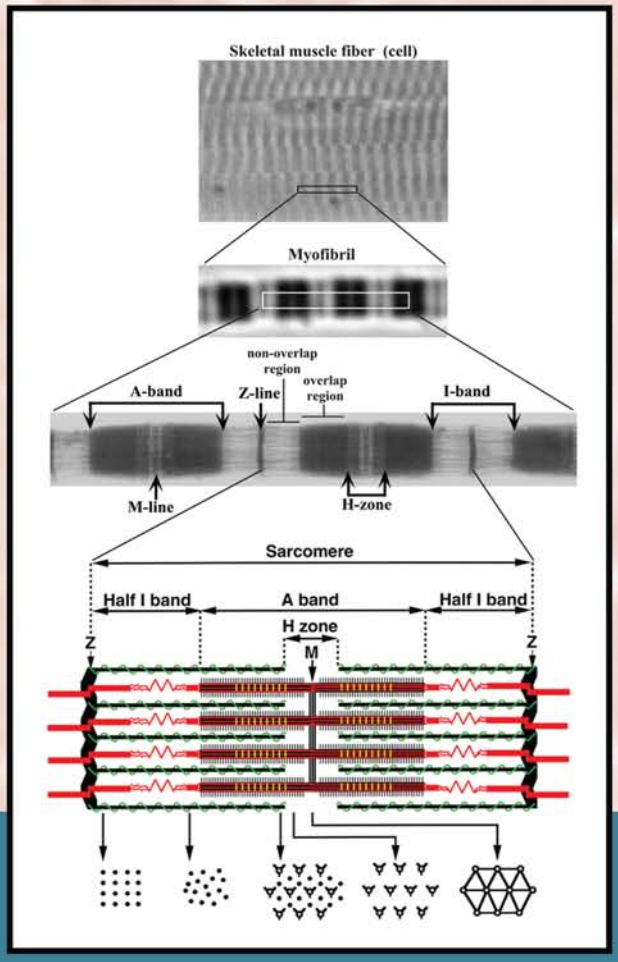
Edited by

Min Du
Richard J. McCormick

Applied Muscle Biology and Meat Science



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Preface

Skeletal muscle is the single largest organ mass in the vertebrate body. As a source of food, it represents most of the value in a meat-producing animal. Both the growth characteristics of muscle and the quality of the resulting meat determine the ultimate worth of a carcass. In recent years, tremendous strides have been made in the field of muscle biology, particularly in our understanding of the mechanisms controlling skeletal muscle growth and development, and the impact these factors have on meat production and quality. Many of the past difficulties that meat and animal scientists have experienced when attempting to address specific problems (e.g., stress susceptibility and poor meat quality in swine) have been due to a lack of understanding the underlying biological mechanisms driving muscle growth, metabolism, and its conversion to meat. This book is designed to provide the advanced reader with state-of-the-art knowledge about skeletal muscle and meat, and also as a platform for further investigation of specific issues. At the same time, this comprehensive review provides the newcomer with a sound background in applied muscle biology and meat science.

Chapter 1 provides a detailed analysis of muscle structure with an emphasis on muscle proteins, microstructure, membrane composition, and contraction. This discussion lays the groundwork for succeeding chapters.

An understanding of the growth and development of skeletal muscle and some of its components such as fat and connective tissue is crucial to our ability to manipulate production of the meat animal and subsequent meat quality characteristics. Chapters 2 through 7 address issues relevant to muscle growth and development. Accordingly, myogenesis and the biology and nature of satellite cells are reviewed. Adipogenesis, essential for muscle development and growth and so critical to meat quality, is discussed in Chapter 3. Chapter 4 reviews current issues related to the fetal stage of muscle development, with emphasis on the sensitivity of the fetus to maternal nutritional status and resulting fetal programming of skeletal muscle. The characteristics of muscle fiber types and mechanisms of *in vivo* protein degradation are the subjects of Chapters 5 and 6. Finally, a discussion of collagen and other matrix constituents and their contribution to muscle growth and quality concludes this part of the book.

Chapters 8 through 14 review key aspects of postmortem changes in mammalian and fish muscle that are responsible for meat quality characteristics. Thus, the first chapters in this middle section discuss, arguably, the two most important biological changes in postmortem muscle: proteolysis and glycolysis. A recurrent problem in the production of pork has been superior lean growth coupled with the development of poor quality "acid meat." Chapter 10 reviews recent research related to alterations in protein signaling pathways that are related to development of this genotype in pigs. Color has been recognized for decades as one of the premier quality traits of meat. Chapter 11 presents a comprehensive review regarding mechanisms governing meat color. Chapter 12 discusses multiple factors responsible for lipid oxidation in meat and strategies for its prevention. Recognition of the beneficial properties of specific fatty acids in meat has prompted interest in components such as ω-3 long-chain polyunsaturated fatty acids and conjugated linoleic acids. Chapter 13 reviews recent progress in nutritional management for enrichment of these meat constituents and the subsequent impact on meat quality. This section concludes with Chapter 14, a comprehensive review of the unique fish myotome and the postmortem changes it undergoes.

The penultimate chapter (Chapter 15) addresses molecular techniques that are increasingly utilized in breeding programs, including molecular mapping and marker-assisted breeding. Finally, the book concludes with an essay (Chapter 16) dedicated to animal welfare and the ethics of using animals for food, an especially timely discussion given the concerns of animal rights activists.

In summary, this book focuses on biological changes in skeletal muscle and meat. Given recent developments in energy costs and distribution, and changes in the commodities markets driven by the demand for biofuels, the challenges for animal production agriculture will only increase. Increased appreciation for the underlying biological mechanisms related to animal and meat production can only help solve the challenges. Thus, this book is suitable as a text for advanced courses in applied muscle biology and meat science; it further serves as a reference for scientists and industry personnel involved in meat science, as well as animal growth and development.

About The Editors

Dr. Min Du earned a B.S. in food science from Zhejiang University in 1990, an M.S. in meat science from China Agricultural University in 1993, and a Ph.D. in meat science from Iowa State University in 2001. He pursued post-doctoral studies in the Department of Biochemistry, University of Alberta, Canada, focusing on cell signaling events controlling skeletal muscle growth. He joined the faculty of the Animal Science Department, University of Wyoming, in 2003. His current research examines molecular mechanisms controlling skeletal muscle growth and meat quality, with a particular emphasis on fetal muscle development.

Dr. Du has published numerous peer-reviewed articles and abstracts, and has made multiple invited presentations. He is the section editor for the *Journal of Animal Science* and *Journal of Muscle Foods*, and is a reviewer for nearly 20 scientific journals and funding agencies. Dr. Du has taught in his native China and currently teaches meat science courses to undergraduate students and a unique course on cell signaling to graduate students.

Dr. Richard J. McCormick received B.S. and M.S. degrees from the Animal Science Department, University of Connecticut, and a Ph.D. from the Animal Science Department, Kansas State University. Prior to joining the faculty of the Animal Science Department of the University of Wyoming in 1985, he worked as a guest scientist at the Federal Meat Research Institute in Kulmbach, Germany. For the past 20 years, his research interests have focused on connective tissue biology in skeletal and cardiac muscle. He has published extensively on collagen biochemistry related to meat science, muscle biology, and cardiac and exercise physiology. Most recently, Dr. McCormick initiated genomic studies in models of cardiac (myocardial infarction) and lung (bovine and human high altitude disease) pathologies. He has taught numerous courses to undergraduate and graduate students in the areas of biology and food science and technology, and has lectured extensively in Europe, South America, and Asia.

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1 Muscle Structure and Function

Darl R. Swartz, Marion L. Greaser, and Marie E. Cantino

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1.1 INTRODUCTION

Muscle is the tissue that moves us about and transports the liquids within us. There are two general types of muscle — striated and smooth — with this nomenclature derived from the microscopic appearance. Striated muscle has striations while smooth does not. Striated muscle can be further subdivided into skeletal and cardiac. The common feature of the muscle types is that the cells are enriched in proteins that interact in a cyclic fashion to produce force and/or shorten the cell. These proteins are myosin in the thick filament and actin in the thin filaments. If the cell is connected to other muscle cells around a lumen, as in smooth and cardiac muscle, it will decrease the lumen volume and thus move things about within us. If it is attached to the skeleton, as for skeletal muscle, it will move the skeleton and thus allow change in our position or location. All three types of muscle are used in meat products, with smooth and cardiac mostly found in comminuted products and skeletal found in intact or near intact meat products as well as comminuted products. Skeletal muscle is by far the most important in animal agriculture and is the main focus of this chapter.

1.2 HISTOLOGY AND GROSS ANATOMY

Skeletal muscle, as the name implies, is generally attached to the skeleton. It contains the other tissue types (nerve, epithelia, connective tissue) to a much lesser extent than other tissues and organ systems. The cells of striated muscle are unique in that they are multinucleate, very large, and extremely elongate, reaching several millimeters to centimeters in length. This cylindrical and extended length of the cells results in the muscle cell being called a fiber. The parallel organization of muscle fibers within a group or fascicle gives anisotropy to the tissue or the so-called "grain" that is observed in a steak. Anisotropy imparts unique mechanical properties to near-intact muscle foods, as the resistance to breakage (such as when meat is chewed) depends on the angle of shear. The origins of this anisotropy and the striations are discussed in much greater detail in subsequent sections. Like other cells, the muscle cell is enveloped in a membrane and contains intracellular organelles such as the Golgi, mitochondria, nuclei, and an endoplasmic reticulum. The amount of these elements is much less than in other cell types as the primary function of muscle is to shorten and thus it contains very large quantities of the contractile proteins actin and myosin. Also, unlike other cells, striated muscle has an extremely low to no propensity to divide; renewal involves a myoblast-like cell called the satellite cell.

The individual muscle cells are encased in collagenous connective tissue that can be subdivided into the portion that is in intimate contact with the cell called the basement membrane and a more distal portion called the endomysium (Figure 1.1). A group or fascicle of muscle cells is surrounded by a more robust collagenous connective tissue network called the perimysium. An anatomical muscle consists of a group of fascicles and is surrounded by the epimysium. It is through the epimysium that the main product of muscle (shortening) is coupled to bone via the myotendinous junction. The collagen fibers of endo-, peri-, and epimysium intermesh such that contraction at the individual cell level is transmitted from the endomysium to the epimysium and thus to tendon and bone. The efferent and afferent nerve fibers and blood vessels penetrate the epimysium and bifurcate throughout the muscle, primarily within the perimysium. Generally, the nerve fibers and blood vessels are grouped together and subsequently bifurcate to supply each single muscle fiber with a nerve and the capillary network that feeds the muscle fibers.

The anatomical names of muscles are related to size, shape, attachments, fiber orientation, location, and action. The morphological types of muscle are related to their general form and muscle fiber orientation/organization. Muscle with fibers oriented mostly along the long axis is termed "strap" and "fusiform." Those with the fibers oriented at an oblique angle to the muscle, but mostly one angle, are termed "pennate." Those with multiple oblique angles are termed "bipennate," "multipennate," and "circumpennate." Most anatomy textbooks diagram all muscles as strap when, indeed, strap muscles are not very common. For intact to near-intact muscle foods, it is important

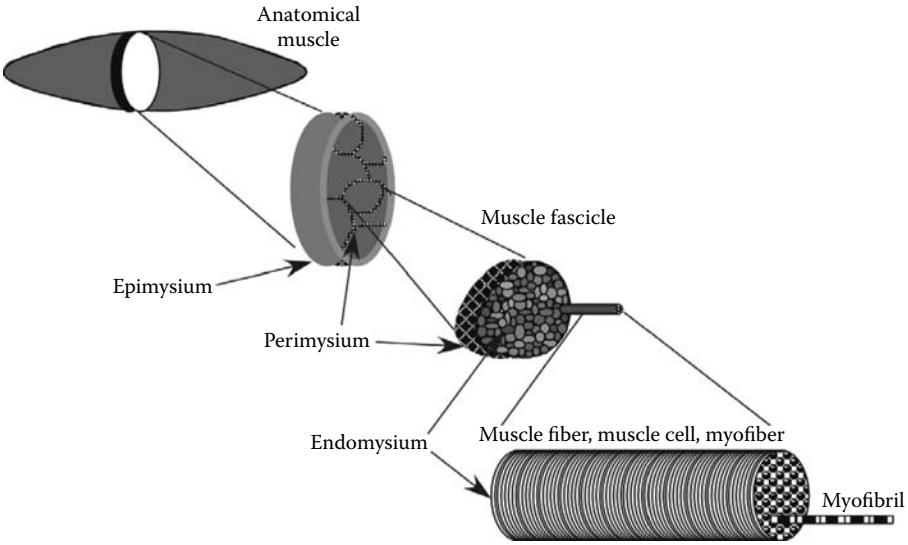

FIGURE 1.1 Organization of striated muscle and its collagenous connective tissue layers.

to understand the morphological muscle type as this may impact the tenderness. For example, the tender psoas major is a strap-type muscle. From a functional perspective, strap muscles develop the greatest extent and speed of shortening but the lowest force per cross-sectional area of the muscle (not the fiber *per se*), while circumpennate muscles develop the least extent and speed of shortening but the greatest force/cross-sectional area of the muscle. These biomechanical features arise, partially, from the fact that the speed of muscle shortening is related to the number of force-producing units in series while the force is related to the cross-sectional area of the muscle fibers for a given myosin isoform (see below). The sum of the fiber cross-sectional area for a strap muscle is equal to the muscle cross-sectional area, while for the circumpennate muscle, it is the area of a cylinder through the center of the muscle.

The microscopic appearance of a skeletal muscle cell is primarily determined by its intracellular components and their organization (Figures 1.2 and 1.3). The cell can be divided into its constituent elements, which include the outer membrane called the sarcolemma, intracellular membrane networks that include the T-tubules and sarcoplasmic reticulum, as well as mitochondria, nuclei, and myofibrils. The myofibril is a string-like structure made of repeating units called sarcomeres. The sarcomere is delimited by the α-actinin containing Z-lines to which the actin-containing thin filaments attach. Myosin-containing thick filaments are in the center of the sarcomere and form the A-band. In the center of the A-band is the M-line that contains myomesins and part of titin involved in formation of the A-band and positioning of the thick filaments in the center of the sarcomere. Thick and thin filaments interdigitate in the overlap region and the extent of overlap increases as sarcomere length decreases. The I-band includes a dense Z-line at its center and the low-density non-overlap regions of the thin filaments from the adjacent half sarcomeres on each side. Classically, the sarcomere is described as having thin-filament-containing I-bands flanking the thick-filament-containing A-band but this does not convey the functional aspect of the overlap region.

As can be seen in Figures 1.2 and 1.3, the proteins of the sarcomere are organized into a near-crystalline lattice, and the form of these proteins and their interactions with themselves and other proteins result in a highly organized structure. The fascinating feature of muscle structure and function is that one can start with most of the structures of individual proteins, organize them into their respective polymers as governed by their interprotein interactions, build the individual filaments (thick and thin) and their attachments (Z-line and M-line), build a sarcomere, and link these sarcomeres in series to form the myofibril. These myofibrils can be associated and aligned,

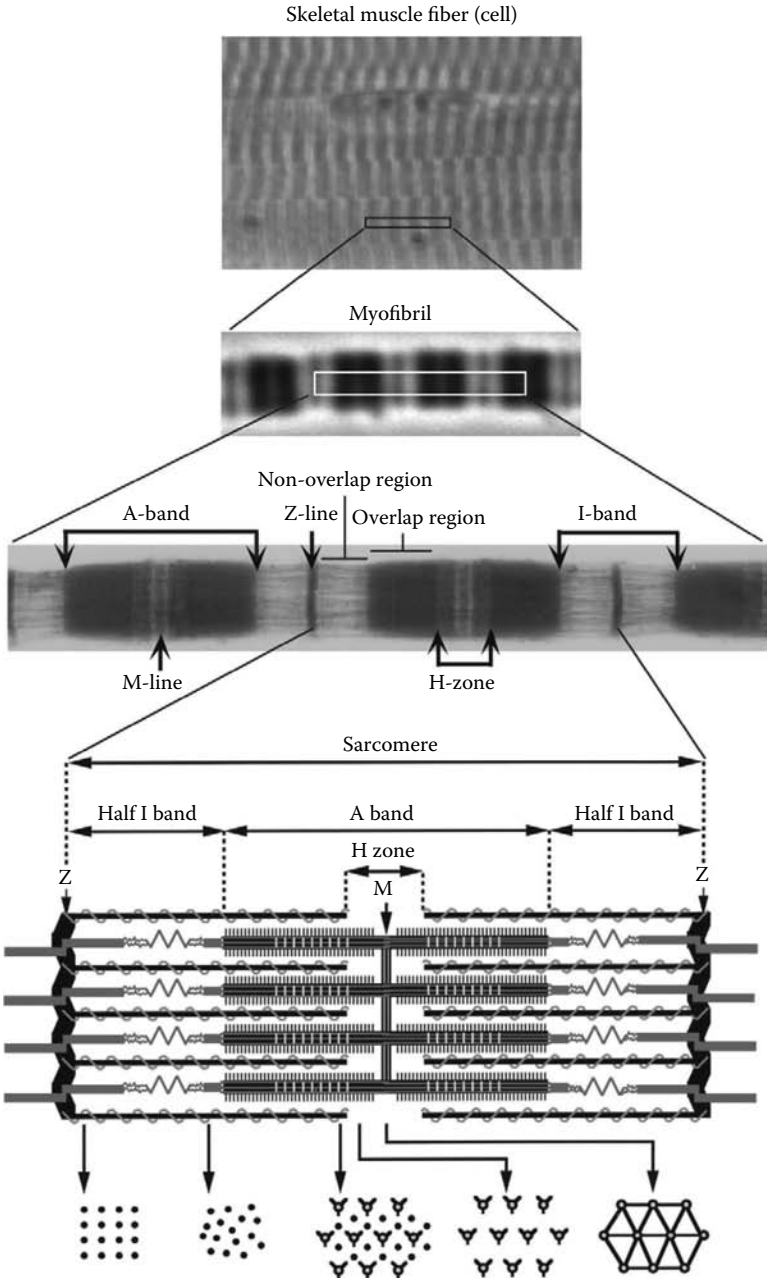

FIGURE 1.2 Vertebrate skeletal muscle fiber, myofibril, and sarcomere organization. Skeletal muscle fiber and myofibril imaged by phase-contrast microscopy (top) and single myofibril whole mount imaged with HVEM after positive staining (middle). Cartoon of sarcomeric organization in both longitudinal and cross-section (bottom).

primarily by extra-sarcomeric proteins, and the muscle cells can be arranged to generate a model of muscle organization consistent with that observed at the anatomic, light, and electron microscope (EM) levels. This molecule-to-macroscopic approach will be used for this chapter. The x-ray crystal structures available for many of the major myofibrillar proteins will be combined with electron micrographs of the ultrastructural organization to illustrate and describe the individual proteins of

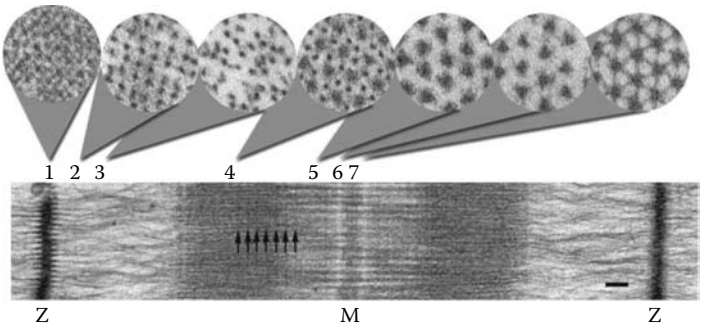

FIGURE 1.3 Filament organization within the sarcomere as seen in electron micrographs of positively stained sections of rabbit psoas muscle. Different regions of the near-crystalline lattice of the sarcomere shown in cross-section (top) and in longitudinal section (bottom) are: 1, Z-line; 2, I-band near Z-line; 3, I-band; 4, overlap region of the A-band; 5, non-overlap region of A-band; 6, bare zone of A-band adjacent to M-band; 7, M-band. Arrows note the stripes associated with MyBP-C. Bar = 100 nm.

the sarcomere, their interactions with themselves and other proteins, their function, and to build their respective subsarcomeric structures. We then build the sarcomere, the myofibril, adding some membrane elements, and describe how all these elements work together to elicit muscle contraction upon stimulation from the nerve.

1.3 MYOFIBRILLAR PROTEINS

The myofibril is an insoluble (at near-physiological salt levels) protein assembly composed of more than 30 different proteins. It is isolated by homogenizing muscle (preferably post rigor) in a buffered 0.1 M saline solution followed by cycles of low-speed centrifugation. Most of the membrane elements and cystosolic proteins remain in the supernatant while the nuclei and myofibrils are in the pellet. The proteins of the myofibril interact with each other by non-covalent bonds. Many of the individual protein-protein domain interactions are weak but the number of domains interacting is large, thus resulting in a very high overall affinity and providing stability to the protein complexes that maintain the organization of the muscle structures. The major proteins and their approximate levels in isolated myofibrils are shown in Table 1.1, and an SDS-PAGE gel of fast and slow bovine myofibrils is shown in Figure 1.4 to aid in visualizing the relative amount of the different proteins and their approximate monomeric mass. A description of the structure and function of the individual myofibrillar proteins follows, with subsequent organization of these proteins into their subsarcomeric structures. We do not focus on the numerous isoforms of the myofibrillar proteins and use isoforms differences only to highlight the functions of the proteins.

1.3.1 Myosin

The molecular motor that produces muscle force and contraction is the actin-activated ATPase, myosin. There are numerous myosin classes within this large protein family (Foth et al. 2006). The form contained in the contractile apparatus of all muscle types, and contributing nearly half of the myofibrillar protein content in vertebrate striated muscle, is myosin II. There are several muscle myosin types, differing in the speed with which they hydrolyze ATP and their regulation. We describe here some of their common structural features; additional details may be found in other reviews of muscle, myosin, and myosin filament structure (e.g., Squire 1981; Squire et al. 2005; Craig and Woodhead 2006). Myosin isoform variations in different fiber types are also described in more detail elsewhere in this volume.

TABLE 1
Major Myofibrillar Proteins

Protein	Subunits	MW (kDa)	% MF protein (w/w)
Myosin	Heterohexamer:	520	43
	Heavy chain	220	
	Essential (proximal) light chain	23	
	Regulatory (distal) light chain	20	
Actin	Homopolymer	43	22
Titin	Homohexamer?	3,200–3,700	10
Nebulin	Monomer	900	5
Troponin	Heterotrimer:	73	5
	Troponin T	31	
	Troponin I	23	
	Troponin C	18	
Tropomyosin	Homo/heterodimer:	66	5
	Alpha-tropomyosin	33	
	Beta-tropomyosin	33	
MyBP-C	Monomer	130	2
MyBP-H	Monomer	55	<1
Myomesin (1-3)	Homodimer	165–185	2
α-actinin	Homodimer	100	2
Tropomodulin	Monomer	40	<1
CapZ	Heterodimer:	68	<1
	Alpha-subunit	36	
	Beta-subunit	32	
Creatine kinase	Homodimer	43	<1
Adenylate kinase	Monomer	22	
Desmin	Homopolymer	54	<1
Synemin	Heteropolymer (w/desmin)	185	<1
Filamin	Homodimer	240	<1
Telethonin/T-cap	Monomer	19	<1
Myopalladin	Monomer	145	<1
T-cap	Monomer	19	<<1
Dystrophin	Monomer	427	<<1

Myosin II consists of two identical heavy chains and two pairs of light chains (Figure 1.5A and B), with a combined molecular weight of around 520 kDa. The C-terminal α-helical portions of each 220-kDa myosin heavy chain dimerize to form a coiled-coil rod, approximately 150 nm in length. This region is also involved in the formation of thick filaments and is discussed further below. Toward their N-terminal ends, the two heavy chains separate and each terminates in a globular region. Enzymatic digestion divides the molecule within the rod portion into light meromyosin (LMM) and heavy meromyosin (HMM). Heavy meromyosin can be further broken down to yield subfragments 1 and 2 (S1 and S2). Each S1 fragment (also referred to as the myosin head or crossbridge) includes the globular portion of the heavy chain, with sites that bind MgATP and actin, and an α-helical neck that binds two different types of light chains (Figure 1.5B), ranging from 20 to 23 kDa.

The S1 head alone is sufficient to produce movement of actin filaments *in vitro* (Toyoshima et al. 1987), confirming that muscle contraction is a direct result of conformational changes that occur within this fragment. The vertebrate myosin S1 crystal structure (Figure 1.5B), first solved in

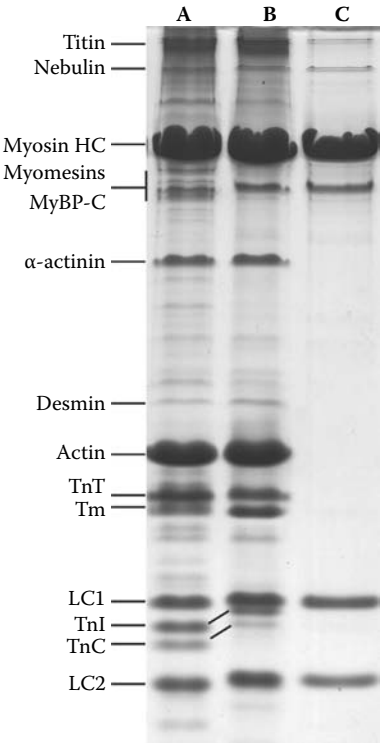

FIGURE 1.4 SDS-PAGE of myofibrillar proteins and crude myosin. Bovine cutaneous trunci myofibrils (fast muscle, lane A), bovine masseter myofibrils (slow muscle, lane B), and myosin (lane C) were subjected to SDS-PAGE.

1993 (Rayment et al. 1993), has helped identify the structural motifs important for transduction of chemical energy (Geeves et al. 2005). The globular region of S1 is known as the catalytic or motor domain because it contains the polypeptide sequences most fundamental to energy transduction in muscle. Two subdomains (upper and lower 50K) are separated by a cleft that is associated with actin binding. Adjacent to this area is a G-protein-like nucleotide binding pocket with a P-loop that coordinates binding of MgATP. Partial ATP hydrolysis and subsequent release of one or both hydrolysis products (ADP, inorganic phosphate Pi; see Figure 1.21) is closely associated with a change in the conformation of the S1 head termed the power stroke. The resulting stable complex of actin and myosin, free of nucleotide, is called the rigor complex.

The neck region of each S1 is the binding domain for two calmodulin-like light chains. The essential (or alkali) light chain binds near the N-terminal end of each neck, and is so named because it is necessary for ATPase activity and actin binding by myosin. The regulatory light chain (also referred to as lightchain 2 or the DTNB light chain) binds at the C-terminal end of the neck, nearest to S2. The functions of the light chains are not well understood, especially in vertebrate striated muscle, although recent studies are beginning to shed light on how they contribute to contractile properties (Moss and Fitzsimons 2006; Hernandez et al. 2007). Because the S1 neck acts as a lever arm during the crossbridge cycle, its structure plays a role in determining the mechanical properties of the muscle as a whole, with its length limiting the distance actin moves per ATP hydrolyzed (and therefore, muscle velocity), and its stiffness affecting the load-bearing properties of each crossbridge. The light chains are thought to not only increase the stiffness of the lever arm, but also to have important regulatory functions. Vertebrate smooth muscle and many invertebrate muscles are referred to as "myosin regulated" because their activation requires modification of the light chains by phosphorylation or calcium binding. Evidence is accumulating that a similar

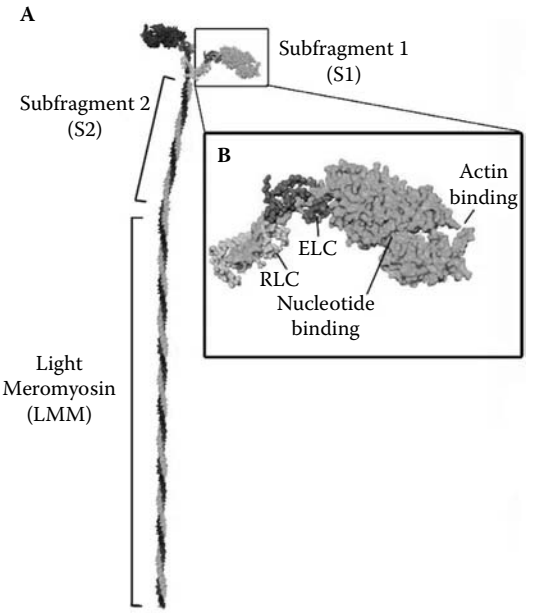

FIGURE 1.5 (See color insert following page 148.) Myosin structure in vertebrate striated muscle. The myosin molecule (A) includes two heavy chains (rendered in light and dark green) and two light chains (magenta and cyan), and can be divided into three subfragments as shown. The crystal structure of S1 (B) shows locations of light chains and binding sites for actin and nucleotides. Images were rendered with MacPyMol using pdb file 2MYS for S1 and 2FXO for S2.

mechanism, while not obligatory, plays an important role in tuning the activation of vertebrate striated muscle.

1.3.2 Myosin Binding Protein-C (MyBP-C)

Other proteins besides myosin and titin are present in the crossbridge region of thick filaments. Myosin binding protein-C (MyBP-C, also called C-protein) and a smaller related protein, myosin binding protein-H, are members of the immunoglobulin superfamily. For many years these proteins were known to bind at regular positions along the thick filaments of striated muscle (Craig and Offer 1976; Bennett et al. 1986) (Figure 1.3) but generated limited interest because they were not required for activation of contraction. The discovery that mutations in the gene for cardiac MyBP-C are associated with familial hypertrophic cardiomyopathies has stimulated renewed study of how these proteins contribute to normal muscle function, especially in the heart (De Tombe 2006; Granzier and Campbell 2006; Oakley et al. 2007).

Three isoforms of MyBP-C have been identified and are encoded by different genes. One form, MyBP-C3, is found only in cardiac muscle. MyBP-C1 and MyBP-C2 (formerly MyBP-X) are found mainly in fast and slow skeletal muscle, respectively. Each isoform consists of a single polypeptide chain (extended length approximately 50 nm), containing at least seven repeating immunoglobulin I (Ig) type domains and three fibronectin (FN) domains. These are numbered from the N-terminus as domains C0 to C10. Repeats toward the C-terminus (C7 to C10) bind to the LMM portion of myosin, and also to another myosin binding protein, titin (see below). The N-terminus of MyBP-C has affinity for the S-2 portion of myosin and for actin (Kulikovskaya et al. 2003). The cardiac isoform is larger than the skeletal, due to a proline charge-rich insert near the center of the sequence and addition of a cardiac-specific IgI domain. It also contains additional phosphorylation sites near the N-terminus.

MyBP-C binds at seven to nine locations in each half of the thick filament, separated by gaps of 43 nm, the same as the axial helical repeat of myosin heads on the filament surface (Figures 1.2, 1.3, and 1.17). Two to four molecules of MyBP-C are thought to bind at each axial position. Two models have been proposed for their arrangement. In the trimeric collar model (Winegrad 1999), three molecules of MyBP-C consecutively dimerize to form a ring around the myosin filament at each axial location. The alternative model aligns C-terminal repeats of MyBP-C parallel to the thick filament axis (Squire et al. 2003). In both models, the N-terminus can extend away from the backbone to interact with myosin S2, actin, or both. Thus far, high-resolution EM studies have been unable to rule out either model, and it is possible that the arrangement differs with muscle or fiber type (Flashman et al. 2008).

It now seems likely that MyBP-C functions in regulation, assembly, and structural stability of the contractile apparatus. Thick filament assembly and stability are promoted by interactions of the C-terminus domains of MyBP-C with titin and are impaired by its absence. Regulatory functions are associated with residues near the MyBP-C N-terminus that bind myosin S2. Studies of both intact muscle fibers and isolated myosin filaments suggest that in its unphosphorylated state, MyBP-C helps tether the S2 portion of myosin to the thick filament backbone; in cardiac muscle, phosphorylation of MyBP-C domains C1-C2 is proposed to decrease their association with myosin S2, allowing S1 to move closer to actin prior to activation (Levine et al. 2001). It is also possible that binding of its N-terminus to actin creates a strut that maintains the positions of the thin filaments midway between adjacent thick filaments in the overlap region in relaxed striated muscle (Squire et al. 2003).

1.3.3 Myomesins

Three separate genes code for related proteins found in the M line/band (Schoenauer et al. 2008). Myomesin 1 (185 kDa) is found in all striated muscles; myomesin 2 (165 kDa, also called M-protein) is found in fast twitch fibers; and myomesin 3 (162 kDa) is restricted to slow and intermediate fiber types. All three proteins have unique N-terminal regions followed by two immunoglobulin-like (Ig) domains, 5 fibronectin domain 3 like (FN3) domains, and 5 more Ig domains. BLAST sequence comparisons, however, indicate only 40% to 50% identity among the myomesins, with the longest span being less than 15 amino acids. Myomesin 1 has been proposed to be the major thick filament cross-linking protein, with a function analogous to α-actinin in the Z-line (Lange et al. 2005). The protein binds to myosin through its N-terminal domain and to titin via the most C-terminal three FN3 domains. All three myomesins form only homodimers through their carboxyl-terminal regions.

1.3.4 Creatine Kinase and Adenylate Kinase

Creatine kinase (CK) is also localized at the M-line. This enzyme catalyzes both the forward and reverse reaction of creatine phosphate + ADP to yield creatine and ATP. CK binds to myomesin through the latter's Ig domains 6 to 8 (Hornemann et al. 2003). Only the MM form of creatine kinase is found in the M-line and its binding affinity for myomesin is pH dependent, being weak at high pH (>7.5) and strong at lower pH (pH 6.0). Adenylate kinase (formerly called myokinase) interconverts 2ADP to AMP + ADP and has been found near the M-band. The interaction of adenylate kinase at the M-band has been proposed to occur through binding to Fhl2/DRAL, which in turn attaches to the carboxyl-terminal region of titin (Lange et al. 2002). The tethering of creatine kinase and adenylate kinase near the site of ATP usage appears beneficial for muscle function.

1.3.5 Actin

Actin is the most abundant protein of muscle on a molar basis, being near 600 μM within the myofilament lattice. It acts as both a structural element of the sarcomere and as a co-factor for activation of the myosin motor domain ATPase. It polymerizes to form the major component of the thin

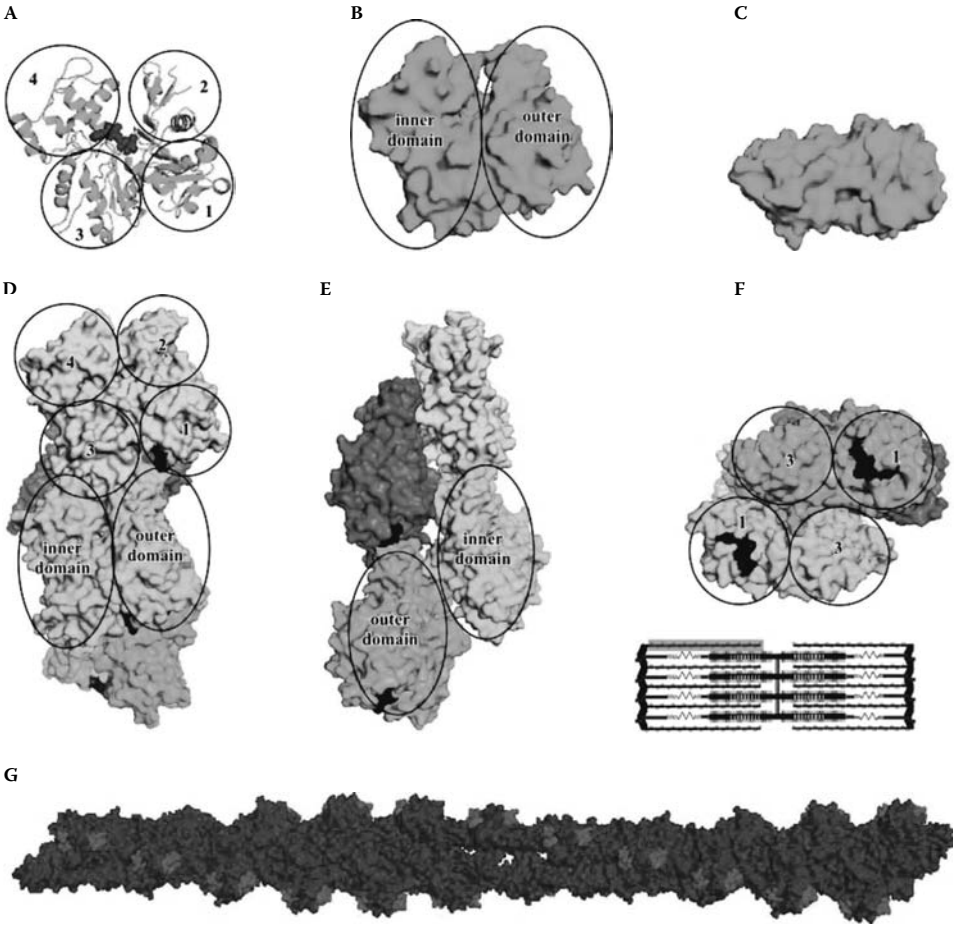

FIGURE 1.6 (See color insert following page 148.) Actin monomer and polymer structure. The crystal structure of G-actin is shown in α-carbon cartoon view (A) and surface rendered views (B and C). The subdomains are noted, as well as the inner and outer domains. Tilting of the structure 90° into the page shows the bottom (subdomains 1 and 3) and that the molecule is mostly flat (C). The crystal structure of an F-actin filament containing four monomers is shown (D through F). Each subunit is colored differently and the subdomains and domains are noted. The C-terminal three residues for all monomers is colored in black for reference. View (D) shows the filament from the front, (E) from the side, and (F) from the barbed (+) end of the filament. A smaller view of a longer filament is shown in (G) with the α-actinin binding sites colored magenta. The barbed (+) end is to the left and pointed (-) end is to the right. Note the spiral of the binding sites resulting from the helical nature of the filament. Images were adapted from Pirani et al. (2006) supplemental files.

filament in an ATP-dependent manner. The crystal structure of G-actin was solved several years ago (Kabsch et al. 1990), and different views of the structure are shown in Figure 1.6A through F. The general anatomy of the protein is more planar than spherical. It is subdivided into four subdomains numbered 1 through 4 that are named in a clockwise fashion with subdomain 2 starting in the upper right and progressing to 1, 3, and then 4 in the upper left. Subdomains 3 and 4 are called the inner domain of actin, whereas 1 and 2 are called the outer domain (Holmes et al. 1990). MgATP/ADP is bound in a cleft formed between subdomains 2 and 4. The planar nature is obvious when the structure is viewed from subdomains 1 and 3 (Figure 1.6C) or 2 and 4. Polymerization of G-actin into fibrous or F-actin is complex, being dependent on concentrations of salt, divalent cations, G-actin, and ATP (Carlier 1991). It follows a sequential process in which subdomains 2 and 4 of one monomer interact with 1 and 3 of another (yellow and cyan monomers in Figure 1.6D and E) to form a dimer. Subdomains 3 and 4 of another monomer, rotated about 170° about its vertical axis, associate

with subdomain 4 of the lower G-actin and subdomain 3 of the upper G-actin of the dimer (magenta monomer in Figure 1.6D and E). If the cleft is considered the top of the molecule, dimerization involves a stacking of one monomer on the other while formation of the trimer involves addition of a monomer (after rotation of about 170°) to the side of this stack. Subsequent addition of monomers occurs by primarily adding additional monomers onto the existing trimer (green monomer Figure 1.6D and E). The process is rate limited by dimer and trimer formation. This polymerization results in the filament having polarity with the clefts all in the same direction. This end of the polymer is termed the minus (–) end of the filament because it grows more slowly than the other plus (+) end. When the filament is "decorated" with rigor myosin S1, the minus end appears pointed and is thus called the pointed end while the other end appears barbed and is called the barbed end. For most illustrations, we will orient the pointed end at the top or right of the figure.

The arrangement of actin monomers in the filament (Figure 1.6G) can be described as either a left-handed single stranded helix (sometimes referred to as "short pitch") or as a right-handed double stranded helix ("long pitch"). In electron micrographs the most prominent features are a 6 nm striation along the filaments resulting from the turns of the left-handed helix, and a 38 nm periodicity reflecting the crossover points of the two strands of the right-handed helix. The filament can be described as a twisted double strand of pearls with the twist of the two strands giving the long pitch and the twist, in going from pearl to pearl, the short-pitch. The filament, without associated proteins, is dynamic in that it can grow and shorten, depending on the conditions and G-actin level. In striated muscle, there are proteins tightly associated with either the plus end (CapZ) or minus end (tropomodulin) and along the side (nebulin and tropomyosin) that stabilize the filament.

The diameter of the filament is about 10 nm and its length in muscle is primarily determined by nebulin (see below). There are a few proteins that bind to the ends of the filament and involve both the inner and outer domains (McGough 1998). The majority of proteins bind along the filament and interact primarily with the outer domain. At the sarcomere level, these proteins include myosin, tropomyosin, troponin (TnT and TnI), α-actinin, nebulin, and possibly MyBP-C. For myosin and α-actinin, two actin monomers are bound along the long axis of the filament but to different domains of each monomer. The surface regions on F-actin involved in binding tropomyosin, myosin, and α-actinin are shown in Figure 1.7, and it is apparent that there is overlap of some of these sites. F-actin's interactions with other proteins are numerous and are discussed further in sections on the substructures of the sarcomere.

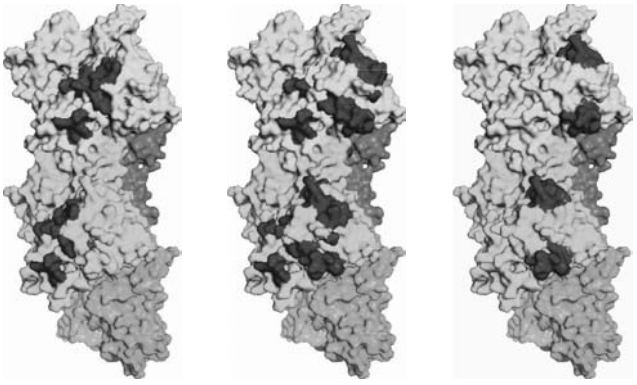

FIGURE 1.7 (See color insert following page 148.) Binding sites on the actin filament. The F-actin model from Figure 1.6 was modified by coloring the amino acid residues (dark blue) thought to be involved in the binding of tropomyosin in the blocked state, S1 in rigor, and α-actinin. Note that similar residues are involved for all three proteins. The residues involved are from McGough (1998) and images were rendered as in Figure 1.5.

1.3.6 Nebulin

Nebulin is also a large protein with a size in the 750 to 850 kDa range that is primarily associated with the thin filament (McElhinny et al. 2003). The name comes from its nebulous function at the time of its discovery. Unfortunately, the role of this protein in muscle still remains mysterious. More than 45 mutations have been detected in the human nebulin gene, and most of these lead to nemaline myopathy, a muscle disorder where microscopic rods appear in the muscle cells. Nebulin is composed of a large number of repeating domains and it is alternatively spliced. It contains an SH3 domain at the C-terminus, and this sequence is proposed to bind to a proline-rich sequence near the N-terminal end of titin in the Z-line. The proteins α-actinin, desmin, and CapZ also bind to the nebulin carboxyl end. A ~35 amino acid repeat occurs 185 times in nebulin. Each of these repeats contains a central "SDXXYK" amino acid sequence that is believed to anchor the nebulin to each actin monomer along the filament at 5.5 nm intervals. There is also a "WLKGIGW" sequence at every seventh module (22 super repeats) in the center of the 185 repeat section, and this corresponds to the 38.5 nm spacing of the troponin complexes along the thin filament. The repeating structure is thought to be α-helical. Nebulin likely follows a helical path on either side of the actin double strand (Figure 1.2, shown in green). The amino terminus binds to tropomodulin near the free end of the thin filament.

Nebulin has been proposed to function as a molecular ruler to determine the thin filament length. Support for this idea comes from the observation that the size of the nebulin correlates with the thin filament lengths when comparing different species. Thus, bovine and human thin filaments, with lengths of 1.3 microns (μm), have larger nebulins than mouse with filament lengths of 1.05 μm. The protein has also been shown to bind to calmodulin. Nebulin knockout mice have altered calcium regulation and markedly up-regulated levels of sarcolipin, a protein that alters the activity of the sarcoplasmic reticulum calcium pump. However, direct interaction of the two proteins has not been demonstrated.

1.3.7 Tropomyosin

Tropomyosin is a parallel homo- or heterodimer that is a component of the thin filament (Perry, 2001). Dimerization is via formation of an α-helical coiled coil involving hydrophobic interactions associated with its amino acid heptad repeat. The non-polar (N) and polar (P) amino acid repeat (NPPNPPP) results in a non-polar side chain at average intervals of 3.5 amino acids, very similar to the 3.6 amino acid repeat of the α helix. This results in a hydrophobic strip along one side of the α helix. Tropomyosin is a stiff, elongate molecule having a length of about 40 nm and a diameter of about 2.5 nm (Figure 1.8). Under low salt conditions, it polymerizes end-to-end involving an overlap of about 9 amino acids at both the N- and C-termini. It binds along the long axis of the actin filament, and the end-to-end interactions between the tropomyosins are such that it is akin to a linear co-polymer with F-actin. The orientation of the binding of tropomyosin to actin is such that its sequence is anti-parallel to the polarity of the actin filament (N-terminus of tropomyosin at the (–) or pointed end). While the overall binding affinity of tropomyosin to F-actin is high, its interaction with individual actin monomers is relatively weak. This allows for some minor movements of tropomyosin on the surface of F-actin that are important in regulation of contraction. The major binding sites on F-actin are on the outer domain of the filament, with other sites more toward the inner domain that are associated with un-blocking of actin for interaction with myosin (Figure 1.8). The mole ratio of actin monomers to the tropomyosin dimer is 7 to 1. Tropomyosin is involved in stabilizing the thin filament but its primary function is to regulate myosin's interaction with actin. This involves movement of tropomyosin on the surface of the actin filament that is mediated by troponin (see below). Troponin binds to tropomyosin in the C-terminal third of the molecule and slightly into the N-terminus of the overlapping tropomyosin. This binding is mostly through the TnT subunit of troponin, with TnT being anti-parallel with respect to the tropomyosin sequence. Tropomodulin binds near the N-terminal 14 amino acid residues of tropomyosin and inhibits tropomyosin end-to-end interactions.

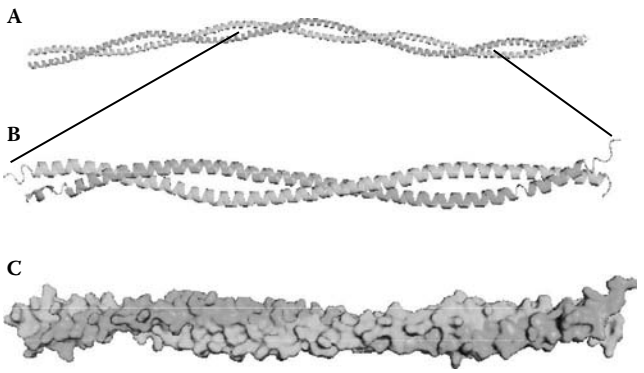

FIGURE 1.8 (See color insert following page 148.) Tropomyosin structure. A low-resolution structure of tropomyosin in α-carbon cartoon view is shown in (A), and a higher-resolution structure of part of the molecule (B). The high-resolution structure is also shown in surface view in (C). One monomer is colored cyan and the other green. Note the coiling of the monomers to form the coiled-coil dimer.

1.3.8 Troponin

Troponin (Tn) is an additional thin-filament associated protein whose primary function is to control the location of tropomyosin on the thin filament in a calcium-dependent manner and thus control contraction (Farah and Reinach 1995). It is a heterotrimeric protein, being made of subunits called TnT, TnI, and TnC. Biochemical studies over the past 40 years determined many of the interactions of the subunits with the thin filament and the subunit interactions within the heterotrimer. Troponin T primarily binds to tropomyosin (Perry 1998). Troponin T can be divided into two domains called TnT1 and TnT2. Tropomyosin binding is mainly by TnT1 whereas TnI and TnC binding is mostly by TnT2. Troponin I is inhibitory to actin activation of myosin ATPase activity and interacts primarily with actin (Perry 1999). It too can be subdivided into an N-terminal domain that is involved in structural interactions with TnC and TnT and a C-terminal domain that is involved in regulatory interactions with actin and TnC. The switching of this domain between actin and TnC is part of the molecular switch involved in regulation of contraction. Troponin C binds calcium and is considered the calcium binding switch for turning on contraction (Grabarek et al. 1992). As with TnI, TnC can also be sub-divided into a regulatory and a structural domain, but with the opposite polarity. The C-terminal globular domain is involved in structural interactions with the C-terminal region of TnT and the far N-terminal region of TnI. This domain binds two calcium ions with low micromolar affinity or two magnesium ions with low millimolar affinity. The N-terminal globular domain binds two calcium ions with micromolar affinity for the fast skeletal isoform but only one calcium for the slow/cardiac isoform. This "regulatory" calcium binding is associated with structural changes in the N-domain that result in exposure of a hydrophobic region. This calcium exposed hydrophobic region is a high affinity site for a part of the regulatory domain of TnI.

The troponin trimer forms a club-like structure with a length of about 25 nm and a diameter of about 5 nm (Flicker et al. 1982). The handle is made of the N-terminal region of TnT (TnT1), and the club part is made of the so-called core-domain of Tn, which includes the C-terminal region of TnT (TnT2) and all of TnI and TnC. Crystal structures of the core domain were recently obtained (Vinogradova et al. 2005) and are shown in Figure 1.9. There are numerous contacts between the subunits and much of TnI and TnT are not resolved in the crystal. The structural interactions that are involved in formation of the trimer are demonstrated by the coiled-coil formed between TnT and TnI, as well as the interaction of the C-terminal domain of TnC with the N-terminal domain of TnI. The C-terminal domain of TnC is held in the structure like chopsticks, with TnI being one stick and the TnIT coiled-coil being the other. In the calcium-saturated structure (Figure 1.9C–F), the N-terminal domain of TnC has structural differences from the apo state that are associated with the exposure of a

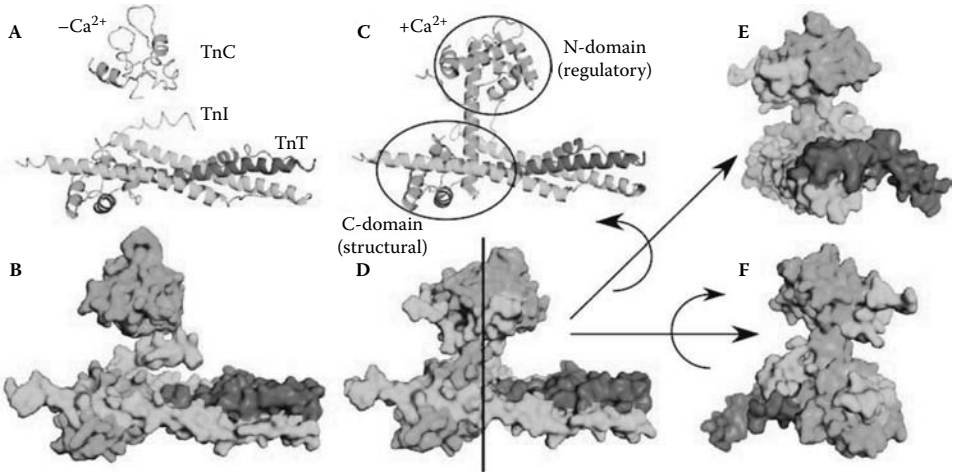

FIGURE 1.9 (See color insert following page 148.) Structure of the troponin core domain. Most of TnC (green) and part of TnI (cyan) and TnT (magenta) are shown as α-carbon cartoon structures (A and C) or surface views (B, D, E, and F) The core domain without regulatory calcium bound is shown (A and B) and with regulatory calcium bound (C through F). Views (E) and (F) are viewed rotated 90° either out of (E) or into (F) the page. Note in (A) and (C) the greater amount of structure detected in the calcium-bound state as a result of calcium-dependent regulatory interactions between TnC and TnI.

hydrophobic region. Nestled within this region is an α-helical 9 or 10 amino acid region of TnI. There is no direct competition of actin for the same amino acid residues in TnI; rather, the actin binding regions are thought to flank the TnC binding region (Luo et al. 2002). Troponin binds to the thin filament primarily via TnT, through TnT1's interaction with tropomyosin and via TnI's interaction with actin. Weaker interactions of TnT2 with actin also may be involved in binding Tn to actin (Pearlstone and Smillie 1981). There are suggestions that TnI also binds to tropomyosin at specific sites, further enhancing its interaction with the thin filament (Geeves et al. 2000; Zhou et al. 2000). The mole ratio of actin monomers to troponin heterotrimer is about 7:1, while that of the troponin heterotrimer to tropomyosin is 1:1. The so-called regulated thin filament thus has a stoichiometry of 7:1:1.

1.3.9 Tropomodulin

Tropomodulin is a low copy number protein that binds tropomyosin and actin and thus is a component of the thin filament (Fischer and Fowler 2003). It was originally discovered in red blood cells, and subsequent isoforms were found in skeletal muscle. It has an N-terminal tropomyosin binding domain and a C-terminal actin binding domain. Tropomodulin's affinity (in K_d) for tropomyosin is about 1 μM, while its affinity for actin is about 0.3 μM. As noted above, tropomodulin binds to tropomyosin's N-terminus. Its interaction with actin is such that it binds to the pointed (–) end of the actin filament, likely across the end of the filament (Fowler et al. 2003). Tropomodulin binds to the actin–tropomyosin complex with a very high affinity ($K_d < 50$ pM). Considering its interactions, one would predict a mole ratio of 2 molecules of tropomodulin per thin filament. Because of its extremely high affinity for actin–tropomyosin, it essentially prevents addition or loss of G-actin monomers from the pointed (–) end of the filament.

1.3.10 CapZ

Another low copy number protein bound to the thin filament is CapZ (Cooper and Schafer 2000). It is a heterodimer made of α- and β-subunits. It binds tightly to the barbed (+) ends of actin filaments with a K_d of about 1 nM. As the name implies, it caps actin filaments and is found at the Z-line of the sarcomere. It

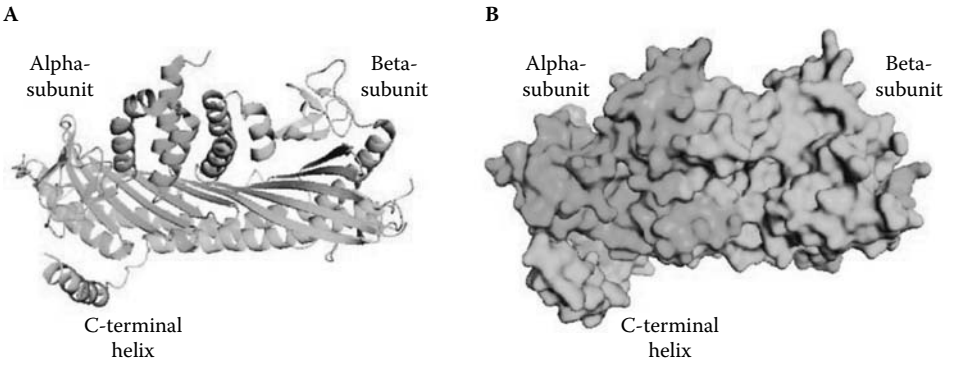

FIGURE 1.10 (See color insert following page 148.) Structure of CapZ. The α-subunit is colored green and β-subunit cyan in carbon cartoon (A) and surface (B) views. Note the similarity in general structure between the subunits and the C-terminal extension in the β-subunit that is thought to be involved in binding to the barbed (+) end of actin filaments.

also appears to bind α-actinin, and this interaction may stabilize its localization to the Z-line. CapZ was recently crystallized (Yamashita et al. 2003) and this structure is shown in Figure 1.10. The molecule is elongate, being about 9 nm long and 5 nm wide. There are extensive interactions between the subunits in the heterodimer, and the structure of the individual subunits is strikingly similar, although there is limited sequence similarity. Both subunits have a far C-terminal region that likely protrudes from the main core of the protein. The proposed interactions with the actin filaments involve binding to the barbed (+) end G-actin monomers, mediated primarily by the far C-terminal regions of CapZ (Narita et al. 2006). The likely mole ratio of CapZ to thin filaments is 1:1.

1.3.11 α-ACTININ

α-actinin is an anti-parallel homodimer found in the Z-line (Otey and Carpen 2004). It has a length of about 35 nm and a diameter of about 4 nm. A model structure for α-actinin was developed from the individual structures of its domains (Tang et al. 2001) and is shown in Figure 1.11. The monomer has three major domains; near the N-terminus is a globular actin binding domain composed of a pair of calponin homology domains. This is followed by four triple α-helical, spectrin-like domain repeats. This is also called the rod domain of α-actinin. Near the C-terminal is a calmodulin-like domain, which in the muscle isoform does not bind calcium. The anti-parallel nature of the molecule results in an actin binding domain at each end, with these domains being rotated approximately 180° about the short axis of the molecule. As the name implies, the actin binding domain binds to actin, and the residues on actin involved in this binding have some overlap with residues involved in myosin and tropomyosin binding (see Figure 1.7). This likely results in competition between α-actinin and these other proteins for binding to actin, as suggested by biochemical experiments (Zeece et al. 1979). α-actinin also binds to titin and this involves the C-terminal calmodulin-like domain and spectrin repeats 2 and 3 (Young et al. 1998). Binding to nebulin occurs at the ends (Pappas et al. 2008) and to CapZ in the rod domain (Papa et al. 1999). These numerous interactions with other sarcomeric proteins result in very high affinity binding of α-actinin to the Z-line of the sarcomere. Studies with myofibrils *in vitro* demonstrate that α-actinin can readily be exchanged into the Z-line and the apparent dissociation rate is 0.01 min^{-1}, in accord with its high affinity for the various proteins at the Z-line (Swartz 1999).

1.3.12 TITIN

Titin is a giant protein with a monomer size of over 3 million Daltons (for review, see Granzier and Labeit 2007). It was so named after the Greek Titans who were giants. It also is sometimes referred

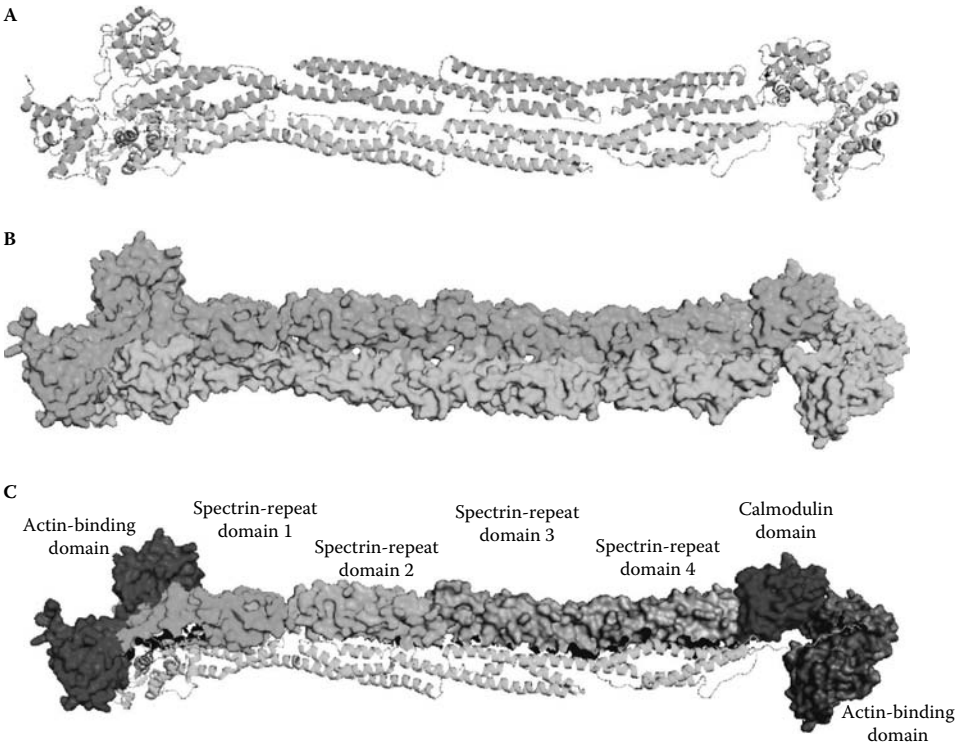

FIGURE 1.11 (See color insert following page 148.) Model structure and the domain organization of α-actinin dimer. One monomer is colored green while the other is colored cyan in the α-carbon cartoon (A) and surface (B) views. The complete domain structure of one of the monomers is shown (C) with the actin-binding domain in red, spectrin repeats in shades of green, and the calmodulin-like domain in blue. The actin-binding domain of the other monomer is shown in dark salmon to demonstrate its location opposite the calmodulin domain.

to as connectin. A single titin molecule extends from the Z-line (amino terminus) to the M-line (carboxyl terminus) in the sarcomere, a length of more than 1 μm (Figure 1.2, shown in red). Thus, the groups of titin molecules have an opposite polarity in each half sarcomere. The arrangement of titin in the myofibril has been determined based on antibody staining of permeabilized cells with monoclonal antibodies. An example is shown in Figure 1.12, where the extra mass from the monoclonal 9D10 is clearly visible in the middle I-band region and a dense line is found in the middle of each half A-band using monoclonal 856. Several studies using many different monoclonals show similar pairs of antibody stripes at equivalent positions in each half sarcomere. Titin filaments are not visible by electron microscopy in intact myofibrils except after extreme stretch where the thick and thin filaments are pulled beyond overlap and so-called "gap filaments" can be observed in the gap between the A- and I-bands. There are also structures referred to as "end filaments" that appear to be formed by aggregates of several titin molecules emerging from the ends of thick filaments after the thick filaments are mechanically removed from the sarcomere (Figure 1.13).

The structure of the protein is extremely complex, with large numbers of repeating domains. Most of the mass consists of 90 to 100 amino acid modules (as many as 280 total) that are 7 stranded β-sheets. These modules are of two types — those similar to the backbone-folding pattern of Ig and another set with similarity to the third domain of fibronectin (FN3). The portion of titin that lies in the A-band has both FN3 and Ig domains but the I-band region contains only the Ig-type domains.

Because titin is linked at both the Z-lines and the M-band, the structure must be elastic in nature to accommodate changes in sarcomere length during contraction and relaxation. In skeletal muscle, the elasticity arises from two sources. First, as a muscle is extended from its rest length,

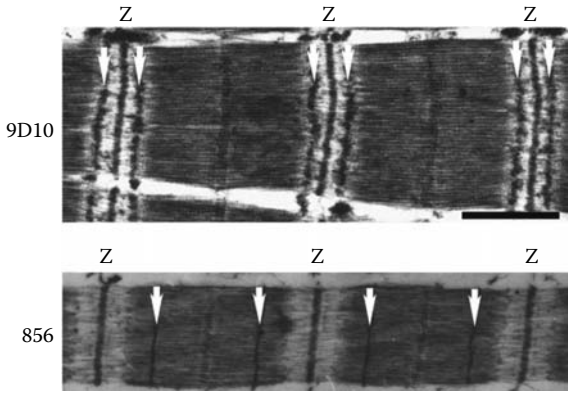

FIGURE 1.12 Titin location within the sarcomeremicron. Two sarcomeres sections of rabbit psoas muscle treated with two different monoclonal antibodies to titin prior to preparation for transmission EM. Because the antibody binds at only one position within each titin molecule, a dense band appears at only one location in each half sarcomere (white arrows). Bar = 1 μm.

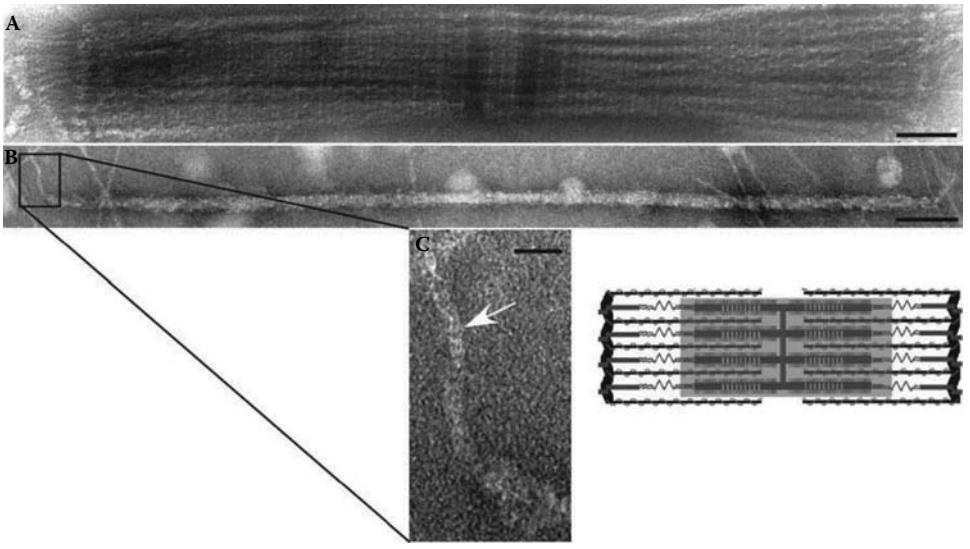

FIGURE 1.13 End filaments of the thick filament. Isolated A-band segment (A) and isolated thick filament (B) from goldfish bodywall muscle. Due to negative staining, protein structures in these electron micrographs appear as light areas on a dark background. End filaments can be seen protruding from the ends of the myosin filaments in both (A) and (B). Note the fine 4 nm striation in the end filament enlargement (C, arrow), thought to arise from the intrinsic domain repeat in this region of the titin molecule. Bars = 100 nm (A and B) or 20 nm (C).

the supercoiled titin molecules are straightened. Recent evidence indicates that this straightening occurs much like that of a carpenter's ruler that has both rigid and flexible regions. With greater extension forces, a specialized amino acid sequence called the PEVK region (positioned near the middle of the half I-band) lengthens. The PEVK is so named because more than 75% of the amino acids in this region consist of proline, glutamic acid, valine, and lysine. Most of the PEVK contains a repeating structure that includes a large number of 26 to 28 amino acid modules and several glutamic-acid-rich regions. While the Ig and FN3 modules are structurally stable in living muscle, the PEVK region is believed to be intrinsically disordered. Because this region is particularly sensitive

to protease action (such as during the postmortem period), SDS gel electrophoresis often reveals two titin bands (see Figure 1.4) — the T1 (corresponding to the full-length protein) and T2 (the A-band end of the molecule).

As a muscle cell is stretched under resting conditions (i.e., with magnesium and ATP present and calcium levels less than 10^{-6} M), the resisting force gradually increases with an increase in sarcomere length. The force measured is often referred to as passive tension (see below). The increased tension with sarcomere extension also allows titin to maintain the A-band in the center of the sarcomere. Passive tension varies between different muscles, due in part to the presence of different-sized titins (between 3.2 and 3.7 MDa). There is a single titin gene in higher vertebrates, and the size differences arise through alternative splicing in the middle I-band and PEVK sequence regions.

Unlike the I-band portions discussed above, the A-band portion of titin is believed to remain at fixed length during physiological stretch. The Ig (I) and FN3 (F) domains have 6 super repeats with the pattern I-F-F-I-F-F in the so-called D (distal) region of the A-band and 11 super repeats with the pattern I-F-F-I-F-F-F-I-F-F-F in the C-region found in the middle of each half A-band. The spacing of the start position of the 11 super repeats corresponds to the spacing (43 nm) of MyBP-C in the sarcomere and is consistent with the length of 11 modules (4 nm length determined by x-ray and NMR of an isolated expressed titin module). The precise domain patterns of the titin modules have led to the view that titin serves a ruler function for the assembly of the thick filament. Recent evidence suggests that titin runs parallel to the long axis of the thick filament on the surface of the shaft. A-band titin also contains a kinase domain. It has been shown to phosphorylate telethonin (also know as T-cap) but the kinase and T-cap are half a sarcomere apart in the assembled myofibril. No other protein substrates have been identified to date.

Titin interacts with a plethora of other proteins. These include telethonin/T-cap, filamin, nebulin, α-actinin, and small ankrin 1 in the Z-line region; obscurin, actin, αß crystalline, Fhl2/DRAL, MARP, and calpain-3 in the I-band region; myosin and MyBP-C in the A-band region; and MURFs, Ca/calmodulin kinase, Nbr1, Fhl2/DRAL, lamin, calpain-3, and myomesin in the M-line region. Some of the listed proteins provide structural anchors for titin. Others have been proposed to function in signaling and/or protein turnover. However, the functional significance of most of these interactions is currently unknown.

1.3.13 Obscurin

Obscurin is a large myofibrillar protein with a confusing localization pattern (Kontrogianni-Konstantopoulos, and Bloch 2005). It appears to be bound at the Z-line in developing muscle but localizes to the M-line in adults. Similar to titin, the myomesins, and MyBP-C, obscurin contains Ig and FN domains (68 and 2, respectively). In addition, there is a calmodulin domain, a RhoGEF domain (GEF = GDP/GTP exchange factor) with an associated pleckstin homology (PH) domain, and 2 serine-threonine kinase domains. The amount of the protein is very small — on the order of one tenth as much as nebulin. Obscurin has been found to associate with the Z-line region of titin through two specific Ig domains. It also binds to small ankrin 1 and thus may help tether the sarcoplasmic reticulum to the myofibril and perhaps the myofibrils to the sarcolemma. The RhoGEF domain has been suggested to function in G protein coupled signaling.

1.4 SUBSARCOMERIC STRUCTURES

The sarcomere of the myofibril can be divided into several substructures made from the proteins described above. This includes the thin and thick filaments that make up most of the sarcomere. There are also structures involved in organization of the thin (Z-line) and thick filaments (M-band) into their respective I-bands and A-bands. The organization of these subsarcomeric structures are described below.

1.4.1 Thick Filament

Myosin II molecules associate together to form the myosin or thick filaments found in muscle cells. Whereas the globular N-terminus of myosin determines its motor activity, the C-terminal rod portion is essential for filament formation. Each LMM rod associates with rods of neighboring molecules to form the filament backbone, probably as a result of interactions between charged amino acids at ~4 nm intervals along the rod (McLachlan and Karn 1983; Straussman et al. 2005). Polymerization of myosin into thick filaments *in vitro* is salt, pH, and myosin concentration dependent (Davis 1988). Polymerization starts by formation of longitudinal dimers, association of the dimers to form the bare zone, and then addition of dimers at the ends of the newly formed bare zone. Filaments formed *in vitro* can have highly variable lengths, pointing out the importance of thick-filament associated proteins (i.e., titin) in determining *in vivo* filament lengths. In vertebrate skeletal muscle, filament lengths are quite uniform within each fiber, with around 300 myosin molecules per 1.6 μm filament. The molecules at each end of the filament face in opposite directions, with their C-terminal rods pointing toward the center (Figure 1.14). This bipolar anti-parallel arrangement results in a central bare zone devoid of projecting heads (Figure 1.14B and C)**.** At the center of each filament are additional proteins that tether adjacent thick filaments together and form the M-band (described below). Several models of myosin rod packing have been proposed, some with and some without grouping of monomers into subfilaments (Wray 1979; Chew and Squire 1995). Structural data from vertebrate striated muscle so far do not clearly favor one particular model but EM features suggest that the rods lie nearly parallel to the filament and may surround a narrow hollow core (Squire et al. 1998; Kensler 2005). The organization of myosin in vertebrate smooth muscle filaments has not been fully characterized but probably is based on anti-parallel packing of tails in a side-polar arrangement along the filament so that heads emerge on opposite faces or sides of each filament (Xu et al. 1996). Many invertebrate thick filaments also include the protein paramyosin in their core.

Although the rod portions of the myosin molecules are packed so that some lie buried within the filament backbone, the S1 heads must all be exposed at the filament surface in order to bind to

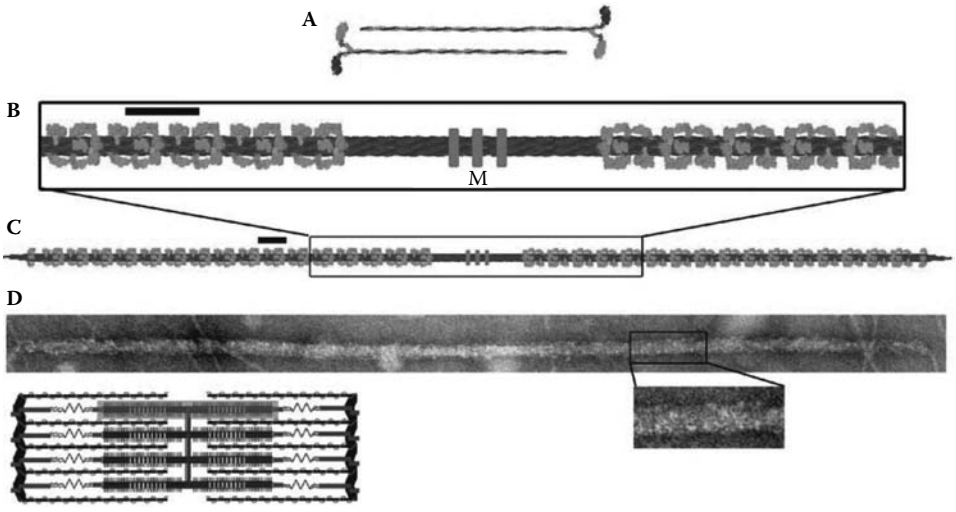

FIGURE 1.14 The filament is formed by association of myosin monomers along their LMM tails, which point in opposite directions in each half filament (A). The absence of motor domains in the center results in a bare zone (B). In vertebnrates, sequential polymerization of about 300 molecules gives rise to a bipolar filament of about 1.6 μm in length (C). Images were rendered as described in Figure 1.5 and the myosin filament arrangement was adapted from the vertebrate cardiac myosin filament structure of Al-Khayat et al. (2006). A transmission electron micrograph of a negatively stained thick filament from goldfish bodywall shows the full thick filament length (D) and at higher magnification, projections of motor domains and their apparent spiral appearance on the surface of the filament. Bar = 50 nm.

adjacent actin filaments. In relaxed muscle, the S1 heads project off the backbone in regular arrays (Figure 1.14B, C, D) that have been extensively studied by low angle x-ray diffraction and EM, and differ by muscle type (smooth vs. striated) and species (Squire et al. 2005; Craig and Woodhead 2006). For example, in insect filaments there are four myosins emerging at each level (crown), whereas in scallop there are seven. Successive crowns are evenly spaced and rotated, so that the heads form a helical pattern running along the surface of the filament. In vertebrate striated muscle, three pairs of myosin heads emerge at each crown but the spacing and rotations of successive crowns vary, with this pattern repeating every three crowns, about 43 nm.

The orientations of the individual myosin S1 heads in relaxed muscle have been the subject of many studies because they represent a starting point for the contractile event, and their highly ordered and periodic arrangement makes them accessible to analysis by x-ray diffraction (Huxley and Brown 1967; Yagi et al. 1977; Squire et al. 2006; Oshima et al. 2007) and EM image processing (Kensler and Stewart 1983; Cantino and Squire 1986; Craig et al. 1992; Al-Khayat et al. 2006; Zoghbi et al. 2008). Moreover, recent work suggests that in myosin-regulated muscles, this arrangement may help maintain the relaxed state. In smooth muscle, myosins with unphosphorylated regulatory light chains have very low ATPase activity and are therefore effectively turned off. Structural studies of smooth muscle HMM suggest that in the unphosphorylated off state, intramolecular interactions inhibit actin binding by one head and ATP hydrolysis by the other (Wendt et al. 2001). This smooth muscle interacting head motif appears consistent with the observed EM structures in relaxed thick filaments of myosin-regulated tarantula muscle (Woodhead et al. 2005). The argument is made that this arrangement is a common feature of all thick filaments that are myosin regulated.

But do such head-head interactions also occur in vertebrate striated muscle? In these systems, activation is initiated by calcium binding to troponin but thick filament protein interactions that stabilize the resting head configuration could play a secondary role in maintaining the relaxed state. Due to perturbations in the helical arrangement, the arrangement of heads in thick filaments of vertebrate striated muscle is more difficult to resolve. Structural studies of myosin filaments from vertebrate skeletal muscle have supported models with heads from the same molecules closely associated but with variations in orientation at different levels within each three-crown repeat (Kensler 2005b; Al-Khayat et al. 2006; Oshima et al. 2007). Most recently, two independent EM studies have modeled crossbridge orientations in mammalian cardiac thick filaments at 4 nm resolution or better. Both find that the smooth muscle interacting-head motif fits the observed EM images at two of three crowns in the myosin helix (Al-Khayat et al. 2008; Zoghbi et al. 2008). In cardiac muscle, it is not expected that this arrangement would prevent activation (as it may in myosin-regulated systems) but could lower its probability at low calcium, thus reducing diastolic pressure and optimizing performance of the heart. These intramolecular interactions, if shown to be widely conserved, could help explain the universal occurrence of two-headed myosins in muscle.

As noted above, the LMM portion of the α-helical rod is essential for filament formation and provides the majority of the mass found in the filament backbone. The S2 domain of the rod is more soluble, associating only weakly with the filament backbone, and single myosin molecules imaged using EM often show a bend near the LMM–HMM junction. These and other data suggest that S2 has considerable mobility *in situ*. During relaxation, it appears to lie close to the filament backbone, maintaining the ordered array of helical filaments. During activation, release of S2 from the filament surface would increase the number of target actin binding sites on adjacent filaments where S1 can bind. This mobility may also help the contractile system adapt to increases in interfilament distance that occur as the muscle shortens.

1.4.2 Thin Filament

The thin filament of skeletal muscle contains primarily F-actin. Bound along the length of the F-actin filament are nebulin, tropomyosin, and troponin (Figure 1.15). The barbed (+) end binding CapZ (Figure 1.15a) and α-actinin is located at the Z-line while the pointed (–) end binding

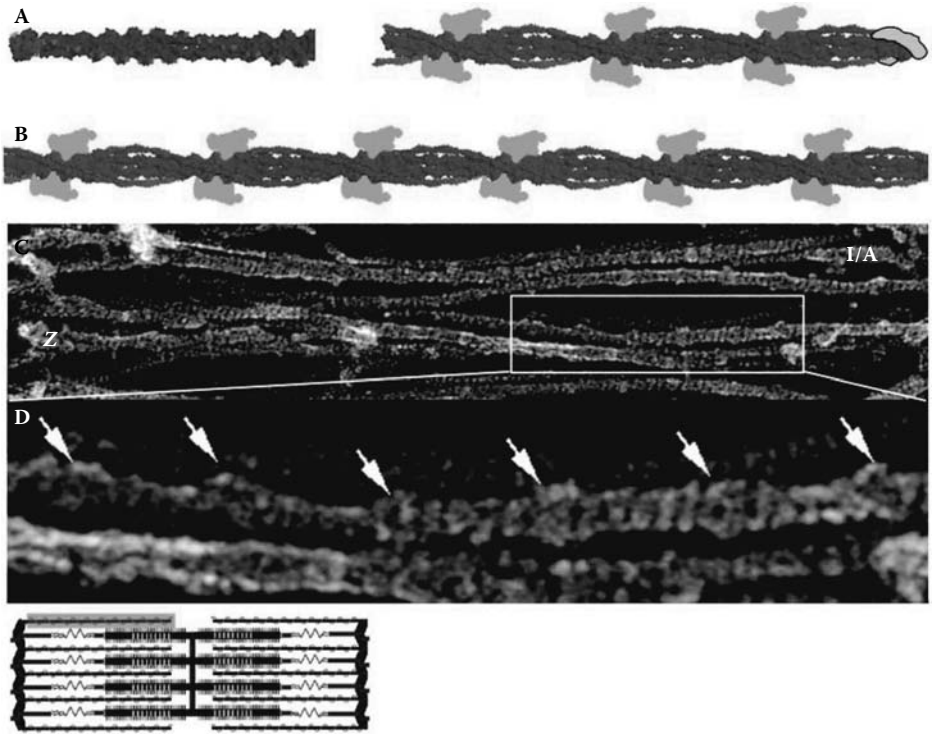

FIGURE 1.15 (See color insert following page 148.) Thin filament structure. Cartoons of the thin filament (A and B) and transmission electron micrographs (C and D) of thin filaments in the non-overlap region from plaice fin muscle after freeze fracture show the protein organization along the filament. Cartoons of the ends (Z-line and tip in the overlap region) of the thin filaments (A) show the capping of the filament. For the plus or Z-line end (leftmost), actin is colored blue and CapZ is colored red. For the minus or tropomodulin end, actin is blue, Tm is red, Tn is green, and tropomodulin is cyan. Arrows in D denote the troponin bulges. The rotation of each successive troponin pair around the filament axis has been omitted to simplify the rendering.

tropomodulin is at the opposite end. The interactions of primarily nebulin, CapZ, and tropomodulin stabilize the F-actin filament from polymerization/depolymerization (Littlefield and Fowler 1998). There may be transient interactions of MyBP-C with actin but these are not well resolved. Myosin also interacts with the thin filament in an ATP-dependent transient fashion, moving toward the barbed (+) end of the thin filament.

Thin filament length in striated mammalian muscle is not the same between muscles within a species or between species. It ranges from 1.0 to 1.3 μm in length. This relatively stable structure and its anatomy allow one to calculate the mole ratio of proteins within the thin filament. For a 1 μm thin filament, there are about 400 actin monomers, 56 troponins and tropomyosins, 2 nebulins, 2 tropomodulins, and 1 CapZ. The amount of α-actinin is dependent on Z-line width, which varies with fiber type but ranges from 2 to 8.

The azimuthal position (position with respect to the cross-section of the filament) of tropomyosin on the actin filament can change, depending on calcium level and strong (rigor) crossbridges (Vibert et al. 1997). This feature was initially inferred from x-ray diffraction studies on muscle and led to the steric blocking model of thin filament regulation (Squire and Morris 1998). More recent studies using EM reconstruction of reconstituted thin filaments (actin+tropomyosin+troponin), confirming this model and adding more detail, are discussed below. The azimuthal location of nebulin is not well resolved. Early studies suggested that the actin binding domains bind to the inner domain, primarily in subdomains 3 and 4 (Pfuhl et al. 1994). More recent studies with F-actin and a 3-module actin-binding fragment of nebulin suggest that it binds in potentially three different locations — the extreme outer

domain, the outer domain, and the inner domain (Lukoyanova et al. 2002). Some of these sites are occupied by tropomyosin, so there is potential for steric clashing of these molecules on F-actin.

Some of the change in the location of tropomyosin on actin is mediated by troponin. Troponin is spaced at about 38 nm intervals along each strand of the two-stranded long-pitch helix. Troponins on opposite strands are staggered axially by about 2.5 nm (Figure 1.15A-D). The azimuthal location of troponin with respect to the actin has been difficult to determine. Recent studies using EM reconstruction and single-particle analysis have led to two different models for troponin's location with respect to actin and tropomyosin. One has troponin bound to actin+tropomyosin with the regulatory domain of TnI interacting with the actin and controlling the location of tropomyosin on the same long-pitch strand. In this model, the troponin core domain moves with the tropomyosin that it controls (Narita et al. 2001). The other model has the troponin on the opposite long-pitch strand controlling the position of tropomyosin; that is, the regulatory domain of TnI spans the short-pitch helix (across the filament) to control the location of tropomyosin (Pirani et al. 2006). This model also suggests that part of TnT spans the short pitch and further influences the location of tropomyosin. With this model, the troponin structural domain (TnIT coiled-coil and C-terminal of TnC) does not move; only the regulatory domain of TnI moves to allow for tropomyosin movement on the surface.

1.4.3 Z-Line/Z-Disc

The Z-line bisects the I-band and is a critical structural element that links the contractile activity of the individual thin filaments to contractions at the ends of the muscle cell (Vigoreaux 1994). In cross-section, it appears as a circle or disc and is sometimes called the Z-disc. At the EM level, in cross-section within the Z-disc, it appears as either a square lattice or as a basket-weave lattice (see Figures 1.2 and 1.3). For the square lattice, the corners are the actin filaments and the connections between the corners are called Z-links that are mostly made of α-actinin. For the basket weave, the "weave" are the Z-links and their points of intersection are the actin filaments. These two different appearances are thought to be the result of expanding the basket-weave lattice into the square and thus straightening out the Z-links (Yamaguchi et al. 1985). In longitudinal-section, the Z-line appears with a zigzag line connecting the actin filaments (Figure 1.16a–c). The actin filaments from the opposing half sarcomeres overlap at the Z-line (Yamaguchi et al. 1983), allowing α-actinin to link two actin filaments of opposite polarity (Figure 1.16a). The width of the Z-line is fiber type specific with fast muscles having a narrow (about 50 nm) Z-line and slow/cardiac muscles having a wide (about 140 nm) Z-line. Narrow Z-lines appear to have two Z-links while slow bovine sternomandibularis has six Z-links (Luther et al. 2002). The longitudinal spacing between the links is about 18 nm. Independent of fiber type, the width of the Z-line changes with sarcomere length, being wider at long sarcomere length than short to accommodate the constant volume nature of the filament lattice. This is readily observed in light microscopy of isolated myofibrils using phase-contrast. The Z-line is quite visible in sarcomeres at 3 μm but barely visible at 2.2 μm. However, these apparent changes in width are overestimated because of the contrast method. Additional structural proteins within the Z-line include titin and nebulin. The N-terminal of titin penetrates the Z-line and extends across the complete width. It interacts with the C-terminal, calmodulin-like domains of α-actinin through its Z-repeats. Nebulin interacts with the Z-line and penetrates to the level of the first Z-bridge (Millevoi et al. 1998).

There is emerging evidence that the Z-line is a potential nexus for several signal transduction pathways (Frank et al. 2006) and that there are up to an additional 10 proteins that localize to the Z-line or its periphery (Faulkner et al. 2001). These proteins typically are present in low copy number, many bind to α-actinin, and some are involved in various signal transduction processes and potentially myofibrillogenesis. Additional work needs to be done to document that many of these are *bona fide* structural elements of the Z-line and/or that they are transiently associated with the Z-line in a docking fashion. Some that are well characterized include γ filamin, telethonin, and myopalladin. Filamin was localized to the Z-line more than 25 years ago (Gomer and Lazarides 1981). More recent studies suggest that this localization involves both the actin binding domains of filamin and through an indirect

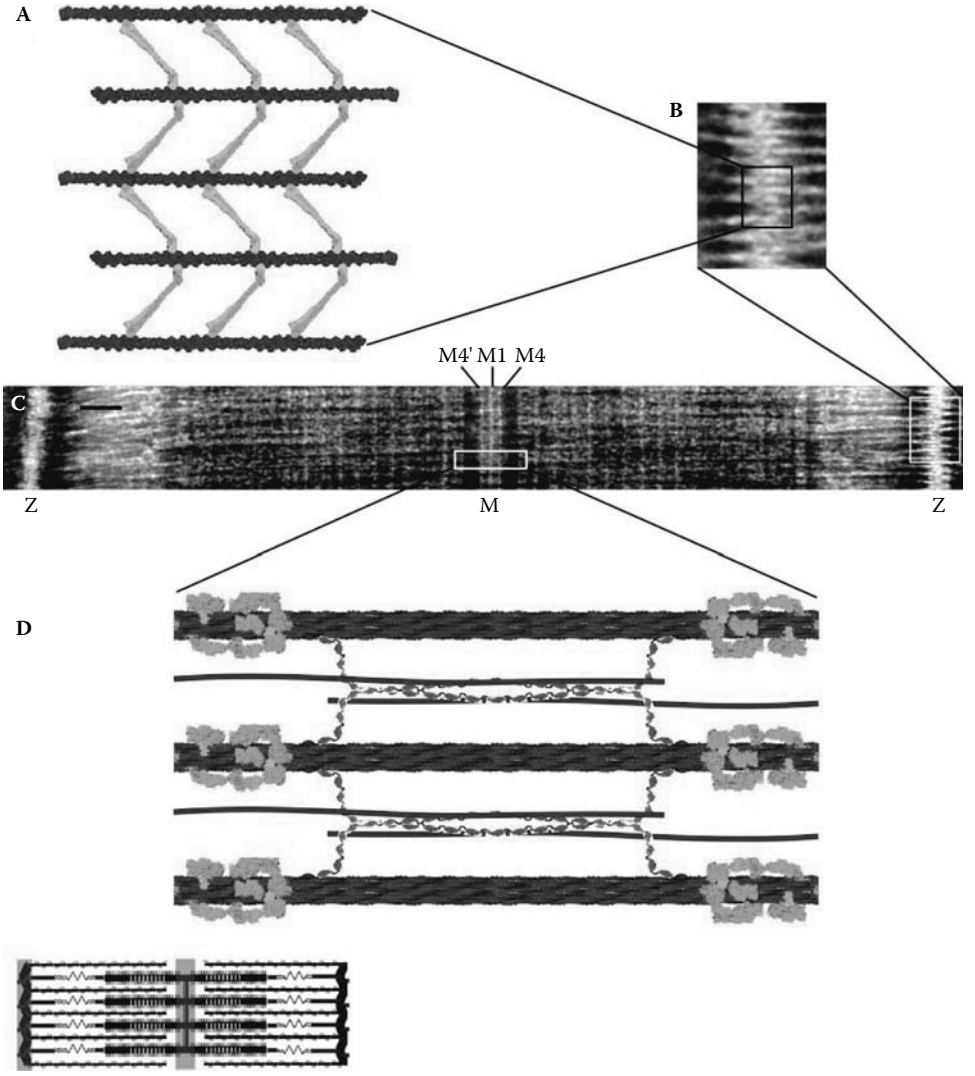

FIGURE 1.16 (See color insert following page 148.) Structure of the Z-line/Z-disc and M-band. A cartoon of the Z-line (A) was made using F-actin structures from Figure 1.8 and the α-actinin structure in Figure 1.13. F-actin is in blue, CapZ in red, and α-actinin in cyan and green. This zigzag appearance is evident in electron micrographs from negatively stained sections of frog semitendinosus muscle (B and C). The M-band as envisioned in cartoon form (D) was made using Fn and Ig domain models for myomesin and the thick filament cartoons in Figure 1.14. The general organization was interpreted from Lange et al. (2002). Myosin is colored green, titin blue, Ig domains magenta, Fn domains orange, and myosin binding domain black. Myomesin bridges are shown more widely spaced than expected for clarity. The M-lines (M4′, M1, and M4) are noted. Bar in (C) = 100 nm.

interaction with α-actinin via a protein called myotilin (Van der Ven et al. 2000). Telethonin/T-cap is localized to the Z-line through its interaction with the N-terminus of titin (see above). Myopalladin is a recently discovered Z-line 145 kDa protein that links the C-terminal Src homology domains of nebulin to the C-terminal calmodulin-like domain of α-actinin (Bang et al. 2001).

1.4.4 I-Band

The I-band was initially so-named because it is isotropic in refractive index when viewed in the polarizing microscope. In phase contrast and EM images, it is the low-density region of the

sarcomere bisected by the Z-line (Figure 1.2). As such, a single I-band includes one Z-line and the region of the thin filaments on each side of the Z-line (longitudinally adjacent half sarcomeres) that are not in the overlap region. The width of the I-band decreases as the sarcomere shortens and this feature, with a lack of change in the A-band width, led to the sliding filament hypothesis for muscle contraction. The thin filaments within a half I-band have the same axial repeat or twist. In well-aligned sarcomeres in EM longitudinal sections, as shown in Figure 1.16C, this alignment of adjacent filaments produces perpendicular I-band stripes at 38 nm intervals due to the additional mass of troponin. In cross-section, the actin filaments have a square lattice near the Z-line (out to about 100 nm) and a hexagonal lattice near the A-band (see Figures 1.2 and 1.3). The center-to-center spacing between adjacent thin filaments is about 25 nm in the overlap region but varies with sarcomere length to maintain a constant volume.

1.4.5 M-Band

The electron microscopic appearance of the M-band is related to the types and distributions of myomesins (Agarkova et al. 2003). Five prominent lines spaced 22 nm apart are visible in muscle sections, and these are labeled M6, M4, M1, M4′, and M6′, with M1 at the center. The M4, M4′ lines are present in all muscles (Figure 1.16C and D) but the other lines vary with muscle type and developmental stage. The M1-band is present in fast muscles containing myomesin 2, and M6, M6′ occurs in muscles containing myomesin 3. The binding to titin and the near proximity of this interaction to the titin kinase domain suggests that the myomesins may have some involvement in titin stretch signaling. Myomesins are also thought to be the mass associated with the M-bridges observed in cross-section (Figures 1.2 and 1.3). Creatine kinase (MM isoform) also binds to the M-band to contribute additional mass.

1.4.6 A-Band

In striated muscle, thick filaments lie parallel to each other in clusters that give rise to the A-bands seen in the light microscope (Figure 1.2). The separation between thick filaments is around 48 nm in vertebrate fibers at rest, but decreases as a muscle is stretched. In most species, the filaments are connected at their centers by the M-bridges, which maintain them in a regular hexagonal array. The myomesin-dependent M-bridges and M-lines also keep the filaments in vertebrate striated muscle in close axial register, so that bare zones, myosin crowns, and myosin binding proteins are in nearly perfect alignment (Figures 1.2, 1.3, 1.16, and 1.17). In minimally disrupted A-bands, this alignment creates the appearance of stripes running across each A-band. Weak stripes can be detected

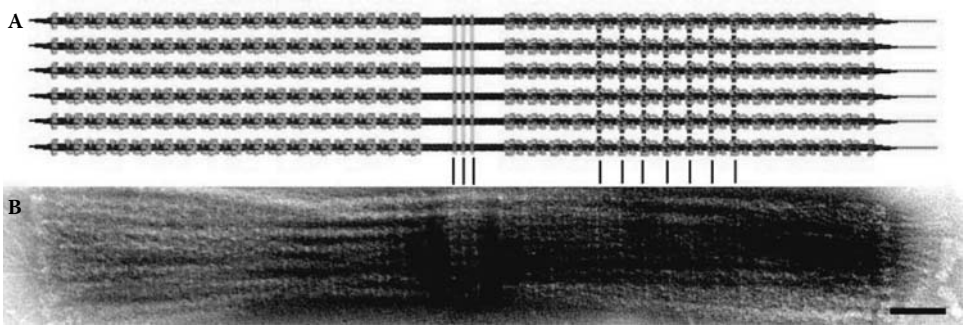

FIGURE 1.17 (See color insert following page 148.) Structure of the A-band. A cartoon of the A-band is shown (A) with thick filaments in green (from Figure 1.14C), M-line forming the M-band in cyan, MyBP-C in dark blue, and titin in red. A transmission electron micrograph of a negatively stained mechanically isolated A-band from goldfish bodywall shows the A-band structures noted in the cartoon (B). Bar = 100 nm.