THE ROLE OF FILAMIN IN THE MORPHOGENESIS OF THE SKELETAL MUSCLE SARCOMERE

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ABSTRACT

During chicken skeletal myogenesis in tissue culture, filamin is found on stress fibers in myoblasts and early myotubes. Approximately one day after fusion and shortly before α-actinin transits to Z lines, filamin disappears from the cells. The disappearance of filamin is correlated with a cessation of its synthesis. Approximately six days after fusion, filamin reappears at the Z lines of myogenic cells, shortly before desmin and vimentin transit to the Z line. In adult muscle, filamin is found at the periphery of the Z disk, along with desmin and vimentin. Peptide map analysis of the various filamins shows that gizzard and fibroblast filamins are identical while myoblast filamin is quite similar to these two filamins. Cultured myotube and adult myofibril filamins are virtually identical to each other and are quite different polypeptides when compared to gizzard, fibroblast and myoblast filamins. Analysis of terminally differentiated slow and fast muscle shows that both muscle types contain identical, skeletal muscle type filamins although in the slow muscle, filamin is distributed additionally on the I band. The molar filamin to actin ratio is 1:25 in gizzard and fibroblast, 1:54 in myoblasts, 1:820 in fast skeletal myofibrils and 1:82 in slow skeletal myofibrils.

These results offer several new insights into eucaryotic molecular morphogenesis. From the disappearance of filamin during myogenesis, we see that a morphogenetic process may involve the temporary removal of a family of proteins. The different distributions of identical filamin polypeptides in slow and fast muscle indicates that filamin may be synthesized at different times and rates in the two myogenic processes. It appears that, at least in the case of the skeletal muscle sarcomere, temporal control of protein synthesis may be an important part of eucaryotic molecular morphogenesis.

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Chapter 1.

INTRODUCTION

Morphogenesis

Biological morphogenesis remains one of the most fascinating problems in science. How do we almost always get five fingers on each hand? How do the cells at the elbow know how far away the cells of the shoulder are so as to make the upper arm the proper length? How do they know along which axis to make the hinge so the arm folds in the proper direction? Such questions can be reduced to a consideration of how a developing tissue counts, determines lengths and determines angles. Precise answers to these questions are at present impossible to obtain because multicellular morphogenesis involves many unknown variables. Cell division rates, stiffness, adhesiveness and extent and direction of cell motility can vary from point to point in a developing tissue in an unknown fashion.

The morphogenesis of subcellular structures is a much more approachable problem than multicellular morphogenesis. One of the best characterized subcellular morphogenetic systems is the T4 bacteriophage (for review see Wood, 1980). This virus is composed of a hollow tubular stalk with a DNA-containing head at one end and a set of leg-like tail fibers at the other. The morphogenesis of T4 involves the sequential addition of proteins to form a complete head, a complete stalk and complete tail fibers. Subsequently, these three subassemblies are joined to form a complete structure. Under different conditions the same set of proteins will form different structures, such as heads of different shapes.

In eucaryotes a comparable system is the cytoskeleton, which is composed of a set of proteins that can combine to make different structures. To study cytoskeletal morphogenesis we would want to choose a structure that contains an isolatable protein that can be easily used to study the binding of other proteins to it in vitro.

Such a protein is actin, a ubiquitous protein that will readily polymerize to form long filaments (for review see Pollard and Craig, 1982) that can be precipitated

by centrifugation. This allows a simple assay for the binding of actin crosslinking proteins to actin (for review see Craig and Pollard, 1982): a mixture of polymerized (F-) actin is mixed with a putative actin-binding protein, the mixture is centrifuged and the supernatant and pellet electrophoresed on SDS-polyacrylamide gels. With the proper controls, appearance of the protein in the pellet indicates that it is an actin-binding protein.

There are only three isolatable actin-containing cytoskeletal structures with a clearly visualizable morphology that are currently being intensively studied. These are the human erythrocyte cytoskeleton, the avian intestinal brush border microvillus and the skeletal muscle sarcomere. Other cytoskeletal structures, although intensively studied, do not meet the above criteria. For instance, no definitive isolation procedure has been developed for stress fibers, microtubules or intermediate filaments that do not involve disruption of the structure.

The Human Erythrocyte Cytoskeleton

A well-studied system involving the crosslinking of actin filaments is the human red blood cell cytoskeleton. When red blood cells are depleted of hemoglobin by hypotonic lysis, and the resulting membranes extracted with detergents such as Triton X-100, an insoluble structure remains. This cytoskeleton consists primarily of four proteins, bands 1, 2, 4.1 and 5 (Yu et al., 1973). Band 1 is the 240,000 dalton α subunit of spectrin, band 2 the 220,000 dalton β subunit, and band 5 is actin (Marchesi, 1979). Spectrin is thought to be one of the proteins responsible for anchoring particles in the plane of the membrane. Spectrin can be released from red cell membranes by extraction with low ionic strength buffers at 0°C, allowing intramembrane particles to cluster (Elgsaeter and Branton, 1974). The fact that clustering can occur only in the absence of spectrin indicates that there is a

physical connection between the spectrin-containing submembrane cytoskeleton and the intramembrane particles.

Biophysical studies and direct electron microscopic visualization show that spectrin is a flexible molecule 970 Å long, composed of two strands forming a loose double helix (Shotton et al., 1979). One strand is the α subunit composed of five sequentially linked α-helical domains; the other strand is the β subunit similarly composed of four domains (Speicher et al., 1980). The two strands are non-covalently linked along their length (Morrow et al., 1980). Purified spectrin in solution (Ungewickell and Gratzer, 1978) and spectrin on the red cell membrane (Ji et al., 1980) form tetramers composed of a double helix containing an α and a β spectrin linked end-to-end with a second, identical double helix.

The binding of spectrin to red cell membranes has been well characterized. Spectrin can be labeled with $^{32}PO_4$ or ^{125}I and membranes can be pelleted by centrifugation, allowing a convenient and simple binding assay. Spectrin binds much better to 'inside-out' membrane vesicles than to the external surface of red cell membranes, indicating a specificity for the cytoplasmic side. The saturable binding site is proteinacious since treatment of membranes with 0.1 M acetic acid or protease irreversibly inhibits the binding (Bennett and Branton, 1977). SH reagents, the absence of monovalent salts and treatment of membranes with antibodies against bands 3, 4.1 and 4.2 also inhibit the binding of spectrin to membranes (Litman et al., 1980). The protein to which spectrin binds was found in the following manner: chymotryptic digestion of inside out membranes releases a 72,000 dalton peptide that inhibits the binding of spectrin to membranes (Bennett, 1978). Comparison of peptide maps of this fragment with peptide maps of other membrane proteins showed that the 72 K dalton fragment was derived from band 2.1 (Luna et al., 1979, Yu and Goodman (1979). Antibodies to the fragment specifically immunoprecipitated band 2.1 and partially purified band 2.1 inhibited the binding of spectrin

to membranes (Bennett and Stenbuck, 1979), indicating that band 2.1 was indeed the membrane binding site for spectrin.

Band 2.1 (ankyrin) is not an integral membrane protein because it can be released from membranes by treatment with high salt (Tyler et al., 1979; Bennett and Stenbuck, 1979). Thus the binding of spectrin to band 2.1 does not explain the apparent link between spectrin and intermembrane particles. However, immunoprecipitation of band 2.1 from detergent-treated red blood cells coprecipitated 10–15% of the total cellular band 3 (Bennett and Stenbuck, 1979), which is an anion transporter located within the intermembrane particles (Cabantchik et al., 1978). This suggests that band 2.1 mediates the binding of spectrin to an intermembrane particle.

Despite initial reports to the contrary, spectrin and actin alone will not form a complex (Ungewickell et al., 1979; Fowler and Taylor, 1980); gel formation requires the addition of band 4.1 to tetrameric spectrin and F-actin in the absence of Ca^{++}. While dimeric spectrin has one band 4.1/actin binding site, tetrameric spectrin has two and can thus crosslink actin (Brenner and Korn, 1979). In the red blood cell cytoskeleton actin is probably mostly present as 10 subunit oligomers, with approximately five spectrin/band 4.1 complexes bound per oligomer (Brenner and Korn, 1980). Band 2.1 and band 4.1 bind saturably to spectrin and do not compete with each other for binding sites (Tyler et al., 1980). Examination of mixtures of purified proteins by electron microscopy clearly shows that spectrin dimers bind head to head to form a tetramer, band 2.1 binds near the heads, band 4.1 binds near the tails and F-actin filaments bind to complexes of spectrin and band 4.1 (Tyler et al., 1979; Cohen et al., 1980; Branton et al., 1982; Bennett, 1982).

The study of the erythrocyte cytoskeleton has generated several new ideas about the nature of subcellular cytoskeletons. First, we now realize that a cytoskeleton can be physically linked to proteins within the plasma membrane and therefore

can modulate the activity of receptors, pumps and other membrane-associated proteins. Second, it is apparent that proteins which crosslink actin filaments play a major role in actin-containing cytoskeletons. Finally, we see that linker proteins such as band 2.1 and band 4.1 can be used to modulate the interactions of other proteins. In the erythrocyte cytoskeleton, the quantity of band 2.1 could regulate the extent of interaction between the cytoskeleton and the plasma membrane, while the quantity of band 4.1 could independently regulate the extent of cross-linking of the cytoskeleton and thus its rigidity. Despite the wealth of knowledge of the protein components of the erythrocyte cytoskeleton, very little is known about how they assemble to form the structure.

The Brush Border Microvillus

Another well-studied structure is the chicken intestinal brush border microvillus. Brush borders can be isolated with high purity. After extraction with detergents, only a small number of proteins remain insoluble: actin (Tilney and Mooseker, 1971), three proteins with molecular weights 110, 95 and 68 K dalton, with molar ratios approximately one-tenth that of actin, and calmodulin (Howe et al., 1980). The 100 K dalton protein forms projections from the central bundle of parallel actin filaments to the membrane of the microvillus (Matsudaira and Burgess, 1979). Fimbrin, the 68 K dalton component, binds to and crosslinks actin independent of Ca^{++} concentration (Bretscher, 1981; Glenney et al., 1981). Villin, the 95 K dalton component, binds to and crosslinks actin filaments only in the absence of calcium, much like α-actinin (Mooseker et al., 1980). However, dissimilar peptide maps and the absence of immunological crossreactivity show that villin and α-actinin are distinct polypeptides (Mooseker and Stephens, 1980). At high calcium concentrations villin fragments actin filaments into short segments and binds to

the 'barbed' end of F-actin so as to prevent further polymerization (Bretscher et al., 1981; Glenney et al., 1981; Nunnally et al., 1981; Cribbs et al., 1982).

Like the erythrocyte cytoskeleton, the brush border cytoskeleton consists of crosslinked actin filaments which are linked to the plasma membrane via mediating proteins. The importance of actin crosslinking in this structure is shown by the fact that, of the four major non-actin proteins in the structure, two crosslink actin. The reason for having two different actin-crosslinking proteins in this structure is unknown. As in the case of the erythrocyte cytoskeleton, little is known about the morphogenesis of the brush border microvillus cytoskeleton.

The Skeletal Muscle Sarcomere

Skeletal muscle sarcomere development is an excellent system for studying eucaryotic molecualr morphogenesis (Lazarides, 1981).

Although a developed sarcomere is considerably more complicated than the erythrocyte cytoskeleton or the brush border microvillus, it contains actin and actin-binding proteins and has the advantage that its morphogenesis can be studied in vivo and in vitro in cells cultured under defined conditions. The in vivo process is well documented by electron microscopic studies. The process can be duplicated using relatively pure, synchronized cultured cells which facilitates the study of the biochemistry of myogenesis. A large fraction of the proteins synthesized by myogenic cells are sarcomere-associated structural proteins. Many of these proteins have been isolated, partially characterized and localized within the myofibril by techniques such as indirect immunofluorescence. These include actin (Huxley, 1963; Hanson and Lowy, 1963; Granger and Lazarides, 1978), myosin (Huxley, 1963; Pepe, 1966; Dabrowska et al., 1977; Granger and Lazarides, 1978; Bandman et al., 1981), troponins (Endo et al., 1966; Ohtsuki et al., 1967; Matsuda et al., 1981; Toyota and Shimada, 1981), desmin (Lazarides and Hubbard, 1976; Lazarides, 1978;

Lazarides and Balzer, 1978; Granger and Lazarides, 1978), vimentin (Granger and Lazarides, 1979), α-actinin (Masaki et al., 1967; Granger and Lazarides, 1978), filamin (Bechtel, 1979), C-protein (Offer et al., 1978; Moos et al., 1975; Pepe and Drucker, 1975; Shafiq et al., 1982), a parvalbumin-like protein (Heizmann et al., 1977), I-protein (Ohaski et al., 1977a; Ohashi et al., 1977b; Maruyama et al., 1977); M-protein (Masaki and Yoshizaki, 1972; Masaki and Takaiti, 1974), a calcium-activated protease (Dayton and Schollmeyer, 1981), Z-protein (Ohaski and Maruyama, 1979, 1981; Ohashi et al., 1982), creatine kinase (Turner et al., 1973; Walliman et al., 1977; Dym et al., 1978), synemin (Granger and Lazarides, 1980) amorphin (Chowrashi and Pepe, 1982), and T tubule components (Rosenblatt et al., 1981), among others. The ability to study the time dependence of the expression of these proteins and their mRNAs using synchronized cultured cells will facilitate eventual studies on the genetic control of sarcomere morphogenesis. Finally, the finished product, a myofibril, can be easily isolated.

The first morphological aspect of myogenesis begins at Hamburger-Hamilton (Hamburger and Hamilton, 1951) stage 14 (Allen and Pepe, 1965; Fischman, 1967). Presumptive myoblasts and the mitochondria within them elongate between this stage and stage 16, and thereby become distinguishable from mesenchymal cells. Myoblasts fuse with one another to form multinucleated myotubes; myoblasts may also fuse with myotubes to enlarge them. A few short (less than 1 μm) thin filaments are seen in the myotube. By stage 18 the number of thin filaments has increased, and myosin filaments have appeared. Both probably self-assemble from actin and myosin respectively. The thick filaments, morphologically identical to those of adult muscle, are always found in clusters associated with thin filaments, indicating that the second major step toward the morphogenesis of a sarcomere might be an interaction between actin and myosin. Because cross sections through developing muscle show even the smallest of these aggregates to be hexagonally packed, it

has been suggested that thick filaments come into lateral register by interacting via the intervening actin filaments. This is supported by the fact that, in saponin-permeabilized cultured myotubes containing only a few myosin filaments, which were treated with heavy meromyosin to decorate actin filaments, most of the actin associated with the myosin has the same spatial polarity as the actin associated with myosin in the mature myofibril (Shimada and Obinata, 1977). Actin filaments in early myotubes rarely exceed a length of 1.1 μm. Along with myosin, polyribosomes and clusters of glycogen granules also appear at this stage.

By stage 24 small (0.5 μm diameter) myofibrils appear, usually at the periphery of the cell. The formation of myofibrils probably requires the presence of, among other things, Mg^{++} (Konierczny et al., 1982). However, these incipient myofibrils lack Z and M lines and H zones although tubules have been seen surrounding the sarcomeres at 1.5 μm intervals at approximately the future positions of the Z lines (Paterson et al., 1974). These tubules are projections of the endoplasmic reticulum forming the sarcoplasmic reticulum and invaginations of the sarcolemma forming the transverse tubule system. By stage 28 the M and Z lines, and H zones appear and sarcomeres are morphologically indistinguishable from those found in adult muscle. The myofibrils then line up so their Z lines are in register. It has been suggested that the collars of desmin around Z discs may be involved in this step (Granger and Lazarides, 1978). Theories for the formation of Z discs include the claim that Z lines are derived from dense bodies, formed at the cell membrane, that are released and float into the cytoplasm (Heuson-Stiennon, 1965). Other studies (Auber, 1969) propose that the Z lines arise as a condensation of filaments already existing in the cytoplasm of the myoblast, or that the Z lines are derived from desmosomes (Hagopian and Sprio, 1970). Close examination of Z lines by transmission electron microscopy shows that the actin filaments from the I band do not simply terminate at the Z line but appear to be looped together to form

a square lattice in the plane of the Z disc (Garamvolgyi, 1963, Franzini-Armstrong and Porter, 1964; Kelly, 1967).

Pulse label experiments using cultured myoblasts from chick (Coleman and Coleman, 1968; Turner et al., 1974; Merlie et al., 1975; Devlin and Emerson, 1978; Gard and Lazarides, 1980; Affara et al., 1980a,b), calf (Whalen et al., 1976) and Drosophila (Fyrberg and Donady, 1979) show several patterns of protein synthesis during myogenesis. The synthesis rates of some proteins increase during myoblast fusion, sometimes by as much as 500-fold. Examples of these proteins are desmin, the muscle-specific (α) actin, myosin heavy and light chains, troponins, tropomyosins, creatine kinase and aldolase isozymes. Isotope dilution experiments (Allen et al., 1979) show that the synthesis rates of α-actinin, myosin and tropomyosin all begin to increase simultaneously. Using cell-free translation systems, it has been shown that the increase of synthesis of these proteins occurs as a result of an increase in the amount of mRNA coding for them (Paterson et al., 1974; Devlin and Emerson, 1974). Some proteins are synthesized at approximately constant rates, while other, such as the nonmuscle (β and γ) actins, are synthesized at decreasing rates during myogenesis. Finally, some proteins, such as tropomyosins and myosin light chains, undergo isozymic changes during differentiation (Roy et al., 1979). For example, pulse chase experiments using cultured myoblasts show that the half life of myosin synthesized in myoblasts (three days) is approximately half that of myosin synthesized in myotubes (six days) (Rubinstein et al., 1976; Zani et al., 1978). This is true of myosin synthesized in myoblasts and chased into myotubes, indicating that rather than an increase in proteolysis there is a change in the myosin itself. By blocking cell fusion with lowered Ca^{++} (Emerson and Beckner, 1975; Moss and Strohman, 1976; Vertel and Fischman, 1976) or DMSO (Blau and Epstein, 1979) it has been shown that the increase in production of myosin that occurs at the time of cell fusion occurs independently of cell fusion.

A few mutations are known that affect sarcomere morphogenesis. There are nematodes with altered body wall myosins (Epstein et al., 1974; Harris et al., 1977; Schadrat et al., 1977) that disrupt sarcomere morphology. Lethal mutations of chickens (Asmundson, 1945) and mice (Pai, 1965a,b) involve a general degeneration of skeletal muscle. Two Wing Up (Wup) mutants in Drosophila experience degeneration of almost fully developed muscles (WupA/WupA), no myofibril development (WupB/WupB) or weak muscles with crooked Z bands (WupB/+) (Hotta and Benzer, 1972; Mogami et al., 1981); a similar mutant is flt0 (Koana and Hotta, 1978).

After a functional sarcomere appears in a muscle fiber it has not stopped differentiating. Innervation of the myofiber causes it to become either slow or fast (Samaha et al., 1980; Kelly and Rubenstein, 1980). Changes occur in myosin isozymes (Rushbrook and Stracher, 1979; Whalen et al., 1979; Gauthier et al., 1982; Bandman et al., 1982), tropomyosin isozymes (Montarras et al., 1981, 1982) and troponin isozymes (Matsuda et al., 1982) in skeletal muscle of the young chick.

The study of sarcomere morphogenesis is still in its infancy. The total protein composition of the developed structure is unknown; new structural proteins are still being found. As is the case for erythrocyte cytoskeletons and the microvillus, nothing is known about what regulates the size of the structure, both longitudinally and diametrically.

One of the logical first approaches to studying the morphogenesis of the skeletal muscle sarcomere is to examine the behavior of the actin-binding proteins. There are four main classes of actin-binding proteins: depolymerizing proteins, filament fragmenting and capping proteins, contractile proteins such as myosin and tropomyosin and finally crosslinking proteins (for review see Craig and Pollard, 1982). The latter class of proteins has the greatest probability of directly contributing to the morphogenesis of an actin-containing structure. Two actin crosslinking proteins are known in

skeletal muscle: α-actinin (Masaki et al., 1967; Gard and Lazarides, 1980) and filamin (Bechtel, 1979).

α-Actinin

α-Actinin is a dimer of two 100,000 dalton actin-binding proteins that has several similarities to filamin. It was first purified from skeletal muscle (Ebashi et al., 1964; Ebashi and Ebashi, 1965) and was found to both accelerate the polymerization of actin and to form a gel with actin (Maruyama and Ebashi, 1965). Antibodies to this protein stain Z lines in skeletal muscle (Masaki et al., 1967). Low ionic strength extraction of skeletal myofibrils caused the apparent partial disappearance of phase-dense Z lines and the release of, among other proteins, α-actinin. Incubation of the extracted myofibrils with pure α-actinin caused the phase-dense Z lines to reappear (Robson et al., 1970). At 37°C α-actinin competes with tropomyosin for binding sites on F-actin while at 0°C it will displace tropomyosin, binding to F-actin at a molar saturation of 1 α-actinin/10 actin monomers (Robson et al., 1970; Goll et al., 1972). This saturation value can be derived from electron micrographs showing 300 x 20 Å rod-like α-actinin molecules crosslinking two parallel F-actin molecules like rungs of a ladder, the α-actinin rungs have a spacing of 370-400 Å (Podubnaya et al., 1975).

α-Actinin is found in a large variety of tissues. It can be purified from heart and smooth muscle as well as skeletal muscle (Ebashi et al., 1966; Langer and Pepe, 1980; Feramisco and Burridge, 1980). In cultured rat embryo cells, antibodies to skeletal α-actinin stain stress fibers with a 1.6 Å periodicity, in 0.4 Å patches that complement the 1.2 Å tropomyosin containing segments (Lazarides and Burridge, 1975), while in the columnar epithelial cells of the intestine α-actinin is found on terminal web roots (Geiger et al., 1979). Despite the immunological crossreactivity, α-actinins from different tissues are not homogeneous. Smooth and skeletal mussle

α-actinins show "considerable chemical and immunological differences" (Bretscher et al., 1979), and non-muscle (HeLa cell or human platelet) α-actinins are inhibited from binding to F-actin by micromolar quantities of Ca^{++}, unlike muscle α-actinins (Burridge and Feramisco, 1981; Rosenberg et al., 1981). During myogenesis α-actinin has a punctate distribution on stress fibers in myoblasts and early fused myotubes. One to two days after fusion α-actinin transits to the primitive Z discs (Gard and Lazarides, 1980). In adult Z discs α-actinin is found in the central domain (Granger and Lazarides, 1978).

Filamin

Filamin was first discovered as a major protein component of high salt extracts of chicken gizzard (Wang et al., 1975). Purification by ammonium sulfate fractionation, gel and ion exchange chromatography (Shizuta et al., 1976; Wang, 1977; Wallach et al., 1978; Feramisco and Burridge, 1980; Davies et al., 1982) gave a single band migrating at 250,000 daltons on SDS-polyacrylamide gels while sedimentation equilibrium analysis showed that the purified material was a dimer with molecular weight 500,000 dalton (Shizuta et al., 1976; Davies et al., 1977). Antibodies raised against this protein stained, using indirect immunofluorescence techniques, stress fibers in mouse 3T3 cells and primary cultures of chick gizzard or dorsal root ganglion (Wang et al., 1975). The antibodies were not staining myosin as shown by a lack of crossreactivity between anti-filamin and anti-myosin antibodies by double immunodiffusion. In cultured PtK2 rat kangaroo cells the fibrous patterns shown by indirect immunofluorescence labeling for filamin have been shown to correspond to the stress fibers observed in whole mount transmission electron microscopy of the same cells (Webster et al., 1978). Filamin and actin, but not myosin, are found by indirect immunofluorescence in microspikes, ruffles and in areas of cell-cell contact of rat fibroblasts (Heggeness et al., 1977). Filamin is also observed

on the basal side of chicken intestinal brush borders (Bretscher and Weber, 1978a,b). Two cell types that lack filamin are neurons and glial cells (Shaw et al., 1981).

Purified filamin interacts with purified filamentous (F) actin to form an oligomeric complex (Wang and Singer, 1977). When solutions of the two proteins at concentrations on the order of 0.5 mg/ml are mixed, they immediately interact to form a rigid gel. The gelation occurs over a wide variety of salt, divalent cation, ATP and EDTA concentrations.

Addition of tropomyosin to the F-actin prior to the addition of filamin abolishes the gelling effect of filamin (Maruyama and Ohashi, 1978; Zeece et al., 1979). Addition of tropomyosin to a preformed gel has no effect on the gel. This would suggest two possible uses of filamin within a cell: to control the amount of actin free to interact with myosin, by crosslinking actin filaments into a large complex, or as a modulator of a second type of actin containing filament, the first being those containing tropomyosin (Lazarides, 1976).

Unlike tropomyosin, α-actinin binds to actin at a site sufficiently far away from the filamin binding site so as to bind noncompetitively with filamin (Zeece et al., 1979), although it does compete with tropomyosin (Robson et al., 1979; Goll et al., 1979). However, immunofluorescence of capping cells shows that α-actinin accumulates under caps, and is thus probably involved in the capping process, while filamin does not change its distribution during the capping process (Geiger and Singer, 1979). Thus filamin and α-actinin are not necessarily located on the same population of actin filaments within a cell. Filamin can also be shown to interact with actin by virtue of the fact that, in concentrations below that required for gelation, it reduces the ability of F-actin to activate skeletal muscle heavy meromyosin (HMM) ATPase (Davies et al., 1977a).

Mammalian filamin, also called actin-binding protein (Hartwig and Stossel, 1975; 1982), forms gels with actin in the same manner as chicken filamin although

with slightly different kinetics (Boxer and Stossel, 1976; Hartwig and Stossel, 1976; Weihing, 1976; Schloss and Goldman, 1979; Jennings et al., 1981; Rosenberg et al., 1981; Rosenberg and Stracher, 1982; Carroll et al., 1982). This protein has the same molecular weight as chicken filamin, binds anti-filamin antibody, and is phosphorylated, like chicken filamin, in a cyclic AMP-dependent manner (Wallach et al., 1978).

Interestingly enough, both filamin and actin-binding protein are similar to human erythrocyte spectrin in their molecular weights, solubility properties and ability to form complexes with actin (Hartwig and Stossel, 1975). Although filamin and spectrin both resemble long rods when visualized by electron microscopy, filamin does not bind to the spectrin-binding sites on inside-out human red blood cell membranes (Tyler et al., 1980), has a different amino acid composition (Wang, 1977) and does not crossreact with antibodies to spectrin (Glenney et al., 1982). Similarly, antibodies to filamin do not crossreact with spectrin.

Direct visualization of avian filamin by electron microscopy shows it to be a long flexible rod, 980 Å long and 27 Å wide, forming 1930 Å long dimers (Castellani et al., 1981). Mammalian macrophage actin-binding protein has a similar flexible shape and forms 1620 Å long dimers (Hartwig and Stossel, 1981). Filamin and actin-binding protein bind to F-actin at a minimum of 350-400 Å intervals (approximately 1 filamin/14 actin monomers) (Schloss and Goldman, 1979; Hartwig and Stossel, 1981) to link two parallel actin filaments along their length or to link perpendicular actin filaments (Hartwig et al., 1980).

A Ca^{++}-activated protease, purified from porcine skeletal muscle, has been shown to cleave filamin into two subunits with molecular weights of 240,000 and 10,000 daltons (Davies et al., 1978a). The larger of the two can bind to actin as demonstrated by its ability to inhibit the activation of HMM ATPase by actin.

However, this large subunit cannot induce actin to form a gel. Because the activation of protease requires approximately 1 mM Ca^{++}, a level much higher than normally found in cytoplasm it has been suggested that the release of stored or extracellular Ca^{++} into the cell could allow control of the assembly and disassembly of actin bundles within a subcellular region.

Another interesting property of filamin is that it can be phosphorylated (Davies et al., 1977b; Davies et al., 1978). Homogenized normal rat kidney fibroblasts, guinea pig vas deferens or homogenized vas deferens incubated with [γ-^{32}P] ATP all show a phosphorylated 250,000 dalton protein that can be precipitated with antibody to chicken gizzard filamin. Purified filamin can be phosphorylated by cAMP-dependent protein kinase isolated from beef skeletal muscle. The effect of filamin phosphorylation on filamin-actin interactions is unknown.

Recently filamin has been shown to exist in skeletal muscle (Bechtel, 1979). Double immunofluorescence on myofibrils shows filamin to be localized at the Z line, in myofibrils from chicken skeletal muscle and in cultured rat skeletal muscle (L6) cells. Filamin is also present on the Z lines of chick heart muscle (Koteliansky et al., 1981). Filamin exists in myoblasts as part of filamentous structures. Because of its ability to crosslink actin, filamin probably plays an important role in determining the structure of a sarcomere much as spectrin is involved in the structure of an erythrocyte cytoskeleton and villin and fimbrin are involved in determining the structure of a microvillus.

This thesis is an attempt to study morphogenesis at the subcellular level by examining the behavior of an actin crosslinking protein during the morphogenesis of an actin-containing structure. Specifically, it consists of an examination of the synthesis, location, type and amount of filamin during skeletal myogenesis. I will try to correlate the morphological changes observed in developing sarcomeres

with the presence or absence of filamin as determined by immunofluorescence, immunoautoradiography and metabolic labeling (to examine the synthesis of filamin), the type of filamin as determined by two-dimensional peptide mapping and the quantity of filamin as determined by immunoprecipitation and densitometry of stained SDS-polyacrylamide gels. Finally, I will use some of these techniques to examine the role filamin may play in affecting the physiological properties of a sarcomere.

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Chapter 2.

THE SYNTHESIS AND DEPLOYMENT OF FILAMIN IN CHICKEN SKELETAL MUSCLE

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The Synthesis and Deployment of Filamin in Chicken Skeletal Muscle

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Summary

During myogenesis in vitro the actin-binding protein filamin is present in myoblasts and early fused cells and is associated with α-actinin-containing filament bundles, as judged by double immunofluorescence using antibodies specific for these two proteins. Approximately one day after cell fusion, yet before the development of α-actinin-containing Z line striations, filamin disappears from the cells. Later in myogenesis, several days after the appearance of α-actinin-containing Z line striations, filamin reappears and accumulates in the cells. Double immunofluorescence with antibodies to filamin and vimentin (or desmin) reveals that the newly appearing filamin localizes now to the myofibril Z line and is visible there shortly before vimentin or desmin becomes associated with the Z line. Immunofluorescent localization of filamin in isolated chicken skeletal myofibrils and Z disc sheets indicates that filamin has the same distribution as desmin and vimentin; it surrounds each myofibril Z disc and forms honeycomb-like networks within each Z plane of the muscle fiber. Filamin may thus be involved in the transition of desmin and vimentin to the Z disc. Analysis of whole-cell extracts by SDS-polyacrylamide gel electrophoresis and by immunoautoradiography shows that filamin is present in myoblasts and in myotubes early after cell fusion. Concomitant with the absence of filamin fluorescence during the subsequent few days of myogenesis, the quantity of filamin is markedly reduced. During this time, metabolic pulse-labeling with ^{35}S-methionine reveals that the synthetic rate of filamin is also markedly reduced. As filamin fluorescence appears at the Z line, the quantity of filamin and its synthetic rate both increase. The removal of filamin from the cells suggests that filamin either may not be required, or may actually interfere with a necessary process, during the early stages of sarcomere morphogenesis. These results also indicate that the periphery of the Z disc is assembled in at least two distinct steps during myogenesis.

Introduction

Filamin is a high molecular weight actin-binding protein (250,000 daltons) found in smooth muscle. The protein can be isolated from alveolar macrophages and leukemic leukocytes (Hartwig and Stossel, 1975), as well as from smooth muscle cells (Wang et al., 1975), and has the ability to interact with actin filaments in vitro (Hartwig and Stossel, 1975; Shizuta et al., 1976; Stossel and Hartwig, 1976; Wang and Singer, 1977; Brotschi et al., 1978). In immunofluorescence this protein is localized along actin filament bundles, microspikes and membrane ruffles in a variety of nonmuscle cells (Wang et al., 1975). More recently filamin was identified in avian and mammalian skeletal muscle cells and was shown to be a component of myofibril Z discs (Bechtel, 1979).

With the development of a new technique for the isolation of avian muscle Z discs (Granger and Lazarides, 1978), we have shown that these structures are composed of at least two distinct domains: a central one that contains α-actinin and actin, and a peripheral one composed of actin and the intermediate filament subunits, desmin and vimentin (Granger and Lazarides, 1979). Using antibodies to α-actinin as probes for the newly assembling Z discs and antibodies to desmin and vimentin as probes for intermediate filaments, we could show that these two domains of the Z disc assemble sequentially during myogenesis (Gard and Lazarides, 1980). α-Actinin is first localized in a punctate pattern along actin filament bundles in patterns similar to those seen in nonmuscle cells (Gard and Lazarides, 1980; Jockusch and Jockusch, 1980). During this time desmin and vimentin exist in a cytoplasmic filamentous network that exhibits no obvious association with the newly assembling Z discs. Later in myogenesis and several days after the development of α-actinin-containing Z line striations, desmin and vimentin become associated with the peripheries of the Z discs (Gard and Lazarides, 1980).

To determine the behavior of filamin during the assembly of the Z disc we have investigated the localization and biosynthesis of this molecule with respect to α-actinin and desmin (or vimentin) during myogenesis in vitro, and its localization in isolated Z discs.

Results

Presence and Disappearance of Filamin during the Early Stages of Myogenesis in Vitro

Immunofluorescence using directly conjugated antisera to skeletal muscle α-actinin reveals that α-actinin is first distributed in focal deposits along the phase-dense actin filament bundles as previously described (Gard and Lazarides, 1980). In Figure 1 double immunofluorescence with antibodies to filamin and to α-actinin reveals that these two molecules coexist on actin filament bundles during this early stage of myofibril assembly. The first recognizable α-actinin-containing Z lines become visible as early as three days after culture, depending upon the culture conditions (Gard and Lazarides, 1980). By the third day

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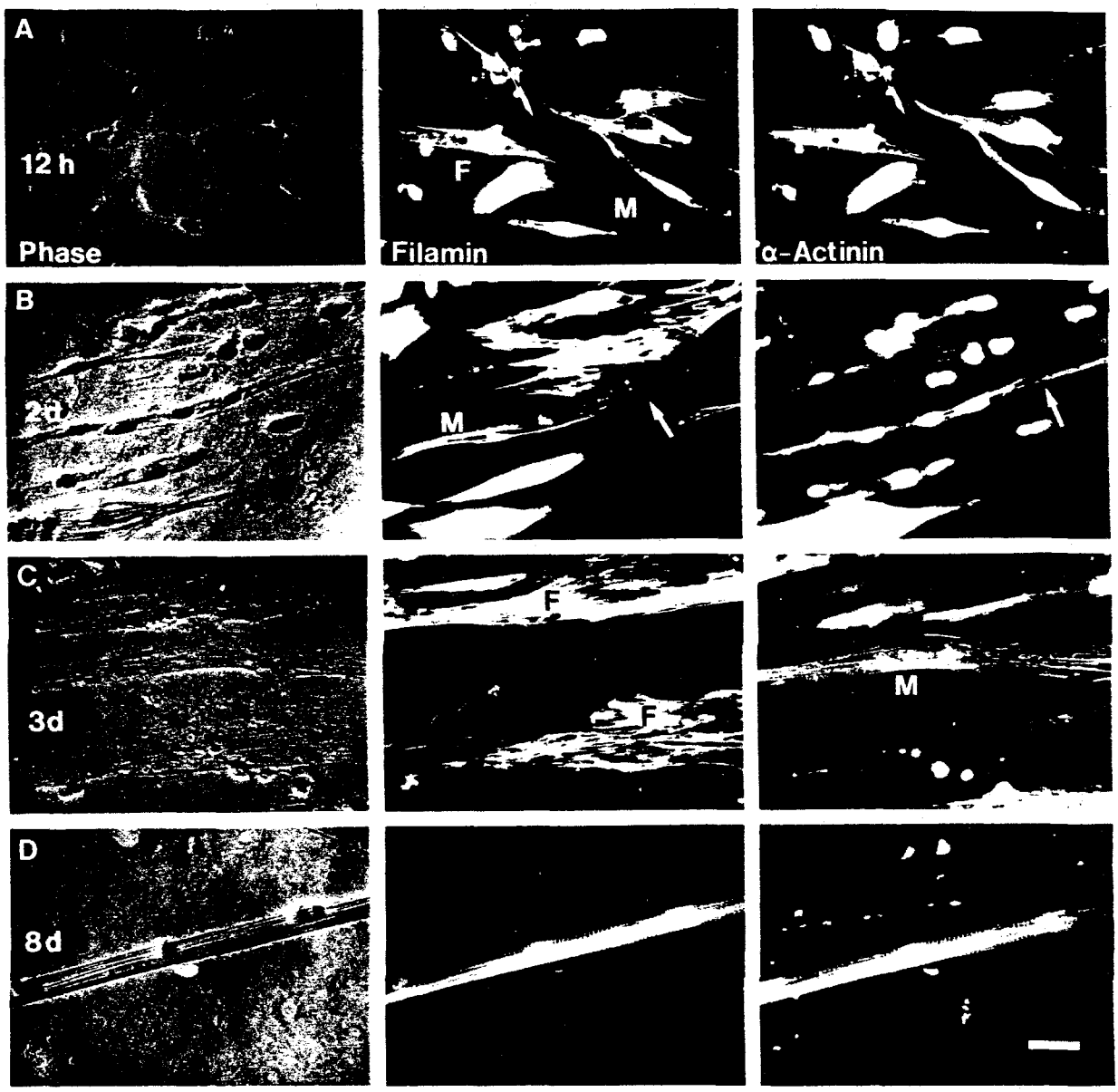

Figure 1. Double Immunofluorescent Localization of Filamin and α-Actinin during Myogenesis

(A) Twelve hours after plating, myoblasts (M) and fibroblasts (F) contain α-actinin and filamin.

(B) Two days after plating, a myotube (M) contains both filamin and α-actinin. However, the distribution of the two molecules is not coincidental, filamin being absent or markedly reduced in a subset of actin filament bundles (arrows).

(C) Three days after plating, filamin is completely absent from myotubes (M) while remaining in fibroblasts (F); note that the myotube depicted in this figure shows two different developmental stages: α-actinin in actin filament bundles and in Z line striations.

(D) Eight days after plating, α-actinin and filamin are both located at the myofibril Z line. Bar = 20 μm.

of culture and well before the appearance of α-actinin-containing Z line striations, the filamin fluorescence disappears, and remains absent during the subsequent three days of differentiation (Figure 1C). Close comparison of the double immunofluorescent images of filamin and α-actinin near the time of filamin disappearance indicates that filamin does not disappear uniformly from all the stress fibers in a given myotube; rather it disappears from some actin filament bundles while remaining on others.

The absence of filamin fluorescence between the second and the sixth days of myogenesis was observed using four different fixation and cell permeabilization protocols for indirect immunofluorescence (see Experimental Procedures). All four fixation protocols yielded identical results for filamin, under conditions that clearly reveal the localization of α-actinin, desmin and vimentin, as well as tropomyosin, myosin

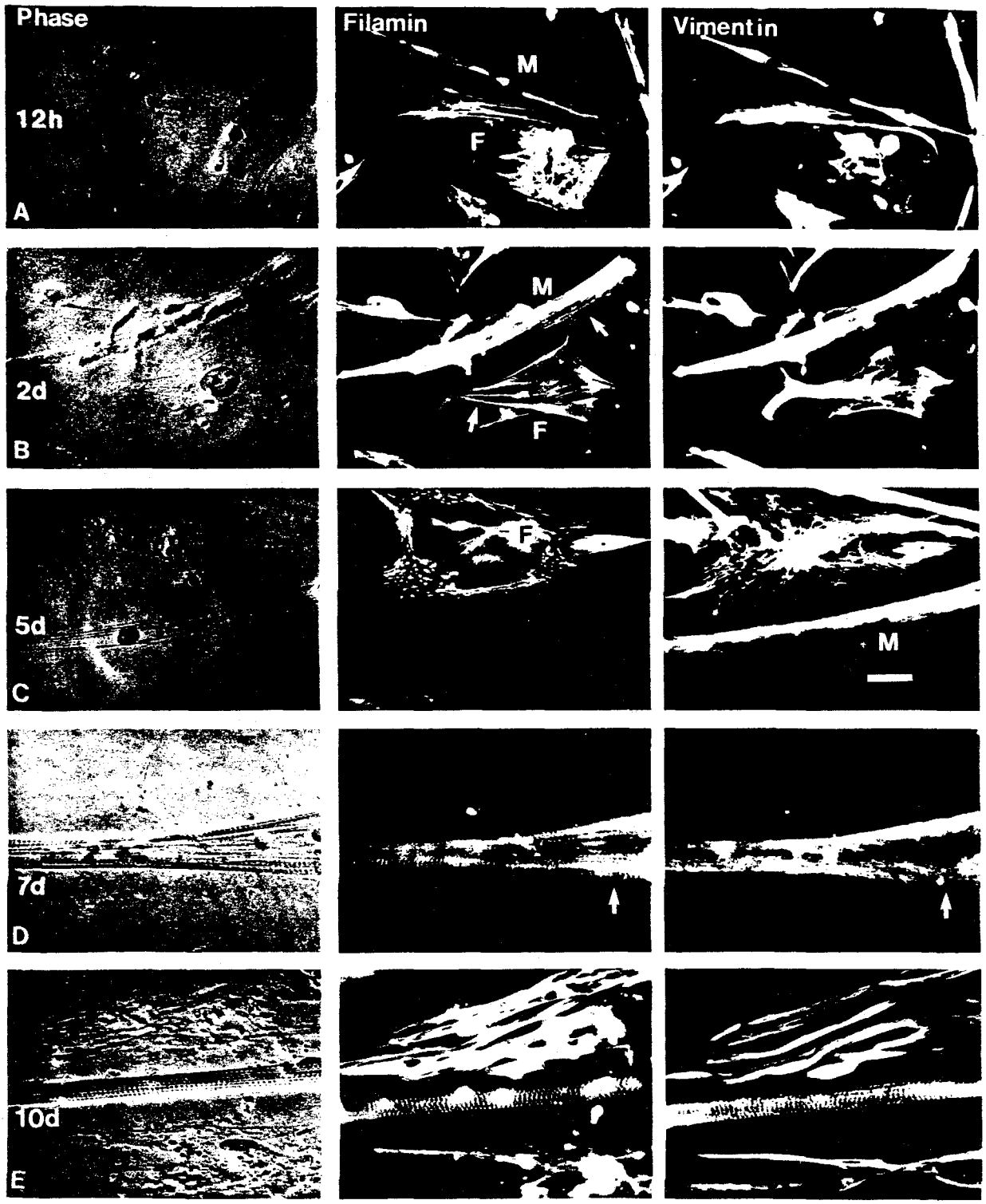

Figure 2. Double Immunofluorescent Localization of Filamin and Vimentin during Myogenesis

(A) Twelve hours after plating, myoblasts (M) and fibroblasts (F) contain vimentin and filamin. The distribution of the two antigens is different in both cell types.

(B) Two days after plating, filamin is present in actin filament bundles (arrows) in a multinucleate myotube (M) and fibroblasts (F). Vimentin shows a much more irregular pattern.

(C) Three days after plating, filamin is absent from a myotube (M) while still present in a fibroblast (F). This fibroblast exhibits a geodesic dome-like structure (Lazarides, 1976) containing filamin but not vimentin. Vimentin still exists in an irregular cytoplasmic filamentous network.

(D) Seven days after plating, filamin reappears in the myotube. The absence of any diffuse filamin fluorescence, and the presence of filamin-containing Z lines in areas of the cell where vimentin is still in its characteristic cytoplasmic irregular network (arrows), indicate that filamin upon its resynthesis rapidly associates with the Z line before vimentin (and therefore desmin; see Gard and Lazarides, 1980).

(E) Ten days after plating, myotubes contain both filamin and vimentin at the Z line. Bar = 20 μm.

and actin (data not shown). It appears therefore that the filamin antigen is not selectively masked or extracted from the cells by the fixation technique during its period of disappearance from the cytoplasm.

Reappearance of Filamin at the Later Stages of Myogenesis in Vitro

As previously reported (Gard and Lazarides, 1980), during the earlier stages of myofibril assembly (days 0–6) vimentin (and desmin) is distributed as a fine filamentous network filling the sarcoplasm. Double immunofluorescence during this time, with antibodies to filamin and vimentin, indicates that vimentin exists in this filamentous network both during the time that filamin is associated with actin filament bundles and during filamin's subsequent disappearance (Figure 2).

After approximately seven days in culture, many myotubes exhibit numerous individual myofibrils, visible by both phase-contrast microscopy and α-actinin Z line fluorescence (for example see Figure 1D). At this time filamin fluorescence becomes visible in the cytoplasm. Figure 1D shows that the newly appearing filamin localizes now to the myofibril Z line, as revealed by the coincident double immunofluoresence images obtainable with α-actinin and filamin antibodies and Z lines by phase-contrast microscopy. Comparison of the localization of the newly appearing filamin with that of vimentin (or desmin) shows that filamin associates with the Z lines shortly before the transition of vimentin to this structure (Figure 2D). Figure 2D shows two regions of the myotube's cytoplasm, one in which filamin is associated with Z lines while vimentin still exists in a cytoplasmic filamentous form, and another where the two molecules exhibit coincidental distribution of the Z line. Examination of a large number of cells has indicated that in every case the association of filamin with the Z line precedes the transition of vimentin (and therefore desmin; Gard and Lazarides, 1980) to this structure. After longer times in culture (ten days) myotubes have filamin, α-actinin, vimentin and desmin on every Z line (Figure 2E), as is the case with myofibrils from adult chicken skeletal muscle. Figure 3 shows the localization of filamin and desmin at the Z line at higher magnification.

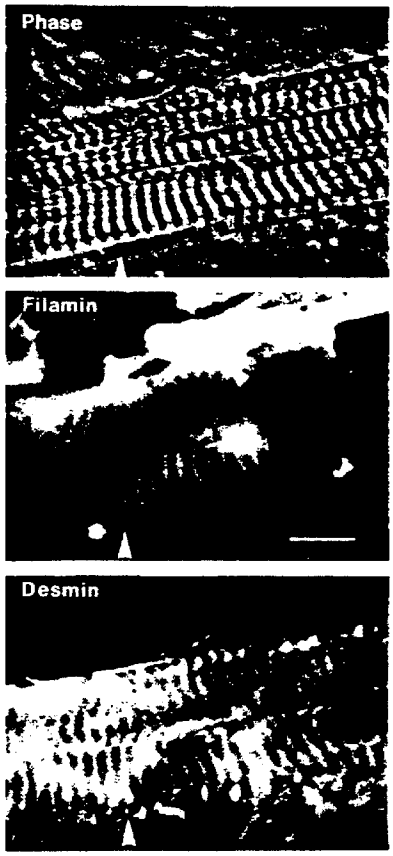

Figure 3. Double Immunofluorescent Localization of Filamin and Desmin in a Fully Differentiated Myotube

A careful comparison shows both sets of striations to be localized at phase-dense Z lines (arrows). Bar = 10 μm.

Filamin Localization in Z Disc Sheets

Affinity-purified antibodies to chicken gizzard filamin were used in indirect immunofluorescence to determine the localization of filamin in isolated Z disc sheets (Granger and Lazarides, 1978). As previously observed (Bechtel, 1979), filamin was found to be a component of isolated chicken skeletal myofibrils (results not shown). Figure 4A shows that extraction of myofibrils for short periods of time (45 min) with 0.6 M KI leaves filamin associated with the remaining Z disc scaffolds. Under these conditions desmin, vimentin and small amounts of α-actinin also remain associated with these Z disc scaffolds (Granger and Lazarides, 1979). Figure 4B shows that filamin is localized at the periphery of each Z disc, forming a network within the Z plane. This distribution of filamin in Z disc sheets is indistinguishable from that previously de-

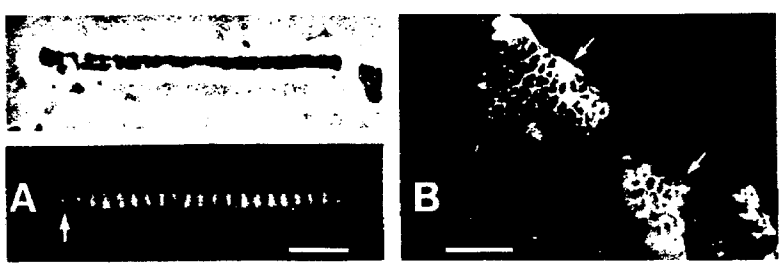

Figure 4. Localization of Filamin in 0.6 M KI-Extracted Myofibrils and in Z Disc Sheets

(A) Filamin is a component of the KI-extracted Z line (arrow). (Top) phase-contrast; (bottom) fluorescence photomicrographs.

(B) Filamin is located at the periphery of each Z disc and occasionally in a spot or line in the center of a Z disc (arrows). Bars = 10 μm.

Figure 5. Synthesis of Filamin during Myogenesis in Vitro

Essentially identical cultures of the indicated ages after plating containing approximately the same number of cells (determined by their nuclear index) were pulse-labeled for 1 hr with 10 μCi ^{35}S-methionine (spec. act. 830 Ci/mmole) per ml of culture media. Cells were harvested, solubilized in SDS sample buffer and frozen. After thawing, aliquots were counted by trichloroacetic acid (TCA) precipitation and an equal number of counts for each time point were loaded on a 10% polyacrylamide-SDS gel. Bands indicated are filamin (F) and myosin (M). Filamin synthesis is detectable at days 0.5, 1, 7 and 10, and is absent from day 2 to day 6.

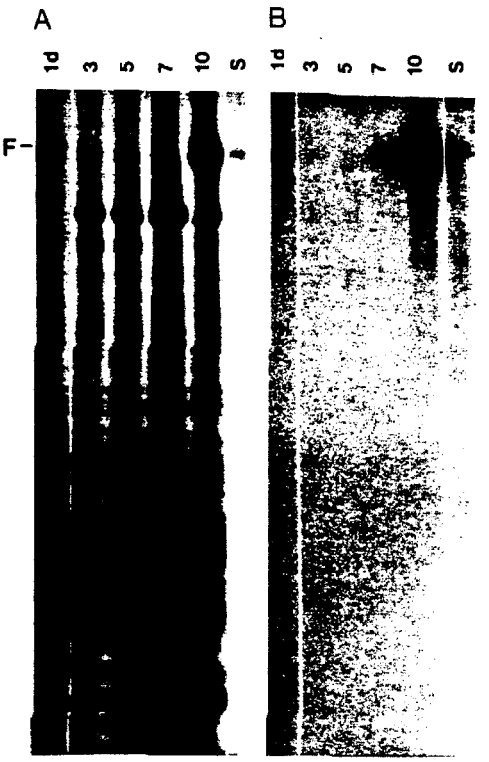

Figure 6. Presence of Filamin during Myogenesis

An equal number of cells (determined by their nuclear index) of the indicated ages after plating were solubilized and run on a 10% polyacrylamide-SDS gel. Approximately equal amounts of protein were loaded in each well. After staining for filamin by immunoautoradiography (Granger and Lazarides, 1979) (B), gels were stained for protein with Coomassie brilliant blue R-250 (A).

(A) Filamin (F) is present a day 1 and day 7 and is absent from day 3 to day 5.

(B) Anti-filamin antibodies react with filamin at day 1 and day 7, and detect little filamin antigen from day 3 to day 5. Note that the filamin antibody does not react with any other proteins in whole-cell extracts. *The same result was obtained with crude or affinity-purified IgG. S*, standard of purified smooth muscle filamin (1 μg).

scribed for desmin and vimentin, and complementary to that of α-actinin. Figure 4B shows that, besides being present at the periphery of the Z disc, filamin is occasionally present at a spot near the center of the Z disc. Similar patterns have been observed for desmin and vimentin (Granger and Lazarides, 1979). These patterns probably correlate with the fissures down the lengths of large myofibrils (as observed by electron microscopy), which are presumably manifestations of myofibril subdivision (Goldspink, 1970, 1971; Shear, 1978).

Prolonged extraction of myofibrils or Z disc sheets with 0.6 M KI (12 hr or more) results in the diminution and ultimate removal of all filamin fluorescence, while desmin and vimentin fluorescence remains unaltered (data not shown).

Expression of Filamin during Myogenesis

For the synthesis studies it was important to ensure that the number of fibroblasts in the cultures used was <1%. This was achieved by multiple preplating of the primary cultures, as well as the addition of Ara-C during the second and third days after plating of secondary cultures. Myotubes labeled for short periods of time with ^{35}S-methionine synthesize several *major proteins, as revealed by one-dimensional SDS* gel electrophoresis. Within the first two days after fusion, filamin can be identified as one of the proteins being synthesized, by its comigration on SDS-polyacrylamide gels with filamin purified from chicken gizzard. By the second day in culture, synthesis of filamin is markedly reduced (Figure 5). Reduced rates of filamin synthesis persist for the next four days, in accordance with the immunofluorescent results reported above. By day 7 in culture, synthesis of filamin is detectable again and continues through the subsequent days of myogenesis (day 10). Quantitation of the amount of synthesis of filamin within the first day of myogenesis and after its reappearance by day 10 indicates that it represents approximately 0.2% of the total synthetic activity of the cells in both cases, while in 4–5 day cultures it is below 0.01%.

Comparable samples to those used to determine *the synthetic rates of filamin were analyzed* by one-dimensional SDS gel electrophoresis, reacted with antibodies to filamin in immunoautoradiography and stained with Coomassie brilliant blue to determine the total quantity of filamin at the different stages of myogenesis. Figure 6 shows that within the first two days of myogenesis filamin is clearly present in total cell extracts. By the second day of myogenesis and for the next five days thereafter the quantity of filamin

drops to almost undetectable levels. However, in the next three days (days 7–10) filamin continues to accumulate and by day 10 it is clearly detectable as a component in total cell extracts.

The immunoautoradiogram of the Coomassie blue-stained gel in Figure 6 demonstrates unambiguously that the antibody to filamin is highly specific for filamin and only detects filamin in total cellular extracts; filamin is present in 1, 7 and 10 day myotubes while it is markedly reduced in 3 day to 5 day myotubes; and the molecular weight of the newly accumulated filamin is the same as chicken gizzard filamin. The small amount of filamin detected at days 3 and 5 is due to the presence of a residual number (<1% of total cells) of contaminating fibroblasts.

These results indicate that a filamin synthesis ceases within two days and remains undetectable for the next five days of myogenesis in vitro; filamin disappears from the cells; and filamin synthesis is reinitiated within seven days of myogenesis in vitro and accumulates in the cells in the ensuing days of myogenesis.

Expression of Filamin during Myogenesis in Vivo

To ensure that the phenomenon of filamin disappearance and reappearance is not an artifact of the in vitro conditions of myogenesis, we examined the localization of filamin in sections of frozen thigh muscle at different stages of chick embryonic development. Since desmin antibodies react specifically with muscle cells, we used these antibodies in double immunofluorescence to unambiguously identify the presence or absence of filamin in myogenic cells. In frozen sections of 11-day-old embryonic thigh muscle, double immunofluorescence with desmin and filamin antibodies reveals that filamin is present in myotubes (Figure 7A). Phase-contrast microscopy indicates that these cells are still at the earlier stages of myofibril differentiation, since no Z line striations are detectable. In Figure 7B double immunofluorescence on frozen sections of 14-day-old embryonic thigh muscles demonstrates that filamin is present in fibroblastic cells but is absent from muscle cells. The presence of phase-contrast Z line striations and the presence of desmin in a cytoplasmic filamentous form clearly iden-

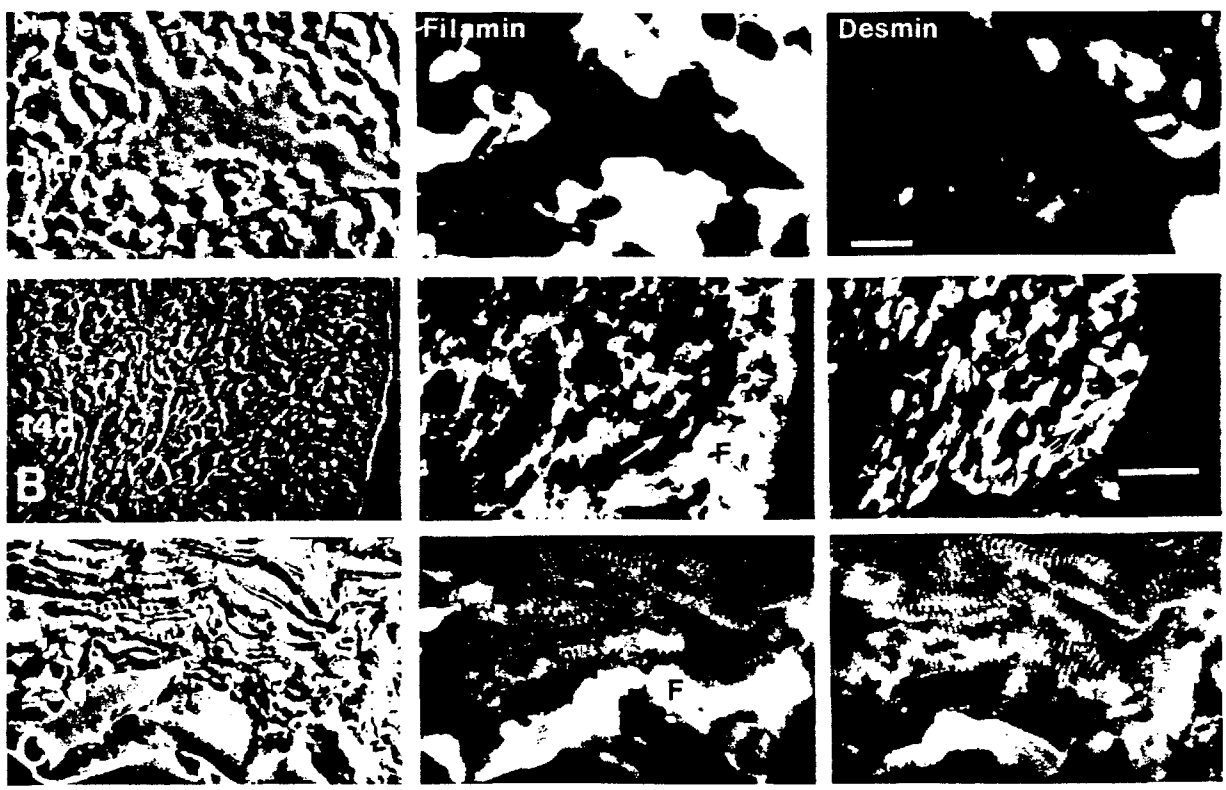

Figure 7. Double Immunofluorescent Localization of Filamin and Desmin during Myogenesis in Vivo

Approximately 8 μm-thick frozen sections were cut from fixed or unfixed pieces of embryonic leg muscle tissue and stained for filamin and desmin.

(A) An 11 day embryo shows myoblasts and fibroblasts containing filamin but no desmin; an occasional myotube contains desmin and filamin. Bar = 25 μm (same magnification for C).

(B) At 14 days, a section containing the periphery of a muscle shows a broad band of filamin-containing fibroblasts in connective tissue (F) and a large area of myotubes that contain desmin but not filamin (arrows). Filamin-containing fibroblasts are interspersed among the myotubes. Bar = 20 μm.

(C) Two days after hatching, chick leg muscle cells contain both filamin and desmin in a typical striated appearance. Fibroblasts (F) contain filamin but not desmin.

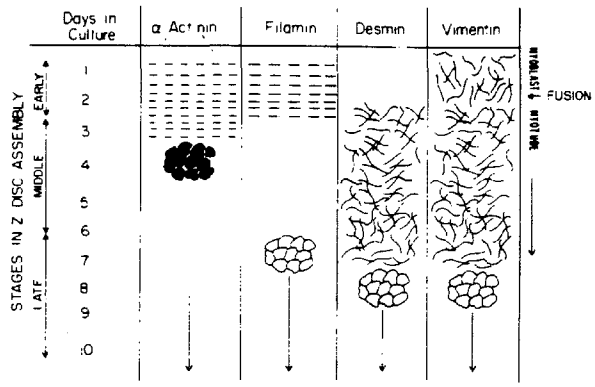

Figure 8. Diagram indicating the Presence and Morphology of α-Actinin, Filamin, Desmin and Vimentin during myogenesis

Dashed lines: speckled appearance of antigen on actin filament bundles; blank: absence of an antigen; wavy lines: tangled appearance of the intermediate filament subunits, desmin and vimentin. Solid patterns: Z line striations (the antigen being found in the interior of adult Z discs); open patterns: Z line striations with the antigen located at the periphery of adult Z discs.

tifies these myotubes as being at a stage before the association of desmin with the Z line. Figure 7C shows that within the first day of hatching both desmin and filamin are found in association with Z lines. These results clearly indicate that the disappearance and reappearance of filamin also occur during normal chick embryonic development in vivo, and that the events observed during in vitro myogenesis accurately reflect the in vivo situation.

Discussion

Distribution of Filamin during Myogenesis

Immunofluorescence studies on the localization of filamin in nonmuscle cells have indicated that this molecule associates with actin filament bundles (Wang et al., 1975), as do the nonmuscle forms of α-actinin (Lazarides and Burridge, 1975), tropomyosin (Lazarides, 1975) and myosin (Weber and Groeschel-Stewart, 1974; Fujiwara and Pollard, 1976). In this paper we have shown that filamin also associates with actin filament bundles during early myogenesis. How then does filamin become associated with the peripheral domain of Z disc? From the observations presented in this paper, it appears that within the first two days of myogenesis, and before the formation of α-actinin-containing Z line striations, filamin disappears from the cells. Later on in myogenesis and shortly before the transition of desmin and vimentin to the Z disc, filamin reappears and associates directly with the Z disc. A summary diagram of the association of these molecules with the Z disc during myogenesis is presented in Figure 8.

Synthesis of Filamin during the Earlier and the Later Stages of Myogenesis

In accordance with the immunofluorescence observations, filamin is synthesized during the first two days after myoblast fusion, but its rate of synthesis decreases dramatically thereafter. The molecular details behind this observation await further experimentation. Similarly, the mechanism that operates to remove filamin from the cytoplasm is also unknown. We presume that once the rate of synthesis of this molecule is decreased, then filamin is removed by protein turnover. However, it is intriguing that this mechanism does not operate simultaneously throughout the myotube's cytoplasmic space. This is shown by the observation that filamin is not removed from all actin filament bundles simultaneously. Since the process of myofibril assembly is asynchronous within a cell, such a selective removal suggests that filamin is removed when a specific point in the early stage of myofibril assembly is reached. Later on in myogenesis, corresponding to the immunofluorescent reappearance of filamin in the myotubes, the synthesis of filamin increases. This molecule is clearly detectable as one of the proteins being synthesized by the tenth day of myogenesis, both by pulse-labeling experiments and by immunoautoradiography on whole-cell extracts. Again, the molecular details that culminate in an increase in the rate of synthesis of this protein are presently unknown. It is also unknown whether the filamins expressed early and late in differentiation are actually the same gene product or whether the filamin expressed late in myogenesis is due to the expression of a new filamin gene.

Possible Regulatory Roles of the Disappearance and Reappearance of Filamin

A considerable amount of evidence has demonstrated that filamin is an actin-binding protein capable of interacting with actin filaments (Hartwig and Stossel, 1975, 1978; Shizuta et al., 1976; Stossel and Hartwig, 1976; Wang and Singer, 1977; Brotschi et al., 1978). Under certain in vitro conditions, filamin markedly reduces the actin-activated myosin ATPase (Davies et al., 1977) and competes with tropomyosin for binding sites on actin filaments (Maruyama and Ohashi, 1978; Zeece et al., 1979). It is therefore possible that, at the time or shortly after the time of filamin disappearance, a myosin–actin filament interaction and the activation of the myosin ATPase by actin become vital in order to bring about a reorganization of the assembling sarcomeres. Actin filaments complexed with filamin may not be competent to activate the myosin ATPase, and the removal of filamin may be a necessary prerequisite for the subsequent assembly of sarcomeres. This is in accordance with the observation reported here that within a day after the removal of filamin the Z lines appear, as shown by the striated localization of α-actinin.

The reappearance of filamin later on in myogenesis suggests that this molecule is necessary for the final assembly of the Z disc. Since upon its reappearance filamin associates with the Z disc shortly before the association of desmin and vimentin with this structure,

filamin may regulate some part of this process. We have previously shown that actin is present in both the central and the peripheral domains of the Z disc (Granger and Lazarides, 1978). Filamin may therefore bind to an actin site at the periphery of the Z disc. Such a filamin–actin interaction at the periphery of the Z disc may be a prerequisite for the association of desmin and vimentin with this structure. This could explain the persistent copurification of small amounts of actin (Hubbard and Lazarides, 1979) and filamin (our unpublished observations) with desmin when the latter is purified from smooth muscle by cycles of polymerization and depolymerization.

Experimental Procedures

Filamin Purification and Antibody Preparation

Filamin was purifed according to the method of Wang (1977). Briefly, chicken gizzard smooth muscle was homogenized in 0.3 M KCl, 2 mM K_2ATP, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.05 M imidazole, pH 6.9, 0.1 mM phenylmethyl sulfonylfluoride (PMSF). The extract was clarified at 10,000 × g and the supernatant was precipitated with 16 g ammonium sulfate/100 ml. The pellet was suspended in 0.6 M KCl, 2 mM $MgCl_2$, 1 mM ethylenediamine tetraacetate (EDTA), 0.1 mM DTT, 0.01 M Tris–HCl, pH 7.4, clarified and run on a Biogel A-15m column. The filamin peak fractions were pooled and precipitated at 50% ammonium sulfate saturation, and the precipitate was pelleted and resuspended in 0.02 M Tris–acetate, pH 7.6, clarified and run on a Whatman DE-52 ion exchange column with a KCl gradient in the Tris–acetate buffer. The filamin purified by this procedure showed no detectable impurities as judged by SDS-polyacrylamide slab gel electrophoresis. Gel electrophoresis in the presence of SDS was performed as previously described (Hubbard and Lazarides, 1979).

For immunization of rabbits, filamin was electrophoresed on a 7.5% SDS-polyacrylamide slab gel. Gels were briefly stained with Coomassie brilliant blue R-250 and destained; the filamin band was cut out and neutralized in several changes of phosphate-buffered saline (PBS). Bands were homogenized in a motor-driven Teflon homogenizer, precipitated with 1% $AlCl_3$, neutralized with NaOH and emulsified with Freund's adjuvant. Approximately 0.7 mg of protein was injected subcutaneously into a female New Zealand white rabbit. The immunization protocol was essentially as previously described for vimentin antibodies (Granger and Lazarides, 1979). Blood serum was precipitated at 50% ammonium sulfate saturation and the partially purified IgG was dialyzed against PBS with 0.01 M sodium azide. For the affinity purification of filamin antibodies, 200 μg of filamin were coupled to 2 ml of CNBr-activated Biogel A-15m as described (Lazarides and Lindberg, 1974). Unbound protein was washed off in a sintered glass funnel with 0.1 M $NaHCO_3$, then with 0.1 M $NaHCO_3$, 0.5 M NaCl and the reaction was terminated by incubating the beads in 0.5 M ethanolamine, pH 9.6, for 2 hr at room temperature (rt) with gentle shaking. Beads were then washed with 0.1 M $NaHCO_3$, then alternately with 0.1 M sodium borate, 0.5 M KCl, pH 9.0, and 0.1 M sodium acetate, 0.5 M KCl, pH 4.0, and finally with PBS at rt. One milliliter each of ammonium sulfate-cut preimmune and immune serum was added to a slurry of 2 g beads in 5 ml PBS, and this mixture was gently shaken overnight at 4°C. The beads were then placed into columns and washed with PBS until the OD_{280} of the outflow was below 0.002. The columns were then washed with PBS, 0.6 M NaCl until the outflow OD_{280} was below 0.002. Bound antibody was eluted with 0.6 M NaCl, 0.2 M glycine, pH 2.4, and immediately neutralized with NaOH. Affinity-purified antibodies were dialyzed against PBS, 5 mM NaN_3 and stored at 4°C. One milliliter of serum would yield typically 100 μg of affinity-purified antibody. Affinity-purified preimmune serum gave no immunofluorescent reaction.

Filamin antibodies gave a single precipitin line against partially purified filamin in Ouchterlony immunodiffusion (data not shown). Crude or affinity-purified filamin antibodies reacted only with one band at 250,000 daltons in whole chicken myotube extracts, as determined by immunoautoradiography on 10% polyacrylamide gels. This band comigrated with purified filamin (see text). Under the same conditions preimmune sera did not bind to any proteins in the gel. Immunoautoradiography was performed as described previously (Granger and Lazarides, 1979).

Antibodies to α–actinin, desmin and vimentin were those used in previous studies (Lazarides and Burridge, 1975; Granger and Lazarides, 1978, 1979). Rhodamine B isothiocynate (RBITC) conjugation of these antisera was as previously described (Gard and Lazarides, 1980), following the method of Cebra and Goldstein (1965) and Brandtzaeg (1973). $OD_{280/560}$ ratios were 4.3 for RBITC anti-α–actinin, 1.3 for RBITC anti-vimentin and 2.0 for anti-desmin.

Immunofluorescence

Cells grown on collagen-coated glass coverslips were treated by one of the following procedures. All fixations preserved the apparent distribution of vimentin and α–actinin, while showing the disappearance of filamin at the appropriate time.

—0.5% Triton X-100, 0.37% formaldehyde in PBS, 37°C, 5 min; 3.7% formaldehyde in PBS, 37°C, 15 min; PBS, rt, 10 min; 0.1% Trition X-100 in PBS, rt, 10 min and all washes in this buffer.

—0.5% Triton X-100, 0.37% formaldehyde in PBS, 37°C, 5 min; 3.7% formaldehyde in PBS, 37°C, 15 min; PBS, 10 min, 95% ETOH, rt, 10 min, PBS, 10 min and washes in PBS.

—3.7% formaldehyde in PBS, 37°C, 15 min; PBS, 10 min; 95% ETOH, rt, 10 min; PBS, rt, 10 min and washes in PBS.

—100% acetone, rt, 15 min; PBS, rt, 10 min and washes in PBS.

Double immunofluorescence and antibody incubations were as previously described (Granger and Lazarides, 1979; Gard and Lazarides, 1980). Immunofluorescence on Z disc sheets and myofibrils was as previously described (Granger and Lazarides, 1979). Other details of microscopy and photography were performed as described (Granger and Lazarides, 1979; Gard and Lazarides, 1980).

Preparation of Tissue Material

Z disc sheets and myofibrils were prepared from adult glycerinated pectoralis or thigh skeletal muscle as described (Granger and Lazarides, 1979). Since prolonged extractions removed all of the filamin fluorescence, Z disc sheets were prepared after 12 hr of extraction at rt. Myofibrils and KI-extracted myofibrils were prepared as described (Granger and Lazarides, 1979).

Frozen sections of embryonic muscle were obtained as described previously (Lazarides and Hubbard, 1976). Sections were approximately 8 μm thick. Tissue was excised from embryos or hatched chicks, cut into 2 mm cubes and either immediately frozen in Tissue-Tek O.C.T. compound or fixed at rt in 3.7% formaldehyde-PBS for 1 hr and then frozen in O.C.T. compound. Sections were picked up on glass coverslips and were immersed immediately in 95% ETOH at rt for 10 min. Prior to incubation with antibodies, the sections were rinsed with PBS.

Preparation of Myogenic Cultures

Primary and secondary cultures of embryonic chicken thigh muscle were prepared from 10-day-old embryos as previously described (Gard and Lazarides, 1980). Metabolic labeling of cells with ^{35}S–methionine was as previously described (Gard and Lazarides, 1980). Cultures were rinsed with PBS and harvested with a rubber policeman into 0.5 ml of SDS sample buffer and were placed immediately in a boiling water bath for 2 min. Details and quantitation of filamin levels at different stages of myogenesis were the same as previously described for desmin and vimentin (Gard and Lazarides, 1980). In Figure 5 the area of the gel corresponding to a filamin standard was cut from the different time points and the gel slices were solubilized overnight in a toluene-based scintillation mixture containing 5% NCS and 0.5% distilled water. Results are reported as the percentage of total (TCA-precipitable) protein synthesis.

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Note Added in Proof

Antibodies to native chicken gizzard filamin give identical results in the immunofluorescence studies on the disappearance of filamin as those reported here with antibodies to filamin purified by SDS gel electrophoresis prior to immunization.

Chapter 3.

SWITCHING OF FILAMIN POLYPEPTIDES DURING MYOGENESIS *IN VIVO*

ABSTRACT During chicken skeletal myogenesis in vitro, the actin binding protein filamin is present at first in association with actin filament bundles both in myoblasts and in myotubes early after fusion. Later in mature myotubes it is found in association with myofibril Z discs. These two associations of filamin are separated by a period of several days, during which the protein is absent from the cytoplasm of differentiating myotubes (15). To characterize the two classes of filamin polypeptides we have compared by two dimensional peptide mapping ^{125}I- labeled filamin immunoprecipitated from myoblasts and fibroblasts to filamin immunoprecipitated from mature myotubes and adult skeletal myofibrils. Myoblast filamin is highly homologous to fibroblast and purified chicken gizzard filamins. Mature myotube and adult myofibril filamins are highly homologous to each other but exhibit extensive peptide differences from the other three classes of filamin. Comparison of peptide maps from immunoprecipitated ^{35}S-methionine-labeled filamins also shows that fibroblast and myoblast filamins are highly homologous to each other but show substantial peptide differences from mature myotube filamin. Filamins from both mature myotubes and skeletal myofibrils exhibit a slightly higher electrophoretic mobility than gizzard, fibroblast and myoblast filamins. Short pulse labeling studies show that mature myotube filamin is synthesized as a lower molecular weight variant and is not derived from a higher molecular weight precursor. These results suggest that myoblast and mature myotube filamins are distinct gene products and that during skeletal myogenesis in vitro one class of filamin polypeptides is replaced by a new class of filamin polypeptides, and that the latter is maintained into adulthood.

INTRODUCTION

The development of myofibrils in skeletal muscle is an excellent system to study molecular morphogenesis in a eucaryotic system. The process has been studied by electron microscopy (11), immunofluorescence using antibodies to various myofibril proteins (13, 35) and biochemical identification of the contractile proteins present at different stages (8, 36). However the molecular details of the assembly of the different myofibril substructures are at present poorly understood.

Filamin is a high molecular weight (250,000 dalton) actin-binding protein originally isolated from avian smooth muscle (39) and is closely related to an actin binding protein isolated from mammalian macrophages (20). Immunofluorescence studies have shown that filamin is associated with actin filament bundles in the cytoplasm of cultured fibroblasts and in vitro studies have shown that it interacts with actin filaments causing their gelation by crosslinking (3, 20, 33, 34, 40).

A protein with approximately the same molecular weight as filamin has been partially purified also from adult skeletal muscle (1). This protein is antigenically related to gizzard filamin; by immunofluorescence it is localized at myofibril Z-lines. We have previously studied the expression and distribution of filamin during myogenesis in vitro with respect to other known Z-disc proteins such as α-actinin, desmin and vimentin (15). By immunofluorescence, we observed that in myoblasts and early after the onset of fusion, filamin is found in association with actin filament bundles. However within three days after the onset of fusion, and before the development of α-actinin containing Z-line striations, filamin disappears from the cytoplasm. Several days after the development of α-actinin-containing Z-line striations, filamin is resynthesized and becomes associated with Z-lines (15).

In this paper we have isolated myoblast and mature myotube filamins by immunoprecipitation and have compared them by two dimensional peptide mapping to each

other as well as to adult skeletal muscle, adult smooth muscle and embryonic fibroblast filamins. We show that myoblast and mature myotube filamins differ from each other both in their peptide maps and in their electrophoretic mobilities. On the other hand, myoblast filamin is highly homologous to adult gizzard and fibroblast filamin, while mature myotube filamin is highly homologous to adult skeletal muscle filamin. These results suggest that myoblast and mature myotube filamins are distinct gene products, and that during myogenesis the synthesis of the myoblast filamin is switched off and replaced later on in myogenesis by a new class of filamin polypeptides.

MATERIALS AND METHODS

Cell Culture

Chicken embryonic fibroblasts and embryonic myogenic cells were grown as previously described (13), with the following modifications: To obtain myogenic cultures sufficiently pure for this study, primary cell cultures were preplated twice for 1/2 h on plastic tissue culture dishes (Falcon 3003, Oxnard, CA) and then plated onto collagen-coated petri dishes at a density of 1×10^7 cells/100 mm dish. After 18 h, secondary cultures were made by rinsing the cells three times with calcium-and magnesium-free Earle's balanced salt solution, removing the myoblasts by the addition of trypsin for 3 min at 37°C, neutralizin the trypsin with growth medium and washing the released cells by sedimentation twice in growth medium. Cells were again preplated twice for 1/2 h at 1×10^7 cells/100 mm dish and then plated onto collagen-coated dishes at a density of 3×10^6 cells/100 mm dish. Chick embryo fibroblasts were obtained by trypsinization of the first preplates after 3 days of incubation in complete medium and were grown in complete medium on non-collagenized petri dishes. Metabolic labeling of cell cultures with ^{35}S-methionine was as previously described (13; see figure legends for details).

Sample Preparation

Skeletal myofibrils were prepared from adult chicken pectoral muscle by trimming a piece of muscle free of fat and connective tissue, and homogenizing it in a Lourdes blender at top speed for 30 sec in ice cold 20 mM Tris/HCl, 100 mM KCl, 5 mM ethylenegly bis-(β-amino ethylether) N,N,N',N' tetraacetic acid (EGTA), pH 7.5. Myofibrils were then purified by filtering the homogenate twice through two layers of cheesecloth followed by centrifugation at 1500xg for 5 min. The pellet was then immediately resuspended in 1% sodium dodecyl sulfate (SDS), 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5 and boiled for 1 min at a concentration of approximately 10 mg wet weight of myofibrils per ml.

Adult chicken gizzard was trimmed free of fat and connective tissue and homogenized for 20 sec at top speed in a Lourdes blender in ice cold 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5 at a concentration of approximately 1 mg wet weighted gizzard/ml, 20% SDS was added to a final concentration of 1% and the extract was then boiled for 1 min.

For immunoprecipitation, cell cultures were rinsed at room temperature with phosphate buffered saline (PBS), scraped into 1% SDS, 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5 and boiled for 1 min; typically cells from one 100 mm culture plate were solubilized in 1 ml. Chicken gizzard filamin was purified as previously described by Wang (38), with the modification that all buffers contained an additional 1 mM EGTA.

Some tissue or cell samples were treated with proteolysis inhibitors after solubilization in SDS. A solution of 25 mM phenyl methyl sulfonyl fluoride [PMSF (Sigma, St. Louis, MO.)], 0.5 M p-tosyl-L-arginine methyl ester [TAME (Sigma)] and 50 mM o-phenanthroline (Matheson, Coleman and Bell, Norwood, OH) in 100% ethanol was added to the samples at a concentration of 4 µl/ml of sample.

Antibody Preparation

Antifilamin antibodies used in this study were prepared in rabbits using native chicken gizzard filamin as antigen. Blood serum was precipitated at 50% ammonium sulfate saturation at 0°C and the partially purified IgG was dialyzed against PBS in the presence of 10 mM sodium azide. The final protein concentration was approximately 20 mg/ml, assuming $E^{280}_{1\ mg/ml} = 1.4$. The IgG was further purified by dialysis against 10 mM $NaPO_4$ pH 7.5 for 2 days and then removing the insoluble material by centrifugation at 10,000xg for 15 min. One milliliter of the supernatant was then passed through a column containing 4 ml of Whatman DE-52 (Whatman Inc., Clifton, NJ) ion exchange resin equilibrated and run in the above buffer. The purified IgG was collected in the column flow-through peak; this was typically 4 ml containing 1 mg/ml protein. The IgG fraction was then dialyzed against PBS/10 mM NaN_3. Affinity purified antiserum was prepared as described (15). Typical concentrations were 0.2 mg/ml protein.

Immunoprecipitation

Cells or myofibrils solubilized in 1% SDS were diluted with 4 volumes of ice cold 1.25% Nonidet P-40 [NP-40 (Particle Data Laboratories LTD, Elmhurst, Ill.)], 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5 to a final detergent concentration of 0.2% SDS and 1% NP-40. The solution was then clarified at 10,000xg for 10 min in a SS34 rotor. 2 ml of the above supernatant containing approximately 0.2 mg total protein (fibroblasts and myoblasts) or 2 mg total protein (myotubes and myofibrils) was added to either 20 µl of ammonium sulfate purified anti-filamin antiserum, 150 µl of DEAE purified IgG or 60 µl of affinity purified IgG. After gentle rocking at 4°C for 2 h, 150 µl of a 10% w/v solution of fixed Staphylococcus aureus was added (23, 24). Prior to incubation, the bacteria were washed twice in 20 mM Tris/Cl, 130 mM NaCl, 5 mM EGTA, pH 7.5, resuspended to 10% w/v in the same buffer containing 1% SDS and then placed in a

boiling water bath for 1 min. After pelleting in an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY) for 4 min, the bacteria were washed an additional three times in precipitation buffer (20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, 0.1% SDS, 1% NP-40).

The bacteria were rocked with the immunoprecipitation solutions for 2 h at 4°C and then pelleted in a Sorvall J6 centrifuge using a JS5.2 rotor (Dupont Instruments, Newtown, CT) for 15 min at 2,000 x g. The pellets were resuspended at 4°C in precipitation buffer and washed by pelleting three times in an Eppendorf centrifuge for 4 min. Immunoprecipitates to be run on SDS polyacrylamide gels were resuspended in 50 µl sample buffer (1% SDS, 0.5% β-mercaptoethanol, 20 mM Tris/HCl, pH 6.8, 0.2 mM ethylenediamine tetracetate (EDTA), 10% glycerol, 0.005% bromphenol blue), boiled for 2 min and cleared of bacteria by centrifuging for 5 min in an Eppendorf centrifuge. Immunoprecipitates to be iodinated (see below) were washed additionally twice in 100 mM sodium phosphate, pH 7.5, after having been washed in precipitation buffer and then resuspended in 100 µl of 0.5 M sodium phosphate, pH 7.5 containing 1% SDS, boiled for 15 sec, and cleared of bacteria by spinning twice for 5 min in an Eppendorf centrifuge.

Iodination

Iodination of proteins in polyacrylamide gel slices was performed by the method of Elder et al. (10). For the peptide maps shown in this study proteins were iodinated in solution following the method of Greenwood et al. (19). 0.3 mCi of carrier free ^{125}I [New England Nuclear O33H (Boston, MA)] in 10 µl of 0.5 M $NaPO_4$, pH 7.5 was added to the 100 µl of immunoprecipitate in 0.5 M $NaPO_4$ pH 7.5, 1% SDS, followed by 20 µl of 1 mg/ml chloramine T (Sigma, St. Louis, MO). The reaction proceeded for 2 min at room temperature and was stopped by the addition of 10 µl of 10 mg/ml sodium metabisulfite. Thirty µl of 5x SDS sample buffer was added, the mixture was boiled for 15 sec and then loaded on a SDS polyacrylamide gel and electrophoresed. After

removing the dye front, the gel was stained and destained normally, and the filamin bands were then cut out.

The advantages of this method over the method of Elder et al. are that the inclusion of the excess immunoglobulin in the iodination mixture appears to act as a carrier, so that differences in the quantity of precipitated filamin have a negligible effect on the total protein concentration. The Elder et al. method has the added disadvantage of generating iodinated variants of contaminants present in the polyacrylamide gel slice which then give a reproducible background pattern in all of the peptide maps. Finally, performing the iodination on the protein in solution in the presence of 1% SDS should help to eliminate differences in labeling due to differences in protein tertiary structure or the presence of the polyacrylamide matrix.

Peptide Mapping

Polyacrylamide gel slices containing either ^{125}I- or ^{35}S-labeled proteins were washed for 48 h in two changes of 10% methanol and then for 6 h in 100% methanol at room temperature; they were then dried under vacuum. Following the method of Elder et al. (10), digestion of labeled proteins in gel slices was carried out by adding 400 µl of 0.05 mg/ml protease in 200 mM ammonium bicarbonate to each dried gel slice for 12 h at 37°C. Six hundred microliters of freshly prepared protease solution was then added and the digestion allowed to proceed for an additional 12 h, after which time the eluted peptides were lyophilized. Proteases used were thermolysin (Sigma P1512), alpha-chymotrypsin (Worthington Type CDS, Freehold, NJ) and trypsin-TPCK (Millipore Worthington TRTPCK, Bedford, MA). Two dimensional peptide mapping was performed on cellulose 20x20 cm Chromagram sheets [Eastman 13255 (American Scientific Products, McGraw Park, IL)]. The electrophoresis buffer for the first dimension was 11.4:10:379 acetic acid:formic acid:water and the chromatography buffer for the second dimension was 5.5:3.3:1:3 butanol:pyridine:acetic acid:water. Autoradiography was

on Kodak AR-5 film (Eastman Kodak, Rochester, NY) using, for ^{125}I, DuPont Cronex Lightening-Plus intensifying screens (E. I. DuPont de Nemours and Co., Wilmington, DE). ^{35}S peptide maps were sprayed with Enhance (New England Nuclear) and the X-ray film was preflashed (26).

Quantitation of Radioactivity

Ten microliters of a cell extract containing ^{35}S-labeled proteins was added to 1 ml of 4 mg/ml L-methionine (Sigma), 0.1 mg/ml bovine serum albumin (Sigma) in 1 N sodium hydroxide. After a 10 min incubation at 37°C, 2 ml of ice-cold 30% trichloroacet acid was added and the mixture allowed to stand on ice for 2 h with occasional vortexing. The precipitated protein was collected by filtration through GF/C filters (Whatman), washed with 3% trichloroacetic acid and dried. All samples were counted in Aquasol-2 (New England Nuclear) using an LS-233 scintillation counter (Beckman Instruments Inc., Fullerton, CA). Counting efficiency for ^{35}S was 100% with the full width isoset, using ^{35}S-methionine from NEN as a standard.

Quantitation of Protein

Purified chicken gizzard filamin was quantitated using the Biorad protein assay (Biorad Laboratories, Richmond, CA). Ovalbumin (Sigma A5503) weighed on a calibrated Mettler H 34 (Mettler Instruments Corp., Hightstown, NJ) scale was used to construct a curve of O.D.595 vs. weight of protein. To determine the amount of protein in 1% SDS extracts, the assay was performed using 1 ml of the diluted Biorad dye reagent mixed with 5 µl of sample in 1% SDS. Ovalbumin was weighed as above, boiled in 1% SDS and then used to construct a standard curve.

Gels stained with Coomassie blue were scanned with a Joyce Loebl MK III C densitomet (National Instrument Laboratories Inc., Rockville, MD), using a 0-1 O.D. wedge. Several scans were made across each filamin band, the traces being recorded on paper. The

area under each peak was cut out, weighed and an average taken. Scans of known quantities of filamin (as determined above) were used to construct a standard curve for each gel.

Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was based on a modification of the method of Laemmli (25) as described by Hubbard and Lazarides (22). Gels contained 10% acrylamide and 0.13% N,N'-methylene bisacrylamide.

RESULTS

Immunoprecipitations

Filamin for peptide maps was obtained by immunoprecipitation with anti-filamin antibody and fixed Staphylococcus aureus following the method developed by Kessler (23, 24). The method was altered to effect a complete solubilization of the protein to ensure an accurate representation of the filamin in a given tissue or culture; this solubilization consisted of boiling the sample in 1% SDS for 2 min. After diluting the sample to 0.2% SDS and adding NP-40 to 1% final concentration (see Methods) antibody was added, followed by the fixed Staphylococcus aureus bacteria. The immunoprecipitates were then electrophoresed on SDS-polyacrylamide gels.

To ensure accurate representation of the filamin present in a given tissue or cell type, an excess of antibody over antigen and an excess of fixed Staphylococcus aureus over antibody were used to precipitate all of the filamin present in a given extract. The saturation point of fixed Staphylococcus aureus over antibody was determined by precipitating serial dilutions of antibody; a two-fold excess of the Staphylococcus aureus was then used for all subsequent precipitations. Similarly, the saturation point of antibody for filamin was determined by precipitating serial dilutions of filamin with a fixed amount of antibody. These precipitates were run on SDS-polyacrylamide gels

along with known quantities of purified chicken gizzard filamin, and a Joyce-Loebel densitometer was used to scan the gels (see Methods). In this manner we determined that the amount of purified chicken gizzard filamin that could be precipitated by 20 µl of ammonium sulfate purified antibody was at least 13 µg (data not shown). Fig. 1a, lane 4, shows an immunoprecipitation starting from 50 µg of purified gizzard filamin thereby showing the saturation level of the antibody. Bands below filamin are degradation products of filamin. For quantities of filamin below the saturation point, the gel scans show that we obtained at least 95% recovery of filamin in the immunoprecipitations (data not shown).

Gel scans of filamin immunoprecipitated from known quantities of myoblast, fibroblast, adult gizzard or adult skeletal muscle protein in 1% SDS extracts gave the following approximate quantities expressed as g filamin/g total protein: myoblast 2 x 10^{-3}; fibroblast 1 x 10^{-2}; gizzard 3 x 10^{-2}; skeletal myofibrils 1 x 10^{-3}. As shown in Fig. 1, purified chicken gizzard, fibroblast and myoblast filamins all have the same electrophoretic mobility (Fig. 1a, lanes 3, 4, 5, 8 and 9). Filamin immunoprecipitated from 7-14 day old cultured chick myotubes has the same electrophoretic mobility as filamin immunoprecipitated from adult chicken pectoral muscle myofibrils (lanes 6 and 7). This apparent molecular weight is lower than that of gizzard, myoblast or fibroblast filamin by approximately 5000. A protein with an apparent molecular weight slightly lower than that of purified chicken gizzard filamin occasionally appears in immunoprecipitates of cultured myoblasts using both immune (Fig. 1a, lane 5) and preimmune (Fig. 1a, lane 11) sera. Although there is less of this protein in the preimmune than in the immune immunoprecipitate, in other experiments the opposite is seen. Thus we believe that this is a nonspecific contaminant rather than a filamin associated protein or a filamin degradation product. A similar protein is also occasionally seen in immunoprecipitates from fibroblasts (Fig. 1a, lanes 8 and 14). A different protein with an electrophoretic mobility near that of myosin heavy chain is also nonselectively immunoprecipitated

from myofibrils and occasionally from myoblasts and myotubes (Fig. 1a, lanes 7 and 13). This protein appears in immunoprecipitates from myofibrils using immune or preimmune antibodies to other myofibril proteins such as α-actinin and synemin and is also precipitated by fixed Staphylococcus aureus alone (Fig. 1b), lane 3).

To demonstrate that the protein immunoprecipitated from skeletal muscle is immunologically related to purified chicken gizzard filamin, the anti-chicken gizzard filamin antibody was affinity purified using immobilized chicken gizzard filamin. This was then used to immunoprecipitate filamin from an SDS solubilized myofibril preparation as shown in Fig. 1b. This antibody precipitates the lower molecular weight myofibril filamin, showing that the same antibodies that bind to purified gizzard filamin will bind to the lower molecular weight myofibril filamin molecules.

Synthesis of Skeletal Muscle Filamin

Using cultures of skeletal myoblasts and myotubes essentially free of fibroblasts (99% fibroblast free), an experiment was performed to determine if myoblasts synthesize only the high molecular weight variant of filamin whereas mature myotubes synthesize only the lower molecular weight variant. Fig. 1c shows an autoradiograph of filamins immunoprecipitated from cell cultures metabolically labeled with ^{35}S-methionine and electrophoresed on a 10% polyacrylamide SDS gel. Cells were labeled for 5 min and then immediately solubilized by boiling in SDS. Lane 1 shows the filamin from pulse labeled myoblasts, lane 3 shows the filamin from myotubes and lane 2 is a mixture of lanes 1 and 3. These results have also been obtained when the cell samples were treated with the protease inhibitors TAME, PMSF and o-phenanthroline immediately after solubilizat indicating that the lower molecular weight of myotube filamin is not due to proteolysis during the immunoprecipitation.

^{125}I Peptide Maps

Figure 2 shows peptide maps of immunoprecipitated filamins iodinated in solution.

Precipitations, iodinations and digestions were performed in parallel for all samples. Maps generated by three proteases, trypsin, chymotrypsin and thermolysin, all show that fibroblast, skeletal myoblast and mature skeletal myotube filamins are related to chicken gizzard filamin as purified by the method of Wang (38). Cultured chick embryo fibroblast filamin appears essentially identical to purified chicken gizzard filamin when the pairs 2a and b, 2e and f and 2i and j are compared. Cultured chick skeletal myoblast filamin (2c, 2g and 2k) is closely related, but not identical, to purified gizzard and fibroblast filamin. With each protease a small number of differences can be seen between myoblast and gizzard or fibroblast filamin. For instance there is a spot (*) in the tryptic map of myoblast filamin (Fig. 2c) that is not present in the tryptic maps of gizzard or fibroblast filamins (Figs. 2a and 2b). Finally, the protein precipitated from 10 day old cultured chicken skeletal muscle myotubes by anti-chicken gizzard filamin antibodies shows a large number of peptide differences from gizzard, fibroblast and myoblast filamins. This can be seen by comparing Fig. 2a, b and c with 2d; 2e, f and g with 2h and 2i, j and k with 2l.

To further examine the similarities and differences between filamins from early and late stage myogenic skeletel muscle cells, peptide maps were made of mixtures of cultured myoblast and myotube filamins as shown in Fig. 3 to more accurately determine which peptides from the myoblast and myotube maps comigrate. An analysis of the tryptic peptides (Fig. 3a, c) shows 10 spots that match, 13 spots unique to the myoblast filamin and 14 spots unique to the myotube filamin. Similarly, in the chymotryptic maps 19 spots match, 13 are unique to myoblasts and 16 are unique to myotubes (Fig. 3e, g). With both proteases the matching and unique peptides were fairly evenly distributed over the maps, indicating no segregation of any of the three classes of peptides according to charge, size, or hydrophobicity. For unknown reasons some matching spots differ in intensity between maps (arrows, Fig. 3a and c). Because of the large percentage of nonmatching spots in both the tryptic and chymotryptic peptide maps, myoblast and

myotube filamins appear to be quite different polypeptides despite their antigenic similarity.

A similar analysis of the purified chicken gizzard filamin peptides in Fig. 2 shows that all but one of 24 tryptic peptides exactly match the 24 fibroblast filamin peptides, and of 27 gizzard filamin chymotryptic peptides, all match the fibroblast filamin chymotryp peptides. A comparison of purified gizzard filamin with myoblast filamin shows that 16 tryptic and 23 chymotryptic peptides match. At least eight gizzard and fibroblast filamin tryptic peptides do not correspond to any myotube filamin spots. Similarly four gizzard filamin chymotryptic peptides do not comigrate with any myotube filamin peptide. Of the seven tryptic peptides present in myoblast but not in purified gizzard filamin, five are peptides common to myoblast and myotube filamins and two are unique to myoblasts. Similarly nine chymotryptic peptides are present in myoblast but not gizzard or fibroblast filamins. Six of these peptides are common with myotube filamin chymotryptic peptides and three are not.

Peptide maps of filamins iodinated by the method of Elder et al. (10) rather than the method described above gave results identical to those described here for the similarities and differences of the various filamins (data not shown). Similar results were also obtained when comparing peptide maps of filamins digested under different conditions such as varying the protease concentration from 50 to 1000 μg/ml, or concentration of the digestion buffer from 50 to 200 mM ammonium bicarbonate.

Finally, a comparison of iodinated cultured skeletal myotube and adult myofibril filamins (Fig. 4) shows very few differences between the two filamins when they are digested with three different proteases. This indicates that the filamin present in the late stage of *in vitro* myogenesis is indistinguishable from the corresponding filamin present in *in vivo* myogenesis.

^{35}S Peptide Maps

The close similarity of fibroblast and skeletal myoblast filamins and their difference

to myotube filamin, shown by peptide maps of filamins labeled with ^{125}I in vitro, are also seen in peptide maps of filamins metabolically labeled with ^{35}S-methionine (Fig. 5). Because the digested proteins were metabolically labeled, differences observed in these maps cannot be artifacts of an in vitro labeling system. An analysis of chymotryptic peptides of myoblast and fibroblast filamins shows that out of 40 peptides, only one is different between the two, while a comparison of myoblast and myotube filamins shows 30 matching peptides, with an additional 10 peptides unique to myoblasts and 11 unique to myotubes. No peptides from myotube filamin comigrate with the two peptides that distinguish fibroblast from myoblast filamin. The close similarity of fibroblast and myoblast ^{35}S peptide maps shows that the difference between myoblast and myotube ^{35}S filamin maps cannot be due to random variations caused by the mapping procedure.

DISCUSSION

Patterns of protein synthesis change markedly during skeletal myogenesis. At the time myoblasts fuse to form myotubes several proteins begin to be synthesized, such as the α variant of actin (42), desmin (13), the acetylcholine receptor (29), the sodium channel (12) and creatine kinase and aldolase isozymes (9, 36). Other proteins exhibit changes from one variant to another well after functional myofibrils have developed. These proteins include some myosin light chains (5, 14, 43), myosin heavy chains (21, 32, 44), tropomyosins (30) and troponin T (28, 35). Finally, a small number of proteins exhibit changes of variants between myoblasts and late embryonic myotubes, for example, myosin light chains exhibit a loss of three minor variants between day 10 and day 18 in chick embryos (14).

We have previously shown that filamin exhibits a novel pattern of expression, and possibly a different mode of regulation of its expression during myogenesis (15). This protein is present in skeletal myoblasts and early fused myotubes in association with actin filament bundles as shown by immunofluorescence. However, with two days

after the onset of fusion and before the transition of α-actinin from a punctate distribution along the actin filament bundles to striated Z lines, filamin disappears from the cells. Several days after the appearance of α-actinin containing Z line striations, filamin reappears at the Z lines and maintains this distribution into adulthood. These studies, in conjunction with previous studies (1, 39), have also established that filamins from gizzard, fibroblast, myoblast, mature myotube and adult skeletal muscle are antigenically related.

In the present study we have isolated filamins from cultured chicken skeletal myoblasts and myotubes by immunoprecipitation and compared them to each other and to filamins immunoprecipitated from adult gizzard, adult skeletal muscle and fibroblasts Tryptic, chymotryptic and thermolytic peptide maps have shown that myoblast and mature myotube filamins labeled with ^{125}I *in vitro* exhibit substantial peptide differences (Figs. 2 and 3), implying that they are distinct polypeptides, which may be either distinct gene products or arise as a result of differential RNA processing of the same gene product. Myoblast and mature myotube filamins also exhibit differences in their chymotryptic peptide maps when metabolically labeled with ^{35}S-methionine prior to immunoprecipitation (Fig. 5). These latter results show that the presence of peptide differences between these two forms of filamin is not due to a peculiar charge modification of any proteins during the iodination procedure. Comparison of ^{125}I-labeled peptides from myoblast and mature myotube filamins with those of adult gizzard, adult skeletal muscle and cultured fibroblast filamins, shows that myoblast filamin is highly homologous to the gizzard and fibroblast proteins while mature myotube filamin is highly homologous to the adult skeletal muscle protein. However, even in the case of gizzard, fibroblast and myoblast filamins, a close examination of their peptide maps reveals the presence of some peptide differences. At present we do not know whether these differences represent heterologous amino acid sequences or posttranslational modifications of identical sequences. These small peptide differences provide convenient markers in the peptide maps which enabled us to be confident that myoblast, myotube and myofibril filamins

were not contaminated to any substantial extent by fibroblast filamin. This was especially important in the case of mature myotubes and adult myofibrils where filamin represents a much lower percentage of total protein compared to gizzard, fibroblasts or myoblasts. However, the extensive peptide differences between myotube or myofibril filamins and fibroblast filamin and the absence from their peptide map of peptides characteristic of fibroblast filamin render this possibility unlikely. The results presented here have been consistently obtained with several different preparations of immunoprecipitated filamins from all five sources. In addition, closely similar peptide maps have been obtained with the antigens either iodinated directly in the gel slices according to the procedure of Elder et al. (10) or iodinated after immunoprecipitation, dissociation of the immune complexes by SDS, and subsequent purification of the ^{125}I-labeled filamin by SDS gel electrophoresis. Thus we feel confident that the peptide homologies and differences seen between the five forms of the filamins indicate that myoblast filamin is homologous to gizzard and fibroblast filamins, and that mature myotube filamin is highly homologous to adult skeletal myofibril filamin but is very different from myoblast filamin. These results, in conjunction with the immunofluorescence results obtained earlier (15), suggest that the synthesis of the myoblastic form of filamin ceases with a concomitant removal of the molecule from the cytoplasm, presumably by protein turnover, and that later in myogenesis a different filamin polypeptide is synthesized and becomes localized at the Z-disc. The homology of mature myotube and adult myofibril filamins suggests that this form of filamin does not change substantially subsequent to its association with the Z-disc.

The Z-line form of filamin also exhibits a slightly greater electrophoretic mobility in SDS polyacrylamide gels compared to its myoblast counterpart. A calcium-activated protease purified from skeletal muscle has been shown to cleave filamin into proteins of 240,000 and 9,500 molecular weight (7). Other studies have shown that platelet filamin is cleaved by an endogenous calcium-activated protease (41) to an unknown set of cleavage

products. Immunoprecipitation of filamin from extracts containing a variety of protease inhibitors prepared from myoblasts or mature myotubes metabolically labeled with ^{35}S-methionine for a short period of time showed that myotubes exclusively synthesize the lower molecular weight filamin (Fig. 1c). This observation argues that the myotube form of filamin is synthesized as a polypeptide slightly shorter than myoblast filamin and that this electrophoretic difference among them is not due to a higher susceptibility of the myotube molecule to proteolysis.

The differences in peptide maps between adult skeletal muscle filamin and adult smooth muscle (gizzard) filamin correlates with the differences observed between these two muscle types for other structural and contractile proteins such as the intermediate filament protein desmin (31), actin (37), myosin (4) and α-actinin (2). Such differences may be due to the existence of distinct genetic programs for the assembly of smooth and skeletal muscle.

It has been shown previously that purified chicken gizzard filamin inhibits the actin-activated myosin ATPase (6) and blocks the binding of tropomyosin to actin (27, 45). The similarity between myoblast and gizzard filamins supports our previous hypothesis that filamin is removed from early myogenic cells because it blocks the interaction between tropomyosin or myosin and actin which is necessary for sarcomere assembly. The synthesis of a new form of filamin later in myogenesis and its association with Z-lines indicates that there is a functional difference between the myoblast and the Z-line forms of this protein. As we have demonstrated previously, skeletal muscle filamin associates with the periphery of the myofibril Z-disc (15) which also contains actin and the intermediate filament proteins desmin, vimentin and synemin (16-18). The differences between Z-line and myoblast filamins might restrict the former to binding only the actin present at the periphery of the Z-disc, or to mediate the binding of desmin and vimentin to the Z-disc.

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FIGURE LEGENDS

FIGURE 1 Filamin was immunoprecipitated and then electrophoresed on 10% SDS-polyacrylamide gels (1a, b and c). 1a and b are Coomassie stained gels; 1c is an autoradiogran 1a molecular weight markers are purified chicken skeletal muscle myofibrils (lane 1) showing myosin heavy chain (M) (200,000 daltons) and actin (A) (43,000 daltons); 10 d cultured chick myotubes (lane 2) and purified chicken gizzard filamin (lanes 3, 9 and 15 250,000 daltons). Lanes 4-8 show immunoprecipitations using rabbit antifilamin antisera; lanes 10-14 show parallel precipitations of the same antigens with preimmune sera. The heavy bands above actin in the immunoprecipitates are immunoglobulin heavy chains. In Fig. 1a filamin is precipitated from purified chicken gizzard filamin, starting with 50 μg of filamin (lane 4 immune serum, lane 10 preimmune serum), cultured myoblasts (lane 5 immune serum, lane 11 preimmune serum), 10 d cultured myotubes (lane 6 immune serum, lane 12 preimmune serum), purified skeletal myofibrils (lane 7 immune serum, lane 13 preimmune serum) and cultured chick fibroblasts (lane 8 immune serum, lane 14 preimmune serum). Fig. 1b shows filamin immunoprecipitated from skeletal myofibrils using antibody affinity purified with purified chicken gizzard filamin. Lane 1 is a purified gizzard filamin marker, lane 2 is the immunoprecipitate from the myofibrils and lane 3 is a control precipitate using PBS instead of affinity purified antiserum. The affinity purified antiserum precipitates both the lower molecular weight myofibril filamin and a small amount of contaminating fibroblast filamin. Fig. 1c shows immunoprecipitated filamin from myoblasts (lane 1) and pure myotubes (lane 3) labeled with ^{35}S-methionine at 40 μCi/ml for 5 min; lane 2 is a mixture of lanes 1 and 3. Myoblasts and myotubes were both from a single 100 mm culture dish originally plated at 3×10^6 cells/plate.

FIGURE 2 Two-dimensional peptide maps of iodinated gizzard filamin (first column), chick embryo fibroblast filamin (second column), chick skeletal myoblast filamin (third

column) and 10-day-old cultured chick myotube filamin (fourth column). Digests were performed with Trypsin-TPCK (top row), α-chymotrypsin (middle row) and thermolysin (bottom row). Peptide maps were of typically one-fifth of the immunoprecipitated filamin (0.2 μg) labeled at approximately 0.2 moles I/mole protein. Electrophoresis was from left to right and ascending chromatography from bottom to top. Maps contained 5,000 cpm ^{125}I labeled peptides and were exposed at -70°C with intensifying screens typically for 48 h. Star in Fig. 2c shows a peptide present in myoblast and absent from gizzard or fibroblast filamin.

FIGURE 3 Peptide maps comparing iodinated chick skeletal myoblast filamin (first column) with 10-day-old myotube filamin (third column) by means of a coelectrophoresis map showing a combination of the two filamins (second column). Proteases are trypsin-TPCK in the top row and α-chymotrypsin in the bottom row. Tracings (fourth column) show spots unique to myoblasts (O), unique to myotubes (●) or common to both (⊖). Arrows (3a and 3c) show an example of matching spots that differ in intensity.

FIGURE 4 A comparison of 10-day-old cultured chick skeletal myotube filamin (left column) and filamin from purified adult skeletal myofibrils (right column); both were labeled with ^{125}I. Proteases used were trypsin-TPCK (top row), α-chymotrypsin (middle row) and thermolysin (bottom row). Quantities of ^{125}I-filamin used for peptide maps and exposure times were as in Fig. 2.

FIGURE 5 Chymotryptic digests of ^{35}S-methionine labeled filamins isolated from chick embryo fibroblasts (a) chick skeletal myoblasts (b) and 10-day-old cultured chick skeletal myotubes (c). Two 100 mm plates containing approximately 5×10^6 cells of each type were labeled for 18 h in methionine-free minimum essential medium with

0.2 mCi ^{35}S-methionine (1000 Ci/mM) in 5 ml medium in each plate. Approximately 3×10^8 cpm of total TCA precipitable counts were recovered from each pair of plates. Three µg of each filamin was immunoprecipitated using 300 µl of 10% w/v Staphylococcus aureus and 40 µl of ammonium sulfate purified antifilamin antiserum antibody (see Methods) and electrophoresed on a 10% SDS-polyacrylamide gel which was stained and destained normally. Filamin bands were cut out and washed for 48 h in 10% methanol, dried and digested with α-chymotrypsin as described in Materials and Methods. Maps contain approximately 2×10^4 cpm ^{35}S and were exposed for 15-30 days. Electrophoresis was from left to right and chromatography from bottom to top. Arrows in 5b indicate three of the peptides present in fibroblast and myoblast filamins and absent from myotube filamins. Similarly, arrows in 5c indicate peptides unique to myotube filamin.

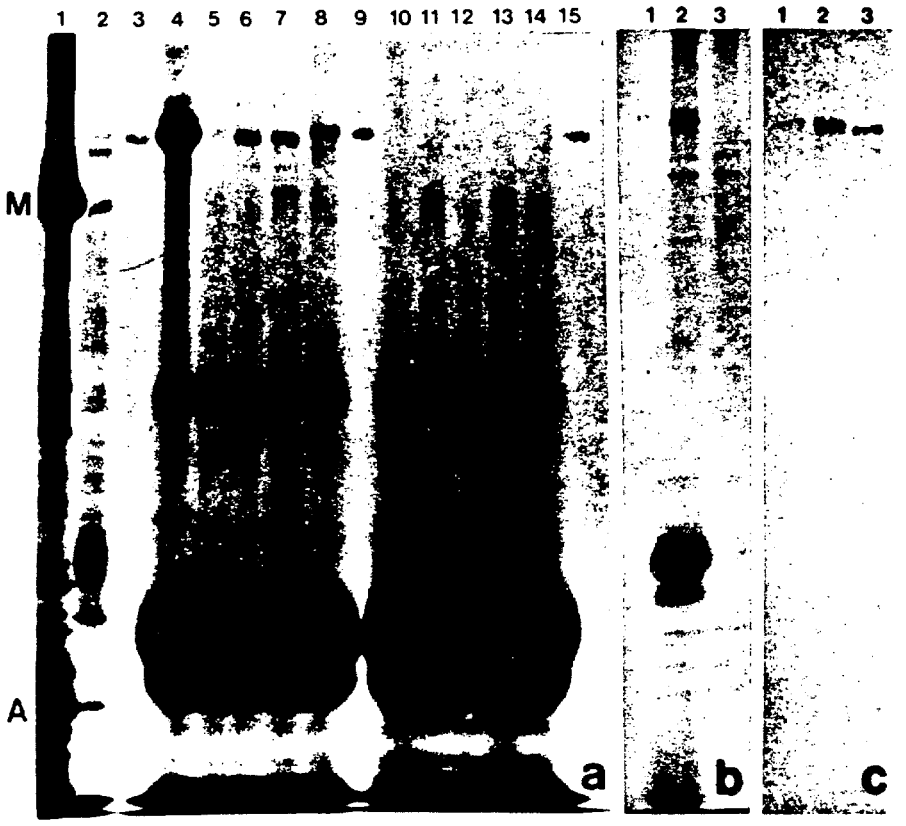

Figure 1

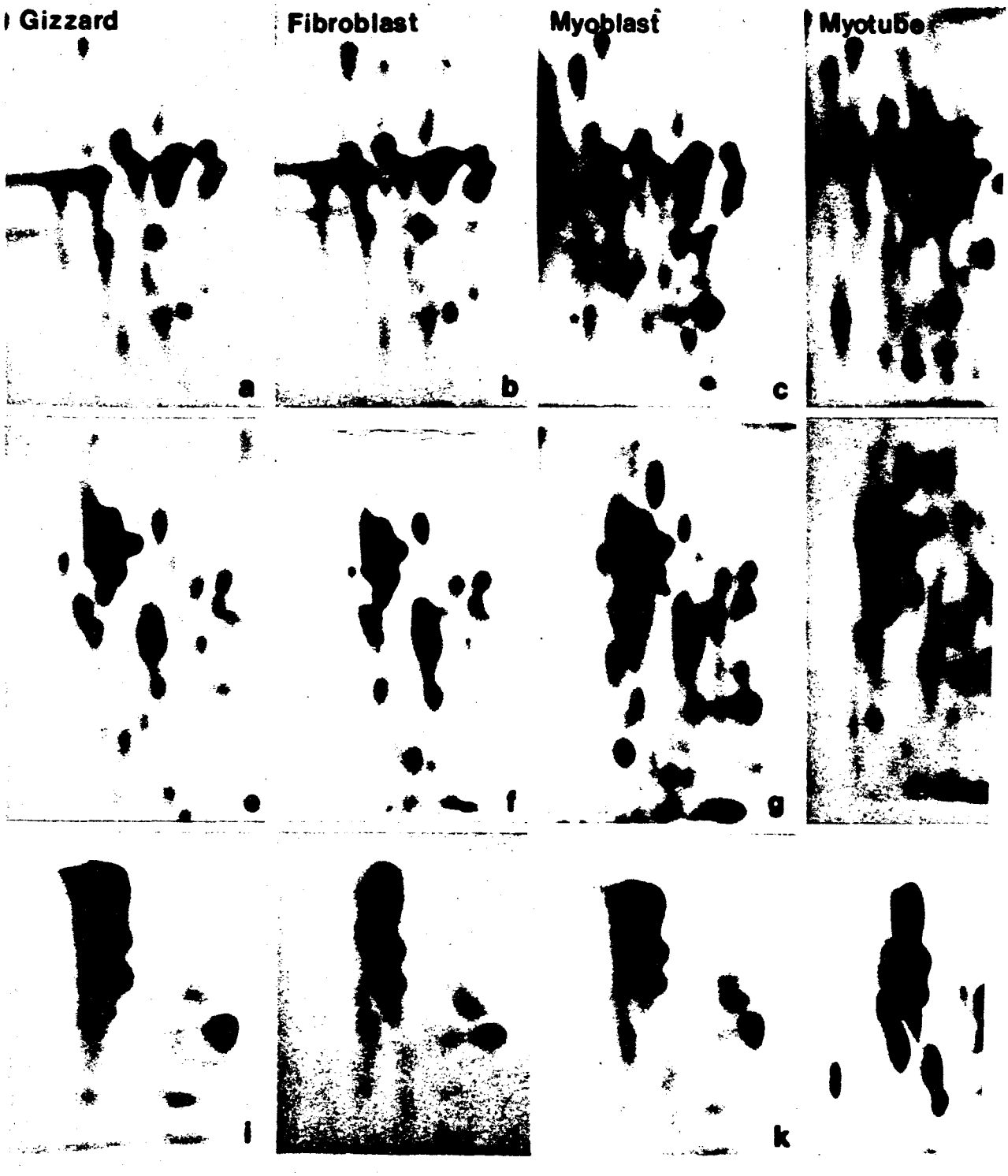

Figure 2

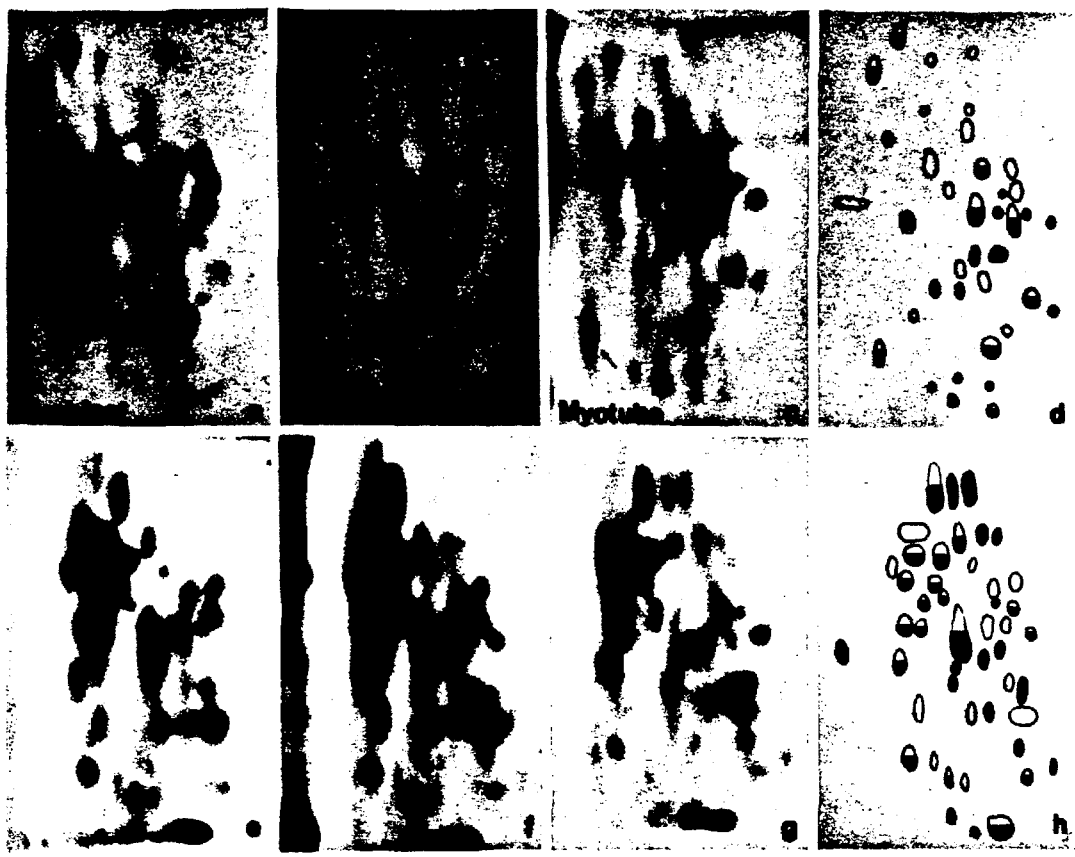

Figure 3

Figure 4

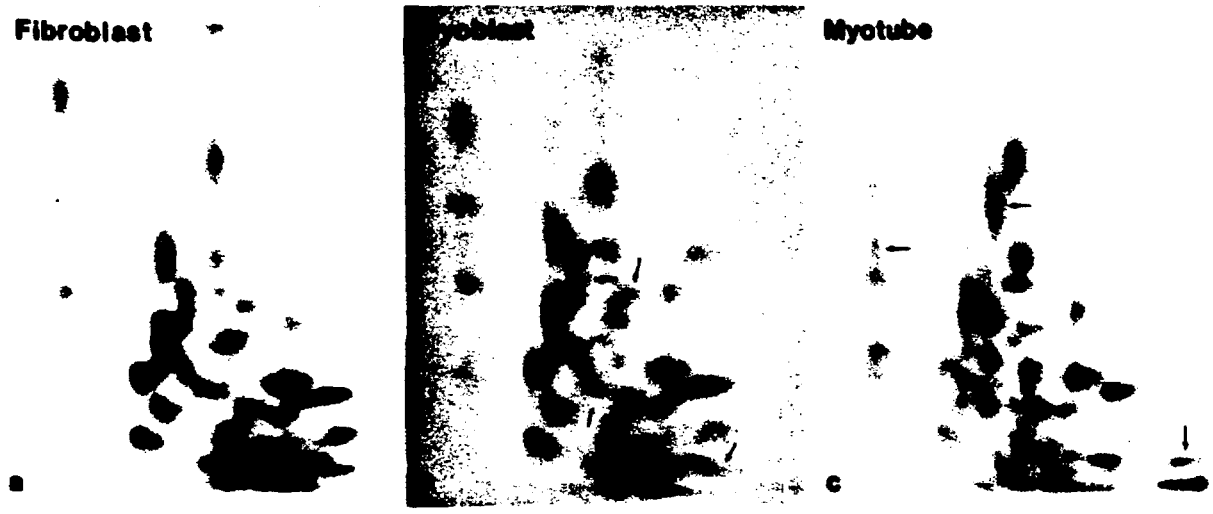

Figure 5

Chapter 4.

HOMOLOGOUS FILAMIN POLYPEPTIDES HAVE DIFFERENT DISTRIBUTIONS IN SLOW AND FAST MUSCLE FIBERS

ABSTRACT The high molecular weight actin binding protein filamin is located at the periphery of the Z disc in the fast adult chicken pectoral muscle (Gomer and Lazarides, Cell 23, 524-532, 1981). In contrast, we have found that in the slow anterior latissimus dorsi (ALD) muscle, filamin is additionally located throughout the I band as judged by immunofluorescence with affinity purified antibodies on myofibrils and cryosections. The Z line proteins desmin and α-actinin, however, have the same distribution in ALD as they do in pectoral muscle. Quantitation of filamin and actin from the two muscle types shows that there is approximately ten times as much filamin per actin in ALD myofibrils as in pectoral myofibrils. Filamin immunoprecipitated from ALD has an electrophoretic mobility in SDS-polyacrylamide gels identical to that of pectoral myofibril filamin and slightly greater than that of chicken gizzard filamin. Two-dimensional peptide maps of filamin immunoprecipitated and labeled with ^{125}I show that ALD myofibril filamin is virtually identical to pectoral myofibril filamin and is distinct from chicken gizzard filamin.

INTRODUCTION

Filamin is a 250,000 dalton actin binding protein originally isolated from avian smooth muscle (48,55) and is closely related to an actin binding protein isolated from mammalian leukocytes (21). Filamin binds to F-actin filaments and crosslinks them, forming a gel under a large variety of conditions (7,10,11,22,23,35,38,44,51,57,59). Antibodies to filamin stain stress fibers, membrane ruffles and microspikes in a variety of nonmuscle cells grown in tissue culture (56). Filamin is located on Z lines of chicken thigh muscle (5), chicken pectoral muscle (16) and chicken heart (32).

We have previously studied the expression and distribution of filamin in avian myogenic cells (16). By double immunofluorescence filamin is present on stress fibers in unfused myoblasts and early fused myotubes, and disappears from the cells shortly before the transition of α-actinin from stress fibers to Z lines. Several days later filamin reappears in the myotubes at the Z lines, preceding the transition of desmin and vimentin from cytoplasmic filaments to Z lines. Filamin is located on the periphery of glycerinated and KI extracted Z discs along with desmin and vimentin. The disappearance of filamin can also be followed by immunoautoradiography of whole extracts from myogenic cells at different stages of differentiation, and a concomitant temporary absence of filamin synthesis can be seen by metabolic pulse labeling.

We recently used quantitative immunoprecipitation and two-dimensional peptide mapping of proteins labeled with ^{125}I to compare myoblast and mature myotube filamins (17). Chick fibroblast and purified adult gizzard filamin are virtually identical and are closely related to chick myoblast filamin. Mature myotube and adult myofibril filamins are closely homologous but have many peptides different from the other three types. Mature myotube and myofibril filamins have, and

are synthesized as, a lower molecular weight variant compared to myoblast, fibroblast and gizzard filamins. Peptide maps of filamin metabolically labeled with ^{35}S also show a similarity between fibroblast and myoblast filamin and differences with myotube filamin. These results have suggested that myoblast and mature myotube filamins are distinct gene products.

It is well known that adult skeletal muscle fibers are not homogeneous. Two main physiological classes are known, fast and slow (8,25,39,41). Differences between fast and slow muscles can be seen in contraction speed (8,41), ultrastructure (24,25,39,54), innervation (1,8,24,25,45), myosin isozymes (4,25,52), α-actinin isozymes (43,53) and troponin isozymes (13), among other things. In the chicken the anterior latissimus dorsi (ALD) is a slow muscle and the posterior latissimus dorsi and pectoralis are fast muscles. In this study we show that although ALD and pectoral muscle filamins are highly homologous polypeptides, ALD filamin and pectoral filamin have different distributions within myofibrils.

MATERIALS AND METHODS

Cell Culture

Chicken embryonic fibroblasts and embryonic myogenic cells were grown as previously described (15,17).

Myofibril Preparation

Strips of muscle were tied to wooden applicator sticks and glycerinated in 50% glycerol, 1/2X phosphate buffered saline (PBS) and 5 mM ethylene glycol-bis-(β amino ethyl ether) N,N,N',N' tetraacetic acid at -20°C for one year. Myofibrils were obtained by homogenization of glycerinated muscle in a Lourdes blender at top speed for 2 min in PBS/5 mM EGTA at 0°C. Fresh skeletal myofibrils were prepared from adult chicken pectoral muscle by trimming a piece of muscle free

of fat and connective tissue, and homogenizing it in a Lourdes blender at top speed for 30 sec in ice cold C buffer (100 mM KCl, 12 mM NaCl, 4.9 mM K_2HPO_4, 3.6 mM KH_2PO_4, 0.72 mM NaH_2PO_4, 5 mM EGTA pH 7.0 at room temperature). Myoribrils were then purified by filtering the homogenate twice through two layers of cheesecloth followed by centrifugation at 1500 xg for 5 min in a Sorvall HB-4 rotor. The supernatant was discarded and myofibrils were resuspended in C buffer.

Cryosections

Tissue samples for cryosections were taken from freshly killed chickens and frozen in liquid nitrogen. Skeletal muscle samples were stretched with forceps while freezing. Two to 4 micron thick sections were cut on a Tissue Tek II cryostat (Miles Laboratories, Naperville, IL) at -20°C using frozen samples embedded in O.C.T. compound (Tissue-Tek). Sections were picked up onto coverslips and fixed at room temperature for 10 min in C buffer containing 2% formaldehyde (Mallinckrodt Inc., Paris, KY). Sections were then rinsed in C buffer at room temperature for at least 10 min. Alternatively, sections were fixed in 95% ethanol at room temperature for 10 min and then rinsed in C buffer.

Antibody Preparation

Filamin antibodies used in this study were prepared in rabbits using either native chicken gizzard filamin as antigen or filamin further purified by electrophoresis on SDS-polyacrylamide gels prior to use as antigen (16,17). Serum was precipitated at 50% ammonium sulfate saturation at 0°C and the partially purified IgG was dialyzed against PBS in the presence of 10 mM sodium azide. The final protein concentration was approximately 20 mg/ml, assuming $E^{280}_{1\ mg/ml} = 1.4$. The IgG was further purified by dialysis against 10 mM $NaPO_4$ pH 7.5 for two days and then removing the insoluble material by centrifugation at 10,000 xg for 15 min. One milliliter of the supernatant was then passed through a column containing

4 ml of Whatman DE-52 (Whatman Inc., Clifton, NJ) ion exchange resin equilibrated and run in the above buffer. The purified IgG was collected in the column flow-through peak; this was typically 4 ml containing 1 mg/ml protein. The IgG fraction was then dialyzed against PBS/10 mM NaN_3. Affinity purified antiserum was prepared as described (16) using either antiserum described above. Typical protein concentrations were 0.2 mg/ml. Antisera to desmin and α-actinin were as previously described (15,19).

Immunofluorescence

Buffers for immunofluorescence were PBS/5 mM EGTA for glycerol extracted myofibrils and cryosections and buffer C for fresh myofibrils. Coverslips were incubated with antibody for 1 h, rinsed in buffer at room temperature 15-30 min, incubated in a 1:50 dilution of fluorescein isothiocyanate labeled goat anti-rabbit IgG (Miles) for 1 h and then rinsed in the appropriate buffer at room temperature for 30 min. Both antibody incubations were at 37°C for glycerinated myofibrils and cryosections and at room temperature for fresh myofibrils. Coverslips were mounted on slides in PBS/5 mM EGTA/90% glycerol (glycerinated myofibrils and cryosections) or C buffer/90% glycerol (fresh myofibrils). Microscopy and photography were as described (15,19).

Sample Preparation

Myofibrils were prepared from pectoral or ALD muscle as described above. However, instead of resuspending in buffer C, pelleted myofibrils were resuspended in 1% sodium dodecyl sulfate (SDS) 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5 and boiled for 1 min at a concentration of approximately 10 mg wet weight of myofibrils per ml.

Adult chicken gizzard was trimmed free of fat and connective tissue and homogenized for 20 sec at top speed in a Lourdes blender in ice cold 20 mM Tris/HCl,

130 mM NaCl, 5 mM EGTA, pH 7.5 at a concentration of approximately 1 mg wet weighted gizzard/ml, 20% SDS was added to a final concentration of 1% and the extract was then boiled for 1 min. Chicken gizzard filamin was purified as described by Wang (55) with the modification that all buffers contained an additional 1 mM EGTA. Adult chicken pectoral muscle actin was purified according to the method of Spudich and Watt (49). For immunoprecipitation, cell cultures were rinsed at room temperature with phosphate buffered saline (PBS), scraped into 1% SDS, 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5 and boiled for 1 min; typically, cells from one 100 mm culture plate were solubilized in 1 ml.

Immunoprecipitation

Myofibrils solubilized in 1% SDS were diluted with 4 volumes of ice cold 1.25% Nonidet P-40 [NP-40 (Particle Data Laboratories LTD, Elmhurst, Ill.)], 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5 to a final detergent concentration of 0.2% SDS and 1% NP-40. The solution was then clarified at 10,000 xg for 10 min in a SS34 rotor. Two ml of the above supernatant containing approximately 0.2 mg total proteins was added to 60 µl of affinity purified IgG. After gentle rocking at 4°C for 2 hr, 150 µl of a 10% w/v solution of fixed Staphylococcus aureus was added (29,30). Prior to incubation with the samples, the bacteria were washed twice in 20 mM Tris/Cl, 130 mM NaCl, 5 mM EGTA, pH 7.5, resuspended to 10% w/v in the same buffer containing 1% SDS and then placed in a boiling water bath for 1 min. Then, after pelleting in an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY) for 4 min, the bacteria were washed an additional three times in precipitation buffer (20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, 0.2% SDS, 1% NP-40).

The bacteria were rocked with the immunoprecipitation solutions for 2 h at 4°C and then pelleted in a Sorvall J6 centrifuge using a JS5.2 rotor (Dupont

Instruments, Newtown, CT) for 15 min at 2,000 xg. The pellets were resuspended at 4°C in precipitation buffer and washed by pelleting three times in an Eppendorf centrifuge for 4 min. Immunoprecipitates to be run on SDS polyacrylamide gels were resuspended in 50 µl sample buffer (1% SDS, 0.5% β-mercaptoethanol, 20 mM Tris/HCl, pH 6.8, 0.2 mM ethylenediamine tetracetate (EDTA), 10% glycerol, 0.005% bromphenol blue), boiled for 2 min and cleared of bacteria by centrifuging for 5 min in an Eppendorf centrifuge. Immunoprecipitates to be iodinated (see below) were washed additionally twice in 100 mM sodium phosphate, pH 7.5, after having been washed in precipitation buffer and then resuspended in 100 µl of 0.5 M sodium phosphate, pH 7.5 containing 1% SDS, boiled for 15 sec, and cleared of bacteria by spinning twice for 5 min in an Eppendorf centrifuge.

Iodination

Proteins were iodinated in solution following the method of Greenwood et al. (19). 0.3 mCi of carrier free ^{125}I [New England Nuclear O33H (Boston, MA)] in 10 µl of 0.5 M $NaPO_4$, pH 7.5 was added to the 100 µl of immunoprecipitate in 0.5 M $NaPO_4$ pH 7.5, 1% SDS, followed by 20 µl of 1 mg/ml chloramine T (Sigma, St. Louis, MO). The reaction proceeded for 2 min at room temperature and was stopped by the addition of 10 µl of 10 mg/ml sodium metabisulfite. Thirty µl of 5x SDS sample buffer were added, the mixture was boiled for 15 sec and then loaded on a SDS polyacrylamide gel and electrophoresed. After removing the dye front, the gel was stained and destained normally, and the filamin bands were then cut out.

Peptide Mapping

Polyacrylamide gel slices containing ^{125}I-labeled proteins were washed for 48 h in two changes of 10% methanol and then for 6 h in 100% methanol at room temperature; they were then dried under vacuum. Following the method

of Elder et al. (14), digestion of labeled proteins in gel slices was carried out by adding 400 µl of 0.05 mg/ml protease in 200 mM ammonium bicarbonate to each dried gel slice for 12 h at 37°C. Six hundred microliters of freshly prepared protease solution were then added and the digestion allowed to proceed for an additional 12 h, after which time the eluted peptides were lyophilized. The protease used was trypsin-TPCK (Millipore Worthington TRTPCK, Bedford, MA). Two dimensional peptide mapping was performed on cellulose 20x20 cm Chromagram sheets [Eastman 13255 (American Scientific Products, McGraw Park, IL)]. The electrophoresis buffer for the first dimension was 11.4:10:379 acetic acid:formic acid:water and the chromatography buffer for the second dimension was 5.5:3.3:1:3 butanol:pyridine:acetic acid:water. Autoradiography was on Kodak AR-5 film (Eastman Kodak, Rochester, NY) using, for ^{125}I, DuPont Cronex Lightening-Plus intensifying screens (E. I. DuPont de Nemours and Co., Wilmington, DE).

Quantitation of Protein

Purified chicken gizzard filamin or pectoral muscle actin was quantitated using the Biorad protein assay (Biorad Laboratories, Richmond, CA). Ovalbumin (Sigma A5503) was used to construct a curve of O.D.595 vs. weight of protein. To determine the amount of protein in 1% SDS extracts, the assay was performed using 1 ml of the diluted Biorad dye reagent mixed with 5 µl of sample in 1% SDS and 1 ml of water. Ovalbumin was boiled in 1% SDS and then used to construct a standard curve.

Gels stained with Coomassie blue were scanned with a Joyce Loebl MK III C densitometer (National Instrument Laboratories Inc., Rockville, MD), using a 0-1 O.D. wedge. Several scans were made across each band, the traces being recorded on paper. The area under each peak was cut out, weighed and an average taken. Scans of known quantities of filamin or actin (as determined above) were used to construct a standard curve for each gel.

Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was based on a modification of the method of Laemmli (33) as described by Hubbard and Lazarides (27). Gels contained 10% acrylamide and 0.13% N,N'-methylene bisacrylamide or 12.5% acrylamide and 0.10% N,N'-methylene bisacrylamide.

RESULTS

Immunofluorescence

Immunofluorescence using antiserum affinity purified over purified chicken gizzard filamin shows that filamin is exclusively located on the Z line in myofibrils from adult chicken pectoralis major (Fig. 1a and b), posterior latissimus dorsi (PLD) (data not shown) and heart (32). However, myofibrils from the slow anterior latissimus dorsi muscle (ALD) show filamin staining on the I bands in addition to the Z line (Fig. 1d). These results were obtained with myofibrils from glycerol extracted muscle samples or myofibrils from fresh muscle, using antiserum made to either native or SDS denatured to chicken gizzard filamin.

The location of filamin at the I bands in the ALD can also be seen in cryosections of adult muscle. Figure 2 shows longitudinal cryosections of ALD muscle stained with affinity purified filamin antiserum. In this muscle filamin is located both on the I bands and Z lines while in the pectoral muscle it is located only on the Z line. Immunofluorescence on myofibrils and cryosections of the fast skeletal muscle posterior latissimus dorsi and heart also show filamin located only at the Z line. These distributions are seen with either fixed or unfixed tissue (not shown) and in all myofibrils from the ALD.

In the ALD the distribution of filamin is not accompanied by a similar distribution of other Z line associated proteins. The Z line associated intermediate filament

proteins desmin and vimentin are located at the Z line in glycerol extracted ALD myofibrils (18, see also Fig. 1f) or cryosections of ALD (Fig. 2d). The actin binding protein α-actinin, another Z line associated protein, is also located exclusively at the Z line in glycerol extracted (Fig. 1h) or fresh ALD myofibrils or cryosections of ALD (Fig. 2f) (19).

We have previously shown that in glycerol extracted pectoral or thigh muscle extracted with 0.6 M KI, filamin is located at the periphery of the Z disc (16) as is the case for desmin (18) and vimentin (19). Cryosections cut transverse to the myofibrils in fresh ALD show an even distribution of fluorescence (Fig. 3a) indicating that filamin is distributed throughout the I band and possibly the Z line. Similar sections stained for desmin show an open lattice distribution (Fig. 3b) indicating a distribution of this antigen at the periphery of the myofibril as previously shown for leg muscle (34). In glycerinated and then KI extracted thigh muscle α-actinin is located in the interior of the Z disc (18); a similar distribution is seen in transverse cryosections of fresh ALD (Fig. 3c).

Quantitation of Filamin and Actin

By scanning gels of filamin quantitatively immunoprecipitated from known quantities of total protein, we can determine the amount of filamin in a tissue (17). Using this method (Fig. 4) we found that there is 7.0 mg filamin/g total protein in adult chicken ALD myofibrils, as compared to 0.88 mg filamin/g total protein in adult pectoralis major myofibrils

To determine filamin to actin ratios, known quantities of protein from tissues and myofibrils were run on SDS polyacrylamide gels along with known quantities of purified actin (Fig. 5). The results are shown in Table 1. Gizzard, pectoral myofibrils and ALD myofibrils all have roughly equivalent levels of actin, approximately 10% of total protein while, fibroblasts and myoblasts have significantly

lower quantities. Gizzard has the highest level of filamin, approximately 3% of total protein. In fibroblast and ALD myofibrils filamin represents roughly 1% of the total protein. Myoblasts have an even lower level while pectoral myofibrils have the lowest measured level of filamin, less than a tenth of one percent of total protein.

Gel scans of tropomyosin (Fig. 5) from gizzard, ALD and pectoral myofibrils show a qualitatively inverse relationship of tropomyosin and filamin quantities. In gizzard, α-tropomyosin is the band marked with an arrow (Fig. 5, lane 1) and β-tropomyosin is a band slightly above actin that is poorly resolved from the actin band (37). Because the two bands cannot be resolved, in determining actin and tropomyosin levels, we made the assumption that there are equal quantities of α- and β-tropomyosin in gizzard. In ALD (Fig. 5, lane 5) α- and β-tropomyosins are marked with arrows. Pectoral muscle contains only α-tropomyosin (15), which is the band in Fig. 5, lane 4 immediately above ALD α-tropomyosin (37). The ratios of total tropomyosin to actin staining intensity as measured by gel scans is 0.17 for gizzard, 0.39 for ALD myofibrils and 0.54 for pectoral myofibrils. When these numbers are compared with Table 1 it can be seen that there is an inverse relationship between tropomyosin and filamin quantities in the three tissues.

Characterization of ALD Filamin

The electrophoretic mobility of the filamin immunoprecipitated from ALD is slightly greater in SDS-polyacrylamide gels than that of purified chicken gizzard filamin (Fig. 4). We have observed this increased mobility in filamin immunoprecipitated from pectoral muscle or cultured adult myotubes (17).

To further characterize filamin from ALD, two-dimensional peptide maps were performed. Immunoprecipitations, iodinations and proteolytic digestions were performed in parallel for all samples. Filamin was immunoprecipitated from

SDS solubilized fresh adult chicken gizzard, fresh adult chicken pectoral myofibrils and fresh adult chicken ALD myofibrils. Filamin was iodinated in solution in the presence of 1% SDS (see Methods). Maps of tryptic (Figs. 6a,b,c) peptides show that gizzard (Fig. 6a) and pectoral myofibril (Fig. 6c) filamins are related to each other but have many differences. The maps of ALD myofibril filamin (Fig. 6b) show that it is virtually identical to pectoral muscle filamin when comparing 6b and c. Because of the presence of unique peptides in the gizzard map that are not present in the pectoral or ALD myofibril maps (*Figs. 6a) we see that neither of the latter two samples are contaminated with gizzard filamin. Of 45 tryptic peptides in the pectoral myofibril filamin map (Fig. 6c), all match the spots in the corresponding map of ALD filamin (Fig. 6b). However, four spots differ somewhat in intensity (arrows, Figs. 6b and c). These spots are in the lower area of the peptide map, having low R_f values in the chromatography step, and are therefore probably hydrophilic (29).

DISCUSSION

Fast and slow skeletal muscles can be distinguished on the basis of contraction speed (8,25) and type of innervation (1,8,24,25,45,46). Differences also exist with respect to protein composition. Fast and slow muscles have characteristic α-actinins (43,53), tropomyosin subunits (6,8,37), troponins (13), myosin heavy and light chains (3,4,26,36,45) and C-protein subunits (47). In addition, levels of metabolic enzymes are different (12,36,41,50). Fast and slow muscles have different T-tubule and sarcoplasmic reticulum distributions (39,42,54), and differences in Z line thickness (39,46,54).

We have observed here a difference in the location of a structural protein within the contractile apparatus of the sarcomere. In fast muscle filamin is located

on the periphery of the Z disc while in a slow muscle it is additionally located in the I band (Fig. 1).

Figures 1 and 2 show that filamin is distributed evenly throughout the length of the I band rather than being in a specific subset of the I band, such as the A-I junction or the N line. We have observed the distribution of filamin at the I band in all the myofibrils throughout ALD myofibers, rather than, for instance, only those myofibrils at the periphery of the myofiber.

In addition to being evenly distributed along the length of the I band in ALD myfibrils, filamin is evenly distributed along the diameter of the I band. Figure 3b shows that desmin can clearly be seen to occupy a position only on the periphery of ALD myofibrils, and Figure 3c shows that α-actinin can be seen to occupy only the interior part of the diameter of the myofibril. Similar cryosections stained for filamin show neither type of staining but only even patches generally larger than the diameter of a myofibril. This indicates that filamin is located neither at the periphery of the I band in ALD nor at specific sites in the core of the I band, but rather evenly throughout the I band. However, the exact ultrastructural location of filamin within the I band region of ALD is unknown. We do not know whether filamin is randomly binding to all the thin filaments of the contractile apparatus, or to a subset of these thin filaments.

Assuming that there are approximately 400 actin monomers per thin filament in each half of an I band (28,40), and assuming that the vast majority of the actin in a myofibril is in the I band, we see from the results of the gel scans (Table 1) that there is one filamin monomer for every two thin filaments in the I band in pectoral muscle. With the same assumptions, we see that there are approximately five filamin monomers per thin filament in the ALD. If, however, a substantial fraction of myofibril actin is in the Z-disc, the amount of filamin per thin filament will increase correspondingly.

Since the molar saturation value for filamin binding to actin is on the order of 1 filamin/10 actins (21,48,57), these five filamins could bind to approximately 35 adjacent actin monomers. Thus, given the level in which it is present, filamin could conceivably occupy only a fraction of the length of the I band in ALD, rather than being evenly distributed as we observe by immunofluorescence.

Using scans of rabbit pulmonary macrophages, run on SDS-polyacrylamide gels, Stossel and Hartwig (51) found actin and actin-binding protein concentrations to be 10.4 and 1.8%, respectively, of total protein concentration, giving a ratio of approximately 34 mols actin/mol actin binding protein. This is in rough agreement with our figures for gizzard and fibroblast filamin (Table 1), which give a ratio of one filamin monomer per two complete turns of the actin filament helix (28). This is well above the saturation binding values of one filamin monomer per 10 actin monomers per filamin monomer reported for filamin and actin-binding protein (21,48,57).

Little or no gizzard type filamin is present in the ALD myofibrils as shown by absence of its characteristic electrophoretic variant in immunoprecipitates (Fig. 4) and by the absence of characteristic peptides in peptide maps (Fig. 6). The type of filamin in ALD is virtually identical to the type of filamin in fast skeletal muscle (Fig. 6). Although some spots differ in intensity, there is a complete match of spots in the two dimensional tryptic peptide maps. The differences in spot intensity could be due either to an artifact of the labeling and mapping procedure or to differences in levels of posttranslational modification of the peptides. The similarity of fast and slow muscle filamin is in contrast to the differences between fast and slow muscle forms of other actin binding proteins.

In light of the observation that purified gizzard filamin and tropomyosin compete for binding sites on F-actin in vitro (35,59) it is interesting to note that

there is a qualitatively inverse relationship of filamin and tropomyosin quantities in different tissues. Of the three tissues examined, gizzard has the highest filamin to actin ratio and the lowest tropomyosin to actin ratio. Pectoral myofibrils have the lowest filamin to actin ratio and the highest tropomyosin to actin ratio. ALD myofibrils have an intermediate ratio for both proteins. The fact that ALD myofibrils have a lower tropomyosin to actin ratio than pectoral myofibrils indicates that filamin may be displacing tropomyosin from F-actin in the ALD.

Filamin could bind to actin in the I bands of ALD by three general processes. First, the small number of differences we observe between ALD and pectoral filamin (Fig. 6) may be sufficient to cause pectoral filamin to bind only to the periphery of Z lines and allow ALD filamin to bind to I band thin filaments. The second process involves the deposition of filamin on I band thin filaments during myogenesis. We have previously shown that filamin is not present and is not synthesized in myogenic cells during the time in which sarcomeres are first forming (16). We hypothesized that filamin, by blocking the binding of myosin or tropomyosin to actin (16), would interfere with the initial construction of the sarcomere and hence the cell would have to have a mechanism to remove it. During the development of a slow muscle, given the proper innervation (1,8,24,25,45), synthesis of the two filamin forms may overlap rather than temporally separated as in fast muscle. Myoblast filamin could be removed at the same stage in development as in fast muscle but at the same time a small amount of skeletal muscle filamin synthesis could begin. The filamin could then bind to the developing actin filaments. Because only four or five (Table 1) of the 52 tropomyosin binding sites on a thin filament (28) would be taken up by filamin, myogenesis might then proceed normally.

The third general process by which filamin could be found in the I band would be to alter the relative pool sizes in developed myotubes of filamin and a molecule

that competes with filamin for actin binding sites, such as tropomyosin. If components of a given sarcomere are being continuously replaced then a newly synthesized actin filament would be able to bind either tropomyosin or filamin. In a fast muscle, the tropomyosin pool might be in such vast excess over the filamin pool that the actin filament would be saturated with tropomyosin. In a slow muscle, comparable pool size would allow some filamin and some tropomyosin to bind to the actin filament. All of the above hypotheses require a site at the periphery of the Z disc in fast muscle that binds filamin and not tropomyosin.

Finally, we might hypothesize that the presence of filamin in the I band of a slow muscle may be related to some of the observed physiological differences between slow and fast muscles. Although it has been hypothesized that the lower ATPase activities of slow muscle myosin may be directly responsible for the slower twitch times (2,4), the filamin in the I band of a slow muscle may, by displacing tropomyosin from actin filaments, by crosslinking actin filaments, or both, disrupt the semicrystalline contractile apparatus enough to affect contraction speed.

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Table 1

Quantitation of Actin and Filamin in Tissues and Myofibrils

Tissue	mg actin/g total protein[1]	mg filamin/g total protein[1]	mol actin/mol filamin[2]
Gizzard	130	30	52
Fibroblast	41	10	48
Myoblast	18	2.0	108
Pectoral Myofibrils	122	0.88	1650
ALD Myofibrils	106	7.0	164

[1]Values are the result of three separate determinations and have a standard deviation of approximately 15% of the mean.

[2]Assuming molecular weights of 500,000 for filamin and 42,000 for actin.

FIGURE LEGENDS

Figure 1 Immunofluorescence on freshly prepared myofibrils. 1a, c, e and g are phase images and 1b, d, f and h are the corresponding fluorescence images. 1a and b are a pectoral muscle myofibril stained for filamin and 1c and d are an ALD muscle myofibril also stained for filamin. 1e and f are an ALD muscle myofibril stained for desmin and 1g and h are a similar myofibril stained for α-actinin. Bar in figure 1h is 5 microns.

Figure 2 Longitudinal cryosections of fresh ALD muscle stained for filamin, desmin and α-actinin by immunofluorescence. 2a, c and e are phase images and 2b, d and f are the corresponding fluorescence images. 2a and b is a section stained for filamin, 2c and d is a section stained for desmin and 2e and f is a section stained for α-actinin. Bar in figure 2c is 10 microns.

Figure 3 Transverse cryosections of fresh ALD muscle stained for filamin (3a), desmin (3b) and α-actinin (3c). Bar in figure 3c is 5 microns.

Figure 4 A) Coomassie blue stained 10% polyacrylamide SDS gel showing filamin immunoprecipitated from fresh ALD myofibrils. Molecular weight markers are purified gizzard filamin (F) at 250,000 daltons in lanes 1, 4, 7 and 10, myosin heavy chain (M) at 200,000 daltons and actin (A) at 42,000 daltons. Lane 2 shows pectoral muscle myofibrils and lane 3 shows ALD muscle myofibrils, approximately 40 µg of protein were loaded in each lane. Lanes 5, 6 and 8 are immunoprecipitations of filamin from 17, 35 and 70 µg respectively of total ALD myofibril protein using antisera affinity purified with purified gizzard filamin. Lane 9 is an immunoprecipitation as in lane 8 using PBS instead of affinity purified antiserum.

Figure 5 A Coomassie blue stained 12.5% polyacrylamide SDS gel. Lane 1, whole adult gizzard; lane 2, cultured chick embryo fibroblasts; lane 3, cultured myoblasts; lane 4, pectoral myofibrils; lane 5, ALD myofibrils; lane 6, actin standard. Arrows show gizzard α-tropomyosin in lane 1 and ALD β (upper band) and α (lower band) tropomyosins in lane 5.

Figure 6 Two-dimensional peptide maps of filamin from gizzard (5a), ALD myofibrils (5b) and pectoral myofibrils (5c). Filamin was immunoprecipitated, iodinated in the presence of 1% SDS, and digested with Trypsin-TPCK. Peptide maps were of typically 0.1 μg filamin containing 5000 cpm and were exposed at -70 °C with intensifying screens for typically 48 h. In Figure 5a * is an example of a peptide present in gizzard but not ALD or pectoral myofibril filamin. Arrows in Figure 5b indicate spots that differ in intensity between ALD and pectoral myofibril filamins (5b and 5c).

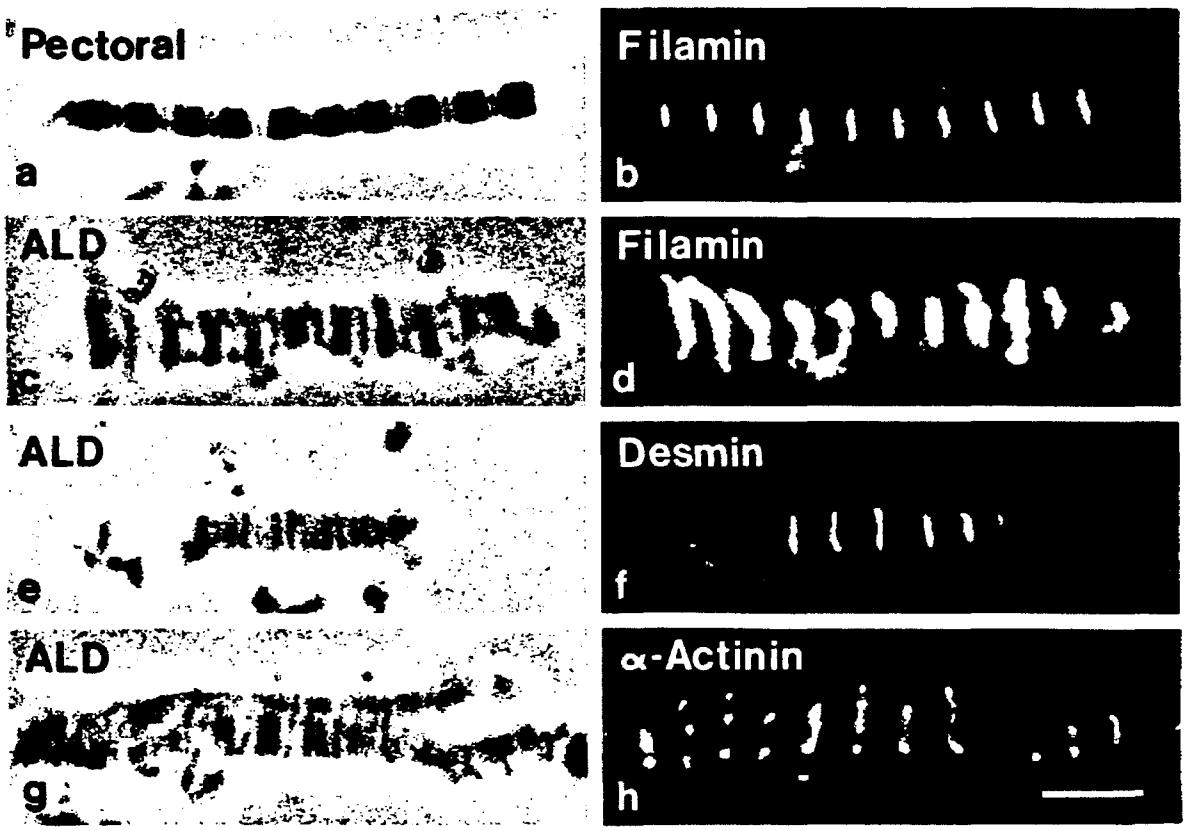
Pectoral
a
Filamin
b
ALD
c
Filamin
d
ALD
e
Desmin
f
ALD
g
α-Actinin
h

Figure 1

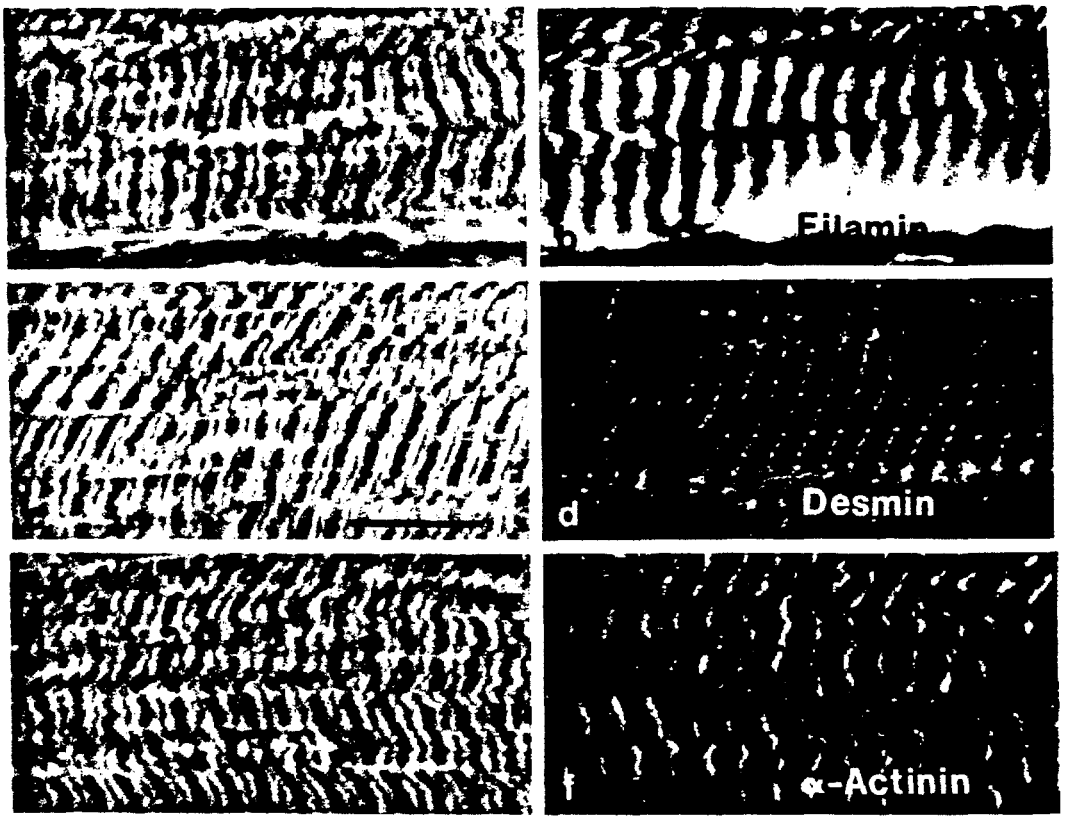

Figure 2

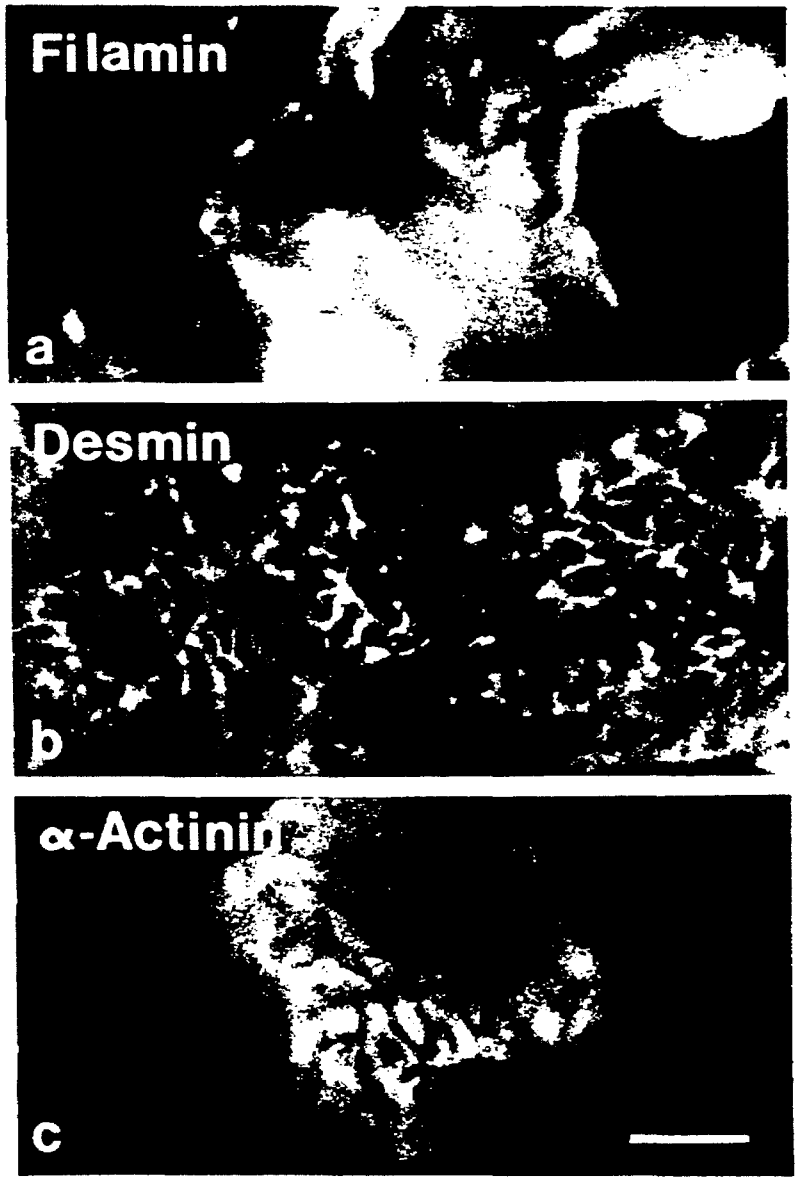

Figure 3

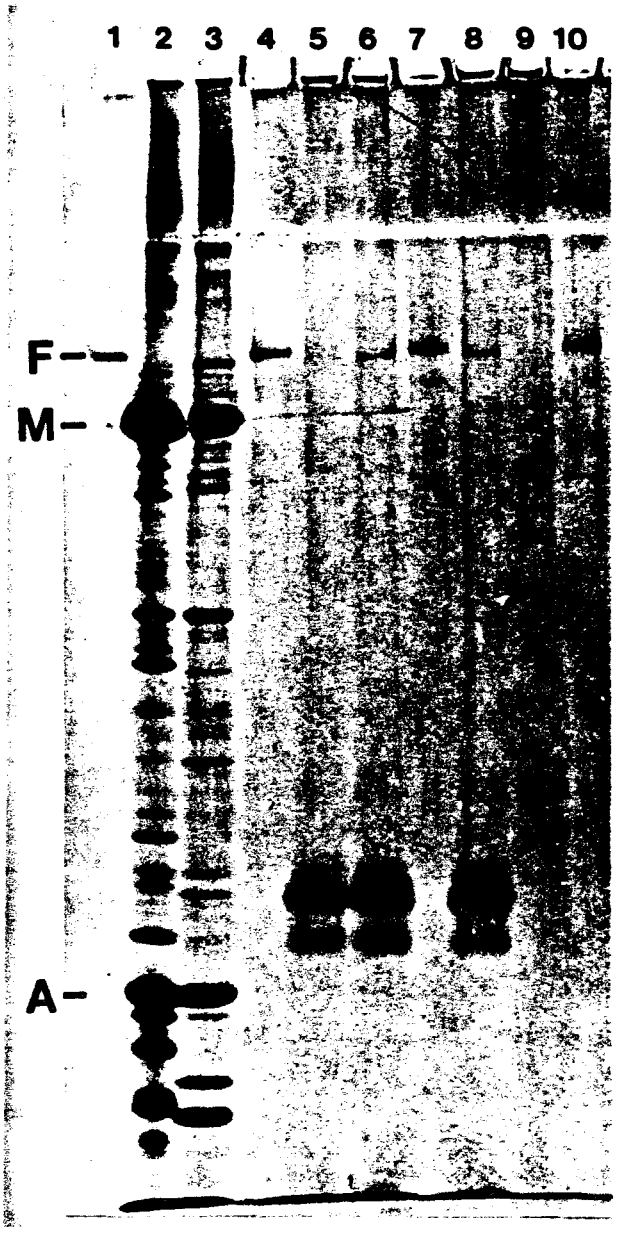

Figure 4

Figure 5

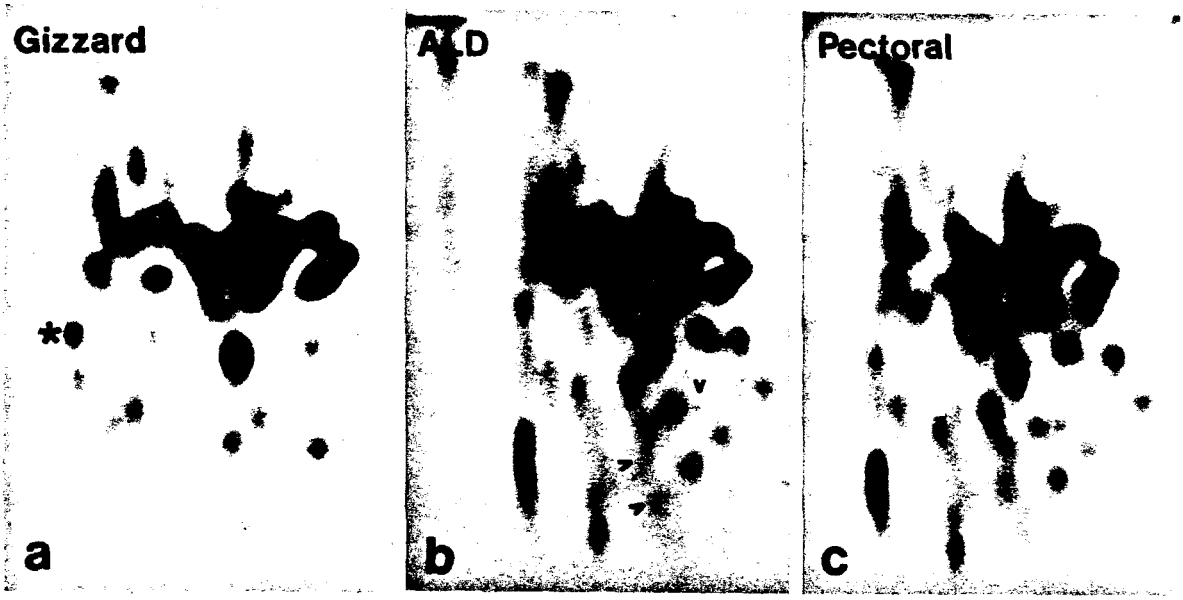

Figure 6

Chapter 5.

CONCLUSION

CONCLUSION

The results of the individual experiments are discussed in Chapters 2,3 and 4. In Chapter 2 I examined the role of filamin in sacromere morphogenesis. I then developed a technique to quantitatively immunoprecipitate a protein from a tissue, and peptide map the immunoprecipated protein. Chapters 3 and 4 use this technique to further examine the type and quantity of filamin during sarcomere morphogenesis.

The main results of the thesis can be summarized as follows. Gizzard and chick embryo fibroblast filamins are identical by peptide map analysis. In the two tissues, the filamin to actin molar ratios are virtually identical, 1 filamin/25 actin monomers although the filamin and actin concentrations are three times higher in gizzard than in fibroblasts. If the actin filaments are homogeneous, this molar ratio would be equivalent to 1 filamin monomer per two turns of the actin double helix.

Like fibroblast filamin, myoblast and early myotube filamin is found on stress fibers. This filamin is quite similar to gizzard and fibroblast filamin by two dimensional peptide map analysis. The amount of filamin in myoblasts, as a percentage of total protein, is one fifth that of fibroblasts. The molar ratio of filamin to actin in these cells is approximately 1:54. If the actin filaments are homogeneous, this would be approximately equivalent to one filamin per four complete turns of the actin double helix. Myoblast filamin is synthesized in myoblasts and early fused myotubes and then ceases being synthesized approximately one day after fusion. Filamin disappears from developing myotubes shortly before alpha-actinin transits from stress fibers to primitive Z disks.

Approximately six days after fusion, when sarcomeres can be clearly seen by phase contrast optical microscopy, skeletal muscle filamin begins to be synthesized. This polypeptide is quite different from gizzard, fibroblast or myoblast filamins by two dimensional peptide mapping. It appears at the periphery of Z disks in the developing muscle shortly before the intermediate filament proteins desmin and vimentin transit from a fibrous cytoplasmic network to the peripheries of Z disks. In fast skeletal muscle, filamin undergoes no further isozymic transition and is present at a concentration of less than 0.1 percent of total myofibril protein, even though the amount of actin is comparable to the amount ot actin in gizzard, 12-13 percent of total

muscle protein. Assuming that all the actin in the myofibril is in the thin filaments of the contractile apparatus, this gives a ratio of approximately one skeletal muscle filamin monomer per two thin filaments.

In slow muscle, filamin is located throughout the I band. This filamin is identical to fast skeletal muscle filamin and is present at a concentration of 0.7 percent of total myofibril protein, giving a molar filamin to actin ratio of 1:82, or five filamin monomers per thin filament.

From these results we see that there is a fundamental difference in the nature of the actin filaments in gizzard, fibroblasts and myoblasts. In fibroblasts and gizzard, the filamin type and filamin to actin ratios are the same despite differences in absolute quantity. In fibroblasts and myoblasts, where actin is present as stress fibers, there is a twofold difference in the filamin to actin ratio and an additional difference in the filamin type. The physiological significance of this remains unknown.

The above results yield several new insights into eucaryotic molecular morphogenesis. First, from the disappearance of filamin during myogenesis, we see that a morphogenetic process may involve the temporary removal of a family of proteins. The removed protein may be blocking a binding site necessary for the morphogenetic process. If so, we might hypothesze that the presence of the protein in other cell types may be 'locking' that cell in a relatively

undifferentiated state. Second, it appears that the stage at which proteins are synthesized and their synthesis rates are very important in determining the final structure. This is illustrated in Chapter 4, where I have shown that the same polypeptide (skeletal muscle filamin) can exist in two different distributions in a type of structure, specifically a sarcomere. All of the hypothesized processes whereby the two different distributions arise require changes in the synthesis rates as a function of time for the various sarcomere proteins, rather than changes in protein variants. I can thus offer the generalization that, at least for some eucaryotic subcellular morphogenetic processes, structures do not form by spontaneous combination of a complete set of proteins present as a pool, but rather by the sequential addition of proteins synthesized by the cell in a strictly defined temporal order.

Experiments not presented in this thesis have been performed to further examine the role of filamin in myogenesis. Initial experiments show that myoblast, fibroblast, gizzard and skeletal muscle filamins are phosphorylated <u>in vivo</u>, and preliminary peptide mapping experiments have been done to compare the phosphopeptides from the various filamins. A purification of skeletal muscle filamin is in progress to allow biochemical comparison of this filamin with stress fiber (gizzard or fibroblast) filamin. I have developed a method to purify filamin from cow uterus smooth muscle. This filamin is

similar to chicken and gizzard filamin by two dimensional peptide mapping and has a slightly lower molecular weight. Like chicken gizzard filamin, this filamin binds to F-actin. Antibodies to this protein stain stress fibers in baby hamster kidney (BHK) cells. This antibody can eventually be used to determine if the results presented in this thesis can also be observed in mammals.