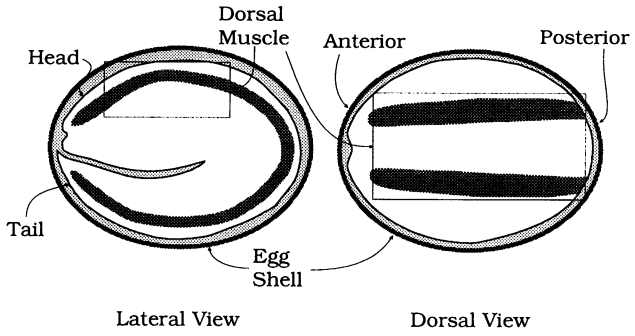

Figure 8. Anatomy of a twofold *C. elegans* embryo. At the twofold stage of embryogenesis, the body-wall muscle is found in four quadrants, two dorsal and two ventral, around the circumference of the animal. This diagram depicts dorsal muscle as it appears in a twofold embryo either from a lateral view or from a dorsal view. Depending on the view, one sees either one or two of the muscle quadrants, respectively. The dorsal muscle extends the entire length of the animal. The boxed areas in these diagrams show the anterior portion of the dorsal muscle. The micrographs in Figure 9 are focused typically on this region of the embryonic body-wall muscle.

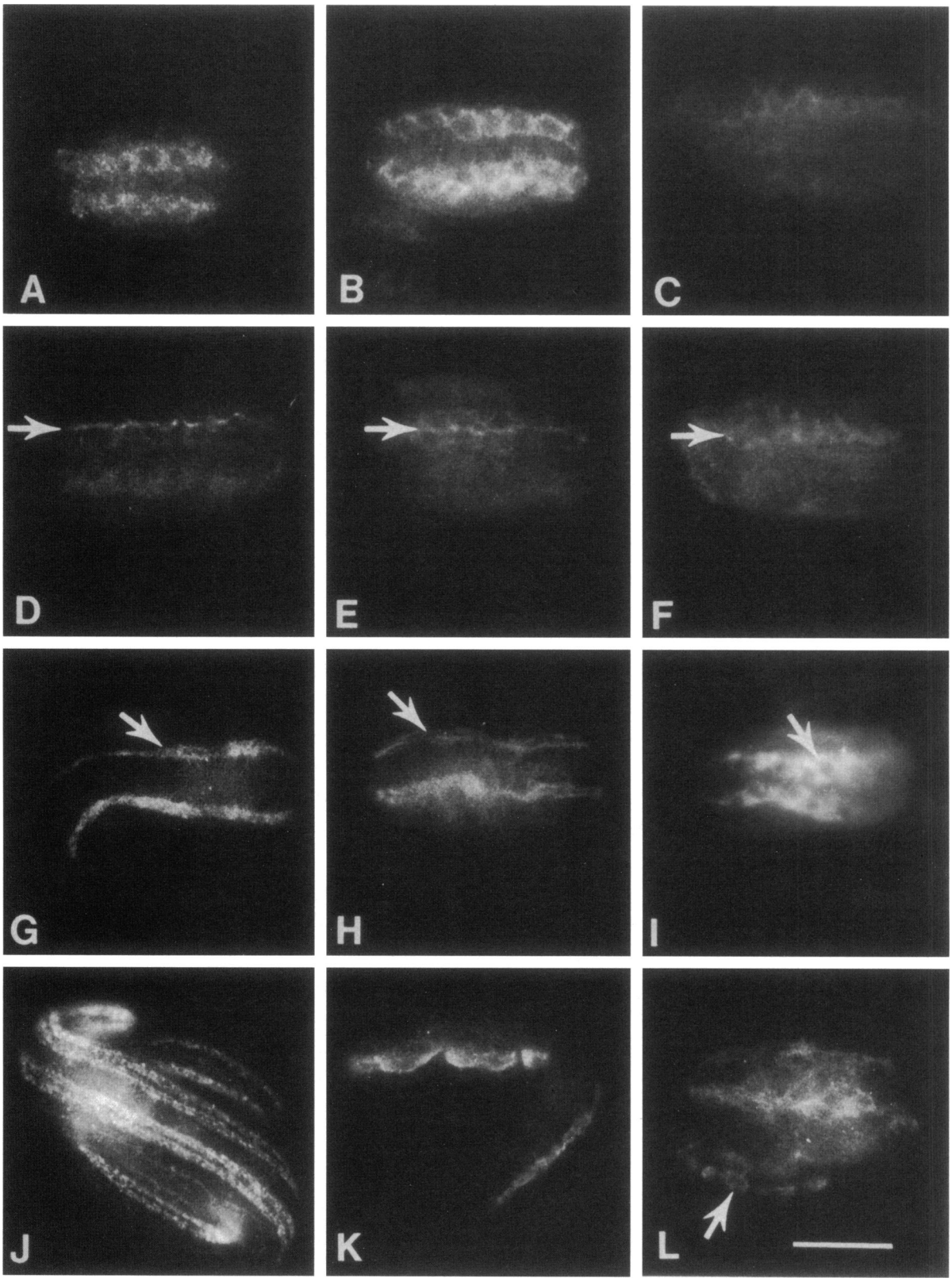

Figure 9.

Existing mutations in the vinculin and β-integrin genes of *C. elegans* eliminate all detectable vinculin and integrin in the mutant animals (Barstead and Waterston, 1991; Gettner *et al.*, 1995; S. Gettner and E. Hedgcock, personal communication; our unpublished results). Previous phenotypic analysis of such mutants has demonstrated that both vinculin and integrin are critical for the formation of a proper linkage between actin and the cell membrane in vivo (Barstead and Waterston, 1991; Coutu-Hresko *et al.*, 1994). In addition, Coutu-Hresko showed that vinculin was disorganized in an integrin mutant, whereas integrin was well organized in a vinculin mutant (Coutu-Hresko *et al.*, 1994). From these data one can conclude that integrin does not depend on vinculin for its organization at the membrane in *C. elegans* body-wall muscle. Although the converse is also true, that vinculin requires integrin for its organization, one cannot determine from these data alone the proximal cause for its failure to organize in an integrin mutant, and so, based on these results alone, the precise relationship between vinculin and integrin is ambiguous.

On the basis of the observed biochemical interactions of integrin and talin in vitro and the colocalization of these proteins in vivo, it had been previously hypothesized that the localization of talin at the membrane depends on integrin. We have directly addressed this hypothesis by examining the organization of talin in cells that are genetically normal in all respects except that, because of a single mutation, they lack all β-integrin. We showed that talin is disorganized in such cells. This result unambiguously shows that talin depends on integrin for its localization to the membrane in vivo.

The work of Volberg *et al.* (1986) and Izzard (1988) suggests that talin can associate with membrane sites independently of vinculin. Our genetic tests support this conclusion. We showed that, just as in wild-type animals, in vinculin mutants talin becomes asymmetrically distributed in the muscle cell, accumulates at sites where adjacent muscle cells contact, organizes into a single stripe at the area of contact between muscle cells in a quadrant, and, finally, organizes into two parallel stripes within each muscle quadrant. The organization of talin in the mutant is not completely normal, however, because it does not progressively accumulate into discrete, partial-dense bodies as it does in wild-type animals.

From our observations we draw the following four conclusions: 1) On the basis of our observation that talin is well organized in a vinculin mutant, we conclude that vinculin is not required for the proper assembly of talin at the membrane at early stages in the assembly of the dense body. Given that the function of these two proteins in vivo is unknown, that both of these proteins are modified post-translationally in vivo (Geiger, 1982; Burn and Burger, 1987; Kellie and Wigglesworth, 1987; Turner *et al.*, 1989; Hagmann *et al.*, 1992), that the function of these post-translational modifications is unknown, that the factors governing the post-translational modification are unknown, and that their associations may be influenced by allosteric changes in their structure (Johnson and Craig, 1994), this result, although in accord with the biochemical data, could not have been predicted from the biochemical data alone. 2) On the basis of our observation that talin is disorganized in an integrin mutant, we conclude that integrin is necessary for the organization of talin at the membrane. Although this result cannot demonstrate, by itself, a direct interaction between integrin and talin, when viewed in conjunction with the biochemical data of the vertebrate homologues, we can conclude credibly that the direct interaction measured for these two proteins in vitro is important in vivo and that the disorganization of talin in an integrin null mutant is a direct result of the absence of integrin at the membrane. 3) Others have shown that talin binds to vinculin (Burridge and Mangeat, 1984),

Figure 9 (cont). Talin is abnormally organized in an integrin mutant but not in a vinculin mutant. Shown here are wild-type animals (A, D, G, and J), vinculin mutants (B, E, H, and K), and integrin mutants (C, F, I, and L). All animals were doubly stained with rabbit antibodies to *C. elegans* talin and with mouse antibodies to either *C. elegans* vinculin or β-integrin. Only the talin pattern is shown, however. Vinculin mutants were negative for staining with the anti-vinculin antibody, and integrin mutants were negative for staining with the anti-integrin antibody. With the exception of panels G and J, all of the panels show a single animal oriented with the anterior to the left and posterior to the right. Each panel shows either one or two dorsal muscle quadrants. At early stages of development (A–C), the distribution of talin in the mutants is the same as in wild-type animals; talin appears as a halo, with no distinct pattern, surrounding the cell nucleus. After ~300 min of embryonic development, talin in wild-type animals begins to organize (D). It first accumulates at the basal surface of the muscle cell adjacent to the hypodermis and then coalesces into a single thin line (arrow). This same early development is seen in the vinculin mutants (E), but not in the integrin mutants (F), in which the talin persists as a halo surrounding the cell nucleus (arrow). At the twofold stage of development (G–I), in both wild-type animals and in vinculin mutants the antibody staining appears as two parallel punctate lines within the muscle (G and H, arrow). Further, talin no longer appears as a halo surrounding the cell nucleus and, instead, appears in a single focal plane adjacent to the hypodermal cells. An integrin mutant at approximately the same stage of development as the animals shown in panels G and H is shown in panel I. Once again, integrin persists as a halo surrounding the cell nucleus of each muscle cell (arrow). Panels J–L illustrate the talin distribution in later stages of development. At the threefold stage of wild-type development, talin is found in discrete-dense bodies (J). In contrast, in a late-staged vinculin mutant (K), although talin is found in its normal position at the basal surface of the muscle cell, it never appears as discrete-dense, partial-dense bodies. Finally, at these late stages, talin is not at all organized in an integrin mutant (L), because it is found throughout the cell surrounding the cell nucleus, just as one sees at the earliest times of muscle development (arrow). Bar, 20 μm.

β-integrin (Horwitz *et al.*, 1986), actin (Kaufmann *et al.*, 1991), and FAK (Chen *et al.*, 1995) in vitro. Whether these three proteins represent the complete set of proteins that interact with talin in vitro or in vivo cannot be known with confidence. Therefore, whether talin has alternative integrin-independent pathways for interaction with the membrane is an open issue. Our observations address this issue directly; that talin is disorganized in mutants that contain no β-integrin unequivocally demonstrates that talin does not have any other significant integrin-independent pathways for interaction with the membrane in vivo. 4) As the organization of talin in a vinculin mutant was not normal at later stages of development, vinculin may be required for the progression of assembly into discrete-dense bodies. Because vinculin mutants fail to complete the morphogenic step of elongation, however, the abnormal appearance of talin at the later stages of embryogenesis may be due indirectly to the consequent aberrant morphology.

The study of these cytoskeletal proteins in a cell as regularly organized as muscle has obvious advantages. More importantly, however, by exploiting the powerful genetic methods available for *C. elegans*, we can address the function of these proteins in vivo. This work is the first step in the genetic characterization of talin in *C. elegans*. Because we could not correlate the genetic location of talin with any known dense-body–affecting mutations, we have no candidate talin mutants, as yet. We are currently undertaking genetic screens for such mutants. From the analysis of talin mutants we can evaluate the role of talin in the assembly of vinculin and other proteins at the dense body.

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