Molecular Analysis of Protein Assembly in Muscle Development

HENRY F. EPSTEIN* AND DONALD A. FISCHMAN

The challenge presented by myofibril assembly in striated muscle is to understand the molecular mechanisms by which its protein components are arranged at each level of organization. Recent advances in the genetics and cell biology of muscle development have shown that in vivo assembly of the myofilaments requires a complex array of structural and associated proteins and that organization of whole sarcomeres occurs initially at the cell membrane. These studies have been complemented by in vitro analyses of the renaturation, polymerization, and three-dimensional structure of the purified proteins.

THE REGULARITY OF MUSCLE STRUCTURE HAS SERVED AS A paradigm for studies of biological organization. Skeletal muscles are composed of muscle fibers which, in turn, contain long cylindrical myofibrils. Each myofibril is made up of repeating assemblies of thick and thin filaments that are visible as striations in the light microscope (Fig. 1a). These near-crystalline assemblies form repeating dark and light bands called sarcomeres. The appearance of striations requires precise assembly of contractile protein filaments, which contain actin (predominately in thin filaments), myosin (principally in thick filaments), and associated proteins (such as tropomyosin, troponin, and α-actinin).

An understanding of the mechanisms controlling muscle assembly is key to many biological problems with practical applications for human well-being. The ultimate defect in cardiac failure is insufficient maintenance of functional myofibrils. One type of familial hypertrophic cardiomyopathy is caused by mutation in the ventricular myosin heavy chain (*1*), the first example of a myofibrillar mutation in humans. Diabetes and starvation lead to extensive disassembly of muscle as myofibrillar proteins degrade (*2*). The increased production of food proteins needed by our increasing population requires more efficient myofibrillar synthesis, assembly, and maintenance in meat animals (*3*).

However, the mechanisms regulating the precision of thick and thin filament protein assembly are only beginning to be revealed. A major challenge is to identify the molecules and molecular reactions that align thick and thin filaments and that control the transverse registration of myofibrils within and across muscle fibers.

In normal homeostasis, hormonal, neuronal, and nutritional signals must somehow be transduced into changes of myofibril assembly (*2*). In development and regeneration, there must be additional mechanisms that control initiation of myosin polymer formation. Self-assembly mechanisms (*4*) do not easily explain these developmental and homeostatic reactions. Actin and myosin cannot be reversibly denatured and renatured, and the spontaneous formation of tropomyosin heterodimers in vitro appears too slow. The action of modifying enzymes and interaction with associated proteins may affect assembly of filament components as in phage morphogenesis (*5*).

H. F. Epstein, Departments of Neurology, Biochemistry, and Cell Biology, Baylor College of Medicine, Houston, TX 77030. D. A. Fischman, Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021.

*To whom correspondence should be addressed.

Isoform Multiplicity

It will be necessary to understand the functional significance of the diversity of the protein building blocks themselves, in order to gain insights into muscle assembly as a dynamic process. Many myofibrillar proteins exist as multiple isoforms—variants in amino acid sequence—within the same organism and even within the same cell. Muscle development is associated with major changes in the expression of distinct isoforms (*6*).

At least four explanations are relevant to possible roles of isoforms in myofibril assembly and muscle development.

1) In the model of differential assembly, the differences in amino acid sequence among isoforms would lead to distinct associations between identical proteins or with other assembling proteins (*7*). The classical demonstration of isoforms exhibiting differential assembly occurs for myosin in *Caenorhabditis elegans*. The myosin molecule is composed of two heavy chains and four light chains, which combine to form a structure with a rod at one end and a globular head. AA and BB myosin heavy chain homodimers are localized in distinct regions of the thick filaments in the body wall muscles of *C. elegans* (*8*). The AA isoform is located exclusively in the central zone, where myosin tails come together, whereas the BB isoform is present in the bilaterally flanking polar regions. Although differences in amino acid sequence have been demonstrated between the A and B myosin heavy chain isoforms, especially in segments that are involved in assembly (*9*), genetic and immunocytochemical experiments show that the presence of a third protein, paramyosin, is necessary for proper myosin assembly (Fig. 2) (*10*).

2) Different isoforms may exhibit distinct functions in their proper biological environment (*11*). Differences in contractile and regulatory function between isoforms have been determined for mammalian myosin, tropomyosin, and troponin-T. In cardiac muscle, the αα and ββ myosin isoforms exhibit threefold differences in the maximum velocity (V_{max}) at which they hydrolyze adenosine triphosphate (ATP) that correlates with differences in contractility between αα-rich and ββ-rich muscle (*12*). In specific skeletal muscle fiber types, the expression of particular tropomyosin and troponin-T isoforms correlates with the cooperativity of Ca^{2+} regulation of tension (*13*).

3) The hypothesis of developmental regulatory cassettes in which the expression of a set of isoforms of different proteins is coregulated could hold for distinct genes or for alternative transcripts of the

same gene. Developmental regulatory cassettes appear to be widespread; nearly all multicellular organisms have developmental stage– or cell type–specific expression of distinct isoforms. The actin gene families of *Dictyostelium* and *Drosophila* contain 17 and 6 members, respectively. Several of the *Dictyostelium* actins appear to be identical in amino acid sequence, but their messenger RNAs (mRNAs) are distinguishable, and their expression varies considerably during early development and cell morphogenesis (*14*). In each cell type or developmental stage, regulatory elements switch specific isoform expression on or off (*15*).

4) The principle of redundancy suggests that the presence of a family of genes in which each encodes a unique isoform would protect the organism against deleterious mutation in one gene. In this model, the protein isoforms would have to be interchangeable in terms of function and assembly, and the patterns of expression would overlap in terms of location and time (*16*). Apparent redundancy is suggested by genetic experiments with the *act-1*, *act-2*, and *act-3* genes each encoding a body-wall muscle actin in *C. elegans*. A deletion mutation that prevents expression of any one of the actin genes has no effect on the phenotype (*17*).

It is likely that no single functional model for protein isoforms will explain all known cases. The utility of the isoform mechanism may be its adaptability to very different levels of function.

Genetic, Molecular, and Structural Approaches Converge

The application of new innovations in genetic, molecular, and structural biology has furthered our understanding of the biophysical mechanisms operating in muscle contraction and the key regulatory interactions underlying commitment and gene expression in myogenesis. Genetic manipulation of the nematode *C. elegans* (*18*) and several *Drosophila* strains (*19, 20*) are providing experimental material for much of the combined efforts. The first molecular cloning and sequencing of a myosin heavy chain gene and an entire myosin heavy chain family was done in *C. elegans* and led to (i) the structural model of the myosin rod (*18, 21*); (ii) evidence for the developmental and functional significance of the myosin heavy chain isoforms and of myosin-associated proteins in thick filaments; (iii) identification of specific proteins of the adhesion plaques active in myofibril initiation; and (iv) the cloning and sequencing of the first neural cell adhesion molecule (N-CAM)–related accessory protein of the thick filament. Research with *Drosophila* provided the first evidence for alternative splicing in the tissue-specific expression of myosin heavy chain isoforms (*19*); indicated the roles of troponin-T, α-actinin, and a specific actin isoform in the assembly of thin filaments in vivo; and demonstrated the necessity of regulated expression of actin and myosin for proper myofibrillar organization (*20*). Both *C. elegans* and *Drosophila* studies have confirmed that thick and thin filaments assemble independently of each other (*18, 20*).

Genetic engineering in vitro has been used to design altered, deleted, or chimeric myofibrillar proteins (*19, 22, 23*). Its successful application in *Drosophila* has permitted physiological testing of engineered proteins, especially the analysis of the interactions of mutated actins in assembly (*20*). Engineered myosin heavy chain segments, tropomyosin, and troponin-C produced in recombinant cells have been analyzed by in vitro aggregation and reconstitution (*22, 23*). The rescue of specific muscle-defective mutant strains by relevant genes engineered in vitro in easily manipulated organisms such as *C. elegans* and *Drosophila* will permit physiologically stringent assays of their myofibrillar function in vivo.

Technological improvements in computer-based imaging are stimulating rapid acquisition of three-dimentional information and permitting direct observations of active assembly. With the generation of specific antibodies, particularly monoclonal reagents, immunofluorescence microscopy has become a standard technique of molecular localization (*24*). As a consequence of advances in video microscopy, it is now possible for researchers to inject fluorescent antibodies or fluorescent myofibrillar proteins into living muscle cells and detect physiological assembly and exchange (*25*). Single-protein filaments may be directly visualized as they assemble in vitro under fully native conditions by video-enhanced differential interference microscopy (*26*). More minute details of filament structure have been obtained by computer-based three-dimensional reconstruction of electron microscope images of negatively stained or unstained hydrated, frozen specimens (*27, 28*). The molecular structure of several myofibrillar proteins has been studied by x-ray diffraction of their crystals (*29*).

Bridging the Gap Between Dynamics and Structure

Understanding the dynamic properties and three-dimensional structures of myofibrillar protein assemblies will allow researchers to predict the supramolecular organization and assembly reactions based on knowledge of the detailed molecular structures and amino acid sequences of the protein monomers. Although this goal has not yet been reached for any muscle protein, progress in both areas has now indicated the most promising approaches.

Four myofibrillar proteins are undergoing analysis by x-ray crystallography; myosin subfragment-1 (the head); troponin-C, the

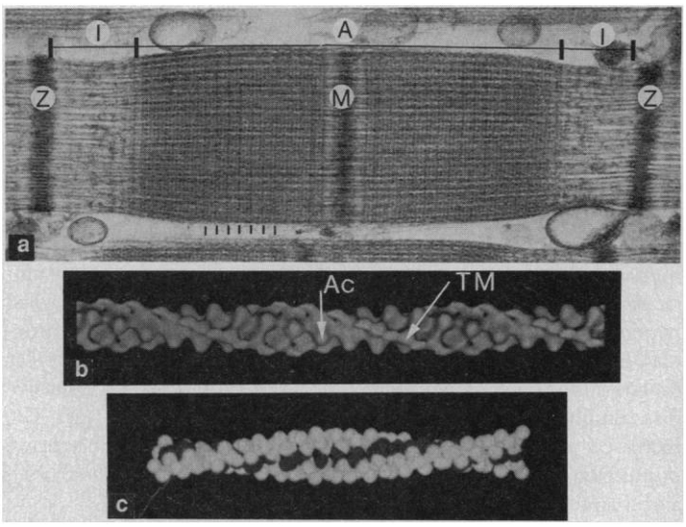

Fig. 1. The myofibril and component structures. (**a**) Electron micrograph of rabbit psoas muscle after rapid freezing, OsO4 fixation, freeze substitution, embedding, and sectioning. The interdigitating thick and thin filaments of the A band (**a**) and the 43-nm C-protein repeats on the thick filament are evident. The thick and thin filaments are cross-linked at the M and Z bands. The I band contains only thin filaments. (Courtesy of H. E. Huxley) (**b**) Space-filling model of reconstituted F actin-tropomyosin filament. The model is derived from electron microscopy of frozen hydrated specimens and computer reconstruction of the images. Note the coiling of tropomyosin (TM) about the right-handed actin helix (Ac) with a pitch of about 36.5 nm. (Courtesy of R. A. Milligan and M. A. Whittaker) (**c**) Space-filling model of a two-stranded α-helical coiled coil. The α-carbon atoms (light) are shown for a portion of the sequence of α_2-tropomyosin (residues 44 to 118). For clarity, only the apolar side chains (dark), which interlock to stabilize the coiled coil, are illustrated. The coiled coil is left-handed, and the individual α-helices are right-handed. [Adapted from (*64*).]

monomeric, globular form of actin (G-actin); and tropomyosin (*28*). Only the troponin-C and G-actin structures are presently known at atomic resolution. The detailed packing of amino acid side chains has recently been determined for G-actin complexed with DNase I. A model for the structure of tropomyosin (Fig. 1c) and for the overall shape of the myosin head have been derived.

At the next level of structure determination, three-dimensional structures of native thick filaments and reconstituted thin filaments have been reconstructed from the electron microscopy of negatively stained or frozen, hydrated specimens (*27, 29*). The structural models produced thus far with electron microscopy have resolutions of about 3 to 4 nm. At this resolution, the contours of protein monomers may be distinguished. The thin filament with its constituents actin, tropomyosin, and troponin is likely to be the first myofibrillar structure to be understood in molecular detail (Fig. 1b). Structural information on myofibrillar proteins will be crucial to explaining and predicting their dynamic properties and supramolecular associations.

A detailed picture of the folding and association of tropomyosin polypeptides into the native α-helical coiled-coil dimer (Fig. 1c) and its interaction with actin and troponin in the assembly of thin filaments is now available. The observation that αβ tropomyosin heterodimers are found preferentially in specific muscles, rather than αα and ββ molecules (*13*), has stimulated a series of studies of the reversible denaturation and renaturation of tropomyosin (*30*). The homo- and heterodimers can all reversibly dissociate to the monomer forms. Heterodimer formation by exchange of monomers is thermodynamically favored but, depending on the experimental conditions, may assemble more slowly or more rapidly than homodimers. Since the observed half-time for αβ formation by exchange is of the order of hours, the folding and association of α and β tropomyosins in muscle may require catalysts.

The genetic and structural dissection of tropomyosin interaction with actin and troponin is providing complementary information about thin filament assembly. The exact register of an integral number of 48 amino acid–residue repeats (about 7.1 nm each) within tropomyosin are necessary for correct binding to actin filaments (*31*). The number of repeats per tropomyosin molecule can be seven (muscle isoform), six (nonmuscle isoform), or five (yeast). Troponin-T is involved in stabilizing tropomyosin-actin interactions, since mutations altering the repeat pattern in tropomyosin are overcome by troponin addition in vitro, and troponin mutations can disrupt thin filaments and their cross-linking by α-actinin in *Drosophila* (*20*). The observation that specific tropomyosin–troponin-T–α-actinin isoform combinations correlate with skeletal muscle fiber types is potentially related to these genetic results (*13*). The location of troponin-T at the head-tail junctions of contiguous tropomyosins along actin filaments is consistent with its importance in stabilizing the overall assembly of muscle thin filaments (*32*).

Kinetics and Equilibria of Reactions of Myofibrillar Proteins

The polymerizations of actin and myosin into synthetic filaments in vitro are classical models of biological assembly (*33*). Although structural considerations and genetic analysis suggest that additional components and reactions are required for thick and thin filament assembly in vivo, the in vitro reactions and polymers can be used to assay the biological properties of genetically engineered myofibrillar proteins. The equilibrium and rate constants determined establish thermodynamic and kinetic constraints on assembly mechanisms that act in vivo. Actin filaments (F-actin) are composed of monomeric subunits of G-actin. The observed equilibria and reaction rates for actin are consistent with the theory of nucleation-dependent polymerization (*34*). Similarly, the helical structure of F-actin is consistent with its nucleation-dependent formation. For actin, polymerization proceeds by

$$A + A \rightleftharpoons A_2$$

$$A_2 + A \rightleftharpoons A_3$$

$$A_3 + A_{n-3} \rightleftharpoons A_n$$

where A is monomeric G-actin, A_3 is the trimeric nucleation structure, and A_{n-3} are F-actin.

For skeletal muscle myosin, the first step is the formation of parallel dimers of myosin molecules staggered by 43 to 44 nm (*34a*)

$$M + M \rightleftharpoons M_2$$

Polymerization ensues by

$$M_2 + M_{i-2} \rightleftharpoons M_i$$

where M is the myosin molecule, and M_{i-2} and M_i are synthetic myosin polymers.

Unlike actin, myosin polymers do not form by simple polar addition of monomers. Instead, the packing of myosin rods results in a bipolar structure. In the case of striated muscle myosin, kinetic and equilibrium studies have not detected a distinct nucleation step for the antiparallel interactions that form bipolar structures. The cooperativity of myosin helix formation is distributed in both the initiation and elongation phases of polymerization (*35*). Varying conditions of pH, ionic strength, Mg^{2+}, and ATP produce stable intermediate structures: bipolar minifilaments (16 to 18 myosins, 0.3 μm length); pH 8.0 filaments (~150 myosins, 0.67 μm), and native-like filaments (~300 myosins, 1.5 μm) (*36*). The minifilaments are similar in length to assemblages produced by depolymerization of native vertebrate thick filaments (*37*). These assemblages represent a stable form of a short bipolar myosin structure.

Myosin interactions become progressively weaker from the central region outward to the ends of both native and pH 8.0 synthetic filaments (*37–39*). This phenomenon results from an increase in dissociation rate with increasing length (*39*). The ionic bonding of myosin at filament ends is so weak that rapid exchange occurs with free myosin. Direct evidence for myosin exchange between synthetic filaments and in muscle has been obtained (*40*). Control of this bonding may be the mechanism of determination of precise filament length in vivo. Indeed, synthetic filaments approximating native lengths can be produced by careful control of myosin and ion concentrations (*36, 39, 41*).

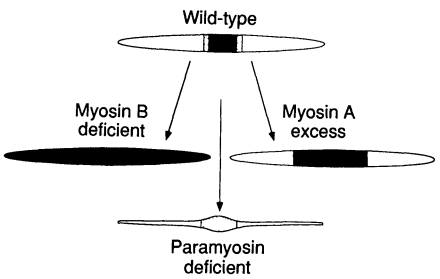

Fig. 2. Differential assembly of myosin isoforms in *C. elegans*. In body wall muscle cells of wild type, thick filaments are about 10 μm in length. Myosins A and B are AA and BB homodimers of myosin heavy chains encoded by the *myo-3* and *unc-54* genes. Myosin A is restricted to a central 1.8-μm–long zone, whereas myosin B is located bilaterally in the polar 4.5-μm-long regions. Bilateral small areas of overlap exist. Myosin A alone (dark), myosin B alone (light), myosins A and B (speckled). In null mutants of *unc-54* such as *e190*, the deficiency of myosin B leads to thick filaments of normal length that have myosin A along their entire length. In duplications of *myo-3*, such as *e1407*, the excess of myosin A leads to spreading of its distribution. In the *e1214* null mutant of *unc-15*, the deficiency of paramyosin leads to a scrambling of myosins A and B in the central zone and the assembly of bilateral tubular structures associated with myosin B (*10*).

Structure and Assembly of Thick and Thin Filaments

Despite the limited structural resolution of filament proteins and filaments and the difficulties in establishing cell-free assays of physiologically significant assembly, available information indicates that the structures of thick and thin filaments and the mechanisms required for their assembly in muscle cells are more complex than in earlier models of simple helical polymers and their self-assembly.

The α-helical coiled coil in myosin heavy chain rods as in tropomyosin is based on a repeating heptapeptide unit within each chain, a-b-c-d-e-f-g, where a and d generally have apolar side chains (Fig. 1c), and the other positions frequently have charged side chains. In the myosins, additional repeats of 28 amino acid residues are found throughout the rods. Extra residues representing "skips" in the 28-residue pattern are detected at only positions 4 and 5, respectively, in the *unc-54* myosin heavy chain and *unc-15* paramyosin rods of *C. elegans*. These skip residues may modulate the coiled-coil pitch and result in a change of twist of the myosin rod that could have profound effects on filament structure. Interactions between myosin rods are strongest at staggers of 98 residues (14.3 nm) and 294 residues (43 nm) (*9, 21*). These staggers agree numerically with the strongest helical repeats detected in a variety of thick filaments by x-ray diffraction (*42*) and electron microscopy (*27, 29*). The challenge will be to extend the one-dimensional analysis to a three-dimensional model of thick filaments.

Although the arrangement of myosin heads appears helical in thick filaments from several species, multiple lines of evidence indicate some form of nonequivalence among the constituent myosin molecules, which may be related to nonmyosin proteins. Examples of these complexities include the 29.0- and 48.0-nm myosin periodicities in the striated adductor of the scallop, the periodic localization of C-protein and other associated molecules to particular sites along myosin in vertebrate skeletal muscles (Fig. 1a), and the interaction of two myosin isoforms and paramyosin in *C. elegans* to produce the wild-type localizations shown in Fig. 2 (*8, 10, 43, 44*).

In vertebrate muscles, at least eight proteins in addition to myosin appear to be associated with the thick filaments: C-protein, H-protein (in mammals) or 86-kD protein (in birds), M-protein, myomesin, M-creatine kinase, adenosine monophosphate (AMP)–deaminase, skelemin, and titin (*44, 45*). None has a demonstrated function in thick filament assembly or A-band formation, but molecular analysis of their sequences and intracellular localization studies suggest a potential morphogenetic role for several of the proteins. Primary sequence analyses of twitchin (the *unc-22* gene product of *C. elegans*), C-protein, titin, skelemin, smooth-muscle myosin light chain kinase, and 86-kD protein indicate that all are intracellular, nonmembrane-associated members of the N-CAM gene family (*46*). All exhibit two sets of repetitive motifs, ~100 amino acids in length, with predicted β-sheet structure. One of the domains belongs to the C2 set of the immunoglobulin gene family, and the other is related to the type III domains of fibronectin (*47*) (Fig. 3). Evidence with N-CAM suggests a role for such domains in intercellular adhesion (*48*); it remains to be seen if these myosin-associated proteins function in comparable intra- or interfilamentous adhesive reactions within the myofibril.

Immunofluorescent studies of cultured embryonic myocytes have demonstrated that titin is one of the earliest markers of sarcomere formation and that C-protein and myomesin appear coincident with A-band formation (*49*). Since a single titin molecule is believed to stretch the full length of each half sarcomere (for example, from M band to Z band), this molecule has been considered a potential length-regulation template during myofibril assembly (*50*), whereas C-protein and myomesin might function in the lateral registration of thick filaments (Fig. 4). However, a central role for twitchin in myofibril assembly is unlikely, since nematode mutants lacking twitchin have normal numbers of myofilaments. These twitchin-deficient mutants can organize sarcomeres when suppressed by missense mutations in the myosin head (*51*). Further, thick filaments assemble in embryonic vertebrate muscle cells independently of the titin-containing complexes (*49*). The highly specific localizations of so many proteins to specific sites about the myosin-containing filaments suggest that the mechanisms of thick filament assembly and A-band organization must be complex.

Mutants of *C. elegans* and *Drosophila* and cultured myocyte models indicate that the assembly of thick and thin filaments are

A **Myosin-associated Ig C-2 family**

```
C-2:    **LTC*****P***L*W****************K/R**********L*L******D*G*Y*C*A*-N
C-pro:  **L*C*****P*P*VTW*K*G***********K/R*********SL*I******D*G*Y*C**V*N
86-kD:  **L*C*****P***I*W****************-K-*********SL*I******D*G*Y*C*AV-N
smLCK:  ****C*****P*P*V*W*K**************--******----*SL*I******D***Y*C*AV-N
Titin:  **L*V*****P*P*V*W*K*G*****--******--*********--L********D*G*Y**TL*-N
Twthn:  ****V*****P*P*V*W*K*G***********K/R**********L*I******D*G*Y***A--N
```

B **Myosin-associated FN III family**

```
C-pro:  PPQ***V*EV******L*W*PP*DDGNA*I*GYTVQK*D****EW**V****R*-TR**V**L**G*EY*FRV*S*N**G*S*E****
86-kD:  PPQ***L*DV******L*W*PP*DNGNS*I*GYTVQK*D****KW**V****T*-TS**I**L**G*TY*FRV*S*N**G*S*T****
smLCK:  PAG***A*DI******L*W*GS*YDGGS*V*SYTVEI*N****KW**L-***R*-TS**V*DL**D*EY*FRV*A*N**G*S*P****
Twthn:  P**-**V*DV******L*W*PP*DDGGA*I**YVVEK*D*****W**V*******T***V**L**G*EY*FRV*A*N**G*S*P****
Titin:  P**-**V**V******L*W**P**DGGS*I*GYIVEK*D*****W******---*****V**L**G*EY*FRV*A*N**G*G*P****
```

Fig. 3. Amino acid consensus alignments of the myosin-associated proteins (located on or about thick filaments) that belong to the immunoglobulin (Ig) C-2 and fibronectin (Fn) type III families. (**A**) The consensus sequence for the Ig C-2 family (C-2) (*46*) is aligned with the predicted amino acid consensus sequences for C-protein (C-pro), 86-kD protein (66)s, smooth muscle light chain kinase (smLCK), titin, and twitchin (Twthn) (*46*). (**B**) The consensus sequences of the type III Fn repeats in these same myosin-associated proteins are aligned according to the consensus (*47*). For each figure, those amino acids that are identical in every member of each family are linked by a vertical bar and underscored with a caret. Those amino acids present in at least three members of each class have also been underscored. Asterisks have been inserted in place of nonidentical amino acids, and dashes have been inserted where the sequences have been shifted to facilitate vertical matches between the protein repeats.

largely independent of one another. Each type of filament assembles in the absence of the other type and in the absence of organized myofibrillar structure (*52*). Mutations affecting either thick or thin filament assembly occur in a greater number of genes than are required to encode the presently known protein components. In *C. elegans*, the assembly of myosin heavy chains (which are the products of *myo-1*, *myo-2*, *myo-3*, and *unc-54*) and of paramyosin (encoded by *unc-15*) require *unc-45* and *unc-82* gene products, respectively (*53*). In *Drosophila*, tropomyosin and troponin-T mutants disrupt normal thin filament–Z band interactions (*20*). Biochemical and localization experiments are compatible with the regulation of actin assembly by the Cap Z protein at the Z band (*54*). Multiple proteins clearly interact in the assembly of myosin and actin to produce thick and thin filaments in muscle.

The assembly of thick and thin filaments, although independent of one another and of overall myofibrillar structure, may require transient nucleation complexes, possibly at special sites such as the cell membrane or the preexisting cytoskeleton (*55*). Multiple mutant strains of *C. elegans* in which thick filament assembly is disrupted accumulate unusually large, thick filaments or multifilament assemblages. Smaller numbers of similar structures accumulate in wild-type *C. elegans*, suppressed nematode mutants, *Drosophila* muscle mutants, and *Limulus* telson muscle (*56*). Preliminary in vitro experiments and genetic studies suggest that the assemblages may be transient assembly complexes (*57*).

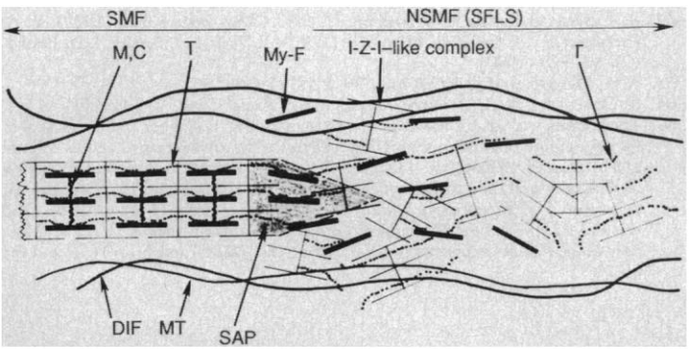

Fig. 4. Proposed model of myofibril assembly based on immunofluorescent and electron micrographic studies of cultured skeletal and cardiac myocytes (*49, 63*) (courtesy of H. Holtzer, H. Ishikawa, and T. Schultheiss). NSMF, nonstriated myofibril; SFLS, stress-fiber–like structure; SMF, striated myofibril; My-F, myosin-containing thick filaments; T, titin; C, C-protein; M, myomesin and M-band protein; SAP, submembranous adhesion plaque; DIF, desmin-containing intermediate filaments; MT, microtubles. NSMFs contain loose arrangements of independently assembled I-Z-I–like complexes and My-Fs. The I-Z-I–like complexes are composed of 0.3-um-wide Z bodies that contain α-actinin and titin linked to filaments composed of actin, tropomyosin, and troponin. SFLS in immature myocytes closely resemble nonmuscle stress fibers. The NSMFs may be initiated by the gradual insertion of muscle-specific isoforms (for example, α-actinin, α-actin, and α-tropomyosin) into SFLS along with unique muscle proteins such as titin and troponin. Hybrid structures containing nonmuscle and muscle isoforms exist transiently. Free My-Fs contain no detectable C-protein, myomesin, or M-protein; these three proteins are first seen when sarcomeres assemble into SMFs. The I-Z-I–like complexes and scattered My-Fs become organized into sarcomeres in close proximity to SAPs. The SAPs may catalyze or stabilize the association of I-Z-I–like complexes and My-Fs. SAPs contain talin, vinculin, and α-actinin. The transverse alignment of thick filaments may be guided and stabilized by C-protein, myomesin, and M-protein. Since nascent myofibrils are attached at each end to SAPs, continued longitudinal growth of the myofibril would necessitate a cyclic detachment of the terminal sarcomere as new units are assembled. In this model, SAPs subserve two functions: (i) provide sites for new sarcomere addition during myofibril elongation, and (ii) serve to transmit tension to the extracellular matrix. The functions of DIFs and MTs in this process are unknown. This model is intended to account for the end-on addition of new sarcomeres in newly formed myofibrils. Another set of interactions may be responsible for the appositional growth of preexisting myofibrils in muscle.

Membrane Involvement in Myofibrillar Organization

Transmission of contractile tension to the vertebrate endoskeleton or invertebrate exoskeleton requires specialized adhesion sites between the myofibrils and extracellular fibrillar components (for example, collagen) across the intervening plasma membrane. In vertebrates, the terminal attachment site, or myotendon junction, contains talin, vinculin, α-actinin, fibronectin, tenascin, the 80-kD protein, and collagen-binding integrins (*58*). Myofibrils appear to be linked transversely to the plasma membrane at periodic adhesion sites, termed costameres, which are in register with the sarcomere repeats of the myofibrils (*59*). Immunofluorescent studies indicate the presence of vinculin, meta-vinculin, a 36-kD vinculin-binding protein, γ-actin, nonmuscle α-actinin, spectrin, and talin on the cytoplasmic face of each costamere. Collagen fibrils insert on the extracellular face, and β_2-containing integrins and col-CAM, present at this location, could serve as transmembrane links between the intra- and extracellular components (*60*). Compositional analysis of invertebrate muscle-hypodermal-cuticular adhesion sites is just beginning, but immunochemical and genetic dissections of these structures in *C. elegans* and *Drosophila* (*18, 53, 61, 62*) indicate that proteins similar to vinculin, α-actinin, and integrins are present at these sites and that null mutations in integrin or vinculin have profound effects on early embryonic morphogenesis (*62*).

Several lines of evidence indicate that the plasma membrane and its associated submembranous cytoskeleton are directly involved in myofibril assembly. The initial stages of vertebrate sarcomere organization, both in vivo and in cell culture, are first detected beneath the sarcolemma (*55*). Nascent myofibrils are associated with stress fiber-like structures (SFLS), composed of nonmuscle contractile protein isoforms (*63*). The early myofibrils are anchored at each end to the cell surface by submembranes adhesion plaques (Fig. 4) that contain many if not all of the proteins identified at costameres (*59, 60*). A schematic model of myofibril assembly based on immunofluorescent analyses of cultured skeletal and cardiac myocytes is presented in Fig. 4. This model should serve to indicate the complexity of myofibrillogenesis, the potential integrating role of the plasma membrane, and the centrality of the adhesion plaque in this assembly process.

REFERENCES AND NOTES

1. G. Tanigawa *et al.*, *Cell* **62**, 991 (1990); A. A. T. Geisterfer-Lowrance *et al.*, *ibid.*, p. 999.
2. A. L. Goldberg *et al.*, *Med. Sci Sports* **7**, 248 (1975); D. Pette and G. Vrbová, *Muscle Nerve* **8**, 676 (1985); R. J. Zeman, R. Ludemann, J. D. Etlinger, *Am. J. Physiol.* **252**, E152 (1987); I. C. Kettelhut, S. S. Wing, A. L. Goldberg, *Diabetes Metab. Rev.* **4**, 751 (1988).
3. "Texas researchers develop four gene-altered calves," *New York Times*, 6 June 1990, p. A1.
4. C. B. Anfinsen, *Science* **181**, 223 (1973); A. Klug, *Harvey Lect.* **74**, 141 (1979); M. Nomura, *Science* **179**, 864 (1973).
5. S. Casjens and J. King, *J. Supramol. Struct.* **2**, 202 (1974); W. B. Wood, *Harvey Lect.* **73**, 203 (1978).
6. A. I. Caplan, M. Y. Fiszman, H. M. Eppenberger, *Science* **221**, 921 (1983).
7. H. F. Epstein, *UCLA Symp. Mol. Cell. Biol. New Ser.* **29**, 653 (1986).
8. D. M. Miller *et al.*, *Cell* **34**, 477 (1983).
9. N. J. Dibb *et al.*, *J. Mol. Biol.* **205**, 602 (1989); H. Kagawa *et al.*, *ibid.* **207**, 311 (1989).
10. H. F. Epstein, I. Ortiz, L. A. T. Mackinnon, *J. Cell Biol.* **103**, 985 (1986).
11. M. Barany, *J. Gen. Physiol.* **50**, 197 (1967).
12. C. A. Dechesne *et al.*, *J. Cell Biol.* **105**, 3031 (1987).
13. D. D. Bronson and F. H. Schachat, *J. Biol. Chem.* **257**, 3937 (1982); F. H. Schachat, D. D. Bronson, O. B. McDonald, *ibid.* **260**, 1108 (1985); M. M. Briggs *et al.*, in *Cellular and Molecular Biology of Muscle Development*, L. H. Kedes and F. E. Stockdale, Eds. (Liss, New York, 1989), p. 597.
14. M. McKeown and R. A. Firtel, *Cell* **24**, 799 (1981).
15. E. A. Fyrberg *et al.*, *ibid.* **19**, 365 (1980).
16. I. Greenwald and H. R. Horvitz, *Genetics* **96**, 147 (1980).
17. R. H. Waterston, D. I. Hirsh, T. R. Lane, *J. Mol. Biol.* **180**, 473 (1984).
18. P. Anderson, *Annu. Rev. Genet.* **23**, 507 (1989); H. F. Epstein, *Mol. Neurobiol.* **4**,

1 (1990); R. H. Waterston, *Cell Motil. Cytoskeleton* **14**, 136 (1989).
19. C. P. Emerson and S. I. Bernstein, *Annu. Rev. Biochem.* **56**, 695 (1987).
20. E. Fyrberg, *Cell Motil. Cytoskeleton* **14**, 118 (1989).
21. A. D. McLachlan, *Annu. Rev. Biophys. Bioeng.* **13**, 167 (1984); H. M. Warrick and J. A. Spudich, *Annu. Rev. Cell Biol.* **3**, 379 (1987).
22. S. E. Hitchcock-DeGregori, *Cell Motil. Cytoskeleton* **14**, 12 (1989).
23. L. A. Leinwand *et al.*, *ibid.*, p. 3.
24. H. Holtzer, J. Marshall, H. Finck, *J. Biophys. Biochem. Cytol.* **3**, 705 (1957); E. Lazarides and K. Weber, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2268 (1974).
25. Y.-L. Wang and D. L. Taylor, *J. Cell Biol.* **82**, 672 (1979); K. Burridge and J. A. Feramisco, *Cell* **19**, 587 (1980).
26. R. D. Allen *et al.*, *Cell Motil. Cytoskeleton* **1**, 275 (1981); S. Inoué, *J. Cell Biol.* **89**, 346 (1981).
27. R. A. Milligan and P. F. Flicker, *J. Cell Biol.* **105**, 29 (1987); J. F. Menetret, R. R. Schröder, W. Hofman, *J. Muscle Res. Cell Motil.* **11**, 1 (1990).
28. D. A. Winkelmann, H. Mekeel, I. Rayment, *J. Mol. Biol.* **181**, 487 (1985); O. Herzberg and M. N. G. James, *Nature* **313**, 655 (1985); W. Kabsch *et al.*, *ibid.*, **347**, 37 (1990); K. C. Holmes *et al.*, *ibid.*, p. 44; G. N. Phillips, Jr., J. P. Fillers, C. Cohen, *J. Mol. Biol.* **192**, 111 (1986).
29. R. W. Kensler and R. J. C. Levine, *J. Cell Biol.* **92**, 443 (1982); M. Stewart and R. W. Kensler, *J. Mol. Biol.* **192**, 831 (1986).
30. H. R. Brown and F. H. Schachat, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2359 (1985); W. C. Bracken *et al.*, *Biopolymers* **27**, 1223 (1988); S. S. Lehrer, Y. Qian, S. Hvidt, *Science* **246**, 926 (1989).
31. S. E. Hitchcock-DeGregori and T. A. Varnell, *J. Mol. Biol.* **214**, 885 (1990).
32. S. P. White, C. Cohen, G. N. Phillips, Jr., *Nature* **325**, 826 (1987).
33. H. E. Huxley, *J. Mol. Biol.* **7**, 281 (1963).
34. F. Oosawa and M. Kasai, *ibid.* **4**, 10 (1962); E. D. Korn, M.-F. Carlier, D. Pantaloni, *Science* **238**, 638 (1987).
34a. J. E. Godfrey and W. F. Harrington, *Biochemistry* **9**, 894 (1970); J. S. Davis, J. Buck, E. P. Greene, *FEBS Lett.* **72**, 199 (1962); A. D. McLachlan and J. Karn, *J. Mol. Biol.* **164**, 605 (1983).
35. J. S. Davis, *Annu. Rev. Biophys. Biophys. Chem.* **17**, 217 (1988).
36. E. Reisler, C. Smith, G. Seegan, *J. Mol. Biol.* **143**, 129 (1980); E. Reisler *et al.*, *Biochemistry* **21**, 701 (1982); R. Josephs and W. F. Harrington, *ibid.* **5**, 3473 (1966); P. K. Chowrashi and F. A. Pepe, *J. Muscle Res. Cell Motil.* **7**, 413 (1986).
37. J. Trinick and J. Cooper, *J. Mol. Biol.* **141**, 315 (1980); R. Niederman and L. K. Peters, *ibid.* **161**, 505 (1982).
38. H. F. Epstein *et al.*, *J. Cell Biol.* **100**, 904 (1985).
39. J. S. Davis, *Biochemistry* **24**, 5263 (1985).
40. A. D. Saad, J. D. Pardee, D. A. Fischman, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9483 (1986); C. S. Johnson, N. M. McKenna, Y.-L. Wang, *J. Cell Biol.* **107**, 2213 (1988).
41. I. Pinset-Harström, *J. Mol. Biol.* **182**, 159 (1985).
42. J. Wray, *Nature* **277**, 37 (1979).
43. P. J. Vibert and R. W. Craig, *J. Mol. Biol.* **165**, 303 (1983); J. E. Dennis *et al.*, *J. Cell Biol.* **98**, 1514 (1984).
44. P. Bennett *et al.*, *J. Muscle Res. Cell Motil.* **7**, 550 (1986).
45. R. Starr and G. Offer, *FEBS. Lett.* **15**, 40 (1971); G. Offer, C. Moos, R. Starr, *J. Mol. Biol.* **74**, 653 (1973); *ibid.* **186**, 393 (1985); H. Takano-Ohmuro *et al.*, *J. Muscle Res. Cell Motil.* **10**, 369 (1989); M. G. Price, *J. Cell Biol.* **104**, 1325 (1987).
46. Twitchin: G. M. Benian *et al.*, *Nature* **342**, 45 (1989); C protein: S. Einheber and D. A. Fischman, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2157 (1990); smooth muscle myosin light chain kinase: N. J. Olson *et al.*, *ibid.*, p. 2284; titin: S. Labeit *et al.*, *Nature* **345**, 273 (1990); 86-kD protein: D. A. Fischman *et al.*, in *Frontiers of Muscle Research*, E. Ozawa, Ed. (Elsevier, Amsterdam, in press).
47. B. A. Cunningham *et al.*, *Science* **236**, 799 (1987); A. F. Williams and A. N. Barclay, *Annu. Rev. Immunol.* **6**, 381 (1988); A. R. Kornblihtt *et al.*, *EMBO J.* **4**, 1755 (1985).
48. G. M. Edelman, *Immunol. Rev.* **100**, 11 (1987).
49. T. Schultheiss *et al.*, *J. Cell Biol.* **110**, 1159 (1990).
50. K. Wang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3685 (1984); A. Whiting *et al.*, *J. Mol. Biol.* **205**, 263 (1989); D. O. Furst *et al.*, *J. Cell Biol.* **105**, 1563 (1989).
51. D. G. Moerman *et al.*, *Cell* **29**, 773 (1982).
52. H. F. Epstein, R. H. Waterston, S. Brenner, *J. Mol. Biol.* **90**, 291 (1974); P. Antin *et al.*, *J. Cell Biol.* **90**, 300 (1981); R. H. Waterston, J. N. Thomson, S. Brenner, *Dev. Biol.* **77**, 271 (1980); J. M. Zengel and H. F. Epstein, *Cell Motil. Cytoskeleton* **1**, 73 (1980); J. W. Mahaffey *et al.*, *Cell* **40**, 101 (1985).
53. R. H. Waterston, in *The Nematode: Caenorhabditis elegans*, W. B. Wood, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), p. 281; L. Venolia and R. H. Waterston, *Genetics* **126**, 345 (1990).
54. J. F. Casella, D. F. Maack, S. Lin, *J. Biol. Chem.* **261**, 10915 (1986); J. F. Casella *et al.*, *J. Cell Biol.* **105**, 371 (1987).
55. D. A. Fischman, in *Myology*, A. G. Engel and B. Q. Banker, Eds. (McGraw-Hill, New York, 1986), p. 5.
56. P. T. O'Donnell and S. I. Bernstein, *J. Cell Biol.* **107**, 2601 (1988); R. J. C. Levine, N. M. Dewey, D. D. Colflesh, *ibid.* **57**, 591 (1973).
57. H. F. Epstein, *Bioessays* **9**, 197 (1988).
58. J. G. Tidball, T. O'Halloran, K. Burridge, *J. Cell Biol.* **103**, 1465 (1986).
59. J. V. Pardo, J. D. Siliciano, S. W. Craig, *Proc. Natl. Acad. U.S.A.* **80**, 1008 (1983).
60. A. Horwitz *et al.*, *Nature*, **320**, 531 (1986); C. A. Buck and A. Horwitz, *Annu. Rev. Cell Biol.* **3**, 179 (1987).
61. G. R. Francis and R. H. Waterston, *J. Cell Biol.* **101**, 1532 (1985); J. M. Mackenzie *et al.*, *Cell* **15**, 751 (1978).
62. R. J. Barstead and R. H. Waterston, *J. Biol. Chem.* **264**, 10177 (1989); E. A. Fyrberg *et al.*, *J. Cell Biol.* **110**, 1999 (1990); T. Volk, L. I. Fessler, J. H. Fessler, *Cell* **63**, 525 (1990).
63. A. A. Dlugosz *et al.*, *J. Cell Biol.* **99**, 2268 (1984).
64. C. Cohen and D. A. D. Parry, *Proteins Struct. Funct. Genet.* **7**, 1 (1990).
65. The authors wish to acknowledge the assistance of K. Gallagher and S. McNeal in the preparation of the manuscript and I. Ortiz for the preparation of figures. We are indebted to H. Huxley, R. Milligan, C. Cohen, and H. Holtzer for graciously providing figures, and to J. Davis, E. Fyrberg, H. Holtzer, and R. Waterston for discussion. H.F.E. gratefully acknowledges support from NIH grants GM-33223 and HL-42267 and the Muscular Dystrophy Association (MDA); D.A.F. gratefully acknowledges support from NIH grants AR-32147 and HL-37675 and the MDA.